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Crosslinked α-elastin biomaterials: towards a processable elastin mimetic scaffold

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Abstract

Elastin is a critical biochemical and biomechanical component of vascular tissue. However, elastin is also highly insoluble and therefore difficult to process into new biomaterials. We present a simple approach for synthesizing elastin-based materials from two commercially available and water-soluble components: α-elastin and a diepoxy crosslinker. Reaction pH was shown to modulate the degree of crosslinking, as demonstrated by materials characterized with a range of swelling ratios (~10–25), enzymatic degradation rates (~8–50% per h in 0.1 u/ml elastase), and elastic moduli (~4–120 kPa). Crosslinking with a combination alkaline and neutral pH process results in materials with the highest degree of crosslinks, as indicated by a swelling ratio of 10, slow degradation rate, and high elastic moduli (~120 kPa). Furthermore, the crosslinked α-elastin materials support vascular smooth muscle cell (VSMC) adhesion and a decreased proliferation rate compared to polystyrene controls. The functional outcomes of the crosslinking reaction, including the dependence of structure–function properties on reaction pH, are discussed. Our approach towards 'processable' elastin-based materials is versatile and could be integrated into existing tissue engineering methodologies to enhance biomaterial performance by providing a natural elastomeric and biofunctional component.

Keywords: Elastin; Biomaterial; Vascular tissue engineering; Degradable; Crosslinking

1. Introduction

One of the greatest challenges in engineering functional small diameter vascular grafts is to mimic key arterial mechanical properties, including strength and compliance. Mismatches in compliance can lead to flow disturbances, and ultimately, graft occlusion [1]. Native arteries derive their unique mechanical properties from a microstructured composite of elastin and collagen (types I and III) in the medial layer [2,3]. It is this intricate structural arrangement that motivates the use of naturally-derived vascular replacements such as autografts and acellular nonautologous grafts [4]. Donor tissue supply shortages and animal-borne pathogens, however, preclude the use of grafted tissues in all cases. This cumulative evidence shows a significant need for new biomaterials that more closely mimic the mechanical properties of native vascular tissue.

Efforts to create vascular replacements using native extracellular matrix components of vessels have largely focused on type I collagen. Collagen is relatively easy to process and can be synthesized by stimulating fibroblasts or vascular smooth muscle cells (VSMC). Indeed, mechanically-conditioned collagen- and cell-based vascular grafts are strong enough to withstand supraphysiological pressures (>2000 mm Hg) [5,6]. However, these grafts have low elastin content and, because elastin is a key contributor to vessel elasticity [3], these

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collagen-based grafts are excessively stiff [1,7]. Efforts to increase graft compliance by increasing elastin production have found only modest success [1,8,9] because (a) elastogenesis is down-regulated in adult cells [10], and (b) elastin is highly insoluble and thus essentially unprocessable [11].

Given elastin's fundamental importance in governing the mechanical properties of native vessels, the development of 'processable' elastin-based biomaterials could advance the design of biofunctional blood vessel replacements. Such elastin-based biomaterials may not need to initially resemble the precise structural organization of native elastin, as the presence of an intact elastin scaffold elastin has been shown to influence the extent of new elastin incorporation [12]. Furthermore, these scaffolds could serve as templates for cellular remodeling and reorganization.

Recombinant protein technologies have allowed the synthesis of well-defined elastin-derived polypeptides, which have driven insightful structure–function studies of tropoelastin [13,14] (the soluble precursor of crosslinked elastin) as well as several discrete elastin domains [15–17]. Several groups have also synthesized adaptations of the tropoelastin amino acid sequence to allow the manipulation of cell adhesion motifs [18,19], thermoresponsive properties [20,21], elastic domains [21], and selective crosslinking sites [21–23]. However, the immunogenicity of these 'synthetic' materials has been relatively unexplored [14,24], and the broad application of these materials is limited by the inherent challenges of synthesizing recombinant proteins (e.g. residual endotoxin, capital cost and expertise, scale-up).

Here, our design goal was to develop a simple synthesis technique for creating elastin-based biomaterials. We chose a versatile system of two commercially available and water-soluble components: α-elastin, a well-characterized elastin digest [25], and ethylene glycol diglycidyl ether (EGDE), a diepoxy crosslinker (Fig. 1a) [26]. In the production of α-elastin, bovine ligament elastin is treated with a mild acid hydrolysis to yield a high molecular weight digest that retains the amino acid composition of native elastin [25]. Despite structural heterogeneities resulting from hydrolysis, α-elastin retains several key physicochemical properties of nascent elastin, including its abilities to coacervate into ordered fibrils [27,28], regulate the VSMC rates of migration [29] and proliferation [29,30], and promote the contractile phenotype of primary VSMC [31]. Despite these advantages, α-elastin-based biomaterials is a relatively unexplored area. The development of processable α-elastin would provide a platform to test the effects of elastin-containing scaffolds on cellular and tissue response, as well as broader issues (e.g. immunogenicity) that are not yet well understood.

