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ECM-based triple layered scaffolds for vascular tissue engineering

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Abstract. The present study focused on the development of three layered small-diameter (<6 mm) extracellular matrix (ECM)-based vessels. These were engineered artificially through the freeze-drying technique. A layer of decellularized bovine aorta (DAM) was deposited on a mandrel and, after lyophilization, it was dipped into a poly-L-lactide acid (PLLA)/polyethylene glycol (PEG) 2000 dichloromethane solution then quickly wrapped with a pre-prepared thin DAM sheet. Mechanical properties of three-layered scaffolds were evaluated by means of uniaxial tensile measurement. Furthermore, human endothelial and smooth muscle cells were seeded on internal and external scaffold surfaces, respectively, and co-cultured for 7 days. Our results demonstrate that i) ECM components provide suitable stimuli for cell adhesion and proliferation, ii) the microporous intermediate PLLA/PEG2000 layer is responsible for the scaffold resistance and iii) the layered deposition technique can be considered a valuable method to obtain layered vascular scaffolds of different sizes and with a good compromise between stiffness and elasticity for optimal cell organization.

Introduction

Cardiovascular diseases, such as coronary artery and peripheral vascular diseases, are the main causes of mortality in Western societies (1). To replace malfunctioning or diseased blood vessels, several reconstructive procedures have been developed with the aim of increasing implant biocompatibility, including the transfer of healthy tissue from one site or individual to another and the use of living tissue prosthetics obtained through the tissue engineering approach (2). Although successful with large diameter (>6 mm) high-flow vessels, autografts, allografts or synthetic prosthetic grafts can not meet clinical demand, especially in low-flow small-diameter vascular grafts (SDVGs) which are often connected with thrombosis, aneurysm formation, calcification, and severe inflammatory reactions (3). Even though autologous grafts have excellent long-term properties, their scarcity is a disadvantage (4). Vascular tissue engineering, however, is a multidisciplinary approach which could be applied to create completely biological and functional SDVGs whenever required. The SDVG must be biocompatible, non-thrombogenic, non-immunogenic, resistant to infection, with appropriate mechanical (such as the ability to withstand long-term hemodynamic stress without failure) and physiological (such as compliance and appropriate vasoconstriction/relaxation response) properties (4). In addition to the improvement of current conduits through the surface coating with angiogenic factors, alternative research approaches are focusing on the construction of new vessel substitutes based on natural materials (5) and extracellular matrix (ECM) proteins that function as intrinsic templates for cell attachment and growth (6-9). Biological scaffold materials derived from naturally occurring ECM have been already shown to promote site-specific tissue remodeling in a variety of body systems (10). The ECM network provides the means by which adjacent cells communicate with each other and with the external environment (11-13), allowing nutrients to be diffused from the blood to the surrounding cells and providing the skeleton for the tissue. These properties together with its biocompatibility make ECM an ideal biomaterial for tissue engineering purposes. ECM harvested from the small intestine, skin, liver, pancreas, and urinary bladder has been successfully used to promote the regeneration of tissues and organs in both animal models and human (14-18). Several types of mono- or multilayer vascular grafts containing collagen and elastin have been obtained using different techniques (9,19-23). Nevertheless, only few studies have dealt with three-layered small-diameter blood vessel substitutes (24-27). Trying to mimic the natural architecture of native blood vessels and to overcome the lack of availability of biological grafts, this study proposes the development of small-diameter vascular grafts whose core is composed of poly-L-lactide acid (PLLA) coated on both

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sides with ECM proteins to support cell adhesion. The ECM component was obtained by homogenization of decellularized bovine aorta (DAM) and then layered on PLLA by the freeze-drying technique. Our findings show that three-layered vascular grafts (TLVGs) possess good mechanical properties and support the adhesion and growth of both endothelial and smooth muscle cells.

