



Some properties of keratin biomaterials: Kerateines

Paulina Hill, Helen Brantley, Mark Van Dyke*

Wake Forest Institute for Regenerative Medicine, Wake Forest University School of Medicine, Medical Center Boulevard, Winston Salem, NC 27157, USA

ARTICLE INFO

Article history:

Received 16 July 2009

Accepted 21 September 2009

Available online 12 October 2009

Keywords:

Keratin
Biomaterial
Protein
Hydrogel
Scaffold
Hair

ABSTRACT

Keratins are a family of structural proteins that can be isolated from a variety of tissues. “Soft” keratins are cytoskeletal elements found in epithelial tissues while protective tissues such as nails, hooves, and hair are composed of “hard” keratins. Hard keratins have been the subject of biomaterials investigations for more than three decades. Numerous methods exist for denaturing these proteins which are characterized by a high sulfur content and extensive disulfide bonding, under either oxidative or reductive conditions, extracting them from tissue and processing them into various physical states such as gels, films, coatings, and fibers. Kerateines or keratases (oxidatively or reductively derived, respectively), alone or in combination with other biomaterials, have been tested in a small number of systems to demonstrate feasibility for medical applications such as wound healing, bone regeneration, hemostasis, and peripheral nerve repair. These investigations have shown generally good compatibility with cells and tissues, but the focus of prior investigations has been fairly narrow, and as a result there is relatively little published data on the general behavior of keratin biomaterials in biological systems beyond cell culture assays. The goal of this study was to produce a reduced form of keratin biomaterial, kerateine, using a typical and well-published technique, and characterize several aspects of its behavior that may have implications to its general use as a biomaterial. Kerateines were extracted from human hair, fabricated into gels and porous scaffolds, characterized, and placed into biological systems to determine their interactions with cells and tissue. Initially, the proteins were analyzed for molecular weight and amino acid content, as well as their ability to facilitate cell adhesion and proliferation. Crosslinked hydrogels were investigated for their hydrolytic stability *in vitro*; the micro-architecture and *in vivo* tissue response of lyophilized gels was also studied. These experiments both confirmed and expanded earlier findings that kerateines demonstrate excellent compatibility in biological systems.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Hair keratins are a family of structural proteins found abundantly in hair and fingernails, and are often referred to as “hard” keratins (as opposed to soft keratins that are found in epithelial tissues). Keratin proteins are self-assembled into fibers in the follicle by a highly proliferative process controlled by more than 30 growth factors and cytokines [1–7]. After extrusion through the skin, the hair fiber is formed into a highly stable structure that is relatively impervious to environmental insult by ambient oxygen catalyzed disulfide crosslinking. This natural fiber system is strikingly similar to synthetic polymers in that it contains structural macromolecules, crosslinkers, plasticizers, and UV stabilizers. Hair morphogenesis results in a highly ornate superstructure that must be taken into account when disassembled during the protein extraction process. Numerous methods for breaking down the

crosslinked structure of hair fibers chemically and extracting keratins have been developed and summarized elsewhere [8]. The keratin proteins extracted from hair can be classified into three broad groups: alpha-, beta-, and gamma-keratins. The alpha-keratins are structural, have an alpha-helical tertiary structure, are low in sulfur content, and have an average molar mass in the range of 60–80 kDa; they reside in the hair fiber cortex. The beta-keratins are primarily protective and form the majority of the cuticle. Beta keratins are difficult to extract and do not form especially useful reconstituted structures. Gamma-keratins are globular, high in sulfur content, and lower in molecular weight (approximately 15 kDa) than the other types of keratins. They function as disulfide crosslinkers, holding the cortical superstructure together. A schematic of a wool fiber, which is similar to a human hair, is shown in Fig. 1.

Keratins are removed from the cortex first by using chemicals to break the disulfide bonds that are prevalent in keratinized tissues. The alpha and gamma-keratins are converted to their non-crosslinked forms by oxidation or reduction, during which cystine is converted to either cysteic acid or cysteine, respectively, and the

* Corresponding author. Tel.: +1 336 713 7266.

E-mail address: mavandyk@wfubmc.edu (M. Van Dyke).

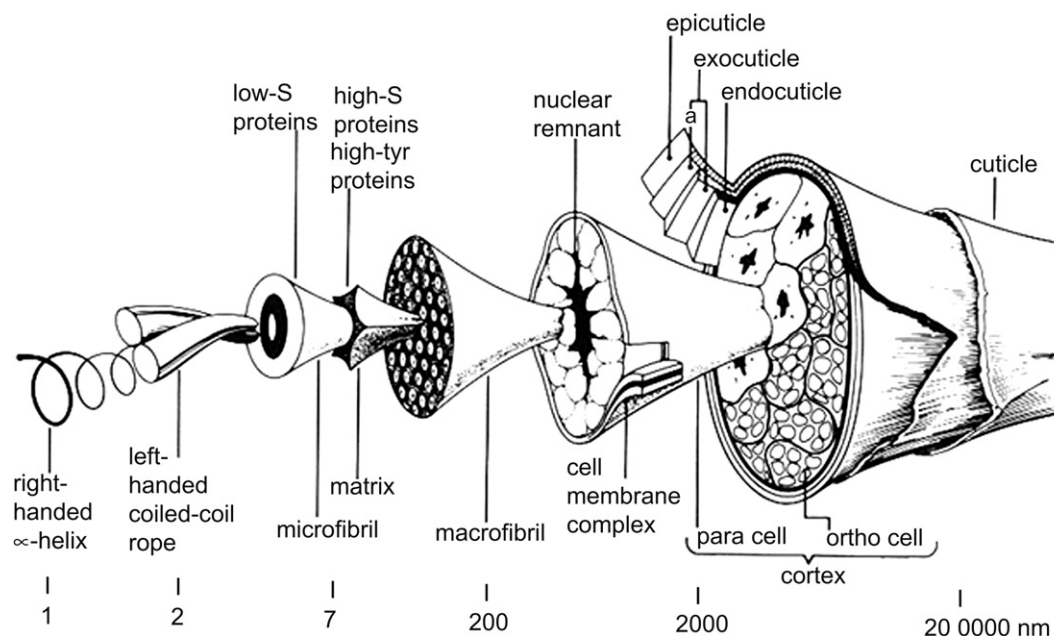


Fig. 1. Schematic of a wool fiber drawn by Bruce Fraser and Tom MacRae [Copyright CSIRO Australia 1996. Reproduced with permission from The Lennox Legacy (Rivett DE, Ward SW, Belkin LM, Ramshaw JAM and Wilshire JFK). Published by CSIRO PUBLISHING, Melbourne Australia]. Human hair has a similar superstructure but varies in the ratio of alpha- to gamma-keratin. Alpha-keratin is composed of the right-handed, alpha-helical proteins that are low sulfur and have a molecular weight of approximately 60 kDa. Gamma-keratin is globular in nature and forms the matrix that holds the microfibrils together and is high in sulfur with a molecular weight of approximately 15 kDa. Oxidative and reductive chemistries can be used to break this structure down and extract both types of keratins from hair fiber.

free proteins extracted with denaturing solvents to produce a solution that can be purified by filtration and dialysis. If the extraction is carried out using an oxidant, the cysteic acid derivatives are referred to as “keratoses”; if a reductant is used, the cysteine-containing proteins are called “kerateines”. While essentially homologous, the nature of sulfur-containing groups imparts some differences to keratoses and kerateines. Keratoses are hygroscopic, non-disulfide crosslinkable, water soluble, and susceptible to hydrolytic degradation at extremes of pH due to polarization of the backbone caused by the electron withdrawing properties of cysteic acid. These characteristics cause keratoses to degrade relatively quickly *in vivo* (i.e. on the order of days to weeks). Kerateines on the other hand, are less polar and therefore slightly less soluble in water. They are more stable at extremes of pH and can be re-crosslinked through oxidative coupling of cysteine groups. This results in biomaterials that can persist *in vivo* for weeks to months.

