8565712

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

January 27, 2025

THIS IS TO CERTIFY THAT ANNEXED IS A TRUE COPY FROM THE RECORDS OF THIS OFFICE OF THE FILE WRAPPER AND CONTENTS OF:

APPLICATION NUMBER: ***14/759,895***
FILING DATE: ***July 08, 2015***
PATENT NUMBER: ***10617790***
ISSUE DATE: ***April 14, 2020***

Certified by

ACTING DIRECTOR OF THE UNITED STATES PATENT AND TRADEMARK OFFICE

PTO-1390 (08-13)
Approved for use through 6/30/2013. OMB 0651-0021
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A SUBMISSION UNDER 35 U.S.C. 371		Attorney Docket No. S1860.70000US01 U.S. Application No. (if known, see 37 CFR 1.5)
International Application No. PCT/US2014/010890	International Filing Date 9 January 2014	Priority Date Claimed 9 January 2013
Title of Invention DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE		
First Named Inventor Ryanne Early		

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). NOTE: The express request under 35 U.S.C. 371(f) will not be effective unless the requirements under 35 U.S.C. 371(c)(1), (2), and (4) for payment of the basic national fee, copy of the International Application and English translation thereof (if required), and the oath or declaration of the inventor(s) have been received.

2. ☐ A copy of the International Application (35 U.S.C. 371(c)(2)) is attached hereto (not required if the International Application was previously communicated by the International Bureau or was filed in the United States Receiving Office (RO/US)).

3. An English language translation of the International Application (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).

4. An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4))
   a. ☐ is attached.
   b. ☐ was previously filed in the international phase under PCT Rule 4.17(iv).

Items 5 to 8 below concern amendments made in the international phase.

PCT Article 19 and 34 amendments

5. ☐ Amendments to the claims under PCT Article 19 are attached (not required if communicated by the International Bureau) (35 U.S.C. 371(c)(3)).

6. ☐ English translation of the PCT Article 19 amendment is attached (35 U.S.C. 371(c)(3)).

7. ☐ English translation of annexes (Article 19 and/or 34 amendments only) of the International Preliminary Examination Report is attached (35 U.S.C. 371(c)(5)).

Cancellation of amendments made in the international phase

8a. ☐ Do not enter the amendment made in the international phase under PCT Article 19.

8b. ☐ Do not enter the amendment made in the international phase under PCT Article 34.

NOTE: A proper amendment made in English under Article 19 or 34 will be entered in the U.S. national phase application absent a clear instruction from applicant not to enter the amendment(s).

The following items 9 to 17 concern a document(s) or information included.

9. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

10. ☒ A preliminary amendment.

11. ☒ An Application Data Sheet under 37 CFR 1.76.

12. ☐ A substitute specification. NOTE: A substitute specification cannot include claims. See 37 CFR 1.125(b).

13. ☐ A power of attorney and/or change of address letter.

14. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13*ter*.3 and 37 CFR 1.821-1.825.

15. ☐ Assignment papers *(cover sheet and document(s))*. Name of Assignee: _____

16. ☐ 37 CFR 3.73(c) Statement *(when there is an Assignee)*.

PTO-1390 (08-13)
Approved for use through 6/30/2013. OMB 0651-0021
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

U.S. APPLN. No. (if known – see 37 CFR 1.5)	INTERNATIONAL APPLICATION No.	ATTORNEY DOCKET No.
	PCT/US2014/010890	S1860.70000US01

17. [X] Other items or information: PCT Published Application w ISR; International Search Report w Written Opinion; Copy of 92*Bis*.

The following fees have been submitted.	CALCULATIONS	PTO USE ONLY
18. [X] Basic national fee (37 CFR 1.492(a)) $280	$ 280.00	
19. [X] Examination fee (37 CFR 1.492(c)) If the written opinion prepared by ISA/US or the international preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4) ..... $0 All other situations $720	$ 720.00	
20. [X] Search fee (37 CFR 1.492(b)) If the written opinion prepared by ISA/US or the international preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4) ..... $0 Search fee (37 CFR 1.445(a)(2)) has been paid on the international application to the USPTO as an International Searching Authority ..... $120 International Search Report prepared by an ISA other than the US and provided to the Office or previously communicated to the US by the IB ..... $480 All other situations $600	$ 480.00	
TOTAL OF 18, 19, and 20 =	$ 1,480.00	
[ ] Additional fee for specification and drawings filed in paper over 100 sheets (excluding sequence listing in compliance with 37 CFR 1.821(c) or (e) in an electronic medium or computer program listing in an electronic medium) (37 CFR 1.492(j)). Fee for each additional 50 sheets of paper or fraction thereof $400		
Total Sheets: 38 - 100 = / Extra Sheets: / 50 = / Number of each addition 50 or fraction thereof (round up to a whole number): / RATE: x $400	$	
Surcharge of **$140.00** for furnishing any of the search fee, examination fee, or the oath or declaration after the date of commencement of the national stage (37 CFR 1.492(h)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	33 - 20 =	13	x $80	$ 1,040.00	
Independent claims	11 - 3 =	8	x $420	$ 3,360.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $780	$ 780.00	
Processing fee of **$140.00** for furnishing the English translation later than 30 months from the earliest claimed priority date (37 CFR 1.492(i)). +				$	
TOTAL OF ABOVE CALCULATIONS =				$ 6,660.00	
[X] **Applicant asserts small entity status.** See 37 CFR 1.27. Fees above are reduced by ½.				- 3,330.00	
[ ] **Applicant certifies micro entity status.** See 37 CFR 1.29. Fees above are reduced by ¾. Applicant must attach form PTO/SB/15A or B or equivalent.					
TOTAL NATIONAL FEE =				$	3,330.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property. +				$	
TOTAL FEES ENCLOSED =				$	3,330.00
				Amount to be refunded: $	
				Amount to be charged: $	

4122183.1

a. [ ] A check in the amount of $ _____ to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees.

c. [X] The Director is hereby authorized to charge additional fees which may be required, or credit any overpayment, to Deposit Account No. 23/2825 as follows:

i. [X] any required fee.

ii. [ ] any required fee except for excess claims fees required under 37 CFR 1.492(d) and (e) and multiple dependent claim fee required under 37 CFR 1.492(f).

d. [X] Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038. The PTO-2038 should only be mailed or faxed to the USPTO. However, when paying the basic national fee, the PTO-2038 may NOT be faxed to the USPTO.

ADVISORY: If filing by EFS-Web, do **NOT** attach the PTO-2038 form as a PDF along with your EFS-Web submission. Please be advised that this is **not** recommended and by doing so your **credit card information may be displayed via PAIR**. To protect your information, it is recommended to pay fees online by using the electronic payment method.

NOTE: Where an appropriate time limit under 37 CFR 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the International Application to pending status.

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File)Transition Applications

[ ] This application (1) claims priority to or the benefit of an application filed before March 16, 2013, and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.

NOTE 1: By providing this statement under 37 CFR 1.55 or 1.78, **this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA**.

NOTE 2: A U.S. national stage application may not claim priority to the international application of which it is the national phase. The filing date of a U.S. national stage application is the international filing date. See 35 U.S.C. 363.

Correspondence Address

[X] The address associated with Customer Number: 23628 **OR** [ ] Correspondence address below

Name					
Address					
City		State		Zip Code	
Country			Telephone		
Email					

Signature	Edward R Gates	Date	July 8, 2015
Name (Print/Type)	Edward R. Gates	Registration No. (Attorney/Agent)	31,616

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Filer:	Edward R. Gates/Kara Verrochi
Attorney Docket Number:	S1860.70000US01

Filed as Small Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Basic National Stage Fee	2631	1	140	140
Natl Stage Search Fee - Report provided	2642	1	240	240
Natl Stage Exam Fee - all other cases	2633	1	360	360
Pages:				
Claims:				
Claims in excess of 20	2615	13	40	520
Independent claims in excess of 3	2614	8	210	1680
Multiple dependent claims	2616	1	390	390

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	3330

Electronic Acknowledgement Receipt

EFS ID:	22862268
Application Number:	14759895
International Application Number:	PCT/US2014/010890
Confirmation Number:	3358
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Customer Number:	23628
Filer:	Edward R. Gates/Kara Verrochi
Filer Authorized By:	Edward R. Gates
Attorney Docket Number:	S1860.70000US01
Receipt Date:	08-JUL-2015
Filing Date:	
Time Stamp:	17:49:48
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	Credit Card
Payment was successfully received in RAM	$3330
RAM confirmation Number	4367
Deposit Account	232825
Authorized User	GATES, EDWARD R.

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. 1.492 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Application Data Sheet	S186070000US01-ADS-ERG.pdf	18156 f9152fd8ccfaaa3486e58372f2b6a11d1ad7c7edb	no	5
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
2		S186070000US01-PAMN-ERG.pdf	34684 fe3897f7bce6c2c35d5ea3c3c04ff88fcedc30e6	yes	7

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Preliminary Amendment	1	1
Specification	2	2
Claims	3	6
Applicant Arguments/Remarks Made in an Amendment	7	7

Warnings:					
Information:					
3		S186070000US01-371APL-ERG.PDF	2825139 11e9e5d022fa63fc2ad4aaa49b8f8f8b5013ebd9	yes	39

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Abstract	1	1
Specification	2	29
Claims	30	32
Drawings-other than black and white line drawings	33	38

	Documents submitted with 371 Applications		39	39	
Warnings:					
Information:					
4	Documents submitted with 371 Applications	S186070000US01-ISR-ERG.pdf	210465 [illegible]	no	8
Warnings:					
Information:					
5	Documents submitted with 371 Applications	S186070000US01-92BIS-ERG.pdf	826146 [illegible]	no	5
Warnings:					
Information:					
6	Transmittal of New Application	S186070000US01-TRN-ERG.PDF	477005 [illegible]	no	3
Warnings:					
Information:					
7	Fee Worksheet (SB06)	fee-info.pdf	39977 [illegible]	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	4431572		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Application Data Sheet

Inventor Information

Inventor Number::	1
Name Prefix::	Ms.
Given Name::	Ryanne
Family Name::	Early
City of Residence::	Fleming Island
State or Province of Residence::	FL
Country of Residence::	US
Street of mailing address::	1909 Hickory Trace Dr.
City of mailing address::	Fleming Island
State or Province of mailing address::	FL
Postal or Zip Code of mailing address::	32003

Correspondence Information

Correspondence Customer Number::	23628

Application Information

Application Type::	Regular
Subject Matter::	Utility
CD-ROM or CD-R?::	None
Sequence submission?::	None
Computer Readable Form (CRF)?::	No

Title::	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
Attorney Docket Number::	S1860.70000US01
Request for Early Publication?::	No
Request for Non-Publication?::	No
Total Drawing Sheets::	6
Small Entity?::	Yes
Petition included?::	No
Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2::	No
Authorization to Permit Access to the Instant Application by the Participating Offices::	No
This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013::	Yes

Representative Information

Representative Customer Number::	23628

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This Application	National Stage of	PCT/US2014/010890	01/09/14
PCT/US2014/010890	An application claiming the benefit under 35 USC 119(e)	61/750555	01/09/13

Foreign Priority Information

Applicant Information

Applicant Number::	1
Applicant Type::	Assignee
Organization Name::	ISE Professional Testing & Consulting Services, Inc.
Street of mailing address::	2007 NW 5th Street
City of mailing address::	Gainesville
State or Province of mailing address::	FL
Postal or Zip Code of mailing address::	32603

Assignee Information Including Non-Applicant Assignee Information

Assignee Number::	1
Organization Name::	ISE Professional Testing & Consulting Services, Inc.
Street of mailing address::	2007 NW 5th Street
City of mailing address::	Gainesville

State or Province of mailing address::	FL
Postal or Zip Code of mailing address::	32603

RegenX Science Exhibit 1004
Page 13

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RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0013

Signature:

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications.			
Signature	/Edward R. Gates/	Date	July 8, 2015
Name	Edward R. Gates	Registration Number	31,616

Docket No.: S1860.70000US01
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Ryanne Early
Application No.:	National Stage of PCT/US2014/010890
Confirmation No.:	N/A
Filed:	Concurrently Herewith
For:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
Examiner:	Not Yet Assigned
Art Unit:	N/A

FIRST PRELIMINARY AMENDMENT UNDER 37 C.F.R. § 1.115

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Madam:

INTRODUCTORY COMMENTS

Prior to examination on the merits, please amend the above-identified U.S. patent application as follows:

Amendments to the Specification begin on page 2 of this paper.

Amendments to the Claims are reflected in the listing of claims which begins on page 3 of this paper.

Remarks/Arguments begin on page 7 of this paper.

AMENDMENTS TO THE SPECIFICATION

Please replace the paragraph beginning on page 1, paragraph [0001] entitled CROSS-REFERENCE TO RELATED APPLICATIONS with the following amended paragraph:

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a national stage filing under 35 U.S.C. § 371 of PCT International application PCT/US2014/010890 designating the United States of America, filed January 9, 2014 which claims the benefit under 35 USC § 119(e) to U.S. provisional application 61/750,555, filed January 9, 2013, the entire contents of which are incorporated herein by reference in their entirety.

AMENDMENTS TO THE CLAIMS

Please replace all prior versions, and listings, of claims in the application with the following list of claims:

1. (Original) A decellularized biomaterial produced by the process that comprises (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

2. (Original) The decellularized biomaterial of claim 1, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

3. (Original) The decellularized biomaterial of claim 1, wherein said process further comprises subjecting said sample to sterilization.

4. (Original) The decellularized biomaterial of claim 1, further comprising devitalized cells.

5. (Original) A tissue graft comprising extracellular matrix components derived from a urodele.

6. (Original) An isolated, decellularized Urodele ECM.

7. (Original) The isolated ECM of claim 6, wherein the ECM is derived from Axolotl tissue.

8. (Original) The isolated ECM of claim 6, wherein the ECM includes basement membrane.

9. (Original) The isolated ECM of claim 6, wherein the ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, a drug or a human agent.

10. (Original) The isolated ECM of claim 6, wherein the agent is a cell, optionally a human cell.

11. (Original) The isolated ECM of claim 6, wherein the ECM is a sheet.

12. (Original) The isolated ECM of claim 11, wherein the sheet includes perforations.

13. (Original) The isolated ECM of claim 6, wherein the ECM is a dry powder.

14. (Original) The isolated ECM of claim 6, wherein the ECM is a reconstituted gel.

15. (Original) The isolated ECM of any one of claims 6-13, therein the ECM is sterile.

16. (Original) A package containing sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM.

17. (Original) The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a sheet of isolated, decellularized Urodele ECM.

18. (Original) The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a dry powder.

RegenX Science Exhibit 1004
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19. (Original) The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a gel.

20. (Original) A sterile medical implant comprising sterile, isolated, decellularized Urodele ECM or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM.

21. (Original) The sterile medical implant of claim 19, wherein the implant is a biocompatible sheet, mesh, gel, graft, tissue or device.

22. (Original) A material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM or a Urodele fraction derived from the isolated, decellularized Urodele ECM.

23. (Original) A tissue culture system comprising (a) an isolated Urodele decellularized ECM, (b) tissue culture medium, and (c) cells xenogenic to the Urodele.

24. (Original) The tissue culture system of claim 22, wherein the cells are human cells.

25. (Original) Tissue culture medium conditioned with isolated Urodele decellularized ECM or a Urodele fraction derived from isolated, decellularized Urodele ECM.

26. (Original) A device comprising at least two sheets of isolated Urodele decellularized ECM laminated to one another.

27. (Original) A product prepared by isolating Urodele ECM from a Urodele, decellularizing the ECM, and sterilizing the decellularized ECM.

28. (Original) A method of preparing a biologic material comprising (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B)

decellularizing the sample forming a decellularlized ECM to remove sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

29. (Original) The method of claim 28, wherein the method further comprises performing the decellularization in a manner to retain structural and functional integrity of the decellularized ECM sufficient to permit the decellularized ECM to be useful as a matrix upon and within which cells can grow.

30. (Original) The method of claim 29, further comprising homogenizing the decellularized ECM to form a powder.

31. (Original) The method of claim 30, further comprising reconstituting the powder as a gel.

32. (Original) The method of claim 29, further comprising sterilizing the decellularized ECM.

33. (Original) The method of claim 29, further comprising attaching the decellularized ECM to an agent xenogenic to a Urodele.

REMARKS

The specification has been amended to update the Related Applications paragraph. No new matter has been added. A first and favorable action is requested.

Applicant believes no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 23/2825 under Docket No. S1860.70000US01 from which the undersigned is authorized to draw.

Dated: July 8, 2015

Respectfully submitted,

Electronic signature: /Edward R. Gates/
Edward R. Gates
Registration No.: 31,616
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 July 2014 (17.07.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/110269 A1

(51) International Patent Classification:
A61K 35/56 (2006.01) *A61F 2/00* (2006.01)
A61K 9/10 (2006.01) *A61L 27/36* (2006.01)
A61K 9/14 (2006.01)

(21) International Application Number:
PCT/US2014/010890

(22) International Filing Date:
9 January 2014 (09.01.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/750,555 9 January 2013 (09.01.2013) US

(72) Inventor; and
(71) Applicant : **EARLY, Ryanne** [US/US]; 1909 Hickory Trace Dr., Fleming island, FL 32003 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to the identity of the inventor (Rule 4.17(i))*

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

(54) **Title**: DECELLULARIZED BIOMATERIAL FORM NON-MAMMALIAN TISSUE

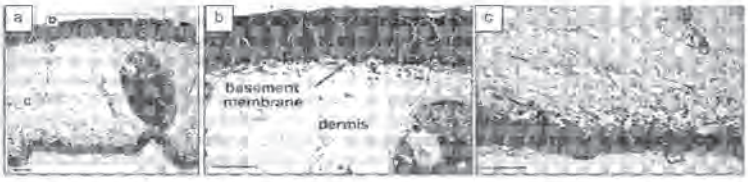


FIGURE 1

(57) **Abstract**: The growth factor profile, connective tissue matrix constituents, and immunoprivileged status of urodele extracellular matrix (ECM) and accompanying cutaneous tissue, plus the presence of antimicrobial peptides there, render urodele-derived tissue an ideal source for biological scaffolds for xenotransplantation. In particular, a biological scaffold biomaterial can be obtained by a process that entails (A) obtaining a tissue sample from a urodele, where the tissue comprises ECM, inclusive of the basement membrane, and (B) subjecting the tissue sample to a decellularization process that maintains the structural and functional integrity of the extracellular matrix, by virtue of retaining its fibrous and non-fibrous proteins, glycoaminoglycans (GAGs) and proteoglycans, while removing sufficient cellular components of the sample to reduce or eliminate antigenicity and immunogenicity for xenograft purposes. The resultant urodele-derived biomaterial can be used to enhance restoration of skin homeostasis, to reduce the severity, duration and associated damage caused by post-surgical inflammation, and to promote progression of natural healing and regeneration processes. In addition, the biomaterial promotes the formation of remodeled tissue that is comparable in quality, function, and compliance to undamaged human tissue.

WO 2014/110269 A1

DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 USC § 119(e) to U.S. provisional application 61/750,555, filed January 9, 2013, the entire contents of which are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

[0002] Tissue engineering efforts are ongoing to produce methods and materials for replacing biological functions, typically repairing or replacing whole tissues or portions thereof. In this regard, wound treatment and skin repair are areas of predominant focus, as the loss of skin integrity due to illness or injury can lead to chronic, life threatening complications.

[0003] Wound healing involves complex interactions between cells, growth factors, and extracellular matrix (ECM) components to reconstitute tissue following injury. The wound healing process in adult mammalian tissue has been well characterized and can be broken down into three stages - inflammation, proliferation, and remodeling.

[0004] Typically, in response to an incision or trauma the body conveys blood, blood products, and proteins into the void (also referred to as the cavity or negative space) formed at the wound. During early inflammation, a wound exudate begins to form under the influence of inflammatory mediators and as a result of vasodilation. Fibrin and fibronectin present in clotting blood provide a scaffold over which cells such as keratinocytes, platelets and leukocytes migrate to the wound site. Bacteria and debris are phagocytosed and removed, and growth factors are released that stimulate the migration and division of fibroblasts.

[0005] The subsequent stage of wound healing involves new tissue formation as fibrous connective tissue, termed granulation tissue (composed of fibroblasts, macrophages and neovasculature) replaces the fibrin clot. New blood vessels are formed during this stage, and fibroblasts proliferate and produce a provisional ECM by excreting collagen and fibronectin. Nearly all mammalian cells require adhesion to a surface in order to proliferate and function properly. The ECM fulfills this function. Initially, the provisional ECM contains of a network of Type III collagen, a weaker form of collagen that is rapidly produced. This is

later replaced by the stronger Type I collagen (which contributes to scar formation). At the same time, re-epithelialization of the epidermis occurs. During this process, epithelial cells proliferate and migrate over the newly forming tissue as proteases such as metalloportineaes (MMPs) and collagenases at the leading edge of the migrating cells help to invade the clot. These enzymes in addition to growth factor signaling (cell-cell interactions) and cell-ECM interactions (signal transduction from interactions between cells, integrins (cell surface receptors), laminin, collagen, fibronectin, and other ECM proteins) stimulate cell migration into the wound and ECM degradation.

[0006] Finally, in the remodeling phase, collagen is remodeled and realigned along tension lines and cells that are no longer needed are removed by apoptosis. Wound contraction occurs as fibroblasts transform into myofibroblasts through their interactions with ECM proteins and growth factors. Myofibroblasts then interact with collagen, vitronectin, and other ECM proteins to contract the wound. As the remodeling phase proceeds, fibronectin and hyaluronic acid are replaced by collagen bundles that lend strength to the tissue.

[0007] By applying biological, chemical and engineering principles to tissue repair and regeneration, tissue engineers have developed transplantable products for use in promoting the tissue repair and regeneration processes. The ability to restore biomechanical function of damaged tissue presents a true challenge. In response, both synthetic and biological scaffold products have been developed that mimic(to some extent) tissue structure and mechanical behavior to promote tissue repair. Such products serve as a temporary replacement, both mechanically and functionally, for damaged, diseased or absent tissue.

[0008] Ideally, transplantable scaffold products should support cell adhesion, proliferation and differentiation and act as an interim synthetic extracellular matrix (ECM) for cells prior to the formation of new tissue. Scaffold materials should be biocompatible, biodegradable and exhibit low antigenicity. The implant should degrade at a rate roughly equal to that of the new tissue formation. Once implanted, the scaffold must have the mechanical properties necessary to temporarily offer structural support until the new tissue has formed. Additionally, scaffold products must be porous, providing an appropriate path for nutrient transmission and tissue ingrowth. Tissue scaffolds also should promote fast healing and facilitate the development or regeneration of new tissue that resembles normal host tissue in both appearance and function. To this end, implanted scaffold products should offer (i)

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bioactive stimulation, e.g., protein and molecular signaling, to encourage cell migration, proliferation and differentiation, and (ii) mechanical or structural support for these processes.

[0009] Today, the development of synthetic scaffolds is an area of active research. Synthetic scaffolds have been manufactured from chemical compounds such as fibrous polymers, gelatin, apatite, and polymer/ceramic composites, polylactic acid, collagen. These scaffolds are designed to mimic the structure of the naturally occurring ECM and have shown some success in bone tissue engineering.

[0010] In addition to synthetic scaffold products, biological scaffolds obtained from mammalian tissues are commercially available for use as allografts (transplanted cell or tissue from a donor of the same species) or xenografts (transplanted cells or tissue from a donor of a different species). Biological scaffolds are composed of mammalian ECM harvested from, for example, dermis, urinary bladder, small intestine, mesothelium, pericardium, bone or aminiotic membrane of various mammals including human (either live donor or cadaver), porcine, bovine and equine. These commercially available products are commonly used for the repair and reconstruction of injured or missing tissues and organs such as soft tissue, tendons, cardiac tissue, neural tissue, chronic wounds, dura mater, bone and cornea.

[0011] Biological scaffold products may comprise skin cells in addition to extracellular matrices produced by tissue and subjected to a decellularization process. They are contacted with a wound site to give mechanical support for cell migration and proliferation as part of the wound healing process. In addition, factors such as growth factors or other proteins also may be provided that promote the wound healing process. The mechanical and material properties of biological scaffolds and the host tissue response to these biomaterials are believed to be influenced by the three dimensional configuration of the material and production processing methods. It further is believed that growth factors, surface topology and the distribution of surface ligands and modulation of the host innate immune response all contribute to the eventual functional performance of biological scaffolds for tissue repair or reconstruction. Tottey *et al.*, *Biomaterials* 32: 128-36 (2011).

[0012] In transplantation the use of human amniotic membrane (AM) has particular advantages, due to the structure of the relatively thick basement membrane, associated devitalized amniotic epithelial cells and stroma, and corresponding growth factor profile and structural protein composition. Meller *et al.*, *Dtsch Arztebl Int*'l 108: 243-8 (2011). For example, AM contains epidermal growth factor (EGF) and keratinocyte growth factor (KGF),

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which are important growth factors for promoting wound healing. In addition, laminin and type VII collagen present in the AM elicit an epitheliotropic effect. AM also is thought to reduce the expression of various growth factors and pro-inflammatory cytokines while releasing anti-inflammatory cytokines such as IL-10, IL-1 receptor antagonists, thus modulating the inflammatory response favorably for wound healing. AM is immunoprivileged, moreover, likely by virtue of low MHC I expression, and so rejection of AM tissue is uncommon. These characteristics make AM an ideal substrate, for instance, with respect to ocular surface reconstruction, in pelvic reconstruction, and in the treatment of ulcers, among other wound-healing applications.

[0013] The use of conventional tissue scaffold products is not without drawbacks, however. Tissue harvesting from human donors can produce undesirable consequences such as donor site morbidity or infection associated with removal of skin for donation. Disease transmission risk and intersample variation are additional drawbacks associated with biological scaffold products. In addition, it may be difficult to obtain sufficient tissue components necessary to cover large areas of damaged tissue. Furthermore, conventional biological and synthetic materials can be costly, not effective in many instances, and limited in availability.

[0014] Accordingly, an abiding exists need for suitable tissue substrate biomaterial for use in transplantation to promote tissue regeneration while restoring functionality. Both the research industry and the medical transplant community would benefit from such a product that is readily available, does not impose additional complications to a donor or recipient (such as requiring an additional surgery to harvest the substrate), and exhibits all or some of the inherent material functionality reflective of the physiochemical, electrochemical, and biochemical properties of natural tissue.

SUMMARY OF THE INVENTION

[0015] The biomaterial of the present invention is obtained from tissue of a urodele. "Urodele" here denotes a salamander of the order Urodela, also known as the order Caudata, in the class Amphibia. In terms of phylogeny the relevant families include *Ambystomatidae*, *Cryptobranchidae*, *Amphiumidae*, *Proteidae*, and *Sirenidae*. See Wiens *et al.*, *Syst. Biol.* 54: 91-110 (2005), the contents of which are incorporated here by reference in their entirety. Accordingly, the urodele category includes, for example, the Pacific Giant Salamander

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(*Andrias davidianus*), the Tiger Salamander (*Ambystoma tigrinum*), and the Mexican Axolotl (*Ambystoma mexicanum*). The present inventor has recognized that the skin and ECM of urodeles possess desirable characteristics analogous to those of AM, making them an ideal source of biomaterial for xenotransplantation.

Urodele ECM and tissue regeneration

[0016] As amphibians, most urodeles begin life as aquatic animals in a larval state and undergo metamorphosis from a juvenile form with gills to an adult, terrestrial, air-breathing form with lungs. During metamorphosis, a urodele's physical features are altered in preparation for life on land. These alterations include caudal fin resorbtion, thickening of the skin, the development of dermal glands and resorption of gills. Sexual maturity also occurs during this time in most urodeles. Some families of urodeles are "neotenic," which means that individuals with such families can exhibit juvenile features, such as gills and fins, even after reaching sexually maturity. Indeed, neotenic urodeles often retain their aquatic (juvenile) form for the duration of their lives. Thus, the Mexican Axolotl normally remains in the neotenic state throughout its adult life although, under certain circumstances, it can undergo metamorphosis and transform into a terrestrial form.

[0017] Axolotls also are known for their ability to regenerate amputated body parts, which typically results in the complete restoration of both the structure and function of the damaged limb or organ. Aquatic axolotls undergo rapid re-epithelialization during wound healing and limb regeneration, both of which are scar-less processes. Similarly, metamorphic terrestrial axolotls retain several larval skin features and also exhibit scar-free wound healing, albeit at a slower rate than their aquatic, pre-metamorphic counterpart.

[0018] The healing process in axolotls varies from that observed in adult mammals. The axolotl process more closely resembles the scar-free healing process of fetal and embryonic wounds. Thus, such wounds likewise exhibit re-epithelialization and basement membrane reformation that occur at a faster rate (is "enhanced") than do the corresponding events in postnatal mammals.

[0019] Moreover, the cutaneous and subcutaneous structures of an axolotl resemble that of the amniotic/chorionic interface, in the sense that axolotl skin is composed of fused ectoderm and mesoderm. Axolotl skin also is rich in growth factors and antimicrobial peptides, similar

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to the AM. Furthermore, axolotl ECM is immunoprivileged and contains collagen III and tissue inhibitors of metalloproteinases (TIMPs), *inter alia*, also in resemblance to AM.

[0020] Of particular importance for transplantation purposes, more generally, is the immunologically privileged state of the human neonate (*e.g.*, fetal dermis) and the AM, a state mirrored by axolotl ECM, whereby immogenicity is rarely manifested. By virtue of the reduced immune response and the generally decreased inflammatory response as compared to adult humans, neonatal and axolotl skin healing alike are not characterized by accelerated tissue resorption, as is observed in adult human wound healing. Rather, the growth factor profile, enzymatic activity, structural composition and immunomodulating effect of urodele and neonate tissues alike favor an appropriately staged removal of structural scaffolding and tissue growth into the resulting negative void space, in addition to enhanced re-epithelialization, during wound healing. This results in an optimal wound healing environment and process. Also, the high concentration of antimicrobial peptides present in AM and urodele tissue further contributes to the favorable environment and enhanced re-epithelialization observed during wound healing.

[0021] A key aspect of the present invention is the inventor's recognition that the growth factor profile, connective tissue matrix constituents, and immunoprivileged status of urodele ECM and accompanying cutaneous tissue, plus the presence of antimicrobial peptides therein, render urodele-derived tissue an ideal source for biological scaffolds for xenotransplantation.

[0022] In accordance with the invention, therefore, a biological scaffold biomaterial is provided that is the product of a process comprising (A) obtaining a tissue sample from a urodele, where the tissue comprises ECM, inclusive of the basement membrane, and (B) subjecting the tissue sample to a decellularization process that maintains the structural and functional integrity of the extracellular matrix, by virtue of retaining its fibrous and non-fibrous proteins, glycoaminoglycans (GAGs) and proteoglycans, while removing sufficient cellular components of the sample to reduce or eliminate antigenicity and immunogenicity for xenograft purposes. Also provided is methodology for using the urodele-derived biomaterial to enhance restoration of skin homeostasis, to reduce the severity, duration and associated damage caused by post-surgical inflammation, and to promote progression of natural healing and regeneration processes. In addition, biomaterial of the invention promotes the formation

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of remodeled tissue that is comparable in quality, function and compliance to undamaged human tissue.

Decellularization

[0023] The biomaterial of the invention is produced by decellularizing a tissue sample obtained from a urodele. The primary constituent of the resulting urodele biomaterial is ECM, possibly with devitalized epithelial cells, which can retain moisture and otherwise protect the wound-healing environment.

[0024] Urodele skin is one example of an appropriate starting material for the present invention. Thus, the starting material that is subjected to decellularization can comprise urodele dermis and basement membrane, with or without epidermis. Even upon decellularization, moreover, the biomaterial of the invention can comprise, with the ECM, adjacent epithelial cells that may be rendered non-viable by the process. Alternatively, non-cutaneous urodele tissues can serve as the starting material of the invention, particularly those comprising a basement membrane or epithelial tissues that form the lining of various body cavities, *i.e.*, parietal mesothelial tissues found, for example in the thoracic cavity, the abdominal cavity, and pericardium. Tissues that contain substantial amounts of fibrous connective tissue, such as cartilage, tendon, bone, dura mater and fascia, also are illustrative of appropriate starting materials of the present invention.

[0025] Effected via any conventional decellularization methodology, urodele tissue decellularization is performed to remove immunogenic cellular antigens that can induce an inflammatory response or immune-mediated tissue rejection, while preserving the structural integrity and composition of the associated ECM. Generally, ECM structural components, many if not all of which remain intact following decellularization, are well-tolerated by xenogeneic recipients. ECM components that may be present in the final biomaterial of the invention include proteins such as collagen (*e.g.*, fibrous collagen I and collagen III, as well non-fibrous collagen IV, collagen V and collagen VII), elastin, fibronectin, laminin, vitronectin, thrombosponsdins, osteopontin and tenascins, plus GAGs (*e.g.*, the proteoglycans, decoran and versican and sulfated GAGs, *e.g.*, heparin sulfate, keratan sulfate, dermatan sulfate and chondroitin sulfate) and growth factors such VEGF, BMP, TGF and FGF. For some indications the post-decellularization material comprises at least collagen IV, laminin,

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sulfated GAGs and one or more growth factors in amounts that approximate pre-decellularization levels when viewed via histological and immunohistological staining.

[0026] Suitable techniques for decellularizing tissues, pursuant to the invention, include physical methods such as freezing, direct pressure application, sonication, and agitation. In addition or in the alternative, chemical methods can be employed, such as alkaline and acid treatments, application of detergents (including amphoteric, cationic, anionic and non-ionic detergents), organic solvents, hypotonic or hypertonic solutions and chelating agents. Enzymatic approaches including protease digestion and treatment with one or more nucleases also may be used to decellularize urodele tissue. In addition or alternatively, the urodele tissue is subjected to cleaning, sterilization, disinfection, antibiotic treatment and/or viral inactivation.

[0027] According to one aspect of the invention, a biomaterial is provided. The material is produced by the process that includes (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft. In some embodiments, decellularizing comprises subjecting said tissue sample to an alkaline treatment. In embodiments, the process can further comprise subjecting said sample to sterilization. In embodiments, the process can further comprise devitalizing cells.

[0028] According to one aspect of the invention, a tissue graft is provided. The graft includes extracellular matrix components derived from a urodele. In embodiments, the extracellular matrix components are substantially free of components that induce an immune response when implanted as a xenograft. In embodiments, the extracellular matrix components are non-toxic.

[0029] According to one aspect of the invention, a decellularized Urodele ECM is provided. In any of the embodiments, the decellularized ECM can be derived from Axolotl tissue. In any of the embodiments, the decellularized ECM can include basement membrane. In any of the embodiments, the decellularized ECM can be infused with, coated with, combined with or attached, covalently or non-coalently, to an agent xenogenic to a Urodele. In any of the embodiments, the agent can be any one or any combination of a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, a drug and/or a human agent (i.e., an agent found in a human,

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isolated, synthetically or recombinantly produced). Further such agents forming part of the invention are described in more detail below. In some embodiments, the agent is a cell, optionally a human cell. In some embodiments, the agent is a progenitor cell, optionally a human progenitor cell. Further such cells forming part of the invention are described in more detail below. In any of the embodiments, the decellularized ECM can take on any variety of shapes, as the material can be formed, laminated, homogenized, gelled, etc. In some embodiments, the ECM is a sheet. The sheet optionally can include perforations. The sheet optionally can include a backing and/or an adhesive. The backing may be biodegradable or may be non-biodegradable. In some embodiments, the decelularized ECM is a dry powder. In some embodiments the decelularized ECM is a reconstituted gel. In any of the embodiments, the ECM can be sterile.

[0030] According to one aspect of the invention, a package is provided. The package contains sterile, decellularized Urodele ECM or a Urodele fraction derived from the sterile, decellularized Urodele ECM. The package can contain any of the ECMs described above. For example, the package can include a sheet, a dry powder or a reconstituted gel of decellularized Urodele ECM. The package can contain any product, for example any implant, that comprises sterile, decellularized Urodele ECM or a Urodele fraction derived from the sterile, decellularized Urodele ECM. Such an implant may be made in whole or only in minor part of the ECM of the invention.

[0031] The invention also provides a sterile medical implant comprising decellularized Urodele ECM or a Urodele fraction derived from the decellularized Urodele ECM. Examples of such medical implants include a biocompatible sheet, mesh, gel, graft, plug, tissue or device. Devices include, for example, coated stents, bone replacements, joint replacements, implantable hardware and the like. The implant can be fabricated entirely or in part from the ECM. The implant also can encapsulate, can be infused with, coated with, impregnated with, laminated with, or covalently or non-covalently attached to the ECM of the invention.

[0032] The invention also provides a material, the material being coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM or a Urodele fraction derived from the isolated, decellularized Urodele ECM. The material can be natural or synthetic. Examples are metals, plastics, ceramics and fibers.

[0033] According to one aspect of the invention, a tissue culture system is provided. The system comprises (a) an isolated Urodele decellularized ECM, (b) tissue culture medium, and

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(c) cells xenogenic to the Urodele. The cells may be from animal, and in some embodiments, the cell is a mammalian cell. The cell can be any type of cell capable of culture. In embodiments, the cell is a human cell, optionally a progenitor cell.

[0034] According to one aspect of the invention, a conditioned tissue culture medium is provided. The medium, which can be any commonly used in liquid tissue culture, is conditioned with isolated Urodele decellularized ECM or a Urodele fraction derived from isolated, decellularized Urodele ECM. Numerous liquid tissue culture media are commercially available and well known to those of ordinary skill in the art.

[0035] According to one aspect of the invention, a device is provided. The device is at least two sheets of isolated Urodele decellularized ECM laminated to one another. The sheets can be from the same or different tissue. The sheets can an orientation and can be oriented in the same direction or oriented at angles to one another. The sheets can further comprise any agent xenogenic to the Urodele, which agent may be coated on, infused or impregnated within, or otherwise attached to one or more of the laminated sheets.

[0036] In any embodiment described above involving a sheet, the sheet may further comprise a backing and/or adhesive.

[0037] According to one aspect of the invention, a product is provided. The product is prepared by isolating Urodele ECM from a Urodele, decellularizing the ECM, and sterilizing the decellularized ECM. The preparation of the device can further involve any one or more of the following steps (presented in no particular order): forming the ECM into a shape, homogenizing the ECM, laminating the ECM to a material, combining agents with the ECM such as by coating, impregnating, or otherwise attaching the agent to the ECM, and so on.

[0038] According to one aspect of the invention, a method of preparing a biologic material is provided. The method involves (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample to remove sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft. In embodiments, the method can further involve performing the decellularization in a manner to retain structural and functional integrity of the ECM sufficient to permit the ECM to be useful as a matrix upon and within which cells can grow. In any of the embodiments, the method can further involve homogenizing the ECM to form a particulate or powder. In some embodiments, the method can further involve reconstituting the powder as a gel. In any of the embodiments, the method can further involve sterilizing the

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ECM. In any of the embodiments, the method can further involve attaching the ECM to an agent xenogenic to a Urodele.

[0039] According to another aspect of the invention, the materials such as implants, devices, sheets, gels and powders can be used in methods for treating subjects, where the materials are applied to wounds, surgical beds, and to internal and external tissues, generally, to prevent adhesion, provide tissue support, for example for suturing tissue, for treating a hernia or as a tissue plug, for treating burns and derm-abrasion, as well as other conditions described below.

[0040] In any of the embodiments above, the ECM is or can be isolated.

BRIEF DESCRIPTION OF THE FIGURES

[0041] FIGURE 1 shows native axolotl tissue samples prepared for histological examination, to identify EC elements.

[0042] FIGURE 2 hematoxylin and eosin (H&E) and Alcian Blue staining of native axolotl dermal tissue and human amniotic membrane (40x magnification).

[0043] FIGURE 3 shows immunohistocemical staining via species-specific collagen IV and laminin antibodies of native axolotl dermal tissue and human amniotic membrane tissue, at 40× magnification.

[0044] FIGURE 4 depicts axolotl skin and human amniotic membrane samples prepared for histological examination, to identify EC elements.

[0045] FIGURE 5 illustrates a histological evaluation of H&E-stained, paired native and post-processed sections of axolotl dermal tissue.

[0046] FIGURE 6 presents ELISA data for DNA content in pre- and post-processed axotol tissue, with comparison data from human amniotic membrane tissue.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

[0047] "Antimicrobial polypeptides" (or "AMPs") means small peptides of variable length, sequence and structure with broad spectrum activity against a wide range of microorganisms including bacteria, viruses, fungi, protozoa, parasites, prions, and tumor/cancer cells. (See, e.g., Zaiou, J Mol Med, 2007; 85:317; herein incorporated by reference in its entirety). AMPs have broad-spectrum of rapid onset of killing activities, with potentially low levels of induced resistance and concomitant broad anti-inflammatory effects.

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Anti-microbial polypeptides include defensins, such as a-defensins (e.g., neutrophil defensin 1, defensin alpha 1, neutrophil defensin 3, neutrophil defensin 4, defensin 5, defensin 6), β-defensins (e.g., beta-defensin 1, beta-defensin 2, beta-defensin 103, beta-defensin 107, beta-defensin 110, beta-defensin 136), and θ-defensins. Anti-microbial polypeptides include cathelicidins such as hCAP18.

[0048] "Biocompatible" means that a composition and its normal degradation products *in vivo* are substantially non-toxic and non-carcinogenic in a subject within useful, practical and/or acceptable tolerances.

[0049] "Cytocompatible" means that a composition can sustain the viability and growth of a population of cells.

[0050] "Decellularized ECM" means extra cellular matrix sufficiently free of cellular components to eliminate or reduce antigenicity of the extra cellular matrix to an extent where the matrix would be considered non-toxic as a xenograft.

[0051] "Isolated" when used in connection with the ECM of the invention means separated from other Urodele tissue.

[0052] "Non-toxic" means that a composition, when implanted in a subject, causes little or no adverse reaction or substantial harm to cells and tissues in the body, and does not cause a substantial adverse reaction or substantial harm to cells and tissues in the body, for instance, the composition does not cause necrosis, an infection, or a substantial immune response resulting in harm to tissues from the implanted or applied composition.

[0053] "Progenitor cell" means a cell that can differentiate under certain conditions into a more-differentiated cell type. Non-limiting examples of progenitor cells include stem cells that may be totipotent, pluripotent, multipotent stem cells, or referred to as progenitor cells. Additional non-limiting examples of progenitor cells include perivascular stem cells, blastema cells, and multilineage progenitor cells.

[0054] "Retain structural and functional integrity" used in connection with the ECM of the invention means retaining sufficient structure and function to permit and support the use of the matrix as a substrate for the growth of cells *in vivo* or *in vitro*.

[0055] "Subject" means an animal. In some embodiments the animal is a mammal. The mammal can be a dog, cat, a horse, a cow, a goat, a sheep, a pig or a non-human primate. In any embodiment the mammal can be a human.

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[0056] "Treatment" or "treating" means administration or application to a subject by any suitable dosage means, regimen and route of a composition with the object of achieving a desirable clinical/medical end-point, such as assisting in wound healing, tissue closure, bulking tissue, preventing tissue adhesion, providing structural support to tissue, providing a protective barrier, correcting a defect, etc.

[0057] "Urodele fraction derived from decellularized Urodele ECM" means an extract or isolate of decellularized Urodele ECM maintaining sufficient characteristics of a Urodele in terms of chemical structure and/or relative chemical concentrations of two (or three, or four, or five or more) chemical entities in the extract or isolate to distinguish the extract as obtained from a Urodele by any one or more of electron microscopy, HPLC, immunohistochemistry, and the like.

General Preparative Methodology

[0058] According to the invention, urodele tissue samples obtained for decellularization can be treated in the manner detailed in US 2008/0046095 or US 2010/0104539. Thus, tissue samples may be subjected to cleaning and chemical decontamination. In this manner, a tissue sample is washed for approximately 10 to 30 minutes in a sterile basin containing 18% NaCl (hyperisotonic saline) solution that is at or near room temperature. Visible cellular debris, such as epithelial cells adjacent to the tissue basement membrane, is gently scrubbed away using a sterile sponge to expose the basement membrane. Using a blunt instrument, a cell scraper or sterile gauze, any residual debris or contamination also is removed. Other techniques including, but not limited to, freezing the membrane, physical removal using a cell scraper, or exposing the cells to nonionic detergents, anionic detergents, and nucleases also may be used to remove cells.

[0059] In one embodiment, urodele tissue is decellularized using alkaline treatment.

[0060] The tissue is placed into a sterile container, such as a Nalgene jar, for the next step of chemical decontamination. Thus, each container is aseptically filled with 18% saline solution and sealed (or closed with a top). The containers then are placed on a rocker platform and agitated for between 30 and 90 minutes, which further cleans the tissue of contaminants.

[0061] In a sterile environment using sterile forceps, the tissue is gently removed from the container containing the 18% hyperisotonic saline solution and placed into an empty container. This empty container with the tissue is then aseptically filled with a pre-mixed

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antibiotic solution. Preferably, the premixed antibiotic solution is comprised of a cocktail of antibiotics, such as Streptomycin Sulfate and Gentamicin Sulfate. Other antibiotics, such as Polymixin B Sulfate and Bacitracin, or similar antibiotics available now or in the future, are suitable as well. It is preferred that the antibiotic solution be at room temperature when added so that it does not change the temperature of or otherwise damage the tissue. This container containing the tissue and antibiotics is then sealed or closed and placed on a rocker platform and agitated for, preferably, between 60 and 90 minutes. Such rocking or agitation of the tissue within the antibiotic solution further cleans the tissue of contaminants and bacteria.

[0062] In a sterile environment, the container is opened and, using sterile forceps, the tissue is gently removed and placed in a sterile basin containing sterile water or normal saline (0.9% saline solution). The tissue is allowed to soak in place in the sterile water/normal saline solution for at least 10 to 15 minutes. The tissue may be slightly agitated to facilitate removal of the antibiotic solution and any other contaminants from the tissue.

[0063] In some cases, the present invention involves treating urodele tissue using a chemical sterilization methodology, as illustrated the Tutaplast® and Allowash® procedures, optionally in combination with mechanical processes that gently agitate chemical agents, as in the BioCleanse® system. Thus, urodele tissue is subjected to oxidative and/or alkaline treatments as well as osmotic treatment to break down cell walls, to inactivate pathogens, and to remove bacteria. In addition, tissue may be subjected to delipidization, solvent dehydration (to permit room temperature storage of tissue without damaging the collagen structure) and/or low-dose gamma irradiation to ensure sterility of the final product.

[0064] Efficient cell removal upon decellularization can be verified by various known means, including histological analyses to detect nuclear and cytoplasmic structures, immunohistochemical or immunofluorescent assaying for indicative intracellular proteins, and DNA detection. The nature of desirable components in the final urodele-derived scaffold biomaterial varies depending on the clinical indication being treated. Once a particular indication is identified, the knowledgeable clinician can determine which components in the urodele tissue sample should be retained in the final scaffold product, and standard methodology can be employed to ensure that the desired components are present following decellularization.

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[0065] Samples may be viewed histologically before, during, and/or after decellularization to monitor the process and to confirm that the desired degree of cellular component removal is reached. For instance, tissues can be analyzed for cytoskeletal content to gauge sufficient decellularization. Intracellular protein content also may be assayed to determine if decellularization is sufficient. In addition, the tissue sample thickness and chemical makeup may be monitored to determine when sufficient decellularization has been achieved. Periodic monitoring during processing allows for a real time response to the observed tissue properties.

[0066] In some cases, a sufficiently decellularized tissue comprises no more than 50 ng dsDNA per mg ECM dry weight. Alternatively, for some indications, a sufficiently decellularized tissue lacks visible nuclear material in a tissue section stained with 4',6-diamindino-2-phenylindole (DAPI) or haematoxyilin and eosin (H&E).

[0067] In scenarios where removal of an adjacent epithelial cell layer is required, the presence or absence of epithelial cells remaining in the sample can be evaluated using techniques known in the art. For example, after removal of the epithelial cell layer, a representative tissue sample from the processing lot is placed onto a standard microscope examination slide. The tissue sample is then stained using Eosin Y Stain and evaluated as described below. The sample is then covered and allowed to stand. Once an adequate amount of time has passed to allow for staining, visual observation is done under magnification. The presence of cells and cellular material will appear darker than the areas which have been de-epithelialized.

[0068] Once cellular removal has progressed sufficiently, conventional methods are employed to confirm the retention of desired structural and functional properties of the remaining ECM scaffold. The specific structural testing that should be conducted depends on the intended clinical application of the final scaffold product. In some cases, the urodele tissue starting material may be monitored before, during, and after decellularization to ensure that the desired structural components and configuration are maintained in the final product.

[0069] One method for determining whether the desired ECM components are present involves staining parallel tissue sections and examining them histologically to determine whether the desired constituents and structural orientation of the urodele tissue have been preserved. For instance, urodele tissue can be stained with H&E and immunoperoxidase stain for laminin to assess preservation of ECM and laminin. In general, the three-

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dimensional configuration of ECM components remaining in the final biomaterial scaffold product should approximate that of pre-decellularized material when viewed via histological staining. Another component one can assay for is AMPs, as the ECM of the invention is rich in AMPs.

[0070] Accordingly, the urodele-derived biomaterial of the invention comprises ECM components useful for directing enhanced re-epithelialization and promoting efficient tissue regeneration or wound healing. The inventive biomaterial also serves as a matrix and reservoir for bioactive peptides such as growth factors, adhesion proteins and AMPs. Accordingly, the biomaterial functions effectively as a biological scaffold for tissue regeneration, providing both the necessary bioactive stimulation and structural support. The product can be used as is, cut into smaller pieces or shapes, laminated to itself or other materials, pre-punctured to provide openings for securing attachments, formed into desired three dimensional shapes, as well as other formats, discussed in more detail below.

Powders and Gels

[0071] In embodiments, the scaffold can be further processed into small grains or a powder. The fine particles can be hydrated in water, saline or a suitable buffer or medium to produce a paste or gel. This fine material, paste or gel produced from it may be used for a multitude of purposes, described in greater detail below.

[0072] Although numerous methods exist, two exemplary methods may be used to produce a particulate form of the scaffold. The first method involved lyophilizing the disinfected material and then immersing the sample in liquid nitrogen. The snap frozen material is then reduced to small pieces with a blender so that the particles are small enough to be placed in a rotary knife mill, such as a Wiley mill. A #60 screen can be used to restrict the collected powder size to a desired size, for example less than 250 mm. A Sonic sifter or other classification device can be used to remove larger particles and/or to obtain a particle size distribution within a desired range. A second method is similar to the previous method except the sample is first soaked in a 30% (w/v) NaCl solution for 5 min. The material is then snap frozen in liquid nitrogen to precipitate salt crystals, and lyophilized to remove residual water. This material is then comminuted as described in above. By precipitating NaCl within the sample, it is expected that the embedded salt crystals would cause the material to fracture into more uniformly sized particles. The particles are then suspended in deionized water and centrifuged for 5 min at 1000 rpm three times to remove the NaCl. The suspension is snap

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frozen and lyophilized again. Finally, the powder is placed in a rotary knife mill to disaggregate the individual particles.

[0073] The powder can be hydrated to create a gel, with or without other gelling materials to supplement gelling.

[0074] The powder, paste or gel can be applied without further processing to treat a subject. It can be sprayed, painted, injected or otherwise applied to a wound or surgical site. The gel can be shaped. The powder, paste or gel also can be placed inside a "bag", such as a polymeric synthetic material or a ECM sheet as described herein to produce a larger three-dimensional structure, such as an orthopedic implant for cartilage repair (e.g., knee or TMJ cartilage repair) or an implant for breast reconstruction or augmentation. In such a case, a bag of a desirable size and shape is formed from sheets of ECM material or other biocompatible polymeric material, and then the bag or cover can be filled with the tissue-derived powder or gel described herein. The shape of the device or implant can vary with its intended use. The bag may be molded into a useful shape by any useful molding technique, such as the shape of cartilage for the ear, nose, knee, TMJ, rib, etc., prior to filling the molded bag with the scaffold material described herein. In one example, a biodegradeable polymeric matrix (e.g., PEUU or PEEUU) is sprayed or electrodeposited onto a mold. The resultant molded cover can then be filled with the material. Heat, for example, may be used to seal the cover.

Additives

[0075] In another embodiment, at least one agent xenogenic to a Uroldele is added to the ECM or Urodele fraction thereof before it is implanted in the subject, otherwise administered to the subject or used in cell culture. Generally, the agents include any agent useful in cell culture or as a therapeutic or therapeutic adjuvant. The agents can be coated on, infused into or otherwise covalently or non-covalently attached to or incorporated onto or into the ECM of the invention. The agents also can be otherwise combined with a product that contains the ECM, for example, as by mixing powders of the agent and ECM together. Each agent may be used alone with the ECM of the invention or in combination with other agents. Non-limiting examples of such agents include antimicrobial agents, growth factors, cytokines, chemokines , emollients, retinoids, steroids, and cells, including but not limited to the subject's own cells.

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[0076] In certain non-limiting embodiments, the agent is a growth factor. Non-limiting examples of growth factors include basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factors 1 and 2 (IGF-1 and IGF-2), platelet derived growth factor (PDGF), stromal derived factor 1 alpha (SDF-1 alpha), nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), neurotrophin-3, neurotrophin-4, neurotrophin-5, pleiotrophin protein (neurite growth-promoting factor 1), midkine protein (neurite growth-promoting factor 2), brain-derived neurotrophic factor (BDNF), tumor angiogenesis factor (TAF),corticotrophin releasing factor (CRF), transforming growth factors .alpha. and .beta. (TGF-.alpha. and TGF-.beta.), interleukin-8 (IL-8), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukins, and interferons. Commercial preparations of various growth factors, including neurotrophic and angiogenic factors, are available from R & D Systems, Minneapolis, Minn.; Biovision, Inc, Mountain View, Calif.; ProSpec-Tany TechnoGene Ltd., Rehovot, Israel; and Cell Sciences.RTM., Canton, Mass.

[0077] In certain non-limiting embodiments, the therapeutic agent is an antimicrobial agent, such as, without limitation, an anti-microbial peptide, isoniazid, ethambutol, pyrazinamide, streptomycin, clofazimine, rifabutin, fluoroquinolones, ofloxacin, sparfloxacin, rifampin, azithromycin, clarithromycin, dapsone, tetracycline, erythromycin, ciprofloxacin, doxycycline, ampicillin, amphotericin B, ketoconazole, fluconazole, pyrimethamine, sulfadiazine, clindamycin, lincomycin, pentamidine, atovaquone, paromomycin, diclazaril, acyclovir, trifluorouridine, foscarnet, penicillin, gentamicin, ganciclovir, iatroconazole, miconazole, Zn-pyrithione, and silver salts such as chloride, bromide, iodide and periodate.

[0078] In certain non-limiting embodiments, the therapeutic agent is an anti-inflammatory agent, such as, without limitation, an NSAID, such as salicylic acid, indomethacin, sodium indomethacin trihydrate, salicylamide, naproxen, colchicine, fenoprofen, sulindac, diflunisal, diclofenac, indoprofen, sodium salicylamide; an anti-inflammatory cytokine; an anti-inflammatory protein; a steroidal anti-inflammatory agent; or an anti-clotting agents, such as heparin.

[0079] Other drugs that may promote wound healing and/or tissue regeneration may also be included.

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[0080] The agent may be dispersed within the scaffold by any useful method, e.g., by adsorption and/or absorption. For example, the therapeutic agent may be dissolved in a solvent (e.g., DMSO) and added to the scaffolding. In another embodiment, the agent is mixed with a carrier polymer (e.g., polylactic-glycolic acid microparticles, agarose, a poly(ester urethane) urea elastomer (PEUU) or a poly(ether ester urethane) urea elastomer (PEEUU)), which is subsequently dispersed within or applied to the scaffold. By blending the agent with a carrier polymer or elastomeric polymer, the rate of release of the therapeutic agent may be controlled by the rate of polymer degradation and/or by release from the polymer by diffusion or otherwise. Likewise, a therapeutic agent may be provided in any dissolvable matrix for extended release, as are known in the pharmaceutical arts, including sugar or polysaccharide matrices. The agent also may be included with the powdered ECM and gelled with the powdered ECM. The agent may be covalently attached to the ECM of the invention. The foregoing are meant to be non-limiting examples.

Extracts

[0081] In addition to the decellularized ECM in its native state or ground as a particulate or powder, the invention also provides extracts and isolates of the same. As mentioned above, the Urodele ECM is loaded with antimicrobial peptides, growth promoting factors, collagen and laminins, and Urodele fractions of the ECM are useful according to the invention.

[0082] Extraction buffers are well known in the art. One such buffer is 4 M guanidine and 2 M urea each prepared in 50 mM Tris-HCl, pH 7.4. The powder form of the ECM can be suspended in the relevant extraction buffer (e.g., 25% w/v) containing phenylmethyl sulphonyl fluoride, N-ethylmaleimide, and benzamidine (protease inhibitors) each at 1 mM and vigorously stirred for 24 hours at 4° C. The extraction mixture can then be centrifuged and the supernatant collected. The insoluble material can be washed in the extraction buffer, centrifuged, and the wash combined with the original supernatant. The supernatant can be dialyzed against deionized water. The dialysate can then be centrifuged to remove any insoluble material and the supernatant used immediately or lyophilized for long term storage. Such an isolate will contain growth factors in concentrations specific to Urodeles.

[0083] In another aspect, the extraction is done by conditioning medium. A method of making Urodele tissue-specific extract by taking the powdered ECM, forming a solution thereby generating a supernatant and a particulate, wherein the supernatant is an extract and

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isolating the extract from the particulate. One also could grow cells on the ECM, and isolate the supernatant after a period of time of cell growth.

Synthetic Materials

[0084] Synthetic biocompatible and cyto-compatable material can be combined with the ECM, such as, for example, (a) a structural support for a sheet or a gel of the ECM, (b) a structural support for shaping the ECM, (c) a coating for the ECM (or a coating containing the particulate ECM), a supplemental gelling agent, or (d) a sustained release material for the particulate ECM or an isolate thereof. Such polymers have been known to be applied to other ECM materials as a backing sheet, including materials that are themselves biodegradable. Suitable synthetic material for a matrix can be biocompatible to preclude migration and immunological complications, and can be able to support cell growth and differentiated cell function. Some are resorbable, allowing for a completely natural tissue replacement. Some can be configurable into a variety of shapes and have sufficient strength to prevent collapse upon implantation. Studies indicate that the biodegradable polyester polymers made of polyglycolic acid fulfill all of these criteria (Vacanti, et al. J. Ped. Surg. 23:3-9 (1988); Cima, et al. Biotechnol. Bioeng. 38:145 (1991); Vacanti, et al. Plast. Reconstr. Surg. 88:753-9 (1991)). Other synthetic biodegradable support matrices include synthetic polymers such as polyanhydrides, polyorthoesters, and polylactic acid. Further examples of synthetic polymers and methods of incorporating or embedding cells into these matrices are also known in the art. See e.g., U.S. Pat. Nos. 4,298,002 and 5,308,7

[0085] As a non-limiting example, the powder may be formulated with tri-block co-polymers. See international pubished application WO2012131104 and WO2012131106, each of which is incorporated herein by reference in its entirety. Other examples include poloxamers, which are nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide)). Poloxamers are also known by the trade name Pluronics (BASF). Certain poloxamers are useful as sustained release materials for pharmaceuticals.

[0086] Particles of the invention also may be encapsulated into a polymer, hydrogel and/or surgical sealant. As a non-limiting example, the polymer, hydrogel or surgical sealant may be PLGA, ethylene vinyl acetate (EVAc), poloxamer, GELSITE® (Nanotherapeutics, Inc. Alachua, Fla.), HYLENEX® (Halozyme Therapeutics, San Diego Calif.), surgical sealants

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such as fibrinogen polymers (Ethicon Inc. Cornelia, Ga.), TISSELL® (Baxter International, Inc Deerfield, Ill.), PEG-based sealants, and COSEAL® (Baxter International, Inc Deerfield, Ill.). In another embodiment, the particle may be encapsulated into any polymer known in the art which may form a gel when injected into a subject. As another non-limiting example, the particle may be encapsulated into a polymer matrix which may be biodegradable. Additional examples of polymers for controlled release and/or targeted delivery may also include at least one controlled release coating. Controlled release coatings include, but are not limited to, OPADRY®, polyvinylpyrrolidone/vinyl acetate copolymer, polyvinylpyrrolidone, hydroxypropyl methylcellulose, hydroxypropyl cellulose, hydroxyethyl cellulose, EUDRAGIT RL®, EUDRAGIT RS® and cellulose derivatives such as ethylcellulose aqueous dispersions (AQUACOAT® and SURELEASE®.

Uses

[0087] The decellularized ECMs described herein are useful for growing cells, tissues, organs in virtually any *in vivo*, *ex vivo*, or *in vitro* use. The ECMs can be used as a substrate to facilitate the growth and/or differentiation of cells. In vitro, the ECMs are useful as a cell growth substrate to support the growth in culture of cells, including virtually any type of cells or cell-lines, including stem cells, progenitor cells or differentiated cells. In one embodiment, the cells are cancer cells. In one embodiment, the cancer cells form nodules when grown on the ECMs. Cells on the substrate also may be grown into tissue, organ or body part precursors, or even mature tissues or structures. Cells grown on ECMs may be used for implantation, for wound dressings, for *in vitro* drug testing, for modeling differentiation, etc. The cells may be matched in tissue cell type to the ECM or unmatched. The cells are xenogenic.

[0088] The ECM of the invention is useful *in vivo* as a cell growth scaffold for tissue growth for any useful purpose, including repair, replacement or augmentation of tissue in a subject in either humans or animals. For example, the materials are useful in repair and/or replacement of tissue lost or damaged during trauma or surgery, for example in loss of tissue after tumor removal. The materials are useful for structural repair, such as inguinal hernia repair, parastomal reinforcement, soft tissue reinforcement, surgical staple-line reinforcement during, for example, bariatric surgery or lung resection, umbilical hernia grafts, Peyronie's repair grafts, incision grafts and fistula plugs. The materials are useful for wound dressings, such as for burns, graft and split-thickness graft coverings, ulcers including decubitis ulcers

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and dermal abrasion procedures. The materials are useful for cosmetic purposes, for example in breast, lip or buttock augmentation. An aspect of the invention particularly appealing for anti-adhesion surgical uses is the properties of the basement membrane, which inhibit or prevent adhesion. The presence of the AMPs make the ECM of the invention particularly well suited for the foregoing applications.

[0089] As mentioned above, the materials described herein can be molded or contained within a structure to form desired shapes, such as, for cartilage repair or replacement by seeding the material with, e.g., chondrocytes and/or chondroprogenitor cells. The materials can be ground into a powder and used to reconstitute and/or form gels, as cell culture additives, as a powder, spray, liquid, suspension or coating for application to (a) a wound, (b) an implant, (c) a wound dressing, etc.

[0090] In one embodiment, for example, adipose stem cells are propagated in the cell growth scaffolds described herein. Adipose stem cells are of mesodermal origin. They typically are pluripotent, and have the capacity to develop into mesodermal tissues, such as: mature adipose tissue; bone; heart, including, without limitation, pericardium, epicardium, epimyocardium, myocardium, pericardium, and valve tissue; dermal connective tissue; hemangial tissues; muscle tissues; urogenital tissues; pleural and peritoneal tissues; viscera; mesodermal glandular tissues; and stromal tissues. The cells not only can differentiate into mature (fully differentiated) cells, they also can differentiate into an appropriate precursor cell (for example and without limitation, preadipocytes, premyocytes, preosteocytes). Also, depending on the culture conditions, the cells can also exhibit developmental phenotypes such as embryonic, fetal, hematopoetic, neurogenic, or neuralgiagenic developmental phenotypes.

[0091] In one embodiment, a subject's own cells are dispersed within the matrix. For example, in the production of cartilaginous tissue, chondrocytes and/or chondroprogenitor cells can be dispersed within the matrix and optionally grown ex vivo prior to implantation. Likewise, skin cells of a subject can be dispersed within the scaffolding prior to implantation on a damaged skin surface of a subject, such as a burn or abrasion.

[0092] When used as a gel, a non-limiting example is injecting the gel into a subject at a desirable site, such as in a wound. In one instance, the gel can be injected in a bone breakage or in a hole drilled in bone to facilitate repair and/or adhesion of structures, such as replacement ligaments, to the bone. In another use, finely comminuted particles can be

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sprayed onto a surface of a subject, such as in the case of large surface abrasions or burns. The scaffold can also be sprayed onto skin sutures to inhibit scarring. The ECM of the invention can be place or sutured in place inside the body at a surgical site such as mentioned above. All of these treatments are embraced by the present invention.

[0093] Urodele decellularized ECM can be used also for sustained delivery of therapeutic molecules, proteins or metabolites, to a site in a host. See, for example, U.S. 2004/0181240, which describes an amniotic membrane covering for a tissue surface which may prevent adhesions, exclude bacteria or inhibit bacterial activity, or to promote healing or growth of tissue, and U.S. patent No. 4,361,552, which pertains to the preparation of cross-linked amnion membranes and their use in methods for treating burns and wounds. The ECMs of the invention can be used in the same manner.

Pharmaceutical Formulations

[0094] Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to any other animal, e.g., to non-human animals, e.g. non-human mammals. Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and/or perform such modification with merely ordinary, if any, experimentation.

[0095] The pharmaceutical compositions described herein may be prepared by any method known in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient into association with an excipient and/or one or more other accessory ingredients, and then, if necessary and/or desirable, dividing, shaping and/or packaging the product into a desired single- or multi-use configuration.

[0096] The ECM in accordance with the invention may be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. For example, the composition may comprise between 0.1% and 100% (w/w) of the ECM. When other active agents are included, relative amounts of agents combined with the ECM of the invention will be known to those of ordinary skill in the art, similar to those amounts used in combination with ECM as formulated in the prior art. Relative amounts also may vary, depending upon

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the identity, size, and/or condition of the subject being treated and further depending upon the route by which the ECM is to be administered.

[0097] Pharmaceutical formulations may additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes, but is not limited to, any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, and the like, as suited to the particular dosage form desired. Various excipients for formulating pharmaceutical compositions and techniques for preparing the composition are known in the art. See Remington: THE SCIENCE AND PRACTICE OF PHARMACY (21 st Ed.), A. R. Gennaro, Lippincott, Williams & Wilkins (Baltimore, Md., 2006); incorporated herein by reference in its entirety.

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EXAMPLES

Example 1. Processing axolotl dermis

Axolotl dermis samples can be decellularized by preparing excised samples from healthy or healing axolotl dermal tissue and then subjecting the samples to hypo/hyperosmotic soaks for cell lysis, solvent dehydration, and oven drying. Specific processing of these grafts includes storage in 15- 26% NaCl, multiple hypo/hyperosmotic soaks (utilizing NaCl solutions and water) , and then solvent dehydration using ethanol, and then evaporation of the solvent either with air drying or oven drying at 37° C.

Histological examination of native axolotl dermal tissue was performed to identify the presence of the notable ECM elements, such as the basement membrane. See FIG 1 and FIG. 4. Comparative histological and immunohistochemical analysis of native axolotl dermal tissue and human amniotic membrane was performed to compare the ECM structure and constituents, and to assess relative concentration and distribution of critical constituents. See FIG. 2, FIG. 3, and FIG. 4. Figure 3 shows immunohistocemical staining via species-specific collagen IV and laminin antibodies of native axolotl dermal tissue and human amniotic membrane tissue, at 40× magnification. Figure 2 shows H&E and Alcian Blue staining (40x) of native axolotl dermal tissue and human amniotic membrane, and it demonstrates the comparable histoarchitecture and presence of sulfated glycosaminoglycans in both tissues. Histological evaluation with hematoxylin and eosin-stained, paired native and post-processed sections of axolotl dermal tissue (see FIG. 5)showed post-process preservation of the extracellular matrix histoarchitecture and the absence of cells or any significant concentration of cellular debris.

Example 2. Splitting and lamination of acellular dehydrated axolotl dermis.

Decellularized dehydrated axolotl dermis can be split, via a mechanical splitter, to isolate heterogeneous matrix into homogenous sections. Isolated sections of desired thickness then can be rehydrated and lyophilized to obtain multilayered laminate structures of desired orientation with facial surface features. More specifically, dual-sided basement membrane structure, with interior open porous matrix obtained from the reticular dermis region of the dermal matrix, can be constructed to obtain desired facial surface properties. Alternatively, isolated native section can be used in native form for desired clinical outcome.

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For example, open porous homogenous matrix of the reticular dermis can be used to obtain augmentation of soft tissue structures.

A laminated custom construct with sulfated gags on both facial surface and collagen IV and laminin could be obtained for desirable dual-surface, anti-adhesion and antimicrobial properties for clinical benefit. In addition, multilayer structures could be constructed to prolong in vivo durability of the graft.

Example 3 Preparation of solubilized acellular dehydrated axolotl dermis, pericardium, fascia lata, periosteum, peritoneum, or dura mater

Decellularized dehydrated axolotl native or isolated section of acellular urodele connectivue tissye matrix can be prepared by sectioning decellularized soft tissue structures into 1 cm^2 sections and homogenizing the sections in a Warring blender (~100 grams of tissue) in aqueous 1M glacier acetic acid for 30-60seconds. Preparation of sponge can be obtained by the addition of varying volumes of water followed then neutralization and lypoholization of the slurry in a mold of desired geometric shape. The resultant porosity will correlate to the volume of water added to the matrix. Additionally, a selected range of bioactive extracts can be added to the slurry prior to neutralization, including particulated or small protein constituents extracted from digested human or urodele mineralized and nonmineralized connective tissues, such as demineralized bone matrix, elastin, or bone morphogenic proteins, which can be covalently loaded into constructs. Extracts will be covalently bound with collagen fibers after neutralization and return to physiological condition where fibrillogenisis will occur. Subsequent release of bioactive constituents will occur during proteolyctic degradation in vivo and ensure molecules are not consume or exposed during acute inflammation in vivo. Alternatively, aqueous NaCl can be added to the slurry, prior to neutralization, to obtain a sustained, low viscosity solution for injection, which is stable at room temperature. Injection of slurry through ion-selective membrane will remove salt ions and permit for fibrillogenisis to occur post injection and formation of three-dimensional matrix.

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Example 4 Preparation of sterilized particulated or powder form of mineralized and non-mineralized decellularized and dehydrated urodele connective tissue matrix

Following decellularization of sections of mineralized collagen urodele connective tissue, one can perform a demineralization process, similar to that employed by Urist, and solvent or lyophilization dehydration, cryomilling of sectioned acellular demineralized, mineralized, or non-mineralized urodele connective tissue extracellular matrix, thereby to obtain particulated or powder form of the ECM with preserved histoarhictiecutre and function. The final particle size distribution can be varied depending on duration and sieving, post-cyromilling, between 125 and 850 microns. Low-dose cold gamma irradiation or e-beam irradiation (<25 Kgy) can be employed to sterilize acellular ECM sheets, particulate or powder and custom engineered constructs

Example 5. *In vitro* characterization of acellular mineralized and non-mineralized urodele healthy or healing connective tissue matrix

Through a series of *in vitro* analyses one can verify decellularization and preservation of native or custom engineered functional and structural properties of decellularized extracellular matrix constructs and/or particulate, including multilayer laminated constructs such as a dual-sided basement membrane sheet matrix or isolated native homogenous open porous matrix or solubilized, lyophilized and loaded ECM-derived collagen sponge. DNA content, as a marker for cell debris, can be employed to assess decellularization quantitatively, using a single, ethanol-based extraction technique with a fluorometric dye, Quant-iT PicoGreen (Molecular Probes, USA), in a ratio of 170 µL working solution to 30 µL samples/standards in a 96-well plate. Paired native and post-processed analysis and comparison to commercially available tissue ECM's can be performed to verify acceptability. See Figure 6.

ELISA analysis for quantification of bioactive constituent and native and post process protelyctic resorption profiles can be performed. Upon digestion with collagenase (232 – 262 mg/unit activity), normalized-weight-to-surface-area sections of decellularized and dehydrated urodele ECM tissue or constructs (Sigma, USA), in a pH 7.6 buffer (50 mM Tris-HCl, 200 mM $CaCl_2$, 50 mM NaCl) for 24 hours at 37°C, can be analyzed at various time points to construct a relative resorption curve of pre- and post-process tissue to verify preservation of histoarchitecture. Solubilized collagen following digestion can be assessed,

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using a Sircol kit (Biocolor Ltd., UK), in 100 µL aliquots of acid/salt-washed digests. Specifically, levels of BMP 2/4 and TGF- 1 growth factors or sulfated gags can be assessed, following digestion, by means of commercially available ELISA kits (R&D Systems, Minneapolis, MN). Protein content in 1:10 dilution digests can be measured via a standard Bradford absorbance assay.

Microscopic evaluation of samples can be performed using fixation in 4% paraformaldehyde and paraffin embedding, sectioning at 5 µm, and routine histological staining (Histoserv, Inc., USA). Longitudinal cross sections can be stained with hematoxylin and eosin. Images can be acquired and anyzed using standard brightfield techniques on an Olympus IM inverted microscope. Samples can analyzed using scanning electron microscopy after dehydration in a graded ethanol series (15%, 30%, 50%, 70%, 95%, and 100%), critical-point drying in CO_2, and sputter coating with gold. Samples can be visualized in an FEI Quanta 600 FEG scanning electron microscope, and representative images of scaffold ultrastructure can be acquired.

Direct cell contact methodology (ISO10993, Part 5), for qualitative cell viability assessment at 24 hours, can be conducted for cytotoxicity testing and at extended time points (48 and 72 hours) to gauge cell proliferation and adhesion efficiency. A manual count of non-adherent cells in a hemocytometer, following transfer and trypsinization of the culture wells, can be conducted. A CellTiter 96 assay can performed to quantify viable cells after four days. A live/dead cell staining kit can used as well to visualize scaffolds via fluorescence microscopy at 24 hours and day four, thereby to verify biocompatibility.

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CLAIMS

1. A decellularized biomaterial produced by the process that comprises (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

2. The decellularized biomaterial of claim 1, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

3. The decellularized biomaterial of claim 1, wherein said process further comprises subjecting said sample to sterilization.

4. The decellularized biomaterial of claim 1, further comprising devitalized cells.

5. A tissue graft comprising extracellular matrix components derived from a urodele.

6. An isolated, decellularized Urodele ECM.

7. The isolated ECM of claim 6, wherein the ECM is derived from Axolotl tissue.

8. The isolated ECM of claim 6, wherein the ECM includes basement membrane

9. The isolated ECM of claim 6, wherein the ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, a drug or a human agent.

10. The isolated ECM of claim 6, wherein the agent is a cell, optionally a human cell.

11. The isolated ECM of claim 6, wherein the ECM is a sheet.

12. The isolated ECM of claim 11, wherein the sheet includes perforations.

13. The isolated ECM of claim 6, wherein the ECM is a dry powder.

14. The isolated ECM of claim 6, wherein the ECM is a reconstituted gel.

15. The isolated ECM of any one of claims 6-13, therein the ECM is sterile.

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16. A package containing sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM.

17. The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a sheet of isolated, decellularized Urodele ECM.

18. The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a dry powder.

19. The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a gel.

20. A sterile medical implant comprising sterile, isolated, decellularized Urodele ECM or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM.

21. The sterile medical implant of claim 19, wherein the implant is a biocompatible sheet, mesh, gel, graft, tissue or device.

22. A material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM or a Urodele fraction derived from the isolated, decellularized Urodele ECM.

23. A tissue culture system comprising (a) an isolated Urodele decellularized ECM, (b) tissue culture medium, and (c) cells xenogenic to the Urodele.

24. The tissue culture system of claim 22, wherein the cells are human cells.

25. Tissue culture medium conditioned with isolated Urodele decellularized ECM or a Urodele fraction derived from isolated, decellularized Urodele ECM.

26. A device comprising at least two sheets of isolated Urodele decellularized ECM laminated to one another.

27. A product prepared by isolating Urodele ECM from a Urodele, decellularizing the ECM, and sterilizing the decellularized ECM.

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28. A method of preparing a biologic material comprising (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample forming a decellularlized ECM to remove sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

29. The method of claim 28, wherein the method further comprises performing the decellularization in a manner to retain structural and functional integrity of the decellularized ECM sufficient to permit the decellularized ECM to be useful as a matrix upon and within which cells can grow.

30. The method of claim 29, further comprising homogenizing the decellularized ECM to form a powder.

31. The method of claim 30, further comprising reconstituting the powder as a gel.

32. The method of claim 29, further comprising sterilizing the decellularized ECM.

33. The method of claim 29, further comprising attaching the decellularized ECM to an agent xenogenic to a Urodele.

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FIGURE 1

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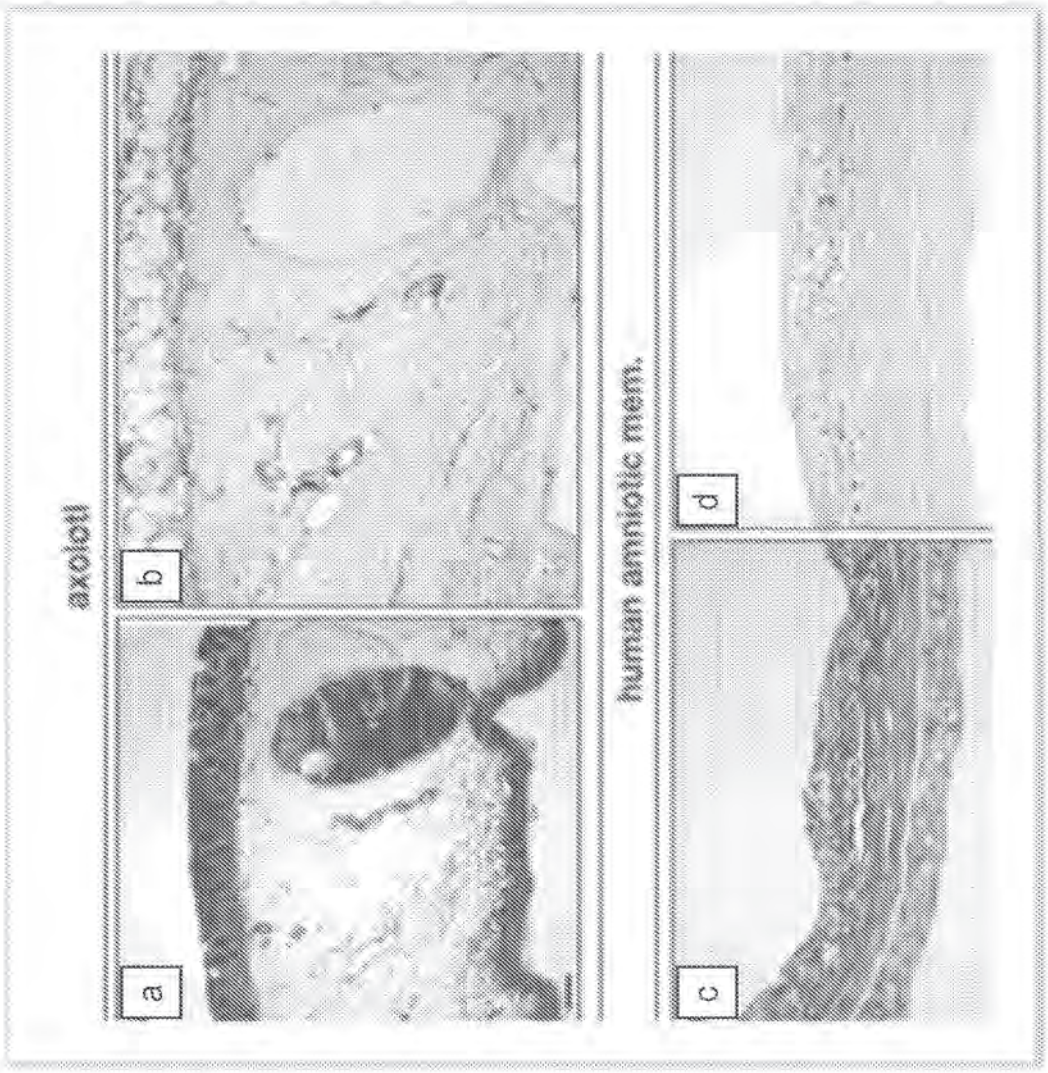


FIGURE 2

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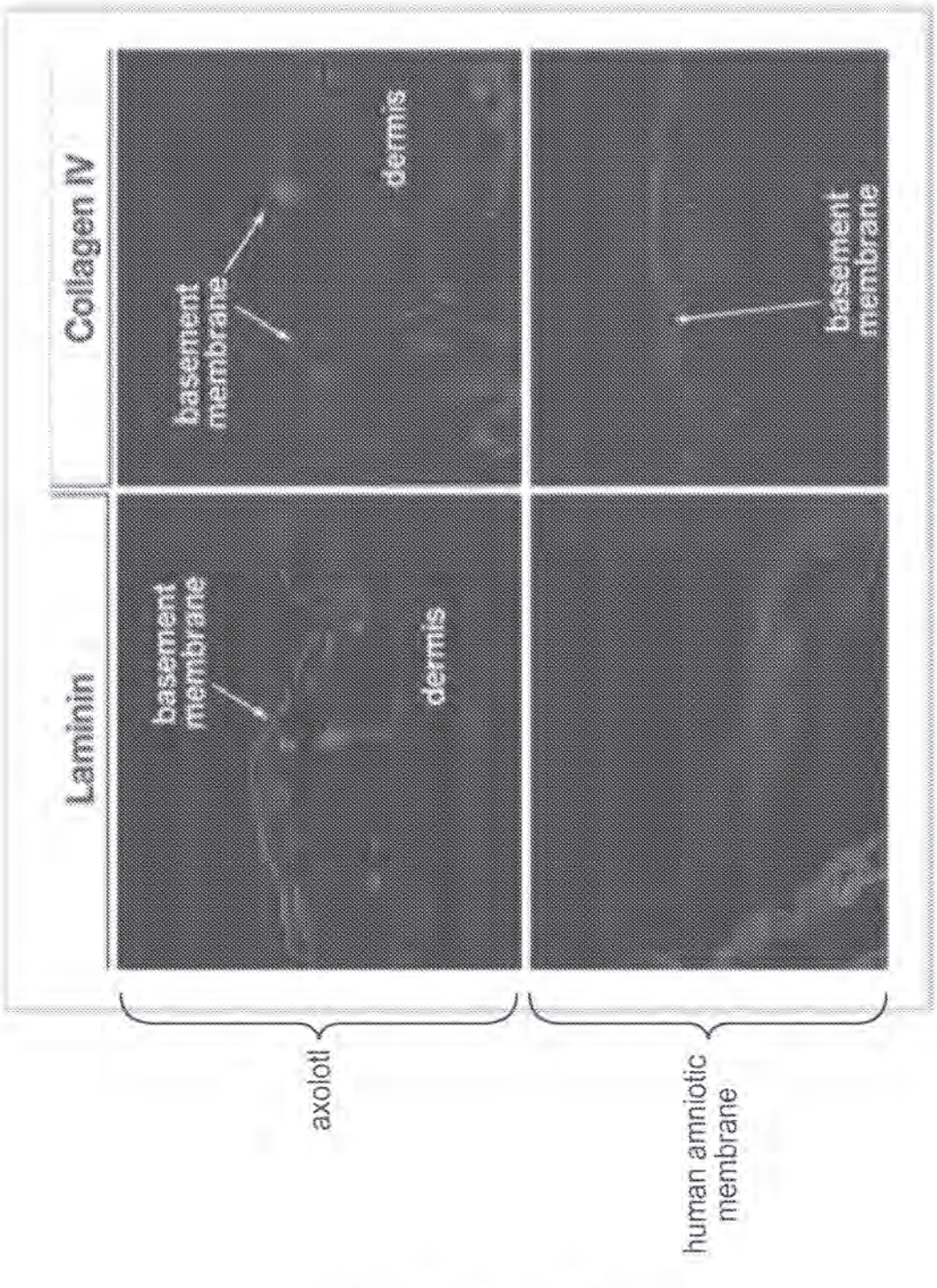


FIGURE 3

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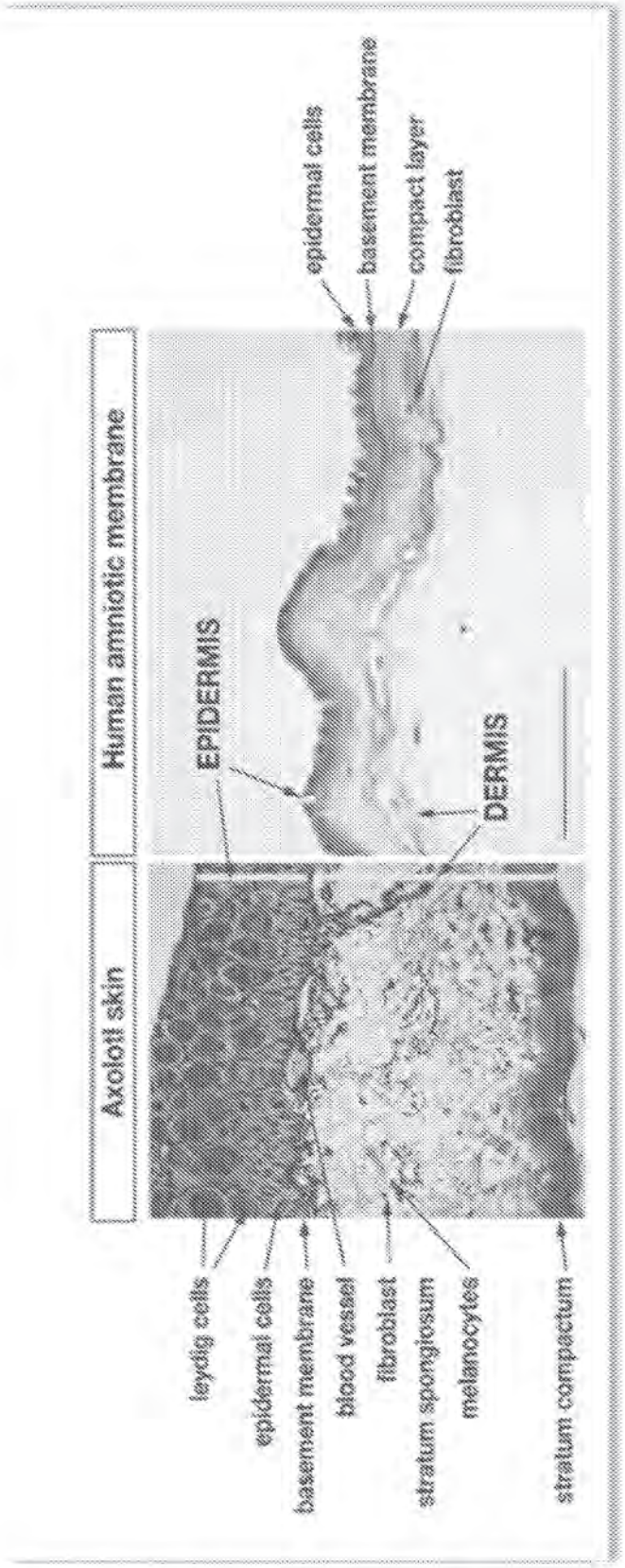


FIGURE 4

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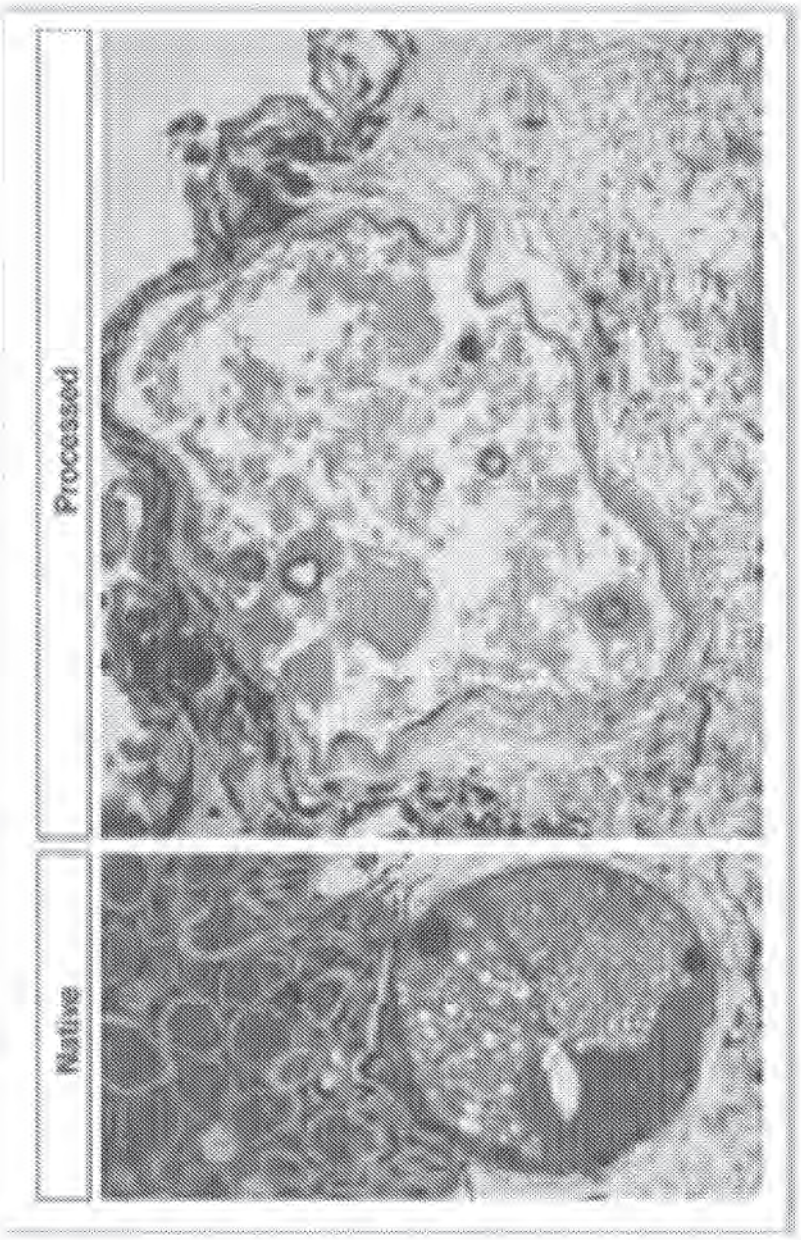


FIGURE 5

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Epoch Plate Reader		Avg. Duplicate			
	Sample ID	260/280	dsDNA conc. ng/ul	dsDNA conc. ug/ul	Percent Reduction
Paired native and post decellurization urodele connective tissue Residual DNA Analysis	A-Native	2	1702.8	1.7028	89.8%
	A-Native	2	1977.5	1.9775	92.8%
	A-Native	2	1618.3	1.6183	92.7%
	A-P1	1.96	173.7	0.1737	
	A-P2	1.95	141.7	0.1417	
	A-P3	1.97	117.9	0.1179	
Commercially available Amnitoic Membrane Products residual Dna	BioDlogic (BioD)	2	1.34	0.00134	
	Biotissue (Amniograft)	1.5	27.81	0.02781	
	Mimedx (Amniofix)	1.26	104.6	0.1046	
	Seed Biotech (Aril)	1.75	3.16	0.00316	

FIGURE 6

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 2014/010890

A. CLASSIFICATION OF SUBJECT MATTER

A61K 35/56 *(2006.01)*
A61K 9/10 *(2006.01)*
A61K 9/14 *(2006.01)*
A61F 2/00 *(2006.01)*
A61L 27/36 *(2006.01)*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 35/56, 31/00, 38/00, 9/10, 9/14, A61F 2/00, A61L 27/36

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DEPATISnet, DWPI, EAPATIS, PAJ, Patentscope, PatSearch (RUPTO internal) PubMed, RUPTO, RUSPAT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/124127 A2 (WAKE FOREST UNIVERSITY HEALTH SCIENCES et al.) 01.11.2007, claims 1, 15, 17-18, 21, p. 2, last paragraph, p. 3, paragraph 3, p. 4, paragraphs 4, 5, 6, p. 7, paragraphs 4, 5, p. 8, paragraphs 3, 5, p. 9, paragraph 4, p. 15, paragraphs 1, 2	1-10, 15-16, 20-25, 27-29, 32-33
Y		11-14, 17-19, 26, 30-31
Y	WO 2005/009498 A2 (SCIMED LIFE SYSTEMS, INC. et al.) 03.02.2005, claims 30-31, p. 13, lines 31-33, p. 16, lines 28-29, p. 26, lines 13-15	11-14, 17-19, 26, 30-31

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 March 2014 (26.03.2014)	15 May 2014 (15.05.2014)
Name and mailing address of the ISA/ FIPS Russia, 123995, Moscow, G-59, GSP-5, Berezhkovskaya nab., 30-1 Facsimile No. +7 (499) 243-33-37	Authorized officer E. Maslova Telephone No. (495)531-64-81

Form PCT/ISA/210 (second sheet) (July 2009)

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

COMMUNICATION IN CASES FOR WHICH NO OTHER FORM IS APPLICABLE

To:
GATES Edward R.
Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue
Boston, Massachusetts 02210-2206
United States of America

Date of mailing *(day/month/year)* 17 April 2015 (17.04.2015)	
Applicant's or agent's file reference S1860.70000	**REPLY DUE** See paragraph 1 below
International application No. PCT/US 2014/010890	International filing date *(day/month/year)* 09 January 2014 (09.01.2014)
Applicant EARLY, Ryanne	

1. [ ] REPLY DUE within _____ months/days from the above date of mailing

   [X] NO REPLY DUE

2. COMMUNICATION:

Send you the copies of requested Forms PCT/ISA/210 and PCT/ISA/237.

Enclosure: 7 sh.

Name and mailing address of the ISA/RU: Federal Institute of Industrial Property, Berezhkovskaya nab., 30-1, Moscow, G-59, GSP-3, Russia, 125993 Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37	Authorized officer T. Vladimirova Telephone No. 499-240-25-91

Form PCT/ISA/224 (January 1994; reprint January 2004)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 101763-0103	FOR FURTHER ACTION	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. PCT/US 2014/010890	International filing date *(day/month/year)* 09 January 2014 (09.01.2014)	(Earliest) Priority Date *(day/month/year)* 09 January 2013 (09.01.2013)
Applicant EARLY, Ryanne		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 2 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of:

☒ the international application in the language in which it was filed.

☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).

c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☐ **Certain claims were found unsearchable** (see Box No. II).

3. ☐ **Unity of invention is lacking** (see Box No. III).

4. With regard to the **title,**

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the **drawings,**

a. the figure of the drawings to be published with the abstract is Figure No. 1

☒ as suggested by the applicant.

☐ as selected by this Authority, because the applicant failed to suggest a figure.

☐ as selected by this Authority, because this figure better characterizes the invention.

b. ☐ none of the figures is to be published with the abstract.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 2014/010890

A. CLASSIFICATION OF SUBJECT MATTER

A61K 35/56 *(2006.01)*
A61K 9/10 *(2006.01)*
A61K 9/14 *(2006.01)*
A61F 2/00 *(2006.01)*
A61L 27/36 *(2006.01)*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 35/56, 31/00, 38/00, 9/10, 9/14, A61F 2/00, A61L 27/36

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DEPATISnet, DWPI, EAPATIS, PAJ, Patentscope, PatSearch (RUPTO internal) PubMed, RUPTO, RUSPAT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/124127 A2 (WAKE FOREST UNIVERSITY HEALTH SCIENCES et al.) 01.11.2007, claims 1, 15, 17-18, 21, p. 2, last paragraph, p. 3, paragraph 3, p. 4, paragraphs 4, 5, 6, p. 7, paragraphs 4, 5, p. 8, paragraphs 3, 5, p. 9, paragraph 4, p. 15, paragraphs 1, 2	1-10, 15-16, 20-25, 27-29, 32-33
Y		11-14, 17-19, 26, 30-31
Y	WO 2005/009498 A2 (SCIMED LIFE SYSTEMS, INC. et al.) 03.02.2005, claims 30-31, p. 13, lines 31-33, p. 16, lines 28-29, p. 26, lines 13-15	11-14, 17-19, 26, 30-31

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 March 2014 (26.03.2014)	15 May 2014 (15.05.2014)
Name and mailing address of the ISA/ FIPS Russia, 123995, Moscow, G-59, GSP-5, Berezhkovskaya nab., 30-1 Facsimile No. +7 (499) 243-33-37	Authorized officer E. Maslova Telephone No. (495)531-64-81

Form PCT/ISA/210 (second sheet) (July 2009)

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

To:
BENT, Stephen A.
FOLEY & LARDNER LLP, 3000 K Street NW, Suite 600
Washington, District of Columbia 20007
United States of America

Date of mailing *(day/month/year)* 15 May 2014 (15.05.2014)	
Applicant's or agent's file reference 101763-0103	**FOR FURTHER ACTION** See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US 2014/010890	09 January 2014 (09.01.2014)	09 January 2013 (09.01.2013)

International Patent Classification (IPC) or both national classification and IPC
A61K 35/56 *(2006.01)*
A61K 9/10 *(2006.01)*
A61K 9/14 *(2006.01)*
A61F 2/00 *(2006.01)*
A61L 27/36 *(2006.01)*

Applicant
EARLY, Ryanne

1. This opinion contains indications relating to the following items:

- [X] Box No. I Basis of the opinion
- [ ] Box No. II Priority
- [ ] Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- [ ] Box No. IV Lack of unity of invention
- [X] Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- [ ] Box No. VI Certain documents cited
- [X] Box No. VII Certain defects in the international application
- [ ] Box No. VIII Certain observations on the international application

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/ FIPS	Date of completion of this opinion	Authorized officer
Russia, 123995, Moscow, G-59, GSP-5, Berezhkovskaya nab., 30-1 Facsimile No. +7 (499) 243-33-37	26 March 2014 (26.03.2014)	E. Maslova Telephone No. (495)531-64-81

Form PCT/ISA/237 (cover sheet) (July 2011)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 2014/010890

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:
   - [X] the international application in the language in which it was filed.
   - [ ] a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. [ ] This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing filed or furnished:
   - a. [ ] forming part of the international application as filed:
     - [ ] in the form of an Annex C/ST.25 text file.
     - [ ] on paper or in the form of an image file.
   - b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
   - c. [ ] furnished subsequent to the international filing date for the purposes of international search only:
     - [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).
     - [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 2014/010890

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	7, 11-14, 17-19, 26, 30-31	YES
	Claims	1-6, 8-10, 15-16, 20-25, 27-29, 32-33	NO
Inventive step (IS)	Claims		YES
	Claims	7, 11-14, 17-19, 26, 30-31	NO
Industrial applicability (IA)	Claims	1-33	YES
	Claims		NO

2. Citations and explanations:

D1 WO 2007124127 A2 01.11.2007
D2 WO 2005009498 A2 03.02.2005

D1 discloses a method of preparing a bioscaffold with the use of decellularizing a tissue, during which an excess material is removed from a tissue sample (claims 1, 21). A tissue of amphibians can be used as the tissue. The representative of the amphibians is the order of Urodele (p. 8, paragraphs 3, 5). When preparing the bioscaffold, it maintains its natural biomechanical integrity (p. 7, paragraph 4; p. 2, last paragraph; p. 3, paragraph 3). Decellularizing the tissue is implemented in order to reduce an inflammatory reaction and immune response (p. 7, paragraph 5). The bioscaffold is used as a transplant, an implant (p. 4, paragraphs 4, 6, claim 18). The bioscaffold can be placed in a package (claim 17). The bioscaffold can comprise growth factors present in a culture medium (p. 9, paragraph 4).

Therefore, the inventions in claims 1, 5, 6, 16, 20, 22, 23, 25, 27, 28 do not meet the criterion of novelty.

D2 discloses a device having a layer of decellularized ECM (claims 30-31).

The invention in independent claim 26 differs from D1 in that the tissue of Urodele is used; the device comprises at least two sheets.

As D1 discloses that a tissue of amphibians is used when preparing decellularized ECM, the use of the tissue of Urodele when preparing ECM is obvious for a person skilled in the art, wherein the application of the necessary number of sheets on the device is also obvious for a person skilled in the art.

Therefore, the invention in independent claim 26 does not meet the criterion of inventive step.

D1 discloses the features of dependent claims 2-4, 8-10, 15, 21, 24, 29, 32-33 relative to decellularizing implemented using, for example, an alkaline treatment (p. 15, paragraphs 1, 2). D1 discloses a bioscaffold that can be sterilized (claim 15); the bioscaffold can comprise human cells,

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Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of V:

growth factors (p. 4, paragraph 5; p. 9, paragraph 4). The bioscaffold also can be used as a transplant, an implant (p. 4, paragraphs 4, 6, claim 18). When preparing the bioscaffold, it maintains its natural biomechanical integrity (p. 7, paragraph 4; p. 2, last paragraph; p. 3, paragraph 3).

Therefore, the inventions in dependent claims 2-4, 8-10, 15, 21, 24, 29, 32-33 do not meet the criterion of novelty.

D2 discloses the features of dependent claims 11-14, 17-19, 30-31 relative to a composition comprising the decellularized ECM that can be made in the form of a sheet (p. 26, lines 13-15) or a gel (p. 13, lines 31-33). The composition can be a powder (p. 16, lines 28-29).

The use of Axolotl tissue in dependent claim 7 is obvious for a person skilled in the art, as D1 (p. 8, paragraphs 3, 5) discloses the use of a tissue of amphibians.

Therefore, the inventions in dependent claims 7, 11-14, 17-19, 30-31 do not meet the criterion of inventive step.

The inventions in claims 1-33 meet the criterion of industrial applicability.

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RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0067

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 2014/010890

Box No. VII Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Dependence of the following claims is incorrect:

- claims 17-19 are dependent on independent claim 16;
- claim 21 is dependent on independent claim 20;
- claim 24 is dependent on independent claim 22.

Form PCT/ISA/237 (Box No. VII) (January 2015)

ePCT Document Upload (Public Services)

IA Number:

PCT/US2014/010890

To:

IB

Date at recipient Office:

08 July 2015 23:04:14 CEST

From:

Kara Verrochi

Message:

Transmittal Letter with 92Bis

The following document(s) have been uploaded via ePCT public services:

- Request for Change under Rule 92bis

Signature: Document(s) signed by Applicant/Agent included in attachment(s)

Pending Processing

IN THE INTERNATIONAL BUREAU OF WIPO

International Application No.:	PCT/US2014/010890
International Filing Date :	09 January 2014 (09.01.2014)
Earliest Priority Date:	09 January 2013 (09.01.2013)
Applicants:	Ryanne Early
Title:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE

International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20
SWITZERLAND

REQUEST UNDER PCT RULE 92BIS TO RECORD ADDITION OF APPLICANT AND CHANGE OF APPLICANT/INVENTOR STATUS

Madam/Sir:

The undersigned agent respectfully requests that the following change be recorded in the above-referenced international application.

Please add the following Applicant *for all designated States*:

ISE PROFESSIONAL TESTING & CONSULTING SERVICES, INC.
2007 NW 5th Street
Gainesville, Florida 32603
United States of America

State of Nationality: US
State of Residence: US

Please change the designation of the following Applicant/Inventor to **Inventor Only** for all designated States:

EARLY, Ryanne
1909 Hickory Trace Drive
Fleming Island, Florida 32003
United States of America

Enclosed is a Power of Attorney form signed by the Applicant/Inventor EARLY, Ryanne. The International Bureau is respectfully requested to contact the undersigned if additional information is required.