Materials and methods

Reagents. All the chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO), with the following exceptions: phosphate-buffered saline (PBS) tablets were purchased from Gibco Invitrogen Corp. (Paisley, UK), sodium chloride from Fluka (Basel, Switzerland); Vectashield Mounting medium for fluorescence with DAPI from Vector Laboratories (Burlingame, CA), Movat pentachromic stain was obtained from Diapath (Bergamo, Italy), Masson trichrome stain from Bio-Optica (Milano, Italy), both endothelial cell growth medium MV2 and smooth muscle cell growth medium 2 from PromoCell (Heidelberg, Germany) and tissue culture Petri dishes from Becton-Dickinson (Franklin Lakes, NJ).

Decellularized aortic matrix (DAM) preparation. Aortas (length 20-25 cm) were harvested from calves and immediately stored in PBS solution containing 1% antibiotic and antimycotic solution. After removal of blood and surrounding tissue, decellularization was carried out using the Meezan's method (28). A single detergent-enzymatic cycle (DEC) of decellularization consists of the following steps: i) treatment with distilled water for 72 h at 4˚C; ii) incubation in 4% sodium deoxycholate for 4 h at room temperature; iii) treatment with 2000 KU (Kunitz Unit) of DNAse I suspended in NaCl solution 1 M for 2 h at room temperature. All steps were carried out with mechanical agitation using a rotating platform. Each decellularization cycle was repeated 2 times. DAMs were stored in PBS containing 1% antibiotic and antimycotic solution at 4˚C until use.

Proteomic characterization. About 3 mg of lyophilized DAM were dissolved in 300 μl of a rehydration buffer (containing 9 M urea, 1% CHAPS, 30 mM DTT, 0.1% Bio-Lyte 3/10 and 0.001% bromophenol blue), applied on a ready Strip IPG (length 17 cm, linear pH gradient 3-10) and focused at 60000 Vh in a Protean IEF cell apparatus (Bio-Rad, Richmond, CA). The strip was incubated in a SDS and DTT-containing buffer and allowed to run down a 2D polyacrilamide gradient gel (6-18%) using a Mod. V161 vertical gel electrophoresis apparatus (BRL, Inc., Rockville, MD). After Coomassie R250 staining, several spots were excised and digested in the gel with trypsin sequencing grade. The extracted tryptic peptides were analyzed by LC-MS/MS (QTof, Micromass UK, Ltd.). Protein identification was made by Mascot (www.matrix-science.com) using a NCBInr 20101016 (mammalia) database.

Three-layered tissue-engineered vascular graft preparation. A homogenate was prepared starting from 1.0 g of DAM, cut into small pieces and made homogeneous in 15 ml of cold 1.6 M acetic acid by milling with Ultra-Turrax (Staufen, Germany). A stainless steel mandrel (10 cm length and 4 mm diameter) was pre-cooled at -70˚C, dipped in DAM homogenate, containing 1.6% glycerol, and quickly removed. This operation was repeated until the layer deposited reached a thickness of nearly 1 mm, followed by lyophilization. The stainless steel mandrel covered by a spongy and dry DAM layer was dipped into a dichloromethane (DCM) solution of PLLA and polyethylene glycol (PEG2000) (PLLA:PEG2000:DCM; 1:1:20 w/w) and immediately wrapped with a preformed DAM sheet of 1 mm thickness previously prepared by lyophilizing a DAM homogenate on a rectangular mould (5x7x0.2 cm). The assembled graft was left at room temperature overnight then immersed in a tube filled with water and kept at -20˚C for 5 h until it was possible to remove the mandrel from the graft entrapped in the ice. After melting the ice, the recovered graft was stored in 80% (v/v) ethanol solution.

Mechanical testing. To evaluate the elasticity of the TLVGs, mechanical tests were carried out. Uniaxial tensile measurement was performed on ten longitudinally cut TLVGs by means of a universal testing machine (CETR Universal Materials Tester UNMT, 1000 N load cell) at 0.1 mm/min until rupture occurred (gripping distance 13 mm). Samples were mounted on the testing device with two grips cushioned with polymeric supports. Stress was defined as the tensile force divided by the initial cross-section area, while the strain was defined as the ratio between the grip displacement and the initial gripping distance. Measurements were taken to evaluate the Young's modulus and referred to collagen (low strain) and PLLA (high strain) response, respectively. The tensile strength (TS) was also calculated. Wall thickness of TLVGs was measured using an optical microscope (Leica DM2000, Leica Microsystems, Wetzlt, Germany). At least three vessels were considered.