Methods for oxidation [9–11] and reduction [12–14] have been in the published literature for more than 100 years and have been summarized in several reviews, most notably by Crewther et al. in 1965 [8]. Investigations of the structure and properties of soluble keratins has continued, but beginning in the 1970’s, the processing of these protein solutions into derivative physical states such as gels and films began appearing in the literature [15–17]. Soon thereafter, the first keratin biomaterials paper appeared wherein a vascular graft coated with a keratin derivative was successfully implanted into a large animal model for 200 days [18]. Since then, keratins have been used in several *in vitro* and preclinical models for wound healing [19,20], fluid resuscitation [21], bone regeneration [22], hemostasis [23], and peripheral nerve repair [24,25]. Overall, the literature on keratin biomaterials is weighted toward patents, with medical applications taking precedence over fundamental studies of biological properties. Only a handful of studies of the basic biological properties of keratin biomaterials have ever been published. While the potential for biomedical use of keratins is demonstrably

high, it would be useful to better understand some of the basic biological characteristics of these unique materials.

The aim of this study was to produce a typical keratin biomaterial from human hair under reductive conditions (i.e. a kerateine) and investigate its general chemical, physical, and biological properties. We hypothesized that kerateine would demonstrate excellent cell and tissue compatibility based on its ubiquitous presence in mammalian systems and highly conserved primary amino acid structure. Utilizing a common extraction method, we created kerateine hydrogels and lyophilized scaffolds and determined the composition, structure, *in vitro* degradation, and cell and tissue interactions with this biomaterial.

2. Methods

2.1. Preparation of kerateine biomaterial

The kerateine used in this study was extracted from human hair based on a protocol first published in 1934 [12]. Briefly, hair from a local barber shop was washed in mild detergent and surface oil removed with organic solvent (hexanes, Fisher Scientific, Pittsburgh, PA). 100 g of clean, dry, de-oiled hair was reduced to break cystine bonds that would otherwise render the proteins insoluble by treatment with 2 L of 1 M thioglycolic acid (Sigma-Aldrich, St. Louis, MO) at pH 10.2 for 12 h. After chemical reduction, a crude fraction of kerateines was extracted using 4 L of 100 mM tris base solution (Sigma-Aldrich), followed by a second extraction with 4 L of deionized (DI) water. The extracts were combined, centrifuged at 6 k rpm, filtered (No. 4 Whatman, Fisher Scientific), and dialyzed against DI water using a 1 kDa nominal low molecular weight cutoff, tangential flow, spiral wound cartridge (Prep/Scale, Millipore, Billerica, MA) connected to a gear pump operating at ca. 1.5 L/min flow rate and 10 psi backpressure. During the dialysis, the pH of the kerateine solution was not allowed to drop below 7.0 by periodically adding small amounts of 0.1 M NaOH solution (Fisher Scientific) to avoid aggregation and precipitation of protein. The total volume of the system was maintained by adding DI water to compensate for loss of permeate. After 5 days, the sample was concentrated 15–20 fold (from approximately 0.3 wt% to 6 wt%) on the dialysis system and the ending concentration of protein determined by lyophilization of a 1.00 g aliquot. The final pH and concentration were adjusted to 7.4 and 5 wt%, respectively, by dropwise addition of a 0.1 M NaOH solution and ultrapure water, respectively. The total thiol content was measured using Ellman’s method [26]. Experiments detailed below were conducted using this solution as the starting material.

2.2. SDS-PAGE analysis

Electrophoretic separation of keratine was performed on a BioRad Mini-Protein II system (BioRad Laboratories, Hercules, CA). The 5 wt% keratine stock solution was diluted to 2.5 mg/mL with ultrapure water. 100 μ L of this solution was mixed with 20 μ L of 5X SDS loading buffer (BioRad) containing 0.6 M β -mercaptoethanol (BioRad). Keratine was denatured by boiling for 5 min in SDS/ β -mercaptoethanol solution and immediately placed on ice. 4 μ L of this cold, denatured solution was loaded onto precast 8–16% gradient Tris–HCl gels (Ready Gel, BioRad). Protein loading into the gel was 8 μ g per lane. Separation was performed at 60 V for 30 min, followed by 120 V for 100 min. After separation, gels were rinsed with ultrapure water for 5 min twice before staining with Bio-Safe Comassie stain (G250, BioRad) for 30 min. Destaining was done overnight in ultrapure water with gentle rotation. Samples were compared to a standard ladder (Benchmark Prestained Protein Ladder, Invitrogen, Carlsbad, CA) and the gel imaged in DIA mode with a Fuji Film LAS-300 imaging system (FujiFilm Life Science, Stamford, CT).

2.3. Amino acid analysis

Quantitative amino acid analyses were performed using the Pico-Tag method as described previously with slight modification [27]. Briefly, keratine samples were diluted from 5 wt% to 1 wt% using ultrapure water. Samples were hydrolyzed using 6 N HCl (Fisher Scientific) in 6 $\times$ 50 mm borosilicate glass tubes (Fisher Scientific) using a vapor-phase hydrolysis method. Samples were dried in the glass tubes which were then placed in a sealed glass vial with glass beads and 500 μ L 6 N HCl. The vials were evacuated and flushed with nitrogen gas, then incubated at 110 $^{\circ}$ C for 24 h. Hydrolyzed amino acids were treated with phenylisothiocyanate (Sigma–Aldrich) to produce phenylthiocarbonyl-amino acid (PTC-AA) derivatives. The PTC-AA were separated and quantified by reverse phase high performance liquid chromatography using a Waters HPLC system. The total amount of each identifiable amino acid was determined and expressed as the mole % $\pm$ standard deviation ($n = 4$).

2.4. Structural analysis

Keratine disks were formed by pipetting 100 μ L of the 5% keratine stock solution into a 96 well tissue culture treated polystyrene plate (Corning Life Sciences, Lowell, MA), freezing in a -80° C freezer, and lyophilizing (Model Freezezone 12, Labconco, Kansas City, MO). The resulting disks (approximately 6 mm $\times$ 4 mm) were retrieved from plate wells, allowed to stand in air for 24 h, and sterilized by γ -irradiation (850 krad) using a ^{60}Co source. Crosslinking of these disks prior to sterilization (as well as the hydrogels described in the next section) was affected by re-oxidizing the cysteine residues to cystine (i.e. reformation of disulfide bonds). This was accomplished simply by allowing the disks to stand in air for 6–10 h. Oxygen in the air was sufficient to catalyze the oxidative crosslinking of cysteine to cystine in these materials. The disks were mounted on stubs, coated with colloidal graphite and analyzed using a Hitachi S-2600 N variable pressure scanning electron microscope (SEM, Hitachi High Technologies Corp., Schaumburg, IL).