Pending Processing

4108923.1

Respectfully submitted,

Edward R. Gates
Registration Number: 31,616
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210
United States of America
Telephone: 617-646-8000
Facsimile: 617-646-8646
E-mail: **PCT@wolfgreenfield.com**

DOCKET NO.: S1860.70000
DATE: 8 July 2015

Pending Processing

4108923.1

ePCT Document Upload (Public Services)

IA Number:

PCT/US2014/010890

To:

IB

Date at recipient Office:

08 July 2015 23:05:25 CEST

From:

Kara Verrochi

Message:

Power of Attorney Form

The following document(s) have been uploaded via ePCT public services:

- Power of Attorney

Signature: Document(s) signed by Applicant/Agent included in attachment(s)

Pending Processing

PCT

GENERAL POWER OF ATTORNEY

(for several international applications filed under the Patent Cooperation Treaty)

(PCT Rule 90.5)

The undersigned person(s):
(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

EARLY, Ryanne
1909 Hickory Trace Dr.
Fleming Island, Florida 32003
United States of America

hereby appoint(s) the following person as: [X] agent [ ] common representative

Name and address
(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

Robert M. Abrahamsen, 40,886; Daryl L. Achilles, Ph.D., 70,515; Eric L. Amundsen, 46,518; Michael J. Attisha, 68,656; C. Hunter Baker, M.D., Ph.D., 46,533; Melissa A. Beede, 54,986; Brandon S. Blackwell, Ph.D., 66,256; O. Tobias Brambrink, 69,335; Tani Chen, Sc.D., 52,728; Yahua Chen, Ph.D., 55,055; Ling Cheung Hughes, Ph.D., 65,065; Thomas S. Chlebeck, 70,616; Heather J. DiPietrantonio, Ph.D., 65,843; Roque El-Hayek, 55,151; Neil P. Ferraro, 39,188; Thomas A. Franklin, 63,456; Andrea J. Gabert, Ph.D., 68,904; Edward R. Gates, 31,616; David S. Gesner, 72,100; Richard F. Giunta, 36,149; James G. Goodberlet, Ph.D., 67,774; Patricia Granahan, Sc.D., 32,227; Lawrence M. Green, 29,384; James M. Hanifin, Jr., 39,213; Girada A. Harmon, Ph.D., 65,996; John S. Harmon, Ph.D., P.E., 67,131; Christopher W. Henry, 60,907; Minita G. Holloway, 67,594; Jason M. Honeyman, 31,624; Gerald B. Hrycyszyn, 50,474; Elisabeth H. Hunt, Ph.D., 67,336; Robert E. Hunt, 39,231; Robert A. Jensen, 61,146; Marc S. Johannes, 64,978; Oona M. Johnstone, Ph.D., 68,177; Jessamine N. Lee, Ph.D., 61,674; Helen C. Lockhart, 39,248; William R. McClellan, 29,409; Andrea D. Merin, 67,217; Katherine J. Miller, Ph.D., 62,080; James H. Morris, 34,681; Timothy J. Oyer, Ph.D., 36,628; Edward F. Perlman, 28,105; Michael J. Pomianek, 46,190; Randy J. Pritzker, 35,986; Michael N. Rader, 52,146; Daniel G. Rudoy, 70,972; Michael Y. Saji, 66,291; Xiaohong Shou, 71,000; Michael T. Siekman, 36,276; Kuangshin Tai, Ph.D., 62,733; Andrew J. Tibbetts, 65,139; Maria A. Trevisan, 48,207; John R. Van Amsterdam, 40,212; Janice A. Vatland, Ph.D., 52,318; Robert H. Walat, 46,324; Patrick R.H. Waller, 41,418; Edmund J. Walsh, 32,950; Jennifer J. Wang, 68,598; Robin A. Weatherhead, Ph.D., 60,141; Daniel T. Wehner, Ph.D., 63,480; Douglas R. Wolf, 36,971; Daniel W. Young, Ph.D., 62,868; and Wei Zhang, 69,059
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
United States of America

to represent the undersigned before [X] all the competent International Authorities
[ ] the International Searching Authority only
[ ] the Authority specified for supplementary search: _____
(please indicate the Authority(ies) specified for supplementary search)
[ ] the International Preliminary Examining Authority only

in connection with any and all international applications filed by the undersigned with the following Office:
US as receiving Office
and to make or receive payments on behalf of the undersigned.

Signature(s) *(where there are several persons, each of them must sign; next to each signature, indicate the name of the person signing and the capacity in which the person signs, if such capacity is not obvious from reading this power):*

Date: 16 October 2014
Day Month Year

Signature

Pending Processing

Form PCT/Model of general power of attorney (for several international applications) (January 2009)

3726940.1

DocCode – SCORE

SCORE Placeholder Sheet for IFW Content

Application Number: 14759895 Document Date: 07/08/2015

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

- Drawing

At the time of document entry (noted above):

- USPTO employees may access SCORE content via eDAN using the Supplemental Content tab, or via the SCORE web page.
- External customers may access SCORE content via PAIR using the Supplemental Content tab.

Form Revision Date: August 26, 2013

VIII-1-1	**Declaration: Identity of the Inventor** Declaration as to the identity of the inventor (Rules 4.17(i) and 51bis.1(a)(i))	In relation to this international application
	Name (LAST, First) Address	EARLY, Ryanne of c/o SEED Biotech, Inc. 3116 Commerce St., Suite A Dallas, Texas 75226 United States of America is the inventor of the subject matter for which protection is sought by way of this international application

PCT REQUEST

Original (for **SUBMISSION**)

0	**For receiving Office use only**	
0-1	International Application No.	PCT/US14/10890
0-2	International Filing Date	09 JAN 2014 09-01-2014
0-3	Name of receiving Office and "PCT International Application"	PCT International Application RO/US
0-4	**Form PCT/RO/101 PCT Request**	
0-4-1	Prepared Using	PCT-SAFE [EASY mode] Version 3.51.060.236 MT/FOP 20131001/0.20.5.21
0-5	**Petition** The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	**Receiving Office (specified by the applicant)**	United States Patent and Trademark Office (USPTO) (RO/US)
0-7	**Applicant's or agent's file reference**	101763-0103
I	**Title of Invention**	DECELLULARIZED BIOMATERIAL FORM NON-MAMMALIAN TISSUE
II	**Applicant**	
II-1	This person is	Applicant and inventor
II-2	Applicant for	All designated States
II-4	Name (LAST, First)	EARLY, Ryanne
II-5	Address	c/o SEED Biotech, Inc. 3116 Commerce St., Suite A Dallas, Texas 75226 United States of America
II-6	State of nationality	US
II-7	State of residence	US

IV-1	**Agent or common representative; or address for correspondence** The person identified below is hereby/ has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	Agent
IV-1-1	Name (LAST, First)	BENT, Stephen A.
IV-1-2	Address	FOLEY & LARDNER LLP 3000 K Street NW, Suite 600 Washington, District of Columbia 20007 United States of America
IV-1-3	Telephone No.	202-672-5300
IV-1-4	Facsimile No.	202-672-5399
IV-1-5	e-mail	ptomailwashington@foley.com
IV-1-5(a)	E-mail authorization The receiving Office, the International Searching Authority, the International Bureau and the International Preliminary Examining Authority are authorized to use this e-mail address, if the Office or Authority so wishes, to send notifications issued in respect of this international application:	as advance copies followed by paper notifications
IV-1-6	Agent's registration No.	29768
IV-2	**Additional agent(s)**	additional agent(s) with same address as first named agent
IV-2-1	Name(s)	AGARWAL, Pavan K.(40888); BECK, George(38072); BERKOWITZ, Benjamin A.(59349); BRINCKERHOFF, Courtenay(37288); ELLIS, William T.(26874); FELDHAUS, John J.(28822); HENSCHEL, Rouget(39221); KAMINSKI, Michael D.(32904); LAW, Glenn(34371); MAEBIUS, Stephen B.(35264); PEET, Richard C.(35792); QUILLIN, George E.(32792); RAWLINS, Andrew E.(34702); REID, Steven M.(54393); SCHORR, Kristel(55600); SHIPLEY, Howard N.(39790); SIMKIN, Michele M.(34717); WEGNER, Harold C.(25258)
V	**DESIGNATIONS**	
V-1	**The filing of this request constitutes under Rule 4.9(a), the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.**	

PCT REQUEST

Original (for **SUBMISSION**)

VI-1	**Priority claim of earlier national application**		
VI-1-1	Filing date	09 January 2013 (09.01.2013)	
VI-1-2	Number	61/750,555	
VI-1-3	Country	US	
VI-2	**Priority document request** The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s):	VI-1	
VI-3	**Incorporation by reference :** where an element of the international application referred to in Article 11(1)(iii)(d) or (e) or a part of the description, claims or drawings referred to in Rule 20.5(a) is not otherwise contained in this international application but is completely contained in an earlier application whose priority is claimed on the date on which one or more elements referred to in Article 11(1)(iii) were first received by the receiving Office, that element or part is, subject to confirmation under Rule 20.6, incorporated by reference in this international application for the purposes of Rule 20.6.		
VII-1	**International Searching Authority Chosen**	Federal Service for Intellectual Property (Rospatent) (Russian Federation) (ISA/RU)	
VIII	**Declarations**	Number of declarations	
VIII-1	Declaration as to the identity of the inventor	1	
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	1	
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	1	
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	-	
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	-	

PCT REQUEST

Original (for **SUBMISSION**)

IX	Check list	Number of sheets	Electronic file(s) attached
IX-1	Request (including declaration sheets)	7	✓
IX-2	Description	28	-
IX-3	Claims	3	-
IX-4	Abstract	1	✓
IX-5	Drawings	6	-
IX-7	TOTAL	45	
	Accompanying Items	Paper document(s) attached	Electronic file(s) attached
IX-8	Fee calculation sheet	✓	-
IX-18	PCT-SAFE physical media	-	✓
IX-20	**Figure of the drawings which should accompany the abstract**	1	
IX-21	**Language of filing of the international application**	English	
X-1	**Signature of applicant, agent or common representative**	S. A. Bent	
X-1-1	Name (LAST, First)	BENT, Stephen A.	
X-1-2	Name of signatory		
X-1-3	Capacity (if such capacity is not obvious from reading the request)		

FOR RECEIVING OFFICE USE ONLY

10-1	**Date of actual receipt of the purported international application**	09 JAN 2014 09-01-2014
10-2	**Drawings:**	
10-2-1	Received	
10-2-2	Not received	
10-3	**Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application**	
10-4	**Date of timely receipt of the required corrections under PCT Article 11(2)**	
10-5	**International Searching Authority**	ISA/RU
10-6	**Transmittal of search copy delayed until search fee is paid**	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	**Date of receipt of the record copy by the International Bureau**	

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92*bis*.1 and Administrative Instructions, Section 422)

To:

EARLY, Ryanne
1909 Hickory Trace Dr.
Fleming island, FL 32003
ETATS-UNIS D'AMERIQUE

Date of mailing *(day/month/year)*
20 May 2014 (20.05.2014)

Applicant's or agent's file reference
101763-0103

IMPORTANT NOTIFICATION

International application No.
PCT/US2014/010890

International filing date *(day/month/year)*
09 January 2014 (09.01.2014)

1. The following indications appeared on record concerning:

☐ the applicant ☐ the inventor ☒ the agent ☐ the common representative

Name and Address

BENT, Stephen, A.
Foley & Lardner LLP
3000 K Street NW, Suite 600
Washington, District of Columbia 20007
United States of America

State of Nationality:
State of Residence:
Telephone No.: 202-672-5300
Facsimile No.: 202-672-5399
E-mail address: ptomailwashington@foley.com

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☐ the person ☐ the name ☐ the address ☐ the nationality ☐ the residence

Name and Address

State of Nationality:
State of Residence:
Telephone No.:
Facsimile No.:
E-mail address:
☐ Notifications by e-mail authorized

3. Further observations, if necessary:
The agent has renounced his apointment. All correspondence will be sent to the applicant identified in the addressee box at the top of this form. Please note the change of the applicant's address.

4. A copy of this notification has been sent to:

☒ the receiving Office
☐ the International Searching Authority
☐ the Authority(ies) specified for supplementary search
☐ the International Preliminary Examining Authority
☐ the designated Offices concerned
☐ the elected Offices concerned
☒ other: BENT, Stephen, A.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. +41 22 338 70 90

Authorized officer
Parianou Christina
e-mail pt01.pct@wipo.int
Telephone No. +41 22 338 74 01

597406

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

February 01, 2014

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *61/750,555*
FILING DATE: *January 09, 2013*
RELATED PCT APPLICATION NUMBER: *PCT/US14/10890*

THE COUNTRY CODE AND NUMBER OF YOUR PRIORITY APPLICATION, TO BE USED FOR FILING ABROAD UNDER THE PARIS CONVENTION, IS *US61/750,555*

Certified by

David J. Kappos

Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

Claims

1. A decellularized biomaterial produced by the process that comprises (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

2. The decellularized biomaterial of claim 1, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

3. The decellularized biomaterial of claim 1, wherein said process further comprises subjecting said sample to sterilization.

4. The decellularized biomaterial of claim 1, further comprising devitalized cells.

5. A tissue graft comprising extracellular matrix components derived from a urodele.

RegenX Science Exhibit 1004
Page 82

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0082

Electronic Acknowledgement Receipt	
EFS ID:	14652874
Application Number:	61750555
International Application Number:	
Confirmation Number:	6396
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	RYANNE EARLY
Customer Number:	22428
Filer:	Stephen A. Bent/Ayesha Wilson
Filer Authorized By:	Stephen A. Bent
Attorney Docket Number:	
Receipt Date:	09-JAN-2013
Filing Date:	
Time Stamp:	15:40:34
Application Type:	Provisional

Payment information:

Submitted with Payment	yes
Payment Type	Credit Card
Payment was successfully received in RAM	$125
RAM confirmation Number	2433
Deposit Account	
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1	Transmittal of New Application	Provisional_App_Transmittal.pdf	86998	no	2
Warnings:					
Information:					
2	Application Data Sheet	ADS.pdf	51592	no	6
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
3		Specification.pdf	625964	yes	14
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Specification		1	12	
	Claims		13	13	
	Abstract		14	14	
Warnings:					
Information:					
4	Fee Worksheet (SB06)	fee-info.pdf	29297	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	793851		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/AIA/14 (08-12)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	
	Application Number	
Title of Invention	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Ryanne		Early	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	GAINESVILLE	State/Province	FL	Country of Residence	US

Mailing Address of Inventor:

Address 1			
Address 2			
City		State/Province	
Postal Code		Country	

Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City		State/Province		Country of Residence	

Mailing Address of Inventor:

Address 1			
Address 2			
City		State/Province	
Postal Code		Country	

All Inventors Must Be Listed – Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

PTO/AIA/14 (08-12)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	
	Application Number	
Title of Invention	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE	

☐ An Address is being provided for the correspondence Information of this application.			
Customer Number	22428		
Email Address	ipdocketing@foley.com	Add Email	Remove Email

Application Information:

Title of the Invention	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE		
Attorney Docket Number		**Small Entity Status Claimed**	☒
Application Type	Provisional		
Subject Matter	Utility		
Suggested Class (if any)		**Sub Class (if any)**	
Suggested Technology Center (if any)			
Total Number of Drawing Sheets (if any)	0	**Suggested Figure for Publication (if any)**	0

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)
☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	◉ Customer Number	○ US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	22428		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.

Prior Application Status			Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)

PTO/AIA/14 (08-12)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	
	Application Number	
Title of Invention	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE	

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button.

Foreign Priority Information:

This section allows for the applicant to claim benefit of foreign priority and to identify any prior foreign application for which priority is not claimed. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55(a).

Remove

Application Number	Country[i]	Filing Date (YYYY-MM-DD)	Priority Claimed
			○ Yes ◉ No

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button.

Authorization to Permit Access:

☒ Authorization to Permit Access to the Instant Application by the Participating Offices

If checked, the undersigned hereby grants the USPTO authority to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the World Intellectual Property Office (WIPO), and any other intellectual property offices in which a foreign application claiming priority to the instant patent application is filed access to the instant patent application. See 37 CFR 1.14(c) and (h). This box should not be checked if the applicant does not wish the EPO, JPO, KIPO, WIPO, or other intellectual property office in which a foreign application claiming priority to the instant patent application is filed to have access to the instant patent application.

In accordance with 37 CFR 1.14(h)(3), access will be provided to a copy of the instant patent application with respect to: 1) the instant patent application-as-filed; 2) any foreign application to which the instant patent application claims priority under 35 U.S.C. 119(a)-(d) if a copy of the foreign application that satisfies the certified copy requirement of 37 CFR 1.55 has been filed in the instant patent application; and 3) any U.S. application-as-filed from which benefit is sought in the instant patent application.

In accordance with 37 CFR 1.14(c), access may be provided to information concerning the date of filing this Authorization.

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

PTO/AIA/14 (08-12)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	
	Application Number	
Title of Invention	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE	

Applicant 1

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

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U.S. PATENT APPLICATION

for

DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE

Inventors: Ryanne Early

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DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE

BACKGROUND OF THE INVENTION

[0001] Tissue engineering efforts are ongoing to produce methods and materials for replacing biological functions, typically repairing or replacing whole tissues or portions thereof. In this regard, wound treatment and skin repair are areas of predominant focus, as the loss of skin integrity due to illness or injury can lead to chronic, life threatening complications.

[0002] Wound healing involves complex interactions between cells, growth factors, and extracellular matrix (ECM) components to reconstitute tissue following injury. The wound healing process in adult mammalian tissue has been well characterized and can be broken down into three stages - inflammation, proliferation, and remodeling.

[0003] Typically, in response to an incision or trauma the body conveys blood, blood products, and proteins into the void (also referred to as the cavity or negative space) formed at the wound. During early inflammation, a wound exudate begins to form under the influence of inflammatory mediators and as a result of vasodilation. Fibrin and fibronectin present in clotting blood provide a scaffold over which cells such as keratinocytes, platelets and leukocytes migrate to the wound site. Bacteria and debris are phagocytosed and removed, and growth factors are released that stimulate the migration and division of fibroblasts.

[0004] The subsequent stage of wound healing involves new tissue formation as fibrous connective tissue, termed granulation tissue (composed of fibroblasts, macrophages and neovasculature) replaces the fibrin clot. New blood vessels are formed during this stage, and fibroblasts proliferate and produce a provisional ECM by excreting collagen and fibronectin. Nearly all mammalian cells require adhesion to a surface in order to proliferate and function properly. The ECM fulfills this function. Initially, the provisional ECM contains of a network of Type III collagen, a weaker form of collagen that is rapidly produced. This is later replaced by the stronger Type I collagen (which contributes to scar formation). At the same time, re-epithelialization of the epidermis occurs. During this process, epithelial cells

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proliferate and migrate over the newly forming tissue as proteases such as metalloportineaes (MMPs) and collagenases at the leading edge of the migrating cells help to invade the clot. These enzymes in addition to growth factor signaling (cell-cell interactions) and cell-ECM interactions (signal transduction from interactions between cells, integrins (cell surface receptors), laminin, collagen, fibronectin, and other ECM proteins) stimulate cell migration into the wound and ECM degradation.

[0005] Finally, in the remodeling phase, collagen is remodeled and realigned along tension lines and cells that are no longer needed are removed by apoptosis. Wound contraction occurs as fibroblasts transform into myofibroblasts through their interactions with ECM proteins and growth factors. Myofibroblasts then interact with collagen, vitronectin, and other ECM proteins to contract the wound. As the remodeling phase proceeds, fibronectin and hyaluronic acid are replaced by collagen bundles that lend strength to the tissue.

[0006] By applying biological, chemical and engineering principles to tissue repair and regeneration, tissue engineers have developed transplantable products for use in promoting the tissue repair and regeneration processes. The ability to restore biomechanical function of damaged tissue presents a true challenge. In response, both synthetic and biological scaffold products have been developed that mimic(to some extent) tissue structure and mechanical behavior to promote tissue repair. Such products serve as a temporary replacement, both mechanically and functionally, for damaged, diseased or absent tissue.

[0007] Ideally, transplantable scaffold products should support cell adhesion, proliferation and differentiation and act as an interim synthetic extracellular matrix (ECM) for cells prior to the formation of new tissue. Scaffold materials should be biocompatible, biodegradable and exhibit low antigenicity. The implant should degrade at a rate roughly equal to that of the new tissue formation. Once implanted, the scaffold must have the mechanical properties necessary to temporarily offer structural support until the new tissue has formed. Additionally, scaffold products must be porous, providing an appropriate path for nutrient transmission and tissue ingrowth. Tissue scaffolds also should promote fast healing and facilitate the development or regeneration of new tissue that resembles normal host tissue in both appearance and function. To this end, implanted scaffold products should offer (i) bioactive stimulation, e.g., protein and molecular signaling, to encourage cell migration, proliferation and differentiation, and (ii) mechanical or structural support for these processes.

[0008] Today, the development of synthetic scaffolds is an area of active research. Synthetic scaffolds have been manufactured from chemical compounds such as fibrous polymers, gelatin, apatite, and polymer/ceramic composites, polylactic acid, collagen. These scaffolds are designed to mimic the structure of the naturally occurring ECM and have shown some success in bone tissue engineering.

[0009] In addition to synthetic scaffold products, biological scaffolds obtained from mammalian tissues are commercially available for use as allografts (transplanted cell or tissue from a donor of the same species) or xenografts (transplanted cells or tissue from a donor of a different species). Biological scaffolds are composed of mammalian ECM harvested from, for example, dermis, urinary bladder, small intestine, mesothelium, pericardium, bone or aminiotic membrane of various mammals including human (either live donor or cadaver), porcine, bovine and equine. These commercially available products are commonly used for the repair and reconstruction of injured or missing tissues and organs such as soft tissue, tendons, cardiac tissue, neural tissue, chronic wounds, dura mater, bone and cornea.

[0010] Biological scaffold products may comprise skin cells in addition to extracellular matrices produced by tissue and subjected to a decellularization process. They are contacted with a wound site to give mechanical support for cell migration and proliferation as part of the wound healing process. In addition, factors such as growth factors or other proteins also may be provided that promote the wound healing process. The mechanical and material properties of biological scaffolds and the host tissue response to these biomaterials are believed to be influenced by the three dimensional configuration of the material and production processing methods. It further is believed that growth factors, surface topology and the distribution of surface ligands and modulation of the host innate immune response all contribute to the eventual functional performance of biological scaffolds for tissue repair or reconstruction. Tottey *et al.*, *Biomaterials* 32: 128-36 (2011).

[0011] In transplantation the use of human amniotic membrane (AM) has particular advantages, due to the structure of the relatively thick basement membrane, associated devitalized amniotic epithelial cells and stroma, and corresponding growth factor profile and structural protein composition. Meller *et al.*, *Dtsch Arztebl Int*'l 108: 243-8 (2011). For example, AM contains epidermal growth factor (EGF) and keratinocyte growth factor (KGF), which are important growth factors for promoting wound healing. In addition, laminin and

type VII collagen present in the AM elicit an epitheliotropic effect. AM also is thought to reduce the expression of various growth factors and pro-inflammatory cytokines while releasing anti-inflammatory cytokines such as IL-10, IL-1 receptor antagonists, thus modulating the inflammatory response favorably for wound healing. AM is immunoprivileged, moreover, likely by virtue of low MHC I expression, and so rejection of AM tissue is uncommon. These characteristics make AM an ideal substrate, for instance, with respect to ocular surface reconstruction, in pelvic reconstruction, and in the treatment of ulcers, among other wound-healing applications.

[0012] The use of conventional tissue scaffold products is not without drawbacks, however. Tissue harvesting from human donors can produce undesirable consequences such as donor site morbidity or infection associated with removal of skin for donation. Disease transmission risk and intersample variation are additional drawbacks associated with biological scaffold products. In addition, it may be difficult to obtain sufficient tissue components necessary to cover large areas of damaged tissue. Furthermore, conventional biological and synthetic materials can be costly, not effective in many instances, and limited in availability.

[0013] Accordingly, an abiding exists need for suitable tissue substrate biomaterial for use in transplantation to promote tissue regeneration while restoring functionality. Both the research industry and the medical transplant community would benefit from such a product that is readily available, does not impose additional complications to a donor or recipient (such as requiring an additional surgery to harvest the substrate), and exhibits all or some of the inherent material functionality reflective of the physiochemical, electrochemical, and biochemical properties of natural tissue.

SUMMARY OF THE INVENTION

[0014] The biomaterial of the present invention is obtained from tissue of a urodele. "Urodele" here denotes a salamander of the order Urodela, also known as the order Caudata, in the class Amphibia. In terms of phylogeny the relevant families include *Ambystomatidae*, *Cryptobranchidae*, *Amphiumidae*, *Proteidae*, and *Sirenidae*. See Wiens *et al.*, *Syst. Biol.* 54: 91-110 (2005), the contents of which are incorporated here by reference in their entirety. Accordingly, the urodele category includes, for example, the Pacific Giant Salamander

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(*Andrias davidianus*), the Tiger Salamander (*Ambystoma tigrinum*), and the Mexican Axolotl (*Ambystoma mexicanum*). The present inventor has recognized that the skin and ECM of urodeles possess desirable characteristics analogous to those of AM, making them an ideal source of biomaterial for xenotransplantation.

Urodele ECM and tissue regeneration

[0015] As amphibians, most urodeles begin life as aquatic animals in a larval state and undergo metamorphosis from a juvenile form with gills to an adult, terrestrial, air-breathing form with lungs. During metamorphosis, a urodele's physical features are altered in preparation for life on land. These alterations include caudal fin resorbtion, thickening of the skin, the development of dermal glands and resorption of gills. Sexual maturity also occurs during this time in most urodeles. Some families of urodeles are "neotenic," which means that individuals with such families can exhibit juvenile features, such as gills and fins, even after reaching sexually maturity. Indeed, neotenic urodeles often retain their aquatic (juvenile) form for the duration of their lives. Thus, the Mexican Axolotl normally remains in the neotenic state throughout its adult life although, under certain circumstances, it can undergo metamorphosis and transform into a terrestrial form.

[0016] Axolotls also are known for their ability to regenerate amputated body parts, which typically results in the complete restoration of both the structure and function of the damaged limb or organ. Aquatic axolotls undergo rapid re-epithelialization during wound healing and limb regeneration, both of which are scar-less processes. Similarly, metamorphic terrestrial axolotls retain several larval skin features and also exhibit scar-free wound healing, albeit at a slower rate than their aquatic, pre-metamorphic counterpart.

[0017] The healing process in axolotls varies from that observed in adult mammals. The axolotl process more closely resembles the scar-free healing process of fetal and embryonic wounds. Thus, such wounds likewise exhibit re-epithelialization and basement membrane reformation that occur at a faster rate (is "enhanced") than do the corresponding events in postnatal mammals.

[0018] Moreover, the cutaneous and subcutaneous structures of an axolotl resemble that of the amniotic/chorionic interface, in the sense that axolotl skin is composed of fused ectoderm and mesoderm. Axolotl skin also is rich in growth factors and antimicrobial peptides, similar

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to the AM. Furthermore, axolotl ECM is immunoprivileged and contains collagen III and tissue inhibitors of metalloproteinases (TIMPs), *inter alia*, also in resemblance to AM.

[0019] Of particular importance for transplantation purposes, more generally, is the immunologically privileged state of the human neonate (*e.g.*, fetal dermis) and the AM, a state mirrored by axolotl ECM, whereby immogenicity is rarely manifested. By virtue of the reduced immune response and the generally decreased inflammatory response as compared to adult humans, neonatal and axolotl skin healing alike are not characterized by accelerated tissue resorption, as is observed in adult human wound healing. Rather, the growth factor profile, enzymatic activity, structural composition and immunomodulating effect of urodele and neonate tissues alike favor an appropriately staged removal of structural scaffolding and tissue growth into the resulting negative void space, in addition to enhanced re-epithelialization, during wound healing. This results in an optimal wound healing environment and process. Also, the high concentration of antimicrobial peptides present in AM and urodele tissue further contributes to the favorable environment and enhanced re-epithelialization observed during wound healing.

[0020] A key aspect of the present invention is the inventor's recognition that the growth factor profile, connective tissue matrix constituents, and immunoprivileged status of urodele ECM and accompanying cutaneous tissue, plus the presence of antimicrobial peptides therein, render urodele-derived tissue an ideal source for biological scaffolds for xenotransplantation.

[0021] In accordance with the invention, therefore, a biological scaffold biomaterial is provided that is the product of a process comprising (A) obtaining a tissue sample from a urodele, where the tissue comprises ECM, inclusive of the basement membrane, and (B) subjecting the tissue sample to a decellularization process that maintains the structural and functional integrity of the extracellular matrix, by virtue of retaining its fibrous and non-fibrous proteins, glycoaminoglycans (GAGs) and proteoglycans, while removing sufficient cellular components of the sample to reduce or eliminate antigenicity and immunogenicity for xenograft purposes. Also provided is methodology for using the urodele-derived biomaterial to enhance restoration of skin homeostasis, to reduce the severity, duration and associated damage caused by post-surgical inflammation, and to promote progression of natural healing and regeneration processes. In addition, biomaterial of the invention promotes the formation

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of remodeled tissue that is comparable in quality, function and compliance to undamaged human tissue.

Decellularization

[0022] The biomaterial of the invention is produced by decellularizing a tissue sample obtained from a urodele. The primary constituent of the resulting urodele biomaterial is ECM, possibly with devitalized epithelial cells, which can retain moisture and otherwise protect the wound-healing environment.

[0023] Urodele skin is one example of an appropriate starting material for the present invention. Thus, the starting material that is subjected to decellularization can comprise urodele dermis and basement membrane, with or without epidermis. Even upon decellularization, moreover, the biomaterial of the invention can comprise, with the ECM, adjacent epithelial cells that may be rendered non-viable by the process. Alternatively, non-cutaneous urodele tissues can serve as the starting material of the invention, particularly those comprising a basement membrane or epithelial tissues that form the lining of various body cavities, *i.e.*, parietal mesothelial tissues found, for example in the thoracic cavity, the abdominal cavity, and pericardium. Tissues that contain substantial amounts of fibrous connective tissue, such as cartilage, tendon, dura mater and fascia, also are illustrative of appropriate starting materials of the present invention.

[0024] Effected via any conventional decellularization methodology, urodele tissue decellularization is performed to remove immunogenic cellular antigens that can induce an inflammatory response or immune-mediated tissue rejection, while preserving the structural integrity and composition of the associated ECM. Generally, ECM structural components, many if not all of which remain intact following decellularization, are well-tolerated by xenogeneic recipients. ECM components that may be present in the final biomaterial of the invention include proteins such as collagen (*e.g.*, fibrous collagen I and collagen III, as well non-fibrous collagen IV, collagen V and collagen VII), elastin, fibronectin, laminin, vitronectin, thrombosponsdins, osteopontin and tenascins, plus GAGs (*e.g.*,the proteoglycans, decoran and versican and sulfated GAGs, *e.g.*, heparin sulfate, keratan sulfate, dermatan sulfate and chondroitin sulfate) and growth factors such VEGF, BMP, TGF and FGF. For some indications the post-decellularization material comprises at least collagen IV, laminin,

sulfated GAGs and one or more growth factors in amounts that approximate pre-decellularization levels when viewed via histological and immunohistological staining.

[0025] Suitable techniques for decellularizing tissues, pursuant to the invention, include physical methods such as freezing, direct pressure application, sonication, and agitation. In addition or in the alternative, chemical methods can be employed, such as alkaline and acid treatments, application of detergents (including amphoteric, cationic, anionic and non-ionic detergents), organic solvents, hypotonic or hypertonic solutions and chelating agents. Enzymatic approaches including protease digestion and treatment with one or more nucleases also may be used to decellularize urodele tissue. In addition or alternatively, the urodele tissue is subjected to cleaning, sterilization, disinfection, antibiotic treatment and/or viral inactivation.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0026] In a preferred embodiment, urodele tissue is decellularized using alkaline treatment.

[0027] According to the invention, urodele tissue samples obtained for decellularization can be treated in the manner detailed in US 2008/0046095 or US 2010/0104539. Thus, tissue samples may be subjected to cleaning and chemical decontamination. In this manner, a tissue sample is washed for approximately 10 to 30 minutes in a sterile basin containing 18% NaCl (hyperisotonic saline) solution that is at or near room temperature. Visible cellular debris, such as epithelial cells adjacent to the tissue basement membrane, is gently scrubbed away using a sterile sponge to expose the basement membrane. Using a blunt instrument, a cell scraper or sterile gauze, any residual debris or contamination also is removed. Other techniques including, but not limited to, freezing the membrane, physical removal using a cell scraper, or exposing the cells to nonionic detergents, anionic detergents, and nucleases also may be used to remove cells.

[0028] The tissue is placed into a sterile container, such as a Nalgene jar, for the next step of chemical decontamination. Thus, each container is aseptically filled with 18% saline solution and sealed (or closed with a top). The containers then are placed on a rocker platform and agitated for between 30 and 90 minutes, which further cleans the tissue of contaminants.

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[0029] In a sterile environment using sterile forceps, the tissue is gently removed from the container containing the 18% hyperisotonic saline solution and placed into an empty container. This empty container with the tissue is then aseptically filled with a pre-mixed antibiotic solution. Preferably, the premixed antibiotic solution is comprised of a cocktail of antibiotics, such as Streptomycin Sulfate and Gentamicin Sulfate. Other antibiotics, such as Polymixin B Sulfate and Bacitracin, or similar antibiotics available now or in the future, are suitable as well. It is preferred that the antibiotic solution be at room temperature when added so that it does not change the temperature of or otherwise damage the tissue. This container containing the tissue and antibiotics is then sealed or closed and placed on a rocker platform and agitated for, preferably, between 60 and 90 minutes. Such rocking or agitation of the tissue within the antibiotic solution further cleans the tissue of contaminants and bacteria.

[0030] In a sterile environment, the container is opened and, using sterile forceps, the tissue is gently removed and placed in a sterile basin containing sterile water or normal saline (0.9% saline solution). The tissue is allowed to soak in place in the sterile water/normal saline solution for at least 10 to 15 minutes. The tissue may be slightly agitated to facilitate removal of the antibiotic solution and any other contaminants from the tissue.

[0031] In some cases, the present invention involves treating urodele tissue using a chemical sterilization methodology, as illustrated the Tutaplast® and Allowash® procedures, optionally in combination with mechanical processes that gently agitate chemical agents, as in the BioCleanse® system. Thus, urodele tissue is subjected to oxidative and/or alkaline treatments as well as osmotic treatment to break down cell walls, to inactivate pathogens, and to remove bacteria. In addition, tissue may be subjected to delipidization, solvent dehydration (to permit room temperature storage of tissue without damaging the collagen structure) and/or low-dose gamma irradiation to ensure sterility of the final product.

[0032] Efficient cell removal upon decellularization can be verified by various known means, including histological analyses to detect nuclear and cytoplasmic structures, immunohistochemical or immunofluorescent assaying for indicative intracellular proteins, and DNA detection. The nature of desirable components in the final urodele-derived scaffold biomaterial varies depending on the clinical indication being treated. Once a particular indication is identified, the knowledgeable clinician can determine which components in the

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urodele tissue sample should be retained in the final scaffold product, and standard methodology can be employed to ensure that the desired components are present following decellularization.

[0033] Samples may be viewed histologically before, during, and/or after decellularization to monitor the process and to confirm that the desired degree of cellular component removal is reached. For instance, tissues can be analyzed for cytoskeletal content to gauge sufficient decellularization. Intracellular protein content also may be assayed to determine if decellularization is sufficient. In addition, the tissue sample thickness and chemical makeup may be monitored to determine when sufficient decellularization has been achieved. Periodic monitoring during processing allows for a real time response to the observed tissue properties.

[0034] In some cases, a sufficiently decellularized tissue comprises no more than 50 ng dsDNA per mg ECM dry weight. Alternatively, for some indications, a sufficiently decellularized tissue lacks visible nuclear material in a tissue section stained with 4',6-diamindino-2-phenylindole (DAPI) or haematoxyilin and eosin (H&E).

[0035] In scenarios where removal of an adjacent epithelial cell layer is required, the presence or absence of epithelial cells remaining in the sample can be evaluated using techniques known in the art. For example, after removal of the epithelial cell layer, a representative tissue sample from the processing lot is placed onto a standard microscope examination slide. The tissue sample is then stained using Eosin Y Stain and evaluated as described below. The sample is then covered and allowed to stand. Once an adequate amount of time has passed to allow for staining, visual observation is done under magnification. The presence of cells and cellular material will appear darker than the areas which have been de-epithelialized.

[0036] Once cellular removal has progressed sufficiently, conventional methods are employed to confirm the retention of desired structural and functional properties of the remaining ECM scaffold. The specific structural testing that should be conducted depends on the intended clinical application of the final scaffold product. In some cases, the urodele tissue starting material may be monitored before, during, and after decellularization to ensure that the desired structural components and configuration are maintained in the final product.

[0037] One method for determining whether the desired ECM components are present involves staining parallel tissue sections and examining them histologically to determine whether the desired constituents and structural orientation of the urodele tissue have been preserved. For instance, urodele tissue can be stained with H&E and immunoperoxidase stain for laminin to assess preservation of ECM and laminin. In general, the three-dimensional configuration of ECM components remaining in the final biomaterial scaffold product should approximate that of pre-decellularized material when viewed via histological staining.

[0038] Accordingly, the urodele-derived biomaterial of the invention comprises ECM components useful for directing enhanced re-epithelialization and promoting efficient tissue regeneration or wound healing. The inventive biomaterial also serves as a matrix and reservoir for bioactive peptides such as growth factors and adhesion proteins. Accordingly, the biomaterial functions effectively as a biological scaffold for tissue regeneration, providing both the necessary bioactive stimulation and structural support.

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ABSTRACT

The growth factor profile, connective tissue matrix constituents, and immunoprivileged status of urodele extracellular matrix (ECM) and accompanying cutaneous tissue, plus the presence of antimicrobial peptides there, render urodele-derived tissue an ideal source for biological scaffolds for xenotransplantation. In particular, a biological scaffold biomaterial can be obtained by a process that entails (A) obtaining a tissue sample from a urodele, where the tissue comprises ECM, inclusive of the basement membrane, and (B) subjecting the tissue sample to a decellularization process that maintains the structural and functional integrity of the extracellular matrix, by virtue of retaining its fibrous and non-fibrous proteins, glycoaminoglycans (GAGs) and proteoglycans, while removing sufficient cellular components of the sample to reduce or eliminate antigenicity and immunogenicity for xenograft purposes. The resultant urodele-derived biomaterial can be used to enhance restoration of skin homeostasis, to reduce the severity, duration and associated damage caused by post-surgical inflammation, and to promote progression of natural healing and regeneration processes. In addition, the biomaterial promotes the formation of remodeled tissue that is comparable in quality, function, and compliance to undamaged human tissue.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Ryanne EARLY

Title: **DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE**

Appl. No.: Unknown

Filing Date: January 9, 2013

PROVISIONAL PATENT APPLICATION TRANSMITTAL

Commissioner for Patents
P.O. Box 1450
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Commissioner:

Transmitted herewith for filing under 37 C.F.R. § 1.53(c) is the provisional patent application of:

Ryanne Early
Gainesville, Florida 32605

[**X**] Applicant claims small entity status under 37 CFR 1.27(c)(1).

Enclosed are:

[**X**] Description, Claim(s), and Abstract (14 pages).

[**X**] Application Data Sheet (37 CFR 1.76).

The adjustment to the number of sheets for EFS-Web filing follows:

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[X]	Small Entity Fees Apply (subtract ½ of above):						=	$125.00
						TOTAL FILING FEE:	=	$125.00
Assignment Recordation Fee:					+	$40.00	=	$0.00
						TOTAL FEE	=	$125.00

The above-identified fees of $125 are paid by credit card via EFS-Web. The Commissioner is hereby authorized to charge any additional fees, as well as to credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment accompany this paper, then the Commissioner is authorized to charge the unpaid amount to the same deposit account.

Please direct all correspondence to the undersigned attorney at the indicated address.

Respectfully submitted,

Date January 9, 2013

By /Stephen A. Bent/

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DOCUMENT MADE AVAILABLE UNDER THE PATENT COOPERATION TREATY (PCT)

International application number:		**PCT/US2014/010890**
International filing date:		**09 January 2014 (09.01.2014)**
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	Filing date:	**09 January 2013 (09.01.2013)**
Date of receipt at the International Bureau:		**02 February 2014 (02.02.2014)**

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a),(b) or (b-*bis*)

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PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

To:
BENT, Stephen A.
FOLEY & LARDNER LLP, 3000 K Street NW, Suite 600
Washington, District of Columbia 20007
United States of America

Date of mailing *(day/month/year)* 15 May 2014 (15.05.2014)	
Applicant's or agent's file reference 101763-0103	**FOR FURTHER ACTION** See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US 2014/010890	09 January 2014 (09.01.2014)	09 January 2013 (09.01.2013)

International Patent Classification (IPC) or both national classification and IPC
A61K 35/56 *(2006.01)*
A61K 9/10 *(2006.01)*
A61K 9/14 *(2006.01)*
A61F 2/00 *(2006.01)*
A61L 27/36 *(2006.01)*

Applicant
EARLY, Ryanne

1. This opinion contains indications relating to the following items:

- [X] Box No. I Basis of the opinion
- [ ] Box No. II Priority
- [ ] Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- [ ] Box No. IV Lack of unity of invention
- [X] Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- [ ] Box No. VI Certain documents cited
- [X] Box No. VII Certain defects in the international application
- [ ] Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/ FIPS Russia, 123995, Moscow, G-59, GSP-5, Berezhkovskaya nab., 30-1 Facsimile No. +7 (499) 243-33-37	Date of completion of this opinion 26 March 2014 (26.03.2014)	Authorized officer E. Maslova Telephone No. (495)531-64-81

Form PCT/ISA/237 (cover sheet) (July 2011)

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 2014/010890

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:
   - [X] the international application in the language in which it was filed.
   - [ ] a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. [ ] This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing filed or furnished:

   a. (means)

      - [ ] on paper
      - [ ] in electronic form

   b. (time)

      - [ ] in the international application as filed
      - [ ] together with the international application in electronic form
      - [ ] subsequently to this Authority for the purposes of search

4. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 2014/010890

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	7, 11-14, 17-19, 26, 30-31	YES
	Claims	1-6, 8-10, 15-16, 20-25, 27-29, 32-33	NO
Inventive step (IS)	Claims		YES
	Claims	7, 11-14, 17-19, 26, 30-31	NO
Industrial applicability (IA)	Claims	1-33	YES
	Claims		NO

2. Citations and explanations:

D1 WO 2007124127 A2 01.11.2007

D2 WO 2005009498 A2 03.02.2005

D1 discloses a method of preparing a bioscaffold with the use of decellularizing a tissue, during which an excess material is removed from a tissue sample (claims 1, 21). A tissue of amphibians can be used as the tissue. The representative of the amphibians is the order of Urodele (p. 8, paragraphs 3, 5). When preparing the bioscaffold, it maintains its natural biomechanical integrity (p. 7, paragraph 4; p. 2, last paragraph; p. 3, paragraph 3). Decellularizing the tissue is implemented in order to reduce an inflammatory reaction and immune response (p. 7, paragraph 5). The bioscaffold is used as a transplant, an implant (p. 4, paragraphs 4, 6, claim 18). The bioscaffold can be placed in a package (claim 17). The bioscaffold can comprise growth factors present in a culture medium (p. 9, paragraph 4).

Therefore, the inventions in claims 1, 5, 6, 16, 20, 22, 23, 25, 27, 28 do not meet the criterion of novelty.

D2 discloses a device having a layer of decellularized ECM (claims 30-31).

The invention in independent claim 26 differs from D1 in that the tissue of Urodele is used; the device comprises at least two sheets.

As D1 discloses that a tissue of amphibians is used when preparing decellularized ECM, the use of the tissue of Urodele when preparing ECM is obvious for a person skilled in the art, wherein the application of the necessary number of sheets on the device is also obvious for a person skilled in the art.

Therefore, the invention in independent claim 26 does not meet the criterion of inventive step.

D1 discloses the features of dependent claims 2-4, 8-10, 15, 21, 24, 29, 32-33 relative to decellularizing implemented using, for example, an alkaline treatment (p. 15, paragraphs 1, 2). D1 discloses a bioscaffold that can be sterilized (claim 15); the bioscaffold can comprise human cells,

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Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of V:

growth factors (p. 4, paragraph 5; p. 9, paragraph 4). The bioscaffold also can be used as a transplant, an implant (p. 4, paragraphs 4, 6, claim 18). When preparing the bioscaffold, it maintains its natural biomechanical integrity (p. 7, paragraph 4; p. 2, last paragraph; p. 3, paragraph 3).

Therefore, the inventions in dependent claims 2-4, 8-10, 15, 21, 24, 29, 32-33 do not meet the criterion of novelty.

D2 discloses the features of dependent claims 11-14, 17-19, 30-31 relative to a composition comprising the decellularized ECM that can be made in the form of a sheet (p. 26, lines 13-15) or a gel (p. 13, lines 31-33). The composition can be a powder (p. 16, lines 28-29).

The use of Axolotl tissue in dependent claim 7 is obvious for a person skilled in the art, as D1 (p. 8, paragraphs 3, 5) discloses the use of a tissue of amphibians.

Therefore, the inventions in dependent claims 7, 11-14, 17-19, 30-31 do not meet the criterion of inventive step.

The inventions in claims 1-33 meet the criterion of industrial applicability.

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 2014/010890

Box No. VII Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Dependence of the following claims is incorrect:

- claims 17-19 are dependent on independent claim 16;
- claim 21 is dependent on independent claim 20;
- claim 24 is dependent on independent claim 22.

VIII-3-1	**Declaration: Entitlement to claim priority** Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application specified below, where the applicant is not the applicant who filed the earlier application or where the applicant's name has changed since the filing of the earlier application (Rules 4.17(iii) and 51bis.1(a)(ii)) Name	In relation to this international application EARLY, Ryanne is entitled to claim priority of earlier application No. 61/750,555 by virtue of the following:
VIII-3-1(i)		the applicant is the inventor of the subject matter for which protection was sought by way of the earlier application

VIII-2-1	**Declaration: Entitlement to apply for and be granted a patent** Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent (Rules 4.17(ii) and 51bis.1(a)(ii)), in a case where the declaration under Rule 4.17(iv) is not appropriate: Name (LAST, First)	In relation to this international application EARLY, Ryanne is entitled to apply for and be granted a patent by virtue of the following:
VIII-2-1(i)		EARLY, Ryanne of c/o SEED Biotech, Inc. 3116 Commerce St., Suite A Dallas, TX 75226 United States of America is the inventor of the subject matter for which protection is sought by way of this international application

ADVANCE E-MAIL

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92*bis*.1 and Administrative Instructions, Section 422)

To:

GATES, Edward R.
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
ETATS-UNIS D'AMERIQUE

Date of mailing *(day/month/year)*
19 November 2014 (19.11.2014)

Applicant's or agent's file reference
S1860.70000

IMPORTANT NOTIFICATION

International application No.
PCT/US2014/010890

International filing date *(day/month/year)*
09 January 2014 (09.01.2014)

1. The following indications appeared on record concerning:

☐ the applicant ☐ the inventor ☒ the agent ☐ the common representative

Name and Address

State of Nationality | State of Residence

Telephone No.

Facsimile No.

E-mail address

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☒ the person ☐ the name ☐ the address ☐ the nationality ☐ the residence

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☒ Notifications by e-mail authorized

3. Further observations, if necessary:
The new agent's file reference is: S1860.70000

4. A copy of this notification has been sent to:

☒ the receiving Office
☐ the International Searching Authority
☐ the Authority(ies) specified for supplementary search
☐ the International Preliminary Examining Authority
☒ the designated Offices concerned
☐ the elected Offices concerned
☐ other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

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A61K 9/14 (2006.01)

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61/750,555 9 January 2013 (09.01.2013) US

(72) Inventor; and
(71) Applicant : **EARLY, Ryanne** [US/US]; 1909 Hickory Trace Dr., Fleming island, FL 32003 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to the identity of the inventor (Rule 4.17(i))*

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

(54) **Title**: DECELLULARIZED BIOMATERIAL FORM NON-MAMMALIAN TISSUE

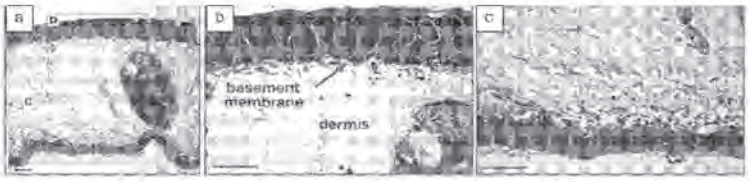


FIGURE 1

(57) **Abstract**: The growth factor profile, connective tissue matrix constituents, and immunoprivileged status of urodele extracellular matrix (ECM) and accompanying cutaneous tissue, plus the presence of antimicrobial peptides there, render urodele-derived tissue an ideal source for biological scaffolds for xenotransplantation. In particular, a biological scaffold biomaterial can be obtained by a process that entails (A) obtaining a tissue sample from a urodele, where the tissue comprises ECM, inclusive of the basement membrane, and (B) subjecting the tissue sample to a decellularization process that maintains the structural and functional integrity of the extracellular matrix, by virtue of retaining its fibrous and non-fibrous proteins, glycoaminoglycans (GAGs) and proteoglycans, while removing sufficient cellular components of the sample to reduce or eliminate antigenicity and immunogenicity for xenograft purposes. The resultant urodele-derived biomaterial can be used to enhance restoration of skin homeostasis, to reduce the severity, duration and associated damage caused by post-surgical inflammation, and to promote progression of natural healing and regeneration processes. In addition, the biomaterial promotes the formation of remodeled tissue that is comparable in quality, function, and compliance to undamaged human tissue.

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DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 USC § 119(e) to U.S. provisional application 61/750,555, filed January 9, 2013, the entire contents of which are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

[0002] Tissue engineering efforts are ongoing to produce methods and materials for replacing biological functions, typically repairing or replacing whole tissues or portions thereof. In this regard, wound treatment and skin repair are areas of predominant focus, as the loss of skin integrity due to illness or injury can lead to chronic, life threatening complications.

[0003] Wound healing involves complex interactions between cells, growth factors, and extracellular matrix (ECM) components to reconstitute tissue following injury. The wound healing process in adult mammalian tissue has been well characterized and can be broken down into three stages - inflammation, proliferation, and remodeling.

[0004] Typically, in response to an incision or trauma the body conveys blood, blood products, and proteins into the void (also referred to as the cavity or negative space) formed at the wound. During early inflammation, a wound exudate begins to form under the influence of inflammatory mediators and as a result of vasodilation. Fibrin and fibronectin present in clotting blood provide a scaffold over which cells such as keratinocytes, platelets and leukocytes migrate to the wound site. Bacteria and debris are phagocytosed and removed, and growth factors are released that stimulate the migration and division of fibroblasts.

[0005] The subsequent stage of wound healing involves new tissue formation as fibrous connective tissue, termed granulation tissue (composed of fibroblasts, macrophages and neovasculature) replaces the fibrin clot. New blood vessels are formed during this stage, and fibroblasts proliferate and produce a provisional ECM by excreting collagen and fibronectin. Nearly all mammalian cells require adhesion to a surface in order to proliferate and function properly. The ECM fulfills this function. Initially, the provisional ECM contains of a network of Type III collagen, a weaker form of collagen that is rapidly produced. This is

later replaced by the stronger Type I collagen (which contributes to scar formation). At the same time, re-epithelialization of the epidermis occurs. During this process, epithelial cells proliferate and migrate over the newly forming tissue as proteases such as metalloportineaes (MMPs) and collagenases at the leading edge of the migrating cells help to invade the clot. These enzymes in addition to growth factor signaling (cell-cell interactions) and cell-ECM interactions (signal transduction from interactions between cells, integrins (cell surface receptors), laminin, collagen, fibronectin, and other ECM proteins) stimulate cell migration into the wound and ECM degradation.

[0006] Finally, in the remodeling phase, collagen is remodeled and realigned along tension lines and cells that are no longer needed are removed by apoptosis. Wound contraction occurs as fibroblasts transform into myofibroblasts through their interactions with ECM proteins and growth factors. Myofibroblasts then interact with collagen, vitronectin, and other ECM proteins to contract the wound. As the remodeling phase proceeds, fibronectin and hyaluronic acid are replaced by collagen bundles that lend strength to the tissue.

[0007] By applying biological, chemical and engineering principles to tissue repair and regeneration, tissue engineers have developed transplantable products for use in promoting the tissue repair and regeneration processes. The ability to restore biomechanical function of damaged tissue presents a true challenge. In response, both synthetic and biological scaffold products have been developed that mimic(to some extent) tissue structure and mechanical behavior to promote tissue repair. Such products serve as a temporary replacement, both mechanically and functionally, for damaged, diseased or absent tissue.

[0008] Ideally, transplantable scaffold products should support cell adhesion, proliferation and differentiation and act as an interim synthetic extracellular matrix (ECM) for cells prior to the formation of new tissue. Scaffold materials should be biocompatible, biodegradable and exhibit low antigenicity. The implant should degrade at a rate roughly equal to that of the new tissue formation. Once implanted, the scaffold must have the mechanical properties necessary to temporarily offer structural support until the new tissue has formed. Additionally, scaffold products must be porous, providing an appropriate path for nutrient transmission and tissue ingrowth. Tissue scaffolds also should promote fast healing and facilitate the development or regeneration of new tissue that resembles normal host tissue in both appearance and function. To this end, implanted scaffold products should offer (i)

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bioactive stimulation, e.g., protein and molecular signaling, to encourage cell migration, proliferation and differentiation, and (ii) mechanical or structural support for these processes.

[0009] Today, the development of synthetic scaffolds is an area of active research. Synthetic scaffolds have been manufactured from chemical compounds such as fibrous polymers, gelatin, apatite, and polymer/ceramic composites, polylactic acid, collagen. These scaffolds are designed to mimic the structure of the naturally occurring ECM and have shown some success in bone tissue engineering.

[0010] In addition to synthetic scaffold products, biological scaffolds obtained from mammalian tissues are commercially available for use as allografts (transplanted cell or tissue from a donor of the same species) or xenografts (transplanted cells or tissue from a donor of a different species). Biological scaffolds are composed of mammalian ECM harvested from, for example, dermis, urinary bladder, small intestine, mesothelium, pericardium, bone or aminiotic membrane of various mammals including human (either live donor or cadaver), porcine, bovine and equine. These commercially available products are commonly used for the repair and reconstruction of injured or missing tissues and organs such as soft tissue, tendons, cardiac tissue, neural tissue, chronic wounds, dura mater, bone and cornea.

[0011] Biological scaffold products may comprise skin cells in addition to extracellular matrices produced by tissue and subjected to a decellularization process. They are contacted with a wound site to give mechanical support for cell migration and proliferation as part of the wound healing process. In addition, factors such as growth factors or other proteins also may be provided that promote the wound healing process. The mechanical and material properties of biological scaffolds and the host tissue response to these biomaterials are believed to be influenced by the three dimensional configuration of the material and production processing methods. It further is believed that growth factors, surface topology and the distribution of surface ligands and modulation of the host innate immune response all contribute to the eventual functional performance of biological scaffolds for tissue repair or reconstruction. Tottey *et al.*, *Biomaterials* 32: 128-36 (2011).

[0012] In transplantation the use of human amniotic membrane (AM) has particular advantages, due to the structure of the relatively thick basement membrane, associated devitalized amniotic epithelial cells and stroma, and corresponding growth factor profile and structural protein composition. Meller *et al.*, *Dtsch Arztebl Int*'l 108: 243-8 (2011). For example, AM contains epidermal growth factor (EGF) and keratinocyte growth factor (KGF),

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which are important growth factors for promoting wound healing. In addition, laminin and type VII collagen present in the AM elicit an epitheliotropic effect. AM also is thought to reduce the expression of various growth factors and pro-inflammatory cytokines while releasing anti-inflammatory cytokines such as IL-10, IL-1 receptor antagonists, thus modulating the inflammatory response favorably for wound healing. AM is immunoprivileged, moreover, likely by virtue of low MHC I expression, and so rejection of AM tissue is uncommon. These characteristics make AM an ideal substrate, for instance, with respect to ocular surface reconstruction, in pelvic reconstruction, and in the treatment of ulcers, among other wound-healing applications.

[0013] The use of conventional tissue scaffold products is not without drawbacks, however. Tissue harvesting from human donors can produce undesirable consequences such as donor site morbidity or infection associated with removal of skin for donation. Disease transmission risk and intersample variation are additional drawbacks associated with biological scaffold products. In addition, it may be difficult to obtain sufficient tissue components necessary to cover large areas of damaged tissue. Furthermore, conventional biological and synthetic materials can be costly, not effective in many instances, and limited in availability.

[0014] Accordingly, an abiding exists need for suitable tissue substrate biomaterial for use in transplantation to promote tissue regeneration while restoring functionality. Both the research industry and the medical transplant community would benefit from such a product that is readily available, does not impose additional complications to a donor or recipient (such as requiring an additional surgery to harvest the substrate), and exhibits all or some of the inherent material functionality reflective of the physiochemical, electrochemical, and biochemical properties of natural tissue.

SUMMARY OF THE INVENTION

[0015] The biomaterial of the present invention is obtained from tissue of a urodele. "Urodele" here denotes a salamander of the order Urodela, also known as the order Caudata, in the class Amphibia. In terms of phylogeny the relevant families include *Ambystomatidae*, *Cryptobranchidae*, *Amphiumidae*, *Proteidae*, and *Sirenidae*. See Wiens *et al.*, *Syst. Biol.* 54: 91-110 (2005), the contents of which are incorporated here by reference in their entirety. Accordingly, the urodele category includes, for example, the Pacific Giant Salamander

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(*Andrias davidianus*), the Tiger Salamander (*Ambystoma tigrinum*), and the Mexican Axolotl (*Ambystoma mexicanum*). The present inventor has recognized that the skin and ECM of urodeles possess desirable characteristics analogous to those of AM, making them an ideal source of biomaterial for xenotransplantation.

Urodele ECM and tissue regeneration

[0016] As amphibians, most urodeles begin life as aquatic animals in a larval state and undergo metamorphosis from a juvenile form with gills to an adult, terrestrial, air-breathing form with lungs. During metamorphosis, a urodele's physical features are altered in preparation for life on land. These alterations include caudal fin resorbtion, thickening of the skin, the development of dermal glands and resorption of gills. Sexual maturity also occurs during this time in most urodeles. Some families of urodeles are "neotenic," which means that individuals with such families can exhibit juvenile features, such as gills and fins, even after reaching sexually maturity. Indeed, neotenic urodeles often retain their aquatic (juvenile) form for the duration of their lives. Thus, the Mexican Axolotl normally remains in the neotenic state throughout its adult life although, under certain circumstances, it can undergo metamorphosis and transform into a terrestrial form.

[0017] Axolotls also are known for their ability to regenerate amputated body parts, which typically results in the complete restoration of both the structure and function of the damaged limb or organ. Aquatic axolotls undergo rapid re-epithelialization during wound healing and limb regeneration, both of which are scar-less processes. Similarly, metamorphic terrestrial axolotls retain several larval skin features and also exhibit scar-free wound healing, albeit at a slower rate than their aquatic, pre-metamorphic counterpart.

[0018] The healing process in axolotls varies from that observed in adult mammals. The axolotl process more closely resembles the scar-free healing process of fetal and embryonic wounds. Thus, such wounds likewise exhibit re-epithelialization and basement membrane reformation that occur at a faster rate (is "enhanced") than do the corresponding events in postnatal mammals.

[0019] Moreover, the cutaneous and subcutaneous structures of an axolotl resemble that of the amniotic/chorionic interface, in the sense that axolotl skin is composed of fused ectoderm and mesoderm. Axolotl skin also is rich in growth factors and antimicrobial peptides, similar

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to the AM. Furthermore, axolotl ECM is immunoprivileged and contains collagen III and tissue inhibitors of metalloproteinases (TIMPs), *inter alia*, also in resemblance to AM.

[0020] Of particular importance for transplantation purposes, more generally, is the immunologically privileged state of the human neonate (*e.g.*, fetal dermis) and the AM, a state mirrored by axolotl ECM, whereby immogenicity is rarely manifested. By virtue of the reduced immune response and the generally decreased inflammatory response as compared to adult humans, neonatal and axolotl skin healing alike are not characterized by accelerated tissue resorption, as is observed in adult human wound healing. Rather, the growth factor profile, enzymatic activity, structural composition and immunomodulating effect of urodele and neonate tissues alike favor an appropriately staged removal of structural scaffolding and tissue growth into the resulting negative void space, in addition to enhanced re-epithelialization, during wound healing. This results in an optimal wound healing environment and process. Also, the high concentration of antimicrobial peptides present in AM and urodele tissue further contributes to the favorable environment and enhanced re-epithelialization observed during wound healing.

[0021] A key aspect of the present invention is the inventor's recognition that the growth factor profile, connective tissue matrix constituents, and immunoprivileged status of urodele ECM and accompanying cutaneous tissue, plus the presence of antimicrobial peptides therein, render urodele-derived tissue an ideal source for biological scaffolds for xenotransplantation.

[0022] In accordance with the invention, therefore, a biological scaffold biomaterial is provided that is the product of a process comprising (A) obtaining a tissue sample from a urodele, where the tissue comprises ECM, inclusive of the basement membrane, and (B) subjecting the tissue sample to a decellularization process that maintains the structural and functional integrity of the extracellular matrix, by virtue of retaining its fibrous and non-fibrous proteins, glycoaminoglycans (GAGs) and proteoglycans, while removing sufficient cellular components of the sample to reduce or eliminate antigenicity and immunogenicity for xenograft purposes. Also provided is methodology for using the urodele-derived biomaterial to enhance restoration of skin homeostasis, to reduce the severity, duration and associated damage caused by post-surgical inflammation, and to promote progression of natural healing and regeneration processes. In addition, biomaterial of the invention promotes the formation

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of remodeled tissue that is comparable in quality, function and compliance to undamaged human tissue.

Decellularization

[0023] The biomaterial of the invention is produced by decellularizing a tissue sample obtained from a urodele. The primary constituent of the resulting urodele biomaterial is ECM, possibly with devitalized epithelial cells, which can retain moisture and otherwise protect the wound-healing environment.

[0024] Urodele skin is one example of an appropriate starting material for the present invention. Thus, the starting material that is subjected to decellularization can comprise urodele dermis and basement membrane, with or without epidermis. Even upon decellularization, moreover, the biomaterial of the invention can comprise, with the ECM, adjacent epithelial cells that may be rendered non-viable by the process. Alternatively, non-cutaneous urodele tissues can serve as the starting material of the invention, particularly those comprising a basement membrane or epithelial tissues that form the lining of various body cavities, *i.e.*, parietal mesothelial tissues found, for example in the thoracic cavity, the abdominal cavity, and pericardium. Tissues that contain substantial amounts of fibrous connective tissue, such as cartilage, tendon, bone, dura mater and fascia, also are illustrative of appropriate starting materials of the present invention.

[0025] Effected via any conventional decellularization methodology, urodele tissue decellularization is performed to remove immunogenic cellular antigens that can induce an inflammatory response or immune-mediated tissue rejection, while preserving the structural integrity and composition of the associated ECM. Generally, ECM structural components, many if not all of which remain intact following decellularization, are well-tolerated by xenogeneic recipients. ECM components that may be present in the final biomaterial of the invention include proteins such as collagen (*e.g.*, fibrous collagen I and collagen III, as well non-fibrous collagen IV, collagen V and collagen VII), elastin, fibronectin, laminin, vitronectin, thrombosponsdins, osteopontin and tenascins, plus GAGs (*e.g.*, the proteoglycans, decoran and versican and sulfated GAGs, *e.g.*, heparin sulfate, keratan sulfate, dermatan sulfate and chondroitin sulfate) and growth factors such VEGF, BMP, TGF and FGF. For some indications the post-decellularization material comprises at least collagen IV, laminin,

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sulfated GAGs and one or more growth factors in amounts that approximate pre-decellularization levels when viewed via histological and immunohistological staining.

[0026] Suitable techniques for decellularizing tissues, pursuant to the invention, include physical methods such as freezing, direct pressure application, sonication, and agitation. In addition or in the alternative, chemical methods can be employed, such as alkaline and acid treatments, application of detergents (including amphoteric, cationic, anionic and non-ionic detergents), organic solvents, hypotonic or hypertonic solutions and chelating agents. Enzymatic approaches including protease digestion and treatment with one or more nucleases also may be used to decellularize urodele tissue. In addition or alternatively, the urodele tissue is subjected to cleaning, sterilization, disinfection, antibiotic treatment and/or viral inactivation.

[0027] According to one aspect of the invention, a biomaterial is provided. The material is produced by the process that includes (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft. In some embodiments, decellularizing comprises subjecting said tissue sample to an alkaline treatment. In embodiments, the process can further comprise subjecting said sample to sterilization. In embodiments, the process can further comprise devitalizing cells.

[0028] According to one aspect of the invention, a tissue graft is provided. The graft includes extracellular matrix components derived from a urodele. In embodiments, the extracellular matrix components are substantially free of components that induce an immune response when implanted as a xenograft. In embodiments, the extracellular matrix components are non-toxic.

[0029] According to one aspect of the invention, a decellularized Urodele ECM is provided. In any of the embodiments, the decellularized ECM can be derived from Axolotl tissue. In any of the embodiments, the decellularized ECM can include basement membrane. In any of the embodiments, the decellularized ECM can be infused with, coated with, combined with or attached, covalently or non-coalently, to an agent xenogenic to a Urodele. In any of the embodiments, the agent can be any one or any combination of a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, a drug and/or a human agent (i.e., an agent found in a human,

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isolated, synthetically or recombinantly produced). Further such agents forming part of the invention are described in more detail below. In some embodiments, the agent is a cell, optionally a human cell. In some embodiments, the agent is a progenitor cell, optionally a human progenitor cell. Further such cells forming part of the invention are described in more detail below. In any of the embodiments, the decellularized ECM can take on any variety of shapes, as the material can be formed, laminated, homogenized, gelled, etc. In some embodiments, the ECM is a sheet. The sheet optionally can include perforations. The sheet optionally can include a backing and/or an adhesive. The backing may be biodegradable or may be non-biodegradable. In some embodiments, the decelularized ECM is a dry powder. In some embodiments the decelularized ECM is a reconstituted gel. In any of the embodiments, the ECM can be sterile.

[0030] According to one aspect of the invention, a package is provided. The package contains sterile, decellularized Urodele ECM or a Urodele fraction derived from the sterile, decellularized Urodele ECM. The package can contain any of the ECMs described above. For example, the package can include a sheet, a dry powder or a reconstituted gel of decellularized Urodele ECM. The package can contain any product, for example any implant, that comprises sterile, decellularized Urodele ECM or a Urodele fraction derived from the sterile, decellularized Urodele ECM. Such an implant may be made in whole or only in minor part of the ECM of the invention.

[0031] The invention also provides a sterile medical implant comprising decellularized Urodele ECM or a Urodele fraction derived from the decellularized Urodele ECM. Examples of such medical implants include a biocompatible sheet, mesh, gel, graft, plug, tissue or device. Devices include, for example, coated stents, bone replacements, joint replacements, implantable hardware and the like. The implant can be fabricated entirely or in part from the ECM. The implant also can encapsulate, can be infused with, coated with, impregnated with, laminated with, or covalently or non-covalently attached to the ECM of the invention.

[0032] The invention also provides a material, the material being coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM or a Urodele fraction derived from the isolated, decellularized Urodele ECM. The material can be natural or synthetic. Examples are metals, plastics, ceramics and fibers.

[0033] According to one aspect of the invention, a tissue culture system is provided. The system comprises (a) an isolated Urodele decellularized ECM, (b) tissue culture medium, and

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(c) cells xenogenic to the Urodele. The cells may be from animal, and in some embodiments, the cell is a mammalian cell. The cell can be any type of cell capable of culture. In embodiments, the cell is a human cell, optionally a progenitor cell.

[0034] According to one aspect of the invention, a conditioned tissue culture medium is provided. The medium, which can be any commonly used in liquid tissue culture, is conditioned with isolated Urodele decellularized ECM or a Urodele fraction derived from isolated, decellularized Urodele ECM. Numerous liquid tissue culture media are commercially available and well known to those of ordinary skill in the art.

[0035] According to one aspect of the invention, a device is provided. The device is at least two sheets of isolated Urodele decellularized ECM laminated to one another. The sheets can be from the same or different tissue. The sheets can an orientation and can be oriented in the same direction or oriented at angles to one another. The sheets can further comprise any agent xenogenic to the Urodele, which agent may be coated on, infused or impregnated within, or otherwise attached to one or more of the laminated sheets.

[0036] In any embodiment described above involving a sheet, the sheet may further comprise a backing and/or adhesive.

[0037] According to one aspect of the invention, a product is provided. The product is prepared by isolating Urodele ECM from a Urodele, decellularizing the ECM, and sterilizing the decellularized ECM. The preparation of the device can further involve any one or more of the following steps (presented in no particular order): forming the ECM into a shape, homogenizing the ECM, laminating the ECM to a material, combining agents with the ECM such as by coating, impregnating, or otherwise attaching the agent to the ECM, and so on.

[0038] According to one aspect of the invention, a method of preparing a biologic material is provided. The method involves (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample to remove sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft. In embodiments, the method can further involve performing the decellularization in a manner to retain structural and functional integrity of the ECM sufficient to permit the ECM to be useful as a matrix upon and within which cells can grow. In any of the embodiments, the method can further involve homogenizing the ECM to form a particulate or powder. In some embodiments, the method can further involve reconstituting the powder as a gel. In any of the embodiments, the method can further involve sterilizing the

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ECM. In any of the embodiments, the method can further involve attaching the ECM to an agent xenogenic to a Urodele.

[0039] According to another aspect of the invention, the materials such as implants, devices, sheets, gels and powders can be used in methods for treating subjects, where the materials are applied to wounds, surgical beds, and to internal and external tissues, generally, to prevent adhesion, provide tissue support, for example for suturing tissue, for treating a hernia or as a tissue plug, for treating burns and derm-abrasion, as well as other conditions described below.

[0040] In any of the embodiments above, the ECM is or can be isolated.

BRIEF DESCRIPTION OF THE FIGURES

[0041] FIGURE 1 shows native axolotl tissue samples prepared for histological examination, to identify EC elements.

[0042] FIGURE 2 hematoxylin and eosin (H&E) and Alcian Blue staining of native axolotl dermal tissue and human amniotic membrane (40x magnification).

[0043] FIGURE 3 shows immunohistocemical staining via species-specific collagen IV and laminin antibodies of native axolotl dermal tissue and human amniotic membrane tissue, at 40× magnification.

[0044] FIGURE 4 depicts axolotl skin and human amniotic membrane samples prepared for histological examination, to identify EC elements.

[0045] FIGURE 5 illustrates a histological evaluation of H&E-stained, paired native and post-processed sections of axolotl dermal tissue.

[0046] FIGURE 6 presents ELISA data for DNA content in pre- and post-processed axotol tissue, with comparison data from human amniotic membrane tissue.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

[0047] "Antimicrobial polypeptides" (or "AMPs") means small peptides of variable length, sequence and structure with broad spectrum activity against a wide range of microorganisms including bacteria, viruses, fungi, protozoa, parasites, prions, and tumor/cancer cells. (See, e.g., Zaiou, J Mol Med, 2007; 85:317; herein incorporated by reference in its entirety). AMPs have broad-spectrum of rapid onset of killing activities, with potentially low levels of induced resistance and concomitant broad anti-inflammatory effects.

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Anti-microbial polypeptides include defensins, such as a-defensins (e.g., neutrophil defensin 1, defensin alpha 1, neutrophil defensin 3, neutrophil defensin 4, defensin 5, defensin 6), β-defensins (e.g., beta-defensin 1, beta-defensin 2, beta-defensin 103, beta-defensin 107, beta-defensin 110, beta-defensin 136), and θ-defensins. Anti-microbial polypeptides include cathelicidins such as hCAP18.

[0048] "Biocompatible" means that a composition and its normal degradation products *in vivo* are substantially non-toxic and non-carcinogenic in a subject within useful, practical and/or acceptable tolerances.

[0049] "Cytocompatible" means that a composition can sustain the viability and growth of a population of cells.

[0050] "Decellularized ECM" means extra cellular matrix sufficiently free of cellular components to eliminate or reduce antigenicity of the extra cellular matrix to an extent where the matrix would be considered non-toxic as a xenograft.

[0051] "Isolated" when used in connection with the ECM of the invention means separated from other Urodele tissue.

[0052] "Non-toxic" means that a composition, when implanted in a subject, causes little or no adverse reaction or substantial harm to cells and tissues in the body, and does not cause a substantial adverse reaction or substantial harm to cells and tissues in the body, for instance, the composition does not cause necrosis, an infection, or a substantial immune response resulting in harm to tissues from the implanted or applied composition.

[0053] "Progenitor cell" means a cell that can differentiate under certain conditions into a more-differentiated cell type. Non-limiting examples of progenitor cells include stem cells that may be totipotent, pluripotent, multipotent stem cells, or referred to as progenitor cells. Additional non-limiting examples of progenitor cells include perivascular stem cells, blastema cells, and multilineage progenitor cells.

[0054] "Retain structural and functional integrity" used in connection with the ECM of the invention means retaining sufficient structure and function to permit and support the use of the matrix as a substrate for the growth of cells *in vivo* or *in vitro*.

[0055] "Subject" means an animal. In some embodiments the animal is a mammal. The mammal can be a dog, cat, a horse, a cow, a goat, a sheep, a pig or a non-human primate. In any embodiment the mammal can be a human.

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[0056] "Treatment" or "treating" means administration or application to a subject by any suitable dosage means, regimen and route of a composition with the object of achieving a desirable clinical/medical end-point, such as assisting in wound healing, tissue closure, bulking tissue, preventing tissue adhesion, providing structural support to tissue, providing a protective barrier, correcting a defect, etc.

[0057] "Urodele fraction derived from decellularized Urodele ECM" means an extract or isolate of decellularized Urodele ECM maintaining sufficient characteristics of a Urodele in terms of chemical structure and/or relative chemical concentrations of two (or three, or four, or five or more) chemical entities in the extract or isolate to distinguish the extract as obtained from a Urodele by any one or more of electron microscopy, HPLC, immunohistochemistry, and the like.

General Preparative Methodology

[0058] According to the invention, urodele tissue samples obtained for decellularization can be treated in the manner detailed in US 2008/0046095 or US 2010/0104539. Thus, tissue samples may be subjected to cleaning and chemical decontamination. In this manner, a tissue sample is washed for approximately 10 to 30 minutes in a sterile basin containing 18% NaCl (hyperisotonic saline) solution that is at or near room temperature. Visible cellular debris, such as epithelial cells adjacent to the tissue basement membrane, is gently scrubbed away using a sterile sponge to expose the basement membrane. Using a blunt instrument, a cell scraper or sterile gauze, any residual debris or contamination also is removed. Other techniques including, but not limited to, freezing the membrane, physical removal using a cell scraper, or exposing the cells to nonionic detergents, anionic detergents, and nucleases also may be used to remove cells.

[0059] In one embodiment, urodele tissue is decellularized using alkaline treatment.

[0060] The tissue is placed into a sterile container, such as a Nalgene jar, for the next step of chemical decontamination. Thus, each container is aseptically filled with 18% saline solution and sealed (or closed with a top). The containers then are placed on a rocker platform and agitated for between 30 and 90 minutes, which further cleans the tissue of contaminants.

[0061] In a sterile environment using sterile forceps, the tissue is gently removed from the container containing the 18% hyperisotonic saline solution and placed into an empty container. This empty container with the tissue is then aseptically filled with a pre-mixed

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antibiotic solution. Preferably, the premixed antibiotic solution is comprised of a cocktail of antibiotics, such as Streptomycin Sulfate and Gentamicin Sulfate. Other antibiotics, such as Polymixin B Sulfate and Bacitracin, or similar antibiotics available now or in the future, are suitable as well. It is preferred that the antibiotic solution be at room temperature when added so that it does not change the temperature of or otherwise damage the tissue. This container containing the tissue and antibiotics is then sealed or closed and placed on a rocker platform and agitated for, preferably, between 60 and 90 minutes. Such rocking or agitation of the tissue within the antibiotic solution further cleans the tissue of contaminants and bacteria.

[0062] In a sterile environment, the container is opened and, using sterile forceps, the tissue is gently removed and placed in a sterile basin containing sterile water or normal saline (0.9% saline solution). The tissue is allowed to soak in place in the sterile water/normal saline solution for at least 10 to 15 minutes. The tissue may be slightly agitated to facilitate removal of the antibiotic solution and any other contaminants from the tissue.

[0063] In some cases, the present invention involves treating urodele tissue using a chemical sterilization methodology, as illustrated the Tutaplast® and Allowash® procedures, optionally in combination with mechanical processes that gently agitate chemical agents, as in the BioCleanse® system. Thus, urodele tissue is subjected to oxidative and/or alkaline treatments as well as osmotic treatment to break down cell walls, to inactivate pathogens, and to remove bacteria. In addition, tissue may be subjected to delipidization, solvent dehydration (to permit room temperature storage of tissue without damaging the collagen structure) and/or low-dose gamma irradiation to ensure sterility of the final product.

[0064] Efficient cell removal upon decellularization can be verified by various known means, including histological analyses to detect nuclear and cytoplasmic structures, immunohistochemical or immunofluorescent assaying for indicative intracellular proteins, and DNA detection. The nature of desirable components in the final urodele-derived scaffold biomaterial varies depending on the clinical indication being treated. Once a particular indication is identified, the knowledgeable clinician can determine which components in the urodele tissue sample should be retained in the final scaffold product, and standard methodology can be employed to ensure that the desired components are present following decellularization.

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[0065] Samples may be viewed histologically before, during, and/or after decellularization to monitor the process and to confirm that the desired degree of cellular component removal is reached. For instance, tissues can be analyzed for cytoskeletal content to gauge sufficient decellularization. Intracellular protein content also may be assayed to determine if decellularization is sufficient. In addition, the tissue sample thickness and chemical makeup may be monitored to determine when sufficient decellularization has been achieved. Periodic monitoring during processing allows for a real time response to the observed tissue properties.

[0066] In some cases, a sufficiently decellularized tissue comprises no more than 50 ng dsDNA per mg ECM dry weight. Alternatively, for some indications, a sufficiently decellularized tissue lacks visible nuclear material in a tissue section stained with 4',6-diamindino-2-phenylindole (DAPI) or haematoxyilin and eosin (H&E).

[0067] In scenarios where removal of an adjacent epithelial cell layer is required, the presence or absence of epithelial cells remaining in the sample can be evaluated using techniques known in the art. For example, after removal of the epithelial cell layer, a representative tissue sample from the processing lot is placed onto a standard microscope examination slide. The tissue sample is then stained using Eosin Y Stain and evaluated as described below. The sample is then covered and allowed to stand. Once an adequate amount of time has passed to allow for staining, visual observation is done under magnification. The presence of cells and cellular material will appear darker than the areas which have been de-epithelialized.

[0068] Once cellular removal has progressed sufficiently, conventional methods are employed to confirm the retention of desired structural and functional properties of the remaining ECM scaffold. The specific structural testing that should be conducted depends on the intended clinical application of the final scaffold product. In some cases, the urodele tissue starting material may be monitored before, during, and after decellularization to ensure that the desired structural components and configuration are maintained in the final product.

[0069] One method for determining whether the desired ECM components are present involves staining parallel tissue sections and examining them histologically to determine whether the desired constituents and structural orientation of the urodele tissue have been preserved. For instance, urodele tissue can be stained with H&E and immunoperoxidase stain for laminin to assess preservation of ECM and laminin. In general, the three-

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dimensional configuration of ECM components remaining in the final biomaterial scaffold product should approximate that of pre-decellularized material when viewed via histological staining. Another component one can assay for is AMPs, as the ECM of the invention is rich in AMPs.

[0070] Accordingly, the urodele-derived biomaterial of the invention comprises ECM components useful for directing enhanced re-epithelialization and promoting efficient tissue regeneration or wound healing. The inventive biomaterial also serves as a matrix and reservoir for bioactive peptides such as growth factors, adhesion proteins and AMPs. Accordingly, the biomaterial functions effectively as a biological scaffold for tissue regeneration, providing both the necessary bioactive stimulation and structural support. The product can be used as is, cut into smaller pieces or shapes, laminated to itself or other materials, pre-punctured to provide openings for securing attachments, formed into desired three dimensional shapes, as well as other formats, discussed in more detail below.

Powders and Gels

[0071] In embodiments, the scaffold can be further processed into small grains or a powder. The fine particles can be hydrated in water, saline or a suitable buffer or medium to produce a paste or gel. This fine material, paste or gel produced from it may be used for a multitude of purposes, described in greater detail below.

[0072] Although numerous methods exist, two exemplary methods may be used to produce a particulate form of the scaffold. The first method involved lyophilizing the disinfected material and then immersing the sample in liquid nitrogen. The snap frozen material is then reduced to small pieces with a blender so that the particles are small enough to be placed in a rotary knife mill, such as a Wiley mill. A #60 screen can be used to restrict the collected powder size to a desired size, for example less than 250 mm. A Sonic sifter or other classification device can be used to remove larger particles and/or to obtain a particle size distribution within a desired range. A second method is similar to the previous method except the sample is first soaked in a 30% (w/v) NaCl solution for 5 min. The material is then snap frozen in liquid nitrogen to precipitate salt crystals, and lyophilized to remove residual water. This material is then comminuted as described in above. By precipitating NaCl within the sample, it is expected that the embedded salt crystals would cause the material to fracture into more uniformly sized particles. The particles are then suspended in deionized water and centrifuged for 5 min at 1000 rpm three times to remove the NaCl. The suspension is snap

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frozen and lyophilized again. Finally, the powder is placed in a rotary knife mill to disaggregate the individual particles.

[0073] The powder can be hydrated to create a gel, with or without other gelling materials to supplement gelling.

[0074] The powder, paste or gel can be applied without further processing to treat a subject. It can be sprayed, painted, injected or otherwise applied to a wound or surgical site. The gel can be shaped. The powder, paste or gel also can be placed inside a "bag", such as a polymeric synthetic material or a ECM sheet as described herein to produce a larger three-dimensional structure, such as an orthopedic implant for cartilage repair (e.g., knee or TMJ cartilage repair) or an implant for breast reconstruction or augmentation. In such a case, a bag of a desirable size and shape is formed from sheets of ECM material or other biocompatible polymeric material, and then the bag or cover can be filled with the tissue-derived powder or gel described herein. The shape of the device or implant can vary with its intended use. The bag may be molded into a useful shape by any useful molding technique, such as the shape of cartilage for the ear, nose, knee, TMJ, rib, etc., prior to filling the molded bag with the scaffold material described herein. In one example, a biodegradeable polymeric matrix (e.g., PEUU or PEEUU) is sprayed or electrodeposited onto a mold. The resultant molded cover can then be filled with the material. Heat, for example, may be used to seal the cover.

Additives

[0075] In another embodiment, at least one agent xenogenic to a Uroldele is added to the ECM or Urodele fraction thereof before it is implanted in the subject, otherwise administered to the subject or used in cell culture. Generally, the agents include any agent useful in cell culture or as a therapeutic or therapeutic adjuvant. The agents can be coated on, infused into or otherwise covalently or non-covalently attached to or incorporated onto or into the ECM of the invention. The agents also can be otherwise combined with a product that contains the ECM, for example, as by mixing powders of the agent and ECM together. Each agent may be used alone with the ECM of the invention or in combination with other agents. Non-limiting examples of such agents include antimicrobial agents, growth factors, cytokines, chemokines , emollients, retinoids, steroids, and cells, including but not limited to the subject's own cells.

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[0076] In certain non-limiting embodiments, the agent is a growth factor. Non-limiting examples of growth factors include basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factors 1 and 2 (IGF-1 and IGF-2), platelet derived growth factor (PDGF), stromal derived factor 1 alpha (SDF-1 alpha), nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), neurotrophin-3, neurotrophin-4, neurotrophin-5, pleiotrophin protein (neurite growth-promoting factor 1), midkine protein (neurite growth-promoting factor 2), brain-derived neurotrophic factor (BDNF), tumor angiogenesis factor (TAF),corticotrophin releasing factor (CRF), transforming growth factors .alpha. and .beta. (TGF-.alpha. and TGF-.beta.), interleukin-8 (IL-8), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukins, and interferons. Commercial preparations of various growth factors, including neurotrophic and angiogenic factors, are available from R & D Systems, Minneapolis, Minn.; Biovision, Inc, Mountain View, Calif.; ProSpec-Tany TechnoGene Ltd., Rehovot, Israel; and Cell Sciences.RTM., Canton, Mass.

[0077] In certain non-limiting embodiments, the therapeutic agent is an antimicrobial agent, such as, without limitation, an anti-microbial peptide, isoniazid, ethambutol, pyrazinamide, streptomycin, clofazimine, rifabutin, fluoroquinolones, ofloxacin, sparfloxacin, rifampin, azithromycin, clarithromycin, dapsone, tetracycline, erythromycin, ciprofloxacin, doxycycline, ampicillin, amphotericin B, ketoconazole, fluconazole, pyrimethamine, sulfadiazine, clindamycin, lincomycin, pentamidine, atovaquone, paromomycin, diclazaril, acyclovir, trifluorouridine, foscarnet, penicillin, gentamicin, ganciclovir, iatroconazole, miconazole, Zn-pyrithione, and silver salts such as chloride, bromide, iodide and periodate.

[0078] In certain non-limiting embodiments, the therapeutic agent is an anti-inflammatory agent, such as, without limitation, an NSAID, such as salicylic acid, indomethacin, sodium indomethacin trihydrate, salicylamide, naproxen, colchicine, fenoprofen, sulindac, diflunisal, diclofenac, indoprofen, sodium salicylamide; an anti-inflammatory cytokine; an anti-inflammatory protein; a steroidal anti-inflammatory agent; or an anti-clotting agents, such as heparin.

[0079] Other drugs that may promote wound healing and/or tissue regeneration may also be included.

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[0080] The agent may be dispersed within the scaffold by any useful method, e.g., by adsorption and/or absorption. For example, the therapeutic agent may be dissolved in a solvent (e.g., DMSO) and added to the scaffolding. In another embodiment, the agent is mixed with a carrier polymer (e.g., polylactic-glycolic acid microparticles, agarose, a poly(ester urethane) urea elastomer (PEUU) or a poly(ether ester urethane) urea elastomer (PEEUU)), which is subsequently dispersed within or applied to the scaffold. By blending the agent with a carrier polymer or elastomeric polymer, the rate of release of the therapeutic agent may be controlled by the rate of polymer degradation and/or by release from the polymer by diffusion or otherwise. Likewise, a therapeutic agent may be provided in any dissolvable matrix for extended release, as are known in the pharmaceutical arts, including sugar or polysaccharide matrices. The agent also may be included with the powdered ECM and gelled with the powdered ECM. The agent may be covalently attached to the ECM of the invention. The foregoing are meant to be non-limiting examples.

Extracts

[0081] In addition to the decellularized ECM in its native state or ground as a particulate or powder, the invention also provides extracts and isolates of the same. As mentioned above, the Urodele ECM is loaded with antimicrobial peptides, growth promoting factors, collagen and laminins, and Urodele fractions of the ECM are useful according to the invention.

[0082] Extraction buffers are well known in the art. One such buffer is 4 M guanidine and 2 M urea each prepared in 50 mM Tris-HCl, pH 7.4. The powder form of the ECM can be suspended in the relevant extraction buffer (e.g., 25% w/v) containing phenylmethyl sulphonyl fluoride, N-ethylmaleimide, and benzamidine (protease inhibitors) each at 1 mM and vigorously stirred for 24 hours at 4° C. The extraction mixture can then be centrifuged and the supernatant collected. The insoluble material can be washed in the extraction buffer, centrifuged, and the wash combined with the original supernatant. The supernatant can be dialyzed against deionized water. The dialysate can then be centrifuged to remove any insoluble material and the supernatant used immediately or lyophilized for long term storage. Such an isolate will contain growth factors in concentrations specific to Urodeles.

[0083] In another aspect, the extraction is done by conditioning medium. A method of making Urodele tissue-specific extract by taking the powdered ECM, forming a solution thereby generating a supernatant and a particulate, wherein the supernatant is an extract and

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isolating the extract from the particulate. One also could grow cells on the ECM, and isolate the supernatant after a period of time of cell growth.

Synthetic Materials

[0084] Synthetic biocompatible and cyto-compatable material can be combined with the ECM, such as, for example, (a) a structural support for a sheet or a gel of the ECM, (b) a structural support for shaping the ECM, (c) a coating for the ECM (or a coating containing the particulate ECM), a supplemental gelling agent, or (d) a sustained release material for the particulate ECM or an isolate thereof. Such polymers have been known to be applied to other ECM materials as a backing sheet, including materials that are themselves biodegradable. Suitable synthetic material for a matrix can be biocompatible to preclude migration and immunological complications, and can be able to support cell growth and differentiated cell function. Some are resorbable, allowing for a completely natural tissue replacement. Some can be configurable into a variety of shapes and have sufficient strength to prevent collapse upon implantation. Studies indicate that the biodegradable polyester polymers made of polyglycolic acid fulfill all of these criteria (Vacanti, et al. J. Ped. Surg. 23:3-9 (1988); Cima, et al. Biotechnol. Bioeng. 38:145 (1991); Vacanti, et al. Plast. Reconstr. Surg. 88:753-9 (1991)). Other synthetic biodegradable support matrices include synthetic polymers such as polyanhydrides, polyorthoesters, and polylactic acid. Further examples of synthetic polymers and methods of incorporating or embedding cells into these matrices are also known in the art. See e.g., U.S. Pat. Nos. 4,298,002 and 5,308,7

[0085] As a non-limiting example, the powder may be formulated with tri-block co-polymers. See international pubished application WO2012131104 and WO2012131106, each of which is incorporated herein by reference in its entirety. Other examples include poloxamers, which are nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide)). Poloxamers are also known by the trade name Pluronics (BASF). Certain poloxamers are useful as sustained release materials for pharmaceuticals.

[0086] Particles of the invention also may be encapsulated into a polymer, hydrogel and/or surgical sealant. As a non-limiting example, the polymer, hydrogel or surgical sealant may be PLGA, ethylene vinyl acetate (EVAc), poloxamer, GELSITE® (Nanotherapeutics, Inc. Alachua, Fla.), HYLENEX® (Halozyme Therapeutics, San Diego Calif.), surgical sealants

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such as fibrinogen polymers (Ethicon Inc. Cornelia, Ga.), TISSELL® (Baxter International, Inc Deerfield, Ill.), PEG-based sealants, and COSEAL® (Baxter International, Inc Deerfield, Ill.). In another embodiment, the particle may be encapsulated into any polymer known in the art which may form a gel when injected into a subject. As another non-limiting example, the particle may be encapsulated into a polymer matrix which may be biodegradable. Additional examples of polymers for controlled release and/or targeted delivery may also include at least one controlled release coating. Controlled release coatings include, but are not limited to, OPADRY®, polyvinylpyrrolidone/vinyl acetate copolymer, polyvinylpyrrolidone, hydroxypropyl methylcellulose, hydroxypropyl cellulose, hydroxyethyl cellulose, EUDRAGIT RL®, EUDRAGIT RS® and cellulose derivatives such as ethylcellulose aqueous dispersions (AQUACOAT® and SURELEASE®.

Uses

[0087] The decellularized ECMs described herein are useful for growing cells, tissues, organs in virtually any *in vivo*, *ex vivo*, or *in vitro* use. The ECMs can be used as a substrate to facilitate the growth and/or differentiation of cells. In vitro, the ECMs are useful as a cell growth substrate to support the growth in culture of cells, including virtually any type of cells or cell-lines, including stem cells, progenitor cells or differentiated cells. In one embodiment, the cells are cancer cells. In one embodiment, the cancer cells form nodules when grown on the ECMs. Cells on the substrate also may be grown into tissue, organ or body part precursors, or even mature tissues or structures. Cells grown on ECMs may be used for implantation, for wound dressings, for *in vitro* drug testing, for modeling differentiation, etc. The cells may be matched in tissue cell type to the ECM or unmatched. The cells are xenogenic.

[0088] The ECM of the invention is useful *in vivo* as a cell growth scaffold for tissue growth for any useful purpose, including repair, replacement or augmentation of tissue in a subject in either humans or animals. For example, the materials are useful in repair and/or replacement of tissue lost or damaged during trauma or surgery, for example in loss of tissue after tumor removal. The materials are useful for structural repair, such as inguinal hernia repair, parastomal reinforcement, soft tissue reinforcement, surgical staple-line reinforcement during, for example, bariatric surgery or lung resection, umbilical hernia grafts, Peyronie's repair grafts, incision grafts and fistula plugs. The materials are useful for wound dressings, such as for burns, graft and split-thickness graft coverings, ulcers including decubitis ulcers

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and dermal abrasion procedures. The materials are useful for cosmetic purposes, for example in breast, lip or buttock augmentation. An aspect of the invention particularly appealing for anti-adhesion surgical uses is the properties of the basement membrane, which inhibit or prevent adhesion. The presence of the AMPs make the ECM of the invention particularly well suited for the foregoing applications.

[0089] As mentioned above, the materials described herein can be molded or contained within a structure to form desired shapes, such as, for cartilage repair or replacement by seeding the material with, e.g., chondrocytes and/or chondroprogenitor cells. The materials can be ground into a powder and used to reconstitute and/or form gels, as cell culture additives, as a powder, spray, liquid, suspension or coating for application to (a) a wound, (b) an implant, (c) a wound dressing, etc.

[0090] In one embodiment, for example, adipose stem cells are propagated in the cell growth scaffolds described herein. Adipose stem cells are of mesodermal origin. They typically are pluripotent, and have the capacity to develop into mesodermal tissues, such as: mature adipose tissue; bone; heart, including, without limitation, pericardium, epicardium, epimyocardium, myocardium, pericardium, and valve tissue; dermal connective tissue; hemangial tissues; muscle tissues; urogenital tissues; pleural and peritoneal tissues; viscera; mesodermal glandular tissues; and stromal tissues. The cells not only can differentiate into mature (fully differentiated) cells, they also can differentiate into an appropriate precursor cell (for example and without limitation, preadipocytes, premyocytes, preosteocytes). Also, depending on the culture conditions, the cells can also exhibit developmental phenotypes such as embryonic, fetal, hematopoetic, neurogenic, or neuralgiagenic developmental phenotypes.

[0091] In one embodiment, a subject's own cells are dispersed within the matrix. For example, in the production of cartilaginous tissue, chondrocytes and/or chondroprogenitor cells can be dispersed within the matrix and optionally grown ex vivo prior to implantation. Likewise, skin cells of a subject can be dispersed within the scaffolding prior to implantation on a damaged skin surface of a subject, such as a burn or abrasion.

[0092] When used as a gel, a non-limiting example is injecting the gel into a subject at a desirable site, such as in a wound. In one instance, the gel can be injected in a bone breakage or in a hole drilled in bone to facilitate repair and/or adhesion of structures, such as replacement ligaments, to the bone. In another use, finely comminuted particles can be

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sprayed onto a surface of a subject, such as in the case of large surface abrasions or burns. The scaffold can also be sprayed onto skin sutures to inhibit scarring. The ECM of the invention can be place or sutured in place inside the body at a surgical site such as mentioned above. All of these treatments are embraced by the present invention.

[0093] Urodele decellularized ECM can be used also for sustained delivery of therapeutic molecules, proteins or metabolites, to a site in a host. See, for example, U.S. 2004/0181240, which describes an amniotic membrane covering for a tissue surface which may prevent adhesions, exclude bacteria or inhibit bacterial activity, or to promote healing or growth of tissue, and U.S. patent No. 4,361,552, which pertains to the preparation of cross-linked amnion membranes and their use in methods for treating burns and wounds. The ECMs of the invention can be used in the same manner.

Pharmaceutical Formulations

[0094] Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to any other animal, e.g., to non-human animals, e.g. non-human mammals. Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and/or perform such modification with merely ordinary, if any, experimentation.

[0095] The pharmaceutical compositions described herein may be prepared by any method known in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient into association with an excipient and/or one or more other accessory ingredients, and then, if necessary and/or desirable, dividing, shaping and/or packaging the product into a desired single- or multi-use configuration.

[0096] The ECM in accordance with the invention may be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. For example, the composition may comprise between 0.1% and 100% (w/w) of the ECM. When other active agents are included, relative amounts of agents combined with the ECM of the invention will be known to those of ordinary skill in the art, similar to those amounts used in combination with ECM as formulated in the prior art. Relative amounts also may vary, depending upon

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the identity, size, and/or condition of the subject being treated and further depending upon the route by which the ECM is to be administered.

[0097] Pharmaceutical formulations may additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes, but is not limited to, any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, and the like, as suited to the particular dosage form desired. Various excipients for formulating pharmaceutical compositions and techniques for preparing the composition are known in the art. See Remington: THE SCIENCE AND PRACTICE OF PHARMACY (21 st Ed.), A. R. Gennaro, Lippincott, Williams & Wilkins (Baltimore, Md., 2006); incorporated herein by reference in its entirety.

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EXAMPLES

Example 1. Processing axolotl dermis

Axolotl dermis samples can be decellularized by preparing excised samples from healthy or healing axolotl dermal tissue and then subjecting the samples to hypo/hyperosmotic soaks for cell lysis, solvent dehydration, and oven drying. Specific processing of these grafts includes storage in 15- 26% NaCl, multiple hypo/hyperosmotic soaks (utilizing NaCl solutions and water) , and then solvent dehydration using ethanol, and then evaporation of the solvent either with air drying or oven drying at 37° C.

Histological examination of native axolotl dermal tissue was performed to identify the presence of the notable ECM elements, such as the basement membrane. See FIG 1 and FIG. 4. Comparative histological and immunohistochemical analysis of native axolotl dermal tissue and human amniotic membrane was performed to compare the ECM structure and constituents, and to assess relative concentration and distribution of critical constituents. See FIG. 2, FIG. 3, and FIG. 4. Figure 3 shows immunohistocemical staining via species-specific collagen IV and laminin antibodies of native axolotl dermal tissue and human amniotic membrane tissue, at 40× magnification. Figure 2 shows H&E and Alcian Blue staining (40x) of native axolotl dermal tissue and human amniotic membrane, and it demonstrates the comparable histoarchitecture and presence of sulfated glycosaminoglycans in both tissues. Histological evaluation with hematoxylin and eosin-stained, paired native and post-processed sections of axolotl dermal tissue (see FIG. 5)showed post-process preservation of the extracellular matrix histoarchitecture and the absence of cells or any significant concentration of cellular debris.

Example 2. Splitting and lamination of acellular dehydrated axolotl dermis.

Decellularized dehydrated axolotl dermis can be split, via a mechanical splitter, to isolate heterogeneous matrix into homogenous sections. Isolated sections of desired thickness then can be rehydrated and lyophilized to obtain multilayered laminate structures of desired orientation with facial surface features. More specifically, dual-sided basement membrane structure, with interior open porous matrix obtained from the reticular dermis region of the dermal matrix, can be constructed to obtain desired facial surface properties. Alternatively, isolated native section can be used in native form for desired clinical outcome.

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For example, open porous homogenous matrix of the reticular dermis can be used to obtain augmentation of soft tissue structures.

A laminated custom construct with sulfated gags on both facial surface and collagen IV and laminin could be obtained for desirable dual-surface, anti-adhesion and antimicrobial properties for clinical benefit. In addition, multilayer structures could be constructed to prolong in vivo durability of the graft.

Example 3 Preparation of solubilized acellular dehydrated axolotl dermis, pericardium, fascia lata, periosteum, peritoneum, or dura mater

Decellularized dehydrated axolotl native or isolated section of acellular urodele connectivue tissye matrix can be prepared by sectioning decellularized soft tissue structures into 1 cm^2 sections and homogenizing the sections in a Warring blender (~100 grams of tissue) in aqueous 1M glacier acetic acid for 30-60seconds. Preparation of sponge can be obtained by the addition of varying volumes of water followed then neutralization and lypoholization of the slurry in a mold of desired geometric shape. The resultant porosity will correlate to the volume of water added to the matrix. Additionally, a selected range of bioactive extracts can be added to the slurry prior to neutralization, including particulated or small protein constituents extracted from digested human or urodele mineralized and nonmineralized connective tissues, such as demineralized bone matrix, elastin, or bone morphogenic proteins, which can be covalently loaded into constructs. Extracts will be covalently bound with collagen fibers after neutralization and return to physiological condition where fibrillogenisis will occur. Subsequent release of bioactive constituents will occur during proteolyctic degradation in vivo and ensure molecules are not consume or exposed during acute inflammation in vivo. Alternatively, aqueous NaCl can be added to the slurry, prior to neutralization, to obtain a sustained, low viscosity solution for injection, which is stable at room temperature. Injection of slurry through ion-selective membrane will remove salt ions and permit for fibrillogenisis to occur post injection and formation of three-dimensional matrix.

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Example 4 Preparation of sterilized particulated or powder form of mineralized and non-mineralized decellularized and dehydrated urodele connective tissue matrix

Following decellularization of sections of mineralized collagen urodele connective tissue, one can perform a demineralization process, similar to that employed by Urist, and solvent or lyophilization dehydration, cryomilling of sectioned acellular demineralized, mineralized, or non-mineralized urodele connective tissue extracellular matrix, thereby to obtain particulated or powder form of the ECM with preserved histoarhictiecutre and function. The final particle size distribution can be varied depending on duration and sieving, post-cyromilling, between 125 and 850 microns. Low-dose cold gamma irradiation or e-beam irradiation (<25 Kgy) can be employed to sterilize acellular ECM sheets, particulate or powder and custom engineered constructs

Example 5. *In vitro* characterization of acellular mineralized and non-mineralized urodele healthy or healing connective tissue matrix

Through a series of *in vitro* analyses one can verify decellularization and preservation of native or custom engineered functional and structural properties of decellularized extracellular matrix constructs and/or particulate, including multilayer laminated constructs such as a dual-sided basement membrane sheet matrix or isolated native homogenous open porous matrix or solubilized, lyophilized and loaded ECM-derived collagen sponge. DNA content, as a marker for cell debris, can be employed to assess decellularization quantitatively, using a single, ethanol-based extraction technique with a fluorometric dye, Quant-iT PicoGreen (Molecular Probes, USA), in a ratio of 170 µL working solution to 30 µL samples/standards in a 96-well plate. Paired native and post-processed analysis and comparison to commercially available tissue ECM's can be performed to verify acceptability. See Figure 6.

ELISA analysis for quantification of bioactive constituent and native and post process protelyctic resorption profiles can be performed. Upon digestion with collagenase (232 – 262 mg/unit activity), normalized-weight-to-surface-area sections of decellularized and dehydrated urodele ECM tissue or constructs (Sigma, USA), in a pH 7.6 buffer (50 mM Tris-HCl, 200 mM $CaCl_2$, 50 mM NaCl) for 24 hours at 37°C, can be analyzed at various time points to construct a relative resorption curve of pre- and post-process tissue to verify preservation of histoarchitecture. Solubilized collagen following digestion can be assessed,

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using a Sircol kit (Biocolor Ltd., UK), in 100 µL aliquots of acid/salt-washed digests. Specifically, levels of BMP 2/4 and TGF- 1 growth factors or sulfated gags can be assessed, following digestion, by means of commercially available ELISA kits (R&D Systems, Minneapolis, MN). Protein content in 1:10 dilution digests can be measured via a standard Bradford absorbance assay.

Microscopic evaluation of samples can be performed using fixation in 4% paraformaldehyde and paraffin embedding, sectioning at 5 µm, and routine histological staining (Histoserv, Inc., USA). Longitudinal cross sections can be stained with hematoxylin and eosin. Images can be acquired and anyzed using standard brightfield techniques on an Olympus IM inverted microscope. Samples can analyzed using scanning electron microscopy after dehydration in a graded ethanol series (15%, 30%, 50%, 70%, 95%, and 100%), critical-point drying in CO_2, and sputter coating with gold. Samples can be visualized in an FEI Quanta 600 FEG scanning electron microscope, and representative images of scaffold ultrastructure can be acquired.

Direct cell contact methodology (ISO10993, Part 5), for qualitative cell viability assessment at 24 hours, can be conducted for cytotoxicity testing and at extended time points (48 and 72 hours) to gauge cell proliferation and adhesion efficiency. A manual count of non-adherent cells in a hemocytometer, following transfer and trypsinization of the culture wells, can be conducted. A CellTiter 96 assay can performed to quantify viable cells after four days. A live/dead cell staining kit can used as well to visualize scaffolds via fluorescence microscopy at 24 hours and day four, thereby to verify biocompatibility.

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CLAIMS

1. A decellularized biomaterial produced by the process that comprises (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

2. The decellularized biomaterial of claim 1, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

3. The decellularized biomaterial of claim 1, wherein said process further comprises subjecting said sample to sterilization.

4. The decellularized biomaterial of claim 1, further comprising devitalized cells.

5. A tissue graft comprising extracellular matrix components derived from a urodele.

6. An isolated, decellularized Urodele ECM.

7. The isolated ECM of claim 6, wherein the ECM is derived from Axolotl tissue.

8. The isolated ECM of claim 6, wherein the ECM includes basement membrane

9. The isolated ECM of claim 6, wherein the ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, a drug or a human agent.

10. The isolated ECM of claim 6, wherein the agent is a cell, optionally a human cell.

11. The isolated ECM of claim 6, wherein the ECM is a sheet.

12. The isolated ECM of claim 11, wherein the sheet includes perforations.

13. The isolated ECM of claim 6, wherein the ECM is a dry powder.

14. The isolated ECM of claim 6, wherein the ECM is a reconstituted gel.

15. The isolated ECM of any one of claims 6-13, therein the ECM is sterile.

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16. A package containing sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM.

17. The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a sheet of isolated, decellularized Urodele ECM.

18. The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a dry powder.

19. The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a gel.

20. A sterile medical implant comprising sterile, isolated, decellularized Urodele ECM or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM.

21. The sterile medical implant of claim 19, wherein the implant is a biocompatible sheet, mesh, gel, graft, tissue or device.

22. A material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM or a Urodele fraction derived from the isolated, decellularized Urodele ECM.

23. A tissue culture system comprising (a) an isolated Urodele decellularized ECM, (b) tissue culture medium, and (c) cells xenogenic to the Urodele.