Cytocompatibility evaluation. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords by collagenase digestion, as previously described (29), seeded on fibronectin (1 μg/cm^2) coated dishes and cultured with the endothelial cell growth medium MV2 supplemented with 5% fetal calf serum (FCS), 5 ng/ml epidermal growth factor (EGF), 0.2 μg/ml hydrocortisone, 0.5 ng/ml vascular endothelial growth factor, 10 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml R3 IGF-1 and 1 μg/mL ascorbic acid. Human aortic smooth muscle cells (HAoSMCs) were obtained from Sigma and cultured according to the manufacturer's instructions using the smooth muscle cell growth medium 2 supplemented with 5% FCS, 0.5 ng/ml EGF, 2 ng/ml bFGF, and 5 μg/ml insulin. For the experiments, both HUVECs and HAoSMCs were used at passage 3. TLVGs were sterilized in 80% v/v ethanol solution for 4 h under UV light in a sterile hood and left in PBS overnight in an incubator. After three hours conditioning with appropriate culture medium, HAoSMCs (6x10^5 cell/cm^2) were seeded, longitudinally on the external side of the samples. Every 30 min, vessels were rotated 180 degrees until all surfaces had been completely exposed to cells. Cell medium was added, and seeded vessels were cultured under static conditions (37˚C, 5% CO_2). After five days of culture with HAoSMCs, HUVECs were seeded on the internal side of TLVGs (6x10^5 cells/cm^2) and incubated at 37˚C, 5% CO_2 under static conditions for 48 h, for 4 and 7 days. Throughout the incubation period, the culture medium was exchanged every 2 days.

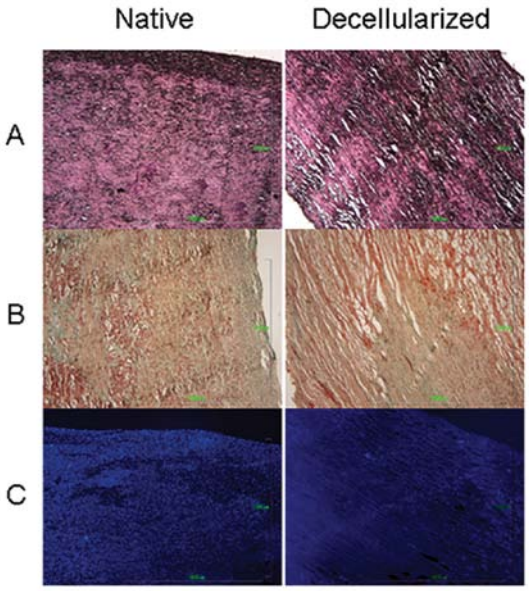

Figure 1. Histological analysis of native and decellularized aorta. (A) Hematoxylin and eosin, (B) Masson trichrome (collagen, green; muscle fibers, red) and (C) DAPI stainings. Magnification, x100.

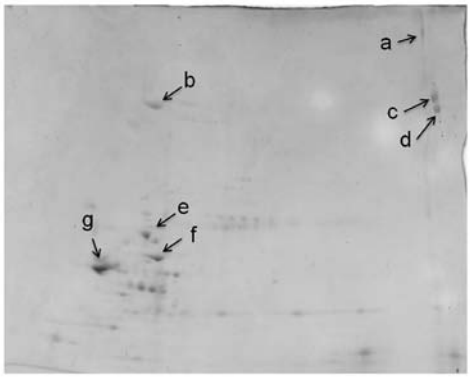

Figure 2. Two dimensional electrophoresis of DAM. Spots: a, myosin-2; b, collagen α1 (VI); c, collagen α1 (I); d, collagen α2 (I); e, vimentin; f, actin γ enteric smooth muscle; g, tropomyosin α1.