2.5. In vitro hydrolytic stability

A 10 wt% keratine solution was prepared by removing water from the 5 wt% keratine stock solution using a rotary evaporation system (Buchi; Fisher Scientific) at 10 mm Hg using a 50 $^{\circ}$ C water bath. 500 μ L of this 10 wt% keratine solution was pipetted into a 15 mL tube (Fisher Scientific), allowed to crosslink, sterilized by γ -irradiation (850 krad), and centrifuged to form a plug at the bottom of the tube. Crosslinking was again affected prior to sterilization simply by allowing the oxygen entrapped in the gel and in the headspace of the vial to catalyze the re-oxidation of cysteine to cystine. This process required 6–10 h. 10 mL of sterile PBS (Invitrogen) was carefully added on top of the gel and the sample placed at 37 $^{\circ}$ C. At each time point ($n = 6$), 1 mL of PBS was removed from the top of each tube and total protein quantified by a Lowry assay (BioRad) to determine the amount of keratine released into solution. The liquid removed from each tube was replaced with 1 mL of fresh PBS to maintain constant volume. Protein concentration was determined by measuring the absorbance at 750 nm (Biomate 3 UV–Vis spectrometer, Thermo Fisher Scientific, Waltham, MA) and converting it to total keratin based on calibration standards prepared from lyophilized keratine. Percent degradation was calculated as grams of keratine released into solution divided by total grams of keratine in the gel plug.

2.6. Cell viability

Cell viability in the presence of keratine was assessed using an MTS assay. MC3T3-E1 mouse pre-osteoblasts (ATCC, Manassas, VA) were seeded at a density of 4,200 cells/cm² in tissue culture treated 48 well polystyrene dishes (Corning Life Sciences) and allowed to grow for 24 h at 37 $^{\circ}$ C in 5% CO₂ (Heracell, Thermo Fisher Scientific). After 24 h, the growth media (low glucose DMEM, 1% penicillin/streptomycin, Invitrogen) was replaced with media (low glucose DMEM, 1% penicillin/streptomycin) containing keratine (1.0 $\times 10^{-6}$ mg/mL to 10 mg/mL) with FBS (10%) and allowed to grow for 48 h. The negative control consisted of cells with serum free

media. After 3 days, cell viability was determined with the Cell Titer 96 Aqueous Non-Radioactive MTS Cell Proliferation Assay (Promega, Madison, WI). Absorbance readings were taken at 490 nm using an ELX-500 UV plate reader (Bio-Tek, Winooski, VT) to determine viability of cells in reference to positive (serum containing) and negative (serum free) controls ($n = 10$).

2.7. Adhesion

Cell adhesion to keratine biomaterials was examined with phase contrast microscopy (Axiovert 200 M, Carl Zeiss Microimaging GmbH, Thornwood, NY) and quantified using the fluorescent dye Calcein AM. Tissue culture treated 48 well plates were coated by overnight incubation with either keratine (100 μ g/mL), fibronectin (5 μ g/mL; Sigma–Aldrich) or left uncoated. Excess solution was removed by aspiration and each well rinsed twice with PBS prior to cell seeding. NIH 3T3 mouse fibroblasts (ATCC) were seeded onto plates at a density of 30,000 cells per well and allowed to adhere for 5 h. Each well was gently rinsed with 500 μ L of PBS to remove non-adherent cells. Attached cells were labeled with 5 μ M Calcein AM (Molecular Probes, Invitrogen, CA) in PBS by incubation at 37 $^{\circ}$ C for 1 h. The relative fluorescence emission was determined using an FLX-500 fluorescent plate reader (Bio-Tek). Background Calcein AM levels in PBS only were measured and subtracted from all readings ($n = 6$).

2.8. Proliferation

Cell proliferation on keratine coatings was assessed using an MTS assay as described above. NIH 3T3 fibroblasts were plated onto keratine, fibronectin or uncoated 48 well plates at 4,000 cells per cm². Seeded cells were cultured for 48 h in serum-containing media (low glucose DMEM, 1% pen/strep). An MTS assay was performed to determine relative cell numbers on each substrate ($n = 6$). The absorbance at 490 nm was measured using an ELX-500 UV plate reader. Cell proliferation on the keratine substrates were compared to uncoated tissue culture treated plates (negative control) and fibronectin coated plates (positive control).

2.9. In vivo degradation and tissue response

All animal procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee. To assess the in vivo biocompatibility of keratine biomaterials, small disks were implanted subcutaneously into mice. Adult male CD1 Swiss-Webster mice (Charles River Laboratories, Wilmington, MA), approximately 30–35 g in weight, were placed under general anesthesia with isoflurane (Abbot Laboratories, North Chicago, IL). The operative site on the back was shaved and cleansed with betadine (Novation Inc., Irving, TX). A 2 cm lateral skin incision was made on the mid-portion of the back and tissue pockets were created by gross dissection laterally using blunt scissors. Sterilized keratine disks (6 mm $\times$ 2 mm as described previously) were implanted into the subcutaneous pocket of each mouse. Skin closures were performed with 5–0 absorbable Vicryl sutures (Ethicon Inc., Somerville, NJ). At 7 days, 1, 2, 3, and 6 months, animals were euthanized and an incision was made on the back of each mouse. Digital photographs of keratine disks were taken in situ and observed grossly for inflammation and capsule formation. The entire implant site, including the disk was excised. Digital photographs of representative explants were taken at each time point to determine the relative degree of in vivo degradation.

2.10. Histology

Tissue explants were fixed in 10% Neutral Buffered Formalin (NBF, Fisher Scientific) for 24 h, dehydrated in increasing concentrations of ethanol (Fisher Scientific) and embedded in paraffin (Fisher Scientific). Cross-sections (5 μ m thick) were cut on a microtome (Ultramicrotome RM2265, Leica Microsystems Inc., Bannockburn, IL), mounted on StarFrost[®] glass slides (Engelbrecht, Edermünde, Germany), and air dried. Sections were stained with hematoxylin & eosin (H&E, Fisher Scientific) and Masson's Trichrome (Fisher Scientific) to assess the presence and thickness of fibrous tissue, cellular response, collagen deposition and vascularization.

2.11. Statistical analysis

All in vitro assays were analyzed by a single factor Analysis of Variance (ANOVA) using SigmaStat 3.1 (Systat Software Inc., San Jose, CA) to determine statistically significant differences. Post-hoc testing was performed using the Student–Newman–Keuls method. Values of $p \leq 0.05$ were considered to be significant.

3. Results

3.1. SDS-PAGE analysis

Analysis of the molecular weight of keratine samples prior to crosslinking showed a characteristic banding pattern as described

by other investigators [28]. The gel image shows a large, broad band of keratines at the head of the lane that typically do not disassociate, even under strongly reducing, denaturing conditions. Characteristic bands for α -keratines at 64 and 43 kDa represent the distinctly different acidic and basic families. Low molecular weight gamma-keratines can be seen in lesser abundance at 10 and 16 kDa (Fig. 2).

3.2. Amino acid analysis

The amino acid makeup of keratine samples was consistent with earlier reports in the literature for wool and human hair keratins (Table 1), with the exception of arginine [29,30]. It is not known why arginine is low, but reported results vary significantly between fiber types and methods of keratin extraction. A total of 17 amino acids were identified in keratine extracts. Tryptophan is irreversibly destroyed in the hydrolysis step and cannot be quantified. Asparagine and glutamine side chains are hydrolyzed to form Aspartic and Glutamic Acid, respectively. As expected, serine, glutamic acid and cysteine were the most abundant amino acids present in keratine extracts. The cysteine content was consistent

Table 1

Amino acid analysis of keratine biomaterials derived from human hair. Values are expressed as the mole % of the total identifiable amino acids $\pm$ standard deviation ($n = 4$).