To crosslink the α-elastin fragments, we chose to adapt established epoxy-based approaches [30,32,33].

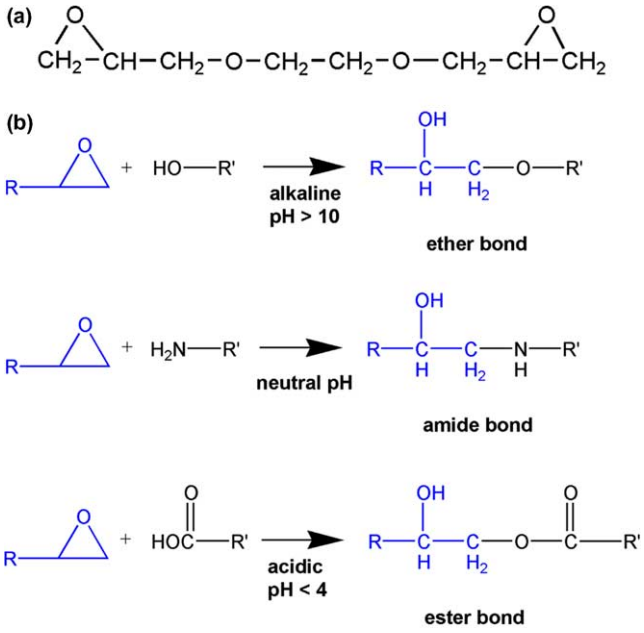

Fig. 1. Schematic of epoxy reaction dependence on pH. (a) Structure of ethylene glycol diglycidyl ether (EGDE). (b) The reactivity of epoxy functional groups depends on the reaction pH. Under alkaline conditions (pH > 10), epoxy groups predominately react with hydroxyl groups to yield stable ether bonds. Under neutral conditions (pH ~ 7), epoxy groups react with amines to form stable amide bonds. Lastly, under acidic conditions (pH < 4), epoxy groups predominately react with carboxylic acids to generate hydrolytically degradable ester bonds [26].

Zhao et al. [33] developed a clever double-step diepoxyoctane crosslinking procedure for synthesizing hyaluronan hydrogels. The first step was carried out under alkaline conditions to form ether linkages between hyaluronan's hydroxyl groups and then the second step was carried out under acidic conditions to form ester linkages between hyaluronan's carboxyl groups. In work with elastin, Ito et al. [30,32] developed a related process to create insoluble thin films of coacervated α-elastin using a polyglycidyl ether (i.e. polyepoxy) crosslinker. However, Ito et al. focused exclusively on characterizing cellular behavior on the crosslinked elastin films and did not adequately explore the chemistry, processing or material properties of this unique system.

Therefore, we have developed a pH-dependent crosslinking scheme to yield insoluble α-elastin biomaterials. We hypothesized that reaction pH could be used to modulate the physical properties of the materials that are controlled by the degree of crosslinking. Specifically, we predicted that the crosslinking could be controlled by forming three types of crosslinks (i.e. ether, amide, ester) between the elastin fragments in a pH-dependent manner (Fig. 1b). We expected that under alkaline pH, EGDE may predominately react with the hydroxyl groups of α-elastin's serine, threonine and tyrosine groups to yield stable ether bonds. At neutral pH, we

expected EGDE to react with the amines found on lysine as well as the fragment N-termini to form stable amide bonds. Finally, we expected that under acidic conditions, EGDE may react predominately with the carboxylic acids of aspartic acid, glutamic acid and also the fragment C-termini to form hydrolytically degradable ester bonds [26].

We characterized several physical properties that are well known to be affected by crosslink density, including swelling, in vitro enzymatic degradation in elastase, and elastic modulus. Furthermore, studies of VSMC attachment and proliferation were used to confirm the predicted VSMC response to α-elastin substrates. These results suggest that crosslinked α-elastin could provide a platform for complex composite materials with both mechanical and bioactive properties that closely mimic native vascular tissue.

2. Materials and methods

Standard reagents were obtained from Sigma-Alrich (St. Louis, MO) unless otherwise noted.

2.1. Crosslinking of α-elastin

To crosslink the α-elastin fragments, we chose the diepoxy crosslinker ethylene glycol diglycidyl ether (EGDE, Fluka, St. Louis, MO, Fig. 1a) because of its hydrophilicity and short length (~1 nm). We explored several crosslinking approaches (Fig. 2): single-step treatments of three different pH ranges (acidic, neutral, and alkaline) as well as double-step treatments that are essentially combinations of single-step treatments in two different pH ranges. Two bulk shapes of crosslinked α-elastin materials were developed. For the majority of the characterization studies, we formed the elastin materials into discs (~1 mm thick and ~4 mm in diameter) that were easily handled and transported. For the cell studies, flat thin films (<0.5 mm thick) were formed on the bottom of tissue culture 12-well plates.