24. The tissue culture system of claim 22, wherein the cells are human cells.

25. Tissue culture medium conditioned with isolated Urodele decellularized ECM or a Urodele fraction derived from isolated, decellularized Urodele ECM.

26. A device comprising at least two sheets of isolated Urodele decellularized ECM laminated to one another.

27. A product prepared by isolating Urodele ECM from a Urodele, decellularizing the ECM, and sterilizing the decellularized ECM.

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28. A method of preparing a biologic material comprising (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample forming a decellularlized ECM to remove sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

29. The method of claim 28, wherein the method further comprises performing the decellularization in a manner to retain structural and functional integrity of the decellularized ECM sufficient to permit the decellularized ECM to be useful as a matrix upon and within which cells can grow.

30. The method of claim 29, further comprising homogenizing the decellularized ECM to form a powder.

31. The method of claim 30, further comprising reconstituting the powder as a gel.

32. The method of claim 29, further comprising sterilizing the decellularized ECM.

33. The method of claim 29, further comprising attaching the decellularized ECM to an agent xenogenic to a Urodele.

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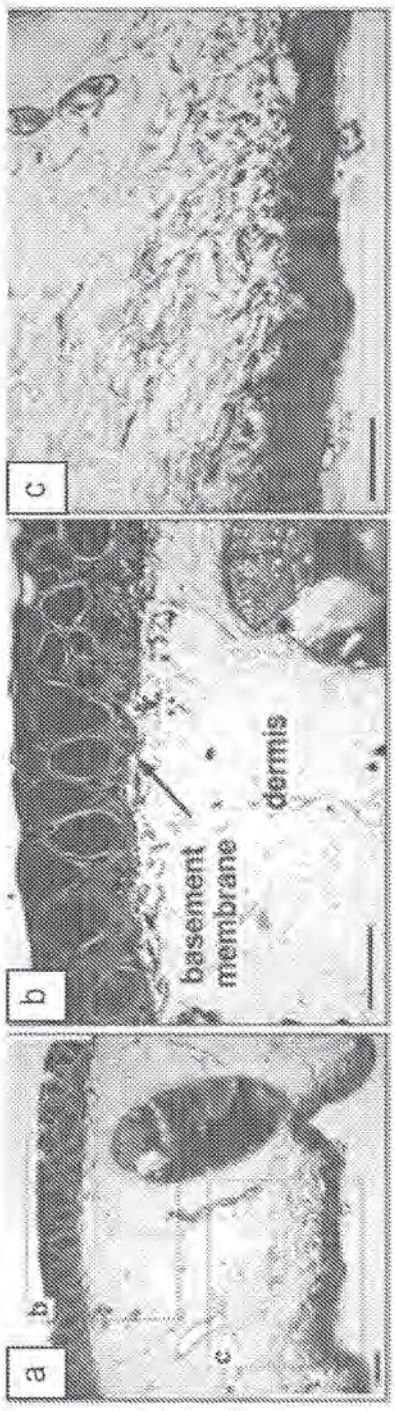


FIGURE 1

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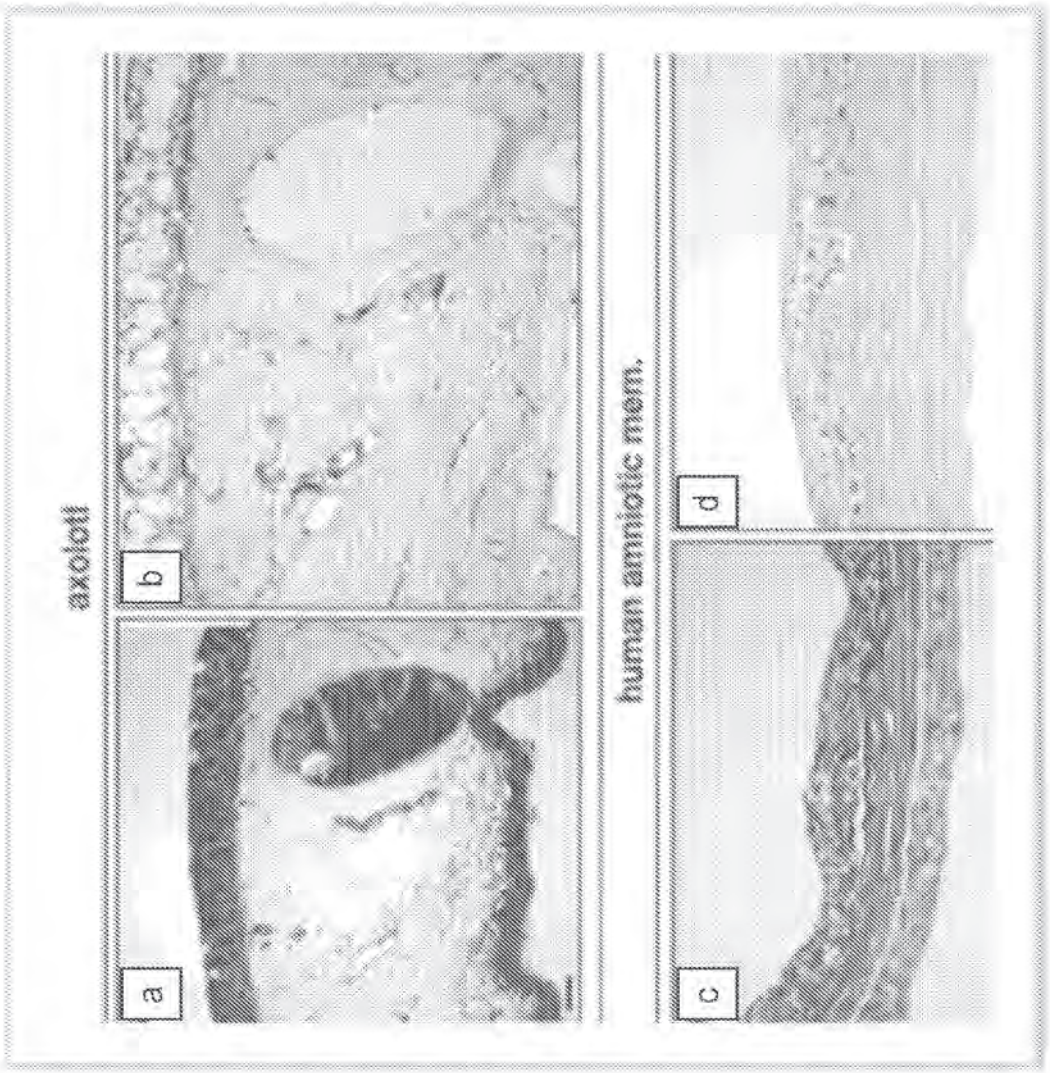


FIGURE 2

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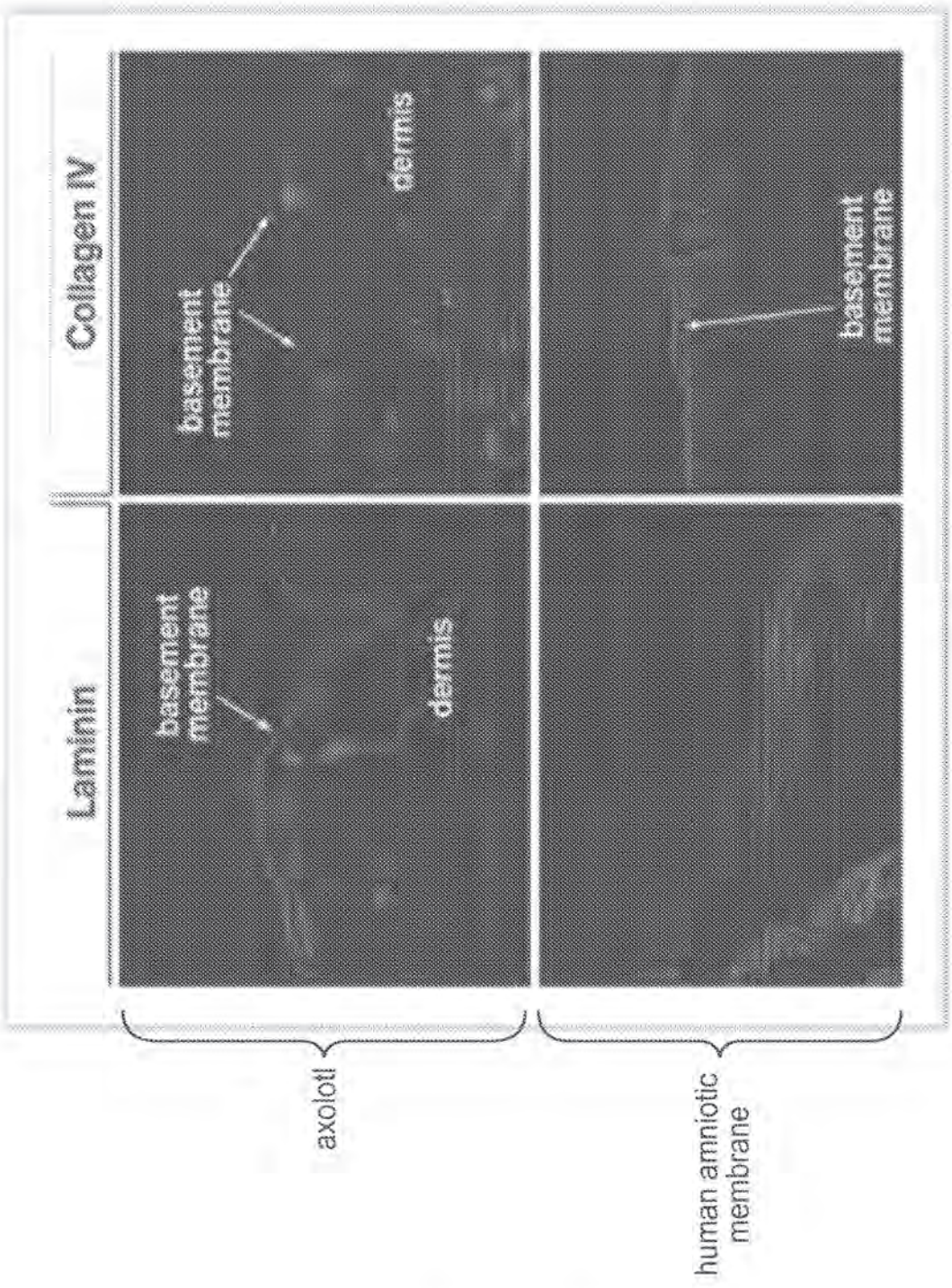


FIGURE 3

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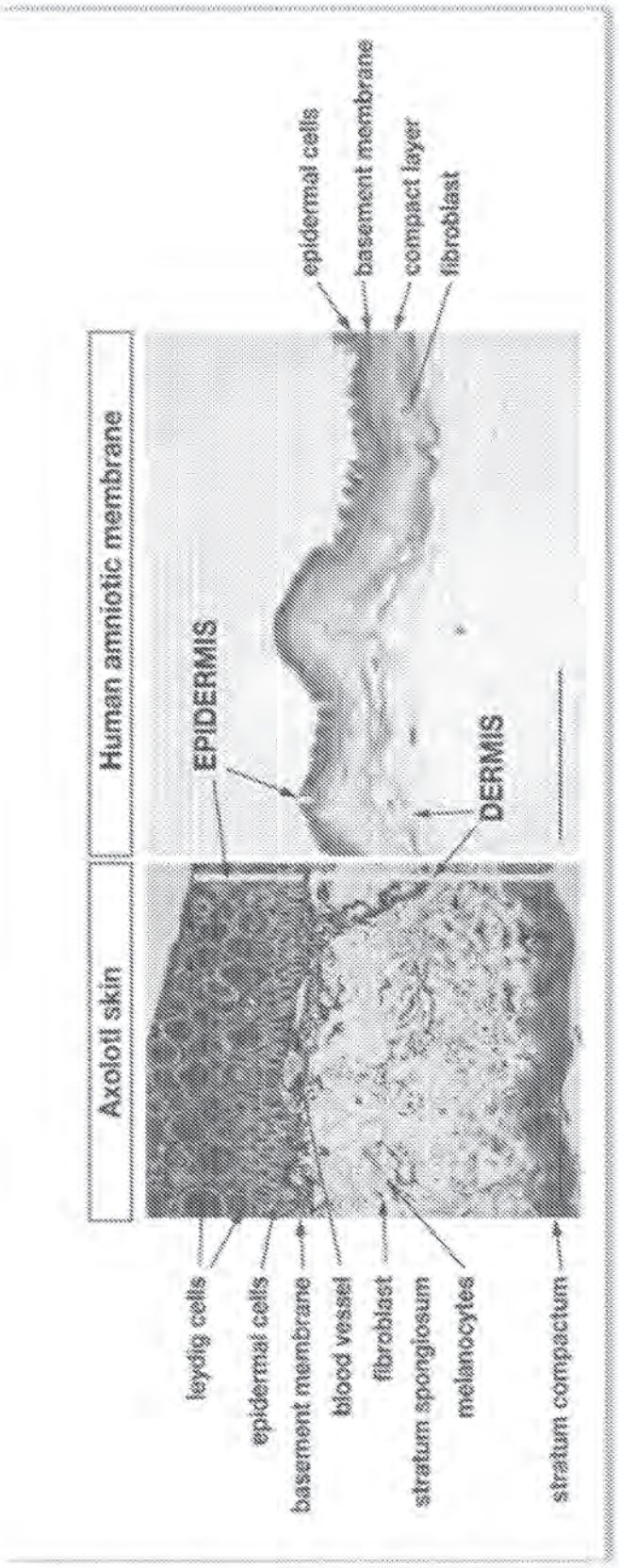


FIGURE 4

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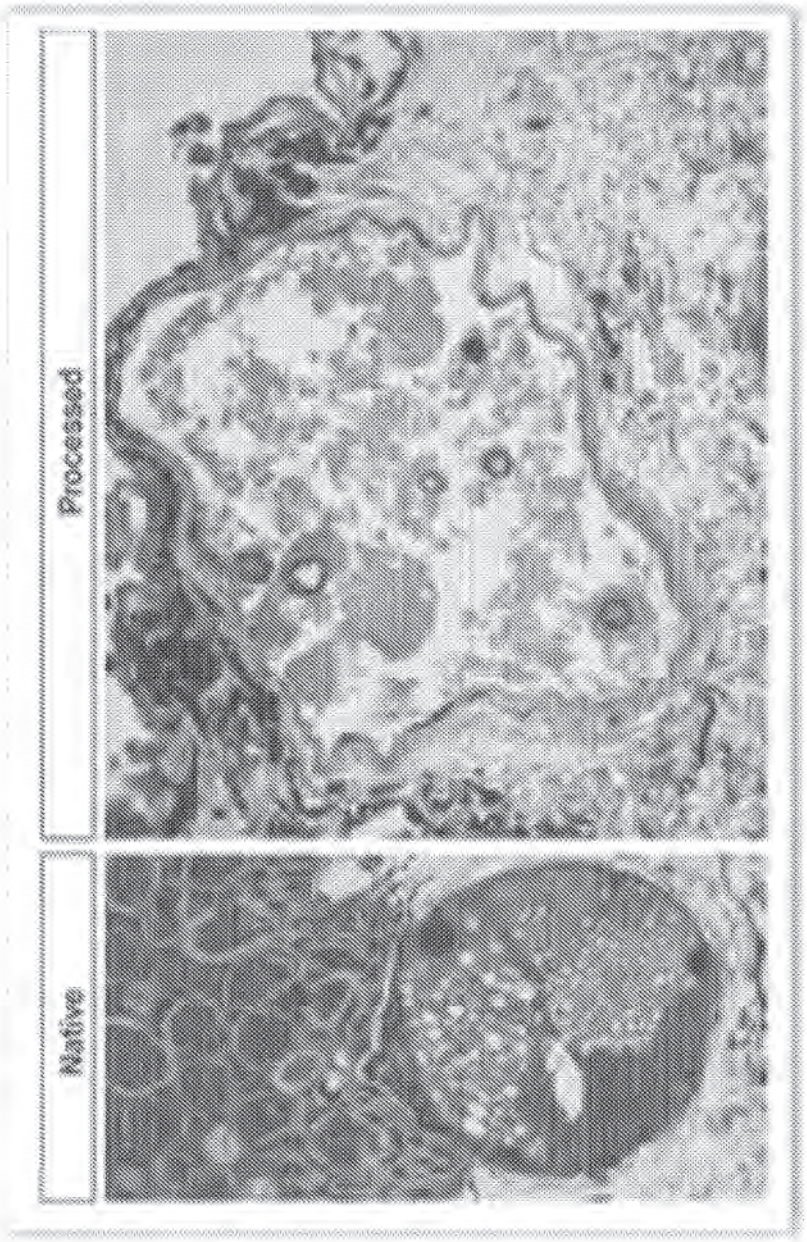


FIGURE 5

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Epoch Plate Reader		Avg. Duplicate			
	Sample ID	260/280	dsDNA conc. ng/ul	dsDNA conc. ug/ul	Percent Reduction
Paired native and post decellurization urodele connective tissue Residual DNA Analysis	A-Native	2	1702.8	1.7028	89.8%
	A-Native	2	1977.5	1.9775	92.8%
	A-Native	2	1618.3	1.6183	92.7%
	A-P1	1.96	173.7	0.1737	
	A-P2	1.95	141.7	0.1417	
	A-P3	1.97	117.9	0.1179	
Commercially availabie Amnitoic Membrane Products residual Dna					
	BioDlogic (BioD)	2	1.34	0.00134	
	Biotissue (Amniograft)	1.5	27.81	0.02781	
	Mimedx (Amniofix)	1.26	104.6	0.1046	
	Seed Biotech (Aril)	1.75	3.16	0.00316	

FIGURE 6

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 2014/010890

A. CLASSIFICATION OF SUBJECT MATTER

A61K 35/56 *(2006.01)*
A61K 9/10 *(2006.01)*
A61K 9/14 *(2006.01)*
A61F 2/00 *(2006.01)*
A61L 27/36 *(2006.01)*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 35/56, 31/00, 38/00, 9/10, 9/14, A61F 2/00, A61L 27/36

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DEPATISnet, DWPI, EAPATIS, PAJ, Patentscope, PatSearch (RUPTO internal) PubMed, RUPTO, RUSPAT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/124127 A2 (WAKE FOREST UNIVERSITY HEALTH SCIENCES et al.) 01.11.2007, claims 1, 15, 17-18, 21, p. 2, last paragraph, p. 3, paragraph 3, p. 4, paragraphs 4, 5, 6, p. 7, paragraphs 4, 5, p. 8, paragraphs 3, 5, p. 9, paragraph 4, p. 15, paragraphs 1, 2	1-10, 15-16, 20-25, 27-29, 32-33
Y		11-14, 17-19, 26, 30-31
Y	WO 2005/009498 A2 (SCIMED LIFE SYSTEMS, INC. et al.) 03.02.2005, claims 30-31, p. 13, lines 31-33, p. 16, lines 28-29, p. 26, lines 13-15	11-14, 17-19, 26, 30-31

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 March 2014 (26.03.2014)	15 May 2014 (15.05.2014)
Name and mailing address of the ISA/ FIPS Russia, 123995, Moscow, G-59, GSP-5, Berezhkovskaya nab., 30-1 Facsimile No. +7 (499) 243-33-37	Authorized officer E. Maslova Telephone No. (495)531-64-81

Form PCT/ISA/210 (second sheet) (July 2009)

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

U.S. APPLICATION NUMBER NO.	FIRST NAMED INVENTOR	ATTY. DOCKET NO.
14/759,895	Ryanne Early	S1860.70000US01

23628
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

INTERNATIONAL APPLICATION NO.	
PCT/US2014/010890	
I.A. FILING DATE	PRIORITY DATE
01/09/2014	01/09/2013

CONFIRMATION NO. 3358
371 ACCEPTANCE LETTER

OC000000077143408

Date Mailed: 08/31/2015

NOTICE OF ACCEPTANCE OF APPLICATION UNDER 35 U.S.C 371 AND 37 CFR 1.495

The applicant is hereby advised that the United States Patent and Trademark Office, in its capacity as a Designated / Elected Office (37 CFR 1.495), has ACCEPTED the above identified international application for national patentability examination in the United States Patent and Trademark Office.

The United States Application Number assigned to the application is shown above. A Filing Receipt will be issued for the present application in due course. **THE DATE APPEARING ON THE FILING RECEIPT AS THE "FILING DATE or 371(c) DATE" IS THE DATE ON WHICH THE LAST OF THE 35 U.S.C. 371 (c)(1) and (c)(2) REQUIREMENTS HAS BEEN RECEIVED IN THE OFFICE. THIS DATE IS SHOWN BELOW.** The filing date of the above identified application is the international filing date of the international application (Article 11(3) and 35 U.S.C. 363)

07/08/2015
DATE OF RECEIPT OF 35 U.S.C.
371(c)(1) and (c)(2) REQUIREMENTS

The following items have been received:

- Indication of Small Entity Status
- Copy of the International Application filed on 07/08/2015
- Copy of the International Search Report filed on 07/08/2015
- Preliminary Amendments filed on 07/08/2015
- U.S. Basic National Fees filed on 07/08/2015
- Priority Documents filed on 07/08/2015
- Application Data Sheet (37 CFR 1.76) filed on 07/08/2015

Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.495(c).

- Properly executed inventor's oath or declaration for the following inventor(s) has not been submitted: **Ryanne Early**

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

MARILYN J YOUNGER

Telephone: (571) 272-8183

FORM PCT/DO/EO/903 (371 Acceptance Notice)

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 14/759,895

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	140		N/A	
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A	240		N/A	
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	360		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	40 minus 20 =	* 20	x 40 =	800	OR		
INDEPENDENT CLAIMS (37 CFR 1.16(h))	11 minus 3 =	* 8	x 210 =	1680			
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			0.00			
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				390			
* If the difference in column 1 is less than zero, enter "0" in column 2			TOTAL	3610		TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)	OR	RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

UNITED STATES PATENT AND TRADEMARK OFFICE

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Alexandria, Virginia 22313-1450
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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
14/759,895	07/08/2015		3680	S1860.70000US01	33	11

CONFIRMATION NO. 3358

23628
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

FILING RECEIPT

OC000000077143407

Date Mailed: 08/31/2015

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Ryanne Early, Fleming Island, FL;

Applicant(s)

ISE Professional Testing & Consulting Services, Inc., Gainesville, FL;

Assignment For Published Patent Application

ISE Professional Testing & Consulting Services, Inc., Gainesville, FL

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a 371 of PCT/US2014/010890 01/09/2014
which claims benefit of 61/750,555 01/09/2013

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

If Required, Foreign Filing License Granted: 08/27/2015

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 14/759,895**

Projected Publication Date: 12/10/2015

Non-Publication Request: No

Early Publication Request: No
**** SMALL ENTITY ****

Title

DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE

Preliminary Class

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: Yes

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

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Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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RegenX Science Exhibit 1004
Page 159

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0159

the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

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NOT GRANTED

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MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

Substitute for Form PTO-1360
(For use with Form PTO/SB/06)

Application Number	Filing Date
14759895	

Applicant(s): Ryanne Early

* May be used for additional claims or amendments

CLAIMS	AS FILED		AFTER FIRST AMENDMENT		AFTER SECOND AMENDMENT	
	Indep	Depend	Indep	Depend	Indep	Depend
1	1					
2		1				
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15		8				
16	1					
17		(1)				
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Total Indep	11		0		0	
Total Depend	29	↵	0	↵	0	↵
Total Claims	40		0		0	

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	Indep	Depend	Indep	Depend	Indep	Depend
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Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 08/28/2015

MYOUNGER	SALE	#00000001	Mailroom Dt:	07/08/2015	232825	14759895
	01	FC : 2617		70.00 DA		
	02	FC : 2615		280.00 DA		

To: Patents_eOfficeAction@WolfGreenfield.com,WGS_eOfficeAction@WolfGreenfield.com,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 23628

Aug 31, 2015 05:21:55 AM

Dear PAIR Customer:

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206
UNITED STATES

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Application	Document	Mailroom Date	Attorney Docket No.
14759895	M903	08/31/2015	S1860.70000US01
	APP.FILE.REC	08/31/2015	S1860.70000US01

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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/759,895	07/08/2015	Ryanne Early	S1860.70000US01

CONFIRMATION NO. 3358

23628
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

PUBLICATION NOTICE

OC000000079257205

Title:DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE

Publication No.US-2015-0352257-A1
Publication Date:12/10/2015

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Office of Public Records. The Office of Public Records can be reached by telephone at (703) 308-9726 or (800) 972-6382, by facsimile at (703) 305-8759, by mail addressed to the United States Patent and Trademark Office, Office of Public Records, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently http://pair.uspto.gov/. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

To: Patents_eOfficeAction@WolfGreenfield.com,WGS_eOfficeAction@WolfGreenfield.com,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 23628

Dec 11, 2015 05:49:07 AM

Dear PAIR Customer:

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
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UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 23628 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

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Application	Document	Mailroom Date	Attorney Docket No.
14759895	NTC.PUB	12/10/2015	S1860.70000US01

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PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/759,895	Filing Date 07/08/2015	☐ To be Mailed

ENTITY: ☐ LARGE ☒ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 = *		X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 = *		X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT 07/08/2015

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 40	Minus	** 20	= 20	X $40 =	800
Independent (37 CFR 1.16(h))	* 11	Minus	***3	= 8	X $210 =	1680
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	2480

AMENDMENT

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
/KIM P. DOZIER/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/759,895	07/08/2015	Ryanne Early	S1860.70000US01	3358

23628 7590 09/09/2016
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

EXAMINER
DAVIS, RUTH A

ART UNIT	PAPER NUMBER
1651	

NOTIFICATION DATE	DELIVERY MODE
09/09/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents_eOfficeAction@WolfGreenfield.com
WGS_eOfficeAction@WolfGreenfield.com

Office Action Summary	Application No. 14/759,895	Applicant(s) EARLY, RYANNE	
	Examiner RUTH DAVIS	Art Unit 1651	AIA (First Inventor to File) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 2 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1-33* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☐ Claim(s) _____ is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☒ Claim(s) *1-33* are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____

4) ☐ Other: _____

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

DETAILED ACTION

Election/Restrictions

REQUIREMENT FOR UNITY OF INVENTION

As provided in 37 CFR 1.475(a), a national stage application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept ("requirement of unity of invention"). Where a group of inventions is claimed in a national stage application, the requirement of unity of invention shall be fulfilled only when there is a technical relationship among those inventions involving one or more of the same or corresponding special technical features. The expression "special technical features" shall mean those technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art.

The determination whether a group of inventions is so linked as to form a single general inventive concept shall be made without regard to whether the inventions are claimed in separate claims or as alternatives within a single claim. See 37 CFR 1.475(e).

WHEN CLAIMS ARE DIRECTED TO MULTIPLE CATEGORIES OF INVENTIONS

As provided in 37 CFR 1.475(b), a national stage application containing claims to different categories of invention will be considered to have unity of invention if the claims are drawn only to one of the following combinations of categories:

(1) A product and a process specially adapted for the manufacture of said product; or

(2) A product and process of use of said product; or

(3) A product, a process specially adapted for the manufacture of the said product, and a use of the said product; or

(4) A process and an apparatus or means specifically designed for carrying out the said process; or

(5) A product, a process specially adapted for the manufacture of the said product, and an apparatus or means specifically designed for carrying out the said process.

Otherwise, unity of invention might not be present. See 37 CFR 1.475(c).

Restriction is required under 35 U.S.C. 121 and 372.

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

In accordance with 37 CFR 1.499, applicant is required, in reply to this action, to elect a single invention to which the claims must be restricted.

Group I, claim(s) 1 – 4, 6 – 15, 27, drawn to a biomaterial.

Group II, claim(s) 5, 20 – 22, 26, drawn to a tissue graft.

Group III, claim(s) 16 - 19, drawn to a package.

Group IV, claim(s) 23 - 24, drawn to a culture system.

Group V, claim(s) 25, drawn to a culture media.

Group VI, claim(s) 28 - 33, drawn to a method for making a biomaterial.

The groups of inventions listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Groups I - VI lack unity of invention because the groups do not share the same or corresponding technical feature.

Applicant is advised that the reply to this requirement to be complete must include (i) an election of a species or invention to be examined even though the requirement may be traversed (37 CFR 1.143) and (ii) identification of the claims encompassing the elected invention.

The election of an invention or species may be made with or without traverse. To preserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable on the elected invention or species.

Should applicant traverse on the ground that the inventions have unity of invention (37 CFR 1.475(a)), applicant must provide reasons in support thereof. Applicant may submit evidence or identify such evidence now of record showing the inventions to be obvious variants

or clearly admit on the record that this is the case. Where such evidence or admission is provided by applicant, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other invention.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to RUTH DAVIS whose telephone number is (571)272-0915. The examiner can normally be reached on M-F 9:00 -4:00 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Renee Claytor can be reached on 571-272-8394. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/RUTH DAVIS/
Primary Examiner, Art Unit 1651

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BIB DATA SHEET

CONFIRMATION NO. 3358

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
14/759,895	07/08/2015 RULE	424	1651	S1860.70000US01

APPLICANTS
ISE Professional Testing & Consulting Services, Inc., Gainesville, FL;

INVENTORS
Ryanne Early, Fleming Island, FL;

** **CONTINUING DATA** **********
This application is a 371 of PCT/US2014/010890 01/09/2014
which claims benefit of 61/750,555 01/09/2013

** **FOREIGN APPLICATIONS** **********

** **IF REQUIRED, FOREIGN FILING LICENSE GRANTED** ** SMALL ENTITY **
08/27/2015

Foreign Priority claimed	35 USC 119(a-d) conditions met	Verified and Acknowledged	Met after Allowance	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
☐ Yes ☑ No	☐ Yes ☐ No	/RUTH A DAVIS/ Examiner's Signature	☐ rad Initials	FL	6	33	11

ADDRESS
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206
UNITED STATES

TITLE
DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE

FILING FEE RECEIVED		
3680	FEES: Authority has been given in Paper No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit

To: Patents_eOfficeAction@WolfGreenfield.com,WGS_eOfficeAction@WolfGreenfield.com,
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UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 23628 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

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Application	Document	Mailroom Date	Attorney Docket No.
14759895	CTRS	09/09/2016	S1860.70000US01

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Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

Docket No.: S1860.70000US01
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Ryanne Early
Application No.:	14/759,895
Confirmation No.:	3358
Filed:	July 8, 2015
For:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
Examiner:	R. A. Davis
Art Unit:	1651

Certificate of Electronic Filing Under 37 CFR 1.8
I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: November 9, 2016 Electronic Signature for Nicole M. Forgrave: /Nicole M. Forgrave/

AMENDMENT AND RESPONSE TO RESTRICTION REQUIREMENT

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Prior to examination on the merits, please amend the above-identified U.S. Patent application as follows:

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments being on page 8 of this paper.

AMENDMENTS TO THE CLAIMS

1. (Withdrawn) A decellularized biomaterial produced by the process that comprises (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample to retain structural and functional integrity while removing sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

2. (Withdrawn) The decellularized biomaterial of claim 1, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

3. (Withdrawn) The decellularized biomaterial of claim 1, wherein said process further comprises subjecting said sample to sterilization.

4. (Withdrawn) The decellularized biomaterial of claim 1, further comprising devitalized cells.

5. (Original) A tissue graft comprising extracellular matrix components derived from a urodele.

6. (Withdrawn) An isolated, decellularized Urodele ECM.

7. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is derived from Axolotl tissue.

8. (Withdrawn) The isolated ECM of claim 6, wherein the ECM includes basement membrane.

9. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is infused with, coated with,

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or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, a drug or a human agent.

10. (Withdrawn) The isolated ECM of claim 6, wherein the agent is a cell, optionally a human cell.

11. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is a sheet.

12. (Withdrawn) The isolated ECM of claim 11, wherein the sheet includes perforations.

13. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is a dry powder.

14. (Withdrawn) The isolated ECM of claim 6, wherein the ECM is a reconstituted gel.

15. (Withdrawn) The isolated ECM of any one of claims 6-13, therein the ECM is sterile.

16. (Withdrawn) A package containing sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM.

17. (Withdrawn) The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a sheet of isolated, decellularized Urodele ECM.

18. (Withdrawn) The package of claim 17, wherein the sterile, isolated, decellularized Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a dry powder.

19. (Withdrawn) The package of claim 17, wherein the sterile, isolated, decellularized

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Urodele ECM or a Urodele fraction derived from the sterile, isolated, decellularized Urodele ECM is a gel.

20. (Original) A sterile medical implant comprising sterile, isolated, decellularized Urodele ECM or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM.

21. (Original) The sterile medical implant of claim 20, wherein the implant is a biocompatible sheet, mesh, gel, graft, tissue or device.

22. (Original) A material coated with, impregnated with, encapsulating, or having attached thereto isolated, decellularized Urodele ECM or a Urodele fraction derived from the isolated, decellularized Urodele ECM.

23. (Withdrawn) A tissue culture system comprising (a) an isolated Urodele decellularized ECM, (b) tissue culture medium, and (c) cells xenogenic to the Urodele.

24. (Withdrawn) The tissue culture system of claim 22, wherein the cells are human cells.

25. (Withdrawn) Tissue culture medium conditioned with isolated Urodele decellularized ECM or a Urodele fraction derived from isolated, decellularized Urodele ECM.

26. (Original) A device comprising at least two sheets of isolated Urodele decellularized ECM laminated to one another.

27. (Withdrawn) A product prepared by isolating Urodele ECM from a Urodele, decellularizing the ECM, and sterilizing the decellularized ECM.

28. (Withdrawn) A method of preparing a biologic material comprising (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B)

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decellularizing the sample forming a decellularlized ECM to remove sufficient cellular components of the sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

29. (Withdrawn) The method of claim 28, wherein the method further comprises performing the decellularization in a manner to retain structural and functional integrity of the decellularized ECM sufficient to permit the decellularized ECM to be useful as a matrix upon and within which cells can grow.

30. (Withdrawn) The method of claim 29, further comprising homogenizing the decellularized ECM to form a powder.

31. (Withdrawn) The method of claim 30, further comprising reconstituting the powder as a gel.

32. (Withdrawn) The method of claim 29, further comprising sterilizing the decellularized ECM.

33. (Withdrawn) The method of claim 29, further comprising attaching the decellularized ECM to an agent xenogenic to a Urodele.

34. (New) The tissue graft of claim 5, wherein the extracellular matrix components derived from a Urodele are produced by a process that comprises (A) obtaining a tissue sample from a urodele, which tissue sample comprises extracellular matrix, and (B) decellularizing the sample.

35. (New) The tissue graft of claim 34, wherein the tissue sample is decellularized to remove sufficient cellular components of the tissue sample to reduce or eliminate antigenicity of the biomaterial as a xenograft.

36. (New) The tissue graft of claim 35, wherein said decellularizing comprises subjecting said tissue sample to an alkaline treatment.

37. (New) The tissue graft of claim 34, wherein said process further comprises subjecting said sample to sterilization.

38. (New) The tissue graft of claim 34, wherein the Urodele is an Axolotl.

39. (New) The sterile medical implant of claim 20, wherein the implant is isolated, decellularized Urodele ECM.

40. (New) The sterile medical implant of claim 39, wherein the Urodele ECM includes basement membrane.

41. (New) The sterile medical implant of claim 20, wherein the Urodele ECM is derived from Axolotl tissue.

42. (New) The sterile medical implant of claim 20, wherein the Urodele ECM is infused with, coated with, or attached to an agent xenogenic to a Urodele that is a growth factor, a cytokine, a chemokine, a protein, a carbohydrate, a sugar, a steroid, an antimicrobial agent, a synthetic polymer, an adhesive, a drug or a human agent.

43. (New) The sterile medical implant of claim 20, further comprising a human cell and wherein the Urodele ECM is infused with, coated with, or attached to the human cell.

44. (New) The sterile medical implant of claim 20, wherein the Urodele extracellular matrix (ECM) or a sterile Urodele fraction derived from the isolated, decellularized Urodele ECM is a sheet.

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45. (New) The sterile medical implant of claim 20, wherein the Urodel ECM is particles of the Urodel ECM.

46. (New) The sterile medical implant of claim 20,, wherein the Urodel ECM is a reconstituted gel.

47. (New) The material of claim 22, wherein the Urodele ECM is Axolotl ECM.

48. (New) The device of claim 26, wherein the Urodele ECM is Axolotl ECM.

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REMARKS

Claims 1-33 were previously pending in this application. Claims 1-4, 6-19, 23-35 and 27-33 are withdrawn. Claims 5, 20, 21, 22 and 26 are maintained, with claim 21 amended to correct a dependency. Claims 34-48 are newly added. Support for new claims 34-44 and 46-48 is found in the originally filed claims. Support for claim 45 is found at least at paragraph 72 of the application as filed. As a result, claims 5, 20-22, 26 and 34-48 are pending for examination with claims 5, 20, 22 and 26 being independent claims. No new matter has been added.

Restriction Requirement

In response to the restriction requirement set forth in the Office Action mailed September 9, 2016, Applicant hereby elects a tissue graft and Group II (claims 5, 20-22, 26), drawn to a tissue graft for continued examination. All the claims read on the elected tissue graft. This election is made without traverse.

Applicant believes no fee is due with this response. However, if there is a fee occasioned by this response, including an extension fee, the Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith to our Deposit Account No. 23/2825, under Docket No. S1860.70000US01, from which the undersigned is authorized to draw.

Dated: November 9, 2016

Respectfully submitted,

By: /Edward R. Gates/

Edward R. Gates
Registration No.: 31,616
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

RegenX Science Exhibit 1004
Page 182

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0182

Electronic Acknowledgement Receipt

EFS ID:	27462810
Application Number:	14759895
International Application Number:	
Confirmation Number:	3358
Title of Invention:	DECELLULARIZED BIOMATERIAL FROM NON-MAMMALIAN TISSUE
First Named Inventor/Applicant Name:	Ryanne Early
Customer Number:	23628
Filer:	Edward R. Gates/Nicole Forgrave
Filer Authorized By:	Edward R. Gates
Attorney Docket Number:	S1860.70000US01
Receipt Date:	09-NOV-2016
Filing Date:	08-JUL-2015
Time Stamp:	15:37:03
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	S186070000US01-TRN-ERG.pdf	24636 67177a862b3642d1331857 4b594830d51 e20c9c	no	1

Warnings:

Information:					
2		S186070000US01-ERRRAMN-ERG.pdf	39200 599bd4d911bea0f31c87c9a0f5fa2c39d0fce87b	yes	8

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Response to Election / Restriction Filed	1	1
Claims	2	7
Applicant Arguments/Remarks Made in an Amendment	8	8

Warnings:	
Information:	
Total Files Size (in bytes):	63836

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	14/759,895-Conf. #3358
Filing Date	July 8, 2015
First Named Inventor	Ryanne Early
Art Unit	1651
Examiner Name	R. A. Davis
Attorney Docket Number	S1860.70000US01

ENCLOSURES (*Check all that apply*)

- [ ] Fee Transmittal Form
  - [ ] Fee Attached
- [ ] Amendment/Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
  - [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
  - [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) (please identify below):

Amendment and Response to Restriction Requirement

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Edward R. Gates/		
Printed name	Edward R. Gates		
Date	November 9, 2016	Reg. No.	31,616

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FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 14/759,895	ATTY. DOCKET NO.: S1860.70000US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: July 8, 2015	CONFIRMATION NO.: 3358
				FIRST NAMED INVENTOR: Ryanne Early	
Sheet	1	of	2	GROUP ART UNIT: 1651	EXAMINER: Ruth A. Davis

U.S. PATENT DOCUMENTS

Examiner's Initials #	Cite No.	U.S. Patent Document		Name of Patentee or Applicant of Cited Document	Date of Publication or Issue of Cited Document MM-DD-YYYY
		Number	Kind Code		
		4,298,002		Ronel et al	11-03-1981
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Examiner's Initials #	Cite No.	Foreign Patent Document			Name of Patentee or Applicant of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	Translation (Y/N)
		Office/ Country	Number	Kind Code			
		WO	03/082201	A2	Anthrogenesis Corporation	10-09-2003	
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		WO	2007/124127	A2	Wake Forest University Health Sciences	11-01-2007	
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		WO	2012/131106		Ingell Technologies Holding B.V.	10-04-2012	

OTHER ART – NON PATENT LITERATURE DOCUMENTS

Examiner's Initials #	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	Translation (Y/N)
		Extended European Search Report for European Application No. 14737892.1 mailed September 28, 2016 (S1860.70000EP00).	
		International Search Report and Written Opinion for International Application No. PCT/US2014/010890 mailed May 15, 2014 (S1860.70000WO00).	
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FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 14/759,895	ATTY. DOCKET NO.: S1860.70000US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: July 8, 2015	CONFIRMATION NO.: 3358
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Sheet	2	of	2	GROUP ART UNIT: 1651	EXAMINER: Ruth A. Davis

		ZAIOU, Multifunctional antimicrobial peptides: therapeutic targets in several human diseases. Journal of Molecular Medicine. 2007 Apr; 85(4):317-29. Epub 2007 Jan 10.	

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Confirmation Number:	3358
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First Named Inventor/Applicant Name:	Ryanne Early
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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Foreign Reference	WO03082201A2.pdf	4251760 [illegible]	no	72
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Information:					
2	Foreign Reference	WO2005009498.pdf	6615831 823101fa6bd61759bb9dcbd4fa0d09015c5f7 7fd0	no	41
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3	Foreign Reference	WO2007124127.pdf	7218168 [illegible] 2613e	no	46
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4	Foreign Reference	WO12131104.pdf	529360 [illegible] c9d2	no	60
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5	Foreign Reference	WO12131106.pdf	536477 [illegible] ab33	no	57
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6	Other Reference-Patent/App/Search documents	S186070000EP00_EESR_mailed_09-28-2016.pdf	240222 [illegible] 88a9	no	7
Warnings:					
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7	Other Reference-Patent/App/Search documents	S186070000WO00_ISR_WO_Mailed_05-15-2014.pdf	1203692 [illegible] 9d7ef	no	7
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8	Other Reference-Patent/App/Search documents	S186070000WO00_IPRP_Mailed_07-23-2015.pdf	977471 [illegible] 54	no	7
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Information:					

9	Non Patent Literature	Meller_et_al_DTSCH_aRZTEBL_Intl_2011_v108_p243.pdf	461236 b5b8cffa9f87afd3d9f988ee4d4a13903dcd9f05081	no	7
Warnings:					
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10	Non Patent Literature	Vacanti_et_al_J_Pediatr_Surg_1988_v23_p3.pdf	2096134 2970295036dbe629482295c1a97eb3ddc62100e8	no	7
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11	Non Patent Literature	Vacanti_et_al_Plast_Reconstr_Surg_1991_v88_p753.pdf	501165 88410efb532866f4922ab6a2e26ab3d1912326f7c	no	7
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12	Non Patent Literature	Wiens_et_al_Systematic_Biology_2005_V54_p91.pdf	6193627 e5013d6dc129c807c7763a3c21c07cf85b799af99	no	20
Warnings:					
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13	Non Patent Literature	Zaiou_J_Mol_Med_2007_v85_p317.pdf	279339 680ef8a8924de2ff46a8f57f163ba6053b15e32	no	13
Warnings:					
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14	Non Patent Literature	Cima_et_al_Biotechnol_Bioeng_1991_v38_p145.pdf	8808888 f301e3ad63c610488066d1588871e086838b0a32e	no	14
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15	Non Patent Literature	Tottey_et_al_Biomaterials_2011_v32_p128.pdf	8726220 3c337f28d5e885b9236299e61b807bbae6...e858	no	17
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16	Miscellaneous Incoming Letter	S186070000US01-TRN-KZM.pdf	24821 e7f2089c0c2221813a6f0a4de6a1f63d725[illegible]	no	1
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17		S186070000US01-IDS-KZM.pdf	38538 5c6e1ab9a61062d79338747228fdd7732[illegible]	yes	5

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Transmittal Letter	1	3
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[Continued on next page]

(54) Title: COLLAGEN BIOFABRIC AND METHODS OF PREPARATION AND USE THEREFOR



(57) Abstract: The present invention relates to collagenous membranes produced from amnion, herein referred to as a collagen biofabric. The collagen biofabric of the invention has the structural integrity of the native non-treated amniotic membrane, i.e., the native tertiary and quaternary structure. The present invention provides a method for preparing a collagen biofabric from a placental membrane, preferably a human placental membrane having a chorionic and amniotic membrane, by decellularizing the amniotic membrane. In a preferred embodiment, the amniotic membrane is completely decellularized. The collagen biofabric of the invention has numerous utilities in the medical and surgical field including for example, blood vessel repair, construction and replacement of a blood vessel, tendon and ligament replacement, wound-dressing, surgical grafts, ophthalmic uses, sutures, and others. The benefits of the biofabric are, in part, due to its physical properties such as biomechanical strength, flexibility, suturability, and low immunogenicity, particularly when derived from human placenta.

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COLLAGEN BIOFABRIC AND METHODS OF PREPARATION AND USE THEREFOR

[0001] This application is a continuation in part of United States Application No. 10/106,653 filed on March 26, 2002, which is incorporated herein by reference in its entirety.

1. FIELD OF THE INVENTION

[0002] The present invention relates to collagenous membranes produced from amnion, herein referred to as a collagen biofabric. The collagen biofabric of the invention has the structural integrity of the native non-treated amniotic membrane, *i.e.*, the native tertiary and quaternary structure. The present invention provides a method for preparing a collagen biofabric from a placental membrane, preferably a human placental membrane having a chorionic and amniotic membrane, by decellularizing the amniotic membrane. In a preferred embodiment, the amniotic membrane is completely decellularized. The collagen biofabric of the invention has numerous utilities in the medical and surgical field including for example, blood vessel repair, construction and replacement of a blood vessel, tendon and ligament replacement, wound-dressing, surgical grafts, ophthalmic uses, sutures, and others. The benefits of the biofabric are, in part, due to its physical properties such as biomechanical strength, flexibility, suturability, and low immunogenicity, particularly when derived from human placenta.

2. BACKGROUND OF THE INVENTION

2.1 AMNIOTIC MEMBRANE: ANATOMY AND HISTOLOGY

[0003] The placental sac is composed of two layers intimately connected by loose connective tissue. They are known as the amniotic and chorionic layers (*See* FIG. 1). The amniotic layer is the most internal of the two layers and comes into contact with the amniotic fluid that surrounds the fetus and together they form the amniotic sac. The amniotic layer is avascular and lined by simple columnar epithelium overlying a basal membrane and it measures 30-60 microns in thickness. The chorionic membrane is the outer layer of the sac and it is heavily cellularized. The vascular tree originates in the placenta and extends to the placental membranes through the chorionic layer. The chorionic layer is separated from the amniotic layer by loose connective tissue and combined, the two layers measure 120-180 microns. The placental membranes have a collagen matrix that is heavily laden with

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mucopolysaccarides and they are believed to serve primarily as a protective sac for the developing fetus. The membranes also maintain a barrier for infectious and immnunologic agents present in the maternal circulation. Placental membranes have both active and passive transports. Most small molecules and proteins can travel freely through them but large proteins such as IgM cannot cross through the basal layer.

[0004] Preservation of the placental membranes (which are either from animal or human sources) in either 95% ethyl alcohol or glycerol mixed in 50-50% proportions with tissue culture media has been utilized for preservation of the amniotic membrane prior to freezing. These preservatives eliminate the vitality of the placental tissues making the nuclei pyknotic but the collagen matrix and basal membranes are preserved. Interestingly, both forms of preservation also eliminate the antigenicity of the transplanted membranes and also any potentially virulent agents. Preservation is usually accomplished after the amniotic membranes are carefully separated from the chorion. The side of the amniotic membrane with the basal lamina and epithelium is shiny and the opposite side facing the chorion is dull.

2.2 PREVIOUS CLINICAL APPLICATIONS OF AMNIOTIC MEMBRANES

[0005] Possible potential problems with xenogenic tissues (tissues from other species) carrying zoonotic diseases or causing cross-species rejection have made these tissues less desirable. Allogenic grafts, or grafts from different individuals of the same species, continue to be the preferred source for human graft materials. The scarcity of human donor tissues for grafting is a growing problem that has stimulated the development of new materials for tissue grafting. Most often these sources of biological raw material are scarce, difficult to obtain, and very costly. The collagen sheet from human amnion, however, has desirable biomechanical characteristics useful in tissue graft applications. Thus, amniotic membranes are a good source of allogenic graft material.

[0006] The fetal membrane including the amniotic and chorionic membrane has been used in surgeries documented as early as 1910 and has been reviewed by Trelford and Trelford-Sauder in 1979 (*See* Trelford and Trelford-Sauder, 1979, *Am. J. Obstet. Gynecol.* 833). In 1910 Davis was the first to report the use of fetal membranes as a surgical material in skin transplantation for example on burned and ulcerated skins (Davis *et al.*, 1910, *Johns Hopkins Med. J.*; 15: 307). These studies were mainly in animals and human trials proved disappointing. Since then the use of amniotic membranes in surgery has been expanded (*See, e.g.*, Stern *et al.*, 1913, *JAMA*, 13: 973-4; Sabella *et al.*, 1913 *Med. Records NY*, 83: 478-80;

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de Rotth *et al.*, 1940 *Arch. Opthalmol*, 23: 522-5; Sorsby *et al.*, 1946, *Br. J. Opthamlol.* 30: 337-45). It is now utilized as a biological dressing for burned skin, skin wounds, and chronic ulcers of the leg, as an adjunctive tissue in surgical reconstruction of artificial vagina, and for repairing omphaloceles (*See e.g.*, Trelford and Trelford-Sauder, 1979, 134 *Am. J. Obstet. Gynecol.* 833; Colocho *et al.*, 1974 *Arch. Surg.* 109: 370-3; Faulk *et al.* 1980, *Lancet*, 1156; Prasad *et al.*, 1986, *J. Trauma*, 26: 945-6; Subrahmanyan *et al.*, 1995, *J. Plastic Surg.* 48: 477-8; Gruss *et al.*, 1978, *J. Can. Med. Assoc.* 118: 1237-46; Ward *et al.*, 1984, *Br. J. Plastic Surg.* 37: 191-3; Dhall, 1984, *J. Obstet. Gynaecol.* 91: 279-82). It has also been used to prevent tissue adhesion in surgical procedures of the abdomen, head and pelvis (Gharib *et al.*, 1996, *Pediatr. Surg. Int.* 11: 96-9; Rennekampff *et al.*, 1994, *Invest. Surg.* 7: 187-93). In the 1940s, several authors reported the beneficial role of the amniotic membrane in treating a variety of ocular surface disorders (*See e.g.*, de Rotth *et al.*, 1940 *Arch. Opthalmol*, 23: 522-5; Sorsby *et al.*, 1946, *Br. J. Opthalmol.* 30: 337-45; Lavery *et al.*, 1946, *Trans. Opthalmol. Soc. UK*, 66: 668).

[0007] Numerous attempts in the field to optimize the preparation and preservation of the amniotic membranes for use in transplantation have been previously described (*see e.g.*, Dua *et al.*, 1999, *Br. J. Opthalmol.* 83: 748-52 ("Dua") for a review). Various preparation of amniotic membranes have included preservation by saline and antibiotic mixtures, alcohol dehydration with or without separation of the amniotic layer from the chorionic layer. However, all of the methods described in Dua, and in the references described above still carry shortcomings that need to be addressed by improvements in preparation and preservation of amniotic membranes.

[0008] More recently, methods have been disclosed which rely on freezing for preservation of the amniotic membrane for application in tissue graft surgical procedures to correct corneal epithelial defects. *See e.g.*, U.S. Patent Nos. 6,152,142 and 6,326,019B1 ("Tseng"). Tseng discloses an amniotic membrane that is mounted on a substrate and preserved in a mixture of Dulbecco's Modified Eagle Medium and glycerol and frozen at -80°C. The process of freezing the tissue at any time during its preparation makes the Tseng amniotic membrane brittle, and even more brittle after the steps of thawing and activation. In addition, the thawing and activation steps add time required for the handling of the amniotic membrane. Furthermore, because of the brittleness of the Tseng amniotic membrane caused by the freezing step in the preservation and preparation process, a structural support or backing is required to ensure structural integrity of the Tseng amniotic membrane during storage. This presents the added difficulty of separating the preserved amniotic membrane

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from the backing, which, due to its brittleness can be difficult to handle and separate intact. Separation of the amnion membrane from the backing thus increases the likelihood of rupture of the membrane, and increases the length of time required to activate the amniotic membrane to allow for thorough impregnation of the activation solution into the frozen amniotic membrane prior to performing the surgical procedure, leading to increased preparation time in the surgical suite. Storage and shipping are also complicated by the requirement of -80°C freezing. Finally, the membranes of Tseng are not generally decellularized; as a result, the amniotic membranes so prepared are typically opaque and do not have a uniform structural composition.

[0009] More recently, Yui *et al.*, U.S. Patent Nos. 5,618,312, (the "'312 Patent") described the preparation of collagen sheets from stratum compactum of tissue membrane. Although the material described is primarily collagen, it is weak enough due to its processing to require a separate step of crosslinking in order to render it strong enough for medical use, *e.g.*, suturable. One method for cross-linking described in the '312 Patent employs high heat treatment, preferably at 100° to 110°C, which is believed to adversely affect the native conformation of collagen. Yui *et al.*, U.S. Patent No. 5,876,451 describe a similar collagen material derived from placenta. This material, however, is treated during preparation with proteases as part of a decellularization step, which likely results in destruction and/or disruption of native conformation of the components of the matrix; thus, the resulting collagen matrix does not maintain its native conformation.

[0010] There thus remains a need in the art for an improved amniotic membrane for use in medical, diagnostics, and cosmetic applications, which has improved structural characteristics. The present invention provides such a biofabric, comprising collagen that, unlike prior collagen-based biofabrics, retains the native collagen quaternary structure. As a result, the biofabric of the present invention is easily prepared, easily used, and is strong enough for medical and surgical purposes, and provides a superior substrate for wound healing.

3. SUMMARY OF THE INVENTION

[0011] The present invention relates, in part, to the discovery by the inventors of a novel method of preparation of a collagen biofabric from placenta, preferably a human placenta, that results in a novel collagen biofabric with improved physical and biophysical properties. Preferably the method of preparation involves minimal manipulation of the amniotic membrane. The collagen biofabric of the invention unlike those described in the

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prior art, due to the way by which it is processed, has an intact native tertiary and quaternary structure. The present invention also provides a placental-derived amniotic membrane or biofabric having superior characteristics of increased tensile strength, suturability, and reduced immunogenicity resulting in reduced host-graft rejection. The present invention also provides a placental-derived amniotic membrane or biofabric that can be stored as dehydrated sheets without freezing or cryopreservation. Preferably, the placental-derived amniotic membrane is derived from a human placenta for use in human patients. However, the same methods can be employed using placentas from various animal species for veterinary use in animal patients.

[0012] The present invention provides a method of preparing a collagen biofabric comprising providing a placenta, preferably a human placenta, separating the amnionic and chorionic layers from each other, and decellularizing the amniotic membrane while preserving the architecture of the underlying extracellular matrix. The method further entails washing and drying the decellularized membrane. This method yields a dehydrated, decellularized biofabric that can remain stable under sterile storage conditions at room temperature and that is subsequently rehydrated and grafted to or implanted into a subject.

[0013] In a specific embodiment, the placenta is from a human female who has undergone a cesarean section delivery or natural delivery. In preferred embodiments, common seriological and bacteriological assays known to one skilled in the art are used to determine if the placenta is from a donor that is free of a communicable disease. In a specific embodiment, the placenta has been tested to be free of at least one communicable disease. In another embodiment, the source of the placenta is known including medical history, blood type, immunologic data, and genotype characteristics of the donor. Although, the placenta can be from any mammal, preferably the donor is a human female. In some embodiments, the placenta is exsanguinated using common methods known to one skilled in the art prior to separating the amniotic membrane from the chorionic membrane.

[0014] The decellularization of the amniotic membrane in accordance with the methods of the invention comprises removing all visible cellular material and cellular debris from the amniotic membrane, *e.g.*, from the maternal side of the amniotic membrane and the fetal side of the amniotic membrane. The decellularization of the amniotic membrane should not result in disruption of the structural integrity of the amniotic membrane or alter the biochemical composition. Accordingly, decellularizing the amniotic membrane in accordance with the methods of the invention does not constitute freezing at any point in preparation of the amniotic membrane or contacting the membrane with a protease.

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Preferably, the amniotic membrane is decelluarized using a weak detergent containing solution, *e.g.*, a solution comprising 0.01-1.0% deoxycholic acid sodium salt monohydrate, a nonionic detergents, Triton X-l00, an anionic detergent, or sodium dodecyl sulfate or a combination thereof.

[0015] Once the amniotic membrane is decellularized in accordance with the methods of the invention, the membrane may be washed and dried, preferably with low heat under vacuum.

[0016] In some embodiments, the invention provides a collagen biofabric comprising a dehydrated, decellularized and substrate-free amniotic membrane, so that the membrane has native tertiary and quaternary structure. In other embodiments, the invention provides a collagen biofabric comprising a decellularized substrate-free amniotic membrane, comprising of collagen, elastin and fibronectin. In yet other embodiments, the invention provides a collagen biofabric comprising a dehydrated, decellularized, uniform, translucent, and substrate-free amniotic membrane, with the provision that the amniotic membrane has never been contacted with a protease.

[0017] In some embodiments, the biofabric further comprises one or more biomolecules, *e.g.*, therapeutic agents, including but not limited to, antibiotics, hormones, growth factors, anti-tumor agents, anti-fungal agents, anti-viral agents, pain medications, anti-histamines, anti-inflammatory agents, anti-infectives, wound healing agents, wound sealants, cellular attractants and scaffolding reagents, and the like. In a specific example, the collagen biofabric may be impregnated with one or more growth factors, for example, fibroblast growth factor, epithelial growth factor, *etc.* The biofabric may also be impregnated with one or more small molecules, including but not limited to small organic molecules such as specific inhibitors of particular biochemical processes *e.g.*, membrane receptor inhibitors, kinase inhibitors, growth inhibitors, anti-cancer drugs, antibiotics, *etc.* In some embodiments, the collagen biofabric is impregnated with a biomolecule, during production or during preparation for surgery depending on its intended use.

[0018] In some embodiments, the invention encompasses a laminate comprising at least two layers of the biofabric of the invention, and methods of preparing same. In other embodiments, the invention encompasses shaping the laminates into complex three dimensional scaffolds depending on the intended use, including but not limited to sheets, fibers, spheres, tubes.

[0019] In one embodiment, the invention encompasses a method of preparing an amniotic membrane laminate comprising: providing a placenta comprising an amniotic

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membrane and a chorionic membrane; separating the amniotic membrane from the chorionic membrane; and decellularizing the amniotic membrane. In another embodiment, the method further comprises washing the decellularized amniotic membrane at least once; layering at least two of the decellularized amniotic membranes in contact with each other so that an amniotic membrane laminate is formed; and drying the decellularized amniotic membrane laminate. Alternatively, in another embodiment, the method for preparing an amniotic membrane laminate comprises, drying at least two amniotic membranes prepared in accordance with the methods of the invention, and layering the at least two amniotic membranes in contact with each other so that an amniotic membrane laminate is formed.

[0020] In some embodiments, the amniotic membrane layers produced in accordance with the methods of the invention may be placed in contact with each other in the presence of an adhesive to form an amniotic membrane laminate. The adhesive used in accordance with the methods and compositions of the invention may be any biological glue known to one skilled in the art, preferably a biocompatible glue, including but not limited to, natural glue, *e.g.*, fibronectin, fibrin, synthetic glue. In other embodiments, the amniotic membrane layers prepared in accordance to the methods of the invention are cross-linked to each other to form an amniotic membrane laminate. Any cross-linking reagent and method known to one skilled in the art is within the scope of the present invention, including but not limited to, chemical cross-linking, peptide cross-linking, UV cross-linking, radiation cross-linking, fibronectin cross-linking, fibrinogen cross-linking, hydrogel cross-linking. In other embodiments, the amniotic membrane laminates produced in accordance with the methods of the invention do not comprise an adhesive.

[0021] In some embodiments, the invention encompasses using the collagen biofabric as a surgical graft. In a specific embodiment, the invention encompasses use of the surgical graft in a surgical procedure in a subject, preferably a human, comprising placing the graft directly on the surgical site.

[0022] The invention provides a collagen biofabric, an amniotic membrane laminate, or a three-dimensional scaffold further comprising one or more hydrogel compositions, and methods of preparing same. The hydrogel composition may comprise a polymer inlcuding but not limited to polyvinyl alcohol, polyethylene glycol, hyaluronic acid, and derivative and analogs thereof.

[0023] In some embodiments, the collagen biofabric of the invention, aminates, three-dimensional scaffolds, or hydrogel compositions thereof may be further populated with cells,

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such as stem cells, differentiated adult cells, progenitor cells, and the like, preferably human so that the cells are uniform and confluent.

[0024] The invention provides methods of high level production of the collagen biofabric, laminates thereof, three-dimensional scaffolds thereof, and hydrogel compositions thereof, particularly but not limited to, commercial scale production. The invention solves difficulties in producing large-scale quantities of amniotic membranes for use in clinical trials and commercial sales.

[0025] The invention encompasses compositions comprising a collagen biofabric of the invention suitable for drug delivery; tissue engineering; urological related uses, *e.g.*, correction of urinary incontinence; ocular uses, *e.g.*, for the treatment of an ocular surface disorder, and as an ophthalmic surgical graft; vascular uses, *e.g.*, blood vessel repair, construction and replacement of a blood vessel; cardiological uses, *e.g.*, as a prosthetic device in constructing diseased valves; neuronal-related uses, *e.g.*, repair of injured nerves, especially severed peripheral nerves, as a dural substitute, and as a prostheses around nerve anastosmosis; bone related uses, *e.g.*, for the treatment of orthopedic defects, as a bone replacement; dermatological uses, *e.g.*, for the treatment of wounds (external and internal), acute and chronic wounds, congenital wounds, and burns; for the treatment of skin conditions, *e.g.*, skin lesions, aged skin, wrinkles, fine lines, thinning, reduced skin elasticity, rough skin, and sun damaged skin; as a wound dressing; and for the treatment of wound infections.

[0026] In a specific embodiment, the invention encompasses a method for treating and/or preventing an eye related disease or disorder, *e.g.*, ocular surface disease, in a subject, comprising using the collagen biofabric of the invention, for example, by placing the biofabric as a surgical graft on the diseased corneal surface of the subject. In another embodiment, the invention encompasses a method of treating and/or preventing a skin condition in a subject, comprising using a biofabric of the invention for example, by contacting the skin with the biofabric. In yet another embodiment, the invention encompasses treating a wound and/or burn in a subject comprising contacting the wound and/or burn with a biofabric of the invention. In another specific embodiment, the invention encompasses a method of correcting urinary incontinence in a subject, preferably, a human, comprising using a collagen biofabric of the invention as an implant.

[0027] In other embodiments, the invention encompasses a method of delivering a therapeutic agent to a subject comprising contacting the subject with a collagen biofabric of the invention. The invention further encompasses methods of delivering cells to a subject

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comprising populating a collagen biofabric or laminate of the invention with living cells for example in tissue engineering.

[0028] The invention provides a surgical graft comprising the biofabric of the invention for use in a surgical procedure. The surgical graft may be applied to an internal or external site of the subject, preferably a human.

4. BRIEF DESCRIPTION OF THE FIGURES

[0029] **FIG. 1** The chorion and amniotic membrane of a human placenta.

[0030] **FIG. 2 PHOTOMICROGRAPH OF THE COLLAGEN BIOFABRIC**

A. BEFORE PROCESSING

B. AFTER PROCESSING

[0031] **FIG. 3 THE COLLAGEN BIOFABRIC**. The collagen biofabric of the invention is exemplified having a uniform translucent surface with an embossed pattern.

[0032] **FIG.4 MESH FRAME AND THE BIOFABRIC BEING DRIED THEREIN**

5. DETAILED DESCRIPTION OF THE INVENTION

[0033] The present invention provides a collageneous membrane or biofabric derived from the placenta of a mammal, preferably of a human. The collagen biofabric is prepared so as to retain the native collagen conformation, *i.e.*, the native tertiary and quaternary conformation, in the final product. In addition to the collagen biofabric, the present invention also provides methods of making the collagen biofabric, and of using the biofabric in a medical setting.

[0034] The present invention provides a collagen biofabric comprising a dehydrated, decellularized and substrate-free amniotic membrane so that the amniotic membrane has a native tertiary and quaternary structure. In some embodiments the invention provides a decellularized and substrate-free collagen biofabric comprising of collagen, elastin, and fibronectin.

[0035] In some embodiments, the invention provides an amniotic membrane laminate comprising a collagen biofabric of the invention. The amniotic membrane laminate prepared in accordance with the methods of the invention comprises at least two layers of the collagen biofabric that are placed in contact with each other to form the amniotic membrane laminate. In other embodiments, the invention provides a three dimensional scaffold, such as a tube, comprising a collagen biofabric of the invention. In yet other embodiments, the invention

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provides a collagen biofabric of the invention, a laminate thereof, or a three dimensional scaffold thereof further comprising a hydrogel composition.

[0036] The invention thus provides various forms and configurations of the collagen biofabric including but not limited to laminates, three-dimensional scaffolds, and hydrogel compositions. The invention provides any medical useful form of the compositions of the invention. Regardless of the particular form or configuration, the compositions of the invention may further comprise one or more biomolecules, preferably a therapeutic agent. The compositions of the invention comprising a biomolecule have numerous utility in the medical field as described in detail herein. In some embodiments, the invention encompasses populating the compositions of the invention with living cells so that the cells are uniform and confluent. The compositions of the invention populated with cells have numerous utility in the medical and dental field for example for tissue engineering purposes.

[0037] The invention also relates to methods for preparing a collagen biofabric, a laminate thereof, a three-dimensional scaffold thereof, or a hydrogel composition. In a specific embodiment, the invention provides a method of preparing a collagen biofabric from a placenta having an amniotic membrane and a chorionic membrane comprising: separating the amniotic membrane from the chorionic membrane; and decellularizing the amniotic membrane so that the amniotic membrane is not contacted with an enzyme, *e.g.*, a protease. In other embodiments, the method further entails washing and drying the decellularized amniotic membrane. In another specific embodiment, the invention provides a method of preparing an amniotic membrane laminate from a placenta having an amniotic membrane and a chorionic membrane comprising: separating the amniotic membrane from the chorionic membrane; decellularizing the amniotic membrane; and layering at least two of the decellularized amniotic membranes in contact with each other so that an amniotic membrane laminate is formed. In a specific embodiment, the decellularized amniotic membrane is dried prior to layer. In another specific embodiment, the decellularized amniotic membrane is dried after is layered so that at least two of the decellularized amniotic membranes are in contact with each other.

[0038] The invention provides a method of using the collagen biofabric of the invention, laminates thereof, three-dimensional scaffolds thereof, or hyrdogel compositions thereof in a medical, dental and surgical setting. In fact, it is expected that the compositions of the invention have an enhanced therapeutic and clinical utility relative to the other biomaterials known in the art. In some embodiments, the invention provides a method of treating and/or preventing an eye related disease or disorder in a subject using a composition

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of the invention. In a specific embodiment, the invention provides a method of treating and/or preventing an eye related disease or disorder in a subject comprising placing the biofabric on a diseased eye surface of the subject.

[0039] In other embodiments, the invention provides a method of treating and/or preventing a skin condition in a subject, preferably a human, using a composition of the invention. In a specific embodiment, the method comprises contacting the skin of the subject with the composition. In another specific embodiment, the composition is placed directly on the surface of the skin of the subject, which is the site of the skin condition.

[0040] The invention also provides a method for treating a wound or a burn in a subject, preferably a human, comprising contacting the composition at the site of the wound or burn.

[0041] In other embodiments, the invention provides using a composition of the invention (*e.g.*, a collagen biofabric, an amniotic membrane laminate) in a surgical procedure, such as ophthalmic surgery; cardiovascular surgery; periodontal surgery; neurological surgery, dental surgery, and orthopedic surgery.

[0042] The invention provides a method for using a composition of the invention for delivering a biomolecule, preferably a therapeutic agent to a subject, preferably a human. The invention also encompasses a method of delivering cells using a composition of the invention to a subject, preferably a human, wherein the composition has been further populated with cells.

5.1 COLLAGEN BIOFABRIC

[0043] The invention provides a collagenous amniotic membrane, herein referred to as a collagen biofabric. The collagen biofabric of the invention maintains the structural integrity of the native, non-treated amniotic membrane, *i.e.*, retains the tertiary and quaternary structure of the structural proteins in its compositions such as collagen, elastin, and possibly fibronectin. Thus, the collagen biofabric of the invention is composed of the same structural proteins as the native or non-treated amniotic membranes. Prior art methods of producing amniotic membranes require the use of proteases or high heat treatment, as a result these membranes do not maintain the tertiary and quaternary structure of the structural proteins in their composition.

[0044] In a specific embodiment, the present invention provides a dehydrated, decellularized, and substrate-free (*i.e.*, no filter backing) amniotic membrane such that the amniotic membrane has a native tertiary and quaternary structure (*See* FIG. 2A and B). The

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dehydrated decellularized amniotic membrane of the invention is a uniform, *i.e.*, minimal to no cellular material, translucent biofabric, having an appearance as shown in FIG. 3 comprising a left handed triple helix alpha helical sheet of decellularized matrix (*See, e.g.*, Molecular Biology of the Cell, 1989, Alberts *et al.*, ed., Garland Publishing Inc., New York, NY; which is incorporated herein by reference in its entirety).

[0045] The collagen biofabric of the invention may be derived from the amniotic membrane of any mammal, for example, equine, bovine, porcine or catarrhine sources, but is most preferably derived from human placenta. In a preferred embodiment, the biofabric of the invention has the native tertiary and quarternary structure of the collagenous material in its composition.

[0046] The collagen biofabric of the invention in contrast to those described in the prior art is minimally manipulated, *i.e.*, the collagen biofabric of the invention is subjected to at most one chemical or biological treatment or manipulation, *e.g.*, decellularization in a weak detergent. As used herein, "minimally manipulated" refers to a lack of enzymatic treatment, *e.g.*, protease treatment, high heat treatment, harsh chemical treatment, exposure to strong detergents or acids of the amniotic membrane of the invention at any step during the preparation of the collagen biofabric. Protease treatment of the amniotic membrane, as part of a decellularization step, compromises the structural integrity of the biofabric, *e.g.*, affects the tertiary and/or quarternary structure of the collagen material. The minimal manipulation of the amniotic membrane in preparation of the biofabric of the invention, in contrast, results in a product with enhanced mechanical strength and a translucent product relative to those in the prior art.

[0047] The decellularized, substrate-free collagen biofabric of the invention comprises of collagen (including, but not limited to, collagen type I, IV, and II), as well as fibronectin and elastin. This combination is in part responsible for the enhanced mechanical strength of the biofabric of the invention, as prepared in accordance with the methods described herein. As a result of minimal processing and manipulation, the collagen biofabric of the invention retains the composition of the native membrane. Collagen is the primary structural material of vertebrates and it is present in tissues of primarily mechanical function. The collagen molecule consists of three polypeptide chains called alpha chains (each about 1000 amino acids in length) twined around one another as in a three stranded rope forming a regular left-handed superhelix. At least 19 types of collagen have been identified and they all possess the same three-dimensional organization (*See, e.g.*, Lee *et al.*, 2001, *International J. of Pharmaceutics*, 221: 1-22).

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[0048] The amniotic membrane of the present invention is superior in form, biomechanical, and structural features to those reported in the prior art and known to the inventors, in part, based on the discovery of a novel method of preparation of the amniotic membrane and a controlled and abundant source of human placenta, as described herein (*See* Section 5.2).

[0049] In particular, the collagen biofabric of the present invention has one or more of the following characteristics as compared to one or more of the amniotic membranes of the prior art: enhanced tensile strength; superior suturability; reduced immunogenicity resulting in a reduce host-graft rejection response; ease of storage and shipment without the need for freezing or cryopreservation; minimal post-preparation requirement for handling and activation, *i.e.*, rehydration, procedures prior to use; and the ability to be stored at room temperature for extended periods of time while maintaining structural and functional integrity. Table 1 summarizes the advantages of the biofabric of the invention as compared to the ones in the prior art.

[0050] In alternative embodiments, the invention provides a collagen biofabric comprising a dehydrated, decelluarized, and substrate-free chorionic membrane, preferably a human chorionic membrane. It is expected that a collagen biofabric comprising a chorionic membrane will have comparable properties as the collagen biofabric of the invention comprising an amniotic membrane. The invention provides all medically useful forms of the collagen biofabric comprising a chorionic membrane including but not limited to laminates, three-dimensional scaffolds, and hydrogel compositions.

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TABLE 1. BIOFABRICS OF THE INVENTION vs. TRADITIONAL AMNIOTIC MEMBRANE PREPARATIONS

FEATURE	COLLAGEN BIOFABRIC OF THE INVENTION	TRADITIONAL AMNIOTIC MEMBRANES
Seriological Testing	**COMPLETE:** antibody screen (ATY); alanine amino transferase screening (ALT); Hepatitis Core Antibody(nucleic acid and ELISA); Hepatitis B Surface Antigen(nucleic acid and ELISA); Hepatitis C Virus Antibody (nucleic acid and ELISA); HIV-1 and HIV-2; HTLV-1 and HTLV-2; Syphillis test (RPR); CMV antibody test; Hepatitis C and HIV test (nucleic acid test)	**INCOMPLETE:** No CMV test
Preservation	**DRY:** Dehydrated with low-heat vacuum; no need for freezer or refrigeration; shelf storage	**FROZEN:** stored in culture medium; requires dry ice shipping and freezer/refrigeration
Surgical Preparation	**MINUTES:** Prepares within minutes; hydrates directly on the surgical site; *e.g.* on the eye with saline drops; no thawing, soaks, or rinses needed	**LENGTHY:** requires extensive preparation/thawing/soak time; must remove filter backing
Surgical Handling	**SIMPLE:** can be trimmed while dry; and applied to the surgical site and then hydrated in the site	**TEDIOUS:** Must be removed from the filter paper; tends to 'ball up" during surgery; difficult to surgically manipulate
Cellularity	**DECELLULARIZED:** minimal to no epithelial cells; minimal to no cellular debris; faster remodeling	**WITH DEAD CELLS:** dead cells present; clinical evidence suggest longer healing time/higher rejection rate (anecdotal evidence)
Substrate	**NO:** Substrate-free (*e.g.*, no filter backing)	**YES:** supported on nitrocellulose/filter paper

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Sterilization (Optional)	**YES:** Electron beam irradiation; at least18 kGy; increased assurance of tissue safety	**NO:** stored in media containing antibiotics and glycerol (toxic); no formal terminal sterilization
Tissue Clarity	**TRANSLUCENT:** Optically clear	**OPAQUE:** milky or cloudy appearance

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[0051] In a preferred embodiment, the collagen biofabric of the invention is translucent, *i.e.*, optically clear. In another preferred embodiment, the collagen biofabric of the invention is thin and lightweight. In a specific embodiment, the dehydrated collagen biofabric of the invention is 0.3-0.6 mg/cm^2. In a specific embodiment, the collagen biofabric of the invention is at least 30 microns in thickness. In another specific embodiment, the collagen biofabric of the invention is approximately 10-40 microns in thickness.

[0052] The invention encompasses use of the collagen biofabric of the invention in various configurations, *e.g.*, inserts, shields. In general, the biofabric may be configured into any medically useful form. For example, the collagen biofabric may be configured for use in its entirety, *i.e.*, use of the collagen biofabric from an entire placenta. Alternatively, the collagen biofabric may be cut into strips, patches or rolls, or may be woven into threads. In another embodiment, the collagen biofabric, either as a single-thickness sheet or laminate, may be cut at regular intervals and expanded, *e.g.*, to form a mesh.

[0053] In some embodiments, the collagen biofabric is flat, *e.g.*, having a surface with no slope or curvature. In other embodiments the collagen biofabric of the invention is not completely flat and has a dimpled surface, *e.g.*, depression or indentation on the surface.

[0054] In a specific, preferred embodiment, the invention encompasses a laminate comprising at least two layers of the collagen biofabric of the invention. The layers may be physically bound together, for example through the use of a glue, a fastener, or by heat-stamping a portion of the biofabrics, *e.g.*, the periphery of at least one of said layers, to fuse the layers. Because the biofabric has a "grain," the biofabric may be laminated in a variety of ways. In one embodiment, all layers of said laminate have the same grain orientation. In another embodiment, at least one of the layers is in a grain orientation that is rotated about 90 degrees (*i.e.*, is about perpendicular to) the grain orientation of at least one other layer. In a more specific embodiment, said perpendicular layers are adjacent to one another. In yet another embodiment, said laminate comprises at least three layers of the collagen biofabric, wherein the grain structures of each of said at least three layers is rotated about 60 degrees to the grain structure of two of the remaining layers. In another embodiment, said laminate contains at least a first material and a second material, wherein said first material is the collagen biofabric of the invention, and said second material is any other substance that can form a laminate with the collagen biofabric. In a specific embodiment, said second material is a sheet or membrane derived from a membrane other than the amniotic membrane. In another specific embodiment, said second material is a non-natural material, for example, polylactone, polyacetate, plastic film, or the like. In some embodiments, the amniotic

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membrane laminate is multi-layer, comprising at least 6, at least 8, at least 10, at least 20, at least 80, at least 100, at least 1000 amniotic membranes that have been prepared in accordance with the methods of the invention. The amniotic membrane laminate of the invention may contain an unlimited number of layers.

[0055] The laminates have increased structural rigidity that allow the laminate to be shaped into complex three-dimensional structures. Such three-dimensional structures may include sheets, tubes, microspheres.

[0056] The collagen biofabric may also be associated with another material, either as a single sheet or as a laminate. For example, the biofabric may be associated with, *i.e.*, bound to, flexible plastic film, gauze, plastic sheeting, stents, valves, orthopedic devices, bandages, patches, *etc.*

[0057] The collagen biofabric may comprise one or more compounds or substances that are not part of the collagen matrix of the biofabric. For example, the collagen biofabric may be impregnated, either during production or during preparation for surgery, with a biomolecule. Such biomolecules include but are not limited to, antibiotics (such as Clindamycin, Minocycline, Doxycycline, Gentamycin), hormones, growth factors, anti-tumor agents, anti-fungal agents, anti-viral agents, pain medications, anti-histamines, anti-inflammatory agents, anti-infectives including but not limited to silver (such as silver salts, including but not limited to silver nitrate and silver sulfadiazine), elemental silver, antibiotics, bactericidal enzymes (such as lysozome), wound healing agents (such as cytokines including but not limited to PDGF, TGF; thymosin), Hyaluronic acid as a wound healing agent, wound sealants (such as fibrin with or without thrombin), cellular attractant and scaffolding reagents (such as fibronectin) and the like. In a specific example, the collagen biofabric may be impregnated with at least one growth factor, for example, fibroblast growth factor, epithelial growth factor, *etc.* The biofabric may also be impregnated with small organic molecules such as specific inhibitors of particular biochemical processes *e.g.*, membrane receptor inhibitors, kinase inhibitors, growth inhibitors, anticancer drugs, antibiotics, *etc.*

[0058] In yet other embodiments, the collagen biofabric of the invention may be combined with a hydrogel. Any hydrogel composition known to one skilled in the art is encompassed within the invention, *e.g.*, any of the hydrogel compositions disclosed in the following reviews: Graham, 1998, *Med. Device Technol.* 9(1): 18-22; Peppas *et al.*, 2000, *Eur. J. Pharm. Biopharm.* 50(1): 27-46; Nguyen *et al.*, 2002, *Biomaterials*, 23(22): 4307-14; Henincl *et al.*, 2002, *Adv. Drug Deliv. Rev* 54(1): 13-36; Skelhorne *et al.*, 2002, *Med. Device. Technol.* 13(9): 19-23; Schmedlen *et al.*, 2002, *Biomaterials* 23: 4325-32; all of which are

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incorporated herein by reference in their entirety. In a specific embodiment, the hydrogel composition is applied on the collagen biofabric, *i.e.*, discharged on the surface of the collagen biofabric. The hydrogel composition for example, may be sprayed onto the collagen biofabric, saturuated on the surface of the biofabric, soaked with the collagen biofabric, bathed with the collagen biofabric or coated onto the surface of the collage biofabric.

[0059] The hydrogels useful in the methods and compositions of the invention can be made from any water-interactive, or water soluble polymer known in the art, including but not limited to, polyvinylalcohol (PVA), polyhydroxyehthyl methacrylate, polyethylene glycol, polyvinyl pyrrolidone, hyaluronic acid, dextran or derivatives and analogs thereof.

[0060] In some embodiments, the collagen biofabric of the invention is further impregnated with one or more biomolecules prior to being combined with a hydrogel. In other embodiments, the hydrogel composition is further impregnated with one or more biomolecules prior to being combined with a collagen biofabric of the invention. Such biomolecules include but are not limited to, antibiotics (such as Clindamycin, Minocycline, Doxycycline, Gentamycin), hormones, growth factors, anti-tumor agents, anti-fungal agents, anti-viral agents, pain medications, anti-histamines, anti-inflammatory agents, anti-infectives including but not limited to silver (such as silver salts, including but not limited to silver nitrate and silver sulfadiazine), elemental silver, antibiotics, bactericidal enzymes (such as lysozome), wound healing agents (such as cytokines including but not limited to PDGF, TGF; thymosin), Hyaluronic acid as a wound healing agent, wound sealants (such as fibrin with or without thrombin), cellular attractant and scaffolding reagents (such as fibronectin) and the like. In a specific example, the collagen biofabric or the hydrogel composition may be impregnated with at least one growth factor, for example, fibroblast growth factor, epithelial growth factor, *etc.* Preferably, the biomolecule is a therapeutic agent.

[0061] In some embodiments, the hydrogel composition is combined with a laminate comprising the biofabric of the invention.

[0062] The hydrogel/collagen biofabric composition has utility in the medical field including but not limited to, treatment of wounds, burns, and skin conditions (*e.g.*, to treat scarring), cosmetic uses (*e.g.*, cosmetic surgery), and any use as an implant. In some embodiments, the hydrogel/collagen biofabric composition is applied topically to a subject, *i.e.*, on the surface of the skin, for example, for the treatment of a wound. In other embodiments, the hydrogel/collagen biofabric composition may be used in the interior of a subject, for example as an implant, to become a permanent or semi-permanent structure in the body. In some embodiments, the hydrogel compositions in formulated to be non-

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biodegradable. In yet other embodiments, the hydrogel composition is formulated to be biodegradable. In a specific embodiment, the hydrogel composition is formulated to degrade within days. In another specific embodiment, the hydrogel composition is formulated to degrade within months.

[0063] In some embodiments, the collagen biofabric of the invention is populated with cells, so that the cells are uniform and confluent. Cells that can be used to populate a biofabric of the invention include but are not limited to, stem cells, preferably human stem cells, human differentiated adult cells, totipotent stem cells, pluripotent stem cells, multipotent stem cells, tissue specifc stem cells, embryonic like stem cells, committed progenitor cells, fibroblastoid cells. In other embodiments, the invention encompasses populating the biofabric of the invention with specific classes of progenitor cells including but not limited to chondrocytes, hepatocytes, hematopoietic cells, pancreatic parenchymal cells, neuroblasts, and muscle progenitor cells.

5.2 PROCESS FOR PRODUCING COLLAGEN BIOFABRICS

[0064] The present invention provides a method for preparing a collagen biofabric of the invention. In particular, the invention encompasses a method for preparing a collagen biofabric comprising: providing a placenta, comprising an amniotic membrane and a chorionic membrane; separating the amniotic membrane from the chorionic membrane; and decellularizing the amniotic membrane. In a specific embodiment, the method further entails washing and drying the decelluarized amniotic membrane.

[0065] Preferably, the placenta is from a human placenta for use in human subjects. In some embodiments, the invention encompasses placenta from animal species for use in human subject. In other embodiments, the invention encompasses placenta from animal species for veterinary use in animal subjects.

[0066] In a preferred embodiment, the placenta for use in the methods of the invention is taken as soon as possible after delivery of the newborn. In yet another preferred embodiment, the placenta is taken immediately following the cesarean section delivery of a normal healthy infant. Provided the placenta is collected under asceptic conditions. In some embodiments, the placenta is stored for 48 hours from the time of delivery prior to any further treatment. In other embodiments, the placenta is stored for up to 5 days from the time of delivery prior to any further treatment.

[0067] Preferably, the placenta, umbilical cord, and umbilical cord blood are transported from the delivery or birthing room to another location, *e.g.*, a laboratory, for

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further processing. The placenta is preferably transported in a sterile, transport device such as a sterile bag or a container, which is optionally thermally insulated. In some embodiments, the placenta is stored at room temperature until further treatment. In other embodiments, the placenta is refrigerated until further treatment, *i.e.*, stored at a temperature of about 2° to 8°C. In yet other embodiments, the placenta is stored under sterile conditions for up to 5 days before further treatment. In a most preferred embodiment, the placenta is handled and processed under aseptic conditions, as known to one skilled in the art. The laboratory is preferably equipped with an HEPA filtration system (as defined by clean room classification, having a class 1000 or better). In a preferred embodiment, the HEPA filtration system is turned on at least 1 hour prior to using the laboratory room for carrying out the methods of the invention.

[0068] In certain embodiments, the placenta is exsanguinated, *i.e.*, completely drained of the cord blood remaining after birth. In some embodiments, the placenta is 70% exsanguinated, 80% exsanguinated , 90% exsanguinated , 95% exsanguinated , 99% exsanguinated.

[0069] The invention encompasses screening the expectant mother prior to the time of birth, using standard techniques known to one skilled in the art, for communicable diseases including but not limited to, HIV, HBV, HCV, HTLV, syphilis, CMV, and other viral pathogens known to contaminate placental tissue. Preferably, the methods used to screen for a communicable disease follow the regulations as set forth by the Federal Drug Administration. The expectant mother may be screened (*e.g.*, a blood sample is taken for diagnostic purposes) within one month of birth, preferably within two weeks of birth, even more preferably within one week of birth, and, most preferably, at the time of birth. Only tissues collected from donors whose mothers tested negative or non-reactive to the above-mentioned pathogens are used to produce a biofabric of the invention. Preferably, a thorough paternal and medical and social history of the donor of the placental membrane is obtained, including for example, a detailed family history.

[0070] The donor is screened using standard serological and bacteriological tests known to one skilled in the art. Any assay or diagnostic test that identifies the pathogen(s) is within the scope of the method of the invention, but preferable assays are ones that combine high accuracy with capacity for high throughput. In a specific embodiment, the invention encompasses screening the donor using standard techniques known to one skilled in the art for antigens and/or antibodies. A non-limiting example of antigens and antibodies include: antibody screen (ATY); alanine amino transferase screening (ALT); Hepatitis Core

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Antibody(nucleic acid and ELISA); Hepatitis B Surface Antigen; Hepatitis C Virus Antibody; HIV-1 and HIV-2; HTLV-1 and HTLV-2; Syphillis test (RPR); CMV antibody test; and Hepatitis C and HIV test. The assays used may be nucleic acid based assays or ELISA based assays as known to one skilled in the art.

[0071] The invention encompasses further testing the blood from the umbilical cord of the newborn using standard techniques known to one skilled in the art (*See, e.g.*, Cotorruelo *et al.*, 2002, *Clin Lab.* 48(5-6):271-81; Maine *et al.*, 2001, *Expert Rev. Mol. Diagn.*,1(1):19-29; Nielsen *et al.*, 1987, *J. Clin. Microbiol.* 25(8):1406-10; all of which are incorporated herein by reference in their entirety). In one embodiment, the blood from the umbilical cord of the newborn is tested for bacterial pathogens (including but not limited to gram positive and gram negative bacteria) and fungi using standard techniques known to one skilled in the art. In a specific embodiment, the blood type and Rh factor of the blood of the umbilical cord of the newborn is determined using standard techniques known to those skilled in the art. In another embodiment, CBC with differential is obtained from the blood from the umbilical cord of the newborn using standard methods known to one skilled in the art. In yet another embodiment, an aerobic bacterial culture is taken from the blood from the umbilical cord of the newborn, using standard methods known to one skilled in the art. Only tissues collected from donors that have a CBC within a normal limit (*e.g.*, no gross abnormality or deviation from the normal level), test negative for serology and bacteriology, and test negative or non-reactive for infectious disease and contamination are used to produce a biofabric of the invention.

[0072] One exemplary method for preparing a collagen biofabric of the invention comprises the following steps.

[0073] **Step I.** The invention encompasses processing the placental membrane so that the umbilical cord is separated from the placental disc (optionally), and separation of the amniotic membrane from the chorionic membrane. In a preferred embodiment, the amniotic membrane is separated from the chorionic membrane prior to cutting the placental membrane. The separation of the amniotic membrane from the chorionic membrane is preferably done starting from the edge of the placental membrane. In another preferred embodiment, the amniotic membrane is separated from the chorionic membrane using blunt dissection, *e.g.*, with gloved fingers. Following separation of the amniotic membrane from the chorionic membrane and placental disc, the umbilical cord stump is cut *e.g.*, with scissors, and detached from the placental disc. In certain embodiments, when separation of the amniotic and chorionic membranes is not possible without tearing the tissue, the invention

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encompasses cutting the amniotic and chorionic membranes from the placental disc as one piece and then peeling them apart.

[0074] The amniotic membrane is then preferably stored in a sterile saline solution. In some embodiments, the sterile saline solution is buffered. In a specific preferred embodiment, the sterile saline solution for storing the amniotic membrane is a 0.9% sterile NaCl solution. Preferably, the amniotic membrane is stored by refrigeration, at a temperature of at least 4°C. In certain embodiments, the amniotic membrane is refrigerated at a temperature of at least 2°C, at least 6°C, or up to 8°C. At this point, the amniotic membrane may be stored for up to 5 days, provided it is refrigerated and kept covered with sterile saline. Preferably, the separated amniotic membrane is refrigerated for a maximum of 72 hours from the time of delivery prior to the next step in the process.

[0075] **Step II.** Once the amniotic membrane is separated from the chorionic membrane, the invention encompasses decellularizing the amniotic membrane. Any decellularizing process known to one skilled in the art is encompassed by the methods of the invention, with the provision that the process for decellularizing the amniotic membrane of the invention does not include any freezing of the amniotic membrane. As used herein, decellularizing refers to removing all cellular material and cellular debris (*e.g.*, all visible cellular material and cellular debris) from the amniotic membranes of the invention. The decelluarization of the amniotic membrane ensures that substantially all of the cells normally associated with the collagen matrix of the amniotic membrane are removed. Decellularization of the amniotic membrane of the invention that removes "substantially all" of the cells associated with the collagen matrix preferably removes at least 90% of the cells, more preferably removes at least 95% of the cells, and most preferably removes at least 99% of the cells. The amniotic membranes decellularized in accordance with the methods of the invention are uniformly thin, *i.e.*, 10-40 microns in thickness, smooth (as determined by touch) and clear in appearance.

[0076] In a preferred embodiment, decellularization of the amniotic membrane of the invention comprises removing substantially all cellular material and cellular debris from the maternal side of the amniotic membrane followed by removing all cellular material and cellular debris from the fetal side of the amniotic membrane. In a specific embodiment, decellularization of the amniotic membrane of the invention comprises physical scraping in combination with rinsing with a sterile solution. In another specific embodiment, physical scraping of the amniotic membrane comprises scraping with a sterile cell scraper. In yet another specific embodiment, the sterile solution for rinsing the amniotic membrane during

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decellularization is an aqueous solution, a solution comprising a physiological buffer or a saline solution such as for example a 0.9% NaCl solution.

[0077] The decellularization of the amniotic membrane comprises removing native cells and other antigens and cellular debris from the amniotic membrane, and, optionally, treating to inhibit generation of new immunological sites. In decellularizing the amniotic membrane, native viable cells as well as other cellular and acellular structures or components which may elicit an adverse immune response are removed. The decellularization technique employed in accordance with the invention should not result in gross disruption of the anatomy of the amniotic membrane or alter the biomechanical properties of its structural composition, *i.e.*, the structural and biochemical integrity of collagen, elastin, and possibly fibronectin are not affected by the decellularization. Specifically, harsh chemical treatment and protease treatment of the amniotic membrane are not within the scope of the decelluarization technique of the present invention.

[0078] Preferably, the decellularization of the amniotic membrane comprises use of a detergent-containing solution. Detergents that can be used in accordance with the methods of the present invention include, but are not limited to, nonionic detergents, Triton X-100, anionic detergents, sodium dodecyl sulfate. Detergents can be used alone or in combination in the methods of the present invention. Any mild anionic detergent, *i.e.*, a non-caustic detergent, with a pH of 6 to 8, and low foaming, can be used in accordance with the methods of the invention. In a specific embodiment, 0.01-1% deoxycholic acid sodium salt monohydrate is used in the decellularization of the amniotic membrane. Although not intending to be bound by a particular mode of action, decellularization of the amniotic membrane in accordance with the methods of the invention may disrupt cell membranes and aid in the removal of cellular debris from the amniotic membrane. However, steps should be taken to eliminate any residual detergent levels in the amniotic membrane, so as to for example, avoid interference with the later repopulating of the amniotic membrane with viable cells.

[0079] It is essential to limit the protease activity in preparation of the biofabric of the invention. Additives such as metal ion chelators, for example 1,10-phenanthroline and ethylenediaminetetraacetic acid (EDTA), create an environment unfavorable to many proteolytic enzymes. Providing sub-optimal conditions for proteases such as collagenase, may assist in protecting the amniotic membrane compositions such as collagen from degradation during the lysis step. Suboptimal conditions for proteases may be achieved by formulating the hypotonic lysis solution to eliminate or limit the amount of calcium and zinc

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ions available in solution. Many proteases are active in the presence of calcium and zinc ions and lose much of their activity in calcium and zinc ion free environments. Preferably, the hypotonic lysis solution will be prepared selecting conditions of pH, reduced availability of calcium and zinc ions, presence of metal ion chelators and the use of proteolytic inhibitors specific for collagenase such that the solution will optimally lyse the native cells while protecting the underlying amniotic membrane from adverse proteolytic degradation. For example a hypotonic lysis solution may include a buffered solution of water, pH 5.5 to 8, preferably pH 7 to 8, free from calcium and zinc ions and including a metal ion chelator such as EDTA. Additionally, control of the temperature and time parameters during the treatment of the amniotic membrane with the hypotonic lysis solution, may also be employed to limit the activity of proteases.

[0080] It is preferred that the decellularization treatment of the amniotic membrane also limits the generation of new immunological sites. Since enzymatic degradation of collagen is believed to lead to heightened immunogenicity, the invention encompasses treatment of the amniotic membrane with enzymes, *e.g.*, nucleases, that are effective in inhibiting cellular metabolism, protein production and cell division, that minimize proteolysis of the compositions of the amniotic membrane thus preserving the underlying architecture of the amniotic membrane. Examples of nucleases that can be used in accordance with the methods of the invention are those effective in digestion of native cell DNA and RNA including both exonucleases and endonucleases. A non-limiting example of nucleases that can be used in accordance with the methods of the invention include exonucleases that inhibit cellular activity, *e.g.*, DNAase I (SIGMA Chemical Company, St. Louis, Mo.) and RNAase A (SIGMA Chemical Company, St. Louis, Mo.) and endonucleases that inhibit cellular activity, *e.g.*, EcoR I (SIGMA Chemical Company, St. Louis, Mo.) and Hind III (SIGMA Chemical Company, St. Louis, Mo.). It is preferable that the selected nucleases are applied in a physiological buffer solution which contains ions, *e.g.*, magnesium, calcium, which are optimal for the activity of the nuclease. Preferably, the ionic concentration of the buffered solution, the treatment temperature and the length of treatment are selected by one skilled in the art by routine experimentation to assure the desired level of nuclease activity. The buffer is preferably hypotonic to promote access of the nucleases to cell interiors.

[0081] In a specific embodiment, decellularizing the amniotic membrane of the invention comprises the following steps. First, the amniotic membrane is transferred into a clean sterile container, and optionally rinsed with sterile water and dried with sterile gauze. The amnion is then placed on a sterile tray with the maternal side facing upward. Using a

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sterile cell scraper (*e.g.*, 32 cm, PE blade, PS handle, NalgeNunc International), the amnion is partially decellularized by physically removing all visible cellular material from the maternal side of the amniotic membrane. Sterile water is used to assist in the removal of cells and cellular debris, if needed. After completing the partial decellularization on the maternal side of the amniotic membrane, the amniotic membrane is turned over so that the fetal side faces up. All visible cellular debris on the fetal side is gently removed with a cell scraper using minimal pressure on the amniotic membrane to prevent tearing. Sterile water may be used to assist in the removal of the cells and debris.

[0082] In one embodiment, the decellularized amniotic membrane is placed in a sterile container filled with a sterile physiological solution, *e.g.*, sterile 0.9% NaCl solution, before further processing. In accordance with the methods of the invention the next processing step of the amniotic membrane should start no later than 2-3 hours after the amniotic membrane has been placed into the sterile physiological solution. In a specific embodiment, where Step III immediately follows Step II, it is not necessary to place the amniotic membrane into a container with sterile solution.

[0083] The amniotic membrane may be cut during the cleaning process, using *e.g.* a sterile scalpel, to shape it for easier cleaning or to remove the areas that cannot be cleaned.

[0084] **Step III.** Following decellularization, the amniotic membrane is washed to assure removal of cellular debris which may include cellular proteins, cellular lipids, and cellular nucleic acids, as well as any extracellular debris such as extracellular soluble proteins, lipids and proteoglycans. Although not intending to be bound by any mechanism of action, removal of this cellular and extracellular debris reduces the immunogenicity of the amniotic membrane.

[0085] Once the amniotic membranes of the invention are decellularized, the membranes are further washed in order to effectively achieve the complete removal of all visible cellular material and cellular debris from both sides of the amniotic membrane. The solution is preferably an aqueous hypotonic or low ionic strength solution formulated to effectively lyse the native tissue cells. Such an aqueous hypotonic solution may be de-ionized water or an aqueous hypotonic buffer. Preferably the aqueous hypotonic buffer additionally contains additives that provide sub-optimal conditions for the activity of proteases, for example collagenase, which may be released as a result of cellular lysis.

[0086] Preferably, the amniotic membrane is gently agitated in the detergent, *e.g.*, on a rocking platform, to assist in the decellularization. In certain embodiments, the amniotic membrane is agitated for at least 15 minutes, at least 20 minutes, at least 30 minutes, or up to

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120 minutes. The amniotic membrane may, after detergent decellularization, again be physically decellularized as described *supra*; the physical and detergent decellularization steps may be repeated as necessary, as long as the integrity of the amniotic membrane is maintained, until no visible cellular material and cellular debris remain.

[0087] In a specific embodiment, the washing of the amniotic membrane comprises the following steps: the decellularized amniotic membrane is placed into a sterile container which is then filled with a decellularizing solution in an amount sufficient to cover the amniotic membrane; the container with the amniotic membrane and the decellularizing solution is then placed on a rocking platform (*e.g.*, Model 100, VWR Scientific Products Corp., P.O. Box 640169, Pittsburgh, PA 15264-0169). The amniotic membrane in the decellularizing solution is then agitated for between 15 minutes and 120 minutes on the rocking platform. After the agitation step, the amniotic membrane is removed from the container and placed in a clean sterile tray filled with a sterile solution, *e.g.*, 0.9% NaCl solution. Using a new sterile Cell Scraper, residual decellularizing solution is removed and any remaining cellular material is removed form both sides of the amniotic membrane. This step may be repeated as many times as necessary to remove all visible residual cellular material from both sides of the amniotic membrane.

[0088] In certain embodiments, the amniotic membrane is dried immediately (*i.e.*, within 30 minutes) after the decellularization step. Alternatively, when further processing is not done immediately, the amniotic membrane may be refrigerated, *e.g.*, stored at a temperature of 2-8°C, for up to 28 days prior to drying. When the decellularized amniotic membrane is stored for more than three days but less than 28 days, the sterile solution covering the amniotic membrane is preferably changed periodically, *e.g.*, every 1-3 days.

[0089] In certain embodiments, when the amniotic membrane is not refrigerated after washing, the amniotic membrane is washed at least 3 times prior to proceeding to Step IV of the preparation. In other embodiments, when the amniotic membrane has been refrigerated and the sterile solution has been changed once, the amniotic membrane is washed at least twice prior to proceeding to Step IV of the preparation. In yet other embodiments, when the amniotic membrane has been refrigerated and the sterile solution has been changed twice or more, the amniotic membrane is washed at least once prior to proceeding to Step IV of the preparation.

[0090] In specific embodiments, the decellularized amniotic membrane is stored under sterile conditions, and no further processing is performed, *i.e.*, no drying. Prior to

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proceeding to Step IV, it is essential that all bacteriological and seriological testing be assessed to ensure that all tests were negative.

[0091] **Step IV.** The final step of the method of the invention comprises drying the decellularized amniotic membrane of the invention to produce the collagen biofabric. Any method of drying the amniotic membrane so as to produce a flat, dry sheet of collagen may be used. Preferably, however, the amniotic membrane is dried under vacuum.

[0092] In a specific embodiment, an exemplary method for drying the decellularized amniotic membrane of the invention comprises the following steps:

[0093] *Assembly of the* decellularized *amniotic membrane for drying.* The decellularized amniotic membrane is removed from the sterile solution, and the excess fluid is gently squeezed out. The decellularized amniotic membrane is then gently stretched until it is flat with the fetal side faced in a downward position, *e.g.*, on a tray. The decellularized amniotic membrane is then flipped over so that fetal side is facing upwards, and placed on a drying frame, preferably a plastic mesh drying frame (*e.g.*, Quick Count® Plastic Canvas, Uniek, Inc., Waunakee, WI). In other embodiments, the drying frame may be an autoclavable stainless steel mesh. In a most preferred embodiment, about 0.5 centimeter of the amniotic membrane overlaps the edges of the drying frame. In certain embodiments, the overlapping amniotic membrane extending beyond the drying frame is wrapped over the top of the frame, *e.g.*, using a clamp or a hemostat. Once the amniotic membrane is positioned on the drying frame, a sterile gauze is placed on the drying platform of a heat dryer (or gel-dryer) (*e.g.*, Model 583, Bio-Rad Laboratories, 200 Alfred Nobel Drive, Hercules, CA 94547), so that an area slightly larger than the amniotic membrane resting on the plastic mesh drying frame is covered. Preferably, the total thickness of the gauze layer does not exceed the thickness of one folded 4x4 gauze. Any heat drying apparatus may be used that is suitable for drying sheet like material. The drying frame is placed on top of the gauze on the drying platform so that the edges of the plastic frame extend above beyond the gauze edges, preferably between 0.1 - 1.0 cm, more preferably 0.5-1.0 cm. In a most preferred embodiment, the drying frame having the amniotic membrane is placed on top of the sterile gauze with the fetal side of the amniotic membrane facing upward. In some embodiments, another plastic framing mesh is placed on top of the amniotic membrane. A view of the mesh frame and the membrane dried therein is shown in FIG. 4. In another embodiments, a sheet of thin plastic (*e.g.*, SW 182, clear PVC, AEP Industries Inc., South Hackensack, NJ 07606) or a biocompatible silicone is placed on top of the membrane covered mesh so that the sheet

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extends well beyond all of the edges. In this embodiment, the second mesh frame is not needed.

[0094] In an alternative embodiment, the amniotic membrane is placed one or more sterile sheets of Tyvek material (*e.g.*, a sheet of Tyvek for medical packaging, Dupont Tyvek®, P.O. Box 80705, Wilmington, DE 19880-0705), optionally, with one sheet of Tyvek on top of the membrane (prior to placing the plastic film). This alternate process will produce a smoother version of the biofabric (*i.e.*, without the pattern of differential fiber compression regions along and perpendicular to the axis of the material), which may be advantageous for certain applications, such as for example for use as a matrix for expansion of cells, as described herein.

[0095] *Drying the amniotic membrane.* In a preferred embodiment, the invention encompasses heat drying the amniotic membrane of the invention under vacuum. While the drying under vacuum may be accomplished at any temperature from about 0°C to about 60°C, the amniotic membrane is preferably dried at between about 35°C and about 50°C, and most preferably at about 50°C. It should be noted that some degradation of the collagen is to be expected at temperatures above 50°C. The drying temperature is preferably set and verified using a calibrated digital thermometer using an extended probe. Preferably, the vacuum pressure is set to about -22 inches of Hg. The drying step is continued until the collagen matrix of the amniotic membrane contains less than 3-12% water as determined for example by a moisture analyzer. To accomplish this, the amniotic membrane may be heat-vacuum dried, *e.g.*, for approximately 60 minutes to achieve a dehydrated amniotic membrane. In some embodiments, the amniotic membrane is dried for about 30 minutes to 2 hours, preferably about 60 minutes. Although not intending to be bound by any mechanism of action, it is believed that the low heat setting coupled with vacuum pressure allows the amniotic membrane to achieve the dehydrated state without denaturing the collagen.