Amino acid	Mole % $\pm$ SD
Cysteic acid	9.97 $\pm$ 0.55
Aspartic acid	7.63 $\pm$ 0.18
Glutamic acid	11.56 $\pm$ 0.12
Serine	11.66 $\pm$ 0.26
Glycine	6.56 $\pm$ 0.05
Histidine	1.10 $\pm$ 0.03
Arginine	4.92 $\pm$ 0.05
Threonine	6.87 $\pm$ 0.32
Alanine	5.22 $\pm$ 0.09
Proline	9.55 $\pm$ 0.25
Tyrosine	1.20 $\pm$ 0.31
Valine	6.18 $\pm$ 0.21
Methionine	2.49 $\pm$ 0.10
Isoleucine	3.12 $\pm$ 0.07
Leucine	7.09 $\pm$ 0.06
Phenylalanine	1.91 $\pm$ 0.03
Lysine	2.96 $\pm$ 0.05

with free thiol measured after dialysis by Ellman's method, which is typically between 10 and 15 mole percent (data not shown).

3.3. Structural analysis

The micro-architecture of lyophilized keratine biomaterial disks was examined by SEM. Cross sectional images of a 5% keratine extract that had been freeze dried and crosslinked show a continuous porous microstructure (Fig. 3). Keratines were found to form a homogenous network of interconnected pores ranging in size from 30 to 70 μ m.

3.4. In vitro hydrolytic stability

A 10 wt% keratine gel was exposed to PBS at 37 °C and the hydrolytic stability determined over time. At each time point, the total amount of keratine that was released into solution was quantified and expressed as a percentage of the total protein content within the gel. Initially, the amount of protein released into solution was high. Approximately 25% of the keratine gel degraded within the first 7 days and over 50% degraded by 4 weeks. By 3 months, the amount of protein released began to stabilize at about 65%. At 6 months, the total keratine released into solution was 66.1% (Fig. 4).

3.5. Cell viability

To determine whether keratine added to media would affect cell viability, MC3T3-E1 pre-osteoblasts were exposed to keratine diluted in media at concentrations ranging from 1.0×10^{-6} mg/mL to 10 mg/mL. The MTS assay is an established method for quantifying relative cell numbers by measuring the mitochondrial activity in living cells. Keratine biomaterial extracts diluted in media did not adversely affect the viability of MC3T3 when exposed to cells for 48 h at 0.1 mg/mL or less (Fig. 5). At concentrations of 1.0×10^{-6} to 0.1 mg/mL, cell viability was statistically equivalent when compared to serum-containing media. Keratine was found to limit cell viability at concentrations above 1 mg/mL.

3.6. Adhesion

Fibroblast cell adhesion to a keratine substrate was evaluated in vitro. 3T3 fibroblasts were seeded onto keratine coated plates and compared to tissue culture treated plates (uncoated) and

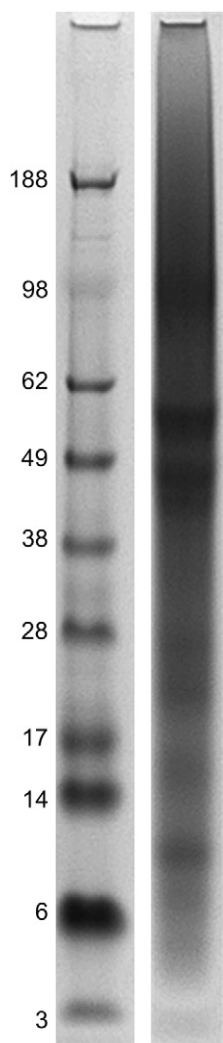


Fig. 2. SDS-PAGE of a dilute keratine solution (1 wt%). The keratine solution was run under reducing conditions and gave the characteristic bands noted in other studies. The complexity of human hair extracts is evident in the number and breadth of bands present.

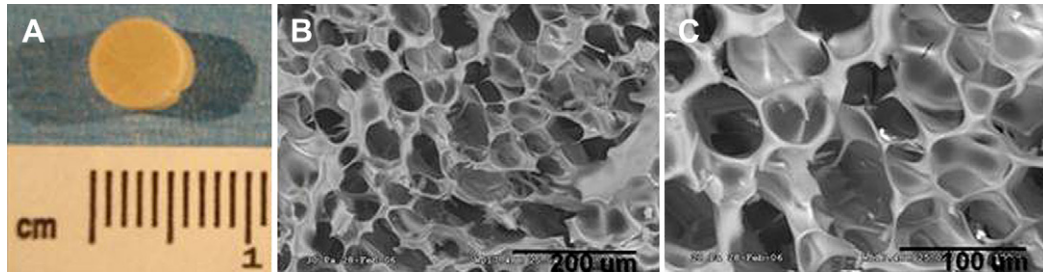


Fig. 3. Images of keratine disks prior to subcutaneous implantation. A digital photo shows a macroscopic view of a 6 mm × 4 mm disk created by lyophilization of a keratine solution (A). Scanning Electron Microscopy (SEM) of lyophilized keratine disks at (B) 100× and (C) 300×. This homogenous porous network was formed by spontaneous re-crosslinking of the proteins upon exposure to air.

fibronectin coated plates. Cell morphology was observed with phase contrast microscopy. Relative adhesion was quantified by measuring the fluorescence emission of Calcein AM stained cells that remained attached on each substrate. Within a few hours after plating, fibroblasts seeded onto keratine and fibronectin substrates were spread out and had a characteristic polygonal morphology. Fibroblasts seeded on uncoated plates had a more spherical, less elongated morphology. Fibroblast adhesion to keratine and fibronectin was significantly better than on an uncoated plate, $p = 0.044$ and $p < 0.001$, respectively (Fig. 6). Adhesion on fibronectin coatings was significantly better than on keratine coated dishes ($p = 0.02$).

3.7. Proliferation

Biocompatible biomaterials are not only non-cytotoxic and supportive of cell adhesion, but in some cases may promote cell proliferation. To assess the ability of a keratine substrate to promote cell proliferation, 3T3 fibroblasts were seeded onto keratine coated plates and cultured for 48 h in serum-containing media. Relative cell viability was determined using the MTS proliferation assay. Fibroblast viability on a keratine substrate was significantly higher than on an uncoated dish and not statistically different than fibronectin (Fig. 7).

3.8. In vivo tissue response

Lyophilized keratine disks approximately 6 mm × 2 mm (Fig. 3A) were implanted onto the backs of immunocompetent

mice. Disks were harvested at 7 days, 1, 2, 3 and 6 months after implantation. Examination of the biomaterial tissue interface showed no gross inflammation or fibrosis at early or late time points (Fig. 8). The in vivo degradation of keratine implants was assessed by measuring the size of disks at time of harvest. Explanted disks did not appear to decrease in size within the first 3 months. At 6 months, however, keratine explants were approximately 4 mm in diameter or approximately 60% of their original size. Histological analysis by H&E and Masson's trichrome confirmed the absence of fibrous capsule formation (Figs. 9 and 10). Cell accumulation on the periphery of the keratine implants was highest at 1–2 months and was a few cell layers thick (Fig. 9). The formation of supportive vasculature could be seen as early as 2 weeks post implantation (arrows; Fig. 9D). By 1 month, many small vessels were present throughout the inner portion of the scaffolds. At later time points, vascular in-growth decreased, particularly in the center of the implants. Host cell migration, consisting of fibroblasts, endothelial cells and inflammatory cells, into keratine scaffolds could be seen as early as 3 days following implantation (data not shown), with cells migrating to the center of the scaffold by day 7 (Fig. 9A). Cell infiltration was high during the first 4 weeks then gradually dropped off. Cell density throughout the scaffolds peaked at 1 month. By 6 months, only a few cells could be seen within the middle portion of the disks. Foreign body giant cells, a classic sign of a chronic inflammatory response, were absent at all time points indicating that keratine biomaterials are highly

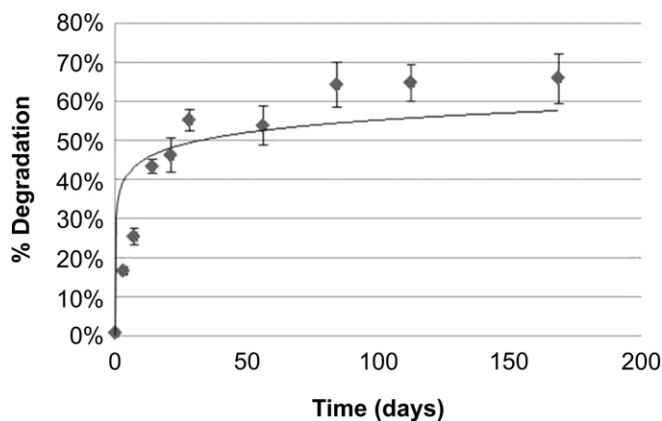


Fig. 4. In vitro hydrolytic stability of keratine biomaterials at 37 °C. A 10% keratine gel was placed in the bottom of a 15 mL falcon tube and exposed to PBS. The amount of keratine released into solution was quantified at each time point ($n = 6$). The degradation rate was rapid within the first month then gradually leveled off. Values are expressed as an average of the % degradation ± standard deviation and the trend line represents a logarithmic fit ($R^2 = 0.7365$).