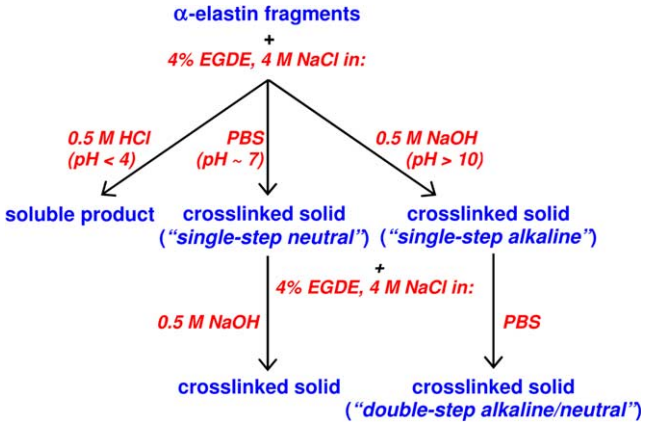

Fig. 2. Schematic of the α-elastin crosslinking approach. The elastin fragments were treated with single-step crosslinking procedures of acidic, neutral or alkaline pH. The acidic single-step procedure yielded a water-soluble product, and was therefore inappropriate for further study. The single-step neutral and alkaline procedures, which each yielded insoluble crosslinked materials, were also investigated in combination with each other. Further characterization was then carried out on three of these materials: 'single-step neutral', 'single-step alkaline' and 'double-step alkaline/neutral'.

To synthesize the disks of crosslinked elastin, 0.1 ml of a solution of 200 mg/ml α-elastin (derived from bovine neck ligament elastin, AE17, Elastin Products, St. Louis, MO) in water was added per well of a 96-well tissue culture plate. Next, ~0.3 ml of phosphate-buffered saline (PBS; pH 7.4) with 4 M NaCl was added per well. The plate was incubated for 2 h at 37 °C to allow complete coacervation. In this case, the small diameter wells of the 96-well plate allow the insoluble coacervate to agglomerate into a single protein mass. The clear supernatant was removed and the protein mass was carefully shaped into a disk with a small Teflon-coated spatula. The elastin masses were rinsed with 4 M NaCl in 0.5 M HCl, PBS or 0.5 M NaOH ('acidic', 'neutral' and 'alkaline' treatments, respectively). Next, we added 0.3 ml/well of crosslinker solution: 4% EGDE and 4 M NaCl in 0.5 M HCl, PBS or 0.5 M NaOH (~0.6 μl EGDE per mg α-elastin). The plate was returned to the 37 °C incubator for 2 h. For the materials crosslinked with a double-step process, the rinsing and crosslinking steps were repeated with a solution of the second pH. Unreacted epoxy groups were quenched with 2 M glycine in PBS overnight at room temperature and finally the crosslinked disks were washed at least once with PBS.

To synthesize thin films of crosslinked elastin, 0.5 ml of a solution of 10 mg/ml α-elastin in water was added to each well of a 12-well tissue culture plate. Next, 1.5 ml of PBS with 4 M NaCl was added per well. The well plate was incubated at 37 °C for 2 h to allow coacervation of the elastin into a uniform film that completely covered the well bottom. The crosslinking and quenching treatments were then carried out as described above (except 1 ml/well of crosslinking solution was used for the 12-well plates; ~8 μl EGDE per mg α-elastin).

2.2. Amino acid analysis

Discs of crosslinked α-elastin (10 mg each) were prepared as above, except that the glycine quenching step was omitted. The crosslinked samples were thoroughly washed in distilled water and then lyophilized. The α-elastin samples (unmodified or crosslinked materials) were subjected to acid hydrolysis and amino acid analysis (Beckman Model 6300 with System Gold software) [34,35]. The amount of elastin was calculated as the sum of the amino acids (in nmoles) multiplied by the average amino acid mass of 85 ng/nmol. Standards for

the elastin crosslinks, desmosine and isodesmosine, were prepared as previously described [36].

2.3. Determination of swelling ratio

To analyze relative degrees of crosslinking, we determined the swelling ratios of the α-elastin materials. The swelling ratio based on mass was calculated by dividing the material's mass after swelling by the material's dry mass. Following crosslinking, the elastin discs (20 mg elastin; 4 mm diameter, 1 mm thick) were swollen overnight in water. The materials were then removed from the water, the excess liquid was blotted from the material's surface and the initial swollen mass was recorded. Next, the materials were dried for at least 24 h at 70 °C with desiccant and then the final dry mass of each material was recorded.