[0096] After completion of the drying process in accordance with the invention, the amniotic membrane is cooled down for approximately two minutes with the vacuum pump running.

[0097] *Packaging and Storing of the Amniotic Membrane.* Once the amniotic membrane is dried in accordance with the methods of the invention as described, the membrane is gently lifted off the drying frame. "Lifting off" the membrane may comprise the following steps: while the pump is still running, the plastic film is gently removed from the amniotic membrane starting at the corner, while holding the amniotic membrane down; the frame with the amniotic membrane is lifted off the drying platform and placed on a

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cutting board with the amniotic membrane side facing upward; an incision is made, cutting along the edge 1-2 mm away from the edge of the frame; the amniotic membrane is then peeled off the frame; and cut in to appropriate sizes as determined by its subsequent use. Preferably, handling of the amniotic membrane at this stage is done with sterile gloves.

[0098] The amniotic membrane is placed in a sterile container, *e.g.*, peel pouch, and is sealed. The biofabric produced in accordance with the methods of the invention may be stored at room temperature for an extended period of time as described *supra*.

[0099] In alternative embodiments, the invention provides a method of preparing a collagen biofabric comprising a chorionic membrane. It is expected that the methods described above would be applicable to the method of preparing a biofabric comprising a chorionic membrane. In a specific embodiment, the invention encompasses a method for preparing a collagen biofabric comprising: providing a placenta, comprising an amniotic membrane and a chorionic membrane; separating the amniotic membrane from the chorionic membrane; and decellularizing the chorionic membrane. In a specific embodiment, the method further entails washing and drying the decelluarized chorionic membrane.

5.2.1 METHODS OF PREPARING THREE-DIMENSIONAL SCAFFOLDS AND LAMINATES

[00100] The invention provides methods of preparing three-dimensional scaffolds, three-dimensional configurations and laminates comprising the collagen biofabric of the invention.

[00101] In some embodiments, the invention provides a method of preparing an amniotic membrane laminate comprising: providing a placenta, preferably a human placenta, comprising an amniotic membrane and a chorionic membrane, separating the amniotic membrane from the chorionic membrane, using methods disclosed herein; decellularizing the amniotic membrane, using methods disclosed herein; washing the decellularized amniotic membrane at least once using methods disclosed herein; layering at least two of the decellularized amniotic membranes in contact with each other so that an amniotic membrane laminate is formed; and drying the amniotic membrane laminate, using methods disclosed herein.

[00102] Alternatively, in another embodiment, the method for preparing an amniotic membrane laminate comprises, drying at least two amniotic membranes prepared in accordance with the methods of the invention, and layering the at least two amniotic membranes in contact with each other so that an amniotic membrane laminate is formed.

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[00103] In some embodiments, the amniotic membrane layers produced in accordance with the methods of the invention may be placed in contact with each other in the presence of an adhesive to form an amniotic membrane laminate. The adhesive used in accordance with the methods and compositions of the invention may be any biological glue known to one skilled in the art, preferably a biocompatible glue, including but not limited to, natural glue, *e.g.*, fibronectin, fibrin, synthetic glue. In other embodiments, the amniotic membrane layers prepared in accordance to the methods of the invention are cross-linked to each other to form an amniotic membrane laminate. Any cross-linking reagent and method known to one skilled in the art is within the scope of the present invention, including but not limited to, chemical cross-linking, peptide cross-linking, UV cross-linking, radiation cross-linking, fibronectin cross-linking, fibrinogen cross-linking, hydrogel cross-linking. In other embodiments, the amniotic membrane laminates produced in accordance with the methods of the invention do not comprise an adhesive.

5.3 STORAGE AND HANDLING OF THE COLLAGEN BIOFABRIC

[00104] The invention encompasses storing the collagen biofabric of the invention as dehydrated sheets at room temperature (*e.g.*, 25°C). In certain embodiments, the collagen biofabric of the invention can be stored at a temperature of at least 10°C, at least 15°C, at least 20°C, at least 25°C, or at least 29°C. Preferably, the collagen biofabric of the invention is not refrigerated. In some embodiments, the collagen biofabric of the invention may be refrigerated at a temperature of about 2 to 8°C. In other embodiments, the collagen biofabric of the invention can be stored at any of the above-identified temperatures for an extended period of time. In a most preferred embodiment, the biofabric of the invention is stored under sterile and non-oxidizing conditions. The biofabric produced according to the methods of the invention can be stored at any of the specified temperatures for 12 months or more with no alteration in biochemical or structural integrity (*e.g.*, no degradation), without any alteration of the biochemical or biophysical properties of the collagen biofabric. The biofabric produced according to the methods of the invention can be stored for several years with no alteration in biochemical or structural integrity (*e.g.*, no degradation), without any alteration of the biochemical or biophysical properties of the collagen biofabric. It is expected that the biofabric of the invention prepared in accordance with the methods of the invention will last indefinitely. The biofabric may be stored in any container suitable for long-term storage. Preferably, the collagen biofabric of the invention is stored in a sterile double peel-pouch package.

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[00105] The invention encompasses handling of the collagen biofabric of the invention in its dry state. In a specific embodiment, the collagen biofabric is trimmed prior to use, for example prior to use as a surgical graft. The invention encompasses any dimensionality of the biofabric of the invention that is compatible for its use, as determined by one skilled in the art. In some embodiments, the invention encompasses a collagen biofabric which is 1x2cm; 2x3 cm; 4x4 cm, 5x5, or 6x8. The biofabric of the invention can be cut into any size needed which is within the limitation of the size of the amniotic membrane.

[00106] The surface orientation of the collagen biofabric of the invention can be visually identified. The collagen biofabric of the invention has a "grid" pattern, which allows for the visual identification of the maternal and detal surfaces by one skilled in the art. In a specific embodiment, the surface orientation of the collagen biofabric is identified under magnification. It will be appreciated by one skilled in the art that the fetal side of the collagen biofabric can be identified by its concave, *i.e.*, recessed, grid pattern. Conversely, the maternal side can be identified by its convex, *i.e.*, elevated grid pattern.

[00107] The collagen biofabric of the invention requires minimal preparation time prior to use. In a preferred embodiment, the collagen biofabric of the invention is ready to use within 5 minutes or less, within 10 minutes or less, within 15 minutes or less. The preparation time of the collagen biofabric of the invention prior to use for example, as a surgical graft, comprises activation by re-hydration of the dehydrated collagen biofabric. In some embodiments, the collagen biofabric of the invention is hydrated while on the surgical site. In other embodiments, the collagen biofabric of the invention is hydrated under sterile conditions in a dish. The invention encompasses hydration of the collagen biofabric of the invention using a sterile physiological buffer. In a specific embodiment, the invention encompasses hydrating the collagen biofabric of the invention with a sterile saline solution, *e.g.*, sterile 0.9% NaCl solution. In some embodiments the sterile saline solution is buffered. In certain embodiments, the hydration of the collagen biofabric of the invention requires at least 2 minutes, at least 5 minutes, at least 10 minutes, at least 15 minutes, or at least 20 minutes. In a preferred embodiment, the hydration of the collagen biofabric of the invention is complete within 5 minutes. In yet another preferred embodiment, the hydration of the collagen biofabric of the invention is complete within 10 minutes. In yet another embodiment, the hydration of the collagen biofabric of the invention takes no more than 10 minutes.

[00108] The collagen biofabric of the invention once hydrated for use, for example as a surgical graft, has enhanced suturability relative to the amniotic membranes in the art, as

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determined by one skilled in the art. The collagen biofabric of the invention does not tear as easily nor is it as friable as the amniotic membranes in the art. The invention encompasses collagen biofabrics that can be sutured effectively.

5.3.1 STERILIZATION

[00109] Sterilization of the biofabric of the invention is preferably done by electron beam irradiation using methods known to one skilled in the art, *e.g.*, Gorham, D. Byrom (ed.), 1991, Biomaterials, Stockton Press, New York, 55-122. Any dose of radiation sufficient to kill at least 99.9% of bacteria or other potentially contaminating organisms is within the scope of the invention. In a preferred embodiment, a dose of at least 18-25 kGy is used to achieve the terminal sterilization of the biofabric of the invention. Sterilization of the biofabric of the invention does not include, however, storage in antibiotics or glycerol.

5.4 METHODS OF USING THE COLLAGEN BIOFABRIC OF THE INVENTION

[00110] The collagen biofabric of the invention has numerous utility in the medical and surgical field due, in part, to its physical properties, such as biomechanical strength, flexibility, suturability, low immunogenicity in comparison to the traditional membranes used in the art. For example, the collagen biofabric of the invention is expected to have an enhanced therapeutic utility for guided tissue regeneration over membranes of the prior art, *e.g.*, synthetic non-resorbable PTFE membranes such as Goretex™; synthetic resorbable membranes formed from glycolide and lactide copolymers; membranes disclosed in WO-88/08305; DE-2631909, U.S. Patent No. 5,837,278.

[00111] The method of preparing the collagen biofabric of the invention ensures the preservation of the tertiary and quaternary structure of the biofabric thus making the biofabric ideal for its intended use in the medical and surgical field. As described in detail below, the invention provides collagen biofabrics whose physical properties allow it to be suitable for uses in a variety of medical and dental applications including but not limited to blood vessel repair, uterus repair, tendon replacements, cornea replacements, artificial skin, treatment of periodontal disease, and wound healing. Depending on its intended use, the invention encompasses use of the collagen biofabric as a two dimensional membrane, *e.g.*, membranes that can be shaped to form tubular vessels; as a three-dimensional scaffold, *e.g.*, an implant, or as a one-dimensional fiber.

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5.4.1 METHODS FOR TREATMENT USING THE COLLAGEN BIOFABRIC OF THE INVENTION

5.4.1.1 METHODS FOR TREATMENT OF SKIN CONDITIONS

[00112] Human skin is a composite material of the epidermis and the dermis. The upper part of the epidermis is the stratum corneum; which is the stiffest layer of the skin, as well as the one most affected by the surrounding environment. Below the stratum corneum is the internal portion of the epidermis. Below the epidermis is the papillary dermis, which is made of relatively loose connective tissues that define the micro-relief of the skin. The reticular dermis, disposed beneath the papillary dermis, is tight, connective tissue that is spatially organized. The reticular dermis is also associated with coarse wrinkles. Underneath the dermis lies the subcutaneous layer.

[00113] The principal functions of the skin include protection, excretion, secretion, absorption, thermoregulation, pigmentogenesis, accumulation, sensory perception, and regulation of immunological processes. These functions are detrimentally affected by the structural changes in the skin due to aging and excessive sun exposure. The physiological changes associated with skin aging include impairment of the barrier function and decreased turnover of epidermal cells, for example, *See*, Cerimele, D. *et al.*, 1990, *Br. J. Dermatol.*, 122 Suppl. 35, p. 13-20. The methods and compositions in the prior art, however, have had limited success in improving skin conditions, *e.g.*, improving skin elasticity and softness, or removing wrinkles.

[00114] The collagen biofabric of the invention has medical as well as a cosmetic applications. The collagen biofabric of the invention has clinical and therapeutic utility in treating skin conditions including but not limited to skin lesions, aged skin, wrinkles, fine lines, thinning, reduced skin elasticity, rough skin, congenital and degenerative skin conditions, collagen VII deficiency, and sun damaged skin. In certain embodiments, the collagen biofabric of the invention can be used as a subcutaneous implant for skin conditions such as acne scars, glabellar furros, excision scars, or any other soft tissue defect known in the art. The collagen biofabric of the invention has clinical and therapeutic utility in treating changes associated with skin aging. In certain embodiments, the collagen biofabric of the invention has utility in improving skin wrinkles, and/or other conditions such as skin elasticity and softness. The collagen biofabric of the invention may be used as an implant, as a laminate, or as a three dimensional rolled up form for the treatment of skin conditions. The collagen biofabric of the invention is expected to have an enhanced clinical utility relative to

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methods known in the art for treating such skin conditions, *e.g.*, U.S. Patent Nos. 5,972,999; 5,418,875; 5,332,579, 5,198,465, in part, due to its stability it may provide a longer lasting effect.

[00115] In a preferred embodiment, the collagen biofabric of the invention is further supplemented with one or more agents known in the art for treating a skin condition. Examples of such agents, include but are not limited to, vitamins, minerals, catechin-based preparations, N-acetlyglucosamine, and glucosamine (*See, e.g.*, Neldner, 1993, *Amer. Acad. Derm. Annl. Mtg.* Wash. DC.; Lubell 1996, *Cosmetic Dermatol*, 9(7): 58-60; Swaine *et al.*, 1995, *J. Am. Board of Family Practice*, 8(3): 206-16; Shan *et al.*, 1994, *Kidney International*, 46(2): 388-95; all of which are incorporated herein by reference in their entirety).

[00116] The collagen biofabric of the invention may be impregnated with any biomolecule with utility in the treatment of a skin condition, including but not limited to, antibiotics (such as Clindamycin, Minocycline, Doxycycline, Gentamycin), hormones, growth factors, anti-tumor agents, anti-fungal agents, anti-viral agents, pain medications, anti-histamines, anti-inflammatory agents, anti-infectives including but not limited to silver (such as silver salts, including but not limited to silver nitrate and silver sulfadiazine), elemental silver, antibiotics, bactericidal enzymes (such as lysozome), wound healing agents (such as cytokines including but not limited to PDGF, TGF; thymosin), Hyaluronic acid as a wound healing agent, wound sealants (such as fibrin with or without thrombin), cellular attractant and scaffolding reagents (such as fibronectin) and the like. In a specific example, the collagen biofabric may be impregnated with at least one growth factor, for example, fibroblast growth factor, epithelial growth factor, *etc.* The biofabric may also be impregnated with small molecules such as small organic molecules such as specific inhibitors of particular biochemical processes *e.g.*, membrane receptor inhibitors, kinase inhibitors, growth inhibitors, anticancer drugs, antibiotics, *etc.*

5.4.1.2 WOUNDS AND BURNS

[00117] The collagen biofabric of the invention is expected to have an enhanced clinical utility as a wound dressing, for augmenting hard and/or soft tissue repair, as compared to other biomaterials known in the art, *e.g.*, those described in U.S. Patent Nos. 3,157,524; 4,320,201; 3,800,792; 4,837,285; 5,116,620, due in part to its physical properties. The collagen biofabric of the invention because it retains collagen's native quaternary structure provides improved tissue in-growth through cell migration into the interstices of the collagen matrix. The biofabric of the invention allows cells to attach and grow into the

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collagen matrix, and to synthesize their own macromolecules. The cells thereby produce a new matrix which allows for the growth of new tissue. Such cell development is not observed on other known forms of collagen such as fibers, fleeces and soluble collagen.

[00118] In some embodiments, the invention encompasses treating a wound by placing the collagen biofabric of the invention directly over the skin of the subject, *i.e.*, on the stratum corneum, on the site of the wound, so that the wound is covered, for example, using an adhesive tape. In other embodiments, the invention encompasses treating a wound using the collagen biofabric of the invention as an implant, *e.g.*, as a subcutaneous implant.

[00119] The invention encompasses enhancing the rate of wound healing by the addition of a macromolecule capable of promoting tissue ingrowth to the collagen biofabric of the invention. Such macromolecules include but are not limited to hyaluronic acid, fibronectin, laminin, and proteoglycans (*See*, *e.g.*, Doillon *et al.* (1987) *Biomaterials* 8:195-200; and Doillon and Silver (1986) *Biomaterials* 7:3-8).

[00120] The invention further encompasses incorporating pharmacologically active agents including but not limited to platelet-derived growth factor, insulin-like growth factor, epidermal growth factor, transforming growth factor beta, angiogenesis factor, antibiotics, antifungal agents, spermicidal agents, hormones, enzymes, enzyme inhibitors in the collagen biofabric of the invention as described herein in section 5.4.2.7 for delivery to the skin, and any biomolecule described above. Preferably the pharmacologically active agents are provided in a physiologically effective amount.

[00121] In some embodiments, the collagen biofabric is further populated by living cells, including but not limited to allogenic stem cells, stem cells, and autologous adult cells, prior to being applied to the site of the wound.

[00122] The collagen biofabric of the invention is particularly useful for the treatment of wound infections, *e.g.*, wound infections followed by a breakdown of surgical or traumatic wounds. In a particular embodiment, the collagen biofabric is impreganted with a therapeutically effective amount of an agent useful in the treatment of a wound infection, including but not limited to, an antibiotic, anti-microbial agent, and an anti-bacterial agent. The collagen biofabric of the invention has clinical and therapeutic utility in the treatment of wound infections from any microorganism known in the art, *e.g.*, microorganisms that infect wounds originating from within the human body, which is a known reservoir for pathogenic organisms, or from environmental origin. A non-limiting example of the microorganisms, the growth of which in wounds may be reduced or prevented by the methods and compositions of the invention are *S. aureus*, *St. epidermis*, beta haemolytic *Streptococci*, *E.*

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coli, *Klebsiella* and *Pseudomonas* species, and among the anaerobic bacteria, the *Clostridium welchii* or *tartium*, which are the cause of gas gangrene, mainly in deep traumatic wounds.

[00123] In other embodiments, the collagen biofabric of the invention is used for wound treatment, including but not limited to epidermal wounds, skin wounds, chronic wounds, acute wounds, external wounds, internal wounds (*e.g.*, the collagen biofabric may be wrapped around an anastosmosis site during surgery to prevent leakage of blood from suture lines, and to prevent the body from forming adhesions to the suture material), congenital wounds (*e.g.*, dystrophic epidermolysis bullosa). In particular, the collagen biofabric has enhanced utility in the treatment of pressure ulcers (*e.g.*, decubitus ulcers). Pressure ulcers occur frequently with patients subject to prolonged bedrest, *e.g.*, quadriplegics and paraplegics who suffer skin loss due to the effects of localized pressure. The resulting pressure sores exhibit dermal erosion and loss of the epidermis and skin appendages.

[00124] The collagen biofabric of the invention may also be used in the treatment of burns, including but not limited to first-degree burns, second-degree burns (partial thickness burns), third degree burns (full thickness burns), infection of burn wounds, infection of excised and unexcised burn wounds, infection of grafted wound, infection of donor site, loss of epithelium from a previously grafted or healed burn wound or skin graft donor site, and burn wound impetigo.

5.4.1.3 TISSUE ENGINEERING

[00125] The invention encompasses use of the collagen biofabric of the invention as a vehicle for the transportation of cultured skin cells, *e.g.*, as a support for epithelial growth and differentiation. In certain embodiments, to populate the biofabric with cells for forming tissue and/or organoids (*i.e.*, resembling in superficial appearance or in structure any of the organs or glands of the body), the biofabric can be treated with cellular adhesion factors to enhance attachment of cells to the biofabric during the process of repopulating the biofabric with such new cells. In certain embodiments, the extent of attachment of cells is increased by treating the amniotic membrane with serum, *e.g.*, human or fetal bovine serum). In other embodiments, the extent of attachment of cells is increased by treating the amniotic membrane with fibronectin.

[00126] The collagen biofabric of the invention may be used in guided tissue regeneration techniques, *e.g.*, to regenerate or replace diseased or damaged tissue. The invention encompasses use of the biofabric of the invention by directly implanting the biofabric at the site of treatment or by the formation of a prosthetic device. The collagen

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biofabric of the invention is particularly useful in any situation, such as following surgery especially oral or dental surgery (as described in more detail in Section 5.4.2.2), where enhanced wound healing and/or replacement of dermis is desirable. The utility of the collagen biofabric of the invention in guided tissue regeneration is due in part to its ability to provide conditions which prevent ingrowth of other tissues into the area where regeneration is required.

[00127] For example, where a substantial portion of a tooth root is removed due to decay or disease, it is desirable that healthy bone regeneration occurs to replace the bone tissue removed. However, it has been found that the cavity left by removal of the bone is quickly filled by connective tissue and that this ingrowth of connective tissue effectively prevents bone regeneration. To prevent such ingrowth, the collagen biofabric of the invention can be surgically inserted around the periphery of the wound cavity. The biofabric prevents or hinders the invasion of the wound cavity by unwanted cell types and thus allows the preferred cells to grow into the cavity, thereby healing the wound.

[00128] In some embodiments, the collagen biofabric has utility as a transplant for ocular surface reconstruction for example in a subject with Stevens Johnson syndrome, limbal stem cell deficiency secondary to a chemical burn, or with chemical and/or thermal burns. The biofabric of the invention is expected to have enhanced clinical utility for example by promoting epithelialization. The biofabric of the invention is expected to have an enhanced clinical utility relative to the amniotic membranes used in the art for tissue engineering purposes, *see, e.g.*, Gomes *et al.*, 2003, *Opthalmology*, 119: 166-73; Ti *et al.*, 2001, *Opthalmology*, 108: 1209-1217; Meller *et al.*, 2000, *Opthalmology*, 107: 980-9; Gris *et al.*, 2002 *Opthalmology*, 109: 508-12; Koizumi *et al.*, 2000, *Invest. Opthal. and Visual Science*, 41: 2506-13; Pires *et al.*, 1999, *Arch. Opthalmol.* 117: 1291-7; Tseng *et al.*, 1998, *Arch. Opthalmol.* 116:431:441; Heiligenhaus *et al.*, 2001 *Invest. Opthal. and Visual Science* 42: 1969-74; Anderson *et al.* 2001, *Br. J. Opthalmol.* 85: 567-75.

[00129] The invention encompasses populating the collagen biofabric with living cells, including but not limited to adult tissue cells, autologous cells, and stem cells. The stem cells for use in the methods of the invention can be totipotent, pluripotent, or differentiated tissue specific cells. Stem cells for use in the methods of the invention can be obtained by standard methods known to one skilled in the art. Preferably, stem cells are collected according to the method disclosed in U.S. Application No. 10/74,976, filed February 13, 2002, which is incorporated herein by reference.

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[00130] The invention encompasses use of the collagen biofabric of the invention (*e.g.*, as three dimensional scaffolds) for the development of bioengineered tissue and organoids, including but not limited, to blood vessels, heart valves, liver, pancreas, and ligaments. Although not intending to be bound by any mechanism of action, the utility of the biofabric of the invention as a bioengineered tissue is due in part, to its hemostatic property in promoting blood coagulation. The biofabric of the invention is particularly useful for vascular prostheses and as a transplant in vessel surgery. The biofabric of the invention can be used, *e.g.*, as a circumferential covering over the anastomotic sites of blood vessels (or vessels to grafts) during vascular surgery procedures to prevent leakage of blood from the suture lines and prevent the body from forming adhesions to the suture material.

5.4.2 METHODS OF USE OF THE COLLAGEN BIOFABRICS IN SURGICAL PROCEDURES

[00131] The invention encompasses use of the collagen biofabric of the invention as a surgical graft. The invention encompasses a surgical graft comprising a collagen biofabric of the invention or a laminate thereof. The invention further encompasses methods of preparing and using the surgical graft.

[00132] In some embodiments, the invention encompasses a method of using the surgical graft in a surgical procedure, so that the surgical graft is applied directly to the surgical site of the subject, *e.g.*, an internal site, or an external site. In some embodiments, the collagen biofabric is used as a surgical graft during a surgical procedure as disclosed in more detail below, for example, to prevent leakage of blood from suture lines and to prevent the body from forming adhesions to the suture materials. In other embodiments, the surgical graft is used as a covering over the anastosomotic sites, *e.g.*, of the GI tract during GI surgery to prevent leakage of intestinal fluid and bile from the suture lines and to prevent the body from forming adhesions to the suture materials.

[00133] The invention encompasses using the biofabric as a graft or dressing to cover burned or surgical skin wounds; to prevent adhesion in all intra peritoneal surgeries or other reconstruction on the serosal surfaces covering the abdomen, chest cavity and pericardium; to reconstruct all mocosal surfaces lining the oral and nasal cavities, respiratory tracts, gastrointestinal tracts, and urogenital tracts; as a substrate to support dural repair in brain surgeries; as a substrate to promote nerve regeneration in the central and peripheral nervous systems; and to reconstruct soft tissues to prevent adhesion in joint or tendon repairs.

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[00134] The invention encompasses impregnating the surgical graft of the invention with one or more biomolecule, preferably a therapeutic agent, depending on the particular intended surgical use. Such biomolecules include but are not limited to, antibiotics (such as Clindamycin, Minocycline, Doxycycline, Gentamycin), hormones, growth factors, anti-tumor agents, anti-fungal agents, anti-viral agents, pain medications, anti-histamines, anti-inflammatory agents, anti-infectives including but not limited to silver (such as silver salts, including but not limited to silver nitrate and silver sulfadiazine), elemental silver, antibiotics, bactericidal enzymes (such as lysozome), wound healing agents (such as cytokines including but not limited to PDGF, TGF; thymosin), Hyaluronic acid as a wound healing agent, wound sealants (such as fibrin with or without thrombin), cellular attractant and scaffolding reagents (such as fibronectin) and the like. In a specific example, the collagen biofabric may be impregnated with at least one growth factor, for example, fibroblast growth factor, epithelial growth factor, *etc.* The biofabric may also be impregnated with small organic molecules such as specific inhibitors of particular biochemical processes *e.g.*, membrane receptor inhibitors, kinase inhibitors, growth inhibitors, anticancer drugs, antibiotics, *etc.*

[00135] The invention further encompasses populating the surgical graft of the invention with living cells, including but not limited to, stem cells, totipotent stem cells, pluripotent stem cells, multipotent stem cells, tissue specifc stem cells, embryonic like stem cells, committed progenitor cells, fibroblastoid cells. In other embodiments, the invention encompasses populating the surgical graft of the invention with specific classes of progenitor cells including but not limited to chondrocytes, hepatocytes, hematopoietic cells, pancreatic parenchymal cells, neuroblasts, and muscle progenitor cells.

5.4.2.1 OPTHALMOLOGY

[00136] The collagen biofabric of the invention has clinical and therapeutic utility in the treatment of an eye related disease or disorder. The collagen biofabric of the invention is particularly useful for the treatment and/or prevention of ocular surface diseases including but not limited to, corneal ulcerations/perforations, bullous keratopathy, ocular dermoids/tumors, primary pterygium, persistent corneal epithelial defect, acute and chronic alkali burns, thermal burns, aniridia, atopic keratitis, idiopathic limbal stem cell deficiency, corneal pannus, neovascularization, rheumatoid corneal melt, ocular cicatricial pemphigoid, leaking filtering bleb, exposed Ahmed valve tube, Serratia cellulitis with subsequent symblepharon, acute and chronic Stevenson Johnson syndrome. The collagen biofabric of the invention is particularly effective in promoting healing of persistent corneal epithelial defects with

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ulceration; promoting epithelialization; facilitation of growth of epithelial and stem cells; reduction of inflamation and pain, inhibition of angiogenesis and scarring; restoration of the epithelial phenotype; and as a substrate alternative to conjunctival autograft during the "bare sclera" removal of pterygia.

[00137] The invention encompasses transplantation using the collagen biofabric of the invention for the treatment of symptomatic bullous keratopathy, preferably in humans, most preferably in humans with poor visual potential. The biofabric of the invention is expected ot have an enhanced therapeutic and clinical utility relative to other standard procedures used in the art for the treatment of symptomatic bullous keratopathy, specifically, conjunctival flap construction. The advantage of transplantation using the biofabric of the invention over standard procedures used in the art for the treatment of symptomatic bullous keratopathy, specifically, in conjunctival flap construction, include for example, reduction of pain, ease of performance, a more cosmetically acceptable appearance, and a reduction in complications such as ptosis and limbal stem cell deficiency. The collagen biofabric of the invention provides an improved alternative to conjunctival flaps for promoting healing of corneal epithelial defects with ulceration; an improved method for conjunctival surface reconstruction for symbelpharon lysis; an improved method for surgical removal of tumors, lesions, or scar tissue from the conjunctival or corneal surface; an improved method for glaucoma surgeries by correcting bleb leakage; an improved substrate alternative to conjunctival autograft during the "bare sclera" removal of pterygia; and an improved method for preventing recurrence of band keratopathy

[00138] The invention also provides for the use of the biofabric as an ophthalmological surgical graft. The invention encompasses preparation of grafts comprising the biofabric of the invention further comprising one or more therapeutic agents that can be delivered to the recipient when attached to the recipient. Examples of therapeutic agents that can be delivered using the biofabric of the invention include but are not limited to pilocarpine, eryhtromycin, gentamicin, vancomycin, tobramycin, netilmycin, polymyxin B sulfate, trimethoprim, amphotericin B, anti-cancer agents, antibiotics, cyclosporin, *etc.*

[00139] The collagen biofabric of the invention also has utility in reducing the corneal haze induced by excimer laser photerefractive/therapeutic keratectomy.

[00140] The collagen biofabric of the invention for use in ophthalmic procedures may be provided in various configurations including but not limited to inserts, shields, particles, gels, aqueous injections, sponges, films.

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[00141] Conventional surgical techniques for ophtalmic grafts, known to one skilled in the art are encompassed within the invention.

[00142] An exemplary protocol for use of a collagen biofabric of the invention as an ophthalmic surgical graft may comprise the following steps: the diseased corneal and/or conjuctival tissues are removed; the surgical graft, prepared in accordance with the methods of the invention is provided under sterile conditions, and trimmed by a free hand technique to cover the corneal epithelium or bare sclera. Using a suture, the graft is anchored to the junction of the bare sclera and conjunctiva; cardinal sutures are placed; and then interrupted sutures are used to create an even distribution of the tension over the graft's surface; so that any space is avoided between the graft and the recipient sclera or cornea. At the junction of the graft and the host, the free edge of the graft must remain under the recipient conjunctival membrane to allow sliding of the host epithelium over the basal lamina at the point of junction with the donor tissue, otherwise the graft is extruded by the recipient.

5.4.2.2 DENTAL

[00143] The collagen biofabric of the invention has particular utility in dentistry, *e.g.*, periodontal surgery, guided tissue regeneration for regeneration of periodontal tissue, guided bone regeneration, and root coverage. The invention encompasses the use of the collagen biofabric of the invention to promote regeneration of periodontal intrabony defects, including but not limited to matched bilateral periodontol defects, interdental intrabony defects, deep 3-wall intrabony defects, 2-wall intrabony defects, and intrabony defects 2 and 3. The collagen biofabric of the invention is expected to have an enhanced therapeutic utility and enhanced clinical parameters for the treatment of periodontal intrabony defects relative to other techniques known in the art, *e.g.*, use of cross-linked collagen membranes such as those disclosed in Quteish *et al.*, 1992, *J. Clin. Periodontol.* 19(7): 476-84; Chung *et al.*, 1990, *J. Periodontol.* 61(12): 732-6; Mattson *et al.*, 1995, *J. Periodontol.* 66(7): 635-45; Benque *et al.*, 1997, *J. Clin. Periodontol.* 24(8): 544-9; Mattson *et al.*, 1999, *J. Periodontol.* 70(5): 510-7) Examples of clinical parameters that are improved using the collagen biofabric of the invention include but are not limited to plaque and gingival index scorings, probing pocket depth, probing attachment depth, and classification of furcation involvement and bony defect, which are known to one skilled in the art.

[00144] The invention also encompasses use of the biofabric of the invention in treating class II furcation defects including but not limited to bilateral defects, paired buccal Class II mandibular molar furcation defects, and bilateral mandibular furcation defect. The

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utility of the collagen biofabric of the invention in treating class II furcation defects can be explained in part by its ability to regenerate lost periodontium in furcation defects. The biofabric of the invention is expected to have an enhanced therapeutic and clinical utility relative to the collagen membranes used in the art for the treatment of class II furcation defects, such as those disclosed in Paul *et al.*, 1992, *Int. J. Periodontics Restorative Dent.* 12: 123-31; Wang *et al.*, 1994, *J. Periodontol.* 65: 1029-36; Blumenthal, 1993, *J. Periodontol.* 64: 925-33; Black *et al.*, 1994, *J. Periodontol.* 54: 598-604; Yukna *et al.*, 1995, *J. Periodontol.* 67: 650-7).

[00145] The invention further encompasses use of the biofabric of the invention in root coverage procedures. The utility of the biofabric of the invention in root coverage can be explained in part due to its ability to replace lost, damaged or disease gingival tissue based on the principles of guided tissue regeneration. The biofabric of the invention is expected to have an enhanced clinical utility in root coverage as compared to collagen membranes in the art traditionally used for root coverage such as those disclosed in Shieh *et al.*, 1997 *J. Periodontol.*, 68: 770-8; Zahedi *et al.*, 1998 *J. Periodontol.* 69: 975-81; Ozcan *et al.*, 1997 *J. Marmara Univ. Dent.* Fa. 2: 588-98; Wang *et al.*, 1997 J. Dent. Res. 78(Spec Issue): 119(Abstr. 106), for reasons cited *supra*.

[00146] The invention further encompasses use of the collagen biofabric in a subject with a periodontal disease including but not limited to, periodontitis and gingivitis. The biofabric of the invention also has clinical utility as an adjunct to scaling and root planning procedures. The invention encompasses treating a subject with a periodontal disease using a collagen biofabric of the invention. An exemplary method for treating a periodontal disease in a subject with using a collagen biofabric of the invention comprises inserting a collagen biofabric, which is preferably impregnated with an antibiotic such as chlorhexidine gluconate, into one or more periodontal pockets in the subject, *e.g.*, greater than or equal to 5mm. Preferably the collagen biofabric is biodegradable.

[00147] The collagen biofabric of the invention for use in dentistry may be impregnated with one or more biomolecules depending on the type of dental disorder being treated. Any biomolecule known in the art for the treatment of dental disorders is encompassed in the methods and compositions of the invention. In a specific embodiment, the collagen biofabric used in the treatment of a dental disorder associated with an infection may be impreganted with one or more antibiotics, including but not limited to doxocyclin, tetracyclin, chlorhexidine gluconate, and minocycline.

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5.4.2.3 NEUROLOGY

[00148] The collagen biofabric of the invention is also useful in repairing injured nerves, particularly in repairing severed peripheral nerves, and neurosurgical procedures. The invention encompasses use of the collagen biofabric, for example, in dural replacements and in peripheral nerve repair. The collagen biofabric of the invention has enhanced clinical utility as a dural substitute or in nerve repair in contrast to other methods used in the art, *e.g.*, Berger *et al.*, 1970, Acta. Neurochir. 23: 141; Ducker *et al.*, 1968, *Mil. Med.* 133: 298; U.S. Patent Nos. 4,778,467; 4,883,618; 3,961,805; 5,354,305, for reasons set forth above.

[00149] In some embodiments, the collagen biofabric of the invention may be used as a prostheses around nerve anastosmosis, for example it can be used to wrap peripheral nerve anastosmosis. The collagen biofabric of the invention has particular utility in nerve repair, for example, by promoting the formation of a longitudinal connective tissue framework across the area of repair and thus giving rise to a longitudinally pattern of sheath cell and axonal regeneration.

[00150] Repair of peripheral nerves is commonly done using sutures in a procedure known as nerorraphy (Jennings *et al.*, 1955, *Surgery*: 206). However, this approach has had limited success since the methods of suturing severed nerves is difficult. The collagen biofabric of the invention may thus provide an alternative to a sutureless method of nerve repair, whereby, for example, the nerve ends are enclosed in a tubular prosthetic device comprising of the biofabric of the invention., and thus bringing the severed ends in close proximity for regeneration.

5.4.2.4 UROLOGY

[00151] The collagen biofabric of the invention has particular utility in the correction of urinary incontinence. Urinary incontinence results from failure of the urethra to remain closed during storage. The cause may be extrinsic, *e.g.*, poor anatomic support of the urethra and bladder neck results in incontinence and responds to pelvic floor resuspension. Urethral failure, alternatively may be intrinsic, *i.e.*, poor urethral function. Traditionally, urinary incontinence is corrected using bulking agents such as collagen, fat, silicone (*See*, review Lightner, 2002, *Current Opinion in Urology*, 12(4): 333-8). The collagen biofabric of the invention has enhanced utility, *i.e.*, improves continence, relative to the bulking agents described in the art. Further the collagen biofabric of the invention has enhanced therapeutic

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utility, *e.g.*, reduced local complications, reduced urinary tract infection, no host reaction, reliable durability, and greater safety.

[00152] The utility of the collagen biofabric of the invention in correcting urinary incontinence is due in part to the physical features unique to the collagen biofabric as described herein, particularly, its non-immunogenicity. In some embodiments, the collagen biofabric is used as an implant, *e.g.*, as a urethral implant. Although not intending to be bound by any particular theory, the collagen biofabric of the invention may have enhances therapeutic utility by increasing, for example, urethral closure pressure and resistance to passive outflow of urine.

5.4.2.5 ORTHOPEDICS

[00153] The collagen biofabric of the invention may be used in orthopedic surgical procedures. In certain embodiments, the collagen biofabric may be used for orthopedic defects, *e.g.*, acquired or congenital defects. Reconstruction of local defects resulting from trauma or surgical resurrection of a bone tumor is a major problem in orthopedic or maxillofacial surgery. Typically, synthetic bone substitutes or collagenous membranes have been used due in part to their osteoinductive activities (*See*, *e.g.*, Rao *et al.*, 1995, *J. Biomater. Sci. Polymer Edn.* 7(7): 623-45). However, a common problem has been a systemic infection as a result of the implanted material. The collagen biofabric of the invention, however, is advantageous over the material used in the art, particularly due to its low immunogenicity as a bone substitute for use in orthopedic defects. In some embodiments, the collagen biofabric may be used as a prosthesis for reconstructing tendons, ligaments and cartilage. In other embodiments, the collagen biofabric of the invention may be used as a prosthesis as a bone replacement.

5.4.2.6 CARDIOVASCULAR SURGERY

[00154] The collagen biofabric of the invention may be used in cardiovascular surgical procedures, for example, as a prostheses for constructing large and small vessels; for repairing congenital malformations of vessels and diseased valves.

[00155] The biofabric of the invention has particular utility as a transplant in vessel surgery, *e.g.*, venous or arterial transplant. The utility of the biofabric in vessel surgery is due, in part, to its non-toxicity, non-immunogenicity; stability; ease of handling; anti-thrombogenic characteristic; minimal implantation porosity of the vessel wall; availability in

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various sizes; reproducibility of the material. In some embodiments, the biofabric of the invention may be used as a valve replacement.

5.4.2.7 DRUG DELIVERY

[00156] The collagen biofabric of the invention can be used as a drug delivery vehicle for controlled delivery of a drug, *e.g.*, a therapeutic agent. In some embodiments the collagen biofabric delivers the one or more therapeutic agents to a subject, preferably a human. The therapeutic agents encompassed within the scope of the invention are proteins, peptides, polysaccharides, polysaccharide conjugates, genetic based vaccines, live attenuated vaccines, whole cells. A non-limiting example of drugs for use in the methods of the invention is antibiotics, anti-cancer agents, anti-bacterial agents, anti-viral agents; vaccines; anesthetics; analgesics; anti-asthmatic agents; anti-inflammatory agents; anti-depressants; anti-arthritic agents; anti-diabetic agents; anti-psychotics; central nervous system stimulants; hormones; immuno-suppressants; muscle relaxants; prostaglandins.

[00157] The collagen biofabric may be used as a delivery vehicle for controlled delivery of one or more small molecules to a subject, preferably a human. In some embodiments the collagen biofabric delivers the one or more small molecules to a subject, preferably a human. As used herein, the term "small molecule," and analogous terms, include, but are not limited to, peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds (*i.e*,. including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, organic or inorganic compounds having a molecular weight less than about 100 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds. Salts, esters, and other pharmaceutically acceptable forms of such compounds are also encompassed.

[00158] In certain embodiments, the collagen biofabric of the invention as a vehicle for drug delivery results in enhanced absorption of the drug; improved pharmacokinetic profile, and systemic distribution of the drug relative to the other drug delivery systems known in the art. By improved pharmacokinetics it is meant that an enhancement of pharmacokinetic profile is achieved as measured, for example, by standard pharmacokinetic parameters such as time to achieve maximal plasma concentration (T_{max}); magnitude of maximal plasma

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concentration (C_{max}); time to elicit a detectable blood or plasma concentration (T_{lag}). By enhanced absorption it is meant that absorption of the drug is improved as measured by such parameters. The measurement of pharmacokinetic parameters are routinely performed in the art.

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6. EXAMPLES

6.1 METHOD OF PRODUCING THE COLLAGEN BIOFABRIC

MATERIALS

[00159] The following materials were used in preparation of the collagen biofabric.

Materials/Equipment

- Copy of Delivery Record
- Copy of Material/Family Health History/Informed Consent
- Source Bar Code Label (Donor ID number)
- Collection # (A sequential number is assigned to incoming material)
- Tissue Processing Record (Document ID #ANT-19F); a detailed record of processing of each lot number is maintained
- Human Placenta (less than 48 hours old at the start of processing)
- Sterile Surgical Clamps/Hemostats
- Sterile Scissors
- Sterile Scalpels
- Sterile Cell Scraper (Nalgene NUNC Int. R0896)
- Sterile Gauze (non-sterile PSS 4416, sterilized)
- Sterile Rinsing Stainless Steel Trays
- Disinfected Processing Stainless Steel Trays
- Disinfected Plastic Bin
- Sterile 0.9% NaCl Solution (Baxter 2F7124)
- Sterile Water (Milli Q plus 09195 or Baxter 2F7113)
- Sterile Specimen Containers (VWR 15704-014)
- Personal Protective Equipment (including sterile and non-sterile gloves)
- Certified Clean Room
- Previously Prepared Decellularizing Solution (D-cell); 0.01-1% deoxycholic acid sodium monohydrate
- Disinfected Bin
- Rocking Platform (VWR Model 100)
- Timer (VWR 21376890)
- Disinfected Plastic Frame Mesh
- PVC Wrap Film
- Vacuum Pump (Schuco-Vac 5711-130)
- Gel Dryer (*i.e.*, heat dryer; BioRad Model 583)
- Disinfected Stainless Steel Cutting Board
- Pouches for Packaging
- Sterile Stainless Steel Ruler (General Tools MFG. Co 1201)
- Traceable Digital Thermometer (Model 61161-364, Control Company)

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- Accu-Seal Automatic Sealer (Accu-Seal, Model 630-1B6)

[00160] The expectant mother was screened at the time of birth for communicable diseases such as HIV, HBV, HCV, HTLV, Syphilis, CMV and other viral, and other pathogens that could contaminate the placental tissues being collected. Only tissues collected from donors whose mothers tested negative or non-reactive to the above-mentioned pathogens were used to produce the collagen biofabric.

[00161] Following normal birth, the placenta, umbilical cord and umbilical cord blood were spontaneously expelled from the contracting uterus. The placenta, umbilical cord, and umbilical cord blood were collected following birth. The materials were transported to the laboratory where they were processed under aseptic conditions in a Clean room having a HEPA filtration system, which was turned on at least one hour prior to processing. Gloves (sterile or non-sterile, as appropriate) were worn at all times while handling the product. All unused (waste) segments of the amnion/chorion and contaminated liquids, generated during tissue processing were disposed of as soon as feasible.

[00162] **STEP I.**

[00163] A sterile *field* was set up with sterile Steri-Wrap sheets and the following instruments and accessories for processing were placed on it.

- sterile tray pack
- sterile Cell Scraper
- sterile scalpel
- disinfected processing tray

[00164] Sterile pack ID # was recorded in the Processing Record.

[00165] The placenta was removed from the transport container and placed onto the disinfected stainless steel tray. Using surgical clamps and scissors, the umbilical cord was cut off approximately 2 inches from the placental disc. The umbilical cord was placed into a separate sterile container for further processing. The container was labeled with Tissue ID Bar Code; and the material and storage solution(s) present (*e.g.*, type of media) were identified. In some cases, the umbilical cord was discarded if not requested for other projects.

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[00166] Starting from the edge of the placental membrane, the amnion was separated from the chorion using blunt dissection with fingers. This was done prior to cutting the membrane.

[00167] After the amnion was separated from the entire surface of the chorion and placental disc, the amniotic membrane was cut around the umbilical cord stump with scissors and detached from the placental disc. In some instances, if the separation of the amnion and chorion was not possible without tearing the tissue, the amnion and chorion were cut from the placental disc as one piece and then peeled apart.

[00168] The chorion was placed into a separate specimen container to be utilized for other projects. The container was labeled with the Tissue ID Bar Code, the material and storage solution(s) present (*e.g.*, type of media) were identified, initialed and dated.

[00169] If any piece of amnion was still attached to the placental disc it was peeled from the disc and cutting off around the umbilical cord with scissors. The placenta was placed back into the transport container to be utilized for other projects.

[00170] The appropriate data was recorded in the Tissue Processing Record.

[00171] The amniotic membrane was kept in the tray with sterile 0.9% NaCl solution. Preferably, the amniotic membrane is stored by refrigeration for a maximum of 72 hours from the time of delivery prior to the next step in the process.

[00172] **STEP II.**

[00173] The amniotic membrane was removed from the specimen container one piece at a time and placed onto the disinfected stainless steel tray. Other pieces were placed into a separate sterile stainless steel tray filled with sterile water until they were ready to be cleaned. Extra pieces of amnion from the processing tray were removed and placed in a separate rinsing stainless steel tray filled with sterile water.

[00174] The amniotic membrane was rinsed with sterile water if grossly contaminated with blood maternal or fetal fluids/materials changing sterile water as needed.

[00175] The amniotic membrane was placed on the processing tray with the maternal side facing upward. Using a sterile Cell Scraper, as much as possible of visible contamination and cellular material from the maternal side of the amnion was carefully removed. (Note: minimal pressure should be applied for this step to prevent tearing the membrane). Sterile water was used to aid in the removal of cells and cellular debris. The amniotic membrane was further rinsed with sterile water in the separate sterile stainless steel rinsing tray.

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[00176] The amniotic membrane was turned over so that the fetal side was facing upward and placed back on the processing tray and rinsed with sterile water. Visible cellular material and debris using the Cell Scraper was gently removed (Note: minimal pressure should be applied for this step to prevent tearing the membrane). Sterile water was used to aid in the removal of cells and cellular debris.

[00177] The amniotic membrane was rinsed with sterile water in between cleaning rounds in separate sterile rinsing trays. The tissue was cleaned as many times (cleaning rounds) as necessary to remove most if not all of visible cellular material and debris from both sides of the membrane. The sterile water was changed in the rinsing trays in between rinses.

[00178] The processing tray was rinsed with sterile water after each cleaning round.

[00179] All other pieces of amnion were processed in the same manner and placed into the same container. Tissue Id Bar Code was affixed, the material and storage solution(s) present (*e.g.*, type of media) were identified, initials date were added.

[00180] The appropriate information and the date were recorded in the Tissue Processing Record.

[00181] **STEP III.**

[00182] The amniotic membrane was removed from the rinsing tray, (or from storage container) excess fluid was gently squeezed out with fingers and the membrane was placed into the sterile specimen container. The container was filled up to the 150 ml mark with D-cell solution ensuring that all of the amniotic membrane was covered and the container was closed.

[00183] The container was placed in the bin on the rocking platform. The rocking platform was turned on and the membrane was agitated in D-cell solution for a minimum of 15 minutes and a maximum of 120 minutes at Setting #6.

[00184] A new sterile field was set up with new sterile instruments and disinfected tray in a same manner as in the Step I. Sterile pack ID # was recorded in the Processing Record.

[00185] After agitation was completed, the rocking platform was turned off and the membrane was removed from the container. The membrane was placed into a new sterile stainless steel processing tray. Sterile 0.9% NaCl solution was added to cover the bottom of the tray.

[00186] Using a new sterile Cell Scraper, residual D-cell and cellular material (if any) was removed from both sides of the tissue. This step was repeated as many times as needed

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to remove as much as possible of visible residual cellular material from the entire surface on both sides. The membrane was rinsed with sterile 0.9% NaCl solution in a separate rinsing tray in between cleaning rounds. The sterile 0.9% NaCl solution was changed in the rinsing trays in between rinses.

[00187] After the last cleaning round was completed, the membrane was rinsed with sterile 0.9% NaCl solution and placed into the new sterile specimen container filled with sterile 0.9% NaCl solution.

[00188] All remaining pieces of amniotic membrane were processed in exactly the same manner.

[00189] When all amniotic membrane pieces were processed and in the container with the sterile 0.9% NaCl solution, the container was placed in the bin on the rocking platform to agitate for a minimum of 5 minutes at setting #6. After agitation was completed, the membrane was removed from the specimen container, the sterile 0.9% NaCl solution was changed in the container and the membrane was placed back into the specimen container.

[00190] The specimen container was labeled with Tissue ID Bar Code and Quarantine label. The material and storage solution(s) present (*e.g.*, type of media) were identified, initialed and dated. The specimen container was placed into a clean zip-lock bag and placed in the refrigerator (2 - 8°C).

[00191] All appropriate data was recorded in the Tissue Processing Record.

[00192] When serology results became available, the appropriate label (Serology Negative or For Research Use Only) was placed on the top of the Quarantine label and those containers were segregated from Quarantined ones.

[00193] **STEP IV.**

[00194] Before proceeding with Step IV, the Tissue Status Review was checked to make sure all applicable test results were negative.

[00195] A sterile field was set up with sterile Steri-Wrap sheet and all sterile and disinfected instruments and accessories were set up in the same manner as in Steps II and III.

[00196] The membrane was removed from the refrigerator and placed into a new sterile stainless steel processing tray. Sterile 0.9% NaCl solution was added to cover the bottom of the tray.

[00197] All visible cellular material and debris (if any) was gently removed using a new sterile Cell Scraper (Note: minimal pressure should be applied for this step to prevent

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tearing the membrane). Sterile 0.9% NaCl solution was used to aid in removal of the cells and debris.

[00198] The membrane was rinsed in the separate sterile stainless steel rinsing tray filled with the sterile 0.9% NaCl Solution. 0.9% NaCl Solution was changed in between cleaning rounds. The membrane was placed into a new sterile specimen container, the container was filled with fresh sterile 0.9% NaCl solution and placed on the rocking platform for agitation for a minimum of 5 minutes at Setting #6.

[00199] The previous step was repeated 3 times and the sterile 0.9% NaCl solution was changed in between each agitation. Appropriate data was recorded in the Tissue Processing Record.

[00200] The membrane was removed from the specimen container one piece at a time, excess fluid was gently squeezed out with fingers and the membrane was placed onto a sterile processing tray. The membrane was gently stretched until flat; ensuring it was fetal side down.

[00201] The frame was prepared by cutting the disinfected plastic sheet with sterile scissors. The size of the frame should be approximately 0.5 cm smaller in each direction than the membrane segment. The frame was rinsed in the rinsing tray filled with sterile 0.9% NaCl solution.

[00202] The frame was placed on the slightly stretched membrane surface and pressed on it gently. It is imperative that the smooth side of the plastic frame faces the tissue.

[00203] Using a scalpel, the membrane was cut around the frame leaving approximately 0.5 cm extending beyond frame edges. The excess membrane was placed back into the specimen container

[00204] The membrane edges that are extended beyond the frame were wrapped over the edges of the frame using clamps or tweezers and put aside on the same tray.

[00205] The next piece of membrane was processed in the same manner. It is important the total area to be dried does not exceed 300 cm^2 per heat dryer. While 'framing out' the piece of membrane, the non-framed pieces should remain in the container in sterile 0.9% NaCl solution.

[00206] The drying temperatures of dryers were set and verified using a calibrated digital thermometer with extended probe. The drying temperature was set at 50°C. The data was recorded in the Tissue Processing Record.

[00207] The vacuum pump was turned on.

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[00208] A sterile gauze was placed on the drying platform of the heat dryer, covering an area slightly larger than the area of the framed membrane. It is important to make sure that the total thickness of the gauze layer does not exceed thickness of one folded 4 x 4 gauze.

[00209] One sheet of plastic framing mesh was placed on top of the gauze. The plastic mesh edges should extend approximately 0.5 - 1.0 cm beyond gauze edges.

[00210] The framed membrane was gently lifted and placed on the heat dryer platform on top of the plastic mesh with the membrane side facing upward. This was repeated until the maximum amount of membrane (without exceeding 300 cm^2) was on the heat dryer platform. (NOTE: fetal side of the amnion is facing up).

[00211] A piece of PVC wrap film was cut large enough to cover the entire drying platform of the heat dryer plus an extra foot.

[00212] With the vacuum pump running, the entire drying platform of the heat dryer was gently covered with the plastic film leaving ½ foot extending beyond drying platform edges on both sides. Care was taken that the film pull tightly against the membrane and frame sheet (*i.e.*, it is "sucked in" by the vacuum) and that there were no air leaks and no wrinkles over the tissue area). The lid was subsequently closed.

[00213] The vacuum pump was set to approximately -22 inches Hg of vacuum. The pump gage was recorded after 2-3 min of drying cycle. The membrane was heat vacuum dried for approximately 60 minutes. Approximately 15 - 30 minutes into the drying process, the sterile gauze layer was replaced in the heat dryer with a new one. The total thickness of the gauze layer must not exceed thickness of one folded 4 x 4 gauze.

[00214] After the change, care was taken so that the plastic film pulled tightly against the membrane and the frame sheet and there were no air leaks and no wrinkles over the membrane area.

[00215] The integrity of the vacuum seal was periodically checked by checking the pump pressure monometer. After completion of the drying process, the heat dryer was opened and the membrane was cooled down for approximately two minutes with the pump running.

[00216] A new sterile field was set up with sterile Steri-wrap and disinfected stainless steel cutting board underneath it. As this point sterile gloves were used. With the pump still running, the plastic film was gently removed from the membrane sheet starting at the corner and holding the membrane sheet down with a gloved hand. The frame was gently lifted with the membrane off the drying platform and placed on the sterile field on the top of the

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disinfected stainless steel cutting board with the membrane side facing upward. Using a scalpel, the membrane sheet was cut through making an incision along the edge 1 -2 mm away from the edge of the frame. The membrane was held in place with a gloved (sterile glove) hand. Gently the membrane sheet was lifted off of the frame by peeling it off slowly and then placed on the sterile field on the cutting board.

[00217] Using scalpel or sharp scissors, the membrane sheet was cut into segments of specified size. All pieces were cut and secured on the sterile field before packaging. A single piece of membrane was placed inside the inner peel-pouch package with one hand (sterile) while holding the pouch with another hand (non-sterile). Care was taken not to touch pouches with 'sterile' hand. After all pieces were inside the inner pouches they were sealed. A label was affixed with the appropriate information (*e.g.*, Part #, Lot #, *etc.*) in the designated area on the outside of the pouch. All pieces of membrane were processed in the same manner. The labeled and sealed peel-pouch packages were placed in the waterproof zip-lock bag for storage until they were ready to be shipped to the sterilization facility or distributor. All appropriate data were recorded on the Tissue Processing Record.

6.2 EVALUATION OF THE COLLAGEN BIOFABRIC FOR HYDRATION/SUTURABILITY

[00218] In order to generate qualitative and quantitative feedback regarding the surgical handling, hydration periods and suturability of the biofabrics of the present invention, amniotic membrane samples prepared according to the methods of the present invention are provided to four experienced, well respected ocular surface surgeons for evaluation. The samples are evaluated by the surgeons by performing tissue grafts on pig eye specimens to determine surgical handling properties and suturability of the biofabrics of the invention.

[00219] The following methodology may be used by each surgeon:

(1) The biofabric is cut dried to fit a single quadrant of the pig's eye;

(2) The cut biofabric is placed on the surface of the pig's eye;

(3) The biofabric is hydrated with sterile saline solution and the graft is allowed to activate, *i.e.*, re-hydrate, on the pig's eye for hydration periods of 2, 5, 10, and 20 minutes;

(4) The biofabric is sutured to the epithelium of the pig's eye with several 9-0 vicryl suture bites; and

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(5) The surgeons make qualitative notes regarding tissue quality, consistency, and suturability of the hydrated amniotic membrane

Equivalents:

[00220] The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

[00221] Various publications, patents and patent applications are cited herein, the disclosures of which are incorporated by reference in their entireties.

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WHAT IS CLAIMED IS:

1. A method of preparing a collagen biofabric from a placenta having an amniotic membrane and a chorionic membrane comprising:

(a) separating the amniotic membrane from the chorionic membrane; and

(b) decellularizing the amniotic membrane so that the amniotic membrane is not contacted with an enzyme.

2. The method of claim 1 further comprising washing and drying the decellularized amniotic membrane.

3. The method of claim 1, wherein the placenta is a human placenta.

4. The method of claim 1, wherein the placenta is from a human female who has undergone a cesarean section delivery or natural delivery.

5. The method of claim 1, wherein the method additionally comprises the step of determining that the placental membrane is from a donor that has been tested for at least one communicable disease.

6. The method of claim 1, wherein the donor is a human female.

7. The method of claim 1, wherein the placenta is provided within 48 hours of birth.

8. The method of claim 1, wherein the method additionally comprises the step of storing the amniotic membrane for up to 72 hours after step (a) and before step (b).

9. The method of claim 8, wherein said storing comprises refrigerating the amniotic membrane obtained in step (a) for up to 5 days.

10. The method of claim 1, wherein the decellularization of the amniotic membrane in step (b) comprises removing all visible cellular material and cellular debris from the amniotic membrane.

11. The method of claim 1, wherein the decellularization of the amniotic membrane in step (b) comprises removal of all visible cellular material and cellular debris

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from the maternal side of the amniotic membrane and the fetal side of the amniotic membrane.

12. The method of claim 1, wherein the decellularization of the amniotic membrane in step (b) comprises physically scraping said membrane.

13. The method of claim 1, wherein the decellularization of the amniotic membrane in step (b) comprises decellularizing the amniotic membrane with a detergent containing solution.

14. The method of claim 13, wherein the detergent containing solution is a solution comprising 0.01-1.0% deoxycholic acid sodium salt monohydrate.

15. The method of claim 13, wherein the detergent in the detergent containing solution is selected from a group consisting of nonionic detergents, Triton X-100, anionic detergents, and sodium dodecyl sulfate or a combination thereof.

16. The method of claim 12, wherein said physical scraping comprises scraping with a cell scraper.

17. The method of claim 1, wherein the decellularization of the amniotic membrane in step (b) is performed in a sterile solution.

18. The method of claim 2, wherein the washing of the amniotic membrane is performed in a sterile solution.

19. The method of claim 2, wherein the drying of the decellularized amniotic membrane is performed at a temperature of about 35°C to about 50 °C.

20. A method of preparing an amniotic membrane laminate from a placenta having an amniotic membrane and a chorionic membrane comprising:

(a) separating the amniotic membrane from the chorionic membrane;

(b) decellularizing the amniotic membrane; and

(c) layering at least two of the decellularized amniotic membranes in contact with each other so that an amniotic membrane laminate is formed.

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21. The method of claim 20, further comprising washing the decellularized amniotic membrane at least once after step (b) and before step (c).

22. The method of claim 20, further comprising drying the decellularized amniotic membrane laminate.

23. The method of claim 20, further comprising assembling at least two of said amniotic membrane laminates into a complex three dimensional scaffold.

24. A collagen biofabric comprising a dehydrated, decellularized and substrate-free amniotic membrane, wherein said amniotic membrane has a native tertiary and quaternary structure.

25. A decellularized and substrate-free collagen biofabric comprising collagen, elastin, and fibronectin.

26. A collagen biofabric prepared by the method of claim 1.

27. The collagen biofabric of claim 24, wherein the amniotic membrane is a human amniotic membrane.

28. The collagen biofabric of any of claims 24-26, wherein the biofabric is about 10 to 40 microns in thickness.

29. The collagen biofabric of any of claims 24-26, wherein the biofabric is further impregnated with one or more biomolecules.

30. The collagen biofabric of claim 29 wherein the biomolecule is selected from the group consisting of antibiotics, hormones, growth factors, anti-tumor agents, anti-fungal agents, anti-viral agents, pain medications, anti-histamines, anti-inflammatory agents, anti-infectives, wound healing agents, wound sealants, cellular attractants and scaffolding reagents.

31. The collagen biofabric of the claim 29 wherein the biofabric is further impregnated with one or more small molecules.

32. The collagen biofabric of any of claims 24-26, wherein the biofabric is further populated with cells, so that said cells are uniform and confluent.

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33. The collagen biofabric of claim 32, wherein the cells are human stem cells or human differentiated adult cells.

34. The collagen biofabric of any of claims 24-26, further comprising one or more therapeutic agents.

35. The collagen biofabric of claim 34, wherein said therapeutic agent is selected from the group consisting of a hormone, a polypeptide, an antibiotic, an antifungal agent, and an enzyme.

36. The collagen biofabric of any of claims 24-26, further comprising one or more hydrogel compositions.

37. The collagen bioabric of claim 36, wherein the hydrogel composition comprises a polymer selected from the group consisting of polyvinyl alcohol, polyethylene glycol, hyaluronic acid, dextran, and derivatives or analogs thereof.

38. A three-dimensional scaffold comprising the collagen biofabric of any of claims 24-26.

39. The three-dimensional scaffold of claim 38, wherein the scaffold is a tube.

40. The three-dimensional scaffold of claim 38 further comprising one or more hydrogel compositions.

41. The three-dimensional scaffold of claim 40, wherein the hydrogel composition comprises a polymer selected from the group consisting of polyvinyl alcohol, polyethylene glycol, hyaluronic acid, dextran, and derivatives or analogs thereof.

42. An amniotic membrane laminate produced by the method of claim 20.

43. An amniotic membrane laminate comprising at least two layers of the collagen biofabric of any of claims 24-26.

44. An amniotic membrane laminate comprising the collagen biofabric of any of claims 24-26.

45. The amniotic membrane laminate of any of claims 42-44, further comprising one or more hydrogel compositions.

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46. The amniotic membrane laminate of claim 45, wherein the hydrogel composition comprises a polymer selected from the group consisting of polyvinyl alcohol, polyethylene glycol, hyaluronic acid, dextran, and derivatives or analogs thereof.

47. A surgical graft comprising the collagen biofabric of any of claims 24-26.

48. A method of using the surgical graft of claim 47 in a surgical procedure, wherein said surgical graft is applied directly to the surgical site of the subject.

49. The method of claim 48, wherein said surgical site is selected from the group consisting of an eye, skin, a serosal surface of the abdomen, a serosal surface of the chest cavity, a serosal pericardium, a mucosal surface of the oral cavity, a mucosal surface of the nasal cavity, a surface of the respiratory tract, a surface of the gastrointestinal tract, a surface of the urogenital tract.

50. The method of claim 48, wherein said surgical graft is applied to an internal site of the subject's body.

51. The method of claim 48, wherein said surgical graft is applied to an external site of the subject's body.

52. The method of claim 48, wherein said subject is human.

53. A method for treatment and/or prevention of an eye disease in a subject, comprising placing the collagen biofabric of any of claims 24-26 on a diseased eye surface of the subject.

54. The method of claim 53, wherein the eye disease is selected from the group consisting of ulcerations/perforations, bullous keratopathy, ocular dermoids/tumors, primary pterygium, persistent corneal epithelial defect, acute and chronic alkali burns, thermal burns, aniridia, atopic keratitis, idiopathic limbal stem cell deficiency, corneal pannus, neovascularization, rheumatoid corneal melt, ocular cicatricial pemphigoid, leaking filtering bleb, exposed Ahmed valve tube, Serratia cellulitis with subsequent symblepharon, acute and chronic Stevenson Johnson syndrome.

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55. A method of using the collagen biofabric of any of claims 24-26 in surgical procedures selected from the group consisting of ophthalmic surgery; cardiovascular surgery; periodontal surgery; neurological surgery, dental surgery, and orthopedic surgery.

56. A method of using the collagen biofabric of any of claims 24-26 in correction of urinary incontinence in a subject

57. A method of delivering a therapeutic agent to a subject comprising contacting the subject with the collagen biofabric of any of claims 24-26.

58. The method of claim 56 or 57 wherein the subject is a human.

59. The method of claim 57, wherein the therapeutic agent is selected from the group consisting of antibiotics, anti-cancer agents, anti-bacterial agents, anti-viral agents; vaccines; anesthetics; analgesics; anti-asthmatic agents; anti-inflammatory agents; anti-depressants; anti-diabetic agents; anti-psychotics; central nervous system stimulants; hormones; immuno-suppressants; muscle relaxants; and prostaglandins.

60. A method of using the collagen biofabric of any of claims 24-26, wherein the preparation time of the collagen biofabric comprises hydration of the collagen biofabric.

61. The method of claim 60, wherein the hydration of the biofabric comprises hydration with a sterile saline solution.

62. The method of claim 60, wherein the biofabric is hydrated for at least 2 minutes prior to use.

63. A method of using an amniotic membrane laminate further comprising populating the laminate with living cells.

64. The method of claim 63, wherein said living cells are selected from the group consisting of adult tissue cells and stem cells.

65. The method of claim 64, wherein said stem cells are totipotent.

66. The method of claim 64, wherein said stem cells are pluripotent.

67. The method of claim 64, wherein said stem cells are tissue specific.

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68. A method for treating or preventing a skin condition in a subject comprising contacting said skin condition with the collagen biofabric of any of claims 24-26.

69. The method of claim 68, wherein said skin condition is selected from the group consisting of a skin lesion, wrinkles, fine lines, skin thinning, reduced skin elasticity, rough skin, acne scars, glabellar furros, excision scar, soft tissue defect, congenital skin condition, degenerative skin condition, collagen VII deficiency, and sun damaged skin.

70. The method of claim 68 further comprising administering one or more therapeutic agents to the subject for the treatment of a skin condition.

71. The method of claim 70, wherein said one or more therapeutic agent is selected from the group consisting of vitamins, minerals, catechin-based preparations, and glucosamine.

72. A method for treating a wound in a subject comprising contacting said wound with a collagen biofabric of any of claims 24-26.

73. The method of claim 72, wherein said wound is selected from the group consisting of an epidermal wound, a skin wound, a pressure ulcer, a chronic wound, an acute wound, an external wound, an internal wound, a congenital wound, a burn wound, a surgical wound, and a wound infection.

74. A method for treating a burn in a subject comprising contacting said burn with a collagen biofabric of any of claims 24-26.

75. The method of claim 74, wherein said burn is selected from the group consisting of first degree burn, second degree burn, third degree burn, an infected burn wound, and a burn wound impetigo.

76. The method of claim 68, 72 or 74, wherein the subject is human.

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FIG. 1

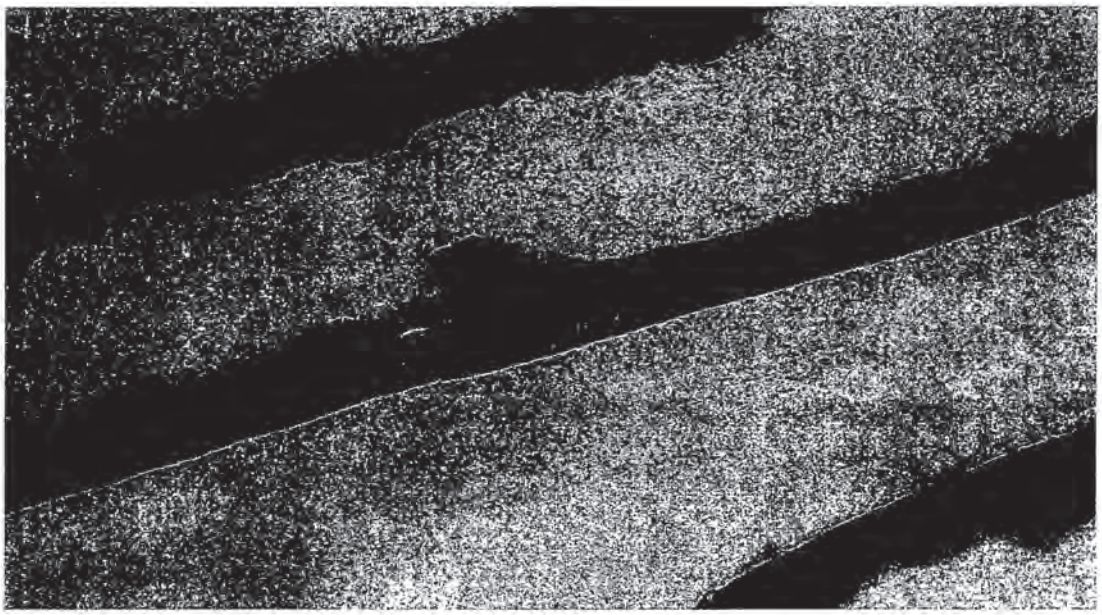


FIG. 2A

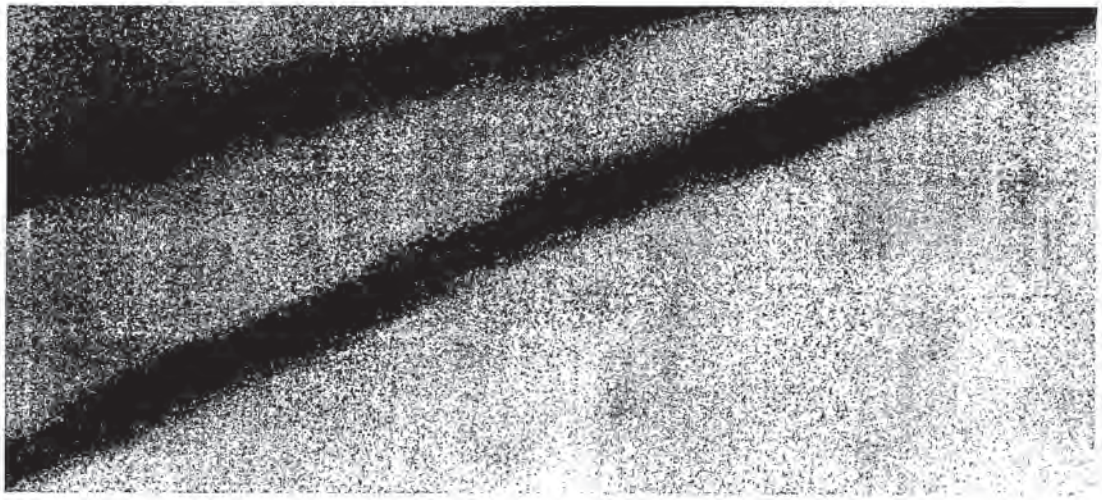


FIG. 2B

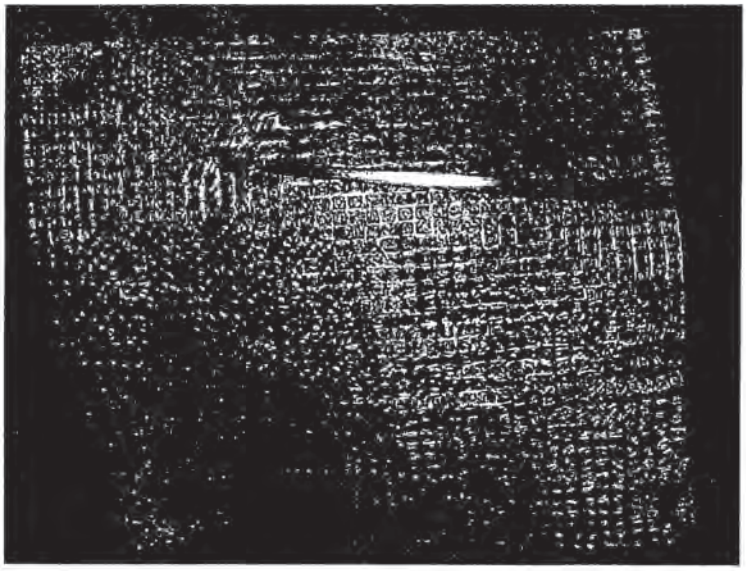


FIG. 3

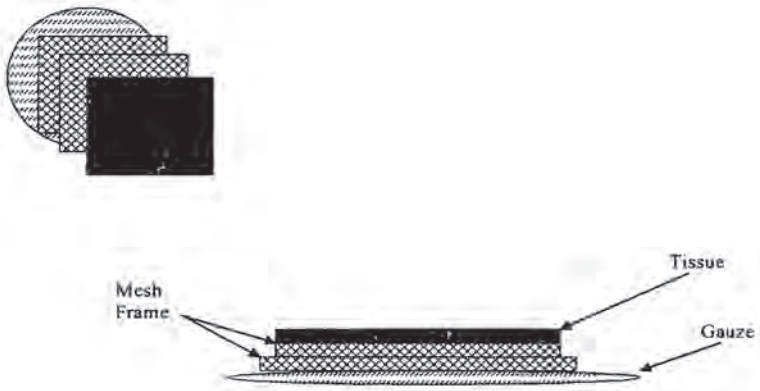


FIG. 4

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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(54) Title: COLLAGEN BIOFABRIC AND METHODS OF PREPARATION AND USE THEREFOR

(57) Abstract: The present invention relates to collagenous membranes produced from amnion, herein referred to as a collagen biofabric. The collagen biofabric of the invention has the structural integrity of the native non-treated amniotic membrane, i.e., the native tertiary and quaternary structure. The present invention provides a method for preparing a collagen biofabric from a placental membrane, preferably a human placental membrane having a chorionic and amniotic membrane, by decellularizing the amniotic membrane. In a preferred embodiment, the amniotic membrane is completely decellularized. The collagen biofabric of the invention has numerous utilities in the medical and surgical field including for example, blood vessel repair, construction and replacement of a blood vessel, tendon and ligament replacement, wound-dressing, surgical grafts, ophthalmic uses, sutures, and others. The benefits of the biofabric are, in part, due to its physical properties such as biomechanical strength, flexibility, suturability, and low immunogenicity, particularly when derived from human placenta.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/09304

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A01N 27/00; A61F 2/00; A61K 35/54; C12N 5/08
US CL : 424/93.7, 423, 582; 435/378,399; 623/315
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
REGISTRY, HCAPLUS

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 6,152,142 A (TSENG, S.C.G.) 28 November 2000 (28.11.2000), Entire document	1-76
Y	US 6,326,019 B1 (Tseng, S.C.G.) 04 December 2001 (04.12.2001), Entire Document, Especially Abstract. Column 7, Line 60 to Column 8, Line Column 10, Line 47.	1-76
Y	US 5,612,028 A (SACKIER, et al.) 18 March 1999 (18.03.1997), Entire Document.	1-76

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 June 2005 (16.06.2005)	04 AUG 2005
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner of Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 305-3230	Authorized officer Dr. Kailash C. Srivastava Telephone No. (703)-308-0196

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Continuation of B. FIELDS SEARCHED Item 3:
DWPI, EPAB, JPAB, PGPB, USOC, USPT, BIOBUSINESS, BIOCOMMERCE, BIOSIS, BIOTECHABS, BIOTECHNO, CABA, CANCERLIT, CAPLUS, CEABA-VTB, CONFSCI, DISSABS, EMBASE, ESBIOBASE, FEDRIP, FROSTI, JICST-PLUS, MEDICONF, MEDLINE, RDISCLOSURE, SCISEARCH, USPI, WPIDS.

Search Strategy:
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(71) Applicant *(for all designated States except US)*: **SCIMED LIFE SYSTEMS, INC.** [US/US]: One Scimed Place, Maple Grove, MN 55311-1566 (US).

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(74) Agents: **STERN, Gidon, D.** et al.; Jones Day, 222 East 41st Street, New York, NY 10017-6702 (US).

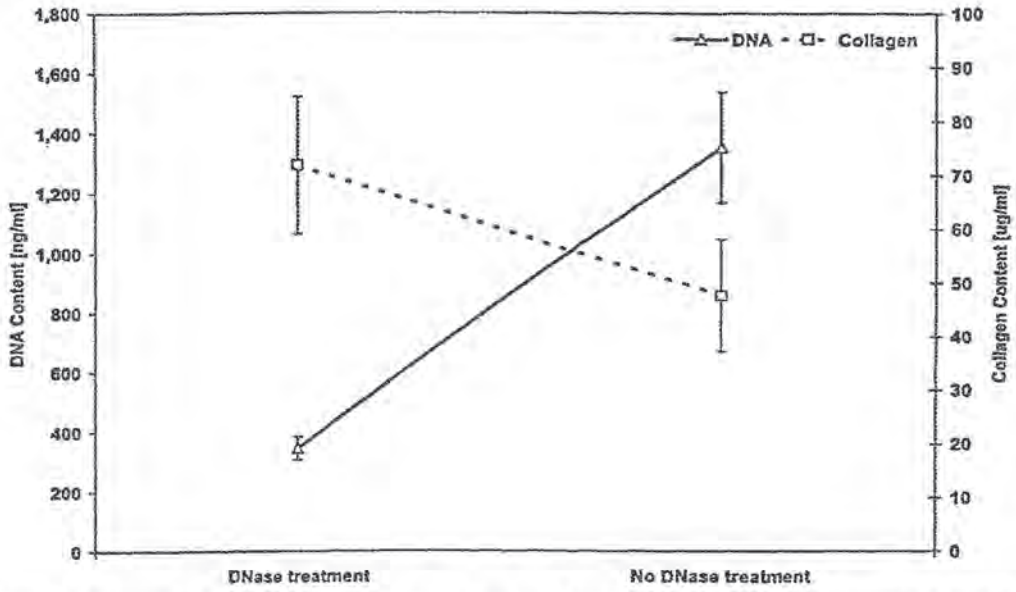
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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[Continued on next page]

(54) **Title:** DECELLULARIZED BONE MARROW EXTRACELLULAR MATRIX



(57) **Abstract:** The invention is directed to compositions comprising decellularized bone marrow extracellular matrix and uses thereof. Methods for repairing or regenerating defective, diseased, damaged or ischemic tissues or organs in a subject, preferably a human, using the decellularized bone marrow extracellular matrix of the invention are also provided. The invention is further directed to a medical device, preferably a stent or an artificial heart, and biocompatible materials, preferably a tissue regeneration scaffold, comprising decellularized bone marrow extracellular matrix for implantation into a subject.

WO 2005/009498 A2

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

DECELLULARIZED BONE MARROW EXTRACELLULAR MATRIX

1. FIELD OF THE INVENTION

The invention relates generally to decellularized bone marrow extracellular matrix, as well as methods for the production and use thereof. In particular, the invention relates to treating defective, diseased, damaged or ischemic tissues or organs in a subject, preferably a human, by injecting or implanting decellularized bone marrow extracellular matrix into the subject. More particularly, the invention is directed to a medical device, preferably a stent or an artificial heart, comprising decellularized bone marrow extracellular matrix for implantation into a subject. Methods for manufacturing and coating a surface of the medical device with decellularized bone marrow extracellular matrix are also provided. The invention further relates to a biocompatible material, preferably a tissue regeneration scaffold, comprising decellularized bone marrow extracellular matrix.

2. BACKGROUND OF THE INVENTION

The healing of damaged or diseased tissues or organs is, in general, imperfect, resulting in scarring and loss of function. The ineffectiveness of pharmacological therapy has led physicians to resort to reconstructive surgery that repairs or reconstitutes soft tissue or skin by introducing an injectable or implantable material. For example, silicon gel, polyester fiber, and polytetrafluorethylene (PTFE) have been extensively used as implants designed to replace diseased or damaged body parts. Other products that have been injected into the human body to correct soft tissue and skin defects include paraffin, petrolatum, vegetable oils, lanolin, and bees wax. Implantation or injection of these synthetic materials, however, can become hazardous to the health of the patient due to leakage, calcium deposit, hematoma, nodules, cellulitis, skin ulcers, and the triggering of autoimmune diseases (*e.g.*, joint swelling and flu-like symptoms).

To reduce immune response caused by the injection or implantation of non-biocompatible materials, hyaluronic acid gel, a non-animal biomaterial, has been used for soft tissue augmentation (Lupton J. and Alster T., 2000, *Dermatol. Surg.* 26:135-7). Bovine collagen has also gained widespread use as an injectable material for soft tissue augmentation. Collagen is the principal extracellular matrix structural protein. Early collagen implants are often solid collagen masses which were cross-linked with chemical

agents, radiation or other means to improve mechanical properties, decrease immunogenicity and/or increase resistance to resorption (see, *e.g.*, Oliver R. *et al.*, 1976, *Clin. Orthop.* 115:291-30; 1980, *Br. J. Exp. Path.* 61:544-549; 1981, *Conn. Tissue Res.* 9:59-62). The main problems associated with solid implants, however, are that they must be implanted surgically and often lose flexibility due to continuing cross-linking *in situ*. In contrast, while injectable collagen implant materials might have improved volume consistency and resistance to physical deformation, they are expensive and time consuming to prepare.

Also, engineered tissue has been used to augment existing tissue. Tissue engineering is an emerging field that studies the repair or regeneration of damaged or diseased tissues or failing or aging body parts with laboratory-grown parts such as bone, cartilage, blood vessels, and skin. Tissue engineering is based upon a relatively simple concept. First, some building material (*e.g.*, extracellular matrix or biodegradable polymer) is shaped as needed (*e.g.*, scaffold), seeded with living cells (*e.g.*, stem cells), and bathed with growth factors. While the cells multiply, they fill up the scaffold and grow into a three-dimensional tissue. Once implanted in the body, the tissue will function as a substitute for the damaged tissue. As blood vessels attach themselves to the new tissue, the scaffold dissolves, and the newly grown tissue eventually blends in with its surroundings.

In response to the need for more efficient and effective implant materials, extracellular matrix (ECM) and other acellular biomaterials have been used as therapeutic scaffolds for cell attachment and proliferation and as templates for tissue repair (Schmidt C. and Baier J., 2000, *Biomaterials* 21:2215-31). Extracellular matrix is a complex structural entity surrounding and supporting cells. The extracellular matrix is found within mammalian tissues and is made up of three major classes of biomolecules: structural proteins (*e.g.*, collagen and elastin), specialized proteins (*e.g.*, fibrillin, fibronectin, and laminin), and proteoglycans (*e.g.*, glycosaminoglycans). Although the exact mechanisms through which ECM facilitates repair or regeneration are not known, the composition and the organization of the components are considered to be important factors that influence cell attachment, gene expression patterns, and cell differentiation.

Successful implants include extracellular matrix from decellularized skin, blood vessels, and submucosal tissue. Abatangelo *et al.* in International Publication No. WO 97/18842 describes the preparation of skin substitutes *in vitro* by seeding keratinocytes with extracellular matrix secreted by bone marrow stem cells partially or completely differentiated into a specific connective tissue. Naughton in U.S. Patent No. 5,830,708 describes methods for soft tissue augmentation by injecting extracellular matrix proteins

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secreted from fibroblasts which were grown on a three-dimensional framework. Bell in U.S. Patent No. 5,800,537 describes a method for producing tissues for grafting using fragmented extracellular matrix particulates.

Bone marrow ECM is a part of the microenvironment that supports hematopoietic development in the bone marrow. Bone marrow ECM consists of accessory cells, cytokines, and extracellular matrix. The extracellular matrix in the bone marrow is comprised mainly of fibronectin, hemonectin, thrombospondin, collagen, laminin and glycosaminoglycans, heparan sulfate, dermantan, chondrotin sulfate (CS), and hyaluronic acid (HA). Recent findings show bone marrow is a source of multipotent adult stem cells (Jiang *et al.*, 2002, *Exp. Hematol.* 30:826-904). The ability to interact positively with stem cells is especially important for improving the healing of tissues, such as but not limited to epithelium tissues, connective tissues, muscle tissues, and nerve tissues.

Restenosis is a closure, re-narrowing or blockage of a peripheral or coronary artery at the same site caused by an effort to open an occluded portion of the artery by angioplasty (a balloon procedure to open an obstruction or narrowing of a blood vessel) or stent (an expandable, slotted metal tube, inserted into a vessel) procedure. Restenosis occurs in 40-50% of patients have angioplasty and in 20-30% with the use of stents (Ino T. *et al.*, 1997, *Acta. Paediatr.* 86:367-71). Restenosis has previously been addressed by providing stents which are seeded with endothelial cells that had undergone retrovirus-mediated gene transfer for either bacterial beta-galactosidase or human tissue-type plasminogen activator (Dichek D. *et al.* in 1989, *Circulation* 80:1347-53) or stents coated with antiplatelet agents, anticoagulant agents, antimicrobial agents, antimetabolic agents (U.S. Patent No. 5,102,417; International Publication No. WO 90/13332). Given the ability of bone marrow ECM to improve healing of tissue, the use of bone marrow ECM as a therapy is likely to improve patient outcome for occluded arteries.

The present inventors have surprisingly found that bone marrow ECM can be isolated, is a rich source of matrix proteins and growth factors, and that injecting or implanting bone marrow extracellular matrix guides tissue repair and regeneration in injured tissue. It is therefore an object of the present invention to provide a pharmaceutical composition, a medical device, as well as a tissue regeneration scaffold comprising a therapeutically significant amount of decellularized bone marrow extracellular matrix.

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3. SUMMARY OF THE INVENTION

To achieve the aforementioned objectives, we have invented a composition comprising decellularized bone marrow extracellular matrix, wherein the bone marrow extracellular matrix has been produced *in vivo* in an animal, preferably a mammal. The composition can be injectable or implantable. Preferably, the decellularized bone marrow extracellular matrix is prepared so the structure of the bone marrow extracellular matrix is maintained after it is decellularized.

In particular, the invention relates to methods for preparing decellularized bone marrow extracellular matrix from a bone marrow sample that has been produced *in vivo*. The bone marrow may be biologically, chemically, pharmaceutically, physiologically and/or mechanically modified prior to decellularization (see U.S. Application No. _____, filed on even date herewith, which is incorporated by reference in its entirety for all purposes and identified by attorney docket number 10177-118).

The bone marrow sample, which comprises an extracellular matrix and a non-extracellular matrix component, is decellularized using a combination of physical, chemical, and biological steps. During the step(s) of decellularization, the bone marrow sample is processed so at least some of the non-extracellular matrix component is removed and the remaining extracellular matrix is sterilized. Non-extracellular matrix components include, but is not limited to, cells, cell components, blood, bone spicules, extracellular matrix antigens, cytokines, serum, and fat.

Preferably, the decellularized bone marrow extracellular matrix is further treated with enzymes to remove nucleic acids and proteins. More preferably, the decellularized bone marrow extracellular matrix is further processed to limit generation of new immunological sites and to promote subsequent repopulation of the extracellular matrix with cells from a recipient after implantation of the decellularized bone marrow extracellular matrix into the recipient. The decellularized bone marrow extracellular matrix may also be dried, concentrated, lyophilized, or cryopreserved. Preferably, the decellularized bone marrow extracellular matrix is suspended in a saline solution as a final product.

The composition comprising decellularized extracellular matrix may further comprise a biological material useful for repairing, regenerating or strengthening tissues. Examples of the biological material include, but are not limited to, erythropoietin (Epo), stem cell factor (SCF), vascular endothelial growth factor (VEGF), transforming growth factor (TGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), cartilage growth factor (CGF), nerve growth factor (NGF), keratinocyte growth factor (KGF), skeletal

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growth factor (SGF), osteoblast-derived growth factor (BDGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), cytokine growth factor (CGF), stem cell factor (SCF), platelet-derived growth factor (PDGF), endothelial cell growth supplement (ECGS), colony stimulating factor (CSF), growth differentiation factor (GDF), integrin modulating factor (IMF), calmodulin (CaM), thymidine kinase (TK), tumor necrosis factor (TNF), growth hormone (GH), bone morphogenic proteins (BMP), matrix metalloproteinase (MMP), tissue inhibitor matrix metalloproteinase (TIMP), interferon, interleukins, cytokines, integrin, collagen, elastin, fibrillins, fibronectin, laminin, glycosaminoglycans, vitronectin, proteoglycans, transferrin, cytotactin, tenascin, and lymphokines.

The invention also relates to the injection or implantation of decellularized bone marrow extracellular matrix into a subject, preferably a human, to treat, manage or prevent the occurrence of defective, diseased, damaged or ischemic tissues or organs. The defective, diseased, damaged or ischemic tissue or organ may result from or is associated with burns, ulcer, wound, bond fracture, diabetes, psoriasis, arthritis, asthma, cystitis, inflammation, infection, ischemia, restenosis, stricture, atherosclerosis, occlusion, stroke, trauma, infarct, vascular disorders, hemophilia, cancer, organ failure (*e.g.*, heart, kidney, lung, liver, intestine), etc. The decellularized bone marrow extracellular matrix can be injected or implanted alone or in conjunction with other therapeutically or prophylactically effective agents or methods to repair, regenerate or strengthen tissues or organs. Preferably, the injected or implanted decellularized bone marrow extracellular matrix is biocompatible. More preferably, the decellularized bone marrow extracellular matrix minimizes or avoids immune response when injected or implanted into a subject. Most preferably, the ECM is sterile and is treated with an antibacterial substance.

The invention also relates to a medical device comprising decellularized bone marrow extracellular matrix. Preferably, the medical device is a stent or an artificial heart. The decellularized bone marrow extracellular matrix can be coated, preferably by spraying or rolling, onto a surface of the medical device. The decellularized bone marrow extracellular matrix can also be used to form a structural component of the medical device. The decellularized bone marrow extracellular matrix can be used alone or in combination with a biologically active material, preferably paclitaxel, or other polymers to coat or form the medical device. The medical device is suitable for insertion in to a subject, preferably a human.

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The invention further relates to a tissue regeneration scaffold comprising decellularized bone marrow extracellular matrix and methods of making the tissue regeneration scaffold.

4. FIGURE

Figure 1 is a graph showing the effect of DNase-treatment on the DNA and collagen contents of bone marrow samples.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to decellularized bone marrow extracellular matrix obtained from a bone marrow sample comprising an extracellular matrix and a non-extracellular matrix component. The bone marrow sample is decellularized using at least a physical, chemical and/or biological step(s), wherein at least some of the non-extracellular matrix component is removed.

Non-extracellular matrix component includes, without limitation, cells, cellular component (*e.g.*, cellular lipids, cellular polysaccharides, cellular debris), antigens, blood, bone spicules, serum, and fat. In preferred embodiments, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99% by weight, by volume or by size of the non-extracellular matrix component is removed.

The remaining decellularized extracellular matrix contains components which serve to attract stem cells to the site and/or provide a substrate and/or soluble factors which will promote the repair, regeneration and/or strengthening of tissues. Preferably, the decellularized bone marrow extracellular matrix is microstructurally and biochemically intact, *i.e.*, retains strength, resiliency, and adhesion properties.

5.1 DECELLULARIZED BONE MARROW EXTRACELLULAR MATRIX

5.1.1 Isolation Of Bone Marrow

Bone marrow (BM) is a soft, spongy tissue that fills the insides of bones. Most blood cells, including red blood cells, platelets and some white blood cells are produced in the bone marrow and are then released into the bloodstream as they become mature. Stem cells found in the bone marrow are either part of a non-adherent hematopoietic component or an adherent stromal cell component. The adherent stromal cell

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component includes multipotent adult progenitor cells (MAPCs) and mesenchymal stem cells (MSCs). MAPCs have the ability to differentiate into many cell types including MSCs, endothelial, epithelial and hematopoietic cells, and MSCs have the ability to self-renew by proliferation and to differentiate into differentiated stromal cells including the osteogenic, chondrogenic, adipogenic, myogenic and fibroblastic lineages (Van Damme A. *et al.*, 2002, *Curr. Gene Ther.* 2:195-209; Verfaillie C. *et al.*, 2002, *Hematology* (Am Soc Hematol Educ Program) 369-91).

Bone marrow represents 3-5% of the total body weight and averages about 1,500 grams in adults. The hematopoietic bone marrow is organized around the vasculature of the bone cavity. Bone marrow can be sampled relatively easily using either a needle aspirate or needle biopsy technique such as those disclosed in U.S. Patent Nos. 4,314,565; 4,481,946; 4,486,188; 4,513,754; 4,630,616; 4,664,128; 5,012,818; 5,257,632; and 5,331,972, all of which are incorporated by reference herein in their entireties.

In a bone marrow aspiration, a special needle (usually a University of Illinois needle) is inserted beneath the skin and rotated until it penetrates the cortex, or outer covering of the bone. A small amount of marrow is suctioned out of the bone by a syringe attached to the needle.

Bone marrow biopsy may be performed immediately before or after bone marrow aspiration. The procedure is performed with a special needle, which has a hollow core, much like a leather punch. In bone marrow biopsy, the needle is inserted, rotated to the right, then to the left, withdrawn, and reinserted at a different angle. This procedure is repeated until a small chip is separated from the bone marrow.

Generally, aspiration withdraws a fluid specimen (0.5 to 500 ml) that contains suspended marrow pustule from the bone marrow and needle biopsy removes a core of actual marrow cells (not fluid). More information on the procedures required to perform and evaluate needle aspiration and biopsy of bone marrow can be found in Hodges A. and Koury M., 1996, *Clin. Lab Sci.* 9:349-53, the content of which is incorporated by reference herein in its entirety.

Bone marrow aspirations and/or bone marrow biopsies can be performed in physicians' offices, outpatient clinics and/or hospital settings. Bone marrow aspirations and/or biopsies should be carried out by trained individuals who are aware of the indications, contraindications, and hazards of the procedure. The operators should follow a standard operating procedure and make an adequate assessment beforehand of clinical and hematological features (*e.g.*, temperature, heart rate, respiration, and blood pressure). For

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the patient's comfort and safety, the posterior iliac crest is generally the preferred site of aspiration. Other common sites include the pelvic bone or the breastbone. The site of puncture should be cleansed first with an antiseptic solution, and the subject given a local anesthetic.

Bone marrow may also be obtained after a terminal procedure. In this case, all marrow is removed from the bones. This may be accomplished with the bones in place or removed from the subject. Sterile surgical or laboratory tools are used to separate the spongy bone containing the marrow from the outer cortical bone.

In preferred embodiments, the bone marrow sample is isolated from a mammal such as a non-primate (*e.g.*, cows, pigs, horses, cats, dogs, rats etc.) or a primate (*e.g.*, monkey and human), most preferably a human. In certain preferred embodiments, the bone marrow sample can be obtained from marrow which has been modified (*e.g.*, manipulation of cells in bone marrow to alter the ECM produced by host cells). In specific embodiments, the bone marrow sample is obtained from a bone that has been conditioned to produce an amount of a biological material that is in an amount different than that produced by a bone absent the conditioning. The bone may be conditioned before or after the bone marrow is harvested.

5.1.2 Decellularization Procedures

Decellularization generally refers to the removal of non-extracellular matrix components, *i.e.*, cells, cellular components, and other non-extracellular matrix components (*e.g.*, blood, antigens, serum, fat) while leaving intact an extracellular matrix component. Preferably, all or substantially all of the cells, cellular components, and non-extracellular components are removed. The macroscopic structure of the ECM does not need to be maintained. However, in preferred embodiments, the microstructure of the extracellular matrix is retained after the bone marrow extracellular matrix is decellularized.

It is believed that the process of decellularization can reduce or eliminate the immune response associated with the cells as well as the cellular components. Acellular vascular tissues have been suggested to be ideal natural biomaterials for tissue repair and engineering (Schmidt C. and Baier J., *supra*). Bell in U.S. Patent No. 5,800,537 describes a method for producing extracellular matrix particulates first by manually and mechanically mincing a tissue source, second by removing the cell remnants from the extracellular matrix of the tissue source in the absence of a high salt solution, and finally by fragmenting the processed extracellular matrix.

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A bone marrow sample consists of cells, blood, bone spicules, fat, and extracellular matrix. The bone marrow sample may be decellularized using one or more physical, chemical and/or biological techniques to remove the cells, blood, bone spicules, and fat (see, *e.g.* U.S. Patent No. 5,192,312; 5,613,982; 5,632,778; 5,843,182; and 5,899,936, which are incorporated herein by reference in their entireties). The physical, chemical and/or biological methods are optimized to preserve as much as possible the composition of biologically active materials and more importantly, the microstructure of the extracellular matrix. The physical, chemical and/or biochemical techniques are to be applied in an appropriate order to purify the extracellular matrix component. The techniques described herein are preferably performed under aseptic conditions.

In certain embodiments, physical forces are used to decellularize the bone marrow samples. For example, vapor phase freezing (slow rate of temperature decline) and liquid phase freezing (rapid rate of temperature decline) can cause the formation of intracellular ice that disrupts the cells. Colloid-forming materials may be added during freeze-thaw cycles to alter ice formation patterns in the samples. Polyvinylpyrrolidone (10% w/v) and dialyzed hydroxyethyl starch (10% w/v) may be added to standard cryopreservation solutions (DMEM, 10% DMSO, 10% fetal bovine serum) to reduce extracellular ice formation while permitting formation of intracellular ice (see U.S. Patent No. 5,613,982). The ruptured cell and resulting cellular debris can be removed by any one or more of the physical, chemical and/or biological techniques described herein.

Similarly, extracellular matrix may be isolated from bone marrow samples using centrifugation. For example, a bone marrow sample is centrifuged with a liquid preparation, preferably Histopaque and more preferably water or saline, which physically separates components of different densities. In a preferred embodiment, the speed for centrifugation ranges from 100 to 10,000 g, and more preferably, from 2,500 to 7,500 g, for between 5 to 20 minutes.

Alternatively, extracellular matrix may be isolated from bone marrow samples using filtration. For example, a bone marrow sample may be filtered using filters of specific pore size. In a specific embodiment, the filter is of a pore size, preferably of 70 to 250 μm, that allows the extracellular matrix to pass through. In another specific embodiment, the filter is of a pore size, preferably of 0.1 to 100 μm, that retains the extracellular matrix and larger components. Filtration is carried out in one step or a series of steps.

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It has been reported that modification of the magnitude of the membrane dipole potential using compounds such as cholesterol, phloretin, and 6-ketocholestanol may also influence binding capacity and disrupts membrane domains. (Asawakarn T. *et al.*, 2001, *J. Biol. Chem.* 276:38457-63). Accordingly, the present invention further relates to methods for physically decellularizing bone marrow sample by agitating cellular membrane potential using electrical (*e.g.*, voltage) means.

Other physical techniques that can be used to decellularize a bone marrow sample include, but are not limited to, mincing, rinsing, agitation, sedimentation, dialysis, cutting, grinding, pressing, electrical stimulation, electromagnetic forces, hydrostatic or hydrodynamic forces, blasting with sound waves, and ultrasonication.

In certain other embodiments, bone marrow samples may be decellularized using at least a chemical techinque. In one embodiment, a bone marrow sample is treated with a solution effective to lyse native cells. Preferably, the solution may be an aqueous hypotonic or low ionic strength solution formulated to effectively lyse the native tissue cells. Such an aqueous hypotonic solution may be de-ionized water or an aqueous hypotonic buffer. The hypotonic lysis solution may include a buffered solution of water, pH 5.5 to 8, preferably pH 7 to 8. In preferred embodiments, the hypotonic lysis solution is free from calcium and zinc ions. Control of the temperature and time parameters during the treatment of the body tissue with the hypotonic lysis solution, may also be employed to limit the activity of proteases. Preferably, the aqueous hypotonic buffer may contain additives that provide suboptimal conditions for the activity of selected proteases, *e.g.*, collagenase, which may be released as a result of cellular lysis. Additives such as metal ion chelators, *e.g.*, 1,10-phenanthroline and ethylenediaminetetraacetic acid (EDTA), create an environment unfavorable to many proteolytic enzymes.

Accordingly, in preferred embodiments, the bone marrow sample is treated with protease inhibitors. General inhibitor solutions manufactured by Sigma and Genotech are preferred. Specifically, 4-(2-aminoethyl)-benzene-sulfonyl fluoride, E-64, bestatin, leopeptin, aprotin, PMSF, Na EDTA, TIMPs, pepstatin A, phosphoramidon, and 1,10-phenanthroline are non-limiting examples of preferred protease inhibitors.

In certain other embodiments, the bone marrow sample is treated with a detergent. In a specific embodiment, the bone marrow sample is treated with an anionic detergent, preferably sodium dodecyl sulfate in buffer. In another specific embodiment, the bone marrow sample is treated with a non-ionic detergent, such as Triton X-100 or 1% octyl phenoxyl polyethoxyethanol, to solubilize cell membranes and fat. In a preferred

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embodiment, the bone marrow sample is treated with a combination of different classes of detergents, for example, a nonionic detergent, Triton X-100, and an anionic detergent, sodium dodecyl sulfate, to disrupt cell membranes and aid in the removal of cellular debris.

Steps should be taken to eliminate any residual detergent levels in the extracellular matrix, so as to avoid interference with the latter's ability to repair, regenerate or strengthen defective, diseased, damaged or ischemic tissues or organs. Selection of detergent type and concentration will be based partly on its preservation of the structure, composition, and biological activity of the extracellular matrix.

Extracellular matrix may also be isolated from a bone marrow sample using at least a biological technique. Preferably, the enzyme treatment limits the generation of new immunological sites. For instance, extended exposure of the bone marrow sample to proteases such as trypsin result in cell death. However, because at least a portion of the type I collagen molecule is sensitive to a variety of proteases, including trypsin, this may not be the approach of choice for collagenous grafts intended for implant in high mechanical stress locations.

In one embodiment, the bone marrow sample is treated with nucleases to remove DNA and RNA. Nucleases are effective to inhibit cellular metabolism, protein production, and cell division without degrading the underlying collagen matrix. Nucleases that can be used for digestion of native cell DNA and RNA include both exonucleases and endonucleases. A wide variety of which are suitable for use in this step of the process and are commercially available. For example, exonucleases that effectively inhibit cellular activity include DNase I and RNase A (SIGMA Chemical Company, St. Louis, MO) and endonucleases that effectively inhibit cellular activity include EcoR I (SIGMA Chemical Company, St. Louis, MO) and Hind III (SIGMA Chemical Company, St. Louis, MO). It is preferable that the selected nucleases are applied in a physiological buffer solution which contains ions, such as magnesium and calcium salts, which are optimal for the activity of the nuclease. It is also preferred that the ionic concentration of the buffered solution, the treatment temperature, and the length of treatment are selected to assure the desired level of effective nuclease activity. The buffer is preferably hypotonic to promote access of the nucleases to the cell interiors.

Other enzymatic digestion may be suitable for use herein, for example, enzymes that disrupt the function of native cells in the bone marrow sample may be used. For example, phospholipase, particularly phospholipases A or C, in a buffered solution, may be used to inhibit cellular function by disrupting cellular membranes of endogenous cells.

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Preferably, the enzyme employed should not have a detrimental effect on the extracellular matrix protein. The enzymes suitable for use may also be selected with respect to inhibition of cellular integrity, and also include enzymes which may interfere with cellular protein production. The pH of the vehicle, as well as the composition of the vehicle, will also be adjusted with respect to the pH activity profile of the enzyme chosen for use. Moreover, the temperature applied during application of the enzyme to the tissue should be adjusted in order to optimize enzymatic activity.

In another embodiment, the bone marrow sample is treated so the cells are removed using immunomagnetic bead separation techniques directed to cell surface markers (*e.g.*, integrins, lineage markers, stem cell markers). Immunomagnetic separation (IMS) technology can isolate strains possessing specific and characteristic surface antigens (Olsvik O. *et al.*, 1994, *Clin. Microbiol Rev.* 7:43-54). Commercially available immunomagnetic separation processes such as Cell Release™ (Sigris Research, Brea, CA) was developed to address the need for a fast, general-purpose way to detach intact cells from beads after immunomagnetic separation.

Subsequent to decellularization protocols, the resultant extracellular matrix is washed at least once with suitable chemical solutions, such as saline, protease, enzymes, detergents, alcohols, acidic or basic solutions, salt solutions, EDTA, etc., to assure removal of cell debris which may include cellular protein, cellular lipids, and cellular nucleic acid, as well as any extracellular debris such as lipids and proteoglycans. Removal of the cellular and extracellular debris reduces the likelihood of the extracellular matrix eliciting an adverse immune response from the recipient upon injection or implantation. For example, the tissue may be incubated in a balanced salt solution such as Hanks' Balanced Salt Solution (HBSS), preferably sterile. The washing process may include incubation at a temperature of between about 2°C and 42°C, with 4°C to 25°C most preferable. The transplant tissue matrix may be incubated in the balanced salt wash solution for up to a day, 3 days, 5 days, a week, two weeks, or a month, with changes in wash solution every second or third day. The composition of the balanced salt solution wash, and the conditions under which it is applied to the transplant tissue matrix may be selected to diminish or eliminate the activity of the nuclease or other enzyme utilized during the decellularization process.

Optionally, an antibacterial, an antifungal or a sterilant or a combination thereof, may be included in the balanced salt wash solution to protect the decellularized bone marrow extracellular matrix from contamination with environmental pathogens. In certain embodiments, the decellularized bone marrow ECM is sterilized by irradiation, ultraviolet

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light exposure, ethanol incubation (70-100%), treatment with glutaraldehyde, peracetic acid (0.1-1% in 4% ethanol), chloroform (0.5%), or antimycotic and antibacterial substances.

The decellularized bone marrow extracellular matrix prepared in accordance with the above is preferably free or substantially free of its native cells, and additionally, cellular and extra-cellular antigen components have been washed out of the extracellular matrix. Preferably, the decellularized bone marrow has been treated in a manner which limits the generation of new immunological sites in the collagen matrix. The ECM is obtained as a slurry of small particles. This slurry, including the slurry solution, may eventually be processed into an implant.