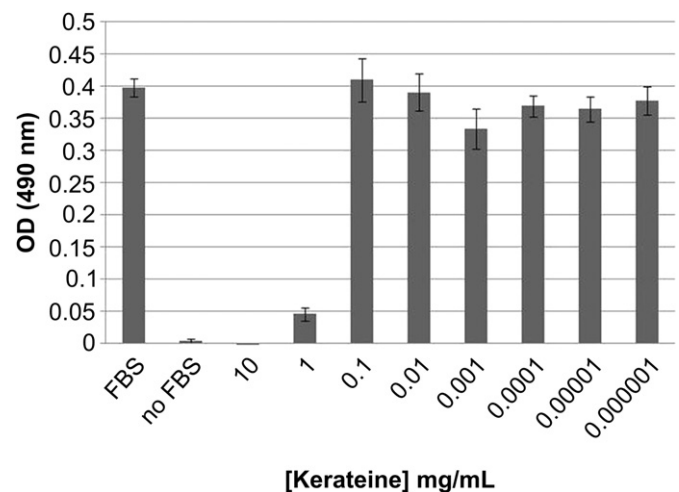


Fig. 5. Viability of MC3T3 pre-osteoblasts grown in keratine-containing media. Cells were exposed to keratine solutions in the presence of FBS for 48 h except for the negative control, which has no serum (no FBS). Relative cell numbers were determined using an MTS assay. Keratine does not hinder cell viability at concentrations ranging from 1.0×10^{-6} mg/mL to 0.1 mg/mL and only inhibits cell proliferation at concentrations of 1 mg/mL or higher. Error bars represent standard error of the mean ($n = 10$).

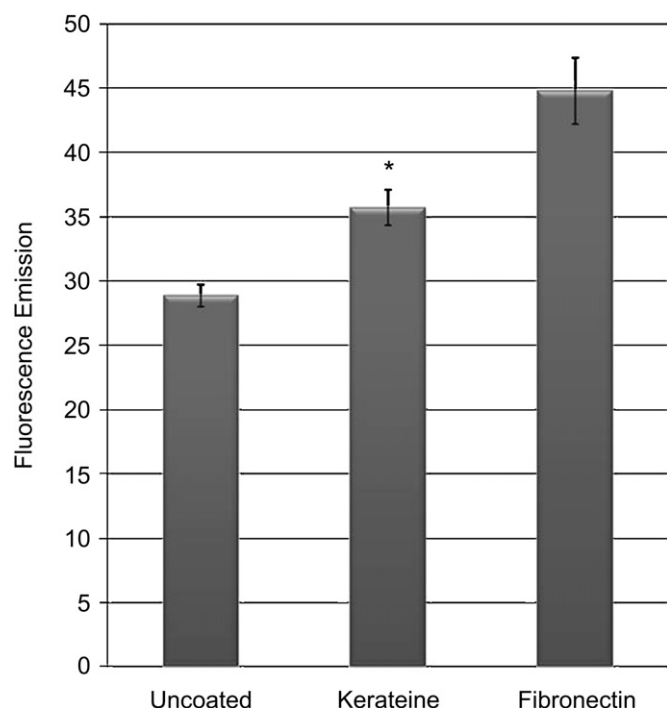


Fig. 6. A keratine biomaterial coating promotes NIH 3T3 fibroblast adhesion. Cells were seeded onto pre-coated dishes and allowed to adhere for 5 h. Unattached cells were washed off and adherent cells labeled with Calcein AM. The fluorescence emission was measured to determine the relative number of adherent cells on each coating. Keratine was found to significantly promote fibroblast cell adhesion over an uncoated dish (* $p < 0.05$, $n = 6$). Adhesion on fibronectin coatings was significantly better than both uncoated and keratine coated dishes.

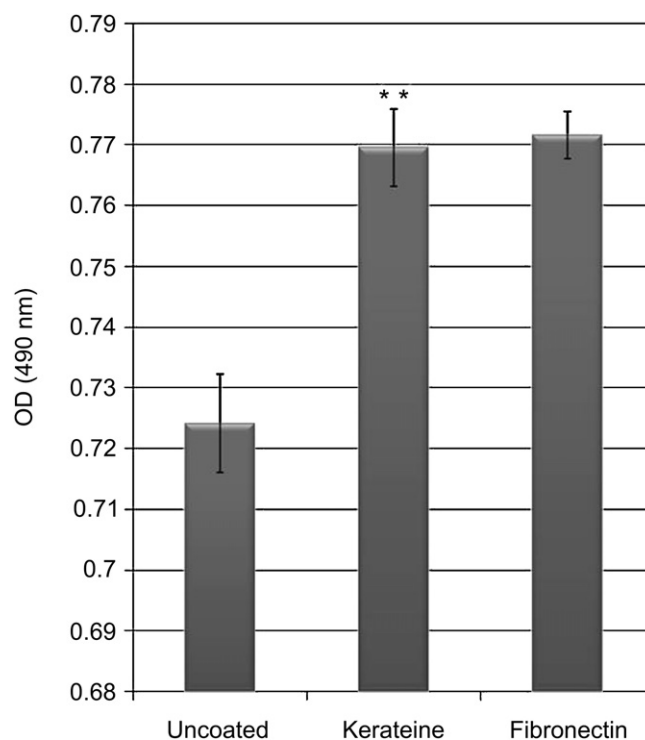


Fig. 7. A keratine substrate stimulates NIH 3T3 fibroblast proliferation. Cells were seeded onto pre-coated dishes and allowed to proliferate for 48 h. Relative cell numbers were determined using the MTS proliferation assay. 3T3 growth on a keratine substrate was significantly higher than on an uncoated dish (** $p < 0.05$, $n = 6$) and not statistically different than fibronectin.

tissue compatible. Masson's trichrome staining showed an increase in collagen deposition as cell infiltration increased. Collagen deposition was highest at 1 month and appeared to stabilize at later time points.

4. Discussion

The first study using a keratin biomaterial was published in 1982. Noishiki and colleagues coated a heparinized keratin derivative onto a polymer stent and implanted it into a dog for more than 200 days without thrombosis [18]. No studies had been published at that time describing a systematic analysis of biocompatibility; and much of the keratin literature was not focused on the basic science, but on potential medical applications. Since then, a few isolated studies have consistently shown good general cell compatibility, despite the many differences in keratin source, extraction, processing methods, fabrication of keratin derivatives, and assay protocols.