Morphological analysis. For scanning electron microscopy (SEM), samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) overnight and then dehydrated with a graded ethanol series. After critical point drying and gold sputtering, they were observed by a scanning electron miscroscope (Stereoscan-205 S; Cambridge Instruments, Cambridge, MA).

For histological analysis, native and decellularized bovine aortic samples, as well as DAM sheet samples were fixed overnight in 4% buffered formaldehyde solution, dehydrated with a graded ethanol series and embedded in paraffin. Then, 5 μm thick sections were obtained by means of a microtome (Reichtert-Jung Histoslide 2000). The second group were TLVG samples, which were frozen in 2-methylbutane cooled by vapor of liquid nitrogen and then sliced (5 μm thick) using a cryomicrotome (Leica CM 1850 UV). These sections were stained with hematoxilin and eosin (H&E), DAPI, Movat pentachromic and Masson trichrome stainings.

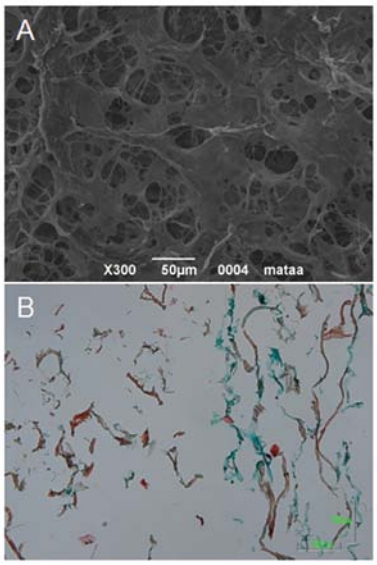

Figure 3. (A) SEM micrograph and (B) Movat pentachromic staining of DAM sheets. Thick bundles of crimped collagen fibers were visible by SEM (magnification, x300), while ECM components were clearly visible by Movat pentachromic staining (collagen, yellowish-orange; elastin, black; fibrins, light red; proteoglycans, blue; muscle layer, red) (magnification, x100).

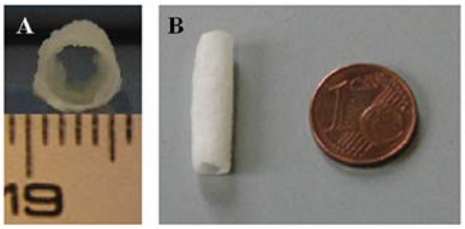

Figure 4. (A) Section and (B) longitudinal views of a TLVG.

Results

The detergent-enzymatic method represents a valid approach to remove cells from bovine aorta. Indeed, vessels completely lacked cellular elements after only two DECs, as demonstrated by H&E and DAPI stainings (Fig. 1A and C). Moreover, Masson trichrome staining (Fig. 1B) revealed the muscle fibers were well-preserved in DAM. Only a minimal amount of DAM (3%) were found to be soluble in 10% acetic acid and the remaining insoluble residue contained mainly collagen type I. The 2D gel of the extractable proteins is shown in Fig. 2, with the arrows indicating the proteins identified by LC/MS-MS analysis as collagen type I (α1 and α2 chains), collagen type VI (α1 chain), vimentin, aortic smooth muscle actin and tropomyosin (α1 chain). Collagen types I and VI are typical ECM proteins and both contain the RGD motif which is involved in cell attachment. The other identified proteins were intracellular components and their presence demonstrated that cell content removal by the detergent-enzymatic treatment was remarkable but incomplete.