In addition, the decellularized extracellular matrix may contain a significant portion of the original tissue mass retaining physical properties in regard to strength and elasticity and has components which are largely collagens but also comprise glycosaminoglycans and proteins closely associated with collagen such as the basement membrane complex, laminin and fibronectin.

The above physical, chemical, and biological techniques may also be used to remove non-extracellular matrix components (*e.g.*, cells, cellular components, blood, bone spicules, extracellular matrix antigens, serum, fat, etc.) prior to removal from the bone marrow cavity. For example, one or more solutions may be perfused through the bone marrow, as blood does normally, to solubilize or force non-extracellular matrix components out of the marrow. More specifically, de-ionized water, a detergent solution, an enzymatic solution (*e.g.*, trypsin, dispase, DNase) or EDTA can be forced through the marrow cavity to remove cells, cell debris, blood or fat prior to harvesting the bone marrow samples.

One aspect of the invention further provides the preservation of the decellularized bone marrow extracellular matrix for later use. The decellularized bone marrow extracellular matrix can be freeze-dried for prolonged storage. Likewise, the decellularized bone marrow extracellular matrix can be air-dried by any known standard techniques. In one embodiment, the decellularized bone marrow extracellular matrix can be concentrated or dehydrated and later reconstituted or rehydrated, respectively, before use.

In yet another embodiment, the decellularized bone marrow extracellular matrix can be lyophilized. The lyophilized ECM may be in the form of an implant which has pores. Characteristics of the pore structure can be controlled by process parameters. In yet another embodiment, the decellularized bone marrow extracellular matrix can be formed as a gel. Preferably, the proteins are temporarily and reversibly denatured. In yet another

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embodiment, the decellularized bone marrow extracellular matrix can be precipitated or co-precipitated with other proteins or biologics.

In certain embodiments, the decellularized bone marrow extracellular matrix is cryopreserved. For general methods of cryopreservation see Brockbank K., *Basic Principles of Viable Tissue Preservation. In: Transplantation Techniques and Use of Cryopreserverd Allograft Cardiac Valves and Vascular Tissue*. D. R. Clarke (ed.), Adams Publishing Group, Ltd., Boston. pp 9-23, which is hereby incorporated by reference herein in its entirety. Cryopreservation of decellularized bone marrow extracellular matrix would assure a supply or inventory of substantially non-immunogenic extracellular matrices which, upon thawing, would be ready for further treatment according to the subsequent steps of this invention, or further processed as desired to provide an implant tissue product.

In another particular embodiment, the decellularized bone marrow extracellular matrix is treated to enhance the ingrowth and attachment of the recipient's own cells. Growth factors, cytokines, genetic material, etc., for example, can be used to incubate the decellularized extracellular matrix prior to its injection or implantation into a subject or prior to its coating on or incorporation into a medical device. Preferred growth factors include, but are not limited to, erythropoietin (Epo), endothelial growth factor (VEGF), transforming growth factor (TGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), cartilage growth factor (CGF), nerve growth factor (NGF), keratinocyte growth factor (KGF), skeletal growth factor (SGF), osteoblast-derived growth factor (BDGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), cytokine growth factor (CGF), stem cell factor (SCF), platelet-derived growth factor (PDGF), endothelial cell growth supplement (ECGS), colony stimulating factor (CSF), growth differentiation factor (GDF), integrin modulating factor (IMF), calmodulin (CaM), thymidine kinase (TK), growth hormone (GH), bone morphogenic proteins (BMP) (*e.g.*, BMP-2, BMP-3, BMP-4, BMP-5, BMP-6 (Vgr-1), BMP-7 (PO-1), BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-14, BMP-15, BMP-16), matrix metalloproteinase (MMP), and tissue inhibitor matrix metalloproteinase (TIMP). Preferred cytokines include, but are not limited to, interleukins (*e.g.*, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-15), lymphokines, tumor necrosis factor (TNF) (*e.g.*, TNF-α, TNF-β), and interferons.

5.2 THERAPEUTIC USE

The invention relates generally to the use of decellularized bone marrow extracellular matrix to repair or regenerate defective, diseased, damaged or ischemic tissues

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or organs in a subject. Pharmaceutical compositions, medical devices and tissue regeneration scaffolds comprising decellularized bone marrow extracellular matrix can be injected or implanted into a subject in need thereof.

In certain embodiments, the decellularized bone marrow extracellular matrix may be used to treat defective, diseased, damaged or ischemic tissues or organs which include, but are not limited to, head, neck, eye, mouth, throat, esophagus, chest, bone, ligament, cartilage, tendons, lung, colon, rectum, stomach, prostate, breast, ovaries, fallopian tubes, uterus, cervix, testicles or other reproductive organs, hair follicles, skin, diaphragm, thyroid, blood, muscles, bone marrow, heart, lymph nodes, blood vessels, large intestine, small intestine, kidney, liver, pancreas, brain, spinal cord, and the central nervous system.

In particular, the decellularized bone marrow extracellular matrix may be used to treat diseases that may benefit from improved angiogenesis, cell proliferation and tissue regeneration. Such diseases or conditions include, but are not limited to, burns, ulcer, wound, bone fracture, diabetes, psoriasis, arthritis, asthma, cystitis, inflammation, infection, ischemia, restenosis, stricture, atherosclerosis, occlusion, stroke, trauma, infarct, vascular disorders, hemophilia, cancer, and organ failure (*e.g.*, heart, kidney, lung, liver, intestine, etc.).

In a specific embodiment, the present invention regenerates or replaces at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, at least 50%, at least 45%, at least 40%, at least 45%, at least 35%, at least 30%, at least 25%, at least 20%, at least 10%, at least 5%, or at least 1% of defective, diseased, damaged or ischemic cells from the affected tissue or organ.

The methods of the present invention is provided for an animal, including but not limited to mammals such as a non-primate (*e.g.*, cows, pigs, horses, chickens, cats, dogs, rats, etc.), and a primate (*e.g.*, monkey such as acynomolgous monkey and a human). In a preferred embodiment, the subject is a human.

The decellularized bone marrow extracellular matrix is useful alone or in combination with other treatment modalities. In certain embodiments, the treatment of the present invention further includes the administration of one or more immunotherapeutic agents, such as antibodies and immunomodulators, which include, but are not limited to, HERCEPTIN®, RITUXAN®, OVAREX™, PANOREX®, BEC2, IMC-C225, VITAXIN™, CAMPATH® I/H, Smart MI95, LYMPHOCIDE™, Smart I D10, ONCOLYM™, rituximab, gemtuzumab, or trastuzumab. In certain other embodiments, the treatment method further comprises hormonal treatment. Hormonal therapeutic treatments

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comprise hormonal agonists, hormonal antagonists (*e.g.*, flutamide, tamoxifen, leuprolide acetate (LUPRON™), LH-RH antagonists), inhibitors of hormone biosynthesis and processing, steroids (*e.g.*, dexamethasone, retinoids, betamethasone, cortisol, cortisone, prednisone, dehydrotestosterone, glucocorticoids, mineralocorticoids, estrogen, testosterone, progestins), antigestagens (*e.g.*, mifepristone, onapristone), and antiandrogens (*e.g.*, cyproterone acetate).

5.2.1 Pharmaceutical Compositions

The decellularized bone marrow extracellular matrix can be formulated into pharmaceutical compositions that are suitable for administration to a subject. Such compositions comprise a prophylactically or therapeutically effective amount of the decellularized bone marrow extracellular matrix as disclosed herein, and a pharmaceutically acceptable carrier.

In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant (*e.g.*, Freund's adjuvant (complete and incomplete) or, more preferably, MF59C.1 adjuvant available from Chiron, Emeryville, CA), excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. Other examples of suitable pharmaceutical vehicles are described in "Remington: the Science and Practice of Pharmacy", 20th ed., by Mack Publishing Co. 2000.

Generally, the ingredients of compositions of the invention are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder

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or water free concentrate in a hermetically sealed container such as an ampule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed from an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The compositions of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

Various delivery systems are known and can be used to administer the compositions of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, 1987, *J. Biol. Chem.* 262:4429-4432), etc. Methods of administering a prophylactic or therapeutic amount of the compositions of the invention include, but are not limited to, parenteral administration (*e.g.*, intradermal, intramuscular, intraperitoneal, intravenous and subcutaneous), epidural, and mucosal (*e.g.*, intranasal, inhaled, and oral routes). Preferably, a prophylactic or therapeutic amount of the compositions of the invention is directly injected at the defective, diseased, damaged or ischemic tissue. The composition comprising decellularized extracellular matrix of conditioned body tissue may be administered by any convenient route, for example, by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents, preferably paclitaxel. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical composition of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir.

In another embodiment, the decellularized bone marrow extracellular matrix can be delivered in a controlled release or sustained release system. In one embodiment, a pump may be used to achieve controlled or sustained release (see Langer, 1990, *Science* 249:1527-1533; Sefton, 1987, *CRC Crit. Ref. Biomed. Eng.* 14:20; Buchwald *et al.*, 1980, *Surgery* 88:507; Saudek *et al.*, 1989, *N. Engl. J. Med.* 321:574). Any technique known to one of skill in the art can be used to produce sustained release formulations comprising the

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decellularized extracellular matrix of the invention. See, *e.g.*, U.S. Patent No. 4,526,938; International Publication Nos. WO 91/05548 and WO 96/20698; Ning *et al.*, 1996, *Radiotherapy & Oncology* 39:179-189; Song *et al.*, 1995, *PDA Journal of Pharmaceutical Science & Technology* 50:372-397; Cleek *et al.*, 1997, *Pro. Int'l. Symp. Control. Rel. Bioact. Mater.* 24:853-854; and Lam *et al.*, 1997, *Proc. Int'l. Symp. Control Rel. Bioact. Mater.* 24:759-760, each of which is incorporated herein by reference in its entirety.

In another embodiment, polymeric materials can be used to achieve controlled or sustained release of the decellularized extracellular matrix material (see, *e.g.*, *Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, 1983, *J. Macromol. Sci. Rev. Macromol. Chem.* 23:61; see also Levy *et al.*, 1985, *Science* 228:190; During *et al.*, 1989, *Ann. Neurol.* 25:351; Howard *et al.*, 1989, *J. Neurosurg.* 71:105); U.S. Patent Nos. 5,679,377; 5,916,597; 5,912,015; 5,989,463; and 5,128,326; International Publication Nos. WO 99/15154 and WO 99/20253). Examples of polymers used in sustained release formulations include, but are not limited to, poly(2-hydroxy ethyl methacrylate), poly(methyl methacrylate), poly(acrylic acid), poly(ethylene-co-vinyl acetate), poly(methacrylic acid), polyglycolides (PLG), polyanhydrides, poly(N-vinyl pyrrolidone), poly(vinyl alcohol), polyacrylamide, poly(ethylene glycol), polylactides (PLA), poly(lactide-co-glucosides) (PLGA), and polyorthoesters. In a preferred embodiment, the polymer used in a sustained release formulation is inert, free of leachable impurities, stable during storage, sterile, and biodegradable. In yet another embodiment, a controlled or sustained release system can be placed in proximity to the target, thus requiring only a fraction of the systemic dose (see, *e.g.*, Goodson, 1984, in *Medical Applications of Controlled Release, supra*, vol. 2, pp. 115-138).

The amount of the pharmaceutical composition which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays and animal models may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

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5.2.2 Medical Devices

The decellularized bone marrow extracellular matrix of the invention may also be used to form a medical or prosthetic device, preferably a stent or an artificial heart, which may be implanted in the subject. More specifically, the decellularized bone marrow extracellular matrix of the invention may be incorporated into the base material needed to make the device. For example, in stent comprising a sidewall of elongated members or wire-like elements, the decellularized bone marrow extracellular matrix can be used to form the elongated members or wire-like elements. On the other hand, the decellularized bone marrow extracellular matrix can be used to coat or cover the medical device.

The medical devices of the present invention may be inserted or implanted into the body of a patient.

5.2.2.1 Types Of Medical Device

Medical devices that are useful in the present invention can be made of any biocompatible material suitable for medical devices in general which include without limitation natural polymers, synthetic polymers, ceramics and metallics. Metallic material is more preferable. Suitable metallic materials include metals and alloys based on titanium (such as nitinol, nickel titanium alloys, thermo-memory alloy materials), stainless steel, tantalum, nickel-chrome, or certain cobalt alloys including cobalt-chromium-nickel alloys such as Elgiloy® and Phynox®. Metallic materials also include clad composite filaments, such as those disclosed in WO 94/16646.

Metallic materials may be made into elongated members or wire-like elements and then woven to form a network of metal mesh. Polymer filaments may also be used together with the metallic elongated members or wire-like elements to form a network mesh. If the network is made of metal, the intersection may be welded, twisted, bent, glued, tied (with suture), heat sealed to one another; or connected in any manner known in the art.

The polymer(s) useful for forming the medical device should be ones that are biocompatible and avoid irritation to body tissue. They can be either biostable or bioabsorbable. Suitable polymeric materials include without limitation polyurethane and its copolymers, silicone and its copolymers, ethylene vinyl-acetate, polyethylene terephtalate, thermoplastic elastomers, polyvinyl chloride, polyolefins, cellulosics, polyamides, polyesters, polysulfones, polytetrafluorethylenes, polycarbonates, acrylonitrile butadiene styrene copolymers, acrylics, polylactic acid, polyglycolic acid, polycaprolactone, polylactic acid-polyethylene oxide copolymers, cellulose, collagens, and chitins.

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Other polymers that are useful as materials for medical devices include without limitation dacron polyester, poly(ethylene terephthalate), polycarbonate, polymethylmethacrylate, polypropylene, polyalkylene oxalates, polyvinylchloride, polyurethanes, polysiloxanes, nylons, poly(dimethyl siloxane), polycyanoacrylates, polyphosphazenes, poly(amino acids), ethylene glycol I dimethacrylate, poly(methyl methacrylate), poly(2-hydroxyethyl methacrylate), polytetrafluoroethylene poly(HEMA), polyhydroxyalkanoates, polytetrafluorethylene, polycarbonate, poly(glycolide-lactide) co-polymer, polylactic acid, poly(ε-caprolactone), poly(β-hydroxybutyrate), polydioxanone, poly(γ-ethyl glutamate), polyiminocarbonates, poly(ortho ester), polyanhydrides, alginate, dextran, chitin, cotton, polyglycolic acid, polyurethane, or derivatized versions thereof, i.e., polymers which have been modified to include, for example, attachment sites or cross-linking groups, *e.g.*, RGD, in which the polymers retain their structural integrity while allowing for attachment of cells and molecules, such as proteins, nucleic acids, and the like.

Furthermore, although the invention can be practiced by using a single type of polymer to form the medical device, various combinations of polymers can be employed. The appropriate mixture of polymers can be coordinated to produce desired effects when incorporated into a medical device. In certain preferred embodiments, the decellularized extracellular matrix is mixed with a polymer.

The decellularized bone marrow extracellular matrix of the invention may also be used alone or in combination with a polymer described above to form the medical device such as through formation of a composite material. The decellularized bone marrow extracellular matrix may be dried to increase its mechanical strength. The dried decellularized bone marrow extracellular matrix may then be used as the base material to form a whole or part of the medical device. In preferred embodiments, the decellularized bone marrow extracellular matrix constitutes at least 5%, at least 10%, at least 25%, at least 50%, at least 80%, at least 90%, at least 95%, at least 99% by weight or by size of the medical device.

Examples of the medical devices suitable for the present invention include, but are not limited to, stents, surgical staples, catheters (*e.g.*, central venous catheters and arterial catheters), guidewires, cannulas, cardiac pacemaker leads or lead tips, cardiac defibrillator leads or lead tips, implantable vascular access ports, blood storage bags, blood tubing, vascular or other grafts, intra-aortic balloon pumps, heart valves, cardiovascular sutures, total artificial hearts and ventricular assist pumps, and extra-corporeal devices such

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as blood oxygenators, blood filters, hemodialysis units, hemoperfusion units and plasmapheresis units.

Medical devices of the present invention include those that have a tubular or cylindrical-like portion. The tubular portion of the medical device need not to be completely cylindrical. For instance, the cross-section of the tubular portion can be any shape, such as rectangle, a triangle, etc., not just a circle. Such devices include, without limitation, stents and grafts. A bifurcated stent is also included among the medical devices which can be fabricated by the method of the present invention.

Medical devices which are particularly suitable for the present invention include any kind of stent for medical purposes which is known to the skilled artisan. Suitable stents include, for example, vascular stents such as self-expanding stents and balloon expandable stents. Examples of self-expanding stents useful in the present invention are illustrated in U.S. Patent Nos. 4,655,771 and 4,954,126 issued to Wallsten and 5,061,275 issued to Wallsten *et al.* Examples of appropriate balloon-expandable stents are shown in U.S. Patent No. 5,449,373 issued to Pinchasik *et al.*

5.2.2.2 Methods Of Coating The Medical Device

In the present invention, the decellularized bone marrow extracellular matrix of the invention, preferably in combination with a biologically active material such as paclitaxel, can be applied by any method to a surface of a medical device to form a coating. Examples of suitable methods are spraying, brushing, swabbing, dipping, rolling, electrostatic deposition and all modern chemical ways of immobilization of bio-molecules to surfaces. Preferably, the decellularized bone marrow extracellular matrix is applied to a surface of a medical device by spraying and rolling. In one embodiment of the present invention, more than one coating method can be used to make a medical device. In certain embodiments, the decellularized extracellular matrix is placed into a carrier in order to apply it to the device surface. Non-limiting examples of carriers include SIBS, PLGA, PGA, collagen, etc.

Furthermore, before applying the coating composition, the surface of the medical device is optionally subjected to a pre-treatment, such as roughening, oxidizing, sputtering, plasma-deposition or priming in embodiments where the surface to be coated does not comprise depressions. Sputtering is a deposition of atoms on the surface by removing the atom from the cathode by positive ion bombardment through a gas discharge. Also, exposing the surface of the device to a primer is a possible method of pre-treatment.

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Coating compositions suitable for applying coating materials to the devices of the present invention can include a polymeric material and preferably a biologically active material dispersed or dissolved in a solvent suitable for the medical device, which are known to the skilled artisan. The solvents used to prepare coating compositions include ones which can dissolve the polymeric material into solution or suspend the polymeric material and do not alter or adversely impact the therapeutic properties of the biologically active material employed. For example, useful solvents for silicone include tetrahydrofuran (THF), chloroform, toluene, acetone, isooctane, 1,1,1-trichloroethane, dichloromethane, and mixture thereof.

The polymeric material should be a material that is biocompatible and avoids irritation to body tissue. Preferably the polymeric materials used in the coating composition of the present invention are selected from the following: polyurethanes, silicones (*e.g.*, polysiloxanes and substituted polysiloxanes), and polyesters. Also preferable as a polymeric material is styrene-isobutylene-styrene (SIBS). Other polymers which can be used include ones that can be dissolved and cured or polymerized on the medical device or polymers having relatively low melting points that can be blended with biologically active materials. Additional suitable polymers include, thermoplastic elastomers in general, polyolefins, polyisobutylene, ethylene-alphaolefin copolymers, acrylic polymers and copolymers, vinyl halide polymers and copolymers such as polyvinyl chloride, polyvinyl ethers such as polyvinyl methyl ether, polyvinylidene halides such as polyvinylidene fluoride and polyvinylidene chloride, polyacrylonitrile, polyvinyl ketones, polyvinyl aromatics such as polystyrene, polyvinyl esters such as polyvinyl acetate, copolymers of vinyl monomers, copolymers of vinyl monomers and olefins such as ethylene-methyl methacrylate copolymers, acrylonitrile-styrene copolymers, ABS (acrylonitrile-butadiene-styrene) resins, ethylene-vinyl acetate copolymers, polyamides such as Nylon 66 and polycaprolactone, alkyd resins, polycarbonates, polyoxymethylenes, polyimides, polyethers, epoxy resins, rayon-triacetate, cellulose, cellulose acetate, cellulose butyrate, cellulose acetate butyrate, cellophane, cellulose nitrate, cellulose propionate, cellulose ethers, carboxymethyl cellulose, collagens, chitins, polylactic acid, polyglycolic acid, polylactic acid-polyethylene oxide copolymers, EPDM (etylene-propylene-diene) rubbers, fluorosilicones, polyethylene glycol, polysaccharides, phospholipids, and combinations of the foregoing.

More preferably for medical devices which undergo mechanical challenges, *e.g.* expansion and contraction, the polymeric materials should be selected from elastomeric polymers such as silicones (*e.g.* polysiloxanes and substituted polysiloxanes), polyurethanes,

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thermoplastic elastomers, ethylene vinyl acetate copolymers, polyolefin elastomers, and EPDM rubbers. Because of the elastic nature of these polymers, the coating composition is capable of undergoing deformation under the yield point when the device is subjected to forces, stress or mechanical challenge.

The term "biologically active material" encompasses therapeutic agents, such as drugs, and also genetic materials and biological materials. The genetic materials mean DNA or RNA, including, without limitation, of DNA/RNA encoding a useful protein stated below, intended to be inserted into a human body including viral vectors and non-viral vectors. The biological materials include cells, yeasts, bacteria, proteins, peptides, cytokines and hormones. Examples for peptides and proteins include vascular endothelial growth factor (VEGF), transforming growth factor (TGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), cartilage growth factor (CGF), nerve growth factor (NGF), keratinocyte growth factor (KGF), skeletal growth factor (SGF), osteoblast-derived growth factor (BDGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), cytokine growth factor (CGF), stem cell factor (SCF), platelet-derived growth factor (PDGF), endothelial cell growth supplement (ECGS), granulocyte macrophage colony stimulating factor (GM-CSF), growth differentiation factor (GDF), integrin modulating factor (IMF), calmodulin (CaM), thymidine kinase (TK), tumor necrosis factor (TNF), growth hormone (GH), bone morphogenic protein (BMP) (*e.g.*, BMP-2, BMP-3, BMP-4, BMP-5, BMP-6 (Vgr-1), BMP-7 (PO-1), BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-14, BMP-15, BMP-16, etc.), matrix metalloproteinase (MMP), tissue inhibitor matrix metalloproteinase (TIMP), cytokines, interleukin (*e.g.*, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-15, etc.), lymphokines, interferon, integrin, collagen, elastin, fibrillins, fibronectin, vitronectin, laminin, glycosaminoglycans, proteoglycans, transferrin, cytotactin, and tenascin. Currently preferred BMP's are BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7. These dimeric proteins can be provided as homodimers, heterodimers, or combinations thereof, alone or together with other molecules. Cells can be of human origin (autologous or allogeneic) or from an animal source (xenogeneic), genetically engineered, if desired, to deliver proteins of interest at the transplant site. The delivery media can be formulated as needed to maintain cell function and viability. Cells include progenitor cells (*e.g.*, endothelial progenitor cells), stem cells (*e.g.*, mesenchymal, hematopoietic, neuronal), stromal cells, parenchymal cells, undifferentiated cells, fibroblasts, macrophage, and satellite cells. Biologically active materials also include non-genetic therapeutic agents, such as:

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- anti-thrombogenic agents such as heparin, heparin derivatives, urokinase, and PPack (dextrophenylalanine proline arginine chloromethylketone);
- anti-proliferative agents such as enoxaprin, angiopeptin, or monoclonal antibodies capable of blocking smooth muscle cell proliferation, hirudin, and acetylsalicylic acid, amlodipine and doxazosin;
- anti-inflammatory agents such as glucocorticoids, betamethasone, dexamethasone, prednisolone, corticosterone, budesonide, estrogen, sulfasalazine, and mesalamine;
- antineoplastic/antiproliferative/anti-miotic agents such as paclitaxel, 5-fluorouracil, cisplatin, vinblastine, cladribine, vincristine, epothilones, methotrexate, azathioprine, adriamycin and mutamycin; endostatin, angiostatin and thymidine kinase inhibitors, taxol and its analogs or derivatives;
- anesthetic agents such as lidocaine, bupivacaine, and ropivacaine;
- anti-coagulants such as D-Phe-Pro-Arg chloromethyl keton, an RGD peptide-containing compound, heparin, antithrombin compounds, platelet receptor antagonists, anti-thrombin antibodies, anti-platelet receptor antibodies, aspirin (aspirin is also classified as an analgesic, antipyretic and anti-inflammatory drug), dipyridamole, protamine, hirudin, prostaglandin inhibitors, platelet inhibitors and tick antiplatelet peptides;
- DNA demethylating drugs such as 5-azacytidine, which is also categorized as a RNA or DNA metabolite that inhibit cell growth and induce apoptosis in certain cancer cells;
- vascular cell growth promoters such as growth factors, vascular endothelial growth factors (VEGF, all types including VEGF-2), growth factor receptors, transcriptional activators, and translational promoters;
- vascular cell growth inhibitors such as antiproliferative agents, growth factor inhibitors, growth factor receptor antagonists, transcriptional repressors, translational repressors, replication inhibitors, inhibitory antibodies, antibodies directed against growth factors, bifunctional molecules consisting of a growth factor and a cytotoxin, bifunctional molecules consisting of an antibody and a cytotoxin;
- cholesterol-lowering agents; vasodilating agents; and agents which interfere with endogenous vasoactive mechanisms;
- anti-oxidants, such as probucol;
- antibiotic agents, such as penicillin, cefoxitin, oxacillin, tobranycin, rapamycin;

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- angiogenic substances, such as acidic and basic fibroblast growth factors, estrogen including estradiol (E2), estriol (E3) and 17-Beta Estradiol;
- drugs for heart failure, such as digoxin, beta-blockers, angiotensin-converting enzyme (ACE) inhibitors including captopril, enalopril, and statins and related compounds; and

In certain embodiments, the medical device of the present invention is covered with one coating layer. In certain other embodiments, the medical device of the present invention is covered with more than one coating layer. In preferred embodiments, the medical device is covered with different coating layers. For example, the coating can comprise a first layer and a second layer that contain different biologically active materials. Alternatively, the first layer and the second layer may contain an identical biologically active material having different concentrations. In one embodiment, either the first layer or the second layer may be free of biologically active material.

5.2.3 Tissue Regeneration Scaffold

One aspect of the invention provides for use alone or through the incorporation of the decellularized bone marrow extracellular matrix into a biocompatible material for implantation into a subject, preferably human. In a preferred embodiment, the biocompatible material is in the form of a scaffold.

The scaffold may be of decellularized bone marrow ECM or combined with natural collagen or synthetic polymer. These scaffolds may be produced by freeze-drying, air-drying, molding or salt leaching. In certain preferred embodiments, the scaffold serves as a template for cell proliferation and ultimately tissue formation. In a specific embodiment, the scaffold allows the slow release of the decellularized bone marrow extracellular matrix into the surrounding tissue. As the cells in the surrounding tissue begin to multiply, they fill up the scaffold and grow into three-dimensional tissue. Blood vessels then attach themselves to the newly grown tissue, the scaffold dissolves, and the newly grown tissue eventually blends in with its surrounding.

5.2.4 Cell Culture Substrate

Certain cell types require a substrate other than culture plastic to grow and retain proper function outside their native environment (*e.g.*, embryonic stem cells, some neural cells). Surfaces coated with extracellular matrix proteins (*e.g.*, laminin, fibronectin, collagen, Matrigel) have been shown to enhance the growth of certain cell types compared to

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the uncoated surface. Also, cells grown on top of or within three-dimensional gels or scaffolds grow and function differently than those on two-dimensional coated or uncoated surfaces.

In certain embodiments, the decellularized bone marrow extracellular matrix of the invention is used for cell culture. In one embodiment, some or all of the decellularized bone marrow extracellular matrix can be solubilized in an acidic solution, preferably 0.05 to 0.5 M acetic acid, with or without an enzyme which breaks apart the collagen molecule (*e.g.*, pepsin, trypsin, dispase, collagenase). The resulting solution containing preferably 0.5 to 15 mg/ml of decellularized bone marrow extracellular matrix will gel when the pH is neutralized (temperature preferably between 25°C to 40°C). During the gelation process the solution can be added to a container to coat the exposed surfaces, seeded with cells to form a gel containing cells, or cast into sheet or slabs.

In another embodiment, a suspension of decellularized bone marrow extracellular matrix or a solution, as described above, can be dried to form a sheet of ECM or adsorbed onto a surface. The thickness will be determined by the concentration of ECM in the liquid and the volume of liquid per surface area of the container in which it is held. A glass or non-stick (*e.g.*, Teflon, silicone) container is preferred to enable the removal of the sheet without destroying its integrity. Although not necessary, the sheet can be cross-linked prior to or after removal from the container in which it was dried. Examples of cross-linking treatments include but are not limited to UV light or other high frequency photon source, aldehydes, carbodiimides, and severe dehydration. Cells may be seeded directly onto the sterilized coated containers or onto the sheets which have been removed.

In yet another embodiment, a suspension of decellularized bone marrow extracellular matrix or a solution, as described above, can be freeze-dried within a container to form a porous coating or layer on the surface of a culture container. The ratio of freezing and concentration of ECM in the liquid will determine the pore structure and pore volume fraction. Altering these properties will allow control of those parameters. Preferably, the freezing temperature will be between -10°C and -196°C.

6. EXAMPLES

6.1 EXAMPLE 1

First, a bone marrow sample is isolated from a pig or cow. Second, the bone marrow sample is filtered to remove bone spicules. Third, the remaining bone marrow

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sample is centrifuged in saline to remove the fat. Fourth, a non-ionic detergent is added to the remaining bone marrow sample to solubilize the cell membranes and any remaining fat. The remaining bone marrow sample is then rinsed with saline and filtered to remove the remaining extracellular matrix components. The rinsing step is repeated at least one time. Further, the remaining bone marrow sample is further treated with DNase and RNase to breakdown remaining nucleic acids and rinsed with saline to remove the remaining extracellular matrix components. Finally, the resulting extracellular matrix is sterilized using standard techniques in the art, *e.g.*, peracetic acid (0.1%).

6.2 EXAMPLE 2

First, a bone marrow sample is removed from the bone marrow cavity and the cortical bone by cutting, scooping, chiseling or sawing. The marrow is then reduced to small pieces by cutting, grinding, chopping, shearing, or crushing. The blood components are removed by rinsing, one or more times, in buffered saline or water. Next, the bone is separated from the ECM and any other remaining components by vigorous agitation (*e.g.*, vortexing, shaking). After allowing the bone fragments to settle to the bottom, the non-bone components are removed by carefully decanting or aspirating (*e.g.*, pipet). This process is repeated by adding more saline or water to the remaining bone. The non-bone components are treated with a combination of non-ionic detergent, acidic solution, alcohol solution, and basic solution to solubilize non-ECM components. Alternatively, the non-blood components are treated with an acidic or enzymatic solution (0.5 M acetic acid with pepsin 1 mg/ml). The solubilized ECM components are then separated by decanted following centrifugation and filtering with a 20 to 100 micron filter. The filtered solids will contain some of the bone marrow ECM. This ECM is processed further for use by lyophilization, precipitation, or gelation.

6.3 EXAMPLE 3

6.3.1 Isolation Procedure

Ribs and sternum from a freshly killed pig are collected and the cortical bone is removed. Large pieces of bone marrow are obtained by piercing, cutting and prying at the cortical bone-bone marrow interface with a sharp, stout knife. The large pieces of bone marrow are subsequently reduced in size to less than 5 mm on a side by cutting, crushing or shearing. Blood which remains in these pieces is rinsed away by multiple rinses using a buffered saline with protease inhibitor Protease-Arrest™ (Genotech, St. Louis, MO). For

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each rinse, the marrow is first placed in the solution and slowly agitated for 30 minutes. The rinsing solution is then removed from the bone marrow pieces with a pipet. Following the final rinse, more buffered saline with protease inhibitor Protease-Arrest™ is added to the bone marrow piece. The marrow is separated from the bone pieces by vigorous agitation with a standard lab vortex mixer. The resulting suspension of marrow is transferred to another container using a pipet. This process is repeated several times until the bone is substantially free of marrow.

Finally, the marrow suspension is centrifuged at about 3,000 g for 15 minutes and the supernatant is removed. Distilled water with protease inhibitor Protease-Arrest™ is added to the pellet and gently agitated to lyse and remove the cells. This suspension is filtered using a 40 micron filter. The filtered solid is collected and processed for use. Optionally, the distilled water rinse and subsequent filtering step can be repeated to remove any remaining cells or cell debris. Aseptic conditions are maintained throughout the isolation procedure.

6.3.2 Production of a Freeze-Dried Porous Scaffold

0.05 M acetic acid is added to the solid material obtained from the isolation procedure discussed above to yield 1 to 6 mg of bone marrow ECM per ml. The mixture is vortexed or homogenized until a suspension is formed. The suspension is transferred to a plastic or metal mold and placed in a -70°C freezer for 4 hours or until frozen. Afterwards, the mold is transferred to a freeze drier and pull a vacuum of < 100 mTorr. The vacuum is held at a temperature between 0°C and 25°C for 12 hours or until all liquid has sublimated. Optionally, the sample can be cross-linked by holding a vacuum of < 300 mTorr at 110°C for 24 hours. Prior to use, the scaffold formed in the mold is rehydrated by placing the scaffold in sterile saline at room temperature for 12 hours. Preferably, prior to rehydration, the scaffold is sterilized by first placing in 1% peracetic acid and 70% ethanol for 12 hours, and then rinsing three times in sterile saline prior to implantation.

6.4 EXAMPLE 4

6.4.1 Isolation Procedure

Ribs and sternum from a freshly killed pig were collected and the cortical bone was removed. Large pieces of bone marrow were obtained by piercing, cutting, and prying at the cortical bone-bone marrow interface with a sharp, stout knife. The large pieces of bone marrow were subsequently reduced in size to less than 5 mm on a side by cutting,

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crushing or shearing. The bone marrow pieces were washed in a buffered saline with protease inhibitor Protease-Arrest™. A DNase solution (10 mg/ml DNase solution (Sigma), 5 mM $MgCl_2 \cdot 6H_2O$, and 0.5 mM $CaCl_2$) was added to cover the bone marrow pieces. The suspension was gently shaken at 37°C for 50 minutes. Afterwards, the DNase solution was removed and buffered saline was added. The suspension was shaken at 4°C for 10 minutes.

The solution was removed and more buffered saline was added. The suspension was vortexed until all fibrous material was removed from the bone marrow pieces. A first supernatant was collected. More buffered saline was added to rinse the bone marrow pieces and a second supernatant was collected.

The first and second supernatant were pooled together and centrifuged at 10,000 RPM for 7 minutes. The pellet was resuspended in 1 ml DNase solution and shaken at 37°C for 20 minutes. After centrifuging the suspension at 10,000 RPM for 7 minutes, the supernatant was discarded. Buffered saline was added and the suspension was shaken at 4°C for 20 minutes. After centrifuging the suspension at 10,000 RPM for 7 minutes, the supernatant was again discarded. The steps of adding the buffered saline, shaking the suspension at 4°C for 20 minutes, centrifuging at 10,000 RPM for 7 minutes, and discarding the supernatant were repeated twice. The suspension was centrifuged at 10,000 RPM for 10 minutes to collect the pellet.

6.4.2 Experimental Design

The PicoGreen® dsDNA quantification assay (Turner BioSystems) was used to determine the DNA content of untreated bone marrow samples in the control group and the DNase-treated bone marrow samples in the experimental group.

The Sircol™ COLLAGEN assay (Biocolor Ltd.) was used to determine the pepsin/acetic acid soluble collagen content of untreated bone marrow samples in the control group and the DNase-treated bone marrow samples in the experimental group

6.4.3 Data

As shown in Figure 1, the average (n = 5) DNA content of DNase treated samples was 4 times lower ($p < 0.005$) than non-DNase-treated samples and approaches the lower detection limit for the assay. In contrast, the average (n = 5) collagen content of DNase treated samples was 1.5 times higher than non-DNase-treated samples, although the difference was not significant ($p = 0.18$).

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Together, the data suggests that the isolation method described *supra* (Section 6.4.1) retains more collagen and causes the DNA in the bone marrow sample to be washed away or fragmented. Therefore, this isolation method lowers the overall amount of cells or cell debris in the remaining collagen or extracellular matrix.

6.4.4 Production of an Air-Dried Sheet

0.05 acetic acid is added to the solid material obtained from the isolation procedure discussed above to yield 1 to 6 mg of marrow ECM per ml. The mixture is vortexted for 5 minutes, ultrasonicated for 2 minutes, or homogenized for less than 1 minute until a suspension is formed. The suspension is transferred to a Teflon, silicone, or glass mold (1 mg/ml suspension at 1 ml/10 cm^2). Afterwards, the mold is transferred to a dryer and dried under a clean, sterile hood at room temperature for 72 hours. Optionally, the sample can be cross-linked by holding a vacuum of < 300 mTorr at 110°C for 24 hours. Prior to use, the scaffold formed in the mold is rehydrated by placing the scaffold in sterile saline at room temperature for 12 hours. Preferably, prior to rehydration, the scaffold is sterilized by first placing in 1% peracetic acid and 70% ethanol for 12 hours, and then rinsing three times in sterile saline prior to implantation.

The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein, will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference.

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What is claimed is:

1. A composition comprising decellularized bone marrow extracellular matrix, wherein the bone marrow extracellular matrix has been produced *in vivo* in an animal.

2. The composition of claim 1, wherein the animal is a mammal.

3. The composition of claim 2, wherein the mammal is selected from the group consisting of cow, pig, horse, chicken, cat, dog, rat, monkey, and human.

4. The composition of claim 3, wherein the human is an adult, adolescent, neonate or fetus.

5. The composition of claim 1, wherein the bone marrow extracellular matrix is arranged in a structure and wherein the structure is maintained after the bone marrow extracellular matrix is decellularized.

6. The composition of claim 1 further comprising a biological material.

7. The composition of claim 6, wherein the biological material is selected from the group consisting of erythropoietin, stem cell factor (SCF), vascular endothelial growth factor (VEGF), transforming growth factor (TGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), cartilage growth factor (CGF), nerve growth factor (NGF), keratinocyte growth factor (KGF), skeletal growth factor (SGF), osteoblast-derived growth factor (BDGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), cytokine growth factor (CGF), stem cell factor (SCF), platelet-derived growth factor (PDGF), endothelial cell growth supplement (ECGS), colony stimulating factor (CSF), growth differentiation factor (GDF), integrin modulating factor (IMF), calmodulin (CaM), thymidine kinase (TK), tumor necrosis factor (TNF), growth hormone (GH), bone morphogenic proteins (BMP), matrix metalloproteinase (MMP), tissue inhibitor matrix metalloproteinase (TIMP), interferon, interleukins, cytokines, integrin, collagen, elastin, fibrillins, fibronectin, laminin, glycosaminoglycans, vitronectin, proteoglycans, transferrin, cytotactin, tenascin, and lymphokines.

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8. A method for treating a defective, diseased, damaged or ischemic tissue or organ in a subject comprising implanting the composition of claim 1 into the subject.

9. A method for treating a defective, diseased, damaged or ischemic tissue or organ in a subject comprising injecting the composition of claim 1 into the subject.

10. A method for augmenting or reconstructing a tissue or organ in a subject comprising implanting the composition of claim 1 into the subject.

11. A method for augmenting or reconstructing a tissue or organ in a subject comprising injecting the composition of claim 1 into the subject.

12. A composition comprising decellularized bone marrow extracellular matrix, wherein the bone marrow extracellular matrix has been produced *in vivo* in an animal and is arranged in a structure, wherein the structure is maintained after the bone marrow extracellular matrix is decellularized.

13. A composition comprising decellularized bone marrow extracellular matrix, wherein said decellularized bone marrow extracellular matrix is produced by a method comprising the steps of:

(a) obtaining from an animal a bone marrow sample that has been produced *in vivo* in the animal, wherein the bone marrow sample comprises an extracellular matrix and a non-extracellular matrix component;

(b) processing the bone marrow sample to remove at least some of the non-extracellular matrix component to obtain decellularized bone marrow extracellular matrix; and

(c) sterilizing the decellularized extracellular matrix.

14. The composition of claim 13, wherein the non-extracellular matrix component comprises cells, cell components, antigens, cytokines, blood, bone spicules, serum, and fat.

15. The composition of claim 13, wherein at least 50% of the non-extracellular matrix component is removed.

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16. The composition of claim 13, wherein at least 80% of the non-extracellular matrix component is removed.

17. The composition of claim 13, wherein at least 95% of the non-extracellular matrix component is removed.

18. The composition of claim 13, wherein the animal is a mammal.

19. The composition of claim 18, wherein the mammal is selected from the group consisting of cow, pig, horse, chicken, cat, dog, rat, monkey, and human.

20. The composition of claim 19, wherein the human is an adult, adolescent, neonate or fetus.

21. The composition of claim 13, wherein the bone marrow extracellular matrix is arranged in a structure and wherein the structure is maintained after the bone marrow extracellular matrix is decellularized.

22. The composition of claim 13 further comprising a biological material.

23. The composition of claim 22, wherein the biological material is selected from the group consisting of erythropoietin, stem cell factor (SCF), vascular endothelial growth factor (VEGF), transforming growth factor (TGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), cartilage growth factor (CGF), nerve growth factor (NGF), keratinocyte growth factor (KGF), skeletal growth factor (SGF), osteoblast-derived growth factor (BDGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), cytokine growth factor (CGF), stem cell factor (SCF), platelet-derived growth factor (PDGF), endothelial cell growth supplement (ECGS), colony stimulating factor (CSF), growth differentiation factor (GDF), integrin modulating factor (IMF), calmodulin (CaM), thymidine kinase (TK), tumor necrosis factor (TNF), growth hormone (GH), bone morphogenic proteins (BMP), matrix metalloproteinase (MMP), tissue inhibitor matrix metalloproteinase (TIMP), interferon, interleukins, cytokines, integrin, collagen, elastin, fibrillins, fibronectin, laminin, glycosaminoglycans, hemonectin, thrombospondin, heparan sulfate, dermantan, chondrotin

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sulfate (CS), hyaluronic acid (HA), vitronectin, proteoglycans, transferrin, cytotactin, tenascin, and lymphokines.

24. The composition of claim 13, wherein the method further comprises the step of enzymatically treating the decellularized bone marrow extracellular matrix.

25. The composition of claim 13, wherein the method further comprises suspending the decellularized bone marrow extracellular matrix in a saline solution.

26. A method for treating a defective, diseased, damaged or ischemic tissue or organ in a subject comprising implanting the composition of claim 13 into the subject.

27. A method for treating a defective, diseased, damaged or ischemic tissue or organ in a subject comprising injecting the composition of claim 13 into the subject.

28. A method for augmenting or reconstructing a tissue or organ in a subject comprising implanting the composition of claim 13 into the subject.

29. A method for augmenting or reconstructing a tissue or organ in a subject comprising injecting the composition of claim 13 into the subject.

30. A medical device suitable for insertion or implantation into a patient, wherein the medical device comprises a surface and a composition comprising decellularized bone marrow extracellular matrix, wherein the bone marrow extracellular matrix has been produced *in vivo* in an animal.

31. The medical device of claim 30, wherein the decellularized bone marrow extracellular matrix is coated onto the surface.

32. The medical device of claim 30, wherein the medical device is non-biodegradable.

33. The medical device of claim 30, wherein the medical device is a stent.

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34. The medical device of claim 30, wherein the medical device is an artificial heart.

35. The medical device of claim 30, wherein the composition further comprises a biologically active material.

36. The medical device of claim 35, wherein the biologically active material is paclitaxel.

37. The medical device of claim 30, wherein the decellularized bone marrow extracellular matrix is produced by a method comprising the steps of:

(a) obtaining from a subject a bone marrow sample having an extracellular matrix and non-extracellular matrix component;

(b) processing the bone marrow sample to remove at least some of the non-extracellular matrix component to obtain decellularized bone marrow extracellular matrix; and

(c) sterilizing the decellularized extracellular matrix.

38. The medical device of claim 37, wherein the non-extracellular matrix component comprises cells, cell components, antigens, cytokines, blood, bone spicules, serum, and fat.

39. The medical device of claim 37, wherein at least 50% of the non-extracellular matrix component is removed.

40. The medical device of claim 37, wherein at least 80% of the non-extracellular matrix component is removed.

41. The medical device of claim 37, wherein at least 95% of the non-extracellular matrix component is removed.

42. The medical device of claim 37, wherein the animal is a mammal.

43. The medical device of claim 42, wherein the mammal is selected from the group consisting of cow, pig, horse, chicken, cat, dog, rat, monkey, and human.

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44. The composition of claim 43, wherein the human is an adult, adolescent, neonate or fetus.

45. The medical device of claim 37, wherein the bone marrow extracellular matrix is arranged in a structure and wherein the structure is maintained after the bone marrow extracellular matrix is decellularized.

46. The medical device of claim 37 further comprising a biological material.

47. The medical device of claim 37, wherein the biological material is selected from the group consisting of erythropoietin, stem cell factor (SCF), vascular endothelial growth factor (VEGF), transforming growth factor (TGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), cartilage growth factor (CGF), nerve growth factor (NGF), keratinocyte growth factor (KGF), skeletal growth factor (SGF), osteoblast-derived growth factor (BDGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), cytokine growth factor (CGF), stem cell factor (SCF), platelet-derived growth factor (PDGF), endothelial cell growth supplement (ECGS), colony stimulating factor (CSF), growth differentiation factor (GDF), integrin modulating factor (IMF), calmodulin (CaM), thymidine kinase (TK), tumor necrosis factor (TNF), growth hormone (GH), bone morphogenic proteins (BMP), matrix metalloproteinase (MMP), tissue inhibitor matrix metalloproteinase (TIMP), interferon, interleukins, cytokines, integrin, collagen, elastin, fibrillins, fibronectin, laminin, glycosaminoglycans, hemonectin, thrombospondin, heparan sulfate, dermantan, chondrotin sulfate (CS), hyaluronic acid (HA), vitronectin, proteoglycans, transferrin, cytotactin, tenascin, and lymphokines.

48. The medical device of claim 37, wherein the method further comprises the step of enzymatically treating the decellularized bone marrow extracellular matrix.

49. The medical device of claim 37, wherein the method further comprises suspending the decellularized bone marrow extracellular matrix in a saline solution.

50. A biocompatible material comprising decellularized bone marrow extracellular matrix, wherein the bone marrow extracellular matrix has been produced in *vivo* in an animal.

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51. The biocompatible material of claim 50, wherein the decellularized bone marrow extracellular matrix is produced by a method comprising the steps of:

(a) obtaining from a subject a bone marrow sample having an extracellular matrix and cellular components;

(b) processing the bone marrow sample to remove at least some of the non-extracellular matrix component to obtain decellularized bone marrow extracellular matrix; and

(c) sterilizing the decellularized extracellular matrix.

52. The biocompatible material of claim 51, wherein the non-extracellular matrix component comprises cells, cell components, antigens, cytokines, blood, bone spicules, serum, and fat.

53. The biocompatible material of claim 51, wherein at least 50% of the non-extracellular matrix component is removed.

54. The biocompatible material of claim 51, wherein at least 80% of the non-extracellular matrix component is removed.

55. The biocompatible material of claim 51, wherein at least 95% of the non-extracellular matrix component is removed.

56. The biocompatible material of claim 51, wherein the animal is a mammal.

57. The biocompatible material of claim 56, wherein the mammal is selected from the group consisting of cow, pig, horse, chicken, cat, dog, rat, monkey, and human.

58. The composition of claim 57, wherein the human is an adult, adolescent, neonate or fetus.

59. The biocompatible material of claim 51, wherein the bone marrow extracellular matrix is arranged in a structure and wherein the structure is maintained after the bone marrow extracellular matrix is decellularized.

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60. The biocompatible material of claim 51 further comprising a biological material.

61. The biocompatible material of claim 60, wherein the biological material is selected from the group consisting of erythropoietin, stem cell factor (SCF), vascular endothelial growth factor (VEGF), transforming growth factor (TGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), cartilage growth factor (CGF), nerve growth factor (NGF), keratinocyte growth factor (KGF), skeletal growth factor (SGF), osteoblast-derived growth factor (BDGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), cytokine growth factor (CGF), stem cell factor (SCF), platelet-derived growth factor (PDGF), endothelial cell growth supplement (ECGS), colony stimulating factor (CSF), growth differentiation factor (GDF), integrin modulating factor (IMF), calmodulin (CaM), thymidine kinase (TK), tumor necrosis factor (TNF), growth hormone (GH), bone morphogenic proteins (BMP), matrix metalloproteinase (MMP), tissue inhibitor matrix metalloproteinase (TIMP), interferon, interleukins, cytokines, integrin, collagen, elastin, fibrillins, fibronectin, laminin, glycosaminoglycans, hemonectin, thrombospondin, heparan sulfate, dermantan, chondrotin sulfate (CS), hyaluronic acid (HA), vitronectin, proteoglycans, transferrin, cytotactin, tenascin, and lymphokines.

62. The biocompatible material of claim 51, wherein the method further comprises the step of enzymatically treating the decellularized bone marrow extracellular matrix.

63. The biocompatible material of claim 51, wherein the method further comprises suspending the decellularized bone marrow extracellular matrix in a saline solution.

64. The biocompatible material of claim 51, wherein the biocompatible material is in the form of a scaffold.

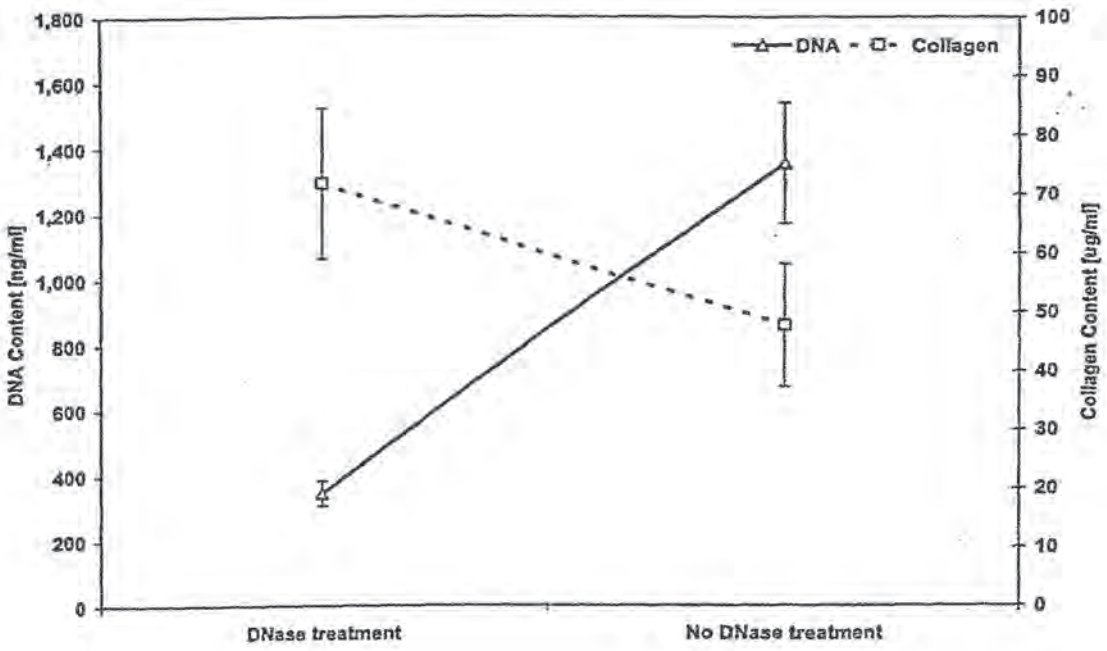
65. The biocompatible material of claim 51, wherein the biocompatible material is suitable for implantation into a patient.

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FIGURE 1



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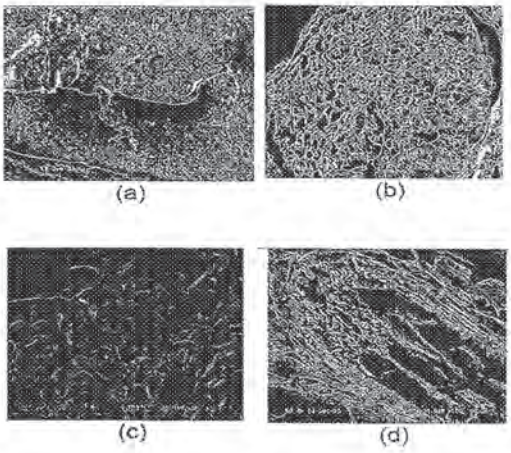
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(54) **Title:** STRUCTURALLY MODIFIED ACELLULAR TISSUE ENGINEERING SCAFFOLDS AND METHODS OF PRODUCTION



(57) **Abstract:** Methods are provided for producing a bioscaffold from natural tissues by oxidizing a decellularized tissue to produce a bioscaffold having pores therein. The pore size and porosity is increased to better accommodate intact cells so that live cells can better infiltrate and inhabit the bioscaffold. The bioscaffold may be freeze-dried or lyophilized, sterilized and (optionally) aseptically packaged for subsequent use. A further aspect of the present invention is a bioscaffold produced by the processes described herein. Methods of treatment using the bioscaffold as a graft or as a biomedical implant for implantation are also provided. Also provided are methods of seeding a bioscaffold with mammalian cells, wherein the seeding carried out either in vitro or in vivo, and wherein a bioscaffold produced as described herein is utilized for said seeding.

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STRUCTURALLY MODIFIED ACELLULAR TISSUE ENGINEERING SCAFFOLDS AND METHODS OF PRODUCTION

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Related Applications

This application claims the benefit under 35 U.S.C. § 119(e) of United States Provisional Patent Application Serial Number 60/794,037, filed April 21, 2006, the disclosure of which is incorporated herein by reference in its entirety.

Field of the Invention

The present invention concerns tissue engineering scaffolds and methods of making the same.

Background of the Invention

Bioscaffold engineering has the potential to vastly improve the treatment of tissue injuries, especially those associated with tumor, trauma, and congenital deficiencies where autograft tissue (i.e., tissue harvested from the recipient patient) or allograft tissue (i.e., tissue harvested from a different subject of the same species) might not be available in sufficient quantity for reconstruction. Current tissue-engineering strategies have relied on scaffolds derived from both synthetic (e.g., polyglycolic acid) and naturally-derived (e.g., collagen) materials to form the cell-scaffold construct. Currently available tissue scaffold products include small intestine submucosa (Restore™, porcine SIS, DePuy Orthopaedics), (CuffPatch™, porcine SIS, Organogenesis), (SIS; Cook Biotech, Inc.), reformulated collagen scaffolds (3D Collagen Composite, BD Biosciences), acellular human dermal collagen matrices (Graftjacket®, Wright Medical Technologies), fetal bovine dermis (TissueMend®, Stryker), and synthetic polymer scaffolds, primarily polyesters (e.g. PGA, PCL, and PLA).

Synthetic scaffolds can produce breakdown products that have been shown to be antimitotic and cytotoxic *in vivo* (Garvin et al. (2003) *Tissue Eng* 9(5):967-979). Many synthetic and naturally-derived scaffolds also lack the initial mechanical strength to permit immediate motion and rehabilitation after implantation, leading to subsequent adhesion formation, decreased range of motion, and poor functional outcomes (Cao et al. (2002) *Plast Reconstr Surg* 110:1280-1289; Buckwalter (1996) *Hand Clin* 12(1):13-24; Buckwalter (1995) *Iowa Orthop J* 15:29-42). As an example, tissue engineered tendons produced *in vitro* have been shown to be weaker than native adult tendons and would not be expected to

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withstand rehabilitation after implantation (Garvin et al. (2003) *Tissue Eng* 9(5):967-979; Calve et al. (2004) *Tissue Eng* 10(5/6):755-761).

Tendon allografts (i.e., tissue taken from an animal of the same species but not of the same genotype) are useful in ligamentous reconstruction due to their availability and lack of donor site pathology (Poehling et al. (2005) *Arthroscopy* 21(7):774-85; Cole et al. (2005) *Arthroscopy* 21(7):786-90). However, these grafts have a micro-architecture that is quite dense and in which appreciable amounts of cellular debris remain, even in freeze-dried grafts. Experience in the preparation and use of allogeneic and xenogeneic (i.e., tissue taken from an animal of a different species) grafts for tissue regeneration has shown that dense grafts with residual cells are difficult to seed *in vitro*, instigate prolonged inflammatory responses *in vivo*, and require longer times to incorporate into native tissue and remodel. In clinical practice, this phenomenon has been observed using human allograft tissue and has been shown to result in a delay of remodeling and integration of implanted allografts in comparison to autograft tissue (Jackson et al. (1996) *Clin Orthop Rel Res* 324:126-33).

Most current strategies for the development of tissue-engineered tendons and ligaments have relied on a construct consisting of cells seeded onto a scaffold. However, the products of recent *in vivo* and *in vitro* studies have not produced a construct with sufficient biomechanical strength to withstand immediate rehabilitation after implantation. It is widely accepted that controlled early restoration of activity can promote healing of soft-tissue injuries and that the prolonged rest or immobilization of soft-tissue injuries can result in delayed recovery and permanent loss of function (Buckwalter (1996) *Hand Clin* 12(1):13-24; Buckwalter (1995) *Iowa Orthop J* 15:29-42).

Methods for removing cells from tissues to create a porous bioscaffold are documented (*see, e.g.*, Badylak (2002) *Semin Cell Dev Biol* 13(5):377-83; U.S. Patent No. 6,893,666; U.S. Patent No. 6,962,814; U.S. Patent No. 6,893,653; U.S. Patent No. 6,866,686; U.S. Patent No. 6,753,181; U.S. Patent No. 6,933,103). A typical method uses a hypo-osmotic solution such as deionized water to burst the cells, followed by a detergent solution, perhaps in combination with an enzyme for disrupting cell adhesion, to remove the cellular debris. A third step is often extraction with a mild basic solution to remove anionic materials such as DNA. The detergent and basic solution are often combined into a single step for convenience. These methods are effective for removal of cellular material without disrupting the native architecture of the extracellular matrix (ECM) or removing many biologically active molecules that mediate cell attachment and growth.

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However, because the cells are essentially broken into small fragments and washed away, the porosity of these bioscaffolds is not ideal for repopulating with new cells, whether they are provided via *in vitro* seeding or by host cell infiltration. This is particularly true if the bioscaffold originated from a dense organ or tissue such as muscle, tendons, ligaments, nerve, blood vessel, cartilage, etc. In fact, most tissues contain a degree of compartmentalization that creates barriers to cell migration. Consequently, seeding cells uniformly throughout such bioscaffolds is extremely difficult, and in some cases impossible.

In addition, ingrowth of seeded or host cells proceeds via a cell-mediated remodeling process that requires considerable time. When a cell seeding approach is used, the subject cells typically attach only to the outside of the bioscaffold and grow outward, away from the bioscaffold.

There remains a need for an improved bioscaffold that addresses these problems and provides an ideal substrate on which cells can grow.

Summary of the Invention

A first aspect of the invention is a method of producing a bioscaffold by decellularizing a tissue to produce a bioscaffold having pores therein. The method incorporates an additional step of oxidizing the bioscaffold during the decellularizing step with an oxidant (*e.g.*, hydrogen peroxide, peracetic acid), with the oxidant preferably being in an aqueous solution, to remove extraneous extracellular matrix material and increase the average pore size therein (for example, in some embodiments, by increasing the average pore size by at least 20, 30, 40 or 50 percent or more). In preferred embodiments, the decellularizing step also includes a detergent.

The average pore size is increased to better accommodate intact cells so that live cells can better re-inhabit the bioscaffold. In some embodiments, the average size of the pores formed after the oxidizing step is at least 10, 20, or 30 microns, or in some embodiments at least 50 or 100 microns or more, depending upon the particular cells which are eventually desired to seed into and populate the scaffold.

Preferably, the advential layer or capsular layer on the tissue is removed (completely, at least in part, or at least a major portion thereof), prior to the decellularizing step, or between the decellularizing and oxidizing step, or after the oxidizing step, so that cell seeding efficiency is facilitated when cells are seeded (*in vitro* before implantation or *in vivo* after implantation) on the bioscaffold.

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The method preferably includes the step of washing said bioscaffold (*e.g.*, with water, saline solution, buffer solution or the like) after said oxidizing step to remove residual oxidant.

The bioscaffold may be freeze-dried or lyophilized after the oxidizing step, sterilized and (optionally) aseptically packaged for subsequent use.

A further aspect of the present invention is a bioscaffold produced by the processes described herein. The bioscaffold preferably has an average pore size that is at least 20, 30, 50, or 100% greater than the average pore size of said extracellular matrix component of said natural animal tissue prior to said treating; and wherein said natural animal tissue is selected from the group consisting of skin, muscle, tendon, bone, meniscus, cartilage, intervertebral discs, and ligament tissue.

Methods of treatment using the bioscaffold as a graft or as a biomedical implant for implantation are provided. The bioscaffold is grafted or implanted in an appropriate configuration into a subject in need thereof, with the particular manner of grafting or implantation depending upon the particular type of tissue being used and the particular condition being treated. Particular applications of the products of the present invention include cardio-vascular tissue engineering, nerve tissue engineering, musculoskeletal tissue engineering, bone, meniscus and cartilage tissue engineering, 3-D cell culture systems, and bioreactor systems.

A further aspect of the present invention is a method of seeding a bioscaffold with mammalian cells (*e.g.*, human, dog, cat, pig, cow, rat, mouse cells, etc.) said seeding carried out either *in vitro* or *in vivo*, wherein a bioscaffold produced as described herein is utilized for said seeding.

Another aspect of the present invention is the use of a bioscaffold as described herein for the preparation of a graft or transplant for carrying out a method of treatment as described herein.

The present invention is explained in greater detail in the drawings herein and the specification set forth below.

Brief Description of the Drawings

Figure 1. SEM images of acellular nerves in cross section. The untreated nerve (a) is dense with no apparent porosity. Such a bioscaffold is difficult to seed with cells. Host cells also have difficulty infiltrating this structure. Nerves treated with 1% (b) and 5% (c)

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peracetic acid at 37°C for 24 hours show graded levels of porosity. Nerve treated with 10% peracetic acid (d) shows large pores, but has lost mechanical integrity.

Figure 2. DAPI staining of both longitudinal and cross sections show the dramatic reduction in cellular density of chicken tendon treated with an oxidant-detergent solution.

Figure 3. Flexor digitorum profundus (FDP) and the decellularized/oxidized FDP tendon scaffold, scanning electron microscopy (SEM), 50X, cross section. The microarchitecture of the untreated tendon is very dense compared to the tendon following treatment with an oxidant-detergent solution, which is characterized by an increase in pore size and porosity.

Figure 4. DNA Content. Fresh-frozen FDP tendon ("untreated") and decellularized/oxidized FDP tendon scaffold ("treated") dry weight DNA content determined using the DNeasy™ assay. There is a significant decrease in the DNA content of the decellularized/oxidized FDP tendon scaffold (67%, $p<0.05$, μg DNA/mg tissue dry weight +/- SEM, two-tailed, Student's t-test with unequal variances) when compared to fresh-frozen FDP tendon, indicating that nuclear material, and therefore cellular material, has been removed.

Figures 5A-5B. Figure 5A: Metabolic Activity Assay (MTS Assay). Metabolic activity for cells exposed to the decellularized/oxidized FDP tendon scaffold is 79% of that observed for cells exposed to media only (control) and was statistically significant ($p<0.05$, abs at λ=490 nm +/- SEM, two-tailed, Student's t-test with unequal variances). **Figure 5B:** Neutral Red Assay. Cell viability for cells exposed to the decellularized/oxidized FDP tendon scaffold is 88% of that observed for cells exposed to media only (control), which was not statistically significant ($p>0.05$, abs at λ=540 nm +/- SEM, two-tailed, Student's t-test with unequal variances).

Figures 6A-6B. Tensile Testing: The ultimate Tensile Stress at Break (MPa) (**Figure 6A**) and Modulus of Elasticity (MPa) (**Figure 6B**) observed for the decellularized/oxidized FDP tendon scaffold were 76% and 78%, respectively, of the values observed for fresh-frozen FDP tendon and were not significantly weaker ($p>0.05$, MPa +/- SEM, two-tailed, Student's t-test with unequal variances).

Figure 7. Decellularized/oxidized FDP tendon scaffold 3 days after subcutaneous implantation in CD1 mice, H&E, 100X, longitudinal section: (a) Fusiform cells, consistent with fibroblasts, are visible at the periphery as well as within the scaffold matrix. Decellularized/oxidized FDP tendon scaffold 21 days after implantation, H&E, 100X,

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longitudinal section: (b) An increase in the number of fibroblast-like cells within the scaffold matrix is apparent after 21 days *in vivo*. There is no evidence of an inflammatory reaction or capsule formation in either micrograph.

Figures 8A-8B. H&E and DAPI staining of freeze-dried human Achilles tendon allograft. **Figure 8A:** Untreated Achilles tendon allograft, midsubstance, longitudinal section, 100X. Left: H&E staining, Right: DAPI staining. **Figure 8B:** Treated Achilles tendon allograft, midsubstance, longitudinal section, 100X. Left: H&E staining, Right: DAPI staining.

Figure 9. DNA content of the decellularized/oxidized freeze-dried human Achilles tendon allograft-derived scaffolds was significantly decreased by 75% (0.110 +/- 0.02 μg DNA/mg tissue dry weight, n=10) after treatment when compared to untreated freeze-dried human Achilles tendon allografts (0.40 + / - 0.14 μg DNA/mg tissue dry weight, n=10), $p<0.05$

Figures 10A-10B. Transmission electron microscopy of untreated **(Figure 10A)** and treated **(Figure 10B)** human Achilles tendon allograft, 51,000X. Microscopy revealed a decrease in fibril density per unit area after treatment of the freeze-dried human Achilles tendon allograft, which represents an increase on pore size and porosity of the scaffold.

Figure 11. Mitochondrial activity determined using the MTS assay (absorbance at λ=490nm) for NIH 3T3 cells exposed to the decellularized/oxidized freeze-dried human Achilles tendon allograft-derived scaffolds was 95% (1.36 +/- 0.31, n=10) of that observed for cells exposed to media only (1.42 +/- 0.31, n=10) a difference which was not statistically significant ($p>0.05$). Cell viability determined using the Neutral Red assay (absorbance at λ=540nm) for NIH 3T3 cells exposed to the decellularized/oxidized freeze-dried human Achilles tendon allograft-derived scaffolds was 92% (0.24 +/- 0.07, n=10) of that observed for NIH 3T3 cells exposed to media alone (0.22 +/- 0.07, n=10, positive control), a difference which was not statistically significant. The decellularized/oxidized scaffold and positive control (cells only) differed significantly ($p<0.001$) from the values obtained for a known cytotoxic material (latex, negative control, n=10) in both assays. The absorbance observed for the negative control was also <10% of the absorbance observed for positive controls in each assay.

Figure 12. Decellularization of porcine arterial segments. (A) Microscopic image of a fresh porcine carotid artery, and (B) decellularized carotid artery, both stained with H&E.

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Figure 13. The removal of adventitia layer of decellularized porcine carotid arteries. (A) Decellularized porcine carotid artery. (B) De-adventitia decelularized porcine carotid artery.

Figure 14. SEM images demonstrating effect of increasing peracetic acid (PA) concentration on decellularized porcine carotid arteries. (A) 0% PA, (B) 3% PA, (C) 6% PA, and (D) 13% PA.

Figure 15. SEM images comparing architecture of the intact meniscus to the scaffold. The pores of the scaffold are larger and promote cellular ingrowth.

Detailed Description of the Preferred Embodiments

Disclosed herein are structural modifications of bioscaffolds via mechanical processes and/or chemical treatments. An architecturally-optimized scaffold can be created that retains the majority of its original biomechanical integrity. The present scaffold may therefore be able to withstand early motion and rehabilitation better than previous scaffolds, which would allow earlier functional recovery and accelerate healing after implantation.

In preferred embodiments, improved bioscaffolds disclosed herein are: 1) derived from natural tissue and amenable to host-cell mediated remodeling *in vivo*, 2) decellularized to minimize inflammatory potential and host immune response, 3) biocompatible, 4) modified to allow seeding, infiltration, and attachment of the patient's own cells prior to or after implantation, 5) have optimum biomechanical integrity to withstand rehabilitation until complete remodeling has occurred, and 6) possess decreased microbial load and are expected to be sterile after processing. These scaffolds promote rapid remodeling, increased strength, improved healing, and early rehabilitation and return to function after implantation. In addition, the availability of these scaffolds eliminates the donor site morbidity, increased surgical time, and poor function associated with autologous tissue harvest.

As used herein in the description of the invention and the appended claims, the singular forms "a," "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, the terms "about" and "approximately" as used herein when referring to a measurable value such as an amount of a compound, dose, time, temperature, and the like, is meant to encompass variations of 20%, 10%, 5%, 1%, 0.5%, or even 0.1% of the specified amount. Also, as used herein, "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative ("or").

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The disclosures of all United States patent references cited herein are hereby incorporated by reference herein in their entirety.

"Bioscaffolds" or "scaffolds" as used herein refer to a substrate on which cells can grow. In preferred embodiments, the bioscaffolds are derived from natural tissues. These scaffolds are useful in both the medical and research settings. For example, the bioscaffolds disclosed herein may be used in cardio-vascular tissue engineering, nerve tissue engineering, musculoskeletal tissue engineering, etc., and/or may be used in 3-D cell culture systems and bioreactor systems.

"Natural tissues" are tissues that are normally found in an animal without human manipulation. Tissues that may be used to carry out the present invention may be from any suitable animal source, including human, other mammalian (*e.g.*, cat, dog, pig, cow, sheep, horse, monkey), avian (*e.g.*, chicken, turkey, duck, goose, etc.), reptile, amphibian, etc. Tissues may be of any suitable type, including but not limited to: blood vessel (*e.g.*, vein, artery) tendon, ligament, fascia, skeletal muscle, smooth muscle (*e.g.*, bladder), cardiac muscle or heart, small intestine, large intestine, kidney, liver, pancreas, nerve (including peripheral nerve), skin, cartilage, meniscus, intervertebral discs, and bone.

More dense tissues (e.g., tissues that bear/transmit/absorb mechanical load as their primary function such as tendon, ligament, bone, meniscus, and intervertebral discs), such as the human Achilles tendon, which is commonly used for ligament reconstruction, may not decellularize to the same extent as less dense tissues. However, we have shown that the same techniques taught herein have proven successful in such dense tissues.

"Subjects" as used herein are preferably human, but also includes other mammals (*e.g.*, cat, dog, pig, cow, sheep, horse, monkey), birds (*e.g.*, chicken, turkey, duck, goose, etc.), reptiles, amphibians, etc.

"Grafting" as used herein refers to the placement of a biocompatable substrate, such as a bioscaffold, into a subject, with or without prior seeding and/or infiltration of cells. Similarly, "transplanting" refers to the taking of a whole or partial organ from one subject and placing it into another, or from one site to another site of the same subject. Cells can be autogeneic (i.e., from the subject to be treated), isogeneic (i.e., a genetically identical but different subject, e.g., from an identical twin), allogeneic (i.e., from a non-genetically identical member of the same species) and/or xenogeneic (i.e., from a member of a different species). Cells may be obtained from a donor (either living or cadaveric) or derived from an established cell line. To obtain cells from a donor (e.g., a potential recipient of a bioscaffold

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graft), standard biopsy techniques known in the art may be employed. Representative techniques are described, for example, in U.S. Patent No. 6,536,567.

Bioscaffolds of some embodiments can be grafted with or without prior seeding with cells. The bioscaffolds of some embodiments an also be used for tissue regrafting as described in U.S. Patent No. 7,108,721 to Huckle et al. The bioscaffolds of some embodiments may also be used in a bioreactor system, such as that described in U.S. Patent Nos. 6,562,616 to Toner et al.; 5,459,069 to Palsson et al.; and 4,937,196 to Wrasidlo et al.

In some embodiments of the invention, the cells employed have been genetically manipulated by the introduction of exogenous genetic sequences or the inactivation or modification of endogenous sequences. For example, genes may be introduced to cause the cells to make proteins that are otherwise lacking in the host. Production of scarce but desirable proteins (in the context of certain tissues) may be enhanced, e.g., nerve growth factor (NGF) in nerve tissues.

Proteins (such as growth factors) or other additives (such as antibiotics, anti-inflammatories, and modulators of the immune response) may also be added to the cell and/or bioscaffold preparations at any time. Also, various treatments may be applied to enhance adherence of cells to the substrate and/or to each other. Appropriate treatments are described, for example, in U.S. Patent No. 5,613,982. Such treatments include the application of various proteins, e.g., growth factors or extracellular matrix proteins to the bioscaffold substrate or to the growing construct. For example, collagen, elastin, fibronectin, laminin, or proteoglycans may be applied to the bioscaffold. The bioscaffold can be impregnated with growth factors such as nerve growth factor (NGF), aFGF, bFGF, PDGF, TGFβ, VEGF, GDF-5/6/7, BMP-1/2/3/4/5/6/7/13/12/14, IGF-1, etc., or these agents may be provided in the culture medium.

In some embodiments, grafting or transplanting may be carried out in order to repair, reconstruct, regenerate, replace, etc., various tissues, especially in applications where sufficient allograft or autograft tissue may not be available, such as tumor, trauma, infection and congenital/developmental deficiencies and diseases. It should be understood by those skilled in the art that scaffolds derived from one tissue may be used to repair or reconstruct damaged tissue of another type. For example, the tendon bioscaffold can be used for tendon reconstruction, but it is also useful in ligament reconstruction.

In some embodiments, examples of tissues that can be repaired, reconstructed, regenerated, replaced, etc., with the bioscaffolds disclosed herein include, but are not limited

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to, the following (inclusive of all tendons and ligaments associated with, crossing, originating or inserting).

In some embodiments, blood vessel scaffolds may be used for: internal and external carotid arteries, internal/external jugular veins, coronary arteries, pulmonary arteries and veins, splenic artery and vein, hepatic artery and vein, renal artery and vein, all gastrointestinal arteries and veins including the superior mesenteric artery and celiac artery and inferior mesenteric artery, the aorta, internal and external iliac arteries, internal and external iliac veins, deep and superficial femoral arteries and veins, popliteal artery and veins, deep and superficial arteries and veins of the upper extremity and lower extremity. All vessels, veins and arteries of the human body. All of the lymphatics of the human body.

In some embodiments, nerve scaffolds may be used for: spinal cord and nerves of the central nervous system. All nerves of the peripheral nervous system.

In some embodiments, tendon scaffolds (e.g., from flexor digitorum profundus (FDP) or Achilles tendon) may be used for: Hand/wrist: extensor tendons, flexor tendons, carpal ligaments, ligaments of the wrist. Ligaments crossing the wrist joint. Ligaments of the phalanges. All tendons and ligaments associated with or crossing the hand, wrist and phalanges. Forearm: interosseous membrane and all associated tendons and ligaments associated with and crossing the elbow. Any previous application which utilized the palmaris longus tendon autograft or allograft. Elbow: lateral/medial collateral ligaments of the elbow. Extensor, pronator, supinator and flexor tendons. Ligaments crossing the elbow joint. All tendons and ligaments associated with or crossing the shoulder. Shoulder (humerus/acromion/ scapula/clavicle): tendons and ligaments of the shoulder, those connected to the clavicle, acromion, scapula and humerus. Rotator cuff tendons. Biceps tendon, triceps tendon, deltoid tendon. Coracoclavicular ligament. Acromioclavicular ligament. Spine: ligaments and tendons of the spine including the anterior longitudinal ligament, posterior longitudinal ligament, ligamentum flavum, intervertebral discs. Pelvis: ligaments and tendons of the pelvis. Hip: extensor/ flexor/ abductor/ adductor/ external and internal rotator tendons of the hip. All tendon and ligaments associated with and crossing the hip joint. Thigh: flexor and extensor tendons of the thigh including those of the quadriceps and hamstring muscle groups, all tendon and ligament structures associated with the thigh. Knee: medial/lateral collateral ligaments of the knee, posterior cruciate ligament (single-bundle and double-bundle reconstruction), anterior cruciate ligament (single-bundle and double-bundle reconstruction), meniscus, posterior lateral corner structures (popliteus tendon and lateral collateral ligament), posterior medial corner (posterior oblique ligament). Injuries

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associated with knee dislocation. All tendon and ligament structures crossing the knee joint. Patellar tendon and quadriceps tendon. Leg: all tendons and ligaments associated with and crossing the leg and associated with the syndesmosis. Ankle: all tendons and ligaments associated with and crossing the ankle joint including but not limited to the anterior tibiofibular ligament, posterior tibiofibular ligament, deltoid ligament, calcaneofibular, Achilles tendon. Any application which previously used the extensor hallicus longus tendon or any other intrasynovial tendon of the body. Foot: All ligamentous and tendon structures associated with the foot, tarsals, metatarsals and phalanges, including pronator tendons, supinator tendons, extensor tendons and extensor tendons.

In some embodiments, cartilage scaffolds may be used for: articular surfaces of the hip, knee, shoulder, ankle and elbow and all other synovial joints normally covered by articular cartilage, as well as those composed of elastic and fibrocartilage.

In some embodiment, meniscus scaffolds may be used for: meniscus replacement. In some embodiments, bone-derived scaffolds may be used for: all bony structures in the human body, including the teeth and skull.

In some embodiments, intervertebral disc scaffolds may be used for: intervertebral discs, joints such as the glenoid, acromioclavicular.

Non-orthopedic applications for the bioscaffolds disclosed herein include: obstetrical and gynecological, neurosurgical, cardiothoracic, urological, ophthalmologic, otolaryngology applications, applications in plastic surgery and reconstruction, transplant surgery, thoracic and gastrointestinal surgery, renal and hepatic surgery, dermatologic applications.

Mechanical Treatments

Mechanical methods for modifying natural tissues to form bioscaffolds are useful in the present invention. Some tissues contain capsular outer tissue layers such that when the cells are removed, the residual extracellular matrix (ECM) of the outer tissue layers presents a barrier to infiltrating or seeded cells. In addition, this capsular layer can have a different composition that is not ideal for cell attachment. As an example, the adventitia of arteries is a particularly tough, dense tissue layer that is impervious to cell seeding, and smooth muscle cells do not readily adhere to the adventitia ECM. This layer can be physically removed, typically by gross dissection, either before or after decellularization and the medial ECM layers exposed, resulting in better cell attachment and improved access to the interstices of the bioscaffold.

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"Adventitia" as used herein refers to the outermost connective tissue covering of an organ, vessel, or other tissue structure. With respect to blood vessel bioscaffolds created by removing the adventitia, the process results in a more accessable matrix and better cell adhesion.

In general, most organs possess a capsular tissue covering. This tissue not only preserves the dimensional integrity of the organ, but also compartmentalizes organ systems and prevents adhesions between tissues. Inclusion of these capsular structures during preparation may lead to inefficient cell seeding of the resulting scaffold. Many types of scaffolds may benefit from the mechanical removal of these outer tissue layers including heart, kidney, liver, tendon, ligament, nerve and cartilage.

Chemical Treatments

Chemical treatments for modifying natural tissues to form bioscaffolds are useful in the present invention. The chemical treatments can be closely controlled so as to increase the pore size in the scaffolds and remove extraneous ECM materials, creating space for infiltrating cells. Increasing the porosity of bioscaffolds is beneficial for many tissue and organ types, particularly for dense tissues such as muscle, tendon, ligament, nerve and cartilage. Virtually any type of bioscaffold may benefit from structural modification by such chemical treatment, so long as it is balanced with the preservation of the minimum mechanical requirements for the intended application, and with the retention of beneficial ECM molecules such as cell adhesion promoters and growth factors. In preferred embodiments, the chemical treatments are performed with oxidants, which dissolve extraneous ECM.

The "extracellular matrix" or "ECM" or "interstitial structure" as used herein is any part of a tissue that is not a part of any cell. The ECM is the defining feature of connective tissue. In animals, the ECM is comprised primarily of collagen. The ECM also contains various proteins, including a wide range of cellular growth factors.

The increase in pore size and porosity improves initial cell seeding and reduces remodeling time. Removal of DNA and other cell matter from the bioscaffolds serves to reduce immunogenic foreign body reaction of the recipient subject. Though not all of the DNA must be removed, the more severe the foreign body reaction, the more likely the bioscaffold is to fibrose. Therefore, in preferred embodiments, DNA is removed such that only a mild or moderate transient foreign body reaction is instigated by grafting or transplanting the prepared bioscaffolds. By increasing pore size and porosity and decreasing

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cellular content in the bioscaffold, cellular in-growth can be enhanced and immunologic response can be reduced.

"Oxidants" that may be used to carry out the present invention are found, for example, in U.S. Patent Nos. 7,029,508; 7,008,593; and 7,008,545. Oxidants may be per compounds or peroxy compounds such as a percarboxylic acid (performic acid, peracetic acid, perpropionic acid, perbenzoic acid, etc.), persulfate, perphosphate, periodate, etc.; peroxy compounds such as alkali and alkaline earth perborate salts, alkali and alkaline earth percarbonate salts, alkali and alkaline earth persulfate salts, hydrogen peroxide, percarboxylic acid and peracetic acid, hypochlorous acid or alkali and alkaline earth hypochlorite salts; etc.; hydroxylamines, ozone, etc., and combinations of the foregoing. The oxidant may comprise one or more of the foregoing in an aqueous solution in, for example, an amount from about 0.01% to about 50% by weight. In preferred embodiments, the oxidant comprises from about 3% to about 30% by weight.

As used herein, "increasing the pore size and porosity" refers to the controlled dissolving of the interstitial structure of the bioscaffold in order to create an average pore size and porosity ideal for efficient cellular infiltration. "Pore size" refers to the two-dimensional measurement of empty or void space present in a tissue, while porosity refers to the three-dimensional measurement of empty space or void volume per total volume. As those skilled in the art will appreciate, the ideal pore size and porosity will vary from tissue to tissue. It will also be understood that the treatments disclosed herein that increase the average pore size in the bioscaffolds disclosed herein also have the effect of increasing the porosity of the tissue.

In some embodiments, average pore sizes are large enough to accommodate an intact cell. For example, in some embodiments the resulting pore sizes are greater than 1 micron, and more preferably greater than 50 microns. In other embodiments, the pore size may be 100 microns or more. In some embodiments, the ideal pore size of scaffolds developed using the processes described herein on bone tissue is from 400-1000 microns. In some embodiments, the ideal pore size of ligament, tendon, and meniscus tissues is from 100-1000 microns. In preferred embodiments, the average pore size is increased by approximately 3 times the cell diameter of the cell intended to be seeded into the bioscaffold. Cells that can be seeded into the bioscaffold include stem cells (pluripotent and/or multipotent), adipose cells, tendon cells, chondrocyte cells, skeletal muscle cells, osteocyte cells, etc., without limitation (See, e.g., U.S. Patent Nos. 6,808,704 to Lanza et al.; 6,132,463 to Lee et al.; and U.S. Patent Application Publication No. 2005/0124003 to Atala et al.). The ideal pore size in

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this preferred embodiment is therefore three times the average diameter of the cell selected to be seeded. In some embodiments, the average pore size is approximately three times a cell diameter of 1 to 30, 40, or 50 or more microns (i.e., 3 to 60, 80, or 100 or more microns).

The ideal pore size may also be represented as a percentage of the average pore size of the tissue before the controlled dissolving of the interstitial structure. In some embodiments, the average pore size is increased by 20, 30, 40, or 50%. In other embodiments, the average pore size is increased by 60, 70, 80, 90, or 100% or more. In other embodiments, the average pore size is increased by 150, 200, 250, 300, or 350% or more. However, the overall structural integrity necessary for the intended application must be considered when selecting for the ideal resultant pore size.

In some embodiments, production of bioscaffolds is conducted by first decellularizing the tissue of interest, for example, by the following procedure:

1. Pre-treat the tissue with deionized (DI) water or other hypotonic solution to disrupt the cells;

2. Optionally, treat the disrupted tissue with an enzyme such as trypsin to break down cell adhesions;

3. Treat the tissue with detergent solution such as 0.5% Triton X-100 (a nonionic surfactant) in ammonium hydroxide;

4. Optionally, but preferably, wash residual reagents out of the bioscaffold with repeated washes of DI water.

In some embodiments, the decellularizing step may be carried out as described in U.S. Patent No. 6,753,181 to Atala, by: mechanically agitating tissue (*e.g.*, a complete organ or a portion thereof) in membrane stripping fluid to disrupt cell membranes while maintaining the interstitial structure of the tissue; treating the isolated tissue in a solubilizing fluid at a concentration effective to extract cellular material from the tissue while maintaining the interstitial structure of the tissue; and optionally washing the isolated tissue in a washing fluid to remove cellular debris while maintaining the interstitial structure of the tissue until the isolated tissue is substantially free of cellular material, to thereby produce a decellularized tissue scaffold (in some embodiments washing may optionally be deferred until after the oxidizing step).

The step of mechanically agitating the tissue may comprise placing the tissue in a stirring vessel having a paddle that rotates at a speed ranging from about 50 revolutions per

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minute (rpm) to about 150 rpm. The step of mechanically agitating the isolated tissue may occur in a non-detergent membrane stripping fluid (e.g., distilled water, physiological buffer or culture medium). The step of treating the isolated tissue in the solubilizing fluid may also occur in a stirring vessel. The treating step may comprise using a solubilizing fluid that is an alkaline solution having a detergent. The treating step may comprise treating the isolated tissue in an alkaline solution (for example, a solution selected from the group consisting of sulphates, acetates, carbonates, bicarbonates and hydroxides, and a detergent selected from the group consisting of Triton X-100, Triton N-101, Triton X-114, Triton X-405, Triton X-705, and Triton DF-16, monolaurate (Tween 20), monopalmitate (Tween 40), monooleate (Tween 80), polyoxethylene-23-lauryl ether (Brij 35), polyoxyethylene ether W-1 (Polyox), sodium cholate, deoxycholates, CHAPS, saponin, n-Decyl β-D-glucopuranoside, n-heptyl β-D glucopyranoside, n-Octyl α.-D-glucopyranoside and Nonidet P-40).

The examples of decellularization techniques provided above are not intended to be limiting, and the invention encompasses the use of essentially any decellularization technique that removes a substantial fraction of the cells while leaving the interstitial structure substantially intact. Alternatively, the decellularization may be combined with the chemical modifications as described below to remove extraneous ECM materials and increase the average pore size.

It will be appreciated by those skilled in the art that certain techniques will be preferred for particular tissue engineered constructs or native tissues, depending upon the properties of these constructs or tissues. An appropriate decellularization technique may be selected and parameters such as temperature and time may be optimized in order to achieve a desired degree of decellularization. In some embodiments of the invention the decellularization process removes at least 50% of the cells. In some embodiments of the invention the decellularization process removes at least 60%, at least 70%, or at least 80% of the cells. In some embodiments of the invention at least 90%, at least 95%, or substantially all of the cells are removed. As described above, there may be a tradeoff between the two goals of achieving a high degree of decellularization and preserving the structure and properties of the extracellular matrix. Thus it is not necessarily preferred to achieve maximal possible decellularization if doing so results in unacceptable damage to the extracellular matrix. The optimum degree of decellularization may depend upon the properties of the construct and the use for which it is intended.

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In preferred embodiments, after decellularization the dense bioscaffold is treated with an oxidant solution to effectively remove extraneous ECM materials and increase the average pore size. Any type of water-soluble oxidant can be used. Preferred oxidants include hydrogen peroxide and peracetic acid. The decellularization and oxidizing steps may also be combined, or the oxidizing step may be performed before the decellularization step.

Typical oxidant concentrations range from 1, 1.5, 2 or 3 to 30, 32, or 34%, and up to 50% (w/v) and will vary depending on the starting density of the tissue and desired degree of porosity. A preferred concentration for nerve tissue is approximately 5% and for tendon is approximately 1.5%, for example. After oxidation, residual chemicals may be removed by repeated washes with DI water and conditioned in a final step by incubation with phosphate buffered saline (PBS). The scaffold may also be freeze-dried and sterilized (e.g., by cold gas) prior to use.

For chemical modification, a batch process can be used to modify large numbers of bioscaffolds at one time. The increase in porosity can be controlled as in any kinetic process by changing oxidant concentration, time, and temperature. Similar to the decellularization step discussed above, there may be a tradeoff between the two goals of achieving adequate porosity and preserving the structure and properties of the extracellular matrix. Thus, it is not necessarily preferred to achieve maximal possible porosity if doing so results in unacceptable damage to the extracellular matrix. The optimum degree of porisity may depend upon the properties of the construct and the use for which it is intended.

In preferred embodiments, the oxidant is added sequentially or simultaneously with the detergent. Simultaneously is close enough in time that combining the two steps (oxidant and detergent) will increase removal of cellular material and DNA. For example, oxidation occurs in conjunction with decellularization and cellular material is only minimally removed from the musculoskeletal dense tissues if the reagents are used separately. In some embodiments, using oxidant and detergent simultaneously increases the removal of DNA and minimizes residual DNA.

The optimized bioscaffolds disclosed herein minimize immunogenic and inflammatory potential of the implanted scaffold in the host after implantation and maximize host cell infiltration into the scaffold *in vivo* or *in vitro*. These disclosed modifications will decrease the time necessary for cellular repopulation *in vivo* or cell seeding *in vitro* and reduce the time necessary for remodeling of collagen fibrils, which subsequently improves the biomechanical integration of the scaffolds *in vivo*. All of these factors have been implicated in the poor incorporation of current grafts used by others in reconstruction of

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tissues. The removal of extraneous cellular material greatly reduces the potential for inflammation and disease transmission upon implantation of the scaffold.

The present invention is explained in greater detail in the following non-limiting Examples.

Examples

Example 1: Nerve Scaffolds. Sciatic nerves were harvested from white New Zealand rabbits. The tissue was placed in deionized (DI) water and shaken at 4°C for 48 hours to break open the cells. Cell fragments were removed by extraction at 4°C with 0.5% Triton X-100 in 0.05% sodium hydroxide solution for 48 hours. Residual reagents were removed by shaking in DI water at 37°C for 96 hours. Efficient removal of the cellular material was confirmed by histological, microscopic, and residual DNA analyses. To increase the porosity and interconnectivity, the acellular nerves were treated at 37°C for 24 hours with peracetic acid at concentrations of 1, 5, and 10 w/v %. Residual oxidant was removed by repeated extractions with DI water. Finally, the bioscaffolds were freeze dried prior to analysis by scanning electron microscopy (SEM).

The SEM results are summarized in **Figure 1.** These data demonstrate the efficacy of oxidant treatment on peripheral nerve bioscaffolds. In cross section, the untreated nerve is dense with no apparent porosity. Treatment with peracetic acid concentration as low as 1% has a dramatic effect on the degree of resulting porosity. However, extensive oxidation can result in loss of mechanical integrity as is shown in **Figure 1d.** Clearly, the kinetic parameters can be adjusted to obtain optimal pore size and interconnectivity without appreciable loss of mechanical strength.

Example 2: Tendon Scaffolds. The feet of 56 day old Leghorn chickens were provided by Wayne Farms (Dobson, NC), placed on ice immediately after receipt, and stored at 4°C until harvest. The feet were cleaned and disinfected using aseptic techniques. The flexor digitorum profundus (FDP) tendon from the long digit was removed and placed into a sterile, 15 ml conical tube (Becton Dickinson, Bedford, MA).

A. Preparation of Decellularized/Oxidized FDP Tendon Scaffolds. Immediately after harvest, FDP tendons were transferred under aseptic conditions from 15 ml conical tubes to individual clean, autoclaved, 100 ml glass, screw-top bottles (Gibco, Grand Island, NY). 100ml of DNase-free/RNase-free, distilled water (Gibco) was added to each sample. The bottle was placed onto a rotating shaker (Barnstead MaxQ400, Dubuque, IA) at 200 rpm,

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37°C, for 24 hours. After 24 hours, the water was discarded and the cycle was repeated. At the conclusion of the second cycle, the water was discarded and 100 ml of 0.05% trypsin (Sigma, St. Louis, MO), 4.0 mM sodium bicarbonate (Sigma), and 0.5 mM tetrasodium EDTA (Sigma), prepared in Hank's Balanced Salt Solution (Gibco) was added. The sample was placed onto the rotating shaker at 200 rpm, 37°C for 1 hour. At the end of the cycle, the trypsin solution was discarded and 100 ml of Dulbecco's Modified Eagle's Medium (DMEM) high-glucose (Gibco) containing 10% fetal bovine serum (FBS) (Valley Labs, Winchester, VA) and 100 I.U./ml Penicillin, 100µg/ml Streptomycin, 0.25 µg/ml Amphotercin B (Gibco) was added in order to halt trypsin digestion of the sample. The sample was placed back onto the rotary shaker at 200 rpm, 37°C, for 24 hours. After 24 hours, the DMEM-FBS solution was discarded and 100 ml of the DNase-free/RNase-free distilled water was added and the sample was placed onto the rotary shaker at 200 rpm, 37°C for 24 hours. The water wash was discarded and 100 ml of 1.5% peracetic acid (Sigma) solution with 1.5% Triton X-100 (Sigma) in distilled, deionized water (diH2O) was added and the sample placed onto the rotary shaker at 200 rpm, 37°C for 4 hours. The solution was discarded and two 100 ml washes with diH2O were performed, each for 12 hours at 37°C and 200 rpm on the rotary shaker. At the end of the second wash, the sample was removed and placed into a clean, sterile 15 ml conical tube and frozen for 24 hours at -80°C. The sample was then freeze-dried (Labconco, Freeze Dry System, Kansas City, MO) for 24 hours before being returned to the freezer and stored at -80°C until further use.

B. Histologic Analysis of Fresh-Frozen FDP Tendons and Decellularized/Oxidized FDP Tendon Scaffolds. Mid-substance portions of fresh-frozen FDP tendon and decellularized/oxidized FDP tendon scaffold were placed in 10% phosphate-buffered formalin at room temperature for 4 hours. The tendons then were processed for histology, embedded in paraffin, and microtomed to obtain 5.0 µm thick, longitudinal sections. The sections were mounted on slides and stained using hematoxylin and eosin (H&E, Sigma) as well as 4,6-diamidino-2-phenylindole (DAPI) (Vector, Burlingame, CA) to identify cellular and nuclear components, respectively. Representative light (H&E) and fluorescence (DAPI) micrographs were taken at 100X magnification.

Abundant cellular material, specifically nuclear material, was evident after H&E and 4',6-diamidino-2-phenylindole (DAPI) staining of longitudinal sections of fresh-frozen Leghorn chicken FDP tendon prior to decellularization/oxidation (**Figure 2**). The low or absent porosity was observed in H&E stained sections of the FDP tendons. After decellularization and oxidation, no nuclear material was evident via H&E staining. DAPI

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staining revealed the presence of DNA and RNA within the decellularized/oxidized tendon scaffolds (**Figure 2**). However, this material was neither organized, nor condensed in appearance as seen in the untreated tendons. An increase in intra-fascicular and inter-fascicular space after treatment was also observed via H&E staining.

C. Scanning Electron Microscopy of Fresh-Frozen FDP Tendons and Decellularized/Oxidized FDP Tendon Scaffolds. Scanning electron microscopy confirmed the dense micro-architecture observed in histologic sections of fresh-frozen FDP tendon, as well as an increase in pore size and porosity following treatment (**Figure 3**). Scanning electron microscopy was performed on fresh-frozen FDP tendons and decellularized/oxidized FDP tendon scaffolds. Specimens of the fresh-frozen FDP tendon and the FDP tendon scaffold were removed directly from storage at -80°C, equilibrated in DNase-free/RNase-free water at 37°C for 30 minutes, patted dry and mounted for analysis. Cross-sectional electronmicrographs were obtained at 25.0 kV, 50 Pa, 50X magnification using a Hitachi S-2600 Scanning Electron Microscope (Hitachi High Technologies America, Pleasanton, CA).

D. Determination of DNA Content in Fresh-Frozen FDP Tendons and Decellularized/Oxidized FDP Tendon Scaffolds. Fresh-frozen FDP tendons (n=16) stored at -80°C for 24 hours were lyophilized for 24 hours. Samples then were weighed and placed into sterile 1.5 ml micro-centrifuge tubes. This process was repeated for the decellularized/oxidized FDP tendon scaffolds which previously had been freeze dried as part of their preparation process (n=8). Total DNA was then isolated from this tissue using a commercially available kit (DNeasy™, Qiagen, Valencia, CA). The DNA concentration in the resulting volume was used to calculate total DNA content at λ=280 nm using a spectrophotometer (Thermo Spectronic, Biomate 3, Rochester, NY), which was then normalized using the initial dry weight of the sample.

DNA content of the FDP tendon scaffolds was significantly decreased by 67.14% (0.1102 +/- 0.0542 μg DNA/mg tissue dry weight, n=8) after treatment when compared to untreated (i.e. fresh-frozen) FDP tendons (0.3354 + / - 0.0200 μg DNA/mg tissue dry weight, n=16), $p<0.001$ (**Figure 4**).

E. In Vitro Biocompatibility of Decellularized/Oxidized FDP Tendon Scaffolds: Direct Contact Method. Representative specimens (approximately 0.04 cm^3 portion/well) of the decellularized/oxidized chicken FDP tendon scaffold (n=10) were placed in the center of sub-confluent murine NIH 3T3 cell monolayers in 96-well plates (Becton Dickinson), which covered one-tenth of the surface area, according to established standards (Pariente et al. (2001) *J Biomed Mater Res* 55:33-39). The same procedure was followed using latex

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(Ansell, Massillon, OH) as a negative control (n=10). Cells not exposed to any foreign material served as a positive control (n=10). The cell-material contact was maintained for 72 hours at 37°C and 5% CO_2.

At the end of the incubation, the test materials were removed and two separate assays were performed to measure metabolic activity (MTS® solution) and cell viability (Neutral Red). Briefly, 40 µL of MTS® solution (Promega, Madison, WI) was added into each well. After a 3 hour incubation at 37°C, the absorbance of the solution was measured at 490 nm using a 96-well plate spectrophotometer (Biotek, ELX800, Winoski, VT). The absorbance obtained was directly proportional to the metabolic activity of the cell populations and inversely proportional to the toxicity of the material.

For the cell viability assay, the media was removed and the cell layers rinsed with 200 µL of cold PBS. 100 µL of neutral red solution (Sigma, 0.005% weight/volume in culture medium) was then added into each well. After a 3 hour incubation period at 37°C, the neutral red solution was removed and dye extraction performed by adding 100 µL of 1% (volume/volume) acetic acid in 50% (volume/volume) ethanol solution into each well. The plates were agitated on a platform shaker (Barnstead) for 5 minutes. Absorbance was measured at λ=540 nm using the 96-well plate spectrophotometer noted above. The absorbance obtained was directly proportional to the viability of the cell populations and inversely proportional to the toxicity of the material. The negative control (cells exposed to latex) for both assays was considered satisfactory if the observed absorbance for both assays was < 10% of that observed for the positive control (cells exposed to media alone).

Mitochondrial activity determined using the MTS® assay (absorbance at λ=490nm) for NIH 3T3 cells exposed to the decellularized/oxidized chicken FDP scaffold was 79% (1.300 +/- 0.049, n=10) of that observed for cells exposed to media only (1.654 +/- 0.057, n=10) and was statistically significant ($p<0.001$, **Figure 5A**). However, cell viability determined using the Neutral Red assay (absorbance at λ=540nm) for NIH 3T3 cells exposed to the decellularized/oxidized chicken FDP scaffold was 88% (0.204 +/- 0.014, n=10) of that observed for NIH 3T3 cells exposed to media alone (0.231 +/- 0.033, n=10, positive control), a difference which was not statistically significant (**Figure 5B**). The decellularized/oxidized scaffold and positive control (cells only) differed significantly ($p<0.001$) from the values obtained for a known cytotoxic material (latex, negative control, n=10) in both assays. The absorbance observed for the negative control was also <10% of the absorbance observed for positive controls
in each assay.

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F. Tensile Testing. Fresh-frozen FDP tendons (n=6) and decellularized and oxidized FDP tendon scaffolds (n=10) were removed from storage at -80°C and allowed to equilibrate in sterile Dulbecco's phosphate buffered saline at 37°C for 30 minutes. Tendons then cut into "dogbone" specimens using a custom made punch (Freeman, Fremont, OH), their ends wrapped with saline-soaked gauze, and placed into custom made grips on an uniaxial load frame (Instron 5544, Needham, MA) for tensile testing. Samples were measured prior to testing with digital calipers to determine cross-sectional area (mm^2) and length (mm). Tendons were pre-loaded to 0.1N, and then pre-conditioned 10 times to 0.1mm extension prior to being loaded to failure at a rate of 10 mm/minute. Ultimate tensile stress at break (MPa) and elastic modulus (MPa) then were determined using the stress-strain curve obtained from the instrument.

Mechanical testing of decellularized/oxidized FDP tendon scaffolds determined that the ultimate stress at break (MPa) was 76% (32.48 +/- 2.98 MPa, n=12) of that observed for fresh-frozen FDP tendons (42.95 +/- 5.04 MPa, n=8) and that the difference was not statistically significant (**Figure 6A**). Similarly, elastic modulus (E) was reduced in decellularized and oxidized FDP tendons (261.12 +/- 17.32 MPa, n=12) to 77% of the value observed for fresh-frozen FDP tendons (335.48 +/- 31.92 MPa, n=6), and that the difference was not statistically significant (**Figure 6B**). Only specimens that failed in the mid-substance region of the tendon and scaffold were included. Specimens that failed at the grips were excluded from calculations.

G. *In Vivo* Cell Infiltration and Inflammatory Response of Decellularized/Oxidized FDP Tendon Scaffolds. To assess inflammatory response and cell infiltration *in vivo,* scaffolds were implanted subcutaneously in 10 week-old, female CD1 mice using aseptic technique. Briefly, a 3 mm incision was made on the dorsum of the mouse and a tissue pocket created using blunt dissection with scissors. Implants (1 per mouse, n=3 per time point) of approximately 1mm thick x 2mm x 2mm in size of decellularized/oxidized tendon scaffolds (ethylene-oxide sterilized) were placed and the wound closed with suture and wound adhesive. The implants were harvested at 3, 7, 14, and 21 days post-implantation and examined grossly and histologically (H&E).

Cell infiltration was observed in H&E-stained sections of explanted decellularized/oxidized FDP tendon scaffolds at 3, 7, 14 and 21 days postimplantation in immune-competent, female, 10 week-old CD1 mice. Representative light micrographs of H&E-stained longitudinal sections of explanted tendon at 3 days (**Figure 7**, left panel) and 21

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days (**Figure** 7, right panel) post-implantation are shown. No evidence of a gross inflammatory reaction or capsule formation was observed at either time of explantation.

H. Statistical Analysis. For DNA content, MTS™, neutral red, and biomechanical analyses, samples were analyzed in replicate, averaged, a standard error of the mean (SEM) was calculated and a comparison was made between groups using a two-tailed, Student's t-test with unequal variances. Differences were considered significant at $p<0.05$.

I. Conclusions. Increased tendon pore size and porosity were produced using chemical oxidation resulting in a scaffold that is more likely to allow cell infiltration and repopulation. Additionally, the tissue-engineered FDP tendon scaffold is biocompatible *in vitro* when assessed via the MTS® and Neutral Red assays. The FDP tendon scaffolds also retained approximately 76-78% of the initial mechanical properties of fresh-frozen FDP tendons, a difference that was not statistically significant. The FDP tendon scaffolds did not exhibit any evidence of a gross inflammatory reaction at 3, 7, 14 or 21 days when implanted subcutaneously in an immunocompetent mouse. Finally, cell infiltration by fibroblast-like host cells was observed at all time points after subcutaneous implantation of FDP tendon scaffolds.

Example 3: Tendon Scaffolds. Experiments with the human Achilles tendon have produced similar results as those reported in Example 2.

A. Preparation of Decellularized/Oxidized Achilles-derived Tendon Scaffolds. Freeze-dried human Achilles tendon allografts from multiple donors were provided and stored at 25°C until use. Freeze-dried human Achilles tendon allografts were transferred under aseptic conditions to individual clean, autoclaved, 1000 ml glass flasks. 1000 ml of DNase-free/RNase-free, distilled water (Gibco) was added to each sample. The flask was placed onto a rotating shaker (Barnstead MaxQ400, Dubuque, IA) at 200 rpm, 37°C, for 24 hours. After 24 hours, the water was discarded and the cycle was repeated. At the conclusion of the second cycle, the water was discarded and 500 ml of 0.05% trypsin-EDTA (Gibco) was added. The sample was placed onto the rotating shaker at 200 rpm, 37°C for 1 hour. At the end of the cycle, the trypsin solution was discarded and 500 ml of Dulbecco's Modified Eagle's Medium (DMEM) high-glucose (Gibco) containing 10% fetal bovine serum (FBS) (Valley Labs, Winchester, VA) and 100 I.U./ml Penicillin, 100µg/ml Streptomycin, 0.25 µg/ml Amphotercin B (Gibco) was added in order to halt trypsin digestion of the sample. The sample was placed back onto the rotary shaker at 200 rpm, 37°C, for 24 hours. After 24 hours, the DMEM-FBS solution was discarded and 1000 ml of the DNase-free/RNase-free

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distilled water was added and the sample was placed onto the rotary shaker at 200 rpm, 37°C for 24 hours. The water wash was discarded and 1000 ml of 1.5% peracetic acid (Sigma) solution with 1.5% Triton X-100 (Sigma) in distilled, deionized water (diH2O) was added and the sample placed onto the rotary shaker at 200 rpm, 37°C for 4 hours. The solution was discarded and three 1000 ml washes with diH_2O were performed, each for 12 hours at 37°C and 200 rpm on the rotary shaker. At the end of the third wash, the sample was removed and placed into a clean, sterile freezer bag and frozen for 24 hours at -80°C. The sample was then freeze-dried (Labconco, Freeze Dry System, Kansas City, MO) for 48 hours before being returned to the freezer and stored at -80°C until further use.