Using the method of O'Donnell and Thompson for the production of keratines [14], and the method of Thompson and O'Donnell for the production of keratoses [31]. Ito et al. generated samples from sheep wool and determined isoelectric point, amino acid composition, and biological responses to keratin-coated materials implanted into animal models, also in 1982 [32]. Polyester meshes were coated with keratin and implanted into the dorsal muscle of rabbits and dogs. Polyester filaments were also coated and implanted into the femoral and jugular veins of dogs. Results showed what the authors characterized as a "mild" foreign body response to the intra-muscular implantation with no foreign body giant cells at 2, 4, and 6 weeks in both models using either type of keratin coating. Implantation into the vasculature showed little to no thrombus formation at 2 h or 2 weeks, respectively, in the

keratose-treated group, while a keratine derivative showed mild thrombus at 2 h and none at 2 weeks.

In another study of the general biocompatibility of keratins, the physical, chemical, and biological properties of an *s*-aminoethylkeratine derivative made from extracted rat hair were investigated [33]. The authors processed the keratine into films and performed standard cell assays with fibroblasts and keratinocytes, as well as immunogenicity studies in rabbits. Cells (species not specified) were tested in direct and indirect contact modes and quantified using an MTT assay. Results showed no cytotoxicity at 5 mg/mL of keratine film and slight toxicity at 20 mg/mL in direct contact with fibroblasts after three days of incubation. In the indirect contact experiment, keratine films were washed in water for three weeks at 37 °C to remove processing chemicals, incubated for 48 h in sterile water at 37 °C at 50 mg/mL, and the water used to prepare media. After incubation with fibroblasts for 48 h, no evidence of cytotoxicity was observed. Keratinocytes were also seeded onto keratine films and showed delayed proliferation, but eventually caught up to the control (cells grown on tissue culture plastic) after a few days. In an experiment to assess immunogenicity, keratine films were implanted into rabbits. The films degraded after two weeks and elicited what the authors characterized as a "weak immunological reaction" (i.e. serum was positive at a 1/10 dilution but not at higher dilutions) which they considered normal for a rat protein implanted in a rabbit model.

In addition to these early investigations of keratin biomaterials, many scientists have assessed general cell compatibility by investigating the attachment and growth of cells on keratin biomaterials. Keratin coatings [34] and films [35,36], keratin-chitosan films [37], compression molded keratin films [38], keratin-hydroxyapatite sponges [22], modified keratin sponges containing growth factors [39], pure keratin scaffolds [40], and keratin-coated hydroxyapatite

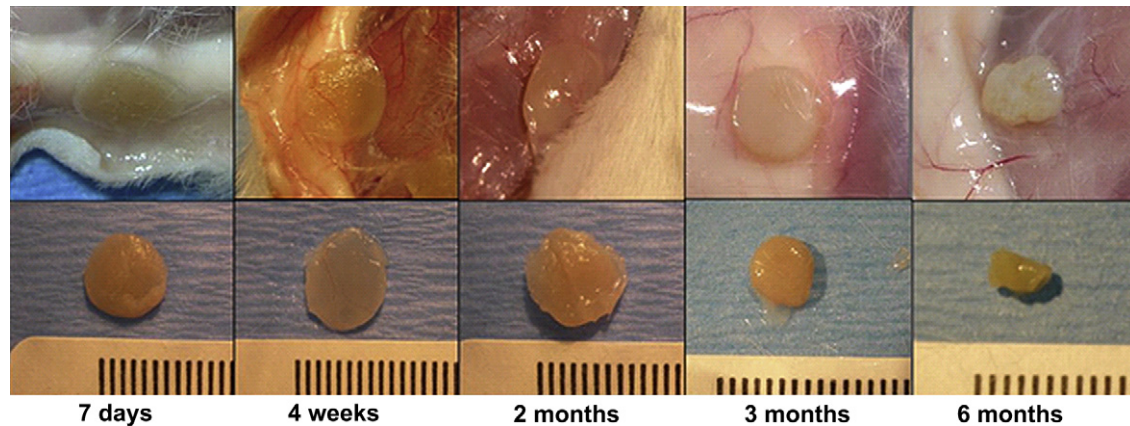


Fig. 8. Keratine disks harvested at 1 week to 6 months following subcutaneous implantation. Gross examination of the implant area did not show any observable inflammation or capsule formation. Implanted keratine disks did not visibly decrease in size until after 3 months. By 6 months the implants could still be easily identified upon harvest, but were smaller in size.

[41] have all been investigated. These materials were primarily derived through reductive chemistries, making keratine the predominant keratin biomaterial tested. Cell types in these studies have included rat mast cells, NIH 3T3 fibroblasts, L929 fibroblasts, MC3T3-E1 pre-osteoblasts, and human fetal osteoblasts. In general, all have shown good attachment and growth of cells on keratine substrates compared to standard controls such as tissue culture plastic or other biomaterials such as collagen. Presently there are no reports in the literature of cells that do not grow well on keratin substrates, although only a limited number have been tested.

In the present study we obtained soluble keratines from human hair that was collected from a local salon. Dialyzed protein samples showed characteristic bands in the SDS-PAGE for alpha- and gamma-keratins and good agreement with published values for amino acid composition with the exception of arginine [8]. General cell compatibility of these samples was demonstrated by marked cell adhesion and proliferation. This was likely due to the amino acid binding motifs expressed by keratins that are specific to the integrin receptors on many cell types. Tachibana et al. were the first to make this observation in a study that demonstrated excellent cell attachment to keratine scaffolds [42].

Structural analysis by SEM showed a highly porous architecture with pore sizes that are conducive to cell infiltration as demonstrated by implantation experiments. These interconnected,

microporous structures likely form spontaneously due to the intrinsic ability of keratins to self-assemble [43,44]. Subcutaneous implantation of these scaffolds showed little initial changes in dimension, but were approximately 60% of their original diameter by 6 months in vivo, which was in general agreement with our in vitro degradation curve. Subcutaneous implant studies published by other investigators demonstrated faster degradation rates [38], but these keratine biomaterials were not disulfide crosslinked as was the case in our experiments. Our crosslinking method results in keratines that are degraded slowly by hydrolysis rather than enzymatic activity. Robust host cell infiltration was observed within the entire thickness of the 6 mm × 4 mm implants at 1 and 4 weeks, thus confirming the interconnectivity observed in SEM analysis, with substantially reduced cell numbers at 2 to 6 months. Interestingly, the infiltrating cells did not mediate a foreign body response that is prevalent with most biomaterials, particularly synthetic polymers [45]. This process typically ensues after coating of the biomaterial with serum proteins. It may not occur with keratin biomaterials to any appreciable level due to their highly hydrated state. The keratine implants used in this study also did not become encapsulated, another typical response to biomaterial implants, or filled with cell-secreted collagen, even after 6 months.