DAM sheets were prepared by lyophilizing a DAM homogenate. A representative SEM micrograph of a DAM sheet is reported in Fig. 3A. It appeared like a sponge made of thick bundles of crimped collagen fibers. As demonstrated

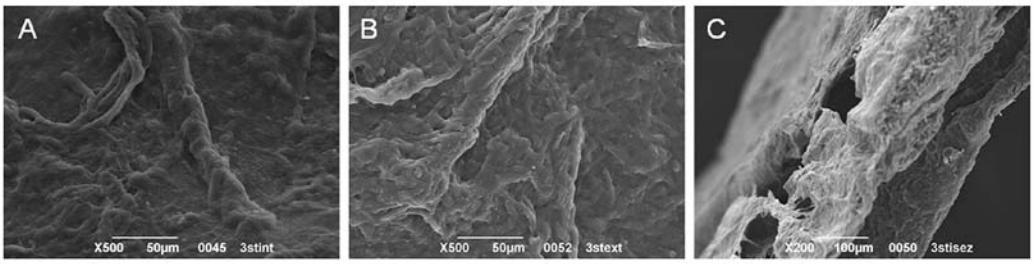

Figure 5. SEM micrographs of (A) internal side, (B) external side and (C) cross-section of a TLVG. In the cross-section, the middle PLLA layer is distinct from the internal and external DAM layers.

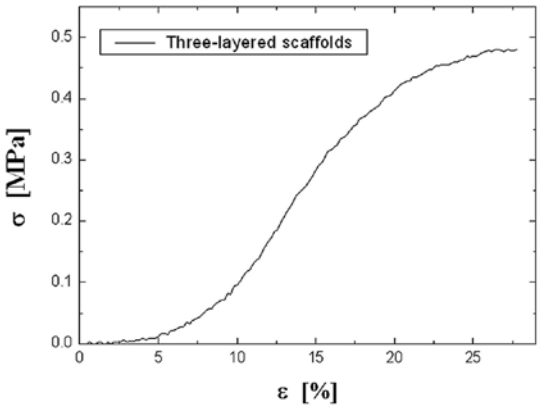

Figure 6. Stress-strain curve acquired in the uniaxial tensile test.

by Movat pentachromic staining (Fig. 3B), DAM sheets were composed of characteristic ECM components, such as collagen (yellowish-orange staining), proteoglycans (blue staining) and muscular structures (red staining).

TLVGs were obtained by wrapping two DAM sheets internally and externally to a PLLA layer. The PLLA central layer was introduced for structural reasons since its mechanical strength and controlled porosity would regulate its degradation time according to the needs of tissue neo-formation (30). Furthermore, PLLA allows for the maintenance of a tubular structure due to its relative strength and rigidity compared to DAM spongy sheets which are soft and mainly made of collagenous fibers. The length of the three-layered vascular grafts prepared was about 3 cm with an internal diameter of ~4 mm (Fig. 4). SEM analysis revealed the presence of a thick bundle of collagen fibers, similar to that already observed in the DAM sheet, in the internal and external surfaces of TLVG (Fig. 5A and B). The cross-section (Fig. 5C) showed each layer apparently separated from each other. Moreover, the porous structure of the PLLA central layer was visible. A stress-strain curve representative of the mechanical behavior of TLVGs is reported in Fig. 6. Young's modulus (1.8±0.01 MPa) was linked to the behavior of ECM collagen fibers, which showed an elastic trend, and to the behavior of the PLLA layer, which showed less elasticity, as revealed by the stress-strain curve (Fig. 6). The addition of PEG2000 modulates the microporosity of the PLLA layer and its stiffness. Moreover, the TS of TLVGs (0.47±0.02 MPa) was linked to the presence of the PLLA layer. Mechanical characterization revealed that TLVGs generally showed a significant increase of the TS with respect to natural vessels of comparable diameter. However, the Young's modulus measured (1.8 MPa) was comparable to that of porcine coronary artery (1 MPa) (31), rabbit aorta (1.25 MPa) (32) and human saphenous or femoral vein (1.50 MPa) (33).