B. Histologic Analysis of Freeze-dried Human Achilles Tendon Allografts and Decellularized/Oxidized Freeze-dried Human Achilles Tendon Allograft-derived Scaffolds. Mid-substance portions of freeze-dried human Achilles tendon allograft and decellularized/oxidized freeze-dried human Achilles tendon allograft-derived scaffold were placed in 10% phosphate-buffered formalin at room temperature for 4 hours. The tendons then were processed for histology, embedded in paraffin, and microtomed to obtain 5.0 μm thick, longitudinal sections. The sections were mounted on slides and stained using hematoxylin and eosin (H&E, Sigma) as well as 4',6-diamidino-2-phenylindole (DAPI) (Vector, Burlingame, CA) to identify cellular and nuclear components, respectively. Representative light (H&E) and fluorescence (DAPI) micrographs were taken at 100X magnification.

Abundant cellular material, specifically nuclear material, was evident after H&E and 4',6-diamidino-2-phenylindole (DAPI) staining of longitudinal sections of freeze-dried human Achilles tendon allograft prior to decellularization/oxidation (**Figure 8A**). Minimal porosity was observed in H&E stained sections of the freeze-dried human Achilles tendon allograft. After decellularization and oxidation, no nuclear material was evident via H&E staining. DAPI staining revealed the presence of DNA and RNA within the decellularized/oxidized tendon scaffolds (**Figure 8B**). However, this material was neither organized, nor condensed in appearance as seen in the untreated tendons. An increase in intra-fascicular and inter-fascicular space after treatment was also observed via H&E staining.

C. Determination of DNA Content in Decellularized/Oxidized Freeze-dried Human Achilles Tendon Allograft-derived Scaffolds and Decellularized/Oxidized Freeze-dried Human Achilles Tendon Allograft-derived Scaffolds. Freeze-dried human Achilles tendon allograft (n=10) stored at -80°C for 24 hours were lyophilized for 24 hours.

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Samples then were weighed and placed into sterile 1.5 ml micro-centrifuge tubes. This process was repeated for the decellularized/oxidized tendon scaffolds which previously had been freeze dried as part of their preparation process (n=8). Total DNA was then isolated from this tissue using a commercially available kit (DNeasy™, Qiagen, Valencia, CA). The DNA concentration in the resulting volume was used to calculate total DNA content at λ=280 nm using a spectrophotometer (Thermo Spectronic, Biomate 3, Rochester, NY), which was then normalized using the initial dry weight of the sample.

DNA content of the decellularized/oxidized freeze-dried human Achilles tendon allograft-derived scaffolds was significantly decreased by 75% (0.110 +/- 0.02 μg DNA/mg tissue dry weight, n=10) after treatment when compared to untreated freeze-dried human Achilles tendon allografts (0.40 + / - 0.14 μg DNA/mg tissue dry weight, n=10), $p<0.05$ **(Figure 9)**.

D. Transmission Electron Microscopy of Freeze-dried Human Achilles Tendon Allografts and Decellularized/Oxidized Freeze-dried Human Achilles Tendon Allograft-derived Scaffolds: Ultra-structural modification.

Transmission electron microscopy revealed a large decrease in fibril density per unit area after treatment of the freeze-dried human Achilles tendon allograft, which represents an increase on pore size and porosity of the scaffold produced using the process described herein **(Figures 10A and 10B)**.

E. In Vitro Biocompatibility of Decellularized/Oxidized Freeze-dried Human Achilles Tendon Allograft-derived Scaffolds: Direct Contact Method. Representative specimens (approximately 0.04 cm^3 portion/well) of the decellularized/oxidized freeze-dried human achilles tendon allograft-derived scaffolds (n=10) were placed in the center of sub-confluent murine NIH 3T3 cell monolayers in 96-well plates (Becton Dickinson), which covered one-tenth of the surface area, according to established standards (Pariente et al. (2001) *J Biomed Mater Res* 55:33-39). The same procedure was followed using latex (Ansell, Massillon, OH) as a negative control (n=10). Cells not exposed to any foreign material served as a positive control (n=10). The cell-material contact was maintained for 72 hours at 37°C and 5% CO_2.

At the end of the incubation, the test materials were removed and two separate assays were performed to measure metabolic activity (MTS® solution) and cell viability (Neutral Red). Briefly, 40 μL of MTS solution (Promega, Madison, WI) was added into each well. After a 3 hour incubation at 37°C, the absorbance of the solution was measured at 490 nm using a 96-well plate spectrophotometer (Biotek, ELX800, Winoski, VT). The absorbance

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obtained was directly proportional to the metabolic activity of the cell populations and inversely proportional to the toxicity of the material.

For the cell viability assay, the media was removed and the cell layers rinsed with 200 µL of cold PBS. 100 µL of neutral red solution (Sigma, 0.005% weight/volume in culture medium) was then added into each well. After a 3 hour incubation period at 37°C, the neutral red solution was removed and dye extraction performed by adding 100 µL of 1% (volume/volume) acetic acid in 50% (volume/volume) ethanol solution into each well. The plates were agitated on a platform shaker (Barnstead) for 5 minutes. Absorbance was measured at λ=540 nm using the 96-well plate spectrophotometer noted above. The absorbance obtained was directly proportional to the viability of the cell populations and inversely proportional to the toxicity of the material. The negative control (cells exposed to latex) for both assays was considered satisfactory if the observed absorbance for both assays was < 10% of that observed for the positive control (cells exposed to media alone).

Mitochondrial activity determined using the MTS assay (absorbance at λ=490nm) for NIH 3T3 cells exposed to the decellularized/oxidized freeze-dried human Achilles tendon allograft-derived scaffolds was 95% (1.36 +/- 0.31, n=10) of that observed for cells exposed to media only (1.42 +/- 0.31, n=10) a difference which was not statistically significant ($p>0.05$, **Figure 11**). Cell viability determined using the Neutral Red assay (absorbance at λ=540nm) for NIH 3T3 cells exposed to the decellularized/oxidized freeze-dried human Achilles tendon allograft-derived scaffolds was 92% (0.24 +/- 0.07, n=10) of that observed for NIH 3T3 cells exposed to media alone (0.22 +/- 0.07, n=10, positive control), a difference which was not statistically significant (**Figure 11**). The decellularized/oxidized scaffold and positive control (cells only) differed significantly ($p<0.001$) from the values obtained for a known cytotoxic material (latex, negative control, n=10) in both assays. The absorbance observed for the negative control was also <10% of the absorbance observed for positive controls in each assay.

Example 4: Arterial Scaffolds. Carotid arterial segments were obtained from large pigs (400 to 600 lb). The blood vessels had an internal diameter of 3 to 4 mm and were cut into segments of approximately 50 mm in length. Vessels were washed in distilled water (D/W) for 24 hours and incubated in 0.05% Trypsin EDTA for 1 hr. Incubation in 10% FBS solution in Dulbecco's Modified Eagles Medium (DMEM) for 24 hours was used to stop the trypsin activity. The vessels were then incubated in decellularization solution (490 mL D/W, 10mL Triton-100X, 3.4 mL ammonium hydroxide) for 3 days in a mechanical rotating shaker

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at 4°C. The decellularization solution was changed daily. The decellularized vessels were placed in D/W for an additional 48 hours to wash away any chemical substances from the matrix and then frozen for 24 hrs in a -80°C freezer, lyophilized, and sterilized with ethylene oxide. Hematoxylin and Eosin (H&E) staining were performed to demonstrate that all cellular components had been removed from the matrix (**Figure 12**).

To remove the adventitia layer of the decellularized vessels, the acellular matrices were individually positioned onto an 18Fr balloon angioplasty catheter. Once inflated, the internal diameters of the decellularized vessels were expanded to 6 mm. Using a dissecting stereo microscopic (2.5X), an incision to the sub-adventitia layer was performed with a 15 gauge blade. At this stage, the adventitia could clearly be identified from the underlying media layer. A surgical curved tip tweezer was then utilized to gently strip off the adventitia layer from the media layer. The de-adventitia decellularized porcine artery was frozen, lyophilized, and sterilized with ethylene oxide. H&E staining was performed to observe the microstructure as shown in **Figure 13.**

In order to increase the porosity of the acellular scaffolds, the decellularized vessels were incubated in peracetic acid (PA) for 24 hours at 37°C in a mechanical shaker (150 RPM). Four concentrations of PA were tested that included 0%, 5%, 10%, and 15%. After 24 hours, the PA solution was removed and followed with 4 days of D/W wash that was changed daily. The vessels were then frozen, lyophilized, and sterilized with ethylene oxide. Scanning electron microscopy (SEM) was performed to analyze the effects of PA on the structural change of decellularized segments (**Figure 14**).

Example 5: Meniscus Scaffolds. Medial menisci from sheep were harvested. The menisci were decellularized and treated with oxidant to increase pore size and porosity using aqueous Triton X-100 and peracetic acid. Tissue was equilibrated in phosphate buffered saline (PBS). The contralateral medial meniscus was used as a control. The central 1/3 body of each meniscus was fixed in 10% formalin and paraffin processed. Sections were cut in the coronal plane and stained with hematoxylin and eosin (H&E) for routine cellular assessment and 4',6-diamidino-2-phenylindole (DAPI) to identify residual nuclear components. Samples were assessed using light microscopy. DNA was then isolated using a commercially available kit (DNEasy, Qiagen, Valencia, CA). The DNA concentration in the resulting volume was used to calculate total DNA content, which is standardized using the initial dry weight of the sample. These values were averaged, a standard deviation was determined, and a comparison made using a Student's unpaired t-test with a p-value <0.05 accepted for

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statistical significance. The architecture of the sheep intact meniscus and the meniscus scaffold was examined using scanning electron microscopy (SEM). Specimens were examined in the coronal plane. Porosity was qualitatively compared between the intact meniscus and meniscus scaffold.

Upon gross inspection, the decellularization and oxidative treatment did not change the general shape and architecture of the meniscus. The histological findings clearly indicate a decrease in cellular and nuclear content. The H&E stained sections also show an increase in porosity. DAPI nuclear staining revealed a decrease in nuclear content in the scaffold compared to the intact fresh frozen meniscus. DNA content analysis showed a decrease in DNA content in the scaffold compared to the intact meniscus.

SEM images confirmed an increase in pore size and porosity in the scaffold compared to the intact meniscus (**Figure 15**). The SEM also showed that the architecture of the meniscus is intact. These results indicate that the decellularization and chemical oxidation treatments decrease DNA content in a meniscus scaffold, and that the extracellular matrix architecture of the meniscus remains intact. This process is able to increase pore size and porosity and decrease nuclear content, providing an improved scaffold that minimizes immunologic response and encourages cellular in-growth.

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

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THAT WHICH IS CLAIMED IS:

1. In a method of producing a bioscaffold by decellularizing a tissue to produce a bioscaffold having pores therein, the improvement comprising:

decellularizing the tissue with an oxidant and detergent simultaneously to remove extraneous material and increase the pore size and porosity therein.

2. The method of claim 1, wherein said oxidant is hydrogen peroxide, peracetic acid, or a mixture thereof.

3. The method of claim 1, wherein said oxidant is in an aqueous solution at a concentration of between 1 and 32% (w/v).

4. The method of claim 1, wherein said oxidant is in an aqueous solution at a concentration of between 3 and 32% (w/v).

5. The method of claim 1, further comprising the step of washing said bioscaffold after said decellularizing step to remove residual oxidant and detergent.

6. The method of claim 5, further comprising the step of freeze-drying said scaffold following said washing step.

7. The method of claim 5, further comprising the step of sterilizing said scaffold following said washing step.

8. The method of claim 1, further comprising the step of removing adventitia from said tissue prior to said decellularizing step.

9. The method of claim 8, wherein said tissue is a blood vessel.

10. The method of claim 1, wherein said tissue is selected from the group consisting of skin, muscle, tendon, bone, meniscus, cartilage, intervertebral discs, and ligament tissue.

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11. The method of claim 1, wherein said tissue is selected from the group consisting of liver, kidney, and pancreas tissue.

12. The method of claim 1, wherein said tissue is nerve tissue.

13. The method of claim 1, wherein said tissue is cartilage tissue.

14. A bioscaffold produced by the process of claim 1.

15. The bioscaffold of claim 14, wherein said bioscaffold is sterile.

16. The bioscaffold of claim 14, wherein said bioscaffold is freeze-dried.

17. The bioscaffold of claim 14 aseptically packaged in a sterile container.

18. A method of treating a subject in need of a bioscaffold implant, comprising implanting a bioscaffold of claim 14 in said subject in a treatment effective configuration.

19. In a method of seeding a bioscaffold with mammalian cells, the improvement comprising utilizing a bioscaffold of claim 14 for said seeding.

20. The method of claim 19, wherein said seeding is carried out by implanting said bioscaffold in a subject.

21. A bioscaffold produced by the process of chemically treating a natural animal tissue,

wherein an extracellular matrix component of said bioscaffold has an average pore size that is at least 20% greater than the average pore size of said extracellular matrix component of said natural animal tissue prior to said treating; and

wherein said natural animal tissue is selected from the group consisting of skin, muscle, tendon, bone, meniscus, cartilage, intervertebral discs, and ligament tissue.

22. The bioscaffold of claim 21, wherein said natural animal tissue is tendon tissue.

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23. The bioscaffold of claim 21, wherein said natural animal tissue is nerve tissue.

24. The bioscaffold of claim 21, wherein said natural animal tissue is arterial tissue.

25. The bioscaffold of claim 21, wherein said natural animal tissue is cartilage tissue.

26. The bioscaffold of claim 21, wherein said process of chemically treating said natural animal tissue comprises oxidizing said natural animal tissue with an oxidant.

27. The bioscaffold of claim 26, wherein said oxidant is hydrogen peroxide, peracetic acid, or a mixture thereof.

28. The bioscaffold of claim 26, wherein said oxidant is in an aqueous solution at a concentration of between 1 and 32% (w/v).

29. The bioscaffold of claim 26, wherein said oxidant is in an aqueous solution at a concentration of between 3 and 32% (w/v).

30. The bioscaffold of claim 21, wherein said bioscaffold is sterile.

31. The bioscaffold of claim 21, wherein said bioscaffold is freeze-dried.

32. The bioscaffold of claim 21 aseptically packaged in a sterile container.

33. A method of treating a subject in need of a bioscaffold graft, comprising grafting a bioscaffold of claim 21 in said subject in a treatment effective configuration.

34. The method of claim 33, wherein said bioscaffold is a tendon bioscaffold.

35. The method of claim 33, wherein said bioscaffold is a tendon bioscaffold, and said subject is in need of tendon reconstruction.

36. The method of claim 33, wherein said bioscaffold is a tendon bioscaffold, and said subject is in need of ligament reconstruction.

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37. A method of seeding a bioscaffold with mammalian cells comprising: providing a bioscaffold of claim 21 for said seeding; and seeding mammalian cells into said bioscaffold.

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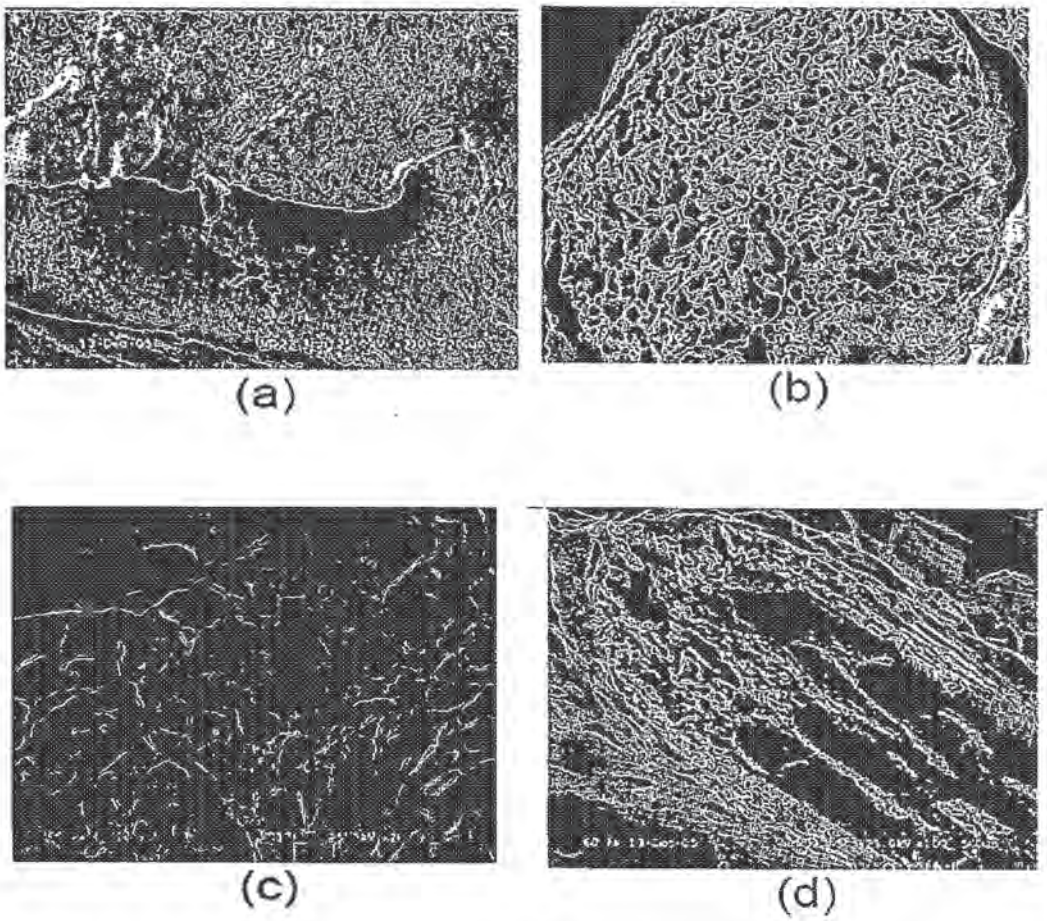


Figure 1

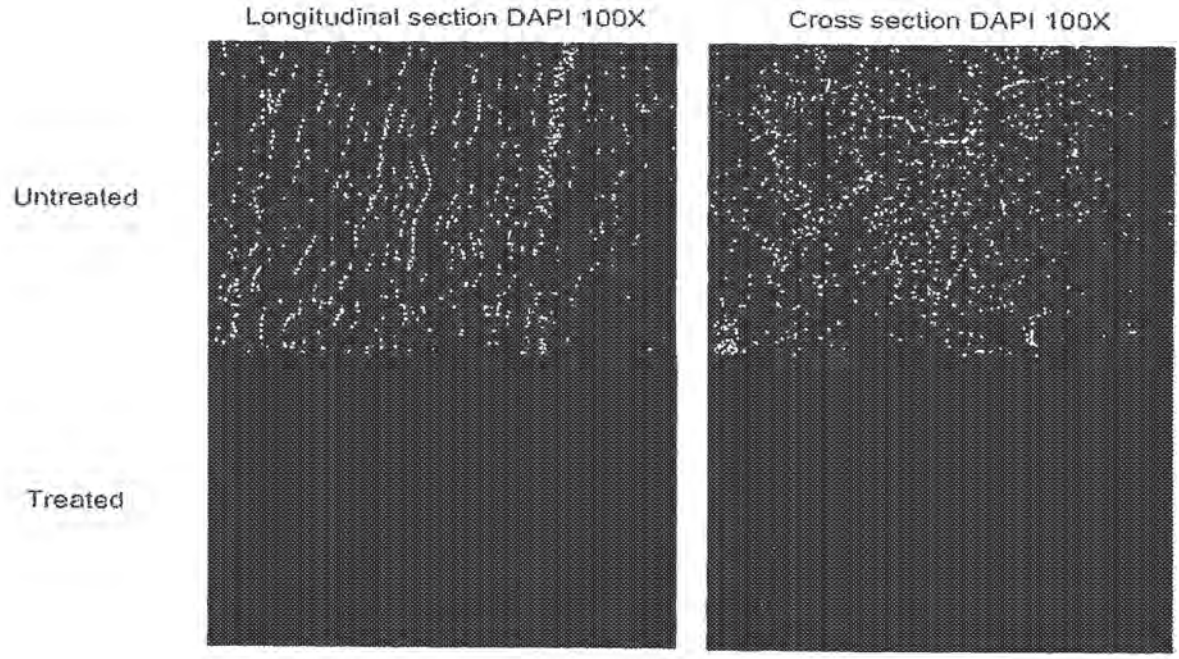
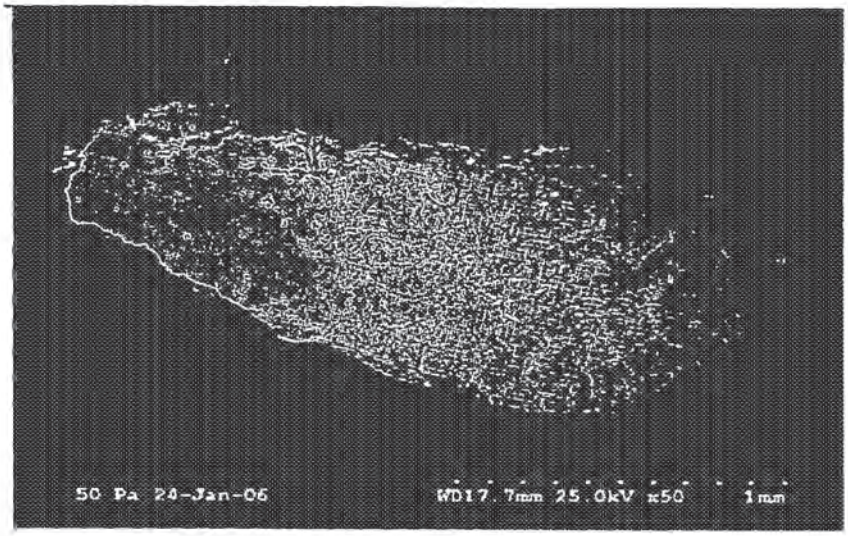
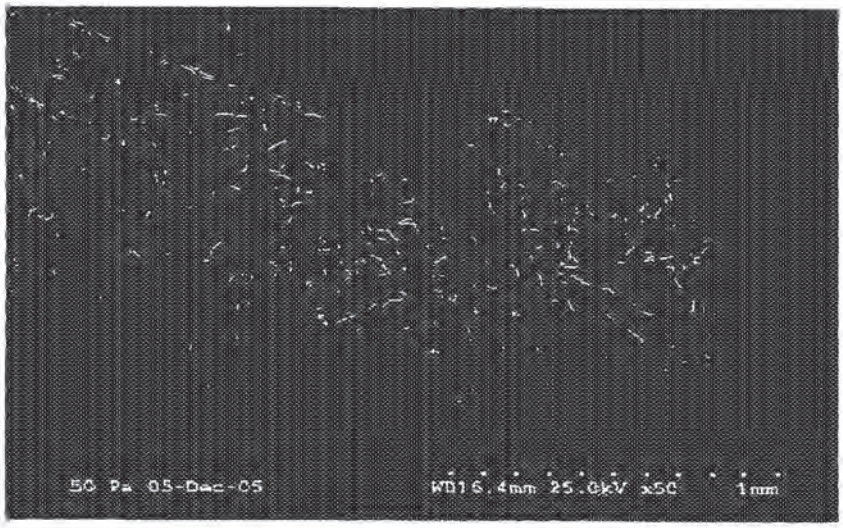

Longitudinal section DAPI 100X
Cross section DAPI 100X
Untreated
Treated

Figure 2



Untreated



Treated

Figure 3

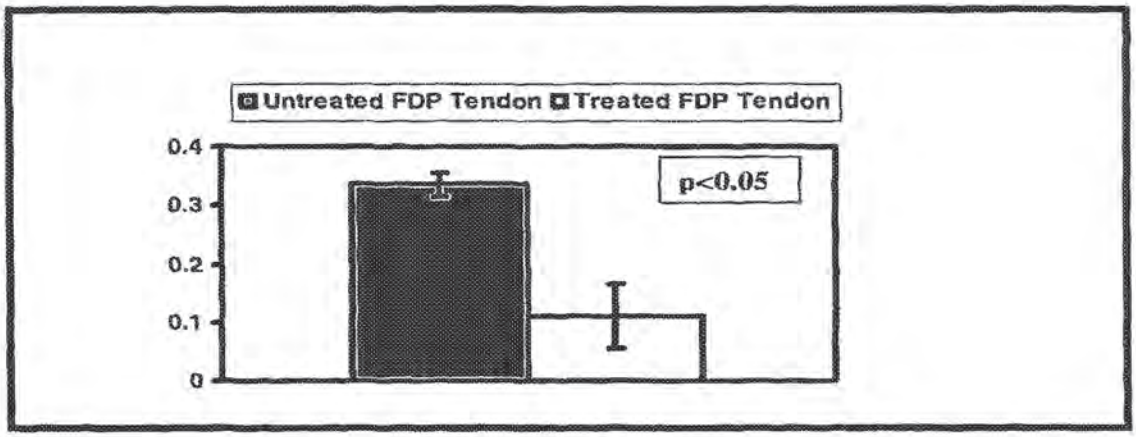


Figure 4

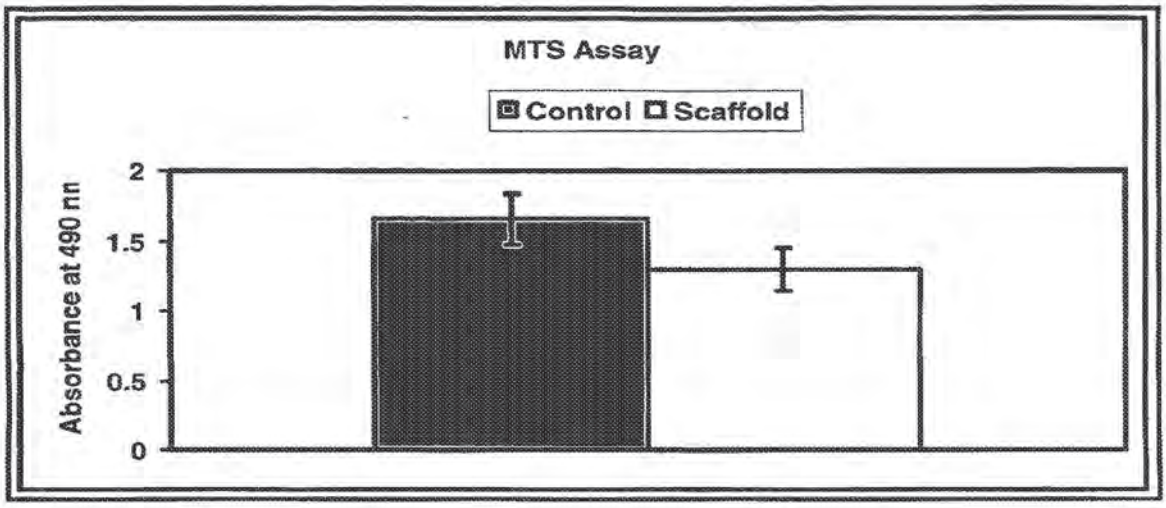


Figure 5A

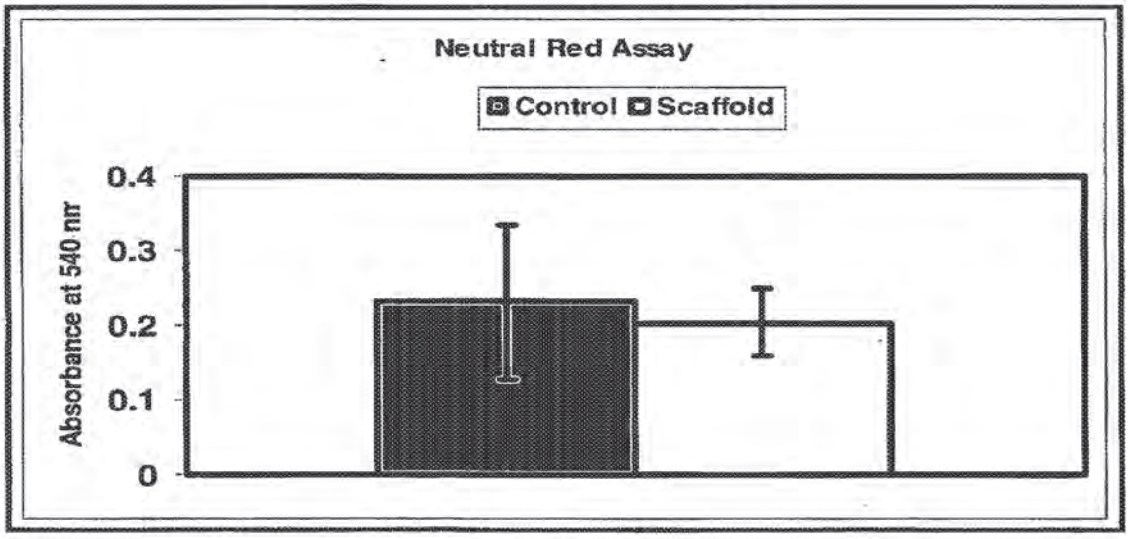


Figure 5B

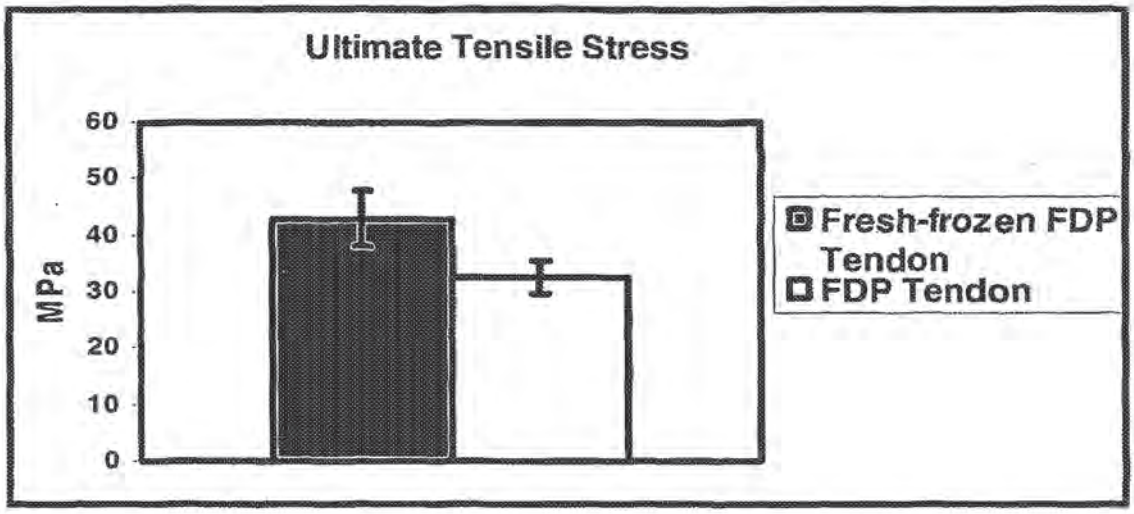


Figure 6A

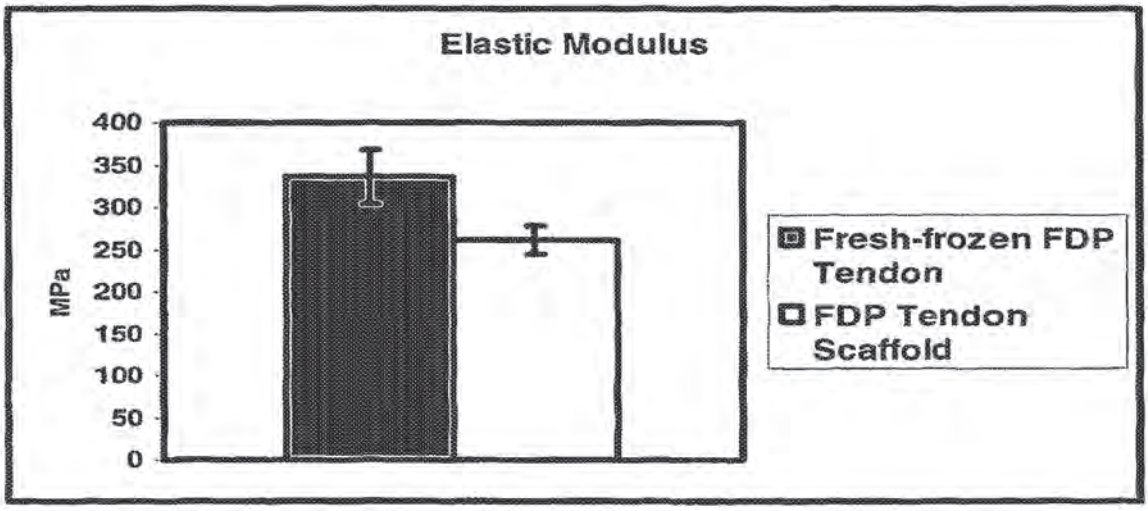


Figure 6B

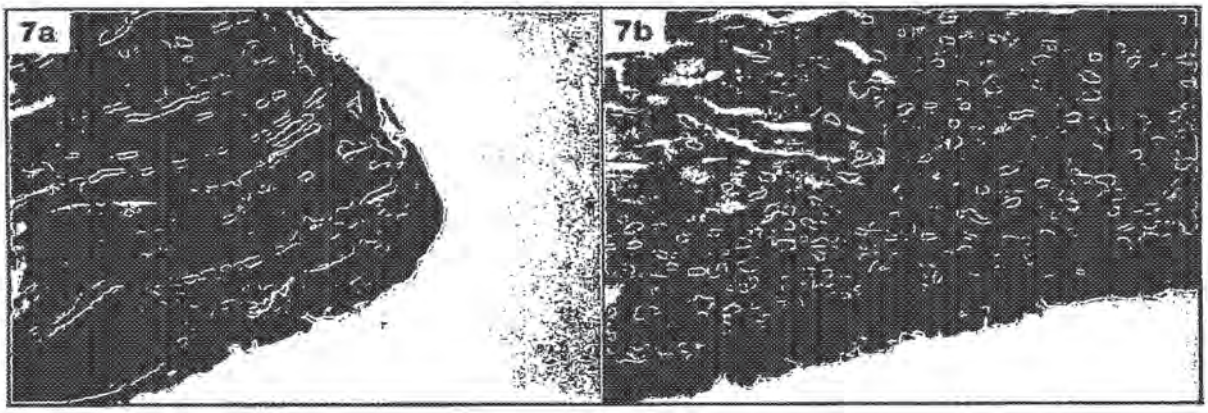


Figure 7

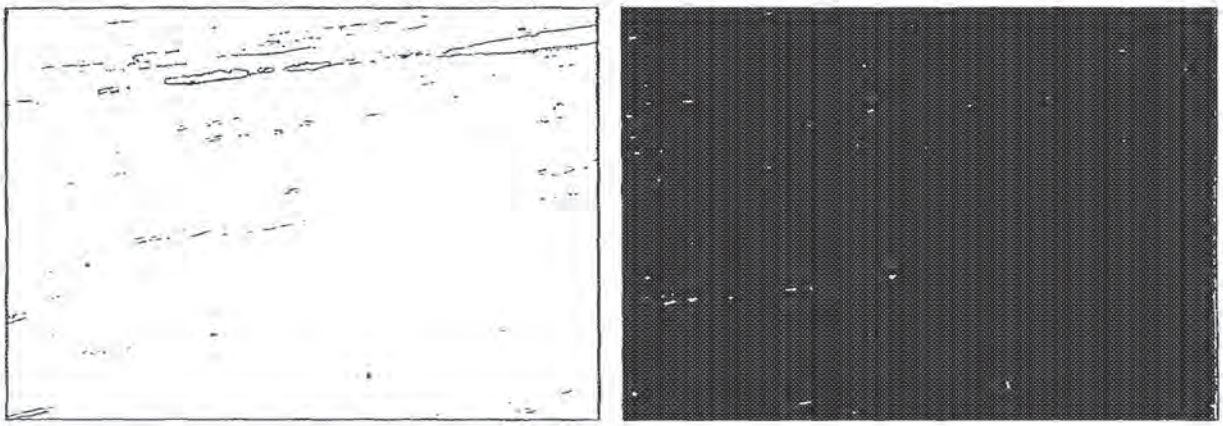


Figure 8A

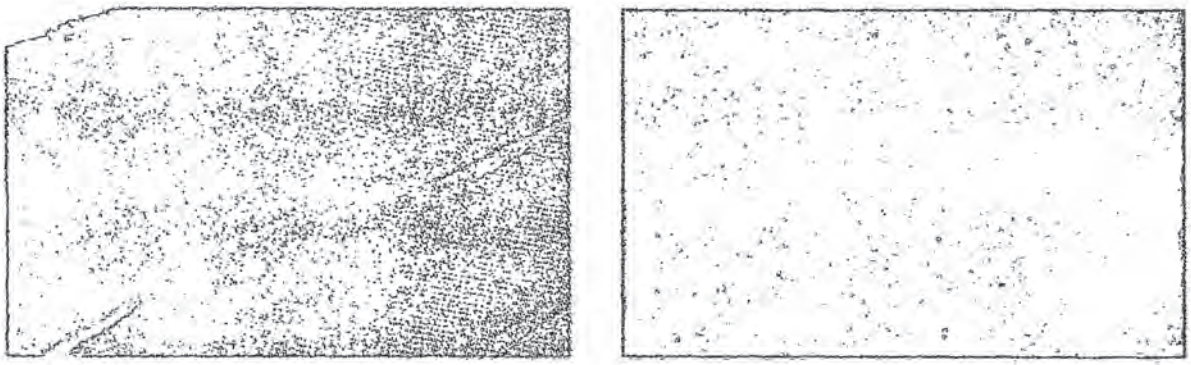


Figure 8B

	Freeze-dried Achilles	Scaffold	% ↓	p-value
DNA Content (ug DNA / mg tissue; x ± SD)	0.40 ± 0.14	0.11 ± 0.02	~75%	<0.05

Figure 9

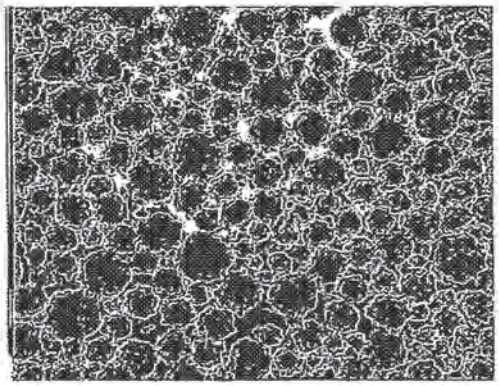


Figure 10A

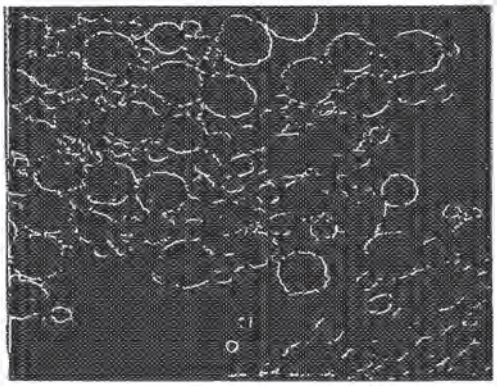


Figure 10B

	*Cells Exposed to Media Only (+ Control) 72 h	Scaffold 72h	% of + Control	p-value
MTS™ Assay (Abs at 490 ηm; x ± SD)	1.42 ± 0.31	1.36 ± 0.17	~95%	>0.05
Neutral Red Assay (Abs at 540 ηm; x ± SD)	0.24 ± 0.07	0.22 ± 0.07	~92%	>0.05

Figure 11

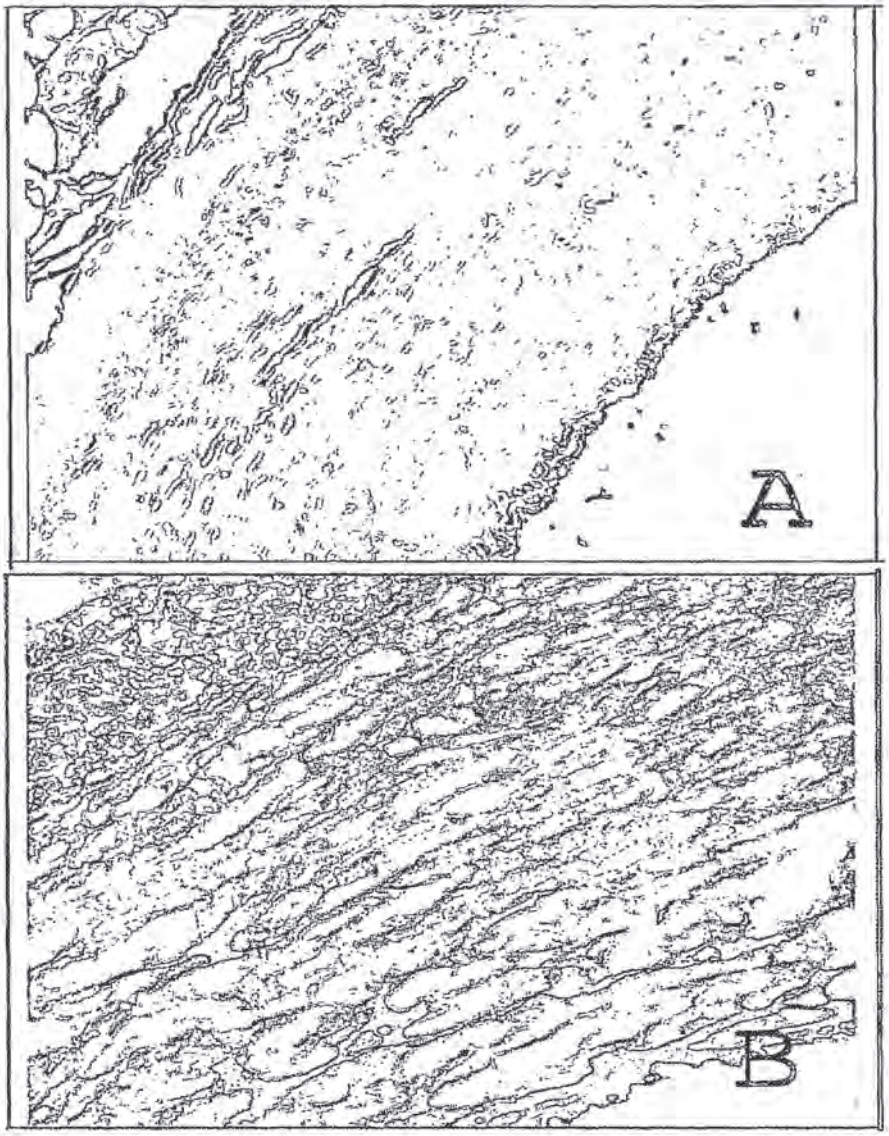
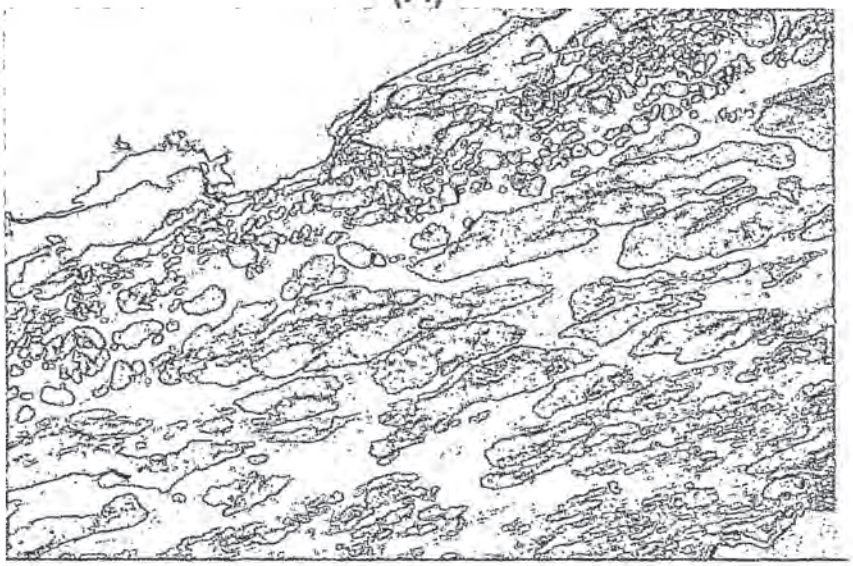


Figure 12

(A)



(B)

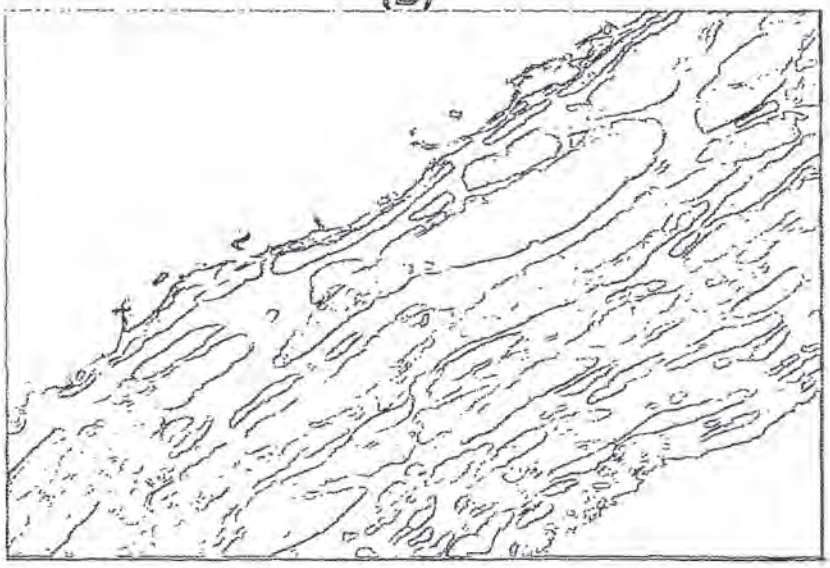


Figure 13

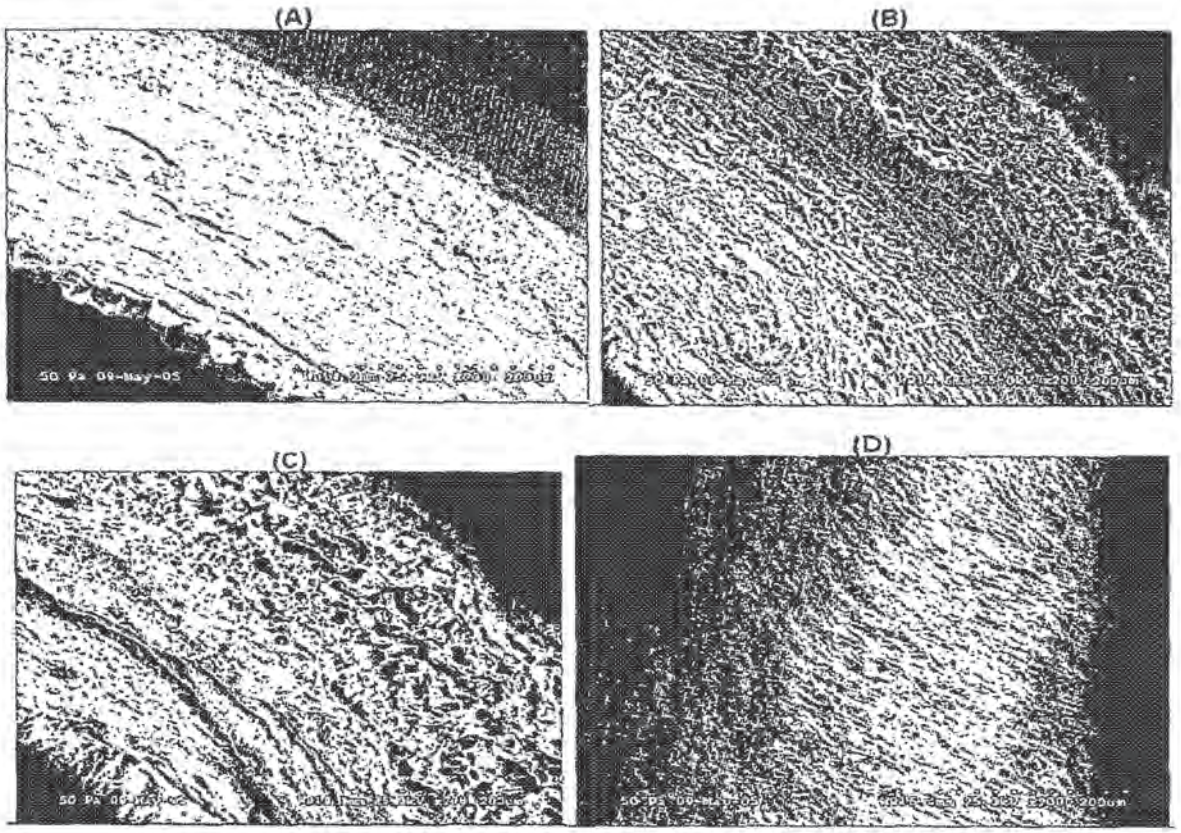


Figure 14

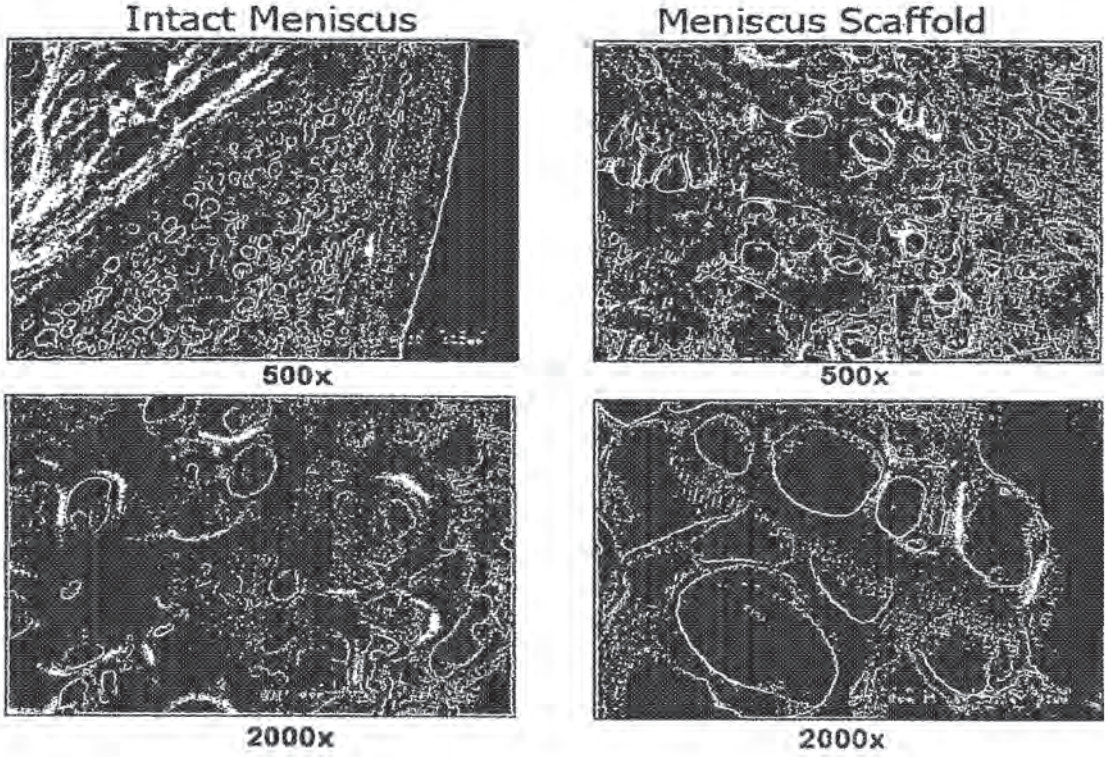


Figure 15

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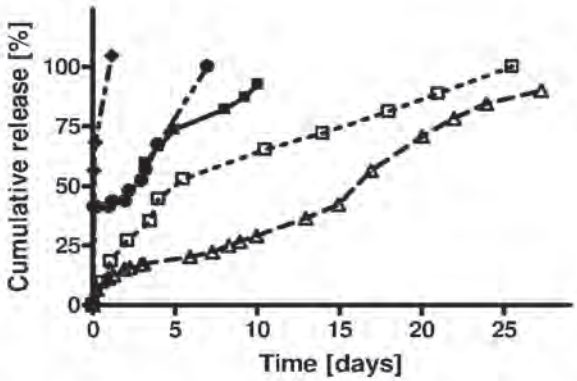
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(54) **Title**: BIODEGRADABLE COMPOSITIONS SUITABLE FOR CONTROLLED RELEASE

Figure 1



(57) **Abstract**: The invention describes a special class of drug-depot forming triblock copolymers which are very suitable for the loading, containment and releasing of sensitive drugs such as proteins from biodegradable, injectable drug depots. Furthermore the invention describes how to visualize these depots for various imaging related purposes. The invention relates to a composition comprising a tri-block copolymer according to formula 1 B-A-B (1), wherein A stands for a linear poly-(ethylene glycol) block and wherein B stands for wherein B stands for a poly(lactide-co-ε-caprolactone) block, wherein the hydroxyl end-groups of the tri-block copolymer are at least partially acylated with an optionally substituted acyl having 2 to 12 C-atoms, C-atoms of the substituents included;an active ingredient, preferably a pharmaceutically active ingredient and a solvent, wherein the block ratio of the tri-block copolymer, which ratio is defined as the ratio between the sum of the average molecular weight of the B- blocks and the sum of the average molecular weight of the A-block ranges from 1.4 to 3.5. This composition is suitable for controlled release of a pharmaceutically active ingredient.

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BIODEGRADABLE COMPOSITIONS SUITABLE FOR CONTROLLED RELEASE

The invention relates to a composition comprising a tri-block copolymer, a solvent and a (pharmaceutically) active ingredient, said composition for use as a medicament, a process for the preparation of said composition and to a method for delivering a (pharmaceutically) ingredient over an extended period to an animal, including a human using said composition. Furthermore this composition can be combined with radiopaque atoms and molecules whereby the composition becomes visible under CT or other imaging techniques.

In recent years, there has been considerable effort to provide systems that are capable of controlled release of drugs in animals, including humans. Some drugs can only be administered to a patient by injection. Controlled release of such drugs has the advantage that the patient does not need to be subjected to multiple injections, but instead only one or a few injections with the controlled release system would suffice.

Some injections into areas of the patient's body are (very) painful and intrinsically increase the risk of infection. Examples of such difficult to dose areas are the eyes, the (synovial) joints, the muscles or the spine. Injection with a drug in a controlled release system will limit the amount of injections needed and will enhance the chance that a patient will continue with the therapy and/or reduce the risk of infection. This will greatly increase the success of the treatment.

Controlled release systems that are very suitable for being injected into such difficult to dose areas are thermogels based on compositions comprising polymers. The polymers have the unique property that at low temperatures they are water soluble, whereas at higher temperatures the polymers become insoluble and form a gel. Preferably, for use as a system that is capable of controlled release, the polymer is soluble in the solvent used at room temperature (e.g. 21°C) and forms a gel once injected into the body (temperature in the range from 30 to 42°C).

A gel (or a hydrogel in the context of the present invention) is a network of polymer chains that are hydrophilic and contain a substantial amount of water (for example between 50 and 99% water, preferably between 66 and 85% water). The gel shows no flow in a vial tilt test: when a glass vial which contains the gel is turned upside down, no flow of the gel is observed during 15 seconds observation time.

Such thermogels are known from for example Zentner *et al.*, Biodegradable block copolymers for delivery of proteins and water-insoluble drugs, Journal of Controlled

Release 72 (2001), 203-215. In this article, the release of several drugs from BAB-type biodegradable thermal gels (marketed under the name ReGel®) is described. This ReGel® polymer is a water soluble, biodegradable polymer at temperatures below the gel transition temperature (so at the injection temperature) and forms a hydrogel inside the patient once injected. The hydrogel forms a controlled release drug depot and according to the article is capable of stabilizing poorly soluble and sensitive drugs, including proteins.

In this article, an example of a drug that is highly soluble in water is given: the in vitro release of g-CSF in 23% w/w ReGel® is described. However, as can be seen from Figure 13, the drug is already released for 60% by the ReGel® system in the first two days. Such so-called burst release is not very advantageous for drugs which require a more constant dosage, such as peptides or anti-inflammatory drugs. For such drugs, a more linear release profile is desired and therefore, it is the object of the invention to provide compositions that are very suitable for controlled release of water soluble drugs (by forming a gel) with a decreased burst release, preferably with a linear release profile.

Within the framework of the invention, with 'water soluble compound' is meant that the solubility of the compound in water measured at 20 °C and at atmospheric pressure (1 bar) is at least 20µg/ml. Solubility in water is determined using the method as described in the Pharmaceutical codex, twelfth edition, page 42, hereby included by reference. For example, the solubility may be determined by shaking an excess of the compound to be dissolved with water at 20 °C at atmospheric pressure (1 bar) until an equilibrium is reached. The solution is then filtered to remove the undissolved compound and the concentration of the dissolved compound is determined by liquid chromatography with UV-absorbance using a calibration curve of the dissolved compound.

This object is achieved by a composition comprising a tri-block copolymer according to formula 1

B-A-B (1)

wherein A stands for a linear poly-(ethylene glycol) block and
wherein B stands for wherein B stands for a poly(lactide-co-ε-caprolactone) block , wherein the hydroxyl end-groups of the tri-block copolymer are at least partially acylated with an optionally substituted acyl having 2 to 12 C-atoms, C-atoms of the substituents included;
an active ingredient, preferably a pharmaceutically active ingredient

and a solvent,
wherein the block ratio of the tri-block copolymer, which ratio is defined as the ratio between the sum of the average molecular weight of the B-blocks and the sum of the average molecular weight of the A-block ranges from 1.4 to 3.5.

Preferably, in the composition of the present invention, the block ratio of the tri-block copolymer ranges from 1.4 to 2.6,
the number average molecular weight (Mn) of the linear poly-(ethylene glycol) block is at least 1250Da and
the acyl is an optionally substituted acetyl, an optionally substituted propionyl, an optionally substituted butyl group, for example an optionally substituted n-butyl or i-butyl group; or an optionally substituted benzoyl group, for example an optionally substituted acetyl, an optionally substituted propionyl or an optionally substituted butyl group.

The inventors have found that a composition according to the invention wherein the hydroxyl end-groups of the tri-block copolymer are at least partially covalently modified (as opposed to ReGel® polymers which have unmodified end-groups) and wherein the block ratio of the tri-block copolymer ranges from 1.4 to 3.5 is very suitable for controlled release of water soluble drugs. The composition according to the invention shows controlled release at a temperature of 30-42 °C (body temperature) for an extended time and hardly shows any burst release of the active ingredient. In many cases, the compositions according to the invention are also capable of a more or less linear release of the active ingredient, preferably both hydrophobic and water soluble drugs, more preferably water soluble drugs.

The terms 'pharmaceutically active ingredient' and 'drug' are used interchangeably herein.

That the composition according to the invention is capable of controlled release for an extended time release with hardly any burst release of the pharmaceutically active ingredient is surprising in view of US 2007/0265356 A1. US 2007/0265356 A1 discloses a thermogelling, aliphatically modified polymer for use in drug delivery. Illustrative embodiments of US 2007/0265356 A1 comprise a composition of matter comprising an aliphatically modified poly(L-lactide-co-ε-caprolactone)-poly-(ethylene glycol)-poly(L-lactide-co- ε-caprolactone), wherein the block ratio of the Mn of the B blocks and the Mn of the A block is 1.13, 1.28, or 1.25 respectively (see Table 1 of US

2007/0265356). US2007/0265356 teaches that phase separation in the body is favourable for drug delivery applications, since it causes spontaneous exclusion of water, thereby increasing the density of the polymeric gel and its stability and extending the drug release.

However, with the composition of the invention, phase separation in the body (temperature from 30 to 42°C) does not occur whereas the composition does show controlled release for an extended time and hardly shows any burst release of the pharmaceutically active ingredient.

Besides a diminished burst release in the first hours or days, also the burst release of the composition of the invention immediately upon injection may be reduced. Another advantage of the composition of the invention may be that the release of the active ingredient, preferably the pharmaceutically active ingredient may be prolonged. Yet another advantage of the composition of the invention may be its ability to stay intact under mild sheer or mild pressure forces inside the body. Yet another advantage of the composition of the invention may be that due to the limited burst release, more drug may be loaded without running the risk of a toxic concentration at the site of injection upon the initial release of the drug.

In the tri-block copolymer according to formula (1)

B-A-B (1)

A stands for a linear poly-(ethylene glycol) block (PEG block). Generally, the number average molecular weight (Mn) of the PEG block in the tri-block copolymer is at least 750Da, for example at least 1000Da, for example at least 1250 Da, 1500 Da and/or preferably at most 5000 Da, for example at most 2000Da. For example, the average molecular weight of the PEG block ranges from 1000 to 5000 Da, preferably from 1000 to 2000 Da, for example from 1000 to 1500 Da. The number average molecular weight of the PEG block is the Mn given by the supplier. The rest of the number average molecular weights as used herein can then be calculated by comparing integrals of the peak of PEG and peaks of incorporated monomers using ^{1}H NMR.

Poly-(ethylene glycol) is a diol also known as poly (ethylene oxide) and both names can be used interchangeably for the purpose of the invention.

B stands for a poly(lactide-co-ε-caprolactone) block (PLCA block), which PLCA block is a copolymer of lactide and ε-caprolactone.Within the framework of the invention the

term lactide refers to all enantiomerically enriched, racemic forms and cyclic ester forms of lactic acid, such as L-lactide, D-lactide and DL-lactide.

The number average molecular weight of each individual PLCA block as determined using ^{1}H NMR as described herein is generally at least 400 Da, for example at least 450 Da, preferably at least 500 Da and/or at most 1500 Da, for example at most 2000 Da, for example at most 2500 Da, preferably at most 3000 Da. For example, the number average molecular weight of the PLCA block ranges from 400 to 3000, for example from 450 to 2000, for example from 500 to 1500 Da.

The block ratio of the tri-block copolymer is defined as the ratio between the sum of the number average molecular weight (Mn) of the B-blocks and the number average molecular weight (Mn) of the A-block, wherein the number average molecular weight of the A-block and of the B-blocks is determined using the information from the supplier for the Mn of the PEG and ^{1}H NMR as described above.

The block ratio may range from at most 3.5, for example at most 3.3, for example at most 3.0, for example at most 2.8, for example at most 2.6, for example at most 2.4 and/or for example at least 1.4, for example at least 1.6, for example at least 1.8, at least 1.9, for example at least 2.0.

Preferably, the block ratio ranges from, for example from 1.4 to 2.6, for example from 1.8 to 2.6, for example from 1.8 to 2.4.

The hydroxyl end-groups of the tri-block copolymer are at least partially acylated with an optionally substituted acyl having 2 to 12 C-atoms, C-atoms of the substituents included. The acyl group may be represented by a compound of formula (2)

$$R^1\text{-}C(O)\text{-} \qquad (2)$$

wherein R^1 stands for an optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl or optionally substituted aryl, preferably for an optionally substituted alkyl or optionally substituted aryl, preferably an optionally substituted alkyl and wherein the R^1-C(O) group is covalently linked to the tri-block copolymer. Examples of R^1 include but are not limited to linear and branched alkyls, for example methyl, ethyl, n-propyl, i-propyl, n-butyl, i-butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl and undecyl; linear and branched alkenyls; and linear and branched alkynyls. R^1 may also stand for the cyclic form of the alkyl, alkenyl or alkynyl. An example of optionally

substituted aryl includes but is not limited to phenyl and halogen, for example iodine substituted phenyls.

Preferably, R^1 stands for an alkyl, preferably for methyl respectively ethyl. (in other words the acyl group is acetyl respectively propionyl).

R^1 may optionally be substituted with a functionalized group, for example with a group containing a heteroatom, for example O, N, for example an NH_2 group, S, for example an SH group, halogen, for example a fluoride, chloride or iodine group, preferably with iodine.

In a special embodiment of the invention, the acyl group may be substituted with a radiopaque atom, for example iodine. For example, an acyl group substituted with a radiopaque atom may be iodobenzoyl.

The radiopaque atoms may be visualized in situ using X-ray, for example 2D or 3D CT (X-ray computed tomography).

The invention therefore also relates to a composition comprising a tri-block copolymer according to formula (1) B-A-B (1)

wherein A stands for a linear poly-(ethylene glycol) block and

wherein B stands for wherein B stands for a poly(lactide-co-ε-caprolactone) block , wherein at least part of the hydroxyl end-groups of the tri-block copolymer are covalently bound to a compound containing radiopaque atoms.

Preferably, the radiopaque atom is iodine.

Preferably, said composition further comprises an active ingredient, preferably a pharmaceutically active ingredient and/or a solvent.

By including a pharmaceutically active ingredient in said composition, it is possible to correlate the degree of gel erosion with the degree of release of pharmaceutically active ingredient from the gel.

Preferably, the block ratio of the tri-block copolymer in said composition, which ratio is defined as the ratio between the sum of the number average molecular weight of the B-blocks and the number average molecular weight of the A-block ranges from 1.4 to 2.6.

By covalently binding the hydroxyl end-groups of the tri-block copolymer to a compound containing radiopaque atoms, many applications of said compositions become available:

For example, it may be visualized where a composition of the invention has been injected. This 3D in situ marking of a location may be useful, for example in radiotherapy for example breast or prostate cancer, where it is necessary to focus the energy beam at exactly the same location for sequential radiation exposures. Current positioning techniques include surgical introduction of small objects of gold, titanium or other electron dense objects that give sufficient contrast in x-ray and CT-imaging. However, these 'contrast agent markers or beckons are permanent and as such less attractive for the patient and may eventually cause scare tissue and bio-mechanic complications.

Also, in situ marking of a location may be useful when during surgery an area is marked, which needs a follow-up. For example, during endoscopic inspection of the colon and other externally accessible parts of the body, small surgical procedures may be performed either on the spot, or during a follow-up session, to burn or cut away tissue, such as small neoplasmas in the colon, which potentially could develop into tumours in the colon. Leaving behind a radiopaque gel depot will greatly facilitate revisiting of the site for inspection for follow-up procedures, thereby using image-guided techniques for positioning.

Treatment and subsequent inspection and follow-up of lesions in the gut, stomach or lung are done at shorter intervals, but again, the in situ marking of a location is desired.

Currently, to actually pin-point and revisit or inspect a previous site of treatment is not easy, as the form, shape and position, for example of the colon, may fold and twist and may alter the external positioning parameters.

Also, the temporary in situ marking of a location in vivo may be useful when successive injections need to be made at or next to previous injection sites. These radiopaque gels are far more compatible to the surrounding tissue than metal objects, and if desired, will erode away over time.

Furthermore, in the composition of the invention, the radiopaque atom stays with the gel since the atom forms part of the tri-block copolymer.This in contrast to other contrasting agents, which will readily diffuse away from the injection site.

As indicated before, the residence time of the composition of the invention can be anything in the range from some days to months.

Preferably, in said compositions, chemical hydrolysis or enzymatic cleavage at

physiological conditions in situ of the tri-block copolymer wherein at least part of the hydroxyl end-groups of the tri-block copolymer is covalently bound to a compound containing one or more radiopaque atoms is limited, for example by choosing a suitable end-group linker and/or a suitable monomer, for example a hydrophobic monomer to linke the compound containing one or more radiopaque atoms to.

Also, the composition of the invention comprising the tri-block copolymer wherein at least part of the hydroxyl end-groups of the tri-block copolymer is covalently bound to a compound containing one or more radiopaque atoms could be used to observe gel-erosion in vivo and to be able to correlate the gel-volume with degree of release of pharmaceutically active ingredient.

The tri-block copolymer wherein at least part of the hydroxyl end-groups of the tri-block copolymer is covalently bound to a compound containing one or more radiopaque atoms may be blended with a tri-block copolymer that does not contain a compound containing one or more radiopaque atoms to fine tune for example i) the quantity, ii) the contrast intensity and iii) the duration of retention on the spot of injection.Modification of the hydroxyl end-groups of the tri-block copolymers in the composition of the invention also offers the possibility to introduce multifunctionality. For example, the hydroxyl group of the tri-block copolymer may be modified with heteroatoms which might increase the affinity of the drug for the micelle (hydrogen bonds) or unsaturation (pi-pi interaction) and/or which are imageable, such as iodo groups, which can be imaged by X-ray or MRI, or may be modified with a compound that interacts with (and could also stabilize) the active ingredient or may be modified with cell-attracting moieties, such as the cell adhesive RGD peptide. The latter may provide a method for recruiting cells into/onto the thermogel after injection into the body.

In another aspect therefore, the invention relates to a method for imaging a position within the body of a warm-blooded species comprising the steps of:

- injecting a composition comprising a tri-block copolymer accordingly to formula (1) B-A-B (1) wherein A stands for a linear poly-(ethylene glycol) block and wherein B stands for a poly(lactide-co-ε-caprolactone) block, wherein at least part of the hydroxyl end-groups of the tri-block copolymer are covalently bound to a compound containing a radiopaque atom of the invention into the body of the warm-blooded species and imaging the composition using conventional imaging methods such as CT or micro CT.

The hydroxyl end-groups of the tri-block copolymer may be acylated using methods known in the art, for example by reaction of the hydroxyl end-groups with an acid chloride or with an anhydride.
For example, acylation of the hydroxyl end-groups of the tri-block copolymer may be done using acetyl chloride (which is an unsubstituted acyl having 2 C-atoms), with propionyl chloride (which is an unsubstituted acyl having 3 C-atoms), etc.
For example, acylation of the hydroxyl end-groups of the tri-block copolymer may be done by reacting with the corresponding anhydride in pyridine at elevated temperature.

With 'at least partially acylated' is meant that the percentage of hydroxyl end-groups that can be acylated (also referred to as the 'degree of modification' or the 'degree of acylation') is higher than 0 and at most 100%; for example the degree of modification of the hydroxyl end-groups is at least 10%, for example at least 25%, for example at least 30%, for example at least 35%, for example at least 40%, for example at least 75% and/or at most 95%, for example at most 90%, preferably 100%.
For the avoidance of doubt, a degree of modification of 100% means that all hydroxyl end-groups of the tri-block copolymer have been modified; a degree of modification of 50% means that half of the hydroxyl end-groups have been modified. The degree of modification, is preferably calculated using ^{1}H nuclear magnetic resonance by comparing integrals of the peaks due to the acylated hydroxyl end-groups and the integrals of the polyethylene glycol (as a measured for the amount of non-acylated hydroxyl end-groups).

The synthesis of the tri-block copolymer as used in the composition of the invention can be done using methods known in the art, for example by ring-opening polymerization or polycondensation reactions. For example, the tri-block copolymer may be synthesized (analogous to) the method described by Seongbong, Jo et al. in 'Reverse thermal gelation of aliphatically modified biodegradable tri-block copolymers', Macromol. Biosci. (2006), pages 923-928, which article is hereby included by reference. Figure 1 as described herein gives the reaction scheme for the synthesis of a tri-block copolymer of PLCA-PEG-PLCA and its acylation of the hydroxyl end-groups with aliphatic acid chloride.

B blocks can be polymerized by using the cyclic monomers mentioned above in a ring-opening polymerization using the hydroxyl end-groups of poly(ethylene glycol) to initiate the polymerization. This is a very controlled and straightforward way of preparing tri-blocks in one step for people skilled in the art. Schemes and details for

similar ring-opening polymerization reactions can be found in several patents or patent applications including and not limited to EP0863745 and WO0018821.

Preparing B blocks by polycondensation reactions using the open form of the cyclic monomers mentioned above, such as lactic acid, glycolic acid, epsilon-hydroxyhexanoic acid and the like is also possible, but less preferred since obtaining well-defined blocks in terms of average molecular weight and end-group functionality with polycondensation reactions is difficult.

Acylation of the hydroxyl end-groups may be done using methods known in the art, for example (analogous to) the method described in Seongbong, Jo et al. in 'Reverse thermal gelation of aliphatically modified biodegradable tri-block copolymers', Macromol. Biosci. (2006), pages 923-928, and as illustrated by figure 1 therein, which article is hereby included by reference.

The number average molecular weight (Mn) of the tri-block copolymer depends on the number average molecular weight of the PCLA and PEG blocks used. The number average molecular weight of the tri-block copolymer may be calculated using ^1H nuclear magnetic resonance. Preferably the Mn of the tri-block copolymer ranges from 3,000 to 5,000 Da.

The active ingredient in the composition of the present invention may be an active ingredient such as any pharmaceutically active ingredient and any diagnostic and any contrast agent and includes those pharmaceutically active ingredients having a prophylactic effect on the animal, including human as well as those pharmaceutically active ingredients that have an effect of alleviating, reducing or even completely eliminating a symptom, or a cause, or a consequence of a disease, such as pain, swelling or inflammation or a disease from the animal, including human.
For example, the pharmaceutically active ingredient may include broad classes of compounds normally delivered into the body. For example, these pharmaceutically active ingredients include but are not limited to anti-infectives (including antibiotics, antivirals, fungicides, scabicides or pediculicides); antiseptics (e.g. benzalkonium chloride, benzethonium chloride, chorhexidine gluconate, mafenide acetate, methylbenzethonium chloride, nitrofurazone, nitromersol and the like); analgesics and analgesic combinations; anorexics; antihelminthics, antiarthritics, antiasthmatic agents; anticonvulsants; antidepressants; antidiabetic agents; antidiarrheals; antihistamines; anti-inflammatory agents, antimigraine preparations; antinauseants; antineoplastics; antiparkinsonism drugs; antipuritics; antipsychotics; antipyretics, antispasmodics; anticholinergics; sympathomimetics; xanthine derivatives; cardiovascular preparations

including potassium and calcium channel blockers; beta-blockers; alpha-blockers and antiarrhythmics; antihypertensives; diuretics and antidiuretics; vasodilators including general coronary, peripheral and cerebral vasodilators; central nervous system stimulants; vasoconstrictors; cough and cold preparations, including decongestants; hormones and steroids (e.g. estrogens, progestins, androgens, adrenocorticoids, corticosteroids and the like); hypnotics; immunosuppressives; muscle relaxants; parasympatholytics; psychostimulants; sedatives and tranquilizers, narcotics (e.g. morphine, meperidine, codeine and the like), local anesthetics (e.g. amide- or anilide-type local anesthetics such as bupivacaine, dibucaine, mepivacaine, procaine, lidocaine, tetracaine and the like); antiemetic agents (e.g. ondansetron, granisetron, tropisetron, metoclopramide, domperidone, scopolamide and the like); antiangiogenic agents (e.g. combrestatine, contortrostatin, anti-VEGF and the like), polysaccharides, immune-modulating, anti-thrombogenic compounds, anti-claudicating drugs, anti-atherosclerotic drugs, antihistamines, anti-cancer drugs (e.g. mechlorethamine, cyclophosphamide, fluorouracil, thioguanine, carmustine, lomustine, melphalan, chloambucil, streptozocin, methotrexate, vincristine, bleomycin, vinblastine, vindesine, dactinomycine, daunorubicin, doxorubicin, tamoxifen, paclitaxel, epirubicin, mitomicin C, cis-platin, carboplatin, and the like and photosensitizers used in photodynamic therapy, vascular drugs, ophthalmic drugs, amino acids, vitamins, neurotransmitters, neurohormones, signaling molecules, psychoactive medicaments, synthetic drugs, semi-synthetic drugs, natural drugs and substances derived from these, or combinations of the above.

The pharmaceutically active ingredient may also be a biological including but not limited to (recombinant) proteins, PEGylated-proteins and peptides (e.g. insulin, erythropoietin, exenatide, glucagon-like-peptide-1, morphogenic proteins (e.g. bone morphogenic proteins, transforming growth factors, fibroblast growth factors, tumor necrosis factors), receptor antagonists (e.g. Interleukin-1-receptor-antagonist), anticancer proteins (e.g. neocarzinostatin, *L*-asparaginase, interleukin-2, bevacizumab and other anti-VEGF agents) prophylactic vaccines, therapeutic vaccines, genetic materials (e.g. nucleic acid sequences, polynucleotides, (antisense) oligonucleotides, plasmids, DNA, RNA, siRNA, microRNA), aptamers, enzymes, antigens, antibodies, antibody fragments, viruses, virus-based materials, cells, cellular substructures, etc.),

Prodrugs, metabolites, derivatives, in-vivo or in in-vitro chemically modified products, in-vivo or in-vitro enzymatic modified products and pharmaceutically active degradation products of the pharmaceutical active ingredients described herein are included in the scope of the invention.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of immune-modifying drugs, anti-inflammatory drugs or growth factors.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of immune-modifying drugs for example cyclosporine, tacrolimus (FK-506), sirolimus or rapamycin.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of steroidal anti-inflammatory drugs, for example prednisone, prednisolon, triamcinolon, clobetasol or betamethason.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of non-steroidal anti-inflammatory drugs, for example aspirin, diclofenac, piroxicam, meloxicam, ibuprofen or a selective COX-2 inhibitor for example celecoxib, valdecoxib, etoricoxib or rofecoxib.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of anticancer agents for example bevacizumab, tamoxifen or interleukin-2.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of anti-viral agents for example acyclovir or oseltamivir.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of anti-bacterial agents for example amoxicillin.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of anti-diabetic agents for example insulin, glucagon-like-peptide-1, exenatide.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of vaccines.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of ophthalmic agents for example Triamcinolone and Bevacizumab.

Preferably, the active ingredient is a pharmaceutically active ingredient effective against forms of neuro-degenerative diseases such as apomorphine, rivastigmine, pramipexole, pioglitazone, memantine and safinamide

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of biologicals including but not limited to growth factors which are very suitable for application in orthopedics and in particular in the prevention or treatment of diseases of intervertebral discs, or cartilage, or bone. Examples of such growth factors include but are not limited to transforming growth factor 3, fibroblast growth factor 18, osteogenic protein 1, bone morphogenic protein 2, bone morphogenic protein 6, bone morphogenic protein 7, interleukin-1-receptor-antagonist .

Preferably, the active ingredient belongs to the class of human growth hormones and its biosimilar derivatives, which can be applied in both pediatric and adult growth disorders, maintanance of sufficient musculature, and for anti-ageing applications.

Preferably, the active ingredient is a pharmaceutically active ingredient effective against inflammation or microbial infections of the inner ear and its connecting tissues, (intratympanic ear diseases).

Preferably, the active ingredient is a pharmaceutically active ingredient effective against forms of diabetes, for example glucagon-like-peptide-1, and its derivatives such as exendin-4 and liraglutide.

For the active ingredient which are water soluble, the drug preferably has a solubility in water of at least 20, for example of at least 100, for example of at least 500µg/ml, for example of at least 1000 µg/ml, for example of at least 5000 µg/ml in water measured at 20°C and at atmospheric pressure (1 bar).

Examples of water soluble active ingredients include small molecules (of up to 5,000 Da), medium sized molecules (of up to 10,000 Da), but also large molecules (of at least 10,000 Da), such as proteins. These water soluble active ingredients may be synthesized chemically, but may also be a biological including but not limited to (recombinant) proteins and peptides (e.g. insulin, erythropoietin, exenatide, glucagon-like-peptide-1, morphogenic proteins (e.g. bone morphogenic proteins, transforming growth factors, fibroblast growth factors, tumor necrosis factors), receptor antagonists (e.g. Interleukin-1-receptor-antagonist), anticancer proteins (e.g. neocarzinostatin, *L*-asparaginase, interleukin-2, bevacizumab and other anti-VEGF agents) prophylactic vaccines, therapeutic vaccines, genetic materials (e.g. nucleic acid sequences, polynucleotides, (antisense) oligonucleotides, plasmids, DNA, RNA, siRNA, microRNA), aptamers, enzymes, antigens, antibodies, antibody fragments, viruses, virus-based materials, cells, cellular substructures, etc.).

Therefore, the invention also relates to a composition according to the invention, wherein the active ingredient is a pharmaceutically active ingredient selected from the group of water soluble drugs, that is drugs that have a solubility in water of at least 20µg/ml as determined using the method described herein.

The invention also relates to a composition, wherein the composition further comprises nano-particles and/ or microparticles (such as liposomes and microspheres) which particles contain any of the Pharmaceutically active ingredients as described above.

The solvent in the composition of the present invention is preferably a solvent that is non toxic, preferably also biocompatible, and approved by regulatory instances, preferably aqueous based or a so-called FDA (Federal Drug Administration) class 3 solvent.
Examples of solvents include but are not limited to water, mixtures of water and an organic solvent like for example ethanol, isopropanol or dimethylsulfoxide (DMSO); aqueous buffer solutions, preferably aqueous buffer solutions that lead to isotonic compositions of the invention, such as PBS (phosphate buffered saline) or Sorenson modified buffer or variants thereof; and organic solvents, such as ethylacetate, acetone, dichloromethane (DCM), dimethylsulfoxide (DMSO), N-methylpyrrolidone (NMP), isopropyl myristate and benzylbenzoate.

The compositions as described in this patent application are of use in all diseases as classified by the World Health Organization, Document ICD-10 (2007) and where the dosing of pharmaceutical active agents as described above is common clinical or experimental practise in all animals including humans.

With 'isotonic' is meant that the solution a solution having an osmotic pressure comparable to or at least compatible with the osmotic pressure of human or animal body fluids, preferably with blood.

Preferably the solvent is water or an aqueous buffer solution, more preferably an aqueous buffer solution that leads to an isotonic composition of the invention.

The pH of the solvent is preferably in the range from 5 to 8, for example in the range from 6.5 to 7.5. More preferably, the pH of the solvent is chosen such that the pH of the composition of the invention is about neutral (in the range from 5 to 8, preferably in the range from 6.5 to 7.5).

With gel temperature is meant the temperature at which the tri-block copolymer in the solvent chosen forms a gel. Vial tilting as described below can be used to determine the gel temperature.

The gel temperature of the composition of the invention is preferably at least 20°C, more preferably at least 25°C, for example at least 30°C and/or preferably at most 36, for example at most 35, for example at most 34°C. For example, the gel temperature of the composition of the invention ranges from 30 to 35°C.

With phase separation temperature is meant the temperature at which the different components present are not capable of forming a homogeneous solution, but instead form separate (liquid) phases. Phase separation can be determined using vial tilting. To this end, the compositions of the invention are vortexed and stored at 5°C. If needed, the samples were subjected to another heating and cooling cycle until all components in the composition of the invention were completely dissolved. The samples were evaluated by vial tilting at 5°C, then allowed to equilibrate at 20°C for 30 minutes after which they were evaluated again. Visual observations were made and noted down. Next, the samples were placed in a water bath and slowly heated (less than 0.5°C/min). At every 1°C increment, samples were evaluated by vial tilting and considered a gel if no flow was observed during a period of 15seconds. Samples were heated until phase separation was observed and the temperature at which phase separation was observed is the 'phase separation temperature'.
Preferably, the phase separation temperature of the composition of the invention is at least 40°C, for example at least 41°C, for example at least 42°C.

Preferably, the composition of the invention has a gel temperature in the range from 25 to 25°C, for example from 30 to 35°C and a phase separation temperature of at least 42°C.

Preferably, for clinical applicability, the tri-block copolymer has a gel window between 25 and 50°C, for example between 30°C and 50°C, for example between 30°C and 42°C. With 'gel window' is meant the 'window between the gel temperature and the phase separation temperature', in other words it is the entire temperature range in which the tri-block copolymer is a gel.

The concentration of the tri-block copolymer in the composition is for example at least 15% w/w, for example at least 17% w/w, for example at least 19% w/w, for example at least 20%w/w, for example at least 21% w/w, preferably at least 22% w/w, preferably at least 23% w/w, preferably at least 24% w/w, preferably at least 25% w/w based on the

amount of solvent and active ingredient present in the composition, more preferably at least 27% w/w, even more preferably at least 30% w/w, for example at least 31% w/w, for example at least 32% w/w, for example at least 33% w/w, for example at least 34% w/w, for example at least 35% w/w, for example at least 36% w/w and/or preferably at most 50% w/w, for example at most 45% w/w, for example at most 44% w/w, for example at most 43% w/w, for example at most 42% w/w, for example at most 41% w/w, for example at most 40% w/w, for example at most 39% w/w based on the amount of solvent and active ingredient present in the composition.
For example, the concentration of the tri-block copolymer in the composition of the invention may range from 15 to 40% w/w, for example from 25 to 35% w/w based on the amount of solvent and active ingredient present in the composition.

If a higher concentration of tri-block copolymer is used, it may be that the composition can be loaded with higher amounts of (hydrophobic) drugs as compared to the loading capacity of the same tri-block copolymer at a lower concentration. This would especially be beneficial for patients having a chronic disease as it would allow to decrease the number of injections a patient requires even further. Also, or furthermore, a higher loading capacity may mean that the volume to be injected can be decreased. This would for example be beneficial for injections into areas where space is limited, for example for injections into joints or into ocular, intra-cranial cavities or intervertebral discs.

The tri-block copolymer in the composition of the invention may be varied on many parameters. Examples of variations that are possible in the tri-block copolymer include but are not limited to the Mn of the tri-block copolymer, Mn of the PCLA block, Mn of the PEG block, the choice of alkyl with which the hydroxyl end-groups of the tri-block copolymer may be covalently modified, the degree of modification of the hydroxyl end-groups of the tri-block copolymer, the block ratio and the weight ratio of ε-caprolactone and lactide in the PCLA block.

The weight ratio of ε-caprolactone to lactide is the weight of ε-caprolactone divided by the weight of lactide used to prepare the B-block with, in case the B-block is PLCA. The weight ratio of ε-caprolactone to lactide is for example from 1/9 to 9/1, for example from 1/4 to 4/1. Preferably the weight ratio of ε-caprolactone to lactide is at least 1/1, for example from 1/1 to 1/0, for example from 1/1 to 9/1.
For the avoidance of doubt, with weight ratio of ε-caprolactone to lactide is 1/0 is meant that only I only ε-caprolactone is present and no lactide. With weight ratio of ε-

caprolactone to lactide is 0/1 is meant that only lactide is present and no ε-caprolactone.

In a special embodiment, the invention relates to a composition of the invention, wherein the block ratio of the tri-block copolymer, which block ratio is defined as the ratio between the sum of the number average molecular weight of the B-blocks and the sum of the number average molecular weight of the A-blocks ranges from 1.8 to 2.4, wherein the ratio of ε-caprolactone /lactide in the tri-block copolymer ranges from 1/1 to 1/0 and wherein at least 10%, preferably at least 25% and most preferably at least 50% of the hydroxyl end-groups of the tri-block copolymer is modified.

The amount of active ingredient in the composition of the present invention depends on the amount to be administered to the animal, including human and on the duration of the release. For example, the amount of active ingredient may be up to and including 50% w/w based on the tri-block copolymer if a high loading of the drug is desired, but may also for example be less, for example an amount of up to and including 40, for example up to and including 30, for example up to and including 20% w/w based on the tri-block copolymer and/or for example at least 0.01% w/w, preferably at least 0.1 % w/w, for example at least 1% w/w based on the tri-block copolymer.

The invention also relates to a composition of the invention, wherein the composition is a pharmaceutical composition further comprising a pharmaceutically acceptable adjuvant, carrier or excipient.

A person skilled in the art knows which carriers can be used as pharmaceutically acceptable carriers. Examples of such pharmaceutically acceptable carriers are both inorganic and organic carrier materials, suitable for oral/parenteral/injectable administration and include water, polyethyleneglycol (PEG), polypropyleneglycol (PPG), cellulose derivatives, such as hydroxypropylmethylcellulose (HPMC), hydroxyethylmethylcellulose (HEMC), hydroxyethylcellulose (HEC); polyvinylpyrrolidone (PVP), polyvinylalcohol (PVA), gelatin, gum arabic, lactose, starch, magnesium stearate, talc, vegetable oils, and the like.

Also, the person skilled in the art knows which pharmaceutically acceptable adjuvants and excipients may be used in the compositions of the invention. Examples of conventional pharmaceutical additives and adjuvants, excipients or diluents, including, but not limited to, water, gelatin of any origin, polyethyleneglycol (PEG), polypropyleneglycol (PPG); cellulose derivatives, such as hydroxypropylmethylcellulose (HPMC), hydroxyethylmethylcellulose (HEMC) or

hydroxyethylcellulose (HEC); polyvinylpyrrolidone (PVP), polyvinylalcohol (PVA), vegetable gums, ligninsulfonate, talc, sugars, starch, gum Arabic, vegetable oils, polyalkylene glycols, flavoring agents, preservatives, stabilizers, emulsifying agents, buffers, lubricants, colorants, wetting agents, fillers, imaging agents, such as contrast agents for X-ray or MRI; and the like, for exampleiodo compounds, such as the commercially available Xenetic® and Hexabrix®.

In another aspect, the invention relates to a composition according to the invention for use as a medicament.

In another aspect, the invention relates to a composition according to the invention for use as a medicament that is injected through a needle of at least 14G, for example at least 18G, for example at least 21G, preferably at least 23G, for example at least 27G, for example at least 31G.

With G is meant 'Gauge' which is a measure for the outer diameter of a needle. Smaller gauge numbers indicate larger outer diameters and as such 'larger needles'. For ease of injection, smaller needles are preferred to larger needles. Inner diameters of a needle depend on both gauge and wall thickness.

Depending on the pharmaceutically active ingredient or combination of pharmaceutically ingredients present in the composition of the invention, different diseases may be targeted, preferably treated using the composition(s) of the present invention.

In yet another aspect, the invention relates to a process for the preparation of a composition of the invention comprising the steps of

- synthesizing the tri-block copolymer and
- mixing the tri-block copolymer with the active ingredient and the solvent.

The order in which the tri-block copolymer and the active ingredient are dissolved in the solvent is in principle not critical; it is for example possible to first dissolve the tri-block copolymer and then the active ingredient, to first dissolve the active ingredient and then the tri-block copolymer or for example to dissolve both the active ingredient and the tri-block copolymer at the same time in the solvent of choice.

However, for active ingredients that are highly soluble in the solvent, preferably first the active ingredient is dissolved in the solvent to form a solution of the active ingredient in the solvent, after which the tri-block copolymer is dissolved into said solution or the polymer is dissolved at a higher concentration, when polymer dissolved a concentrated

drug solution in the buffer of choice is added to get to the right polymer and drug concentration.

For active ingredients that are less soluble in the solvent of choice, the active ingredient may first be dissolved into a solvent in which it is better soluble (for example a hydrophobic drug may be soluble in ethylacetate) together with the polymer, after which the solvent is evaporated and the solvent of choice is added to the (dried) polymer/drug mixture. This will increase the solubility of the active ingredient in the solvent of choice.

The invention can also contain and release micro- or nano-sized particles such as microspheres and liposomes which already contain for instance drugs, nucleotide sequences or imaging agents.

The benefit of combining the invention with these micro-or nano-sized particles is that the invention will ensure localization and gradual release of these particles, instead of a typical migration of such particles from the place of injection.

To facilitate dissolving the tri-block copolymer and/or the active ingredient the composition comprising the tri-block copolymer, the solvent and the active ingredient may be heated for example until the tri-block polymer melts. For example, the heating of the composition may be performed at 50°C.

Preferably, if the active ingredient is sensitive to heat, the tri-block copolymer may be melted (for example at a temperature of about 50°C), dissolved into the solvent of choice after which the obtained solution may be cooled down and the active ingredient may be dissolved in the solvent.

In another aspect, the invention relates to a method for delivering an pharmaceutically active ingredient over an extended period, for example over a period of more than 1 day up to several, e.g. 6, months, to an animal, including a human requiring such treatment which comprises administering to such animal an effective amount of the composition of the invention.

In yet another aspect, the invention relates to a method for delivering an pharmaceutically active ingredient over an extended period, for example over a period of more than 1 day up to several, e.g. 6, months, to an animal, including a human requiring such treatment which comprises administering to such animal an effective amount of the composition of the invention through a needle of at least 14G, for example at least 18G, for example at least 21G preferably at least 23G, for example at least 27G, for example at least 31G.

In the framework of the invention, with animals is meant all animals, including mammals, examples of which include humans. Preferred examples of mammals beside humans include but are not limited to dogs, cats, dromedaris, camels, elephants, goats, mice, guinea pigs, rabbits, pigs, cows, water buffalos, kangaroos, monkeys and horses.

Although the invention has been described in detail for purposes of illustration, it is understood that such detail is solely for that purpose and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention as defined in the claims.

It is further noted that the invention relates to all possible combinations of features described herein, preferred in particular are those combinations of features that are present in the claims.

It is further noted that the term 'comprising' does not exclude the presence of other elements. However, it is also to be understood that a description on a product comprising certain components also discloses a product consisting of these components. Similarly, it is also to be understood that a description on a process comprising certain steps also discloses a process consisting of these steps.

The invention is now elucidated by way of the following examples, without however being limited thereto.

Examples

Measurement methods

The tri-block copolymer composition (PLCA/PEG ratio, cap/lac ratio and DM) was determined with proton nuclear magnetic resonance (^{1}H NMR; Varian, 400 MHz), using deuterated chloroform as solvent and reference. From the integration of various proton signals (due to PEG and incorporated monomers), absolute number average molecular weights M_n were obtained. Mn of the tri-block copolymer is the sum of the molecular weights of the central PEG block and the two polyester blocks (ratio of blocks determined with NMR).

The block ratio as used herein is the weight ratio of the PLCA-blocks to the PEG-block (of known molecular weight) and can be calculated from grams of monomers (lactide + caprolactone) divided by grams of PEG used to synthesize the tri-block copolymer. Final tri-block composition after polymer purification was checked with ^{1}H NMR by comparing integrals of peaks due to PEG and incorporated ring-opened monomers.

The weight ratio of ε-caprolactone to L-lactide can be calculated from ^{1}H NMR by comparing integrals of peaks due to ring-opened lactide and caprolactone
The integrals of peaks due to acyl endgroup and PEG block were used to calculate the degree of modification (range 0-2, which corresponds to a degree of acylation of 0 to 100%).

Example 1: Preparation of the tri-block copolymer #33: acylated (C2-modified) PLCA-PEG-PLCA

Polyethyleneglycol (PEG 1500, 12.5 g, 8.3 mmol) and ca. 100 ml toluene were charged into a 250 ml three-neck round-bottom flask equipped with a magnetic stirring bar. Using a Dean-Stark device with a cooler on top, 40 ml of toluene was distilled off to remove water (from PEG) azeotropically by heating at 150°C at atmospheric pressure (1 bar) under nitrogen.

After cooling down the solution to ca. 80-100°C, L-lactide (2.75 g, 19 mmol) and caprolactone (24.75 g, 217 mmol) were added. 40 ml of toluene was distilled off to dry the monomers by heating at 150°C at atmospheric pressure. Ca. 20 ml of dry toluene was left in the flask for the polymerization.

After cooling down the mixture to ca.80-100°C, tin(II) 2-ethylhexanoate (0.25 ml) was added through one of the necks.

Polymerization was carried out at 120°C for 1 day under nitrogen atmosphere.

After cooling down to room temperature, ca. 70 ml dichloromethane and 5.6 ml triethylamine (40 mmol) were added. Subsequently, 2.5 ml acetyl chloride (33 mmol) was added slowly to the stirred solution, which was cooled with an ice bath. The acylation reaction was continued for a few hours after which dichloromethane was removed by rotavap, and ethyl acetate (ca. 100 ml) was added to the residue. Triethylamine salt was removed by (paper) filtration and the polymer dissolved in the clear filtrate was precipitated by addition of a mixture of hexane and diethyl ether. At ca. - 20 °C (in freezer) the polymer product separated as a waxy solid from which non-solvents could be decanted easily. Finally, the precipitated polymer was dried in vacuo. Yield: ca. 37 g.The PLCA/PEG block ratio was around 2.2; the ε-caprolactone/lactide ratio was 9/1.

Example 2. Preparation of the tri-block copolymer #27

In a manner similar to example 1 an acylated PLCA-PEG-PLCA tri-block was synthesized having a PLCA/PEG ratio of 1.8 and a caprolactone/lactide weight ratio 9/1. The differences with example 1 were: different PLCA/PEG ratio and different caprolactone/lactide weight ratio.

Example 3. Preparation of the tri-block copolymer #17

In a manner similar to example 1 an acylated PLCA-PEG-PLCA tri-block was synthesized having a PLCA/PEG ratio of 1.8 and a caprolactone/lactide weight ratio 9/1. The difference with example 1 was: different PLCA/PEG ratio.

Example 4 Fully C2-modified PLCA-PEG-PLCA (#21)

Polyethyleneglycol (PEG 1500, 10 g, 6.7 mmol) and ca. 110 ml toluene were charged into a 250 ml three-neck round-bottom flask equipped with a magnetic stirring bar. Using a Dean-Stark device with a condenser on top, 45 ml of toluene was distilled off to remove water (from PEG) azeotropically by heating at 150°C at atmospheric pressure under nitrogen.

After cooling down the solution to ca. 80-100°C, L-lactide (2 g, 14 mmol) and caprolactone (18 g, 158 mmol) were added. 40 ml of toluene was distilled off to dry the monomers by heating at 150°C at atmospheric pressure. Ca. 25 ml of dry toluene was left in the flask for the polymerization.

After cooling down the mixture to ca.80-100°C, tin(II) 2-ethylhexanoate (0.2 ml) was added through one of the necks.

Polymerization was carried out at 120°C for 1 day under nitrogen atmosphere.

After cooling down to room temperature, ca. 60 ml dichloromethane and 4.7 ml triethylamine (34 mmol) were added. Subsequently, 2 ml acetyl chloride (28 mmol) was added slowly to the stirred solution, which was cooled with an ice bath. The acylation reaction was continued for a few hours after which dichloromethane was removed by rotavap, and ethyl acetate (ca. 100 ml) was added to the residue. Triethylamine salt was removed by (paper) filtration and the polymer, which was dissolved in the clear filtrate was precipitated by addition of a (1:1) mixture of hexane and diethyl ether. At ca. - 20 °C (in freezer) the polymer product separated as a waxy solid from which non-solvents could be decanted easily. Finally, the precipitated polymer was dried in vacuo. Yield: ca. 27 g.

The PLCA/PEG block ratio is around 2; caprolactone/lactide weight ratio 9/1. Degree of modification (from NMR) is ca. 2.

Example 5. Synthesis comparative tri-block copolymer U5.

This is the polymer that is similar to the one mentioned in the examples of US2007/0265356 (same block ratio, but different caplac).

Polyethyleneglycol (PEG 1500, 12.5 g, 8.3 mmol) and ca. 125 ml toluene were charged into a 250 ml three-neck round-bottom flask equipped with a magnetic stirring bar. Using a Dean-Stark device with a condenser on top, 60 ml of toluene was distilled off to remove water (from PEG) azeotropically by heating at 150°C at atmospheric pressure under nitrogen.

After cooling down the solution to ca. 80-100°C, L-lactide (3 g, 21 mmol) and caprolactone (12 g, 105 mmol) were added. 40 ml of toluene was distilled off to dry the monomers by heating at 150°C at atmospheric pressure. Ca. 25 ml of dry toluene was left in the flask for the polymerization.

After cooling down the mixture to ca.80-100°C, tin(II) 2-ethylhexanoate (0.2 ml) was added through one of the necks.

Polymerization was carried out at 120°C for 1 day under nitrogen atmosphere.

After cooling down to room temperature, ca. 50 ml dichloromethane and 2.5 ml triethylamine (18 mmol) were added. Subsequently, 2.1 ml hexanoyl chloride (15 mmol) was added slowly to the stirred solution, which was cooled with an ice bath. The acylation reaction was continued for a few hours after which dichloromethane was removed by rotavap, and ethyl acetate (ca. 100 ml) was added to the residue. Triethylamine salt was removed by (paper) filtration and the polymer, which was dissolved in the clear filtrate was precipitated by addition of a (1:1) mixture of hexane and diethyl ether. At ca. - 20 °C (in freezer) the polymer product separated as a waxy solid from which non-solvents could be decanted easily. Finally, the precipitated polymer was dried in vacuo. Yield: ca. 27 g.

The PLCA/PEG block ratio of the comparative U5 block copolymer is around 1.2; caprolactone/lactide weight ratio 4/1. Degree of modification (from NMR) is ca. 1.7

Properties of the polymer are listed in table 1 below.

Table 1. Characteristics of tri-block copolymers #33, #27, #21, #17 and U5

Tri-block copolymer	Block ratio (PLCA/PEG ratio)	Weight ratio of ε-caprolactone to lactide (cap/lac ratio)	Mn as determined using ^{1}H NMR	End-group	Degree of acylation (degree of modification, DM) (%)
Comparative U5 polymer	1.2	4/1	3300	Hexanoyl (C6)	75
PLCA/PEG2.2-CL/LA 4/1-C2 (#17)	2.2	4/1	5300	Acetyl (C2)	100
PLCA/PEG2.0-CL/LA 9/1-C2 (#21)	2.0	9/1	4700	Acetyl (C2)	100
PLCA/PEG1.8-CL/LA 9/1-C2 (#27)	1.8	9/1	4200	Acetyl (C2)	100
PLCA/PEG2.2 CL/LA 9/1-C2 (#33)	2.2	9/1	5300	Acetyl (C2)	100

Example 6 Release of model drugs in PBS

This example illustrates the release profile of water soluble and hydrophobic model drugs from tri-block copolymer solutions of the present invention.

The following water soluble model proteins were used:
(1) IgG antibody (Sigma); this is a protein of around 150kDa and has a solubility in water is above 10000µg/ml.
(2) bovine serum albumin (Sigma); this is a protein of around 40kDa and has a solubility in water of above 10000µg/ml.
(3) lysozyme (Sigma); this is a protein of around 16kDa and has a solubility in water of above 10000µg/ml.

The following small molecules were used:

(1) celecoxib (LC Laboratories); this is a non-steroidal anti-inflammatory drug of 386 g/mol and has a solubility in water below 1 ug/ml

(2) triamcinolone acetonide (Sigma); this is a corticosteroid and has a solubility in water below 20 ug/ml

(3) iobitridol (Guerbet); this is a contrast agent of around 700 g/moland has a solubility in water above 5000 ug/ml.

The following peptide was used:

(1) Cyclosporin A (Sigma) ; this is a non-natural cyclic peptideof around 1000 g/mol amd has a solubility is water below 5 ug/ml

Polymer solutions were prepared with the tri-block copolymers described in Table 1. The tri-block copolymers were weighted in 7-ml glass vial and melted at 50°C using a water bath. Protein stock solutions in PBS (phosphate buffer saline; B. Braun, 10 mM, pH = 7.4) were added to the molten polymer to achieve the required polymer content in the solutions containing 1% (w/v) protein. The mixtures of protein, tri-block copolymer and PBS were vortexed for 1 min and placed on a rotating wheel at 4°C overnight.

For the hydrophobic drugs, a mixture drug/polymer was first prepared in ethylaacetate. Ethylacetate was evaporated and PBS was added.

With a syringe, 300 µl of the solutions were placed in glass vials (8.2 x 40 mm). The glass vials were incubated for 30 min at the required release temperature to allow gelation. Pre-heated PBS was added on top of the gel. At regular time intervals, the buffer was removed, followed by the addition of fresh PBS. The removed buffer samples were analyzed for their protein content by either UPLC (for BSA and lysozyme) or bicinchoninic acid assay (BCA®) protein assay (for IgG).

IgG was analysed by BCA® protein assay: IgG release samples (25 µL) were pipetted into a 96-microwells plate and 200 µL of working reagent (BCA reagent A: BCA reagent B, 50/1 v/v) was added. After incubation of the plates for 30 minutes at 37°C, the absorbance was measured at 550 nm with a NovapathTM Microplate Reader (Bio-Rad). Standard protein solutions (concentration range: 0.01-1 mg/mL) were prepared to generate a calibration line.

BSA was analysed by UPLC: BSA release samples were analyzed using a Waters AcquityTM Ultra Performance LC system equipped with an AcquityTM BEH300 C18 1.7 µm column (5 cm), a binary solvent manager, a sample manager with column oven at 50°C and an AcquityTM TUV detector (detection wavelength: 210 nm). After injection of 5 µL release sample, a gradient was run from 80 % A (H2O/ACN 95/5 (% v/v) containing 0.1 % TFA) to 60 % B (100 % ACN with 0.1 % TFA) in 2 minutes at a

flow rate of 0.25 mL/min. Standard protein solutions (concentration range: 0.01-1 mg/mL) were used to obtain a calibration line.