The subdued immunological response to implanted keratin biomaterials is an interesting aspect of the present study and has

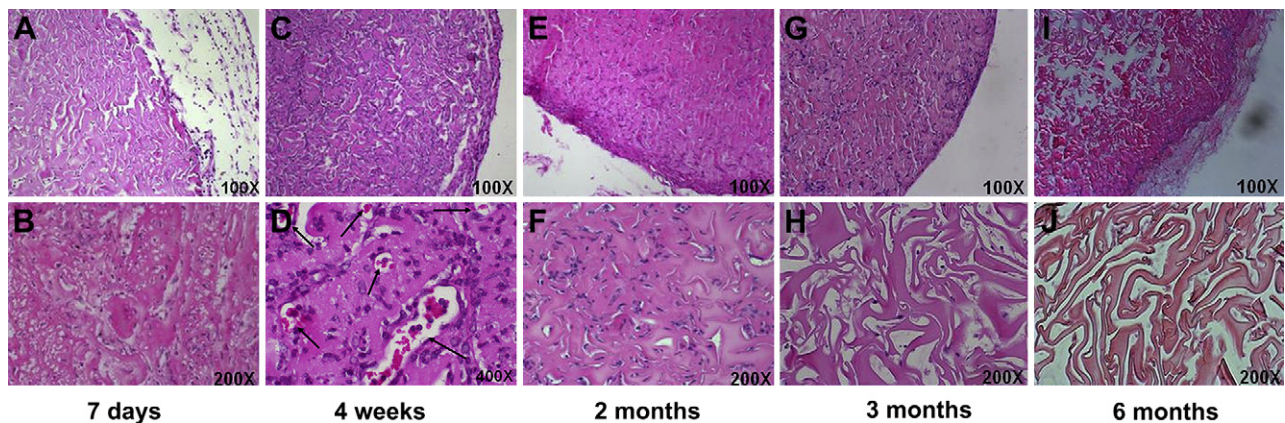


Fig. 9. Hematoxylin & Eosin (H&E) staining of keratine biomaterial implants. At 7 days, host inflammatory cells have begun to infiltrate the scaffold from the surrounding tissue. By 1 month, host cells have migrated to the middle of the scaffold and there is formation of supporting vasculature. By 2 months, there is a high density of cells homogeneously dispersed throughout the scaffold. At 3 months, the cell density within the scaffold has started to decrease and by 6 months the scaffold appears to be more degraded with relatively few cells scattered throughout.

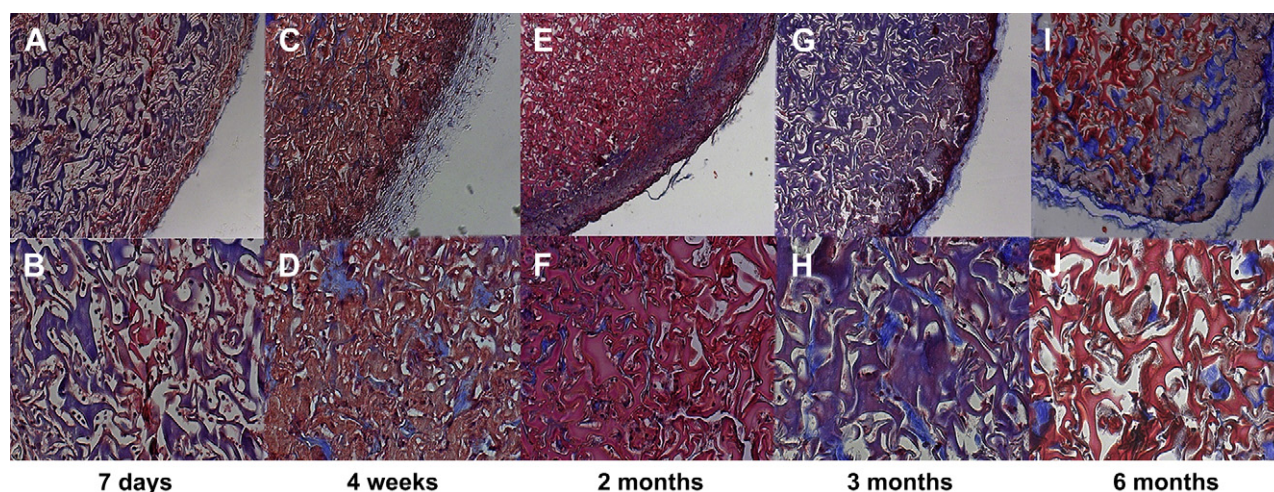


Fig. 10. Masson's Trichrome staining of keratene biomaterial implants for collagen deposition. No fibrotic capsule was present around the implants at any time point. Collagen deposition within the scaffold was present by 4 weeks but did not appear to increase further out to 6 months.

not been widely reported previously. There are only four published investigations of extracted hair keratin's potential immunogenicity. The first is the previously discussed Ito et al. paper in which the acute immune response to keratose and keratene materials coated onto polyester mesh and implanted into the muscle of rabbits and dogs was characterized as "mild" [32]. The second is the previously mentioned Valherie and Gagnieu investigation in which the immunological response to non-crosslinked human hair keratene films implanted subcutaneously in rabbits was characterized as "weak" [33]. The third is reported by Yamauchi et al. wherein the authors state that crosslinked keratene films implanted into the subcutaneous space of mice and retrieved after 18 weeks had degraded less than collagen IV and poly(lactic acid) [46]. In addition, the keratene films did not form adhesions or "abnormal" scar tissue. The fourth is a short communication by Ide and Fujii which reports that mouse hair, human hair, and human nail keratins (extraction method not specified) were assessed for immunogenicity in a mouse model [47]. Sera reactivities were reported to be low for the human proteins, although the authors did note differences between alpha- and gamma-keratin sub-types. The authors further suggested that human keratins "will not cause rejection or allergic reactions." While these results have not been independently confirmed, the basis for such findings could be related to the high sequence homology within the keratin family of proteins. Hesse et al. conducted a comparative analysis of the keratin gene clusters of human, mouse and rat, and reported "remarkable but not perfect synteny" among these clusters [48]. The alpha-keratin protein sequences are generally well conserved, even across different species, which may lead to a more immunologically tolerable biomaterial platform, especially for species with a highly evolved immune system such as humans.

5. Conclusions

Overall our study demonstrates some general properties of keratene and supports its use as a biomaterial. As the number of different cells, tissues, and medical applications that make use of keratin biomaterials widens, the intrinsic characteristics of this novel class of compounds may provide investigators with an alternative platform for many research investigations and the development of medical technologies. As the interactions of keratin biomaterials with specific cell and tissue types become more completely elucidated, enhanced performance may also be possible.

Acknowledgements

The authors gratefully acknowledge the funding support provided by the Errett Fisher Foundation and the US Army. The authors would also like to thank Jacquie Burnell for assistance with histological analysis, Sang Jin Lee for SEM support, and Jillian Rouse for conducting the thiol assays.

Appendix

Figures with essential color discrimination. Figs. 3, 8–10 of this article are difficult to interpret in black and white. The full color images can be found in the on-line version, at [doi:10.1016/j.biomaterials.2009.09.076](https://doi.org/10.1016/j.biomaterials.2009.09.076).

References

- [1] Jones CM, Lyons KM, Hogan BLM. Involvement of bone morphogenetic protein-4 (BMP-4) and Vgr-1 in morphogenesis and neurogenesis in the mouse. *Development* 1991;111(2):531–42.
- [2] Lyons KM, Pelton RW, Hogan BLM. Organogenesis and pattern formation in the mouse: RNA distribution patterns suggest a role for bone morphogenetic protein-2A (BMP-2A). *Development* 1990;109(4):833–44.
- [3] Blessings M, Nanney LB, King LE, Jones CM, Hogan BLM. Transgenic mice as a model to study the role of TGF- β related molecules in hair follicles. *Genes Dev* 1993;7(2):204–15.
- [4] Hardy MH. The secret life of the hair follicle. *Trends Genet* 1992;8(2):55–61.
- [5] Stenn KS, Prouty M, Seiberg. Molecules of the cycling hair follicle – a tabulated review. *J Dermatol Sci* 1994;75:S109–24.
- [6] Rogers GE. Hair follicle differentiation and regulation. *Int J Dev Biol* 2004;48(2–3):163–70.
- [7] Panteleyev AA, Jahoda CAB, Christiano AM. Hair follicle predetermination. *J Cell Sci* 2001;114(19):3419–31.
- [8] Crewther WG, Fraser RDB, Lennox FG, Lindley H. The chemistry of keratins. In: Anfinsen Jr CB, Anson ML, Edsall JT, Richards FM, editors. *Advances in protein chemistry*. New York: Academic Press; 1965. p. 191–346.
- [9] Breinl F, Baudisch O. The oxidative breaking up of keratin through treatment with hydrogen peroxide. *Z Physiol Chem* 1907;52:158–69.
- [10] Earland C, Knight CS. Structure of keratin II: amino acid content of fractions isolated from oxidized wool. *Biochim Biophys Acta* 1956;22:405–11.
- [11] Buchanan JH. A cystine-rich protein fraction from oxidized alpha-keratin. *Biochem J* 1977;167(2):489–91.
- [12] Goddard DR, Michaelis L. A study on keratin. *J Biol Chem* 1934;106:605–14.
- [13] MacLaren JA. The extent of reduction of wool proteins by thiols. *Aust J Chem* 1962;15:824–31.
- [14] O'Donnell JJ, Thompson EOP. Studies on reduced wool IV: the isolation of a major component. *Aust J Biol Sci* 1964;17:973–89.
- [15] Anker CA. Method of preparing keratin-containing films and coatings. US pat no 3,642,498; February 15, 1972.
- [16] Kawano Y, Okamoto S. Film and gel of keratins. *Kagaku To Seibutsu* 1975;13(5):291–2.