HUVECs and HAoSMCs were seeded on the internal and external layers of TLVGs, respectively, and co-cultured for 48 h, 4 and 7 days. SEM micrographs (Fig. 7) and DAPI staining (Fig. 8) showed that TLVGs supported adhesion and proliferation of both cell types. After 48 h, both HUVECs and HAoSMCs appeared flattened and well adhered. At 4 days, endothelial cells produce an almost uniform cellular monolayer, whereas HAoSMCs did not completely cover the external surface. However, smooth muscle cells appeared growing along the collagen fibers. Furthermore, at 7 days, both cell types began to migrate among internal DAM fibers. TLVGs did not collapse along the entire culture period, thus maintaining their tubular shape.

Discussion

The aim of the present study was to prepare small-diameter three-layered blood vessel substitutes by sandwiching a PLLA layer between two ECM layers which are able to foster vascular cell adhesion and proliferation.

It has been reported that an intact ECM plays an important role not only in the maintenance of tissue structure and function, but also for the regulation of vascular cell migration, proliferation and survival (4,26,27). ECM appears relevant for the reconstructive tissue remodeling because its degradation generates a flow of bioactive molecules, as demonstrated by Badylak (34). Herein, the detergent-enzymatic treatment removed cells from bovine aortas without modifying the histo-architecture of the tissue. Indeed, both collagen and elastic fibers were well preserved, as observed by morphological analysis. Since collagen is responsible for vessel strength and elastin for distensibility and recoil (35), these findings support the validity of the decellularization procedure to obtain a suitable ECM scaffold useful in preparing functional small-diameter TLVGs. Consistent with morphological observations, ECM proteins, such as collagen types I and VI containing the RGD motif mediating cell adhesion via interaction with cell membrane integrins, were identified in DAMs by means of 2D gel electrophoresis and mass spectrometry.

Recently, layered vascular grafts have been prepared using decellularized small intestine submucosa (SIS) in combination with arterial elastin and cellularized collagen gels (4,36). A wide variety of constructs with a predefined layering and

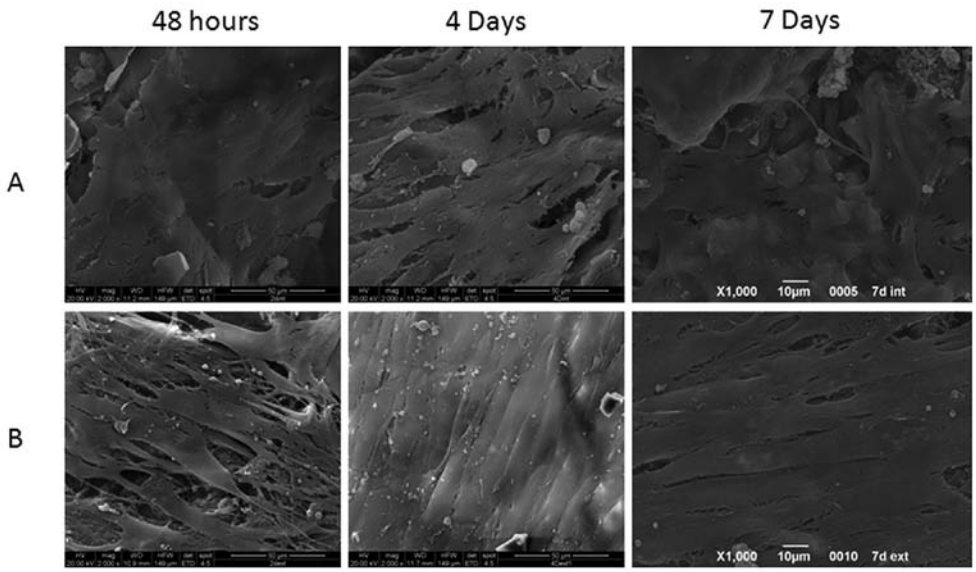

Figure 7. SEM micrographs of TLVGs after (A) HUVECs and (B) HAoSMCs seeding.