Lysozyme was analysed by UPLC: lysozyme release samples were analyzed using a Waters AcquityTM Ultra Performance LC system equipped with an AcquityTM BEH300 C18 1.7 µm column (5 cm), a binary solvent manager, a sample manager with column oven at 50 °C and an AcquityTM TUV detector (detection wavelength: 280 nm). After injection of 5 µL release sample, a gradient was run from 75 % A (H2O/ACN 95/5 (% v/v) containing 0.1 % TFA) to 40 % B (100 % ACN with 0.1 % TFA) in 4 minutes at a flow rate of 0.25 mL/min. Standard protein solutions (concentration range: 0.01-1 mg/mL) were used to obtain a calibration line.

Celecoxib was analysed by UPLC: Celecoxib concentration in the buffer was measured by a Waters AcquityTm) Ultra Performance LC system (UPLC, Waters, Milford MA, USA) equipped with an Acquity™ BEH C18 1.7 µm column (2.1 x 100 mm), a binary solvent manager, a sample manager with column oven at 50 oC and an Acquity™ TUV Detector (detection wavelength: 254 nm). After injection of 7.5 µl release sample, a gradient was run from 100% A (H2O/ACN 95/5% (v/v) containing 0.1% TFA) to 100 % B (MeOH/ACN/H2O 45/45/10% (v/v) containing 0.1% TFA) in 2 minutes and kept at 100% B from 10 min at a flow rate of 0.08 ml/min. The run time was 16 min. A calibration curve was obtained after injection of standard celecoxib solutions in DMSO (0.5 – 100 ug/ml). The chromatograms were analused using Empower Software Version 1154 (Waters, Milford MA, USA). CLB concentration in the release sample was in the range 5-20 µg/ml.

Triamcinolone acetonide (TA) was analysed by UPLC: TA concentration in the buffer was measured by a Waters Acquity™ Ultra Performance LC system (UPLC, Waters, Milford MA, USA) equipped with an Acquity™ BEH C18 1.7 µm column (2.1 x 50 mm), a binary solvent manager, a sample manager with column oven at 50 oC and an Acquity™ TUV Detector (detection wavelength: 240 nm). After injection of 7.5 µl release sample, a gradient was run from 50% A (H2O/ACN 95/5% (v/v) containing 0.1% TFA) to 80 % B (ACN/H2O 95/10% (v/v) containing 0.1% TFA) in 1.5 minutes at a flow rate of 0.25 ml/min. The run time was 3.2 min. A calibration curve was obtained after injection of standard TA solutions in DMSO (0.5 – 100 ug/ml). The chromatograms were analused using Empower Software Version 1154 (Waters, Milford MA, USA). TA concentration in the release sample was in the range 5-20 µg/ml.

Iobitriodol (ITD) was analysed by UPLC: ITD concentration in the buffer was measured by a Waters AcquityTm) Ultra Performance LC system (UPLC, Waters, Milford MA, USA) equipped with an Acquity™ HSS 1.7 µm column (2.1 x 50 mm), a binary solvent manager, a sample manager with column oven at 50 oC and an Acquity™ TUV Detector (detection wavelength: 204 nm). After injection of 7.5 µl release sample, a gradient was run from 100% A (H2O containing 0.1% TFA) to 48 % B (IPA containing 0.1% TFA) in 0.9 minutes at a flow rate of 0.25 ml/min. The run time was 2 min. A calibration curve was obtained after injection of standard ITD solutions in PBS (0.5 – 100 ug/ml). The chromatograms were analysed using Empower Software Version 1154 (Waters, Milford MA, USA). ITD concentration in the release sample was in the range 5-20 µg/ml.

Cyclosporin A (CyA)was analysed by UPLC: CyA concentration in the buffer was measured by a Waters AcquityTm) Ultra Performance LC system (UPLC, Waters, Milford MA, USA) equipped with an Acquity™ BEH C4 1.7 µm column (2.1 x 50 mm), a binary solvent manager, a sample manager with column oven at 50 oC and an Acquity™ TUV Detector (detection wavelength: 220 nm). After injection of 7.5 µl release sample, a gradient was run from 100% A (H2O/ACN 95/5% (v/v) containing 0.1% TFA) to 100 % B (ACN/H2O 95/10% (v/v) containing 0.1% TFA) in 1 minutes at a flow rate of 0.25 ml/min. The run time was 3.6 min. A calibration curve was obtained after injection of standard CyA solutions in DMSO (0.5 – 100 ug/ml). The chromatograms were analysed using Empower Software Version 1154 (Waters, Milford MA, USA). CyA concentration in the release sample was in the range 5-20 µg/ml.

The results of this example are shown in Figures 1 to 4.

Example 7

Synthesis of B-A-B block

The syntheses of the compositions were conducted as per Example 4.

Table 2 Characteristics of tri-block copolymers

ID	Tri-block copolymer	Block ratio (PLCA/PEG ratio)	Weight ratio of caprolactone to lactide (cap/lac ratio)	End group	Degree of acylation / %
A	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{1.8}(C_2)_{2.0}$	1.8	9/1	Acetyl C2	100
B	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{2.0}(C_2)_{2.0}$	2.0	9/1	Acetyl C2	100
C	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{2.2}(C_2)_{2.0}$	2.2	9/1	Acetyl C2	100
D	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{2.4}(C_2)_{2.0}$	2.4	9/1	Acetyl C2	100
E	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{2.6}(C_2)_{2.0}$	2.6	9/1	Acetyl C2	100
F	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{2.8}(C_2)_{2.0}$	2.8	9/1	Acetyl C2	100
G	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{2.0}(C_2)_{0}$	2.0	9/1	-	0
H	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{2.0}(C_2)_{1}$	2.0	9/1	Acetyl C2	50
I	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{1.2}(C_2)_{2.0}$	1.2	9/1	Acetyl C2	100
J	$PEG_{1500}(CAP_{80\%}/LAC_{20\%})_{1.2}(C_6)_{2.0}$	1.2	4/1	Hexanoyl C6	100
K	$PEG_{2000}(CAP_{80\%}/LAC_{20\%})_{1.2}(C_6)_{2.0}$	1.2	4/1	Hexanoyl C6	100

Example 8 Degradation of hydrogel compositions with varying PEG/PLCA ratios

This example shows the degradation profiles for compositions according to the invention. Typically, gels were cast from 20 wt% polymers solution with 3 wt% lysozyme in small vials and allowed to gel at 37 ºC. PBS buffer was added and replaced periodically. The wet gels were weighed and the amount of lysozyme released was determined by the BCA® assay and/or UPLC (BCA® and UPLC were conducted as per example 6). The enzymatic activity of lysozyme was determined by the hydrolysis of the outer cell membrane of Micrococcus Lysodeikticus.

Table 3 Degradation of the B-A-B tri-block copolymers

ID	Structure	Unloaded gels		Protein-loaded gels[1]	
		at 20 wt%	at 25 wt%	BSA	Lysozyme
I	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{1.2}(C_2)_{2.0}$	COMPARATIVE EXAMPLE:does not form a gel at 37°C			
A(#27)	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{1.8}(C_2)_{2.0}$	8 days	14 days	6 days	6 days
B (#21)	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{2.0}(C_2)_{2.0}$	27 days	31 days	21 days	19 days
C (#33)	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{2.2}(C_2)_{2.0}$	31 days	34 days	12 days	12 days
D	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{2.4}(C_2)_{2.0}$	34 days	34 days	11 days	12 days
E	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{2.6}(C_2)_{2.0}$	250 days	250 days	12 days	12 days
F	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{2.8}(C_2)_{2.0}$	COMPARATIVE EXAMPLE: does not dissolve in PBS			

EXAMPLE 8b

Table 4 Different end-groups

ID	Tri-block copolymer	Block ratio (PLCA/ PEG ratio)	Weight ratio of caprolactone to lactide (cap/lac ratio)	End group	Degree of acylation / %
Z1	PEG1500(CAP90%/LAC10%)1.8(C3)2.0	1.8	9/1	C3	100
Z2	PEG1500(CAP90%/LAC10%)2(C3)2.0	2	9/1	C3	100
Z3	PEG1500(CAP90%/LAC10%)1.8(C4)2.0	1.8	9/1	C4	100
A	PEG1500(CAP90%/LAC10%)1.8(C2)2.0	1.8	9/1	Acetyl C2	100
B	PEG1500(CAP90%/LAC10%)2.0(C2)2.0	2.0	9/1	Acetyl C2	100

Tri-block copolymers Z1-Z3 were synthesised in a manner similar to examples 2 and 4.

20% solutions of the tri-block copolymers of Table 4 in PBS were prepared and placed in an oven at 37 °C for thermogelations. The forming of the gel was checked after 1 hour and gels were formed for compositions comprising tri-block copolymers comprising A, B, Z1, Z2 and Z3.. After longer periods of time (several hours) also Z3 showed phase separation and after even longer periods Z2 and Z1 also showed phase separation. Phase separation was not observed for compositions comprising tri-block copolymers A and B.

Example 9 Release of model protein vs PCLA/PEG ratio

This example illustrates the release profile of water soluble model proteins from tri-block copolymer solutions of the present invention.

The method for detecting release of the drug from the compositions was followed as per example 2. Burst release was calculated by plotting the cumulative release versus

$t^{0.5}$. The degradation experiments were conducted as per example 2. The results are presented in Table 4.

Table 5 Characteristics of tri-block copolymers with varying weight ratio -caprolactone to lactide

ID	Structure	Weight ratio ε-caprolac-tone to lactide	Degrada-tion Time	Release of water soluble model protein until	Burst relea-se
M	$PEG_{1500}(CAP_{100\%}/LAC_{0\%})_{1.8}(C_2)_{2.0}$	1/0 ε-caprolactone	> 140 days	~ 25 days	1.8%
N	$PEG_{1500}(CAP_{95\%}/LAC_{5\%})_{2.0}(C_2)_{2.0}$	19/1	> 50 days	~ 25 days	1.6%
B(21)	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{2.0}(C_2)_{2.0}$	9/1	~ 42 days	~ 30 days	1.0%
O	$PEG_{1500}(CAP_{80\%}/LAC_{20\%})_{2.0}(C_2)_{2.0}$	4/1	~ 23 days	~ 16 days	4.9%
P	$PEG_{1500}(CAP_{50\%}/LAC_{50\%})_{2.0}(C_2)_{2.0}$	1/1	~ 22 days	~ 9 days	5.0%

Example 10

Rheology of compositions with different end caps

Composition B (#21) from experiment 9 (which had the minimum burst release profile) was compared by rheological analysis, to the compositions with the same PLCA/PEG ratio but with different degrees of end acylation.

Rheological characterization of the blends was done with a AR-G2 rheometer (TA Instruments, Etten-Leur, The Netherlands) equipped with a 1° steel cone geometry of 20 mm diameter and solvent trap. Polymer blend solutions of 20 % (w/w) were prepared in PBS pH 7.4 at 4°C. 300 µl of the solutions were placed in glass vial (8.2 x 40 mm) and incubated for around 3 hours at 37°C to enable gelation and stabilization. Using a spatula, approximately 70 mg of the sample was placed between the pre-heated (37 °C) plates of the rheometer. Rheological gel characteristics were monitored by oscillatory time sweep experiments. During time sweep experiments G'(shear

storage modulus) and G'' (loss modulus) were measured for a period of 5 min. Also temperature sweep experiments were performed on the polymer solutions. Therefore the plates of the rheometer were pre-cooled at 4 °C. Temperature increase was 1 °C / min. When G''/G' (= tan δ) = 1, the sample is considered as a gel in a rheological point of view. All experiments were performed at constant strain (1%) and frequency (1 Hz).

The results are shown in Table 6

Table 6 Gel characteristics for compositions B and F

	Composition	Gel at 37 °C	Degree of acylation / %
B	21	YES	100
F	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{2.0}(C_2)_1$	YES	50

Example 11Degradation of compositions of tri-block copolymers of the invention with different degrees of acylation

This example illustrates the degradation profile for tri-block copolymers of the invention compared to a model compound that is not acylated. In this example 300 mg of a formulation containing 20% tri-block copolymer and 3 wt% lysozyme was prepared. The procedure was followed according to example 8.

The results are shown in Figure 5 and summarized in Table 7.

Table 7 Degradation times for tri-block copolymers

ID	Polymer composition	Degradation time	Degree of acylation / %
F	$PEG1500(CAP90\%/LAC10\%)_{2.0}(C2)_0$	14 days	0
G	$PEG1500(CAP90\%/LAC10\%)_{2.0}(C2)_1$	14 days	50
B	$PEG1500(CAP90\%/LAC10\%)_{2.0}(C2)_2$	42 days	100

Example 12 Release of lysozyme

This example shows the release profile of lysozyme which was used as a model water soluble protein, from compositions of the invention compared to a composition comprising a tri-block copolymer that is not acylated.

The procedure was followed as per example 6.

The results are shown in Figure 6 and summarized in Table 8.

Table 8 Comparison of controlled release profile for tri-block copolymers with different degrees of acylation.

ID	Composition	Degree of acylation / %	Controlled burst release*
F	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{2.0}(C_2)_0$	0	--
G	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{2.0}(C_2)_1$	50	+
B	$PEG_{1500}(CAP_{90\%}/LAC_{10\%})_{2.0}(C_2)_2$	100	++

--- Very large burst release

- large burst release

+ small burst release

++ very small burst release

Example 13

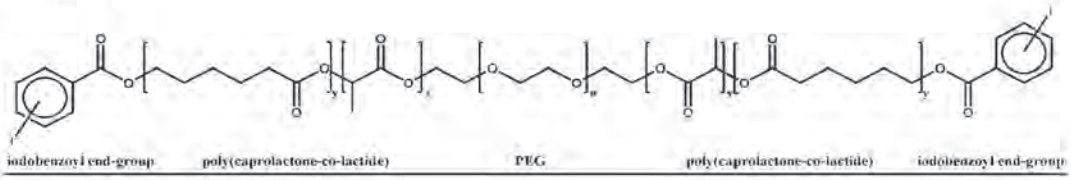
Mathematical analysis of release profiles

The data from the release experiment (example 12) was plotted against the square root of the time of release. The data was fitted using linear regression analysis and the burst release could be determined by extrapolating the lines of best fit to the y axis. The point where the extrapolated line crosses the y axis gives the value for percentage burst release. The results are shown in Figure 7.

Example 14 Synthesis of iodine functionalized tri-block copolymers

This example details a method to covalently bond a radiopaque atom to a tri-block copolymer. The structure of a tri-block copolymer which is acylated is given below in formula (1).

Formula 1



The synthesis of tri-block copolymers wherein at least part of the hydroxyl end-groups of the tri-block copolymer are covalently bound to a compound containing radiopaque atoms was done according to the method previously described in US7740877, examples 1, 2 and 3. The synthesis led to tri-block copolymers (Table 9) with triiodobenzoyl groups (1.8 groups per chain) as confirmed by 1H NMR.

Table 9. Tri-block copolymers used.

ID	ID	PLCA/PEG	Caprolactone / lactide	Degree of acylation (%)	End-group	Number average mol.weight PEG
P (#81)	I-Gel® #1	1.8	9/1	90%	Triiodobenzoyl	1500
Q (#29)	I-Gel® #2	1.0	9/1	90%	Triiodobenzoyl	1500

In the following examples different compositions comprising P and Q were made and the compositions are summarized in Table 10.

Table 10. Overview of compositions used in examples 15-19

Example	Tri-block copolymer (wt %)		
	P	Q	17
15	-	-	100
16	25	-	75
	30	-	70
	50	-	50
	70	-	30
17 a	30	-	70
b	-	30	70
18 a	-	-	100
b	25	-	75
19 a	25	-	75
b	50	-	50

Example 15 Loading of a tri-block copolymer composition with contrast agent Hexabrix™

In order to achieve prolonged release of a pharmaceutically active ingredient, retention in the treatment location (for example a joint) must be maintained for a minimum of 4 weeks. To determine this retention time the tri-block copolymer composition must be visualized. This example shows how loading a tri-block copolymer with a conventional contrast agent does not achieve this goal.

Polymer #17 (see Table 1) was loaded with 15 % Hexabrix™ (Guerbet). Hexabrix™ contrast agent is a low osmolar ionic dimer. Each milliliter contains 393 mg of ioxaglate meglumine, 196 mg of ioxaglate sodium and 0.10 mg of edetate calcium disodium as a stabilizer. The solution contains 3.48 mg (0.15 vmEq) sodium in each milliliter and provides 32% (320 mg/mL) organically bound iodine.

The commercially available Hexabrix™ solution was diluted with phosphate buffer (50 mM phosphate, pH 7.4, 0.07 mM NaCl) at a ratio 15/85 v/v. This diluted Hexabrix™ solution was used to dissolve the polymer, leading to the preparation of a formulation

containing 25 wt% polymer with respect to buffer and Hexabrix™. Each gram of formulation contained 44 mg ioxaglate meglumine.

The loaded tri-block copolymer composition was injected into a chicken knee and visualized using microCT (Skyscan model 1076, Skyscan, Kontich, Belgium) Scans were performed using the following scanner settings: isotropic voxelsize of 35mm, at a voltage of 55kV, a current of 170mA, field of view of 35mm, and a 0.5mm aluminium filter, over 198° with a 0.8 rotation step. 1 hour after injection the visibility of the gel was diminished and a further 1 day later the composition could not be visualized. Make table T=0-1-24hrs showing 100%-50%-0% visualization as relative grey value
The results are also indicated in Table 11.

Table 11: Image-retention of Polymer #17 (25% w/w) loaded with 15% Hexabrix

Time point in hours	Relative grey surface area
T = 0	100%
T = 1	50%
T=24	0%

Example 16 Radiopaque tri-block copolymer compositions

This example shows that a tri-block copolymer wherein at least part of the hydroxyl end-groups of the tri-block copolymer are covalently bound to a compound containing radiopaque atoms can be visualized by microCT. (Skyscan model 1076, Skyscan, Kontich, Belgium) Scans were performed using the following scanner settings: isotropic voxelsize of 35mm, at a voltage of 55kV, a current of 170mA, field of view of 35mm, and a 0.5mm aluminium filter, over 198° with a 0.8 rotation step. Scan time was 10 minutes. In this example different compositions were prepared comprising different weight percentages of tri-block copolymers (Table 10, example 16) wherein the hydroxyl end-groups of the tri-block copolymer are at least partially acylated with an acetyl group or wherein at least part of the hydroxyl end-groups of the tri-block copolymer are covalently bound to a compound containing radiopaque atoms.

The compositions (Table 10, examples 16) were prepared as follows. Both polymers P and #17 were separately dissolved in ethylacetate at a concentration of 500 mg/mL. The solutions were mixed at the desired ratios and the mixtures were transferred into

petri dishes. The solvent was removed under nitrogen flow for 48 hours. To 500 mg polymer blend, phosphate buffer (50 mM, 0.07mM NaCl, 0.02% NaN3, pH 7.4) was added to yield solutions at 25 wt %. The compositions were measured in glass vials and the X-ray intensity plotted on a graph (Figure 8).The compositions were measured in glass vials and the X-ray intensity plotted on a graph (Figure 8).

Example 17 CT imaging in radiopaque tri-block copolymer compositions

This example shows the CT visualization times for different mixtures of tri-block copolymers.

The mixtures of copolymers (Table 10, example 17a and 17b) were prepared, injected into the knee of a rat cadaver and visualized by microCT (Skyscan model 1076, Skyscan, Kontich, Belgium). Scans were performed using the following scanner settings: isotropic voxelsize of 35mm, at a voltage of 55kV, a current of 170mA, field of view of 35mm, and a 0.5mm aluminium filter, over 198° with a 0.8 rotation step. The results are shown in Table 14.

The composition 17a was visible for only a few days whereas composition 17b was visible for 3 weeks (Figure 11).

Example 18 Release profiles of tri-block copolymers wherein the end groups are acylated or are radiopaque substituted acyl groups

This example illustrates the release profile of a pharmaceutically active ingredient from tri-block copolymer compositions and also the degradation profiles for tri-block copolymer compositions comprising a pharmaceutically active ingredient.

OAc-Gell (#17) and a mixture of a I-Gell®#1 (P) and OAc-Gell (#17) were prepared at 25 wt% in 50 mM phosphate buffer at pH 7.4, 0.42% NaCl and 0.05% NaN3. The loading of Celecoxib was 1.25 mg/mL. Release experiments were performed as per example 6 and at 37 °C in PBS buffer containing 0.2% Tween 80. Error bars represent standard error of the mean (n=6). The results are shown in Figure 9 and summarized in Table 12.

Table 12 Overview release time and erosion profile for examples 18a and 18b.

Example	Release time	Erosion time (solid content)
18a	28	28
18b	35	35

Wet weight= weight of the depots, as measured by decanting the PBS buffer and weighing the remaining gel, after which the original vial-weight is subtracted
Dry weight = weight of the freeze dried depots after decanting of the buffer (i.e. polymer weight) and subtracting the weight of the vial
Solid content = polymer concentration in the depots (dry weight/wet weight)

Example 19 Degradation profile for tri-block copolymer compositions

This example shows the in vivo degradation profile for compositions of tri-block copolymers wherein at least part of the hydroxyl end-groups of the tri-block copolymer are covalently bound to a compound containing radiopaque atoms.

Compositions (Table 10, example 19a and 19b) were prepared were prepared by dissolving 25 wt % tri-block copolymer (comprising 75 % P and 25 % #17, or 50 % P and 50 % #17) in 75 % PBS buffer, pH 7.4. 50 mM, 0.15% NaCl of the composition was injected subcutaneously into rats knees. The injected volume of 19b was then visualized and measured using 3D micro-CT imaging (Skyscan model 1076, Skyscan, Kontich, Belgium). The rats were anesthetized using Isoflurane and then placed in a custom made scanner bed, fixing the hind limb in an extended position. Scans were performed using the following scanner settings: isotropic voxel size of 35mm, at a voltage of 55kV, a current of 170mA, field of view of 35mm, and a 0.5mm aluminium filter, over 198° with a 0.8 rotation step. The rat knees and subcutaneous depots were scanned on days 0,1, 4 and 8 and after that weekly until the gel was no longer visible. The scan time was 16 minutes and a frame averaging of 3 was used. Images obtained using the uCT scanner were reconstructed using Skyskan analysis software. The datasets were segmented using a fixed attenuation threshold between air and subchondral bone. Subsequently, regions of interest were drawn around the patellar cartilage and attenuation and volume were calculated. The injected volume of 19a (non-radiopaque), was monitored through the skin with a measuring caliper. The results are shown in Figure 10 and summarized in Table 13 and 14.

Table 13 Overview of erosion monitoring for examples 19a and 19b.

Day	19a		19b	
	Observations	Surface area / %	Observations	Surface area / %
0	Distinct oval shape, sharp edges	100	Higher intensity than for 19a	100
4	Sharp edges maintained	80		70
8	Less sharp edges on interior side	40		65
14	Fractured shape – small light spots in dark area	10		75
21	Not visible	-		50
40	-	-	Still visible	40

Surface area = relative area with respect to surface area at t=0 (set to 100 %)

Table 14. Overview of compositions and microCT characteristics for examples 15-19

Example	Tri-block copolymer (wt %)			Duration of visibility in micro CT	Active ingredient
	P	Q	17		
15	-	-	100	< 24 hours	Hexabrix™
16 a	25	-	75	All visible in micro CT but duration of visibility not measured	-
	30	-	70		-
	50	-	50		-
	70	-	30		-
17 a	30	-	70	< 7 days	-
b	-	30	70	~ 4 weeks	-
18 a	-	-	100	-	Celecoxib
b	25	-	75	-	Celecoxib
19 a	25	-	75	~ 40 days	-
b	50	-	50	~ 77 days	-

Discussion and conclusion

Brief description of the figures.

Fig. 1 shows the release of both hydrophobic-type (celecoxib (CLB), triamcinolone (TA), cyclosporine A (cya) and water soluble (iobitrodol (ITD) and lysozyme (Lys)) active ingredients from 25% w/w U5 (low PLCA/PEG ratio) based on PBS and active ingredient at 37 °C. The comparative U5 polymer that phase separates at 37°C.

In Fig.1, the following legend is used:

Release from U5: Lysozyme (diamonds), ibitridol (circles), Triamcinolone acetonide (squares), Cyclopsorin A (open squares), celecoxib (open triangles)

Figure 2 shows the release of IgG, BSA and lysozyme from a solution of 20% w/w tri-block copolymer #17 based on the amount of solvent and pharmaceutically active ingredient present in the composition at 33°C. Data are shown as average ± standard deviation (n=3).

Figure 3 shows the release of IgG, BSA and lysozyme from a solution of 20% w/w tri-block copolymer #33 based on the amount of solvent and pharmaceutically active ingredient present in the composition at 33°C.

Figure 4 shows the release of model proteins lysozyme, BSA and IgG from tri-block copolymers # 27 and from #21 at 37°C at a concentration of 20%w/w tri-block copolymer based on the amount of solvent and pharmaceutically active ingredient present in the composition. Data are shown as average ± standard deviation (n=3).

Figure 5 shows the degradation profile of the relative wet gel as a function of time for formulations of 20 wt% of polymer and 3 wt % of lysozyme. Measurements were made on 4 samples (n=4)

In Fig. 5 the compositions used are

G PEG1500(Cap90%/Lac10%)2.0 (triangle)

H PEG1500(Cap90%/Lac10%)2.0(C2)1 (diamond)

B (#21): PEG1500(Cap90%/Lac10%)2.0(C2)2 (square)

Figure 6 (Fig. 6) shows the cumulative release profile of lysozyme as a function of time for formulations of 20 wt% of tri-block copolymer and 3 wt% of lysozyme.

In Fig. 6 the compositions used are

G PEG1500(Cap90%/Lac10%)2.0 (triangle)

H PEG1500(Cap90%/Lac10%)2.0(C2)1 (diamond)

B (#21): PEG1500(Cap90%/Lac10%)2.0(C2)2 (square)

Figure 7 (Fig. 7) shows the cumulative release profiles of lysozyme from tri-block copolymer compositions as a function of the square root of time. The formulations comprised 20 wt% of tri-block copolymer and 3 wt% of lysozyme. Lines represent linear fits. The fitted curves correspond to y=mx+c where c is the percentage burst release. Error bars show the standard deviation with n=4.

In Fig. 7 the compositions used are

G PEG1500(Cap90%/Lac10%)2.0 (triangle)

H PEG1500(Cap90%/Lac10%)2.0(C2)1 (diamond)

B (#21): PEG1500(Cap90%/Lac10%)2.0(C2)2 (square)

Figure 8 (Fig. 8) shows the x-ray intensities as measured by the microCT, for compositions shown in Table 10, example 16, whereby the percentage of iodine bound polymer P(#81) is given on the x-axis. The line is added to guide the eye.

Figure 9 (Fig. 9) shows the release profile and gel erosion profiles for example 18. The filled squares indicate composition 18a (OAc-Gell only loaded with Celecoxib) and the empty triangles indicate composition 18b (OAc-Gell / I-Gell®#1 -#81). The compositions contained 25 wt% in 50 mM phosphate buffer, pH 7.4, 0.42% NaCl and 0.05% NaN3. The loading of Celecoxib was 1.25 mg/mL

Figure 10 (Fig.10) shows the erosion profile for compositions 19a (dotted line) and 19b (Solid line). Images of the subcutaneous injected I-Gell depots in rats were taken in-vivo using micro-CT on days 0-1-4-8-14-21-28-35-42-49-56-63-70-77 and the volume of the gel was determined using a caliper as done routinely for volume-estimations of subcutaneous tumor lumps.

Figure 11 (Fig. 11) shows the degradation profile for composition 17b as determined by visualization using microCT.

As can be seen from Figure 1 (Fig. 1), the tri-block copolymer with a low PLCA/PEG ratio of 1.2 maybe be employed to obtain long-term controlled release of hydrophobic molecules (celecoxib, triamcinolone acetonide, cyclosporin A) without burst; whereas that polymer is not suitable to prevent burst release of water soluble molecules (lysozyme, iobitridol) and as such not suitable for use in the composition of the present invention.

As can be seen from Figure 2 (Fig. 2), the tri-block copolymer #17 may be employed in the composition of the invention to get controlled release without too much burst release of water soluble model proteins IgG; BSA and lysozyme.

As can be seen from Figure 3 (Fig. 3), the tri-block copolymer #33 may be employed to get a controlled release without too much burst release of the water soluble pharmaceutically active ingredients (in this case Lysozyme, BSA and IgG).

As can be seen from Figure 4, the tri-block copolymers #21 and #27 are suitable for the controlled release of the tested model proteins: lysozyme and BSA..

The results in Table 3 show that at a block ratio of lower than 1.4, the tri-block co-polymer does not form a gel whereas at a block ratio of above 2.6, the tri-block co-polymer does not dissolve in the buffer solution.

The gel behaviour for the compositions shown in Table 4 indicate that preferably for release of water soluble proteins, the hydroxyl end-groups of the tri-block copolymer in the composition of the invention are at least partially acylated with an optionally substituted acetyl, an optionally substituted propionyl, an optionally substituted butyl, or an optionally substituted benzoyl group. Preferably, the hydroxyl end-groups of the tri-block copolymer in the composition of the invention are at least partially acylated with an optionally substituted acetyl or an optionally substituted propionyl. More preferably, the hydroxyl end-groups of the tri-block copolymer in the composition of the invention are at least partially acylated with an optionally substituted acetyl or an optionally substituted propionyl, most preferably with an optionally substituted acetyl.

As can be seen from Table 5 longer degradation and release are obtained for tri-block copolymers having a weight ratio of -caprolactone to lactide of at least 9/1.

Furthermore it can be seen that a tri-block copolymer having a weight ratio of caprolactone to lactide of at least 1/1 shows low burst release.

Table 6 shows that the at least partially acylated tri-block co-polymers of the invention can form a gel.

As shown in Figure 5 and Table 7, the composition having a degree of acylation of the end-groups of at least 75% for example of 100% have a significantly longer degradation time (42 days) than those compositions having a lower degree of acylation.

As can be seen from Figure 6, the compositions comprising a tri-block copolymer that is at least partially acylated (degree of acylation 50%) or fully acylated (degree of acylation 100%) provide gels with a lower burst release, when compared to compositions comprising a tri-block copolymer that is not acylated. This example clearly demonstrates the surprising improvement in terms of release profile of a water soluble protein from a composition according to the invention comprising an at least partially acylated tri-block copolymer e.g. having a degree of acylation of at least 75% in comparison with the composition comprising a tri-block copolymer lacking acylation.

As can clearly be seen from Figure 7, compositions comprising at least partially acylated (e.g. degree of acylation 50%) or fully acylated end-groups (e.g. degree of acylation 100% tri-block co polymers) have a burst release of less than 2%, which is surprising and significant improvement for use in controlled release of especially water soluble compounds, for example water soluble proteins. The composition comprising non-acylated caprolactone / lactide showed a burst release of 24 % with the same water soluble compounds. which renders this composition useless, and even unsafe, in clinical practise for most protein-based drug therapies.

The conclusion that can be drawn from the mathematical analysis (as shown in Figure 7) of the release profile is that compositions of the invention comprising tri-block copolymers that are at least partially acylated give low or very low burst release compared to compositions comprising tri-block copolymers that are not acylated.

It is also observed, that when using triblock copolymers in the composition of the invention, wherein the number average molecular weight of the A-block is 1000Da, we obtain gels at 37°C that immediately phase separate. When using triblock copolymers in the composition of the invention, wherein the number average molecular weight of the A-block is at least 1250Da (as exemplified by the number average molecular weight

of the A-block of 1500), gels are obtained at 37°C that do not phase separate. Phase separation results in expulsion of free water/buffer from the interior of the gel-matrix, and shrinkage of the gel-matrix expressed as wet weight. Water-dissolved molecules will also exit the gel in large quantities with the expulsion of water-based solvents, hence leading to fast and high quantity release of the loaded water soluble molecules, which we refer to as burst or burst release. Avoiding early stage phase separation of the formulation is essential to keep water soluble drug-molecules inside the gel until they are released by diffusion and/or by gradual degradation of the gel-matrix.

In conclusion the examples presented herein show that the block ratio of the PEG/PLCA tri-copolymer is important and the ratio needs to be greater than 1.2 but less than 2.8 in order to obtain a non-phase separating gel at 37C. Furthermore, tri-block copolymers which are at least partially acylated have unexpected properties in a composition of the invention, when compared to compositions comprising unacylated tri-block copolymers. Notably the at least partial covalent modification of hydroxyl end groups of tri-block copolymers is very suitable for the sustained, controlled release of a pharmaceutically active ingredient, for example a water soluble drug, and results hardly in any burst release of water soluble molecules.

Furthermore, a tri-block copolymer loaded with Hexabrix™ can be used for imaging in microCT studies but the contrast agent diffuses out of the tri-block copolymer within 1 day thereby preventing prolonged imaging of the tri-block copolymer. Surprisingly, a composition comprising a tri-block copolymer wherein at least part of the hydroxyl end-groups of the tri-block copolymer are covalently bound to a compound containing radiopaque atoms is suitable for microCT imaging over longer periods of up to time.

It is shown herein that a composition comprising a tri-block copolymer wherein at least part of the hydroxyl end-groups of the tri-block copolymer are covalently bound to a compound containing radiopaque atoms maintains gel forming, gel erosion and controlled release properties whilst at the same time such composition can be visualized using micro CT.

Furthermore it is shown that it is possible to visualize, e.g. using microCT or CT imaging, a blend of a tri-block copolymer wherein the hydroxyl end-groups of the tri-block copolymer are at least partially acylated with an acetyl group or wherein at least part of the hydroxyl end-groups of the tri-block copolymer are covalently bound to a compound containing radiopaque atoms, while maintaining gel forming, gel erosion and controlled release properties are maintained.

CLAIMS

1. Composition comprising a tri-block copolymer according to formula 1

B-A-B (1)

wherein A stands for a linear poly-(ethylene glycol) block and
wherein B stands for wherein B stands for a poly(lactide-co-ε-caprolactone) block , wherein the hydroxyl end-groups of the tri-block copolymer are at least partially acylated with an optionally substituted acyl having 2 to 12 C-atoms, C-atoms of the substituents included;
an active ingredient, preferably a pharmaceutically active ingredient
and a solvent,
wherein the block ratio of the tri-block copolymer, which ratio is defined as the ratio between the sum of the number average molecular weight of the B-blocks and the number average molecular weight of the A-block ranges from 1.4 to 2.6,
wherein the number average molecular weight (Mn) of the linear poly-(ethylene glycol) block is at least 1250Da and
wherein the acyl group is an optionally substituted acetyl group, an optionally substituted propionyl group, an optionally substituted butyl group or an optionally substituted benzoyl group

2. Composition comprising a tri-block copolymer according to formula (1)

B-A-B (1)

wherein A stands for a linear poly-(ethylene glycol) block and
wherein B stands for a poly(lactide-co-ε-caprolactone) block, wherein at least part of the hydroxyl end-groups of the tri-block copolymer are covalently bound to a compound containing radiopaque atoms.

3. Composition according to claim 2, wherein the composition further comprises an active ingredient, preferably a pharmaceutically active ingredient and a solvent.

4. Composition according to claim 2 or claim 3, wherein the block ratio of the tri-block copolymer, which ratio is defined as the ratio between the sum of the number average molecular weight of the B-blocks and the number average molecular weight of the A-block ranges from 1.4 to 2.6.

5 Composition according to claim 1 or 3 or 4 , wherein the concentration of the tri-block copolymer in the composition of the invention ranges from 21 to 35% w/w based

on the amount of solvent and active ingredient present in the composition, preferably the concentration ranges from 25 to 35% w/w.

6. Composition according to any one of claims 1-5, wherein the acyl group is an acetyl group or a propionyl group.

7. Composition according to any one of claims 1-6, wherein the degree of acylation of the hydroxyl end-groups is at least 75%.

8. Composition according to any one of claims 1-7, wherein the active ingredient is a pharmaceutically active ingredient chosen from the group of steroids, non-steroidal anti-inflammatory drugs, for example a selective COX-2 inhibitor; and biologicals, for example an antibody, nucleotide sequence, protein, antigen, peptide or cell extract.

9. Composition according to anyone of claims 1-8, wherein the active ingredient are microparticles and/or nano-particles, such as microspheres containing drugs or imaging agnets, or liposomes containing siRNA, miRNA, drugs or imaging agents.

10. Composition according to any one of claims 1-9, wherein the active ingredient has a solubility of at least 20µg/ml in water measured at 20°C and at 1 bar pressure.

11. Composition according to any one of claims 1-10, wherein the solvent is water or an aqueous buffer solution, more preferably an aqueous buffer solution that leads to an isotonic composition of the invention.

12. Composition according to any one of claims 1-11, wherein the composition has a gel temperature in the range from 25 to 35°C and a phase separation temperature of at least 42°.

13 Composition according to any one of claims 1-12, wherein the tri-block copolymer has a gel window between 25°C and 42°C.

14 Composition according to any one of claims 1-13, wherein the weight ratio of ε-caprolactone to lactide is at least 1/1.

15. Composition according to any one of claims 1 or 5-9, wherein the acyl group is substituted with a radiopaque atom.

16. Composition according to any one of claims 2-14, wherein the radiopaque atom is iodine.

17. Composition according to any one of claims 1-16, wherein the composition is a pharmaceutical composition further comprising a pharmaceutically acceptable

adjuvant, excipient or carrier, for example a drug-encapsulating nano-particle or microparticle .

18. Composition according to any one of claims 1-17 for use as a medicament.

19. Process for the preparation of a composition of any of claims 1, 3-18 comprising the steps of

- synthesizing the tri-block copolymer
- mixing the tri-block copolymer with the active ingredient and the solvent

Figure 1

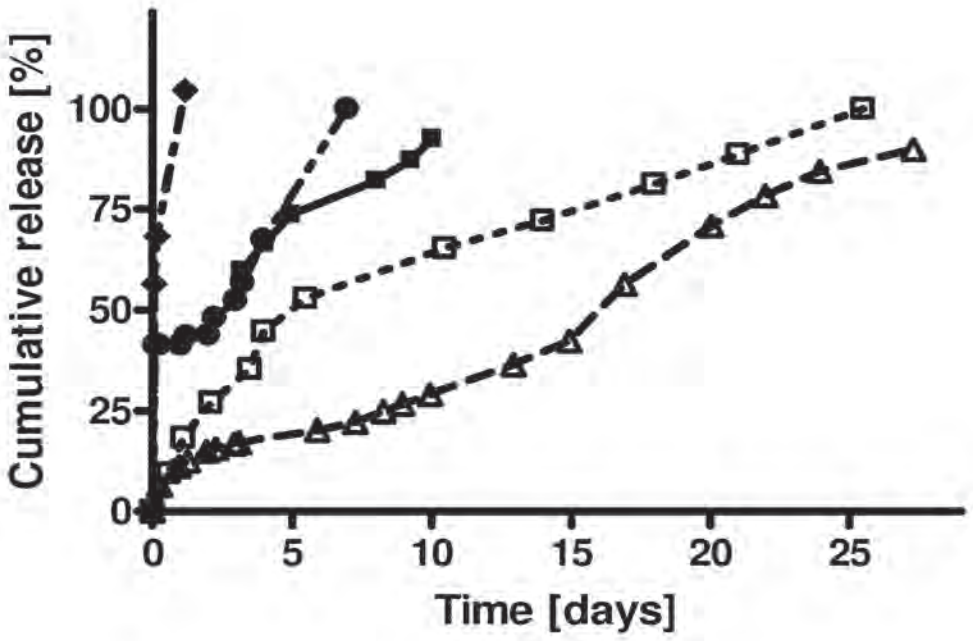


Fig. 2

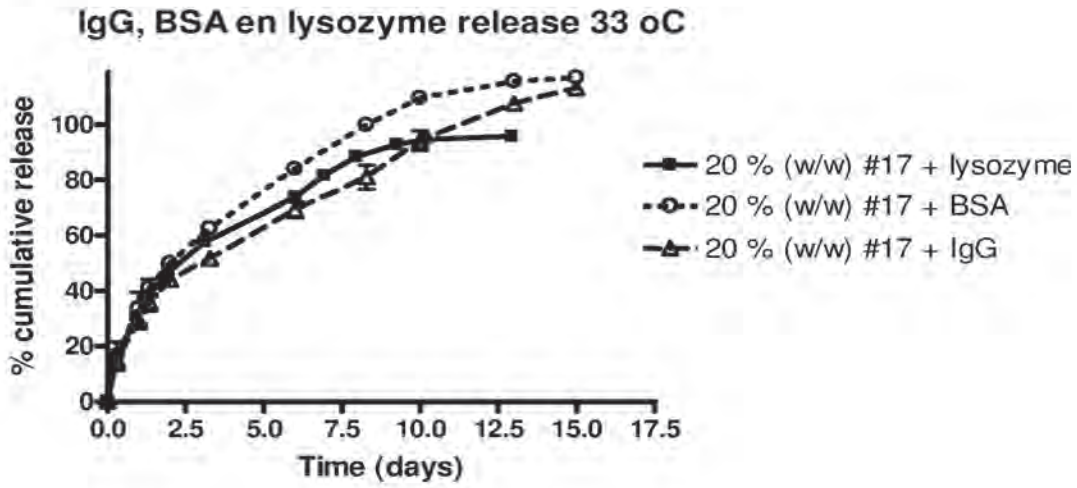

IgG, BSA en lysozyme release 33 oC
% cumulative release
100
80
60
40
20
0
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
Time (days)
20 % (w/w) #17 + lysozyme
20 % (w/w) #17 + BSA
20 % (w/w) #17 + IgG

Fig 3

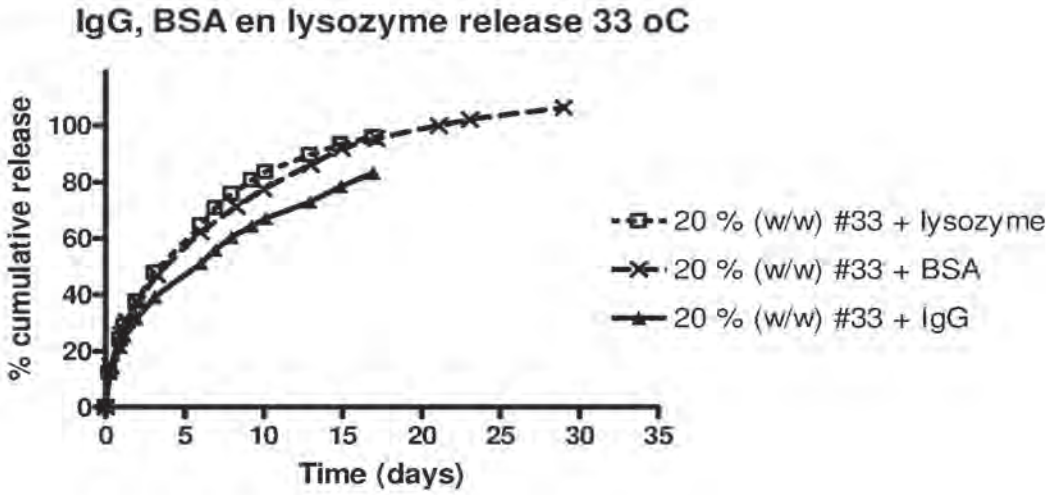

IgG, BSA en lysozyme release 33 oC
% cumulative release
100
80
60
40
20
0
0
5
10
15
20
25
30
35
Time (days)
20 % (w/w) #33 + lysozyme
20 % (w/w) #33 + BSA
20 % (w/w) #33 + IgG

Fig.4

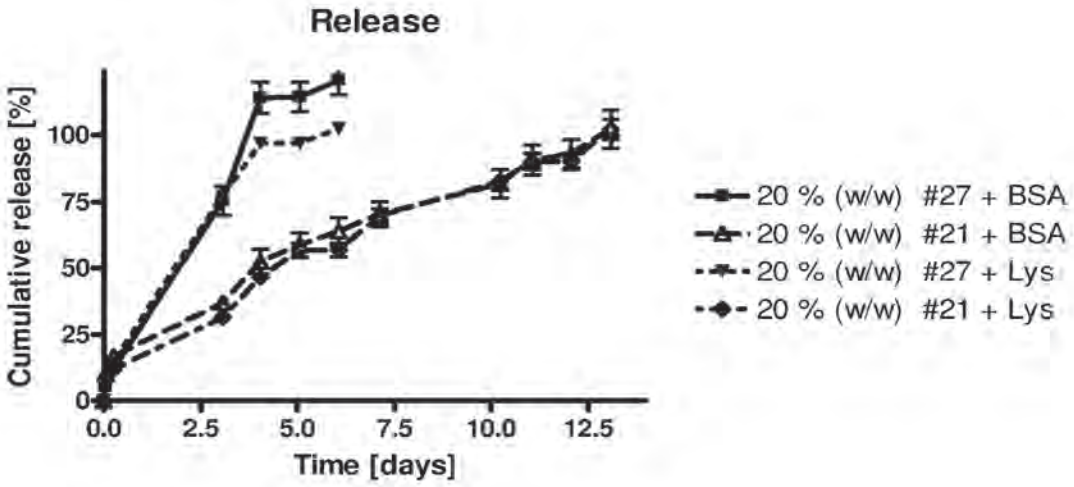

Release
Cumulative release [%]
0
25
50
75
100
0.0
2.5
5.0
7.5
10.0
12.5
Time [days]
20 % (w/w) #27 + BSA
20 % (w/w) #21 + BSA
20 % (w/w) #27 + Lys
20 % (w/w) #21 + Lys

Fig. 5

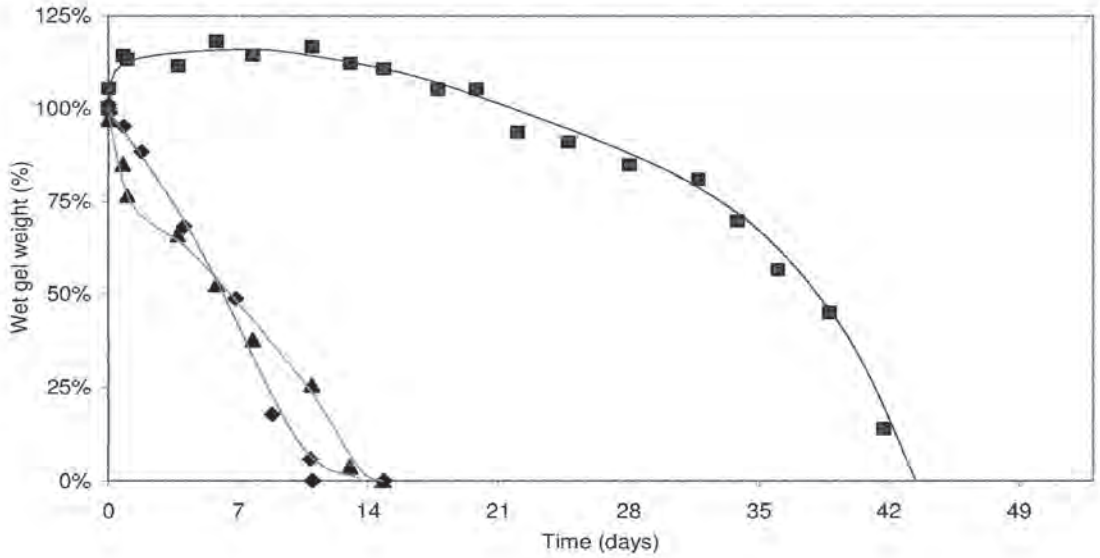


Fig.6

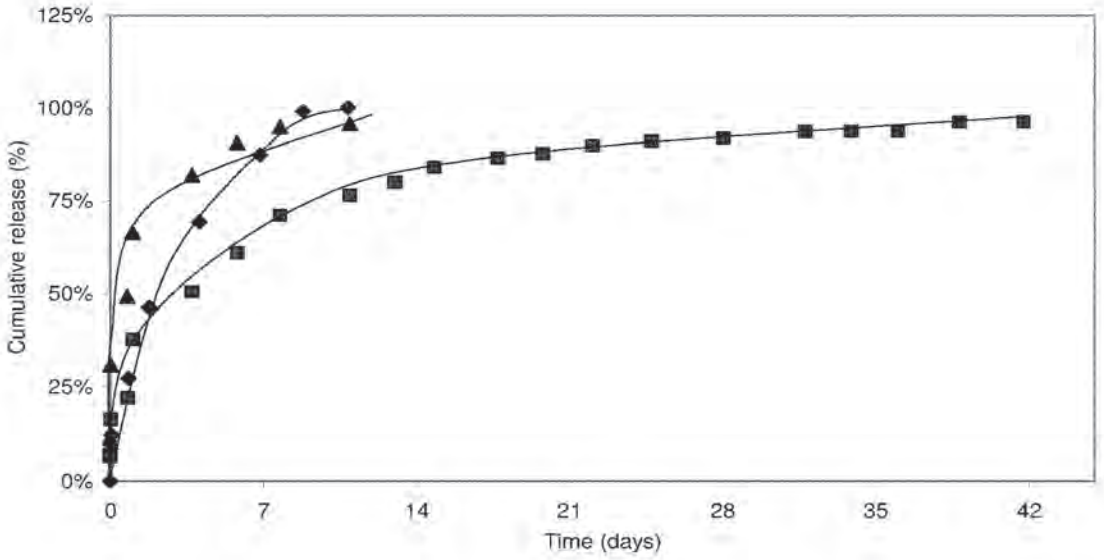

125%
100%
75%
50%
25%
0%
Cumulative release (%)
0
7
14
21
28
35
42
Time (days)

Fig. 7

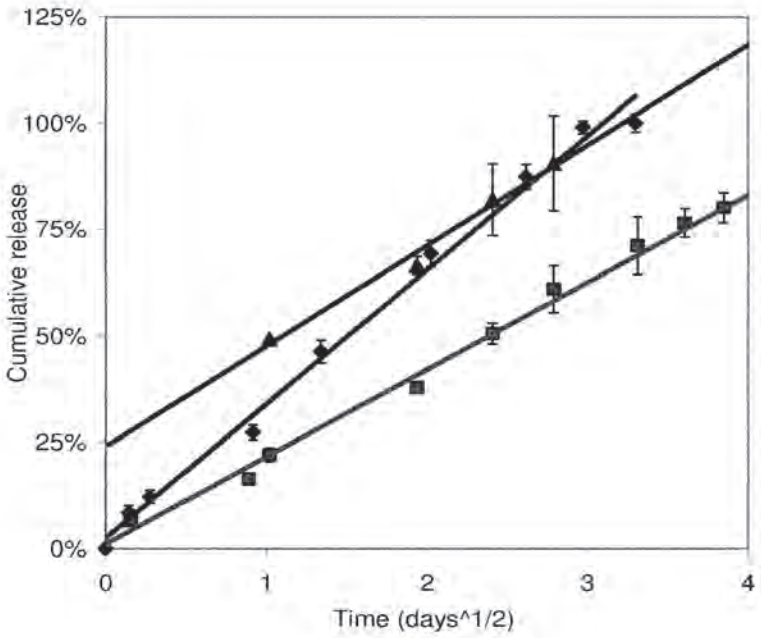


Fig.8

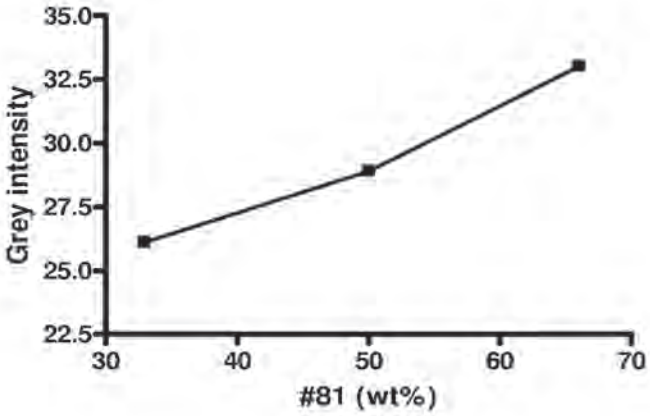
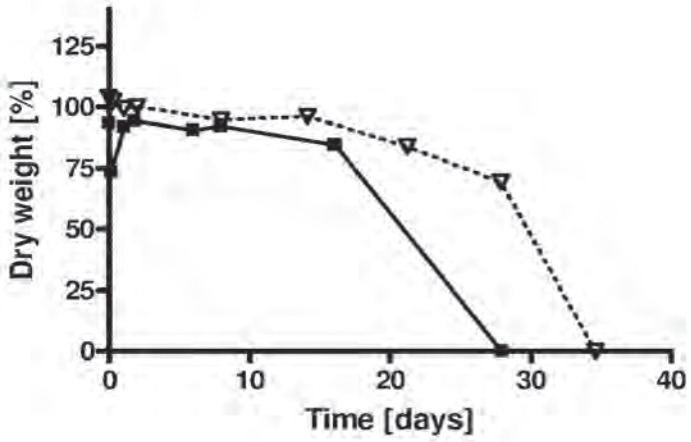
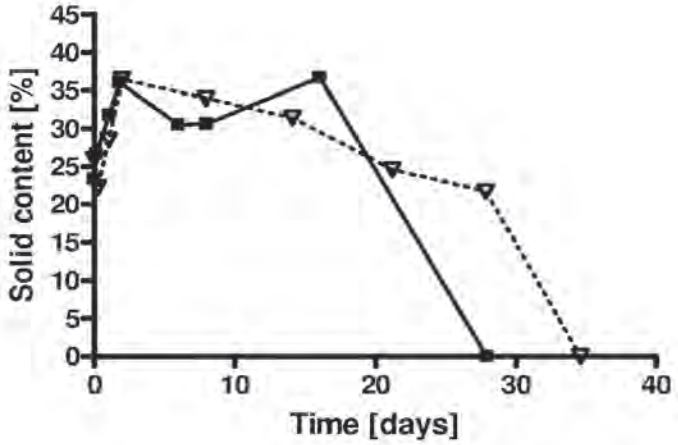
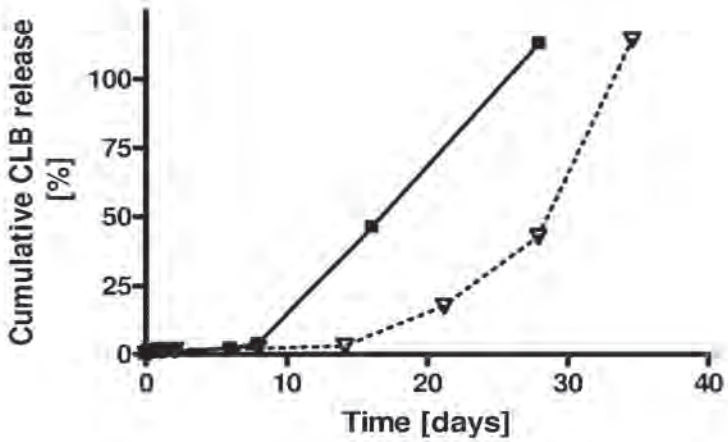


Fig.9

Polymer #17 (squares) ; #17/#81 (75/25) (open triangles)






Cumulative CLB release [%]
100
75
50
25
0
0
10
20
30
40
Time [days]

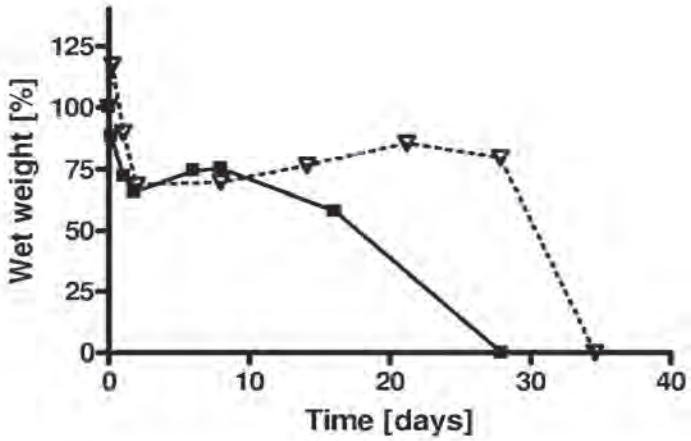

Wet weight [%]
125
100
75
50
25
0
0
10
20
30
40
Time [days]

Fig. 10

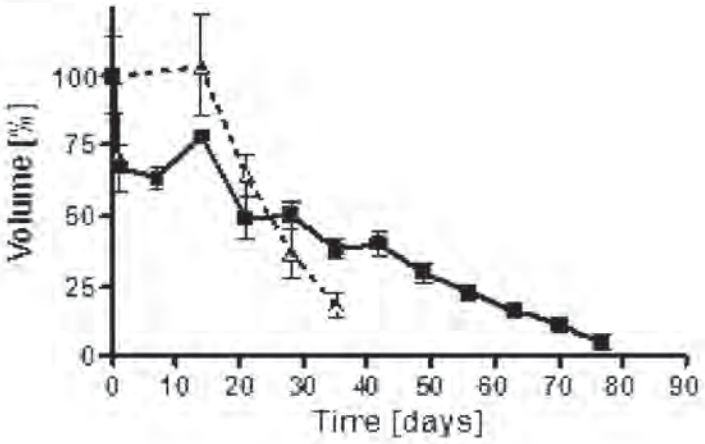
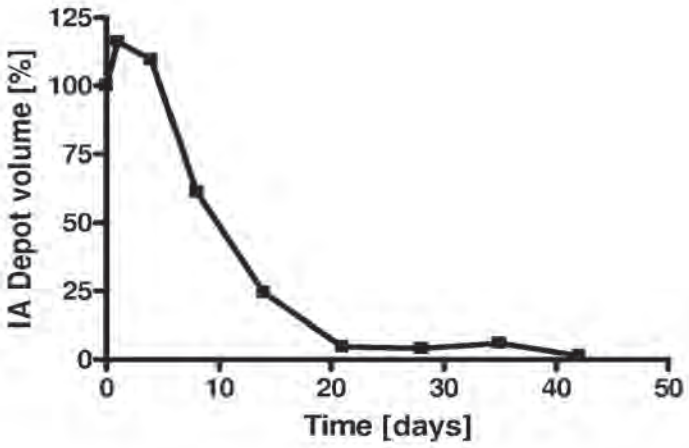


Fig. 11



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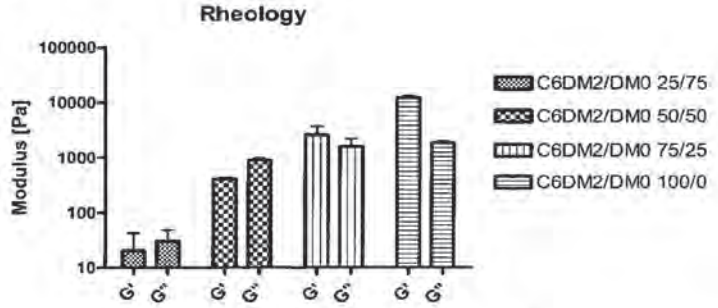
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(54) **Title**: BIODEGRADABLE COMPOSITIONS SUITABLE FOR CONTROLLED RELEASE

Fig 1



(57) **Abstract**: The invention describes simple mixing/blending of a special class of drug-depot forming tri-block copolymers polymers, with the opportunity to cost-effectively tailor drug delivery performances of such biodegradable, injectable depots in a clinical and an industrial setting. Furthermore the invention describes how to visualize these depots for various imaging related purposes The invention relates to a composition comprising (a) an active ingredient, preferably a pharmaceutically active ingredient (b) a solvent and (c) a mixture of at least two types of tri-block copolymers of formula (1) B-A-B (1) wherein B stands for a hydrophobic block and wherein A stands for a hydrophilic block, wherein the mixture is prepared by mixing at least two types of tri-block copolymers having a degree of modification of 100% and wherein the at least two types of B-A-B types of tri-block copolymers differ only on the type of end-group or wherein the mixture is prepared by mixing at least two types of tri-block copolymers, wherein one of the at least two tri-block copolymers has a degree of modification of 00% and one of the at least two tri-block copolymers has a degree of modification of 0% and wherein the at least two types of B-A-B types of tri-block copolymers differ only on the degree of modification of the end-groups. 2

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BIODEGRADABLE COMPOSITIONS SUITABLE FOR CONTROLLED RELEASE

The invention relates to a composition comprising (a) a (pharmaceutically) active ingredient
(b) a solvent and (c) a mixture of at least two types of biodegradable tri-block copolymers, to this composition for use as a medicament, a process for the preparation of said composition and to a method for delivering a (pharmaceutically) active ingredient over an extended period to an animal, including a human.

In recent years, there has been considerable effort to provide systems that are capable of controlled release of drugs in animals, including humans. Some drugs can only be administered to a patient by injection. Controlled release of such drugs has the advantage that the patient does not need to be subjected to multiple injections, but instead only one or a few injections with the controlled release system would suffice.

Some injections into areas of the patient's body are (very) painful. Examples of such difficult to dose areas are the eyes, the (synovial) joints, the muscles or the spine. Injection with a drug in a controlled release system will limit the amount of injections needed and will enhance the chance that a patient will continue with the therapy. This will greatly increase the success of the treatment.

Controlled release systems that are very suitable for being injected into such difficult to dose areas are thermogels based on compositions comprising polymers. The polymers have the unique property that at low temperatures they are water soluble, whereas at higher temperatures the polymers form a gel. Preferably, for use as a system that is capable of controlled release, the polymer is soluble in the solvent used at room temperature (e.g. 21°C) and forms a gel once injected into the body (temperature in the range from 30 to 42°C).

A gel (or a hydrogel in the context of the present invention) is a network of polymer chains that are hydrophilic and contain a substantial amount of water (for example between 50 and 99% water, preferably between 66 and 85% water). The gel shows no flow in a vial tilt test: when a glass vial which contains the gel is turned upside down, no flow of the gel is observed during 15 seconds observation time.

Since different drugs require a different drug administration regime, there is a desire to make thermogels having a tuneable release profile.

Methods to tune release profiles of thermogels of BAB-type tri-block copolymers have

been described by Yu *et al.*, in 'Mixing a sol and a precipitate of block copolymers with different block ratios leads to an injectable hydrogel' in Biomacromolecules, 2009, 10, 1547-1553 and in CN200910049664. Yu *et al.* describe a method to obtain a thermoreversible physical hydrogel by mixing an aqueous solution of an BAB-type triblock copolymer poly(D,L-lactic acid-c-glycolic acid)-b-polyethylene glycol-b-poly(D,L-lactic acid-co-glycolic acid), as indicated PLGA-PEG-PLGA, with a precipitate of a similar copolymer but with a different block ratio. According to Yu *et al.*, this method provides a very convenient approach to design injectable thermogelling biomaterials with a broad adjustable window, which copolymer mixture platform can potentially be used in drug delivery.

Also, WO01/82970 A1 discloses a water-soluble, biodegradable reverse thermal gelation system comprising a mixture of at least two types of tri-block copolymers. The drug release and gel matrix erosion rates of the mixture of copolymers may be modulated by various parameters such as the hydrophobic/hydrophilic component contents, polymer block concentrations, molecular weights and gelation temperatures, and weight ratios of the tri-block copolymer components in the mixture.

However, from a regulatory point of view, every time a different polymer is used in a thermogel blend, the new mixture needs to go through a new regulatory approval process before the national or regional drug approval authority (such as the Federal Drug Administration (FDA) in the US or its European equivalent the European Medicines Agency (EMEA) in Europe). This will prolong the very important time-to-market considerably.

Therefore, it is the object of the invention to provide compositions for which the release profile can easily be tuned, but which have a shorter time to market.

This object is achieved by a composition comprising
(a) an active ingredient, preferably a pharmaceutically active ingredient
(b) a solvent and
(c) a mixture of at least two types of tri-block copolymers of formula (1)

B-A-B (1)

wherein B stands for a hydrophobic block and wherein A stands for a hydrophilic block wherein the at least two types of B-A-B types of tri-block copolymers differ only on the type of end-group and/or on the degree of modification of the end-groups.

Preferably, the invention relates to a composition comprising
(a) an active ingredient, preferably a pharmaceutically active ingredient
(b) a solvent and
(c) a mixture of at least two types of tri-block copolymers of formula (1)

B-A-B (1)

wherein B stands for a hydrophobic block and wherein A stands for a hydrophilic block, wherein the mixture is prepared by mixing at least two types of tri-block copolymers having a degree of modification of 100% and wherein the at least two types of B-A-B types of tri-block copolymers differ only on the type of end-group or wherein the mixture is prepared by mixing at least two types of tri-block copolymers, wherein one of the at least two tri-block copolymers has a degree of modification of 100% and one of the at least two tri-block copolymers has a degree of modification of 0% and wherein the at least two types of B-A-B types of tri-block copolymers differ only on the degree of modification of the end-groups.

By varying the choice and amount of end-groups of the BAB-type tri-block copolymers that are present in the composition of the present invention, the release profile for a drug can easily be varied. Furthermore, since the BAB-type tri-block copolymers of the composition of the invention have the same backbone and only differ in the choice or amount of end-groups, the regulatory approval process of the composition can be considerably shortened if not completely skipped. The composition of the present invention is therefore very suitable for the controlled release of different types of drugs, the release profile of which can be easily varied and adapted to the drug used.

For the avoidance of doubt, the terms 'pharmaceutically active ingredient' and 'drug' are used interchangeably herein.

A further advantage of the composition of the present invention is that the preparation of the tri-block copolymers is much easier. In case of the tri-block copolymers of the invention, the synthesis of the tri-block copolymer having hydroxyl end-groups can be standardized. End-group modification (amount and/or degree) can then be done on the same batch of standardized tri-block copolymer having hydroxyl end-groups.

In case of the tri-block copolymers used in the mixtures described in Yu *et al.*, in 'Mixing a sol and a precipitate of block copolymers with different block ratios leads to an injectable hydrogel', Biomacromolecules, 2009, 10, 1547-1553 and in CN200910049664 and in WO01/82970 A1, every time a different set of tri-block copolymers needs to synthesized in order to be able to tune the release profile for a particular drug.

Furthermore, the compositions of the present invention may have various other advantage besides adaptability of the release profile of the compositions that can be prepared therewith, such as for example that it can provide compositions having variable stability, degradation time, gelling temperature, injectability/viscosity, storage modulus and/or loss modulus.

Furthermore, the composition of the present invention may be more stable than the known mix compositions, since the micelles in the thermogel, by or in which the (pharmaceutically) active ingredients are retained, are more homogeneous. Also, the stability of the active ingredient present in the composition of the invention may be tuned by the method of the invention.

Not only the release profile of the drug may be tuned by using the method of the invention, but also the solubility of the composition can be varied depending on the choice and amount of end-group modification. Solubility of the composition is an important parameter in the formulation of an injectable thermogel.

With 'hydrophilic block' is meant that the block by itself has a solubility in water of at least 0.8% by weight, preferably at least1% by weight. For example PEG is a hydrophilic block.

With 'hydrophobic block' is meant that the block by itself has a solubility in water of at most 0.7% by weight, preferably less than 0.2 % by weight. For example a copolyester block is considered to be hydrophobic.

Modification of the hydroxyl end-groups of the tri-block copolymers in the composition of the invention also offers the possibility to introduce multifunctionality. For example, the hydroxyl group of the tri-block copolymer may be modified with heteroatoms which might increase the affinity of the drug for the micelles (hydrogen bonds) or unsaturation (pi-pi interaction) and/or which are imageable, such as iodo groups, which can be

imaged by X-ray or MRI, or may be modified with a compound that interacts with (and could also stabilize) the active ingredient or may be modified with cell-attracting moieties, such as the cell adhesive RGD peptide. The latter may provide a method for recruiting cells into/onto the thermogel after injection into the body.

Preferably, the invention relates to a composition of the invention wherein the end-group of at least one of the two types of tri-block copolymer is covalently bound to a compound containing radiopaque atoms, for example iodine or barium.

For example in case the endgroup is an acyl group, the acyl group may be substituted with a radiopaque atom, for example iodine. For example, an acyl group substituted with a radiopaque atom may be iodobenzoyl.

The radiopaque atoms may be visualized in situ using X-ray, for example 2D or 3D CT (X-ray computed tomography).

The invention therefore also relates to a composition comprising a tri-block copolymer according to formula (1) B-A-B (1)
wherein A stands for a linear poly-(ethylene glycol) block and
wherein B stands for wherein B stands for a poly(lactide-co-ε-caprolactone) block,
wherein at least part of the hydroxyl end-groups of the tri-block copolymer are covalently bound to a compound containing radiopaque atoms.
Preferably, the radiopaque atom is iodine.

Preferably, said composition further comprises an active ingredient, preferably a pharmaceutically active ingredient and/or a solvent.

By including a pharmaceutically active ingredient in said composition, it is possible to correlate the degree of gel erosion with the degree of release of pharmaceutically active ingredient from the gel.

Preferably, the block ratio of the tri-block copolymer in said composition, which ratio is defined as the ratio between the sum of the number average molecular weight of the B-blocks and the number average molecular weight of the A-block ranges from 1.4 to 2.6.

By covalently binding the hydroxyl end-groups of the tri-block copolymer to a compound containing radiopaque atoms, many applications of said compositions become available:

For example, it may be visualized where a composition of the invention has been injected. This 3D in situ marking of a location may be useful, for example in radiotherapy for for example breast or prostate cancer, where it is necessary to focus the energy beam at exactly the same location for sequential radiation exposures. Current positioning techniques include surgical introduction of small objects of gold, titanium or other electron dense objects that give sufficient contrast in x-ray and CT-imaging. However, these 'contrast agent markers or beckons are permanent and as such less attractive for the patient and may eventually cause scare tissue and bio-mechanic complications.

Also, in situ marking of a location may be useful when during surgery an area is marked, which needs a follow-up. For example, during endoscopic inspection of the colon and other externally accessible parts of the body, small surgical procedures may be performed either on the spot, or during a follow-up session, to burn or cut away tissue, such as small neoplasmas in the colon, which potentially could develop into tumours in the colon. Leaving behind a radiopaque gel depot will greatly facilitate revisiting of the site for inspection for follow-up procedures, thereby using image-guided techniques for positioning.

Treatment and subsequent inspection and follow-up of lesions in the gut, stomach or lung are done at shorter intervals, but again, the in situ marking of a location is desired.

Currently, to actually pin-point and revisit or inspect a previous site of treatment is not easy, as the form, shape and position, for example of the colon, may fold and twist and may alter the external positioning parameters.

Also, the temporary in situ marking of a location in vivo may be useful when successive injections need to be made at or next to previous injection sites. These radiopaque gels are far more compatible to the surrounding tissue than metal objects, and if desired, will erode away over time.

Furthermore, in the composition of the invention, the radiopaque atom stays with the gel since the atom forms part of the tri-block copolymer. This in contrast to other contrasting agents, which will readily diffuse away from the injection site.

As indicated before, the residence time of the composition of the invention can be anything in the range from some days to months.

Preferably, in said compositions, chemical hydrolysis or enzymatic cleavage at physiological conditions in situ of the tri-block copolymer wherein at least part of the hydroxyl end-groups of the tri-block copolymer is covalently bound to a compound containing one or more radiopaque atoms is limited, for example by choosing a suitable end-group linker and/or a suitable monomer, for example a hydrophobic monomer to link the compound containing one or more radiopaque atoms to.

Also, the composition of the invention comprising the tri-block copolymer wherein at least part of the hydroxyl end-groups of the tri-block copolymer is covalently bound to a compound containing one or more radiopaque atoms could be used to observe gel-erosion in vivo and to be able to correlate the gel-volume with degree of release of pharmaceutically active ingredient.

The tri-block copolymer wherein at least part of the hydroxyl end-groups of the tri-block copolymer is covalently bound to a compound containing one or more radiopaque atoms may be mixed blended with a tri-block copolymer that does not contain a compound containing one or more radiopaque atoms to fine tune for example i) the quantity, ii) the contrast intensity and iii) the duration of retention on the spot of injection.

In another aspect therefore, the invention relates to a method for imaging a position within the body of a warm-blooded species comprising the steps of:
- injecting a composition comprising a tri-block copolymer accordingly to formula (1) B-A-B (1) wherein A stands for a linear poly-(ethylene glycol) block and wherein B stands for a poly(lactide-co-ε-caprolactone) block, wherein at least part of the hydroxyl end-groups of the tri-block copolymer are covalently bound to a compound containing a radiopaque atom of the invention into the body of the warm-blooded species and imaging the composition using conventional imaging methods such as CT or micro CT.

In the tri-block copolymer according to formula (1)

B-A-B (1)

A may stand for a linear hydrophilic block, for example for linear poly-(ethylene glycol) block (PEG block). Generally, the number average molecular weight (Mn) of the PEG block in the tri-block copolymer is at least 750Da, for example at least 1000Da, for example at least 1500 Da and/or preferably at most 5000 Da, for example at most 2000Da. For example, the number average molecular weight of the PEG block ranges

from 1000 to 5000 Da, preferably from 1000 to 2000 Da, for example from 1000 to 1500 Da. The number average molecular weight as used herein is defined as the number average molecular weight as determined using ^{1}H nuclear magnetic resonance.

Poly-(ethylene glycol) is a diol also known as poly (ethylene oxide) and both names can be used interchangeably for the purpose of the invention.

In the tri-block copolymer according to formula (1)

B-A-B (1)

B may stand for a (linear) hydrophobic block, for example for a hydrophobic block containing ester and/or carbonate bonds.

B may for example stand for a hydrophobic block comprising at least one, preferably at least two cyclic monomers selected from the group consisting of glycolide, lactide, ε-caprolactone, p-dioxanone (1,4-dioxan-2-one), trimethylene carbonate (1,3-dioxan-2-one), 1,4-dioxepan-2-one (including its dimer 1,5,8,12-tetraoxacyclotetradecane-7,14-dione), 1,5-dioxepan-2-one, 6,6-dimethyl-1,4-dioxan-2-one, 2,5-diketomorpholine, pivalolactone, chi.-diethylpropiolactone, ethylene carbonate, ethylene oxalate, 3-methyl-1,4-dioxane-2,5-dione, 3,3-diethyl-1,4-dioxan-2,5-dione, 6,8-dioxabicycloctane-7-one, β-propiolactone, γ-butyrolactone, δ-valerolactone, ε-decalactone, 3-methyl-1,4-dioxane-2,5-dione, 1,4-dioxane-2,5-dione, 2,5-diketomorpholine, α,α-diethylpropiolactone, 6,6-dimethyl-dioxepan-2-one, 6,8-dioxabicycloctane-7-one and 5,5-dimethyl-1,3-dioxan-2-one, wherein the hydroxyl end-groups of the tri-block copolymer in case they are acylated are acylated with an optionally substituted acyl having 2 to 12 C-atoms, C-atoms of the substituents included.

(Bio)degradation in the context of the present invention may be assessed in vitro by various analytical techniques including size-exclusion chromatography, nuclear magnetic resonance, MALDI-TOF, high pressure liquid chromatography or combinations of those.

The B-blocks may for example comprise monomer combinations comprising between 50 and 100 mol%, for example between 60 and 95 mol%, for example between 75 and 90 mol% glycolide.

The B-blocks may for example comprise monomer combinations comprising between 50 and 100 mol%, for example between 60 and 95 mol%, for example between 75 and 90 mol% ε-caprolactone.

The B-blocks may for example comprise monomer combinations comprising between 50 and 100 mol%, for example between 60 and 95 mol%, for example between 75 and 90 mol% lactide.

The B-blocks may for example comprise monomer combinations comprising between 50 and 100 mol%, for example between 60 and 95 mol%, for example between 75 and 90 mol% trimethylene carbonate.

Other combinations of the listed monomers are also possible, and the skilled person is able to choose them according to the polymer properties that they need for a specific application.

Preferably, the cyclic monomers of the B-blocks are selected from the group consisting of glycolide, lactide, ε-caprolactone, trimethylene carbonate and 1,3-dioxan-2-one, more preferably from the group consisting of lactide and ε-caprolactone. Preferred combinations of cyclic monomers in the B-blocks of the copolymers according to the present invention include but are not limited to:

- glycolide and a monomer of the group of 1,3-dioxan-2-one, 5,5-dimethyl-1,3-dioxan-2-one, 1,4-dioxan-2-one, 1,4-dioxepan-2-one, 1,5-dioxepan-2-one.
- lactide and a monomer of the group of 1,3-dioxan-2-one, 5,5-dimethyl-1,3-dioxan-2-one, 1,4-dioxan-2-one, 1,4-dioxepan-2-one, 1,5-dioxepan-2-one.
- 1,3-dioxan-2-one and a monomer of the group of 5,5-dimethyl-1,3-dioxan-2-one, 1,4-dioxan-2-one, 1,4-dioxepan-2-one, 1,5-dioxepan-2-one.
- ε-caprolactone and a monomer of the group of 1,3-dioxan-2-one, 5,5-dimethyl-1,3-dioxan-2-one, 1,4-dioxan-2-one, 1,4-dioxepan-2-one, 1,5-dioxepan-2-one.

Most preferably, B stands for a poly(lactide-co-ε-caprolactone) block (PLCA block), which PLCA block is a copolymer of lactide and ε-caprolactone. Within the framework of the invention the term lactide refers to all enantiomerically enriched, racemic forms and cyclic ester forms of lactic acid, such as L-lactide, D-lactide and DL-lactide.

The number average molecular weight of each individual B-block, preferably of the PLCA block as determined using ^{1}H NMR as described herein is at least 400 Da, for example at least 450 Da, preferably at least 500 Da, and/or at most 1500 Da, for

example at most 2000 Da, for example at most 2500 Da, preferably at most 3000 Da, for example at most 5000 Da. For example, the number average molecular weight of the PLCA block ranges from 400 to 3000, for example from 450 to 2000, for example from 500 to 1500 Da.

The block ratio of the tri-block copolymer is defined as the ratio between the sum of the number average molecular weight (Mn) of the B-blocks and the number average molecular weight (Mn) of the A-block, wherein the number average molecular weight of the A and of the B-blocks is determined using ^{1}H NMR as described herein.

The block ratio of the tri-block copolymer(s) may range from at least 0.8, for example at least 1.0, for example at least 1.1, for example at least 1.2, for example at least 1.3, for example at least 1.4, for example at least 1.6, for example at least 1.8 to at most 5, for example at most 3, for example at most 2.6, for example at most 2.4, for example at most 2.2, for example at most 1.8, for example at most 1.4.

Preferably, the block ratio ranges of the at least two tri-block copolymers from 1.4 to 2.6, for example from 1.8 to 2.6, for example from 1.8 to 2.4.

The hydroxyl end-groups of the tri-block copolymer are at least partially acylated with an optionally substituted acyl having 2 to 12 C-atoms, C-atoms of the substituents included. The acyl group may be represented by a compound of formula (2)

R^1-C(O)- (2)

wherein R^1 stands for an optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl or optionally substituted aryl, preferably for an optionally substituted alkyl or optionally substituted aryl, preferably an optionally substituted alkyl and wherein the R^1-C(O) group is covalently linked to the tri-block copolymer.
Examples of R^1 include but are not limited to linear and branched alkyls, for example methyl, ethyl, n-propyl, i-propyl, n-butyl, i-butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl and undecyl; linear and branched alkenyls; and linear and branched alkynyls. R^1 may also stand for the cyclic form of the alkyl, alkenyl or alkynyl. An example of optionally substituted aryl includes but is not limited to phenyl and halogen, for example iodine substituted phenyls.
Preferably, R^1 stands for an alkyl, preferably for methyl, ethyl. (in other words the acyl

group is acetyl or propionyl).
R^1 may optionally be substituted with a functionalized group, for example with a group containing a heteroatom, for example O, N, for example an NH_2 group, S, for example an SH group, halogen, for example a fluoride, chloride or iodine group, preferably with iodine.

The hydroxyl end-groups of the tri-block copolymer may be acylated using methods known in the art, for example by reaction of the hydroxyl end-groups with an acid chloride or with an anhydride.
For example, acylation of the hydroxyl end-groups of the tri-block copolymer may be done using acetyl chloride (which is an unsubstituted acyl having 2 C-atoms), with propionyl chloride (which is an unsubstituted acyl having 3 C-atoms), etc.
For example, acylation of the hydroxyl end-groups of the tri-block copolymer may be done by reacting with the corresponding anhydride in pyridine at elevated temperature.

With 'degree of modification' is meant that the percentage of end-groups, preferably hydroxyl end-groups, that is modified. As used herein, 'degree of modification' is also referred – in case the end-groups are acylated as the 'degree of acylation'. The degree of modification may range from 0 and is at most 100%; for example the degree of modification (of one of the tri-block copolymers) of the hydroxyl end-groups is at least 25%, for example at least 30%, for example at least 35%, for example at least 40% and/or at most 95%, for example at most 90%, preferably 100%.
For the avoidance of doubt, a degree of modification of 100% means that all hydroxyl end-groups of the tri-block copolymer have been modified; a degree of modification of 50% means that half of the hydroxyl end-groups have been modified and a degree of modification of 0% means that none of the hydroxyl end-groups have been modified.
The degree of modification, is preferably calculated using ^1H nuclear magnetic resonance by comparing integrals of the peaks due to the acylated hydroxyl end-groups and the integrals of the polyethylene glycol (as a measured for the amount of non-acylated hydroxyl end-groups).

In a preferred embodiment of the invention, one of the at least two tri-block copolymers has a degree of modification of 100% and one of the at least two tri-block copolymers has a degree of modification of 0%. This is preferred since a tri-block copolymer having a degree of modification of 0% is easily reproduced. A tri-block copolymer having a degree of modification of 100% can also be easily reproduced, for instance by using an excess of the compound with which the hydroxyl end-groups are acylated.

Preferably, the at least two types of B-A-B types of tri-block copolymers differ only on the degree of modification of the end-groups. By mixing the B-A-B type of tri-block copolymer having a degree of modification of 100% with a B-A-B type of tri-block copolymer having a degree of modification of 0% in different ratios, different compositions with a reproducible degree of total modification of the end-groups in the composition can be prepared.The reproducibility of the degree of modification of the end-groups in the composition is much higher than if this had to be achieved via synthesis of a single tri-block copolymer, hence a higher reproducibility and accuracy of the medication can be obtained.

Furthermore, by choosing the same (at least) two types of B-A-B tri-block copolymers, which differ only on the degree of modification of the end-groups, regulatory approval only needs to be obtained for these (at least) two types of B-A-B tri-block copolymers; whereas many different compositions having different final degrees of modification of end-groups in the composition can be prepared there from.

In the context of the invention, with 'degree of total modification of the end-groups in the composition' is meant the total amount of end-groups of all tri-block copolymers present that is modified. For example a final degree of modification of the end-groups in the composition of 100% means that all hydroxyl end-groups of the tri-block copolymers in the composition have been modified; a degree of total modification of the end-groups in the composition of 50% means that half of the hydroxyl end-groups of the tri-block copolymers in the composition have been modified.

Alternatively, one of the at least two tri-block copolymers have a degree of modification of 100% . This is another preferred embodiment, since a tri-block copolymers having a degree of modification of 100% can be easily reproduced, for instance by using an excess of the compound with which the hydroxyl end-groups are acylated.

Preferably, the at least two types of B-A-B types of tri-block copolymers differ only on the type of end-group. In a preferred embodiment of the invention, the composition comprises a mixture of a first B-A-B type of triblock copolymer having a specific end-group and a second B-A-B type of triblock copolymer which differs only from the first tri-block copolymer in its end-group.

The synthesis of the tri-block copolymer as used in the composition of the invention can be done using methods known in the art, for example by ring-opening polymerization or polycondensation reactions. For example, the tri-block copolymer wherein the B block is a poly(lactide-co-ε-caprolactone) block (PLCA block) may be

synthesized (analogous to) the method described by Seongbong, Jo et al. in 'Reverse thermal gelation of aliphatically modified biodegradable triblock copolymers', Macromol. Biosci. (2006), pages 923-928, which article is hereby included by reference. Figure 1 as described herein gives the reaction scheme for the synthesis of a tri-block copolymer of PLCA-PEG-PLCA and its acylation of the hydroxyl end-groups with aliphatic acid chloride.

B blocks can be polymerized by using the cyclic monomers mentioned above in a ring-opening polymerization using the hydroxyl end-groups of poly(ethylene glycol) to initiate the polymerization. This is a very controlled and straightforward way of preparing triblocks in one step for people skilled in the art. Schemes and details for similar ring-opening polymerization reactions can be found in several patents or patent applications including and not limited to EP0863745 and WO0018821.

Preparing B blocks by polycondensation reactions using the open form of the cyclic monomers mentioned above, such as lactic acid, glycolic acid, epsilon-hydroxyhexanoic acid and the like is also possible, but less preferred since obtaining well-defined blocks in terms of average molecular weight and end-group functionality with polycondensation reactions is difficult.

Modification of the end-groups of the tri-block copolymer(s) in the composition of the invention may be done using methods known per se. For example, acylation of the hydroxyl end-groups may be done (analogous to) the method described in Seongbong, Jo et al. in 'Reverse thermal gelation of aliphatically modified biodegradable triblock copolymers', Macromol. Biosci. (2006), pages 923-928, and as illustrated by figure 1 therein, which article is hereby included by reference.

The number average molecular weight (Mn) of the tri-block copolymer depends on the number average molecular weight of the B and A blocks used, preferably respectively PLCA and PEG blocks. The number average molecular weight of the tri-block copolymer may be calculated using ^{1}H nuclear magnetic resonance. Preferably the Mn of the tri-block copolymer ranges from 3,000 to 5,000 Da.

The weight ratio of ε-caprolactone to lactide is the weight of ε-caprolactone divided by the weight of lactide used to prepare the B-block with, in case the B-block is PLCA. The weight ratio of ε-caprolactone to lactide is preferably at least 1/1, for example from 9/1 to 1/9, for example from 4/1 to 1/4, for example from 1/1 to 1/0, for example from 1/1 to 9/1.
For the avoidance of doubt, with weight ratio of ε-caprolactone to lactide is 1/0 is meant

that only I only ε-caprolactone is present and no lactide. With weight ratio of ε-caprolactone to lactide is 0/1 is meant that only lactide is present and no ε-caprolactone.

The active ingredient in the composition of the present invention may be an active ingredient such as any pharmaceutically active ingredient and any diagnostic and any contrast agent and includes those pharmaceutically active ingredients having a prophylactic effect on the animal, including human as well as those pharmaceutically active ingredients that have an effect of alleviating, reducing or even completely eliminating a symptom, or a cause, or a consequence of a disease, such as pain, swelling or inflammation or a disease from the animal, including human.
For example, the pharmaceutically active ingredient may include broad classes of compounds normally delivered into the body. For example, these pharmaceutically active ingredients include but are not limited to anti-infectives (including antibiotics, antivirals, fungicides, scabicides or pediculicides); antiseptics (e.g. benzalkonium chloride, benzethonium chloride, chorhexidine gluconate, mafenide acetate, methylbenzethonium chloride, nitrofurazone, nitromersol and the like); analgesics and analgesic combinations; anorexics; antihelminthics, antiarthritics, antiasthmatic agents; anticonvulsants; antidepressants; antidiabetic agents; antidiarrheals; antihistamines; anti-inflammatory agents, antimigraine preparations; antinauseants; antineoplastics; antiparkinsonism drugs; antipuritics; antipsychotics; antipyretics, antispasmodics; anticholinergics; sympathomimetics; xanthine derivatives; cardiovascular preparations including potassium and calcium channel blockers; beta-blockers; alpha-blockers and antiarrhythmics; antihypertensives; diuretics and antidiuretics; vasodilators including general coronary, peripheral and cerebral vasodilators; central nervous system stimulants; vasoconstrictors; cough and cold preparations, including decongestants; hormones and steroids (e.g. estrogens, progestins, androgens, adrenocorticoids, corticosteroids and the like); hypnotics; immunosuppressives; muscle relaxants; parasympatholytics; psychostimulants; sedatives and tranquilizers, narcotics (e.g. morphine, meperidine, codeine and the like), local anesthetics (e.g. amide- or anilide-type local anesthetics such as bupivacaine, dibucaine, mepivacaine, procaine, lidocaine, tetracaine and the like); antiemetic agents (e.g. ondansetron, granisetron, tropisetron, metoclopramide, domperidone, scopolamide and the like); antiangiogenic agents (e.g. combrestatine, contortrostatin, anti-VEGF and the like), polysaccharides, immune-modulating, anti-thrombogenic compounds, anti-claudicating drugs, anti-atherosclerotic drugs, antihistamines, anti-cancer drugs (e.g. mechlorethamine, cyclophosphamide, fluorouracil, thioguanine, carmustine, lomustine, melphalan,

chloambucil, streptozocin, methotrexate, vincristine, bleomycin, vinblastine, vindesine, dactinomycine, daunorubicin, doxorubicin, tamoxifen, paclitaxel, epirubicin, mitomicin C, cis-platin, carboplatin, and the like and photosensitizers used in photodynamic therapy, vascular drugs, ophthalmic drugs, amino acids, vitamins, neurotransmitters, neurohormones, signaling molecules, psychoactive medicaments, synthetic drugs, semi-synthetic drugs, natural drugs and substances derived from these, or combinations of the above.

The pharmaceutically active ingredient may also be a biological including but not limited to (recombinant) proteins, PEGylated-proteins and peptides (e.g. insulin, erythropoietin, exenatide, glucagon-like-peptide-1, morphogenic proteins (e.g. bone morphogenic proteins, transforming growth factors, fibroblast growth factors, tumor necrosis factors), receptor antagonists (e.g. Interleukin-1-receptor-antagonist), anticancer proteins (e.g. neocarzinostatin, *L*-asparaginase, interleukin-2, bevacizumab and other anti-VEGF agents) prophylactic vaccines, therapeutic vaccines, genetic materials (e.g. nucleic acid sequences, polynucleotides, (antisense) oligonucleotides, plasmids, DNA, RNA, siRNA, microRNA), aptamers, enzymes, antigens, antibodies, antibody fragments, viruses, virus-based materials, cells, cellular substructures, etc.),

Prodrugs, metabolites, derivatives, in-vivo or in in-vitro chemically modified products, in-vivo or in-vitro enzymatic modified products and pharmaceutically active degradation products of the pharmaceutical active ingredients described herein are included in the scope of the invention.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of immune-modifying drugs, anti-inflammatory drugs or growth factors.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of immune-modifying drugs for example cyclosporine, tacrolimus (FK-506), sirolimus or rapamycin.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of steroidal anti-inflammatory drugs, for example prednisone, prednisolon, triamcinolon, clobetasol or betamethason.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of non-steroidal anti-inflammatory drugs, for example aspirin, diclofenac, piroxicam, meloxicam, ibuprofen or a selective COX-2 inhibitor for example celecoxib, valdecoxib, etoricoxib or rofecoxib.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of anticancer agents for example bevacizumab, tamoxifen or interleukin-2.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of anti-viral agents for example acyclovir or oseltamivir.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of anti-bacterial agents for example amoxicillin.

Preferably, the active ingredient belongs to the class of human growth hormones and its biosimilar derivatives, which can be applied in both pediatric and adult growth disorders, maintaining sufficient musculature, and for anti-ageing applications.

Preferably, the active ingredient is a pharmaceutically active ingredient effective against inflammation or microbial infections of the inner ear and its connecting tissues, (intratympanic ear diseases).

Preferably, the active ingredient is a pharmaceutically active ingredient effective against forms of diabetes, for example glucagon-like-peptide-1, and its derivatives such as exendin-4 and liraglutide.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of vaccines.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of ophthalmic agents for example Triamcinolone and Bevacizumab.

Preferably, the active ingredient is a pharmaceutically active ingredient effective against forms of neuro-degenerative diseases such as apomorphine, rivastigmine, pramipexole, pioglitazone, memantine and safinamide

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of biologicals including but not limited to growth factors which are very suitable for application in orthopedics and in particular in the prevention or treatment of diseases of intervertebral discs, or cartilage, or bone. Examples of such growth factors include but are not limited to transforming growth factor 3, fibroblast growth factor 18, osteogenic protein 1, bone morphogenic protein 2, bone morphogenic protein 6, bone morphogenic protein 7, interleukin-1-receptor-antagonist.

Preferably, the active ingredient is a pharmaceutically active ingredient effective against forms of diabetes, for example glucagon-like-peptide-1, exenatide, amylin.

The pharmaceutically active ingredient may for example be an agent to suppress or slow down cancerous growth or neovascularisation, such as anti-VEGF agents, si-RNA or aptamers or antistatics, such as rapamycin.

Examples of anticancer agents include doxorubicin, daunorubicin, epirubicin, mitomicin C, paclitaxel, cis-platin, carboplatin, and anticancer proteins such as neocarzinostatin, *L*-asparaginase, and the like and photosensitizers used in photodynamic therapy.

The pharmaceutically active ingredient may for example be an agent to avoid, control, suppress, or eradicate infectious diseases.

Preferably, the active ingredient is a pharmaceutically active ingredient chosen from the group of non-steroidal anti-inflammatory drugs, for example a steroid or a selective COX-2 inhibitor; and biologicals, for example a (monoclonal) antibody, protein, peptide or cell extract.

The solvent in the composition of the present invention is preferably a solvent that is non toxic, preferably also biocompatible, and approved by regulatory instances, preferably aqueous based or a so-called FDA (Federal Drug Administration) class 3 solvent.
Examples of solvents include but are not limited to water, mixtures of water and an organic solvent like for example ethanol, isopropanol or dimethylsulfoxide (DMSO); aqueous buffer solutions, preferably aqueous buffer solutions that lead to isotonic compositions of the invention, such as PBS (phosphate buffered saline) or Sorenson modified buffer or variants thereof; and organic solvents, such as ethylacetate, acetone, dichloromethane (DCM), dimethylsulfoxide (DMSO), N-methylpyrrolidone (NMP), isopropyl myristate and benzylbenzoate.

With 'isotonic' is meant that the solution a solution having an osmotic pressure comparable to or at least compatible with the osmotic pressure of human or animal bodily fluids, preferably with blood.

Preferably the solvent is water or an aqueous buffer solution, more preferably an aqueous buffer solution that leads to an isotonic composition of the invention.
The pH of the solvent is preferably in the range from 5 to 8, for example in the range from 6.5 to 7.5. More preferably, the pH of the solvent is chosen such that the pH of the

composition of the invention is about neutral (in the range from 5 to 8, preferably in the range from 6.5 to 7.5).

With gel temperature is meant the temperature at which the tri-block copolymer in the solvent chosen forms a gel. Vial tilting as described below can be used to determine the gel temperature.

The gel temperature of the composition of the invention is preferably at least 20 °C, more preferably at least 25 °C, for example at least 30 °C and/or preferably at most 36, for example at most 35, for example at most 34 °C. For example, the gel temperature of the composition of the invention ranges from 30 to 35 °C.

With phase separation temperature is meant the temperature at which the different components present are not capable of forming a homogeneous solution, but instead form separate (liquid) phases. Phase separation can be determined using vial tilting. To this end, the compositions of the invention are vortexed and stored at 5 °C. If needed, the samples were subjected to another heating and cooling cycle until all components in the composition of the invention were completely dissolved. The samples were evaluated by vial tilting at 5 °C, then allowed to equilibrated at 20 °C for 30 minutes after which they were evaluated again. Visual observations were made and noted down. Next, the samples were placed in a water bath and slowly heated (less than 0.5 °C/min). At every 1 °C increment, samples were evaluated by vial tiliting and considered a gel if no flow was observed during a period of 15seconds. Samples were heated until phase separation was observed and the temperature at which phase separation was observed is the 'phase separation temperature'.
Preferably, the phase separation temperature of the composition of the invention is at least 25 °C, for example at least 30 °C, for example at least 40 °C, for example at least 41 °C, for example at least 42 °C.

Preferably, for clinical applicability, the tri-block copolymer has a gel window between 30 °C and 50 °C, for example between 30 °C and 42 °C. With 'gel window' is meant the 'window between the gel temperature and the phase separation temperature', in other words it is the entire temperature range in which the tri-block copolymer is a gel.

The concentration of tri-block copolymers in the composition of the invention is in principle not critical, but will generally be from 10 to 40% w/w, preferably from 15 to 35% w/w, for example from 20 to 35% w/w based on the amount of solvent and active ingredient present in the composition..

The ratio in which the tri-block copolymers in the composition of the invention are mixed to obtain the desired release profile of the active ingredient can easily be determined by the skilled person. For example, the weight ratio of the at least two types of tri-block copolymers may range from 0.1/99.9 to 99.9/0.1, for example from 25/75 to 50/50 or from 75/25 to 50/50. Preferably the weight ratio of the at least two types of tri-block copolymers is at least 75/25.

The amount of active ingredient in the composition of the present invention depends on the amount to be administered to the animal, including human and on the duration of the release. For example, the amount of active ingredient may be up to and including 50% w/w based on the tri-block copolymers in the composition if a high loading of the drug is desired, but may also for example be less, for example an amount of up to and including 40, for example up to and including 30, for example up to and including 20% w/w based on the triblock copolymers in the composition and/or for example at least 0.01% w/w, preferably at least 0.1 % w/w, for example at least 1% w/w based on the triblock copolymers in the composition.

The invention also relates to a composition of the invention, wherein the composition is a pharmaceutical composition further comprising a pharmaceutically acceptable adjuvant, carrier, excipient, contrast agent or dye.

The invention also relates to a composition, wherein the composition further comprises nano-particles and/ or microparticles (such as liposomes and microspheres) which themselves contain the pharmaceutically active ingredients as described above.

A person skilled in the art knows which carriers can be used as pharmaceutically acceptable carriers. Examples of such pharmaceutically acceptable carriers are both inorganic and organic carrier materials, suitable for oral/parenteral/injectable administration and include water, polyethyleneglycol (PEG), polypropyleneglycol (PPG), cellulose derivatives, such as hydroxypropylmethylcellulose (HPMC), hydroxyethylmethylcellulose (HEMC), hydroxyethylcellulose (HEC); polyvinylpyrrolidone (PVP), polyvinylalcohol (PVA), gelatin, gum arabic, lactose, starch, magnesium stearate, talc, vegetable oils, and the like.

Also, the person skilled in the art knows which pharmaceutically acceptable adjuvants and excipients may be used in the compositions of the invention. Examples of conventional pharmaceutical additives and adjuvants, excipients or diluents, including, but not limited to, water, gelatin of any origin, polyethyleneglycol (PEG), polypropyleneglycol (PPG); cellulose derivatives, such as

hydroxypropylmethylcellulose (HPMC), hydroxyethylmethylcellulose (HEMC) or hydroxyethylcellulose (HEC); polyvinylpyrrolidone (PVP), polyvinylalcohol (PVA), vegetable gums, ligninsulfonate, talc, sugars, starch, gum Arabic, vegetable oils, polyalkylene glycols, flavoring agents, preservatives, stabilizers, emulsifying agents, buffers, lubricants, colorants, wetting agents, fillers, imaging agents, such as contrast agents for X-ray or MRI; and the like, for example iodo compounds, such as the commercially available Xenetic® and Hexabrix®.

In another aspect, the invention relates to a composition according to the invention for use as a medicament.

In another aspect, the invention relates to a composition according to the invention for use as a medicament that is injected through a needle of at least 18G, for example at least 21G, preferably at least 23G, for example at least 27G, for example at least 30G. With G is meant 'Gauge' which is a measure for the outer diameter of a needle. Smaller gauge numbers indicate larger outer diameters and as such 'larger needles'. For ease of injection, smaller needles are preferred to larger needles. Inner diameters of a needle depend on both gauge and wall thickness.

The invention can also contain and release micro- or nano-sized particles such as microspheres and liposomes which already contain for instance drugs, nucleotide sequences or imaging agents.

The benefit of combining the invention with these micro-or nano-sized particles is that the invention will ensure localization and gradual release of these particles, instead of a typical migration of such particles from the place of injection.

Examples of contrast agents include iodo compounds. Exemplary iodo compounds are commercially available as Xenetic® and Hexabrix®.

Depending on the pharmaceutically active ingredient or combination of pharmaceutically ingredients present in the composition of the invention, different diseases may be targeted, preferably treated using the composition(s) of the present invention.

In another aspect, the invention relates to a method for preparing a composition according to the invention suitable for forming a thermogel comprising the step of mixing at least two types of B-A-B types of tri-block copolymers of formula (1)

B-A-B (1)

wherein B stands for a hydrophobic block and wherein A stands for a hydrophilic block wherein the at least two types of B-A-B types of tri-block copolymers differ only on the type of end-group and/or on the degree of modification of the end-groups.

The was the polymer mixture is prepared is in principle not critical; it is for example possible to dissolve each polymer separately in the solvent of choice or for example it is possible and preferred to dissolve the polymers in a solvent, which solvent is thereafter evaporated. The dried polymer mixture can then be dissolved in the solvent of choice.

In yet another aspect, the invention relates to a process for the preparation of a composition of the invention comprising the steps of
- synthesizing the tri-block copolymer and
- mixing the tri-block copolymer with the active ingredient and the solvent.
The order in which the tri-block copolymers and the active ingredient are dissolved in the solvent is in principle not critical; it is for example possible to first dissolve the tri-block copolymers and then the active ingredient, to first dissolve the active ingredient and then the tri-block copolymers or for example to dissolve both the active ingredient and the tri-block copolymers at the same time in the solvent of choice.
However, for active ingredients that are highly soluble in the solvent, preferably first the active ingredient is dissolved in the solvent to form a solution of the active ingredient in the solvent, after which the tri-block copolymers are dissolved into said solution.
For active ingredients that are less soluble in the solvent of choice, the active ingredient may first be dissolved into a solvent in which it is better soluble (for example a hydrophobic drug may be soluble in ethylacetate), after which the polymers are added and dissolved, after wihich the solvent is evaporated and the solvent of choice is added to the (dried polymer) mixture. This will increase the solubility of the active ingredient in the solvent of choice.
To facilitate dissolving the tri-block copolymer and/or the active ingredient, the composition comprising the tri-block copolymer, the solvent and the active ingredient may be heated for example until the tri-block polymer melts. For example, the heating of the composition may be performed at 50°C.

Preferably, if the active ingredient is sensitive to heat, the tri-block copolymer may be melted (for example at a temperature of about 50°C), dissolved into the solvent of choice after which the obtained solution may be cooled down and the active ingredient may be dissolved in the solvent.

In another aspect, the invention relates to a method for delivering an pharmaceutically active ingredient over an extended period, for example over a period of more than 1 day up to several, e.g. 6, months, to an animal, including a human requiring such treatment which comprises administering to such animal an effective amount of the composition of the invention.

In yet another aspect, the invention relates to a method for delivering an pharmaceutically active ingredient over an extended period, for example over a period of more than 1 day up to several, e.g. 6, months, to an animal, including a human requiring such treatment which comprises administering to such animal an effective amount of the composition of the invention through a needle of at least 18G, for example at least 21G, preferably at least 23G, for example at least 27G, for example at least 30G.

In the framework of the invention, with animals is meant all animals, including mammals, examples of which include humans. Preferred examples of mammals beside humans include but are not limited to dogs, cats, dromedaris, camels, elephants, lama's, goats, mice, guinea pigs, rabbits, pigs, cows, water buffalos, kangaroos, monkeys and horses.

Although the invention has been described in detail for purposes of illustration, it is understood that such detail is solely for that purpose and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention as defined in the claims.

It is further noted that the invention relates to all possible combinations of features described herein, preferred in particular are those combinations of features that are present in the claims.

It is further noted that the term 'comprising' does not exclude the presence of other elements. However, it is also to be understood that a description on a product comprising certain components also discloses a product consisting of these components. Similarly, it is also to be understood that a description on a process comprising certain steps also discloses a process consisting of these steps.

The invention is now elucidated by way of the following examples, without however being limited thereto.

Examples

Measurement methods

The triblock copolymer composition (PLCA/PEG ratio, cap/lac ratio and DM) was

analyzed with proton nuclear magnetic resonance (^{1}H NMR; Varian, 400 MHz), using deuterated chloroform as solvent and reference. From the integration of various proton signals (due to PEG and incorporated monomers), absolute number average molecular weights M_n were obtained. Mn of the tri-block copolymer is the sum of the molecular weights of the central PEG block and the two polyester blocks (ratio of blocks determined with NMR)

The block ratio as used herein is the weight ratio of the PLCA-blocks to the PEG-block (of known molecular weight) and can be calculated from grams of monomers (lactide + caprolactone) divided by grams of PEG used to synthesize the tri-block copolymer. Final triblock composition after polymer purification was checked with ^{1}H NMR by comparing integrals of peaks due to PEG and incorporated ring-opened monomers. The weight ratio of ε-caprolactone to L-lactide can be calculated from NMR by comparing integrals of peaks due to ring-opened lactide and caprolactone The integrals of peaks due to acyl endgroup and PEG block were used to calculate the degree of modification (DM range 0-2, which corresponds to a degree of acylation of 0 to 100%).