- [17] Okamoto S. Formation of films from some proteins. *Nippon Shokuhin Kogyo Gakkaishi* 1977;24(1):40–50.
- [18] Noishiki Y, Ito H, Miyamoto T, Inagaki H. Application of denatured wool keratin derivatives to an antithrombogenic biomaterial: vascular graft coated with a heparinized keratin derivative. *Kobunshi Ronbunshu* 1982;39(4):221–7.
- [19] Rothman J, Band P, Oceta J. Wound healing promoting compositions containing film-forming proteins. PCT pat no 9,102,538; March 7, 1991.
- [20] Van Dyke ME, Nanney LB. Elastomeric biomaterials from human hair keratins as bioactive wound dressings. *Abst Papers Amer Chem Soc* 2002;224(1):U36.
- [21] Widra A. Alpha-keratose as a blood plasma expander and use thereof. US pat no 6,746,836; June 8, 2004.
- [22] Tachibana A, Kaneko S, Tanabe T, Yamauchi K. Rapid fabrication of keratin-hydroxyapatite hybrid sponges toward osteoblast cultivation and differentiation. *Biomaterials* 2005;26(3):297–302.
- [23] Aboushwareb T, Eberli D, Ward C, Broda C, Holcomb J, Atala A, et al. A keratin biomaterial gel hemostat derived from human hair: Evaluation in a rabbit model of lethal liver injury. *J Biomed Mat Res* 2009;90(1):45–54.
- [24] Sierpinski P, Garrett J, Ma J, Apel P, Klorig D, Smith T, et al. The use of keratin biomaterials derived from human hair for the promotion of rapid regeneration of peripheral nerves. *Biomaterials* 2008;29(1):118–28.
- [25] Apel PJ, Garrett JP, Sierpinski P, Ma J, Atala A, Smith TL, et al. Peripheral nerve regeneration using a keratin-based scaffold: long-term functional and histological outcomes in a mouse model. *J Hand Surg* 2008;33(9):1541–7.
- [26] Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959;82(1):70–7.
- [27] Bennett HP, Solomon S. Use of Pico-Tag methodology in the chemical analysis of peptides with carboxyl-terminal amides. *J Chromatogr* 1986;359:221–30.
- [28] Mies HH, Zahn H. Chromatographic and electrophoretic investigations of the properties of unprotected low-sulphur wool keratins. *J Chromatogr* 1987;405:365–70.
- [29] Aluigi A, Zoccola M, Vineis C, Tonin C, Ferrero F, Canetti M. Study on the structure and properties of wool keratin regenerated from formic acid. *Int J Biol Macromol* 2007;41(3):266–73.
- [30] Simmonds DH. The amino acid composition of keratins: part V: a comparison of the chemical composition of merino wools of differing crimp with that of other animal fibers. *Textile Res J* 1958;28:314.
- [31] Thompson EOP, O'Donnel IJ. Studies on oxidized wool I: a comparison of the completeness of oxidation with peracetic and performic acids. *Aust J Biol Sci* 1959;12(3):282–93.
- [32] Ito H, Miyamoto T, Inagaki H, Noishiki Y. Biocompatibility of denatured wool keratin. *Kobunshi Ronbunshu* 1982;39(4):249–56.
- [33] Valherie I, Gagnieu C. Chemical modifications of keratins: preparation of biomaterials and study of their physical, physicochemical and biological properties. Doctoral thesis. Inst Natl Sci Appl Lyon, France; 1992.
- [34] Yamauchi K, Maniwa M, Mori T. Cultivation of fibroblast cells on keratin-coated substrata. *J Biomater Sci Polym Ed* 1998;9:259–70.
- [35] Tanabe T, Okitsu N, Yamauchi K. Fabrication and characterization of chemically crosslinked keratin films. *Mat Sci Eng* 2004;C24(3):441–6.
- [36] Fujii T, Murai S, Ohkawa K, Hirai T. Effects of human hair and nail proteins and their films on rat mast cells. *J Mater Sci Mater Med* 2008;19(6):2335–42.
- [37] Tanabe T, Okitsu N, Tachibana A, Yamauchi K. Preparation and characterization of keratin–chitosan composite film. *Biomaterials* 2002;23(3):817–25.
- [38] Katoh K, Shibayama M, Tanabe T, Yamauchi K. Preparation and physicochemical properties of compression-molded keratin films. *Biomaterials* 2004;25(12):2265–72.
- [39] Tachibana A, Nishikawa Y, Nishino M, Kaneko S, Tanabe T, Yamauchi K. Modified keratin sponge: binding of bone morphogenetic protein-2 and osteoblast differentiation. *J Biosci Bioeng* 2006;102(5):425–9.
- [40] Verma V, Verma P, Ray P, Ray AR. Preparation of scaffolds from human hair proteins for tissue-engineering applications. *Biomed Mater* 2008;3(2):25007. Epub April 15, 2008.
- [41] Belcarz A, Ginalska G, Zalewska J, Rzenski W, Slosarczyk A, Kowalczyk D, et al. Covalent coating of hydroxyapatite by keratin stabilizes gentamicin release. *J Biomed Mater Res B Appl Biomater* 2009;89(1):102–13.
- [42] Tachibana A, Furuta Y, Takeshima H, Tanabe T, Yamauchi K. Fabrication of wool keratin sponge scaffolds for long-term cell cultivation. *J Biotechnol* 2002;93(2):165–70.
- [43] Thomas H, Conrads A, Phan KH, van de Löcht M, Zahn H. In vitro reconstitution of wool intermediate filaments. *Int J Biol Macromol* 1986;8:258–64.
- [44] van de Löcht M. Reconstitution of microfibrils from wool and filaments from epidermis proteins. *Melliand Textilberichte* 1987;10:780–6.
- [45] Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. *Semin Immunol* 2008;20(2):86–100.
- [46] Yamauchi K, Yamauchi A, Kusunoki T, Kohda A, Konishi Y. Preparation of stable aqueous solution of keratins, and physicochemical and biodegradational properties of films. *J Biomed Mater Res A* 1996;31(4):439–44.
- [47] Ide Y, Fujii T. Immunogenicity of hair proteins containing hard keratins. *J Soc Fiber Sci Tech Japan* 2004;60(9):844.
- [48] Hesse M, Zimek A, Weber K, Magin TM. Comprehensive analysis of keratin gene clusters in humans and rodents. *Eur J Cell Biol* 2004;83(1):19–26.