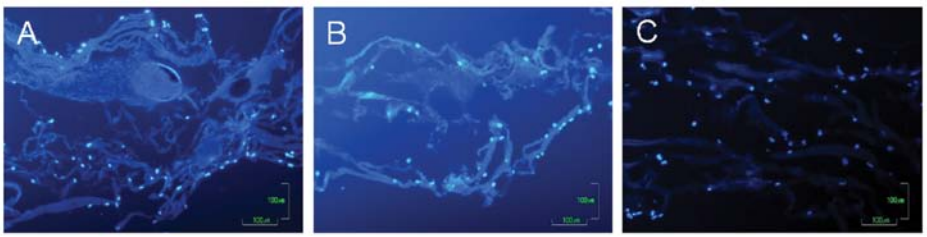

Figure 8. Cross-sections of TLVGs stained with DAPI after (A) 48 h, (B) 4 and (C) 7 days of HUVEC and HAoSMC co-culture. Magnification, x100.

whose size can be tailored on the dimension of blood vessels to be replaced was achieved using this sandwich technique. The layer deposition procedure allowed us to obtain small-diameter TLVGs of different sizes using DAM from bovine aorta containing ECM components, which are naturally present in blood vessels. Our findings show that these novel tailor-made vascular grafts present mechanical properties comparable to those of native vessels (31-33). Furthermore, the lyophilization process makes them easy to store. Levy-Mishali *et al* (37) demonstrated that, on a surface, stiffness is an important parameter since it elicited changes in focal adhesion structure, cytoskeleton assembly, locomotion and spreading of smooth muscle cells. Moreover, the increasing content of semicrystalline PLLA contributed to increasing the strength of the scaffolds (30,37). In contrast, when scaffolds did not provide sufficient resistance to cell contraction forces, they collapsed. Among the biocompatible polymers, the PLLA was a good compromise between stiffness and elasticity in cell organization and had optimal *in vivo* degradation time according to tissue regeneration needs. Moreover, the addition of PEG2000 could modulate the microporosity of the PLLA layer influencing the overall degradation time in biological medium and its stiffness. The longitudinal TS values of our TLVGs (0.47±0.02 MPa) were higher compared to those (0.24±0.10 MPa) of triple layered scaffolds prepared by Koens *et al* (9). Furthermore, the Young's modulus of our TLVGs (1.8 MPa) was comparable to that of porcine coronary artery (1 MPa) (31), rabbit aorta (1.25 MPa) (32) and human saphenous or femoral vein (1.50 MPa) (33). Scaffold strength provides the firmness to resist cell contraction forces, whereas elasticity enables better communication between cells, facilitating parallel organization (27,37,38) and ensuring the maintenance of a tubular shape in our constructs. These findings may be interesting for various tissue engineering applications when scaffold and cells interact and influence one another, while integrating into engineered tissue.

Today, ECM-based materials for vascular tissue engineering can be manufactured and obtained in several forms: multi-laminate sheets (39), particulate suspensions, powered or gel forms (40) assembled into three-dimensional matrices and stored in the hydrated state without being lyophilized (41). Although non-lyophilized or hydrated ECM-based scaffolds have been shown to have excellent biomechanical (strength and loading response), biochemical (degradability) and biocompatibility properties (42), lyophilized forms could present some advantages, such as longer shelf life and easier storage.

The presence of tissue-specific ECM proteins in DAM sheets allowed TLVGs to be colonized *in vitro* by both human endothelial and smooth muscle cells. Indeed, cells adhered well on both sides of TLVGs at 48 h, and a confluent monolayer was visible at 7 days. Furthermore, at 7 days, several cells were also detected in the middle part of TLVGs, confirming that the DAM component provides suitable stimuli for cell adhesion, migration, and growth.

Taken together, our findings indicate that TLVGs can be considered promising tools for blood vessel tissue engineering. By employing the freeze-drying technique, layered vascular scaffolds of different sizes were obtained, in which PLLA determines the mechanical strength whereas DAM sheets are responsible for vascular cell attachment and growth. Further *in vivo* studies will be needed to ascertain the effectiveness of TLVGs to replace blood vessels.

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