Example 1 synthesis of (unmodified) PLCA-PEG-PLCA

In a three-neck round-bottom flask with a Dean Stark trap and condenser on top, 12.5 g PEG 1500 (8.3 mmol) and 125 ml toluene were stirred and heated at 150 °C under a nitrogen atmosphere. PEG was dried azeotropically by distilling off ca. 60 ml toluene/water.

After cooling down the solution to ca. 80-100 °C, 3 g L-lactide (21 mmol) and 12 g caprolactone (105 mmol) were added. The solution was heated again at 150 °C and an additional 40 ml toluene/water was distilled off. The solution was then cooled down to ca. 80-100 °C and 0.2 ml stannous octoate was added. Ring-opening polymerization was carried out at 120 ° C overnight under nitrogen atmosphere. The next day the solution was cooled down and the polymer was precipitated by addition of a 1:1 mixture of hexane and ether. At – 20 °C the polymer solidified and non-solvents were decanted. The polymer was dried in vacuo. Yield: ca. 26 g soft wax.

Unmodified PLCA-PEG-PLCA, having a PLCA/PEG block ratio around 1.2 and a caprolactone/lactide weight ratio 4/1 was obtained. This tri-block copolymer is water-soluble; but does not form a thermogel at 37°C.

Example 2: synthesis of fully C6-modified PLCA-PEG-PLCA

Polyethyleneglycol (PEG 1500, 15.5 g, 10.3 mmol) and ca. 135 ml toluene were charged into a 250 ml three-neck round-bottom flask equipped with a magnetic stirring bar. Using a Dean-Stark device with a condenser on top, 50 ml of toluene was distilled off to remove water (from PEG) azeotropically by heating at 150 °C at atmospheric pressure under nitrogen.

After cooling down the solution to ca. 80-100 °C, L-lactide (3.7 g, 26 mmol) and caprolactone (15 g, 131 mmol) were added. 55 ml of toluene was distilled off to dry the monomers by heating at 150 °C at atmospheric pressure. Ca. 30 ml of dry toluene was left in the flask for the polymerization.

After cooling down the mixture to ca.80-100 °C, tin(II) 2-ethylhexanoate (0.25 ml) was added through one of the necks.

Polymerization was carried out at 120 °C for 1 day under nitrogen atmosphere.

After cooling down to room temperature, ca. 70 ml dichloromethane and 7 ml triethylamine (50 mmol) were added. Subsequently, 5.8 ml hexanoyl chloride (42 mmol) was added slowly to the stirred solution, which was cooled with an ice bath. The acylation reaction was continued for a few hours after which dichloromethane was removed by rotavap, and ethyl acetate (ca. 100 ml) was added to the residue. Triethylamine salt was removed by (paper) filtration and the polymer, which was dissolved in the clear filtrate was precipitated by addition of a (1:1) mixture of hexane and diethyl ether. At ca. - 20 °C (in freezer) the polymer product separated as a waxy solid from which non-solvents could be decanted easily. Finally, the precipitated polymer was dried in vacuo. Yield: ca. 28 g.

The PLCA/PEG block ratio is around 1.2; caprolactone/lactide weight ratio 4/1. Degree of modification (from NMR) is ca. 2.

Example 3 Synthesis of partially C6-modified PLCA-PEG-PLCA with a (DM) degree of endgroup modification of 1.5

Polyethyleneglycol (PEG 1500, 19.5 g, 13 mmol) and ca. 160 ml toluene were charged into a 500 ml three-neck round-bottom flask equipped with a magnetic stirring bar.

Using a Dean-Stark device with a condenser on top, 60 ml of toluene was distilled off to remove water (from PEG) azeotropically by heating at 150°C at atmospheric pressure under nitrogen.

After cooling down the solution to ca. 80-100°C, L-lactide (4.8 g, 33 mmol) and caprolactone (18.7 g, 164 mmol) were added. 60 ml of toluene was distilled off to dry the monomers by heating at 150°C at atmospheric pressure. Ca. 40 ml of dry toluene was left in the flask for the polymerization.

After cooling down the mixture to ca.80-100°C, tin(II) 2-ethylhexanoate (0.3 ml) was added through one of the necks.
Polymerization was carried out at 120°C for 1 day under nitrogen atmosphere.

After cooling down to room temperature, ca. 50 ml dichloromethane and 4.2 ml triethylamine (32 mmol) were added. Subsequently, ca. 3.4 ml hexanoyl chloride (25 mmol) was added slowly to the stirred solution, which was cooled with an ice bath. The acylation reaction was continued for a few hours after which dichloromethane was removed by rotavap, and ethyl acetate (ca. 80 ml) was added to the residue. Triethylamine salt was removed by (paper) filtration and the polymer, which was dissolved in the clear filtrate was precipitated by addition of a (1:1) mixture of hexane and diethyl ether. At ca. - 20 °C (in freezer) the polymer product separated as a waxy solid from which non-solvents could be decanted easily. Finally, the precipitated polymer was dried in vacuo. Yield: ca. 38 g

The PCLA/PEG block ratio is around 1.2; caprolactone/lactide weight ratio 4/1. Degree of modification (from NMR) is ca. 1.5

Example 4 synthesis of fully C12-modified PLCA-PEG-PLCA

C12-modified PLCA-PEG-PLCA was made in the same way as the analogous C6-modified triblock described in example 2. In that case lauroyl chloride was used. Fully C12-modified PLCA-PEG-PLCA is insoluble in (cold) water (temperature of 7°C).

Table 1 summarizes the results of the polymer synthesis and aliphatic modification. The molecular weights of the synthesized polymers were determined by NMR. The NMR spectra of PLCA-PEG-PLCA show characteristic peaks of lactide, caprolactone, and PEG at 5.1, 2.3, and 3.6 ppm, respectively.

TABLE 1

Characterization results of PLCA-PEG-PLCA and aliphatically modified PLCA-PEG-PLCA by NMR

Polymer	PLCA/PEG (block ratio)	Cap/lac ratio (weight ratio of ε-caprolac-tone to lactide)	Degree of Modification (DM or degree of acylation)
PLCA-PEG-PLCA (I)	1.2	4/1	0 (0%)
Partial C6-modified PLCA-PEG-PLCA (IV)	1.2	4/1	1.5 (75%)
Fully C6-modified PLCA-PEG-PLCA (II)	1.2	4/1	2 (100%)
Fully C12- modified PLCA-PEG-PLCA (III)	1.2	4/1	2 (100%)

PLCA/PEG: the ratio of PLCA to PEG

Example 5 blending fully modified and unmodified PLCA-PEG-PLCA

This example illustrates a method for making tri-block copolymer mixtures with various selectable reverse thermal gelation temperatures by mixing two pre-made individual tri-block copolymers. Tri-block copolymers prepared by the method described in Example 1 and 2 (PLCA-PEG-PLCA (I) and C6-modified PLCA-PEG-PLCA (II)) and Example 3 (C12-modified PLCA-PEG-PLCA (III)) were dissolved in Ethylacetate to form 500 mg/ml solutions. Two solutions of Component I and component II or III were mixed together. The mixture was placed in a petri dish under nitrogen flow overnight to evaporate ethyl acetate. The dried polymer mixture was dissolved in PBS, pH 7.4 to prepare different solutions.

Table 2 below summarizes blend compositions of fully modified (C6/C12) and unmodified PLCA-PEG-PLCA and their thermogelation behaviour in aqueous solution at 37 °C. Note that most blends in aqueous solutions phase separate at 37 °C.

Table 2.

Unmodified (wt%)	C6-modified (wt%)	C12-modified (wt%)	20 % solution in PBS at 37 °C	Phase separation at 37°C:NA = not applicable
100			No thermogelling properties	NA
75	25		No gel	No
50	50		No gel	Yes
25	75		Gel	Yes
0	100		Gel	Yes
0		100	Insoluble	NA
25		75	No gel	No
50		50	Gel	Yes
75		25	Gel	Yes

Example 6 Mechanical properties

This example illustrates the measurement of the storage and loss modulus of mixture of tri-block copolymers.

The blends from Table 2 that form gels were tested.

Rheological characterization of the blends was done with a AR-G2 rheometer (TA Instruments, Etten-Leur, The netherlands) equipped with a 1° steel cone geometry of 20 mm diameter and solvent trap. Polymer blend solutions of 20 % (w/w) were prepared in PBS pH 7.4 at 4°C. 300 µl of the solutions were placed in glass vial (8.2 x 40 mm) and incubated for around 3 hours at 37°C to enable gelation and stabilization. Using a spatula, approximately 70 mg of the sample was placed between the pre-heated (37 °C) plates of the rheometer. Rheological gel characteristics were monitored by oscillatory time sweep experiments. During time sweep experiments G' (shear storage modulus) and G'' (loss modulus) were measured for a period of 5 min. Also temperature sweep experiments were performed on the polymer solutions. Therefore the plates of the rheometer were pre-cooled at 4 °C. Temperature increase was 1 °C / min. When G"/G' (= tan δ) < 1, the sample is considered as a gel in a rheological point of view. All experiments were performed at constant strain (1%) and frequency (1 Hz).

Example 7 Drug release and degradation in PBS + 0.2% Tween 80

This example illustrates the drug release profile from tri-block copolymer mixture solutions of the present invention using celecoxib as a model drug of small hydrophobic molecule.

A polymer mixture was prepared as described in Example 5 except that a solution of celecoxib (CLB) in ethyl acetate was used. To the dried mixture of polymer and celecoxib PBS was added to obtain a final concentration of 20% (w/w) polymer based on the amount of celecoxib and PBS and 0.5 w/w% celecoxib on polymer. As described in Example 6, the polymer-celecoxib solutions were transferred with a syringe to a glass vial (8.2 x 40 mm). The vials were placed at 37 °C to allow gel formation. After 30 min 800 µl of PBS (+ 0.2% tween 80) was added. In this experiment Tween 80 was used in order to increase celecoxib solubility to around 100-200 ug/ml. This was necessary to facilate sink conditions.

At predetermined time points, the buffer was removed and kept for celecoxib quantification, the weight of the remaining gel was measured and fresh buffer was added.

For comparison the same experiment was performed with the partially C6- modified PLCA-PEG-PLCA (DM 1.5).

Celecoxib concentration in the buffer was measured by a Waters Acquity™ Ultra Performance LC system (UPLC, Waters, Milford MA, USA) equipped with an Acquity™ BEH C18 1.7 µm column (2.1 x 100 mm), a binary solvent manager, a sample manager with column oven at 50 °C and an Acquity™ TUV Detector (detection wavelength: 254 nm). After injection of 7.5 µl release sample, a gradient was run from 100% A (H2O/ACN 95/5% (v/v) containing 0.1% TFA) to 100 % B (MeOH/ACN/H2O 45/45/10% (v/v) containing 0.1% TFA) in 2 minutes and kept at 100% B from 10 min at a flow rate of 0.08 ml/min. The run time was 16 min. A calibration curve was obtained after injection of standard celecoxib solutions in DMSO (0.5 – 100 ug/ml). The chromatograms were analysed using Empower Software Version 1154 (Waters, Milford MA, USA). CLB concentration in the release sample was in the range 5-20 µg/ml.

The results of this example are shown in Figures 1 to 5.

Example 9 Synthesis of non acylated PLGA-PEG-PLGA (DM 0) and acylated PLGA-PEG-PLGA (DM 2)

To a flask under a nitrogen atmosphere where added 20 g PEG 1500 (13.3 mmol), 13.5 g D-lactide (93.7 mmol), 13.5 g L-lactide (93.7 mmol), 13.5 g glycolide (116 mmol) and 200 mL toluene. 80 mL was distilled off azeotropically using Dean Stark apparatus. After cooling down to room temperature 0.4 g stannous octoate was added and the solution heated under refluxing conditions for 1 day. The resultant polymerization solution was equally divided into batch 1 and batch 2.

Batch 1 was cooled down to room temperature and precipitated in an excess of pentane and ether. The precipitated white product was dried under vacuum to give a solid.

Batch 2 was placed in a flask under a nitrogen atmosphere together with 40 mL dichloromethane, 7 mL triethylamine and the solutions was cooled with an ice-bath. 2 mL acetyl chloride was slowly added to this cold solution. After stirring for 3-4 hours the dichloromethane was removed in vacuo and 80 mL ethyl acetate was added. After filtering off the salts, the product was obtained by precipitation in a pentane / ether mixture. The yellowish polymer was dried under vacuum. The product was characterized by 1H-NMR to confirm the triblock copolymer was fully acylated (DM 2).

Experiment 10 Synthesis of monoacetylated PLGA-PEG-PLGA (DM 1)

10 g of PLGA1500-PEG1500-PLGA1500 (2 mmol; 4 mmol OH) as synthesized in Example 9) was dissolved in 100 mL toluene and dried azeotropically, 80 mL was removed. The reaction was cooled down to room temperature and 10 mL dry dichloromethane, 0.6 mL triethylamine. The reaction was then further cooled using an ice bath and 0.25 mL acetyl chloride 3 mmol) was slowly added allowed to react for 1 day. The work-up was conducted as per experiment 9. A white waxy solid product was obtained. Characterization by 1H-NMR showed that the degree of end-group modification (acetylation) was approximately 1.

Experiment 11Rheological characterization of PLGA-PEG-PLGA compositions of the invention

The conditions for the rheology measurements were used as per Example 6. Plotting the storage modulus versus temperature for DM0, DM1, DM2 and DM0+DM2 shows that compositions DM1 and DM0+DM2 are both gels at 37 °C whereas DM0 and DM2 reach a gel state at either higher or lower temperature respectively. Next to this

significant shift in the temperature of gelation, we also observe a significant difference in G' between DM=1 (430 Pa) and mix of DM=2/DM=0 50-50 (300 PA). The result is shown in Figure 6.

Example 12 Chromatographic method

By mixing polymers in this invention of the same block copolymer composition but with either only hydroxyl-end groups, or only aliphatic end-groups, and at different ratio's, one can generate an average Degree of Modification (DM) of 0.1 to 1.9, in order to fine tune the drug release and other characteristics of the formulation of the invention.

A method to distinguish between a composition comprising a mixture of DM0 and DM2 and a composition comprising also DM1 tri-block copolymer is hydrophilic interaction chromatography (HILIC).

Abrar and Trathnigg (Anal. Bioanal. Chem., 2001, 400, 2531-2537) describe a method for separating polyoxyethylenes according to the number of hydroxyl groups. In the compositions according to the invention, acylated tri-block copolymer compositions have fewer available hydroxyl groups than non-acylated compositions and thus it is expected that the method of Abrar and Trathnigg is suitable for distinguishing compositions of this invention comprising mixtures of tri-block copolymer compositions from compositions comprising a single and partially acylated tri-block copolymer.

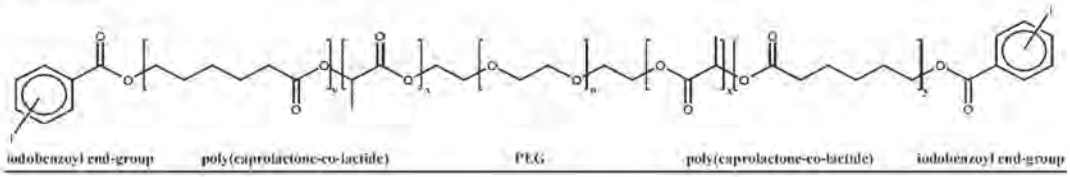
Separation in HILIC is achieved by partitioning the composition between the aqueous layer surrounding the stationary phase and the mobile organic phase. Samples with a higher organic content (acylated) will elute before non-acylated compositions as shown in Figures 7 and 8.

Figure 7 shows that the composition having DM1 comprises at least 3 different components: DM0, DM1 and DM2.
Figure 8 shows that the mixture of DM0 and DM2 comprises only these components. No DM1 is present.

Example 14 Synthesis of iodine functionalized tri-block copolymers

This example details a method to covalently bond a radiopaque atom to a tri-block copolymer. The structure of a tri-block copolymer which is acylated is given below in formula (1).

Formula 1



The synthesis of tri-block copolymers wherein at least part of the hydroxyl end-groups of the tri-block copolymer are covalently bound to a compound containing radiopaque atoms was done according to the method previously described in US7740877, examples 1, 2 and 3. The synthesis led to tri-block copolymers (Table 9) with triiodobenzoyl groups (1.8 groups per chain) as confirmed by 1H NMR.

Table 9. Tri-block copolymers used.

ID	ID	PLCA/PEG	Caprolactone / lactide	Degree of acylation	End-group	Number average mol.weight PEG
P (#81)	I-Gel® #1	1.8	9/1	1.8	Triiodobenzoyl	1500
Q (#29)	I-Gel® #2	1.0	9/1	1.8	Triiodobenzoyl	1500

In the following examples different compositions comprising P and Q were made and the compositions are summarized in Table 10.

Table 10. Overview of compositions used in examples 15-19

Example	Tri-block copolymer (wt %)		
	P	Q	17
15	-	-	100
16	25	-	75
	30	-	70
	50	-	50
	70	-	30
17 a	30	-	70
b	-	30	70
18 a	-	-	100
b	25	-	75
19 a	25	-	75
b	50	-	50

Example 15 Loading of a tri-block copolymer composition with contrast agent Hexabrix™

In order to achieve prolonged release of a pharmaceutically active ingredient, retention in the treatment location (for example a joint) must be maintained for a minimum of 4 weeks. To determine this retention time the tri-block copolymer composition must be visualized. This example shows how loading a tri-block copolymer with a conventional contrast agent does not achieve this goal.

Polymer #17 (see Table 1) was loaded with 15 % Hexabrix™ (Guerbet). Hexabrix™ contrast agent is a low osmolar ionic dimer. Each milliliter contains 393 mg of ioxaglate meglumine, 196 mg of ioxaglate sodium and 0.10 mg of edetate calcium disodium as a stabilizer. The solution contains 3.48 mg (0.15 vmEq) sodium in each milliliter and provides 32% (320 mg/mL) organically bound iodine.

The commercially available Hexabrix™ solution was diluted with phosphate buffer (50 mM phosphate, pH 7.4, 0.07 mM NaCl) at a ratio 15/85 v/v. This diluted Hexabrix™ solution was used to dissolve the polymer, leading to the preparation of a formulation containing 25 wt% polymer with respect to buffer and Hexabrix™. Each gram of formulation contained 44 mg ioxaglate meglumine.

The loaded tri-block copolymer composition was injected into a chicken knee and visualized using microCT (Skyscan model 1076, Skyscan, Kontich, Belgium) Scans were performed using the following scanner settings: isotropic voxelsize of 35mm, at a voltage of 55kV, a current of 170mA, field of view of 35mm, and a 0.5mm aluminium filter, over 198° with a 0.8 rotation step. 1 hour after injection the visibility of the gel was diminished and a further 1 day later the composition could not be visualized. Make table T=0-1-24hrs showing 100%-50%-0% visualization as relative grey value
The results are also indicated in Table 11.

Table 11: retention of Polymer #17 (25% w/w) loaded with 15% Hexabrix

Time point in hours	Relative grey surface area
T = 0	100%
T = 1	50%
T=24	0%

Example 16 Radiopaque tri-block copolymer compositions

This example shows that a tri-block copolymer wherein at least part of the hydroxyl end-groups of the tri-block copolymer are covalently bound to a compound containing radiopaque atoms can be visualized by microCT. (Skyscan model 1076, Skyscan, Kontich, Belgium) Scans were performed using the following scanner settings: isotropic voxelsize of 35mm, at a voltage of 55kV, a current of 170mA, field of view of 35mm, and a 0.5mm aluminium filter, over 198° with a 0.8 rotation step. Scan time was 10 minutes. In this example different compositions were prepared comprising different weight percentages of tri-block copolymers (Table 10, example 16) wherein the hydroxyl end-groups of the tri-block copolymer are at least partially acylated with an acetyl group or wherein at least part of the hydroxyl end-groups of the tri-block copolymer are covalently bound to a compound containing radiopaque atoms.

The compositions (Table 10, examples 16) were prepared as follows. Both polymers P and #17 were separately dissolved in ethylacetate at a concentration of 500 mg/mL. The solutions were mixed at the desired ratios and the mixtures were transferred into petri dishes. The solvent was removed under nitrogen flow for 48 hours. To 500 mg polymer blend, phosphate buffer (50 mM, 0.07mM NaCl, 0.02% NaN3, pH 7.4) was added to yield solutions at 25 wt %. The compositions were measured in glass vials and the X-ray intensity plotted on a graph (Figure 8).The compositions were measured in glass vials and the X-ray intensity plotted on a graph (Figure 8).

Example 17 CT imaging in radiopaque tri-block copolymer compositions

This example shows the CT visualization times for different mixtures of tri-block copolymers.

The mixtures of copolymers (Table 10, example 17a and 17b) were prepared, injected into the knee of a rat cadaver and visualized by microCT (Skyscan model 1076, Skyscan, Kontich, Belgium). Scans were performed using the following scanner settings: isotropic voxelsize of 35mm, at a voltage of 55kV, a current of 170mA, field of view of 35mm, and a 0.5mm aluminium filter, over 198° with a 0.8 rotation step. The results are shown in Table 14.

The composition 17a was visible for only a few days whereas composition 17b was visible for 3 weeks (Figure 11).

Example 18 Release profiles of tri-block copolymers wherein the end groups are acylated or are radiopaque substituted acyl groups

This example illustrates the release profile of a pharmaceutically active ingredient from tri-block copolymer compositions and also the degradation profiles for tri-block copolymer compositions comprising a pharmaceutically active ingredient.

OAc-Gell (#17) and a mixture of a I-Gell®#1 (P) and OAc-Gell (#17) were prepared at 25 wt% in 50 mM phosphate buffer at pH 7.4, 0.42% NaCl and 0.05% NaN3. The loading of Celecoxib was 1.25 mg/mL. Release experiments were performed as per example 6 and at 37 °C in PBS buffer containing 0.2% Tween 80. Error bars represent standard error of the mean (n=6). The results are shown in Figure 9 and summarized in Table 12.

Table 12 Overview release time and erosion profile for examples 18a and 18b.

Example	Release time	Erosion time (solid content)
18a	28	28
18b	35	35

Wet weight= weight of the depots, as measured by decanting the PBS buffer and weighing the remaining gel, after which the original vial-weight is subtracted

Dry weight = weight of the freeze dried depots after decanting of the buffer (i.e. polymer weight) and subtracting the weight of the vial
Solid content = polymer concentration in the depots (dry weight/wet weight)

Example 19 Degradation profile for tri-block copolymer compositions

This example shows the in vivo degradation profile for compositions of tri-block copolymers wherein at least part of the hydroxyl end-groups of the tri-block copolymer are covalently bound to a compound containing radiopaque atoms.

Compositions (Table 10, example 19a and 19b) were prepared were prepared by dissolving 25 wt % tri-block copolymer (comprising 75 % P and 25 % #17, or 50 % P and 50 % #17) in 75 % PBS buffer, pH 7.4. 50 mM, 0.15% NaCl of the composition was injected subcutaneously into rats knees. The injected volume of 19b was then visualized and measured using 3D micro-CT imaging (Skyscan model 1076, Skyscan, Kontich, Belgium). The rats were anesthetized using Isoflurane and then placed in a custom made scanner bed, fixing the hind limb in an extended position. Scans were performed using the following scanner settings: isotropic voxel size of 35mm, at a voltage of 55kV, a current of 170mA, field of view of 35mm, and a 0.5mm aluminium filter, over 198° with a 0.8 rotation step. The rat knees and subcutaneous depots were scanned on days 0,1, 4 and 8 and after that weekly until the gel was no longer visible. The scan time was 16 minutes and a frame averaging of 3 was used. Images obtained using the uCT scanner were reconstructed using Skyskan analysis software. The datasets were segmented using a fixed attenuation threshold between air and subchondral bone. Subsequently, regions of interest were drawn around the patellar cartilage and attenuation and volume were calculated. The injected volume of 19a (non-radiopaque), was monitored through the skin with a measuring caliper. The results are shown in Figure 10 and summarized in Table 13 and 14.

Table 13 Overview of erosion monitoring for examples 19a and 19b.

Day	19a		19b	
	Observations	Surface area / %	Observations	Surface area / %
0	Distinct oval shape, sharp edges	100	Higher intensity than for 19a	100
4	Sharp edges maintained	80		70
8	Less sharp edges on interior side	40		65
14	Fractured shape – small light spots in dark area	10		75
21	Not visible	-		50
40	-	-	Still visible	40

Surface area = relative area with respect to surface area at t=0 (set to 100 %)

Table 14. Overview of compositions and microCT characteristics for examples 15-19

Example	Tri-block copolymer (wt %)			Duration of visibility in micro CT	Active ingredient
	P	Q	17		
15	-	-	100	< 24 hours	Hexabrix™
16 a	25	-	75	All visible in micro CT but duration of visibility not measured	-
	30	-	70		-
	50	-	50		-
	70	-	30		-
17 a	30	-	70	< 7 days	-
b	-	30	70	~ 4 weeks	-
18 a	-	-	100	-	Celecoxib
b	25	-	75	-	Celecoxib
19 a	25	-	75	~ 40 days	-
b	50	-	50	~ 77 days	-

Brief description of the figures.

Figure 1 (Fig. 1) shows the rheological properties at 37 °C of mixtures containing PLCA-PEG-PLCA and PLCA-PEG-PLCA hexanoate. Gels were 20% (w/w). Measurement frequency and strain were 1 Hz and 1%, respectively. Error bars represent standard deviation (n=3)

In Fig. 1 the following abbreviations are used:

C6DM2/DM0 25/75: this is a blend of 25wt% of a 20% w/w solution of tri-block copolymer II in PBS, pH 7.4 and 75wt% of a 20% w/w solution of tri-block copolymer I in PBS, pH 7.4..

C6DM2/DM0 50/50: this is a blend of 50wt% of a 20% w/w solution of tri-block copolymer II in PBS, pH 7.4 and 50wt% of a 20% w/w solution of tri-block copolymer I in PBS, pH 7.4.

C6DM2/DM0 75/25: this is a blend of 75wt% of a 20% w/w solution of tri-block copolymer II in PBS, pH 7.4 and 25wt% of a 20% w/w solution of tri-block copolymer I in PBS, pH 7.4

C6DM2/DM0 100/0: this is a blend of 100wt% of a 20% w/w solution of tri-block copolymer II in PBS, pH 7.4 and 0wt% of a 20% w/w solution of tri-block copolymer I in PBS, pH 7.4

Figure 2 (Fig. 2) shows the rheological properties at 37 oC of mixtures containing PLCA-PEG-PLCA and PLCA-PEG-PLCA laurate. Gels were 20% (w/w). Measurement frequency and strain were 1 Hz and 1%, respectively. Error bars represent standard deviation (n=3).

In Fig. 2, the following abbreviations are used:

C12DM2/DM0 50/50: this is a blend of 50wt% of a 20% w/w solution of tri-block copolymer III in PBS, pH 7.4 and 50wt% of a 20% w/w solution of tri-block copolymer I in PBS, pH 7.4

C12DM2/DM0 75/25: this is a blend of 75wt% of a 20% w/w solution of tri-block copolymer III in PBS, pH 7.4 and 25wt% of a 20% w/w solution of tri-block copolymer I in PBS, pH 7.4

Figure 3 (Fig. 3) shows the weight change of the different gels, i.e. degradation over time of mixtures in PBS, pH 7.4, 37 oC containing 0.2% Tween 80 (+ 0.02% NaN3). Gels were 20% (w/w) containing 0.5% Celecoxib wrt polymer. Error bars represent standard deviation (n=3).

In Fig. 3, the following abbreviations are used:

C6DM2/DM0 75/25: this is a blend of 75wt% of a 20% w/w solution of tri-block copolymer II in PBS, pH 7.4 and 25wt% of a 20% w/w solution of tri-block copolymer I in PBS, pH 7.4

C6DM2/DM0 50/50: this is a blend of 50wt% of a 20% w/w solution of tri-block copolymer II in PBS, pH 7.4 and 50wt% of a 20% w/w solution of tri-block copolymer I in PBS, pH 7.4.

C12DM2/DM0 50/50: this is a blend of 50wt% of a 20% w/w solution of tri-block

copolymer III in PBS, pH 7.4 and 50wt% of a 20% w/w solution of tri-block copolymer I in PBS, pH 7.4

Figure 4 (Fig. 4) shows the cumulative release of celecoxib from the different gels over time of mixtures in PBS, pH 7.4, 37 °C containing 0.2% Tween 80 (+ 0.02% NaN3). Gels were 20% (w/w) containing 0.5% celecoxib wrt polymer. Error bars represent standard deviation (n=3).
In Fig. 4, the following abbreviations are used:
C6DM2/DM0 50/50: this is a blend of 50wt% of a 20% w/w solution of tri-block copolymer II in PBS, pH 7.4 and 50wt% of a 20% w/w solution of tri-block copolymer I in PBS, pH 7.4.

C6DM2/DM0 75/25: this is a blend of 75wt% of a 20% w/w solution of tri-block copolymer II in PBS, pH 7.4 and 25wt% of a 20% w/w solution of tri-block copolymer I in PBS, pH 7.4
C12DM2/DM0 50/50: this is a blend of 50wt% of a 20% w/w solution of tri-block copolymer III in PBS, pH 7.4 and 50wt% of a 20% w/w solution of tri-block copolymer I in PBS, pH 7.4

Figure 5 (Fig.5) shows the cumulative release of celecoxib over time of the partially C6-modified PLCA-PEG-PLCA (DM 1.5) in PBS, pH 7.4, 37 °C containing 0.2% Tween 80 (+ 0.02% NaN3). Gels were 20% (w/w) containing 0.5% celecoxib wrt polymer. Error bars represent standard deviation (n=3).
In Fig. 5, the following abbreviations are used:
DM 1.5: this is a blend of 100wt% of a 20% w/w solution of tri-block copolymer IV in PBS, pH 7.4 and 0wt% of a 20% w/w solution of tri-block copolymer I in PBS, pH 7.4.
C6DM2/DM0 75/25: this is a blend of 75wt% of a 20% w/w solution of tri-block copolymer II in PBS, pH 7.4 and 25wt% of a 20% w/w solution of tri-block copolymer I in PBS, pH 7.4.

Figure 6 (Fig. 6) shows the rheological properties of compositions containing PLGA-PEG-PLGA with or without acylation, and mixtures thereof. Gels were 20 % (w/w). Measurement frequency and strain were 1 Hz and 1 % respectively.

In Fig. 6 the following abbreviations are used:
DM0: PEG1500(GLY33%/LAC67%)2
DM1: PEG1500(GLY33%/LAC67%)2(C2)1

DM2: PEG1500(GLY33%/LAC67%)2(C2)2
1:1: mixture of DM0 and DM2 in ratio of 1 to 1

Figure 7 (Fig. 7) shows a postulated elution profile where t = time and I = Intensity, for a composition consisting of 100% DM1.

Figure 8 (Fig. 8) shows a postulated elution profile for a composition consisting of a 50/50 mixture of tri-block copolymer having a DM of 0 and a tri-block copolymer having a DM of 2.

Figure 9 (Fig. 9) shows the x-ray intensities as measured by the microCT, for compositions shown in Table 10, example 16, whereby the percentage of iodine bound polymer P(#81) is given on the x-axis. The line is added to guide the eye.

Figure 10 (Fig. 10) shows the release profile and gel erosion profiles for example 18. The filled squares indicate composition 18a (OAc-Gell only loaded with Celecoxib) and the empty triangles indicate composition 18b (OAc-Gell / I-Gell®#1 -#81). The compositions contained 25 wt% in 50 mM phosphate buffer, pH 7.4, 0.42% NaCl and 0.05% NaN3. The loading of Celecoxib was 1.25 mg/mL

Figure 11 (Fig.11) shows the erosion profile for compositions 19a (dotted line) and 19b (Solid line). Images of the subcutaneous injected I-Gell depots in rats were taken in-vivo using micro-CT on days 0-1-4-8-14-21-28-35-42-49-56-63-70-77 and the volume of the gel was determined using a caliper as done routinely for volume-estimations of subcutaneous tumor lumps.

Discussions and conclusions

As can be seen in Fig 1, viscoelastic hydrogels were formed for compositions with more than 50% w/w of the C6-acylated PLCA-PEG-PLCA (II), i.e. the storage modulus (G') exceeds the loss modulus (G"). Interestingly, the addition of C6-acylated PLCA-PEG-PLCA led to considerable increase in hydrogel strength as demonstrated by a 10-fold increase of the moduli. This indicates that the micelle interactions, i.e. bridging between the micelles which hold together the network structure became stronger by addition of C6-acylated PLCA-PEG-PLCA. This characteristic shows that the rheology/mechanical strength of the mixture can be tuned using the teaching of the invention.

As can be seen in Fig.2, viscoelastic hydrogels were formed for compositions with at least 50% w/w of the C12-acylated PLCA-PEG-PLCA (III), i.e. the storage modulus (G') exceeds the loss modulus (G"). Interestingly, the addition of C12-acylated PLCA-PEG-PLCA led to considerable increase in hydrogel strength as demonstrated by a 10-fold increase of the moduli. This indicates that the micelle interactions, i.e. bridging between the micelles which hold together the network structure became stronger by addition of C12-acylated PLCA-PEG-PLCA.

Figure 3 shows that the degradation profile of the blends expressed as wet-gel weight loss, and these same samples are evaluated for their release profile of celecoxib as described in figure 4. By blending the same block co polymers with or without C6 end-groups in different ratio's, the formulated gels with drugs and buffer can be tuned to produce a burst release, or not, and they can be tuned to degrade over longer or shorter periods of time. By using C12-endgroups, the same ratio with non-endcapped produces a remarkable higher burst with shorter overall degradation and release time than the C6-endcapped comparable. These results are very interesting for use in chronic drug treatment where multiple injections are needed over the course of time, and where it is advantageous that the polymer is fully degraded shortly after the end of the release of the active pharmaceutical ingredient. So, the degradation profile (burst, speed, total disappearance) of the tri-block copolymer may be tuned using the teaching of the invention.

In figure 4, the cumulative release profile of celecoxib from 3 polymer mixtures is depicted. By varying the polymer end-modification and the type of end-group modification used, we are able to tune the release profile of celecoxib in terms of burst, release time and release rate from 25 to 50 days. It is highly beneficial to be able to tune the release profile in terms of total days of treatment, and the available drug dose per time unit. It is also very beneficial to be able to reduce the burst release to minimize side effects. In some applications one may choose to design a burst release in the first day (10-25% of total release), followed by a gradual dosing over an extended period of time, ie for use of cytostatic drugs in tumour eradication or anti-bacterial medication in case of infections.

As can be seen in figure 5, the cumulative release profile of celecoxib from the partially C6-modified PLCA-PEG-PLCA (DM 1.5) is similar in duration to the one of a blend fully C6-modified/unmodified PLCA-PEG-PLCA (75/25). However the gel prepared with the

polymer mixture according to the invention showed a more regular release rate over that period of time.

As can be seen in Figure 6 a tri-block copolymer composition with having a degree of modification of 1, has the same gelation temperature as measured by G', as a 50 / 50 mixture of tri-block copolymer compositions according to the invention containing DM0 and DM2 tri-block copolymers based on PLGA. We do observe a significant difference in G' between DM=1 (430 Pa) and mix of DM=2/DM=0 50-50 (300 PA). The result is shown in Figure 6.

This shows that the simple mixing of two tri-block copolymers, wherein one of the two tri-block co-polymers has a degree of modification of 100% and one of the two tri-block copolymers has a degree of modification of 0% and wherein the two types of tri-block copolymers differ only on the degree of modification of the end group, exhibit the same physical properties as a tri-block composition with a defined degree of modification of 50%. However, the benefit of mixing is that one can work with a formulation comprising two fully characterized and easy to reproduce block-copolymers (DM=0 or DM=2), and with a mixing step that is easy to plan, execute and reproduce to those skilled in the art. In contrast to the aforementioned practice of the invention, the synthetic approach to achieve comparable DM always has a range of DM=0-1-2 block copolymers, which is more difficult to define, control and reproduce consistently within a pharmaceutical setting.

A tri-block copolymer loaded with Hexabrix™ can be used for imaging in microCT studies but the contrast agent diffuses out of the tri-block copolymer within 1 day thereby preventing prolonged imaging of the tri-block copolymer Table 11, example 15).

Surprisingly, a composition comprising a tri-block copolymer wherein at least part of the hydroxyl end-groups of the tri-block copolymer are covalently bound to a compound containing radiopaque atoms is suitable for microCT imaging over longer periods of up to time, as shown in example 16.

As summarized in Table 8, a composition comprising a tri-block copolymer wherein at least part of the hydroxyl end-groups of the tri-block copolymer are covalently bound to a compound containing radiopaque atoms maintains gel forming, gel erosion and controlled release properties whilst such composition can be visualized using micro CT.

From example 17 and figure 10, it is furthermore shown that it is possible to visualize a blend of a tri-block copolymer wherein the hydroxyl end-groups of the tri-block

copolymer are at least partially acylated with an acetyl group or wherein at least part of the hydroxyl end-groups of the tri-block copolymer are covalently bound to a compound containing radiopaque atoms using micro CT, while maintaining gel forming, gel erosion and controlled release properties. In the given example 17 and figure 10, the blend of polymers with the same tri-block copolymer structure, but with different end-groups at 100% modification (namely tri-iodobenzoyl-group or acetyl-group), shows longer gel stability and longer release of the same hydrophobic compound than the pure 100% acetylated version. This is very beneficial for product development, for manufacturing, and at overall cost level, because it allows us to produce one type of tri-block copolymer at high levels of reproducibility, and subsequently fully modify it with two different end-groups at high reproducibility, then having these different variations fully characterized and qualified for animal and human uses, produce these on stock, and then by simply mixing these high quality products at different ratios, desired release profiles of pharmaceutically active ingredients can be obtained, also having different degradation profiles of the gel-depot, and making visualization of the depots possible in situ, or for other imaging purposes.

CLAIMS

1. Composition comprising
(a) an active ingredient, preferably a pharmaceutically active ingredient
(b) a solvent and
(c) a mixture of at least two types of tri-block copolymers of formula (1)

B-A-B (1)

wherein B stands for a hydrophobic block and wherein A stands for a hydrophilic block, wherein the mixture is prepared by mixing at least two types of tri-block copolymers having a degree of modification of 100% and wherein the B-A-B types of tri-block copolymers differ only on the type of end-group
or wherein the mixture is prepared by mixing at least two types of tri-block copolymers, wherein one of the at least two tri-block copolymers has a degree of modification of 100% and one of the at least two tri-block copolymers has a degree of modification of 0% and wherein the B-A-B types of tri-block copolymers differ only on the degree of modification of the end-groups, and where only tri-block copolymer with a degree of modification of 100% or 0% has been added.

2. Composition according to claim 1, wherein the end-group of at least one of the two types of tri-block copolymer is covalently bound to a compound containing radiopaque atoms

3. Composition according to claim 1 or claim 2,
wherein A stands for a linear poly-(ethylene glycol) block and
wherein B stands for a hydrophobic block comprising at least one, preferably at least two cyclic monomers selected from the group consisting of glycolide, lactide, ε-caprolactone, p-dioxanone (1,4-dioxan-2-one), trimethylene carbonate (1,3-dioxan-2-one), 1,4-dioxepan-2-one (including its dimer 1,5,8,12-tetraoxacyclotetradecane-7,14-dione), 1,5-dioxepan-2-one, 6,6-dimethyl-1,4-dioxan-2-one, 2,5-diketomorpholine, pivalolactone, chi.-diethylpropiolactone, ethylene carbonate, ethylene oxalate, 3-methyl-1,4-dioxane-2,5-dione, 3,3-diethyl-1,4-dioxan-2,5-dione, 6,8-dioxabicycloctane-7-one, β-propiolactone, γ-butyrolactone, δ-valerolactone, ε-decalactone, 3-methyl-1,4-dioxane-2,5-dione, 1,4-dioxane-2,5-dione, 2,5-diketomorpholine, α,α-diethylpropiolactone, 6,6-dimethyl-dioxepan-2-one, 6,8-dioxabicycloctane-7-one and 5,5-dimethyl-1,3-dioxan-2-one, wherein the hydroxyl end-groups of the tri-block

copolymer in case they are acylated are acylated with an optionally substituted acyl having 2 to 12 C-atoms, C-atoms of the substituents included.

4. Composition according to any one of claims 1-3,
wherein A stands for a linear poly-(ethylene glycol) block and
wherein the B stands for a poly(lactide-co-Є-caprolactone) block.

5. Composition according to any one of claims 1-4, wherein the block ratio of the at least two tri-block copolymers, which block ratio is defined as the ratio between the sum of the number average molecular weight (Mn) of the B-blocks and the number average molecular weight (Mn) of the A-blocks ranges from 1.4 to 2.6.

6. Composition according to any one of claims 3-5, wherein the acyl group is an acetyl group or a propionyl group.

7. Composition according to any one of claims 4-6, wherein the weight ratio of Є-caprolactone to lactide is at least 1/1.

8. Composition according to any one of claims 1-7, wherein the solvent is water or an aqueous buffer solution, preferably an aqueous buffer solution that leads to an isotonic composition of the invention.

9. Composition according to any one of claims 1-8, wherein the composition is a pharmaceutical composition comprising a pharmaceutically active ingredient and further comprising a pharmaceutically acceptable adjuvant, carrier, excipient, contrast agent or dye.

10. Composition according to any one of claims 1-9 for use as a medicament.

11. Composition according to any one of claims 1-10 for use as a medicament that is injected through a needle of at least 18G or thinner.

12. Method for preparing a composition according to any one of claims 1-11 suitable for forming a thermogel comprising the step of
mixing at least two types of B-A-B types of tri-block copolymers of formula (1)

B-A-B (1)

wherein B stands for a hydrophobic block and wherein A stands for a hydrophilic block, wherein the at least two types of B-A-B types of tri-block copolymers differ only on the type of end-group and/or on the degree of modification of the end-groups.

13. Process for the preparation of a composition according to any one of claims 1-11 comprising the steps of
- synthesizing the at least two types of tri-block copolymers and
- mixing the at least two types of tri-block copolymers with the active ingredient and the solvent

14. Method for delivering an pharmaceutically active ingredient over an extended period to an animal, including a human requiring such treatment which comprises administering to such animal an effective amount of the composition of any one of claims 1-10.

15. Composition according to any one of claims 1-11, wherein the composition is a pharmaceutical composition further comprising a pharmaceutically acceptable adjuvant, excipient or carrier, for example a drug-encapsulating nano-particle or microparticle.

Fig 1

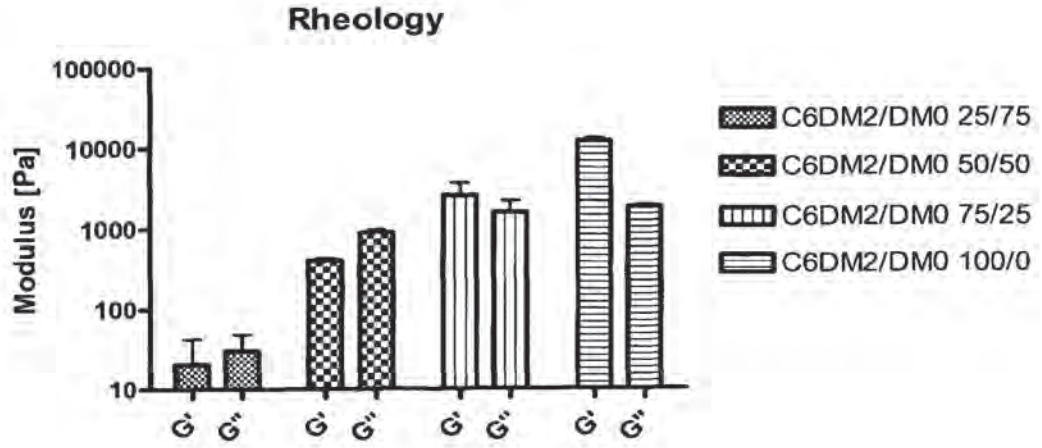
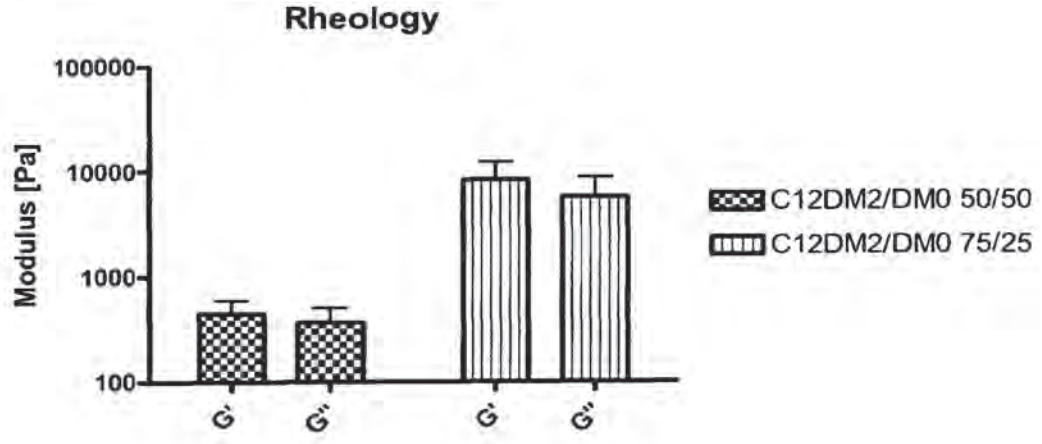


Fig. 2



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Fig. 3

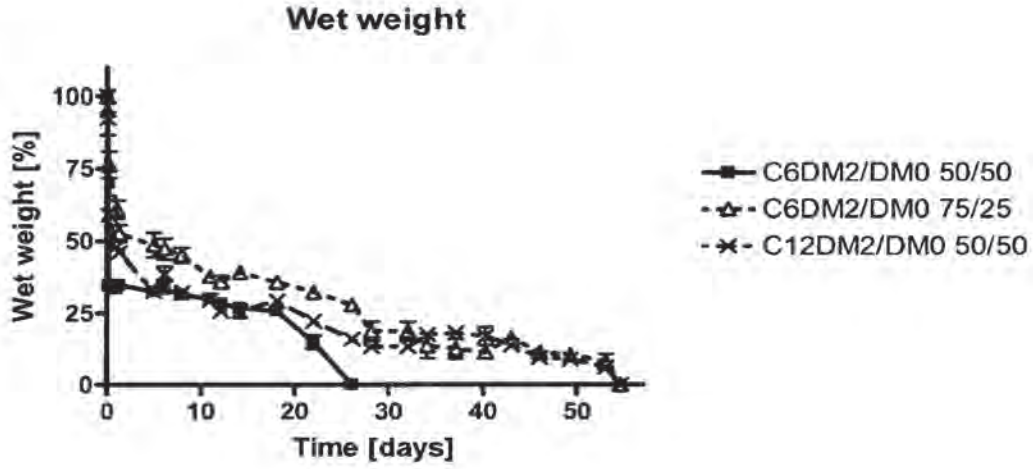
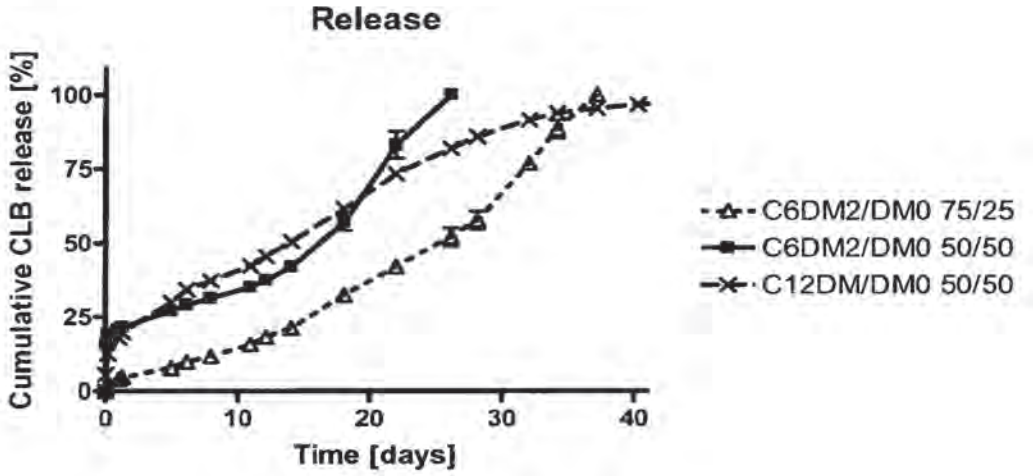
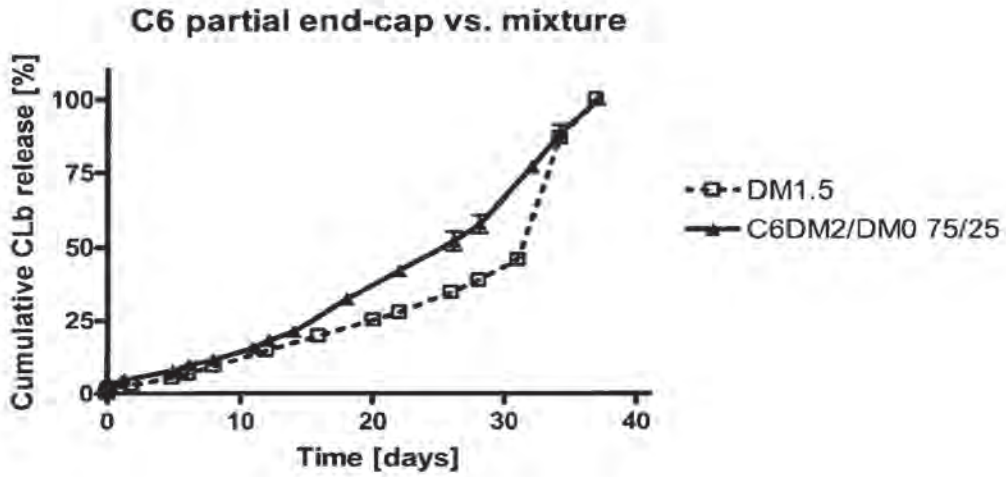


Fig. 4



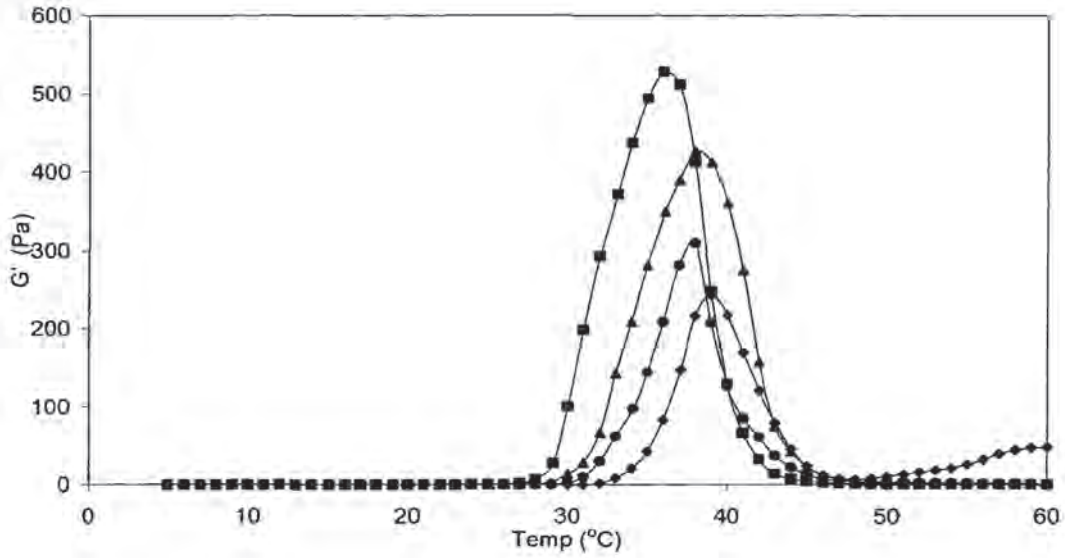
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Fig. 5



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Fig. 6



squares: DM2

triangles: DM1

circles: 1:1 mixture DM0 + DM2

diamonds: DM0

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Fig. 7

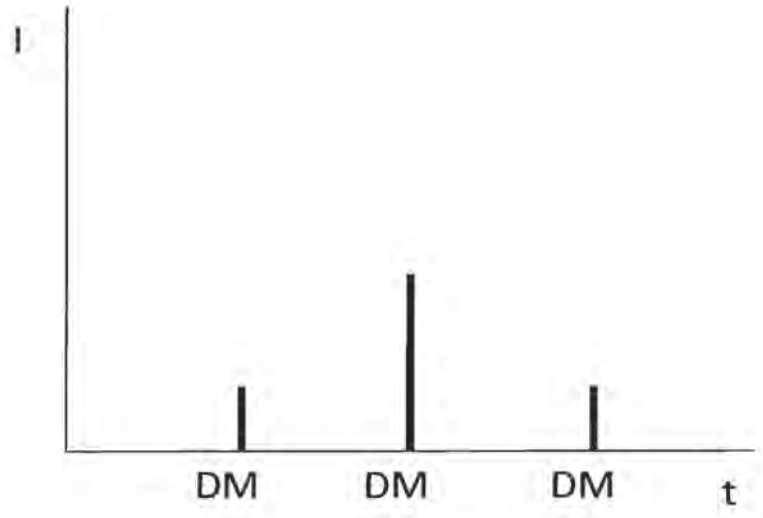
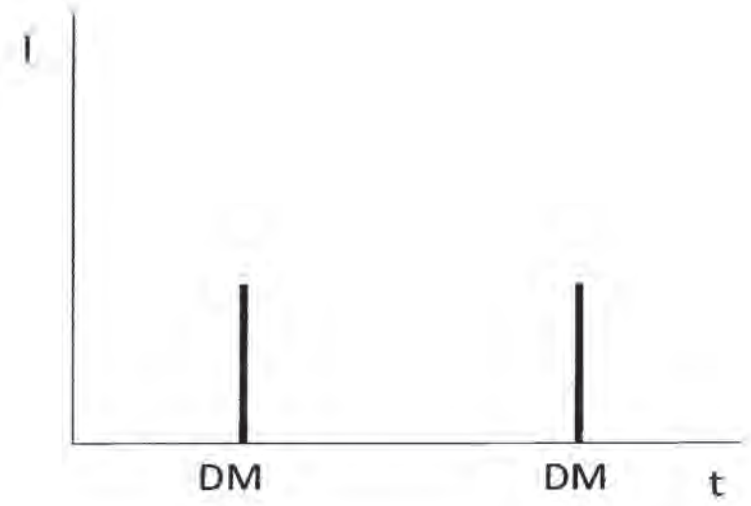


Fig. 8



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Fig. 9

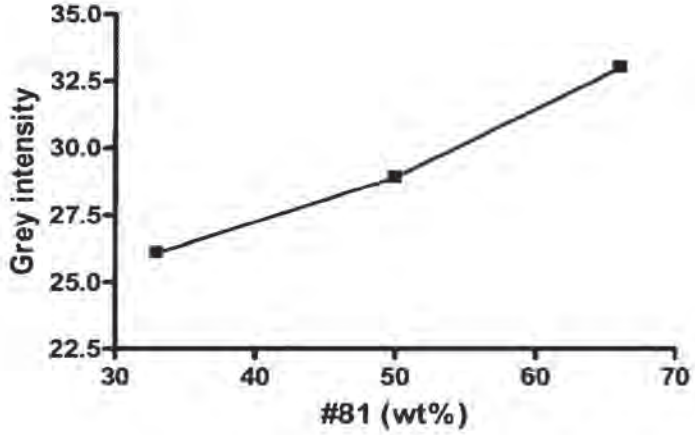
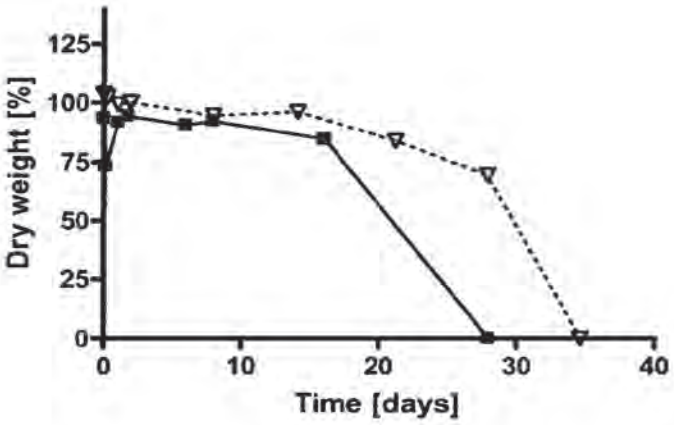


Fig 10a

Polymer #17 (squares) ; #17/#81 (75/25) (open triangles)



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Page 462

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RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0462

Fig. 10b

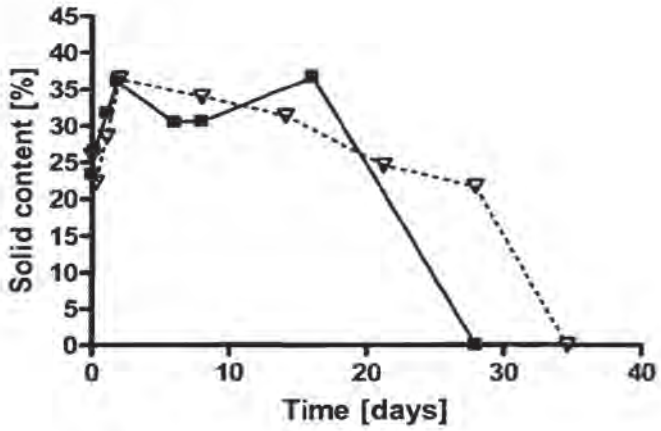
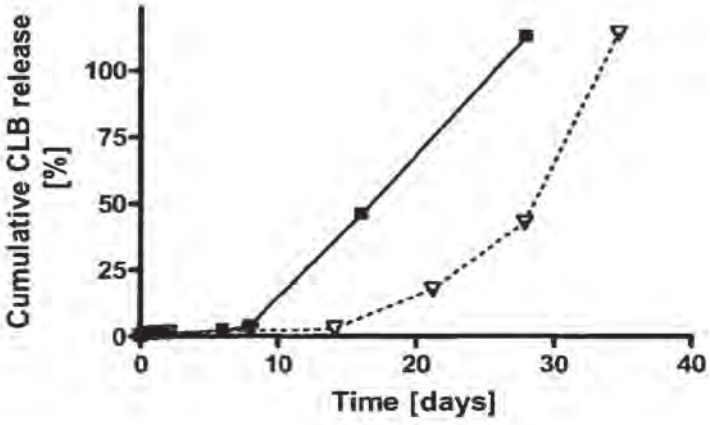


Fig. 10c



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Page 463

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RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0463

Fig. 10d

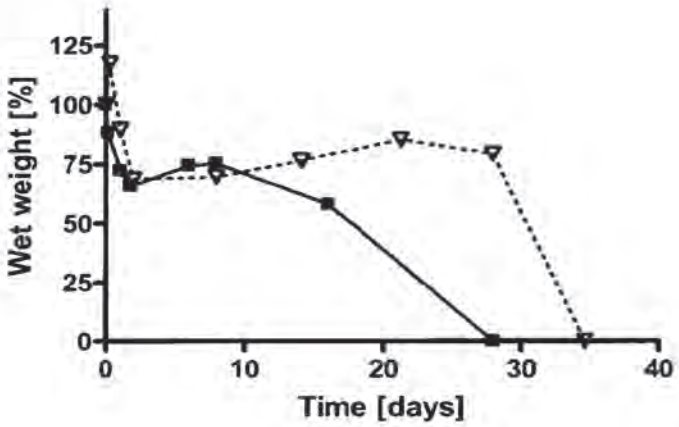
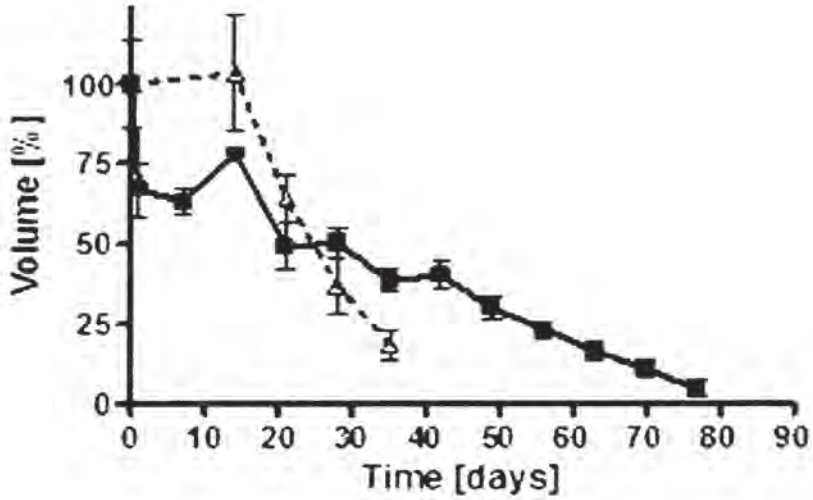


Fig. 11



RegenX Science Exhibit 1004
Page 464

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0464

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/055998

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/06 A61K47/34 A61K49/04 C08G63/08 C08G63/91
C08L67/04
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
A61K C08G C08L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00/38651 A1 (AMGEN INC [US]) 6 July 2000 (2000-07-06)	1,3-15
Y	page 6, line 31 - page 7, line 26 examples 1,2,7-11 figures claims	2

Y	US 2005/036946 A1 (PATHAK CHANDRASHEKHAR P [US] ET AL) 17 February 2005 (2005-02-17) paragraphs [0002], [0026] paragraph [0067] - paragraph [0080] paragraph [0174] - paragraph [0180] examples 24-31 claims	2

	-/--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 July 2012	11/07/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Epskamp, Stefan

1

Form PCT/ISA/210 (second sheet) (April 2005)

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Page 465

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RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0465

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/055998

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2007/265356 A1 (KIM SUNG W [US] ET AL KIM SUNG WAN [US] ET AL) 15 November 2007 (2007-11-15) paragraph [0009] - paragraph [0013] paragraph [0035] examples claims	1-15
A	WO 01/82970 A1 (MACROMED INC [US]) 8 November 2001 (2001-11-08) cited in the application page 3, line 4 - page 4, line 12 examples claims	1-15
A	YU L ET AL: "Mixing a sol and a precipitate of block copolymers with different block ratios leads to an injectable hydrogel", BIOMACROMOLECULES, ACS, WASHINGTON, DC, US, vol. 10, no. 6, 8 June 2009 (2009-06-08), pages 1547-1553, XP002586070, ISSN: 1525-7797, DOI: 10.1021/BM900145G [retrieved on 2009-04-22] cited in the application abstract page 1552, right-hand column, last paragraph - page 2553	1-15

1

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

RegenX Science Exhibit 1004
Page 466

EX-2021
RegenX Science, Inc. v. NeXtGen Biologics, Inc.
0466

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/055998

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0038651 A1	06-07-2000	AU 2708100 A	31-07-2000
		EP 1143929 A1	17-10-2001
		US 6245740 B1	12-06-2001
		US 6979674 B1	27-12-2005
		WO 0038651 A1	06-07-2000
US 2005036946 A1	17-02-2005	US 2005036946 A1	17-02-2005
		US 2010290995 A1	18-11-2010
US 2007265356 A1	15-11-2007	NONE	
WO 0182970 A1	08-11-2001	AR 028382 A1	07-05-2003
		AT 342066 T	15-11-2006
		AU 5544901 A	12-11-2001
		CA 2407298 A1	08-11-2001
		DE 60123772 T2	04-10-2007
		DK 1276505 T3	12-02-2007
		EP 1276505 A1	22-01-2003
		ES 2273834 T3	16-05-2007
		JP 4895457 B2	14-03-2012
		JP 2003531927 A	28-10-2003
		PT 1276505 E	31-01-2007
		TW 539718 B	01-07-2003
		US 7018645 B1	28-03-2006
		US 2004185104 A1	23-09-2004
		WO 0182970 A1	08-11-2001

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European Patent Office
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European Patent Office
80298 MUNICH
GERMANY

Questions about this communication ?
Contact Customer Services at www.epo.org/contact

Gill Jennings & Every LLP
The Broadgate Tower
20 Primrose Street
London EC2A 2ES
ROYAUME UNI

Date
28.09.16

Reference	Application No./Patent No.
FXS00311EP	14737892.1 - 1455 / 2943209 PCT/US2014010890
Applicant/Proprietor	
ISE PROFESSIONAL TESTING & CONSULTING SERVICES INC	

Communication

The extended European search report is enclosed.

The extended European search report includes, pursuant to Rule 62 EPC, the supplementary European search report (Art. 153(7) EPC) and the European search opinion.

Copies of documents cited in the European search report are attached.

☑ 0 additional set(s) of copies of such documents is (are) enclosed as well

Refund of the search fee
If applicable under Article 9 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.

Should you wish to further prosecute this application in the examination phase, your attention is drawn to the provisions of Rule 70a EPC. An invitation to respond to the extended European search report will be issued shortly (R. 70(2) EPC).

Europäisches Patentamt • European Patent Office • Office européen des brevets

The examination is being carried out on the **following application documents**

Description, Pages

1-28 as published

Claims, Numbers

1-13 filed with entry into the regional phase before the EPO

Drawings, Sheets

1/6-6/6 as published

1. Amendments

The amended claims 1-13 filed with entry into the regional phase before the EPO are found to comply with the requirements of Article 123(2) EPC.

2. Cited documents

The following documents (D) cited in the Supplementary European search report are referred to in this communication; the numbering will be adhered to in the rest of the procedure:

D1: WO 03/082201 A (Anthrogenesis Corporation)

3. Novelty

Document D1 discloses (cf. page 1, par. 2; page 4, par. 11 - page 5, par. 11; page 9, par. 33; claims 24-26, 29, 30, 32, 38) a collagenous membrane comprising a decellularised amniotic membrane derived from a mammal, which has the structural integrity of the native non-treated amniotic membrane, *i.e.* the native tertiary and quaternary structure.

The subject-matter of claims 1-13 of the present application differs from the disclosure of D1 in the use of urodele tissue, extracellular matrix or extracellular matrix components and is therefore novel according to Article 54(1) and (2) EPC.

4. Inventive Step

According to the description of the present application (page 4, par. 14), the object of the invention is to provide a suitable tissue substrate biomaterial for use in transplantation to promote tissue regeneration while restoring functionality, which is readily available, does not impose additional complications to a donor or recipient (such as requiring an additional surgery to harvest the substrate), and exhibits all or some of the inherent material functionality reflective of the physiochemical, electrochemical, and biochemical properties of natural tissue.

Document D1, which is considered to represent the most relevant prior art, discloses (cf. page 4, par. 11 - page 5, par. 11) the preparation of a collagen biofabric with improved physical and biophysical properties having increased tensile strength, suturability, and reduced immunogenicity resulting in reduced host-graft rejection.

The subject-matter of independent claims 1, 3, 4, 6, 10, 11, and 12 differs from D1 in the use of urodele tissue, extracellular matrix or extracellular matrix components.

According to the description of the present application (cf. page 6, par. 21), the growth factor profile, connective tissue matrix constituents, and immunoprivileged status of urodele ECM and accompanying cutaneous tissue, plus the presence of antimicrobial peptides therein, render urodele-derived tissue an ideal source for biological scaffolds for xenotransplantation.

In view of D1, the objective technical problem underlying the present application can therefore be considered as to provide a biological scaffold having a growth factor profile, connective tissue matrix constituents, and immunoprivileged status similar to that of amniotic membrane.

The skilled person had no indication that urodele tissue, which has never been used for the preparation of biomaterials, would be a suitable alternative to amniotic membrane tissue. Consequently, the subject-matter of present claims 1-13 does involve an inventive step according to Article 56 EPC.

5. Further objections and remarks

5.1 Each of claims 2, 5, and 6 defines a number of unrelated alternative features which cannot be considered as "alternatives" in accordance with the Guidelines F-IV, 3.7. This style of claim-drafting is considered to obscure the claims, leading to a lack of clarity (Article 84 EPC). The Applicant is requested to either reformulate said claims as individual claims as in the original set of claims or to restrict claims 2, 5 and 6 to one alternative each.

5.2 Product claims 1, 4 and 11 have been drafted as separate independent claims. Under Article 84 in combination with Rule 43(2) EPC an application may contain more than one independent claim in a particular category only if the subject matter claimed falls within one or more of the exceptional situations set out in paragraphs (a), (b) or (c) of Rule 43(2) EPC. However, this is not the case in the present application, since both claims relate to an isolated, decellularized extracellular matrix (ECM) from a urodele. The Applicant is requested to submit one new independent claim instead.

5.3 According to established case law (cf. Case Law Book, 8th Ed. 2016, II.A.7.3), a so-called product-by-process claim is only admissible if it is not possible to define the claimed product in other terms, e.g. by reference to chemical or structural features. In view of claim 4, it seems possible in the present case to define the product without a reference to its manufacture.

5.4 The Applicant is requested to file new claims to overcome the above-mentioned objections. When filing amended claims, the Applicant should at the same time bring the description into conformity with the amended claims (Rule 48(1)(c) EPC and Guidelines F-II, 7.4). The document D1 should be identified in the description and its relevant content should be indicated to meet the requirements of Rule 42(1)(b) EPC.

Care should be taken during revision, especially of the introductory portion and any statements of problem or advantage, not to add subject-matter which extends beyond the content of the application as originally filed (Article 123(2) EPC). In order to facilitate the examination on the conformity of the amended application with the requirements of Article 123(2) EPC, the Applicant is requested to clearly identify the amendments carried out, irrespective of whether they concern amendments by addition, replacement or deletion, and to indicate the passages of the application as filed on which these amendments are based (cf. Guidelines H-III, 2.2, Rule 137(4) EPC). If the Applicant considers it appropriate, these indications could be submitted in handwritten form on a copy of the relevant parts of the application as filed or in form of documents with tracked changes.

Information on Search Strategy - Pilot phase (see OJ 2015, A86)
The type of information contained in this sheet may change during the pilot for improving the usefulness of this new service.

Application Number
EP 14 73 7892

TITLE: DECELLULARIZED BIOMATERIAL FORM NON-MAMMALIAN TISSUE

APPLICANT: ISE PROFESSIONAL TESTING & CONSULTING SERVICES INC

IPC CLASSIFICATION: A61K35/56, A61K9/10, A61K9/14, A61F2/00, A61L27/36

EXAMINER: Heck, Georg

CONSULTED DATABASES: NPL

CLASSIFICATION SYMBOLS DEFINING EXTENT OF THE SEARCH:

IPC:

CPC: A61L27/36/LOW, A61L27/3604, A61L27/3633, A61K35/56, A61K35/58, A61K35/65

FI/F-TERMS:

KEYWORDS OR OTHER ELEMENTS FEATURING THE INVENTION:
Biomaterial comprising extracellular matrix derived from a urodele (order Urodela, also known as Caudata).

EPO FORM P04A42

Europäisches Patentamt
European Patent Office
Office européen des brevets

SUPPLEMENTARY EUROPEAN SEARCH REPORT

Application Number
EP 14 73 7892

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A	WO 03/082201 A2 (ANTHROGENESIS CORP [US]; HARIRI ROBERT J [US]; KAPLUNOVSKY ALEKSANDR M) 9 October 2003 (2003-10-09) * page 1, paragraph 2 * * page 4, paragraph 11 - page 5, paragraph 11 * * page 9, paragraph 33 * * claims 24-26, 29, 30, 32, 38 * -----	1-13	INV. A61K35/56 A61K9/10 A61K9/14 A61F2/00 A61L27/36
			TECHNICAL FIELDS SEARCHED (IPC) A61L A61K

The supplementary search report has been based on the last set of claims valid and available at the start of the search.

Place of search	Date of completion of the search	Examiner
The Hague	8 September 2016	Heck, Georg

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

1

EPO FORM 1503 03.82 (P04C09)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 14 73 7892

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

08-09-2016

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03082201 A2	09-10-2003	AU 2003226018 A1	13-10-2003
		AU 2008202604 A1	03-07-2008
		AU 2011201662 A1	12-05-2011
		CA 2479903 A1	09-10-2003
		CN 1791331 A	21-06-2006
		EP 1575572 A2	21-09-2005
		EP 2700309 A1	26-02-2014
		EP 2702871 A1	05-03-2014
		ES 2526088 T3	05-01-2015
		HK 1085384 A1	21-08-2015
		IL 164220 A	31-10-2012
		JP 4615223 B2	19-01-2011
		JP 5931327 B2	08-06-2016
		JP 2006507851 A	09-03-2006
		JP 2010265298 A	25-11-2010
		JP 2014138590 A	31-07-2014
		KR 20050002906 A	10-01-2005
		MX PA04009188 A	20-06-2005
		NZ 535799 A	25-06-2010
		US 2003187515 A1	02-10-2003
		US 2004048796 A1	11-03-2004
		WO 03082201 A2	09-10-2003
		ZA 200407650 B	30-08-2006

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 101763-0103	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. PCT/US 2014/010890	International filing date *(day/month/year)* 09 January 2014 (09.01.2014)	(Earliest) Priority Date *(day/month/year)* 09 January 2013 (09.01.2013)
Applicant EARLY, Ryanne		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 2 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of:

☒ the international application in the language in which it was filed.

☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).

c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☐ **Certain claims were found unsearchable** (see Box No. II).

3. ☐ **Unity of invention is lacking** (see Box No. III).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the **drawings**,

a. the figure of the drawings to be published with the abstract is Figure No. 1

☒ as suggested by the applicant.

☐ as selected by this Authority, because the applicant failed to suggest a figure.

☐ as selected by this Authority, because this figure better characterizes the invention.

b. ☐ none of the figures is to be published with the abstract.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 2014/010890

A. CLASSIFICATION OF SUBJECT MATTER

A61K 35/56 *(2006.01)*
A61K 9/10 *(2006.01)*
A61K 9/14 *(2006.01)*
A61F 2/00 *(2006.01)*
A61L 27/36 *(2006.01)*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 35/56, 31/00, 38/00, 9/10, 9/14, A61F 2/00, A61L 27/36

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DEPATISnet, DWPI, EAPATIS, PAJ, Patentscope, PatSearch (RUPTO internal), PubMed, RUPTO, RUSPAT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/124127 A2 (WAKE FOREST UNIVERSITY HEALTH SCIENCES et al.) 01.11.2007, claims 1, 15, 17-18, 21, p. 2, last paragraph, p. 3, paragraph 3, p. 4, paragraphs 4, 5, 6, p. 7, paragraphs 4, 5, p. 8, paragraphs 3, 5, p. 9, paragraph 4, p. 15, paragraphs 1, 2	1-10, 15-16, 20-25, 27-29, 32-33
Y		11-14, 17-19, 26, 30-31
Y	WO 2005/009498 A2 (SCIMED LIFE SYSTEMS, INC. et al.) 03.02.2005, claims 30-31, p. 13, lines 31-33, p. 16, lines 28-29, p. 26, lines 13-15	11-14, 17-19, 26, 30-31

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 March 2014 (26.03.2014)	15 May 2014 (15.05.2014)
Name and mailing address of the ISA/ FIPS Russia, 123995, Moscow, G-59, GSP-5, Berezhkovskaya nab., 30-1 Facsimile No. +7 (499) 243-33-37	Authorized officer E. Maslova Telephone No. (495)531-64-81

Form PCT/ISA/210 (second sheet) (July 2009)

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

To:
BENT, Stephen A.
FOLEY & LARDNER LLP, 3000 K Street NW, Suite 600
Washington, District of Columbia 20007
United States of America

Date of mailing *(day/month/year)*	
15 May 2014 (15.05.2014)	
Applicant's or agent's file reference: 101763-0103	FOR FURTHER ACTION: See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US 2014/010890	09 January 2014 (09.01.2014)	09 January 2013 (09.01.2013)

International Patent Classification (IPC) or both national classification and IPC
A61K 35/56 *(2006.01)*
A61K 9/10 *(2006.01)*
A61K 9/14 *(2006.01)*
A61F 2/00 *(2006.01)*
A61L 27/36 *(2006.01)*

Applicant
EARLY, Ryanne

1. This opinion contains indications relating to the following items:

- [X] Box No. I Basis of the opinion
- [ ] Box No. II Priority
- [ ] Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- [ ] Box No. IV Lack of unity of invention
- [X] Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- [ ] Box No. VI Certain documents cited
- [X] Box No. VII Certain defects in the international application
- [ ] Box No. VIII Certain observations on the international application

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/ FIPS Russia, 123995, Moscow, G-59, GSP-5, Berezhkovskaya nab., 30-1 Facsimile No. +7 (499) 243-33-37	Date of completion of this opinion 26 March 2014 (26.03.2014)	Authorized officer E. Maslova Telephone No. (495)531-64-81

Form PCT/ISA/237 (cover sheet) (July 2011)

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 2014/010890

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

[X] the international application in the language in which it was filed.

[ ] a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. [ ] This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing filed or furnished:

a. (means)

[ ] on paper

[ ] in electronic form

b. (time)

[ ] in the international application as filed

[ ] together with the international application in electronic form

[ ] subsequently to this Authority for the purposes of search

4. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 2014/010890

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	7, 11-14, 17-19, 26, 30-31	YES
	Claims	1-6, 8-10, 15-16, 20-25, 27-29, 32-33	NO
Inventive step (IS)	Claims		YES
	Claims	7, 11-14, 17-19, 26, 30-31	NO
Industrial applicability (IA)	Claims	1-33	YES
	Claims		NO

2. Citations and explanations:

D1 WO 2007124127 A2 01.11.2007

D2 WO 2005009498 A2 03.02.2005

D1 discloses a method of preparing a bioscaffold with the use of decellularizing a tissue, during which an excess material is removed from a tissue sample (claims 1, 21). A tissue of amphibians can be used as the tissue. The representative of the amphibians is the order of Urodele (p. 8, paragraphs 3, 5). When preparing the bioscaffold, it maintains its natural biomechanical integrity (p. 7, paragraph 4; p. 2, last paragraph; p. 3, paragraph 3). Decellularizing the tissue is implemented in order to reduce an inflammatory reaction and immune response (p. 7, paragraph 5). The bioscaffold is used as a transplant, an implant (p. 4, paragraphs 4, 6, claim 18). The bioscaffold can be placed in a package (claim 17). The bioscaffold can comprise growth factors present in a culture medium (p. 9, paragraph 4).

Therefore, the inventions in claims 1, 5, 6, 16, 20, 22, 23, 25, 27, 28 do not meet the criterion of novelty.

D2 discloses a device having a layer of decellularized ECM (claims 30-31).

The invention in independent claim 26 differs from D1 in that the tissue of Urodele is used; the device comprises at least two sheets.

As D1 discloses that a tissue of amphibians is used when preparing decellularized ECM, the use of the tissue of Urodele when preparing ECM is obvious for a person skilled in the art, wherein the application of the necessary number of sheets on the device is also obvious for a person skilled in the art.

Therefore, the invention in independent claim 26 does not meet the criterion of inventive step.

D1 discloses the features of dependent claims 2-4, 8-10, 15, 21, 24, 29, 32-33 relative to decellularizing implemented using, for example, an alkaline treatment (p. 15, paragraphs 1, 2). D1 discloses a bioscaffold that can be sterilized (claim 15); the bioscaffold can comprise human cells,

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In case the space in any of the preceding boxes is not sufficient.
Continuation of V:

growth factors (p. 4, paragraph 5; p. 9, paragraph 4). The bioscaffold also can be used as a transplant, an implant (p. 4, paragraphs 4, 6, claim 18). When preparing the bioscaffold, it maintains its natural biomechanical integrity (p. 7, paragraph 4; p. 2, last paragraph; p. 3, paragraph 3).

Therefore, the inventions in dependent claims 2-4, 8-10, 15, 21, 24, 29, 32-33 do not meet the criterion of novelty.

D2 discloses the features of dependent claims 11-14, 17-19, 30-31 relative to a composition comprising the decellularized ECM that can be made in the form of a sheet (p. 26, lines 13-15) or a gel (p. 13, lines 31-33). The composition can be a powder (p. 16, lines 28-29).

The use of Axolotl tissue in dependent claim 7 is obvious for a person skilled in the art, as D1 (p. 8, paragraphs 3, 5) discloses the use of a tissue of amphibians.

Therefore, the inventions in dependent claims 7, 11-14, 17-19, 30-31 do not meet the criterion of inventive step.

The inventions in claims 1-33 meet the criterion of industrial applicability.

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WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 2014/010890

Box No. VII Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Dependence of the following claims is incorrect:

- claims 17-19 are dependent on independent claim 16;
- claim 21 is dependent on independent claim 20;
- claim 24 is dependent on independent claim 22.

PATENT COOPERATION TREATY

ADVANCE E-MAIL

PCT

From the INTERNATIONAL BUREAU

NOTIFICATION CONCERNING TRANSMITTAL OF COPY OF INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY (CHAPTER I OF THE PATENT COOPERATION TREATY)
(PCT Rule 44bis.1(c))

To:

GATES, Edward R.
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
ETATS-UNIS D'AMERIQUE

Date of mailing *(day/month/year)*
23 July 2015 (23.07.2015)

Applicant's or agent's file reference
S1860.70000

IMPORTANT NOTICE

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US2014/010890	09 January 2014 (09.01.2014)	09 January 2013 (09.01.2013)

Applicant
EARLY, Ryanne

The International Bureau transmits herewith a copy of the international preliminary report on patentability (Chapter I of the Patent Cooperation Treaty)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. +41 22 338 82 70

Authorized officer:
Kihwan Moon

e-mail: pt01.pct@wipo.int

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44*bis*)

Applicant's or agent's file reference S1860.70000	**FOR FURTHER ACTION**	See item 4 below
International application No. PCT/US2014/010890	International filing date *(day/month/year)* 09 January 2014 (09.01.2014)	Priority date *(day/month/year)* 09 January 2013 (09.01.2013)
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237		
Applicant EARLY, Ryanne		

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 *bis*.1(a).

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☐	Box No. II	Priority
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☒	Box No. VII	Certain defects in the international application
☐	Box No. VIII	Certain observations on the international application

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44*bis*.3(c) and 93*bis*.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44*bis* .2).

	Date of issuance of this report 14 July 2015 (14.07.2015)
The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No: +41 22 338 82 70	Authorized officer Kihwan Moon e-mail: pt01 pct@wipo.int

Form PCT/IB/373 (January 2004)

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

To:
BENT, Stephen A.
FOLEY & LARDNER LLP, 3000 K Street NW, Suite 600
Washington, District of Columbia 20007
United States of America

Date of mailing *(day/month/year)* 15 May 2014 (15.05.2014)	
Applicant's or agent's file reference 101763-0103	FOR FURTHER ACTION See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US 2014/010890	09 January 2014 (09.01.2014)	09 January 2013 (09.01.2013)

International Patent Classification (IPC) or both national classification and IPC
A61K 35/56 *(2006.01)*
A61K 9/10 *(2006.01)*
A61K 9/14 *(2006.01)*
A61F 2/00 *(2006.01)*
A61L 27/36 *(2006.01)*

Applicant
EARLY, Ryanne

1. This opinion contains indications relating to the following items:

- [X] Box No. I Basis of the opinion
- [ ] Box No. II Priority
- [ ] Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- [ ] Box No. IV Lack of unity of invention
- [X] Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- [ ] Box No. VI Certain documents cited
- [X] Box No. VII Certain defects in the international application
- [ ] Box No. VIII Certain observations on the international application

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

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For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/ FIPS Russia, 123995, Moscow, G-59, GSP-5, Berezhkovskaya nab., 30-1 Facsimile No. +7 (499) 243-33-37	Date of completion of this opinion 26 March 2014 (26.03.2014)	Authorized officer E. Maslova Telephone No. (495)531-64-81

Form PCT/ISA/237 (cover sheet) (July 2011)

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 2014/010890

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

[X] the international application in the language in which it was filed.

[ ] a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. [ ] This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing filed or furnished:

a. (means)

[ ] on paper

[ ] in electronic form

b. (time)

[ ] in the international application as filed

[ ] together with the international application in electronic form

[ ] subsequently to this Authority for the purposes of search

4. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 2014/010890

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	7, 11-14, 17-19, 26, 30-31	YES
	Claims	1-6, 8-10, 15-16, 20-25, 27-29, 32-33	NO
Inventive step (IS)	Claims		YES
	Claims	7, 11-14, 17-19, 26, 30-31	NO
Industrial applicability (IA)	Claims	1-33	YES
	Claims		NO

2. Citations and explanations:

D1 WO 2007124127 A2 01.11.2007

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Therefore, the inventions in claims 1, 5, 6, 16, 20, 22, 23, 25, 27, 28 do not meet the criterion of novelty.

D2 discloses a device having a layer of decellularized ECM (claims 30-31).

The invention in independent claim 26 differs from D1 in that the tissue of Urodele is used; the device comprises at least two sheets.

As D1 discloses that a tissue of amphibians is used when preparing decellularized ECM, the use of the tissue of Urodele when preparing ECM is obvious for a person skilled in the art, wherein the application of the necessary number of sheets on the device is also obvious for a person skilled in the art.

Therefore, the invention in independent claim 26 does not meet the criterion of inventive step.

D1 discloses the features of dependent claims 2-4, 8-10, 15, 21, 24, 29, 32-33 relative to decellularizing implemented using, for example, an alkaline treatment (p. 15, paragraphs 1, 2). D1 discloses a bioscaffold that can be sterilized (claim 15); the bioscaffold can comprise human cells,

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0486

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of V:

growth factors (p. 4, paragraph 5; p. 9, paragraph 4). The bioscaffold also can be used as a transplant, an implant (p. 4, paragraphs 4, 6, claim 18). When preparing the bioscaffold, it maintains its natural biomechanical integrity (p. 7, paragraph 4; p. 2, last paragraph; p. 3, paragraph 3).

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The use of Axolotl tissue in dependent claim 7 is obvious for a person skilled in the art, as D1 (p. 8, paragraphs 3, 5) discloses the use of a tissue of amphibians.

Therefore, the inventions in dependent claims 7, 11-14, 17-19, 30-31 do not meet the criterion of inventive step.

The inventions in claims 1-33 meet the criterion of industrial applicability.

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 2014/010890

Box No. VII Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Dependence of the following claims is incorrect:

- claims 17-19 are dependent on independent claim 16;
- claim 21 is dependent on independent claim 20;
- claim 24 is dependent on independent claim 22.

REVIEW ARTICLE

Amniotic Membrane Transplantation in the Human Eye

Daniel Meller, Mikk Pauklin, Henning Thomasen, Henrike Westekemper, Klaus-Peter Steuhl

SUMMARY

Background: Amniotic membrane transplantation (AMT) has a long tradition in ophthalmic surgery and has become very popular recently because of newly developed methods of tissue preservation.

Methods: We selectively review the literature on recent developments, mechanisms of action, and established indications of AMT in the treatment of various diseases of the ocular surface. We searched the PubMed database for articles that appeared from 1994 to 2009 with the key words "amniotic membrane," "cornea," and/or "conjunctiva."

Results: Amniotic membrane (AM) can function in the eye as a basement membrane substitute or as a temporary graft. It has anti-inflammatory and anti-scarring effects and contains growth factors that promote epithelial wound healing on the surface of the eye. AMT has been found to be a good alternative for corneal and conjunctival reconstruction in many clinical situations, including acute burns, persistent epithelial defects of the cornea, and diseases that cause conjunctival scarring. Nonetheless, there have been no more than a few randomized and controlled trials of AMT to date. Other studies have shown that AM can serve as a culture substrate to expand epithelial progenitor cells for use in ocular surface reconstruction.

Conclusion: AMT is an established technique in the treatment of various diseases of the external eye. In the last few years, AMT has brought about major advances in the reconstructive surgery of the ocular surface.

➤ Cite this as:
Meller D, Pauklin M, Thomasen H, Westekemper H, Steuhl KP: Amniotic membrane transplantation in the human eye. Dtsch Arztebl Int 2011; 108(14): 243–8. DOI: 10.3238/arztebl.2011.0243

Klinik für Erkrankungen des vorderen Augenabschnittes, Zentrum für Augenheilkunde, Universität Duisburg-Essen, Essen: Prof. Dr. med. Meller, Dipl.-Biol. Thomasen, Dr. med. Westekemper, Prof. Dr. med. Steuhl

Institute of General and Molecular Pathology, University of Tartu, Estland: Pauklin

Transplantation of preserved human amniotic membrane (AM) can be considered one of the major new developments in surgery of the ocular surface. Although the first ophthalmological use of AM documented in the international literature took place almost 70 years ago, amniotic membrane transplantation (AMT) has only been performed in larger numbers of patients since 1995, with promising results (1–3).

Various disorders of the ocular surface, including persistent epithelial defects of the cornea, acute chemical burns with long-term loss of integrity of the ocular surface epithelium or conjunctival scarring as a result of the healing of mucous membrane disorders still pose a clinical challenge in ophthalmic surgery. Since modern preservation methods were introduced, the innermost layer of the placenta, the AM, procured in sterile conditions following a Cesarean section, has experienced a renaissance as a basement membrane substitute. Today it is hard to imagine reconstructive surgery of the ocular surface without it (1–3). In 2008, a total of 2308 AMTs were performed in Germany (see http://www.dog.org/?cat=121, in German). The many possible applications of AM established since then range from grafts via patches to a culture substrate/carrier for ex vivo cultivation of ocular surface epithelium. Each type of application yields histologically different integration patterns for AM in the host corneal tissue (4). The most important indications in reconstructive surgery of the ocular surface are persistent epithelial defects of the cornea with corneal ulceration of varying etiology, covering defects after surgical removal of large conjunctival lesions, acute chemical burns, symblepharon and fornix reconstruction in healing conjunctival disorders, and limbal stem cell deficiency of the cornea with simultaneous stem cell grafting (5–7).

This review article presents information obtained from the authors' scientific and clinical activities and a selective search of PubMed literature using the search terms "amniotic membrane," "cornea," and/or "conjunctiva" ranging from 1994 to 2009. It reports new developments, mechanisms of action, and established indications of AMT.

Procurement and histology

The AM is the innermost layer of the placenta, located next to the fetus. Histologically, it is a multilayer membrane approximately 0.02 to 0.5 mm thick. After

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serologically detectable pathogens (HIV-1/2; hepatitis B, C; HTLV-I/II; syphilis) have been ruled out, it is procured under sterile conditions during a Cesarean section, packed in a sterile environment, and swiftly prepared in a sterile working area. Appropriate standardized operating guidelines for procuring and manufacturing human AM from donor placenta are currently being developed with the support of the Tissue Transplantation and Biotechnology Section of the German Society of Ophthalmology (Deutsche Ophthalmologische Gesellschaft, DOG) (e1). When appropriately prepared it consists of a relatively thick basement membrane with devitalized amniotic epithelial cells and an avascular, almost acellular stroma. Cryopreservation involves a cryomedium (glycerin: DMEM medium 1:1), with a storage temperature of –75 °C to –85 °C and maximum storage time of approximately 12 to 24 months. If professionally procured and preserved, AM's biological properties are retained, and as a result this natural matrix can be used to replace damaged stromal tissue of the surface of the eye (4, 5).

Clinical importance of biological properties

Clinical trials suggest that AM transplantation promotes epithelialization and differentiation of the epithelium of the ocular surface (5–9). The most important growth factors that promote wound healing, which have been isolated mainly from the amniotic epithelium but also from the AM stroma, are epidermal growth factor and keratocyte growth factor (8, 9). Structural proteins such as laminin and type VII collagen in the AM basement membrane explain the observed epitheliotropic effects (9, 10). Intrinsic neurotropic substances make AM an ideal substrate for reconstruction of the epithelium of the ocular surface (11, 12).

The inhibition of TGF-ß signal transduction in corneal and conjunctival fibroblasts in vitro explains AM's anti-scarring effect in the treatment of various disorders of the surface of the eye (13). In the initial phase after AMT, there is typically a significant reduction in inflammation. In vitro, AM reduces expression of various growth factors and pro-inflammatory cytokines (14). In addition, anti-inflammatory cytokines such as interleukin-10 and interleukin-1 receptor antagonist are released in the epithelium and stroma of the AM and may modulate inflammatory processes. They may play a role in the healing of acute chemical burns of the cornea covered by AM (15, 16). AM also has an immunomodulatory effect (17), and tissue rejection is therefore rarely observed in clinical use of AM.

The effects of cryopreservation

As the cells of cryopreserved AM are devitalized after thawing, no enzyme activity appears, and no intact RNA can be extracted, these substances are released from the damaged, devitalized cells. Because these factors are removed after application of cryopreserved AM, longer-term use is problematic. It is therefore advisable to replace an AM used as a patch in ocular surface reconstruction at regular weekly intervals until the desired epithelial wound closure occurs. Unlike the generally preferred cryopreservation of AM, other AM preparation procedures which are in use, such as acid pretreatment and subsequent air drying (18), lead to near-complete loss of its biological properties, and as a result their possibilities for clinical application and their efficacy seem limited (19).

Basic surgical techniques

AM has three main aims in clinical use:

- To promote epithelialization
- To reduce pain
- To minimize inflammation of the ocular surface.

This allows procedures to be performed in a sequential manner, such as cornea transplantation with a reduced risk of rejection (5, 20). These aims are achieved using various different surgical procedures for AMT (5, 6).

Inlay or graft technique

In the inlay technique, the AM is applied as a permanent basement membrane substitute. The main indications for this are persistent epithelial defects, corneal ulceration or to cover defects following excision of conjunctival tumors (5, 6, 20–23, e2) *(Box)*. AM is sewn in with the epithelium/basement membrane side

BOX

Indications of amniotic membrane transplantation*

Reconstruction of the corneal surface

- Persistent epithelial defect with corneal ulceration
- Reconstruction of the surface of the conjunctiva
- Acute chemical burns
- Removal of epithelial or subepithelial lesions (band keratopathy, scars, tumors)
- Painful bullous keratopathy
- Partial or complete limbal stem cell deficiency (with stem cell grafting)

Reconstruction of the surface of the conjunctiva

- Acute chemical burns and acute Stevens-Johnson syndrome
- Covering defects after removal of large conjunctival lesions (tumors, conjunctival intraepithelial neoplasia, scars, conjunctival folds parallel to the edges of the eyelids)
- Symblepharon, fornix reconstruction
- Anophthalmia
- Bleb revisions
- Scleral thinning
- Pterygium

*According to (5–7, 16, 21–25). The literature contains no evidence-based, randomized studies comparing AMT with the alternative options for these indications

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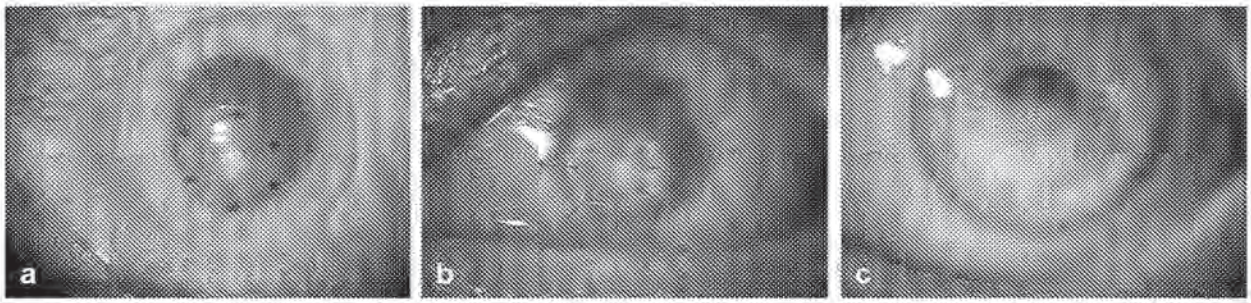


Figure 1: Amniotic membrane transplantation (AMT) in infectious corneal ulceration (area marked with stars): a) Before surgery. b) Three days after AMT. c) Three months after AMT a smooth corneal surface with no irritation has formed, with a paracentral corneal scar. Signs of inflammation have significantly decreased. Modified with the permission of Dr. R. Kaden-Verlag (publishers), Heidelberg

facing outwards, using 10-0 nylon sutures, following wound debridement, so that neighboring recipient epithelial cells can migrate onto the AM and the wound begins to close (5, 6). In deep defects, e.g. corneal ulceration, multiple layers of AM can be used (20, 21). Epithelialization of the AM integrates AM into the host tissue (4). It remains detectable for months, sometimes years, and in defects of the cornea is even colonized by local keratocytes (4).

Onlay or patch technique

In the onlay technique, a large AM is temporarily placed on the surface of the eye as a "natural" patch (5, 6). Unlike the inlay technique, where the AM remains permanently on the cornea, with the onlay technique the AM patch typically becomes detached from the surface of the cornea after one to two weeks. Classical indications range from acute burns to acute herpetic keratitis and the acute stage of Stevens-Johnson syndrome (5, 6, 16, 20, 24). With these indications AM's biological properties, particularly its anti-inflammatory mechanisms of action, are used, although as mentioned above these last only a limited time (8, 9, 19). If delayed wound healing is demonstrated clinically, on the basis of the above-mentioned experimental data it seems to be advisable to change the AM patch weekly until the wound closes as desired. The orientation of the AM plays only a minor role with this surgical procedure: in most cases attaching the AM loosely in the episcleral space or to the bulbar conjunctiva using 8-0 monofilament vicryl sutures is considered sufficient (5, 6).

Inlay and onlay technique

This technique, also called the sandwich technique, is a combination of the two described above and is used mainly in serious disorders of the ocular surface such as deep and extensive corneal ulceration, or in surgical revisions (5, 6). The main purpose of the onlay is to protect the inlay and promote its epithelialization (4, 5). This method is favored due to its high success rate (65% to 80%), and low rate of recurrence (approx. 20% to 35%) (5, 20).

Amniotic membrane as culture substrate and carrier

The AM has only recently been described as a culture substrate for ex vivo expansion of the epithelium of the ocular surface (7, 25, e3–e5). Here the AM functions as a biomatrix and is placed on the surface of the eye together with the cultivated cells (e3, e4). This technique is mainly used to treat limbal stem cell deficiency, but also in reconstruction of major surface defects of the outer eye (7, 25, e5, e6).

Indications of transplantation

So many indications of AMT have already been described in the literature that they would go well beyond the boundaries of this review article (5–7, 16, 24–25, e2, e5, e6). The main indications of AMT are therefore summarized in the *Box*. Unfortunately, reliable clinical data have only been published in small to medium numbers of cases, with a relatively short follow-up period, and overwhelmingly in retrospectively analyzed case series (e6), probably due to the low incidence of individual disorders of the ocular surface. There are no Cochrane Reviews or meta-analyses available from either the Cochrane Library or PubMed on the subjects discussed in this review article. Controlled, randomized, multicenter, long-term trials involving large numbers of patients must be conducted in the future in order to substantiate AMT's clinical potential in reconstructive surgery of the surface of the eye.

Reconstruction of the corneal surface

Persistent epithelial defects and ulceration of the cornea

Disorders of the cornea are one of the most common indications of AMT in the USA, accounting for 41% of cases (9). The treatment of corneal ulcers still poses a major challenge in ophthalmology, despite various conservative and surgical options (5, 20, 21). An intact corneal epithelium is a decisive factor in ocular surface stability. Simple epithelial defects generally heal with no complications. If they are untreated or inappropriately treated, corneal ulceration, descemetocele, and even perforation can quickly develop. AM is used clinically as a basement membrane substitute in patients with persistent epithelial defects both with and without

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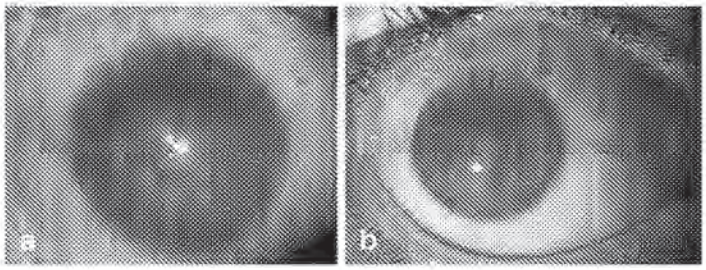


Figure 2: Autologous transplantation of limbal epithelium cultivated ex vivo. a) Before surgery, with complete stem cell deficiency following a chemical burn. Visual acuity sufficient to perceive hand movements. b) Stable, clear corneal surface 4.5 years after reconstruction. Visual acuity has increased to 0.4 long-term. Modified according to Dev Ophthalmol 2010; 45: 57–70, with the permission of Karger-Verlag (publishers), Basel

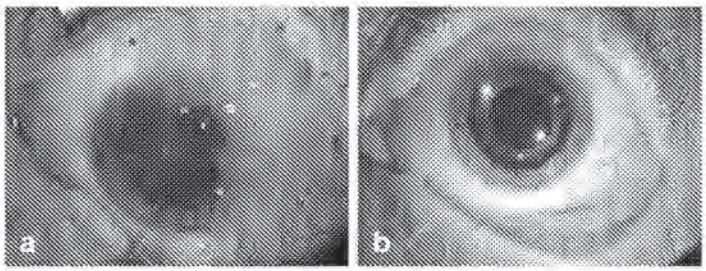


Figure 3: Covering a defect following excision of conjunctival intraepithelial neoplasia: a) Before surgery, with a large conjunctival tumor (see area marked with stars), b) 4 years after tumor excision and AMT there are no signs of recurrence. Intraocular pseudophakia with an irritation (intraocular lens, see area marked with stars) following a cataract operation

corneal ulceration (5, 20). After a median follow-up time of 18 months, Seitz et al. achieved epithelial closure with no recurrence in 65% of cases (5) *(Figure 1)*. The success rate of AMT can be significantly improved using subsequent measures such as inducing upper eyelid ptosis via Botox injection or tarsorrhaphy (5, 20). The reoperation rate then falls from approx. 44% to 30% (20).

A multilayer technique is used to treat deep corneal ulceration, descemetocele, and small corneal perforations (21, e2). Solomon et al. report stable wound closure in 28 of 34 eyes (82%) with these corneal disorders in a retrospective analysis (21). Kruse et al. also achieved stabilization of the corneal surface in 9 of 11 patients with deep ulceration of the cornea, within a follow-up time of 12 months (e2). After AMT, corneal transplantation (keratoplasty) is required for further restoration of vision. Prognosis is substantially improved if an intact surface epithelium has formed on the surface of the eye and signs of inflammation have decreased (5, 20, 21). Turning to complications, calcification is observed in 12% of cases if eye drops containing phosphates are used, and infections in less than 1% of cases (5).

Acute chemical burns

Chemical burns to the ocular surface are a common problem in acute ophthalmological care. These can lead to complete corneal erosion and blood vessel rupture at the limbus and in the conjunctiva. The overriding aims of treatment are to prevent necrosis and scarring and to achieve swift epithelialization. Early AMT improves functional outcomes after chemical burns (2, 16, 24). Symblepharon occurred in only one case of slight to moderate chemical burns treated with prompt AMT (2, 16, 24). In a prospective randomized controlled trial Tamhane et al. documented that the epithelial closure rate was significantly higher in patients treated with an AM in the first week. For severe chemical burns, however, stem cell deficiency of the cornea with long-term, serious loss of visual acuity is often impossible to prevent (16, e7).

Tissue engineering of limbal epithelium using amniotic membrane

The corneal surface is very important to eyesight. Maintaining a functional corneal epithelium is therefore essential to vision. Epithelial stem cells of the corneal epithelium are located in the limbus, which forms an anatomical bridge between the conjunctiva and the cornea. Various disorders of the surface of the eye can lead to limbal stem cell deficiency as a result of destruction of limbal stem cells. Limbal stem cell deficiency is characterized by formation of fibrovascular pannus tissue on the corneal surface, increased glare sensitivity, and loss of vision. Long-term success of surface reconstruction can only be achieved by restoring the limbal stem cell population (e6)

For many years the results of conventional limbus transplantation, which are not yet satisfactory, have given rise to a demand for stem cells from cell cultivation (e6). Further development of methods reliant on cell biology, particularly tissue engineering, now also make it possible to cultivate limbus stem cells. Autologous ex vivo expansion of limbal epithelial cells on AM has only recently been developed as a new procedure (7, 25, e3–e5) *(Figure 2)*: in unilateral stem cell deficiency, in clinical and experimental conditions, a limbal biopsy measuring 1 to 2 mm was taken from the other, healthy eye, and epithelial cells were cultivated and expanded on the AM in vitro. This ex vivo expansion of limbal epithelial cells is less traumatic than conventional autologous limbal transplantation, in which up to 50% of the limbus from the healthy eye is transplanted onto the diseased eye. Tsai et al. were the first to describe their clinical experience in treating limbal stem cell deficiency using AM as a carrier and culture substrate (25). Long-term wound healing and increased visual acuity were achieved in 83% of treated patients. Vascularization as a sign of recurring stem cell deficiency did not occur within the described follow-up period. Many subsequent publications, some of which concern different cell cultivation procedures, have also described notable treatment success (7, e5). Unfortunately, there is currently no standardized procedure available, which makes the published studies harder to compare. Other disadvantages are the small, sometimes heterogeneous patient groups and usually short follow-up

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