2.4. In vitro degradation by elastase

In vitro enzymatic degradation of the crosslinked α-elastin was determined by monitoring the material's mass loss during incubation in elastase. The crosslinked elastin samples (20 mg elastin; 4 mm diameter, 1 mm thick disks) were equilibrated overnight in PBS with 0.2 mg/ml sodium azide as a preservative. The materials were removed from the buffer, excess liquid was blotted from the surface and the initial mass was recorded. The elastin materials were then transferred to 2-ml microcentrifuge tubes and 1.75 ml of 0.01, 0.1 or 1.0 u/ml porcine pancreatic elastase (Sigma-Aldrich) in PBS with 0.2 mg/ml sodium azide was added to each tube and incubated for various time-points (up to 8 h) at 37 °C with mild mixing on a platform shaker. Every 1–2 h, the solution was removed, the elastin discs were blotted, and the masses were carefully determined. The elastase solution was replaced every 2 h.

2.5. Determination of elastic modulus

To determine the elastic modulus on the relatively small crosslinked α-elastin materials, we used a microindentation method, which was developed in our laboratory and described in detail by Jacot et al. [37]. Briefly, a glass fiber with a ~170 μm diameter spherical tip was used to slowly indent the sample's surface. By knowing the spring constant of the glass fiber, the geometry of the tip, and the extent of tip displacement into the material, the elastic modulus can be determined using a Hertzian model of a semi-infinite elastic half space, which reduces to the following equation:

$$E = \frac{3(1-\nu^2)\delta_{\max}k}{2\sqrt{2}ah}$$

where ν is Poisson's ratio, $\delta_{\max}$ is the maximum deflection, k is the spring constant of the glass fiber, a is a function of tip contact radius, and h is indentation. Poisson's ratio was assumed to be 0.499, which is a typical value for elastomers [38]. However, if $\nu = 0.3$ (as is the case for most plastics [38]), elastic modulus values increase by 17%.

Following the crosslinking and quenching treatments, the α-elastin samples were swollen in PBS for at least 1 h, super-glued to an activated glass slide [39] and then resubmersed in PBS during the microindentation analysis. All samples were tested within 48 h of quenching. Each sample was probed at 4–6 separate locations and each crosslinking method was evaluated by testing at least three individual samples of each type.

2.6. Attachment and proliferation of VSMC on crosslinked α-elastin

We investigated the attachment and proliferation of VSMC on double-step alkaline/neutral crosslinked α-elastin thin films. Following the crosslinking and quenching steps, the films were briefly washed with sterile PBS and then the films were sterilized under UV light for 15 min in a laminar flow tissue culture hood. The films were washed with sterile PBS and stored in fresh sterile PBS while collecting the cells.

Bovine aortic smooth muscle cells (Coriell Cell Repositories, Camden, NJ) were maintained in Dulbecco's Modified Essential Medium with low glucose (Gibco, Carlsbad, CA) with 20 mM L-glutamine (Gibco), 1× penicillin/streptomycin (Gibco) and 10% bovine calf serum (Hyclone, Logan, UT). The cells were seeded onto the thin films of crosslinked elastin at 5×10^4 cells/well ($\sim 1.4 \times 10^4$ cells/cm^2). Empty wells served as tissue culture polystyrene (TCPS) controls. Separate plates were selected at 1, 4 and 8 d, rinsed with PBS, drained to remove excess liquid and stored at −20 °C.

To determine the number of cells in each well, a standard acid phosphatase assay [40] was performed. The frozen plates were thawed and rinsed once with PBS. To each well, we added 0.1 ml of an aqueous solution of 10 mM *p*-nitrophenyl phosphate (Calbiochem, San Diego, CA), 13.6 mg/ml sodium acetate and 1 mg/ml Triton X-100. A standard curve was similarly prepared from samples of known cell concentrations in the range of 2.5×10^4 to 2×10^5. The plates and standard samples were incubated at 37 °C for 90 min and then the reactions were developed with 0.1 ml 1 M NaOH. Next, 0.1 ml of each supernatant solution was transferred to a 96-well plate and a plate reader (OpsysMR, Dynex Technologies, St. Paul, MN) was used to determine the absorbance of each sample at 405 nm. Cell numbers were calculated based on the standard curve and are reported in terms of per cent increase from day 0, which was designated as the day that the cells were seeded onto the samples.

2.7. Statistical analysis

We performed one-way ANOVA tests and Student's *t*-tests (with Bonferroni correction) to determine the statistical significance of the differences between results. A significance level of $p < 0.05$ was used as the cutoff.

3. Results

3.1. Crosslinking of α-elastin

Inspired by the pH dependence of epoxy reactions (Fig. 1b), we developed a series of crosslinking approaches based on EGDE treatments within three pH categories (i.e. acidic, neutral, alkaline). Single-step neutral, single-step alkaline and double-step combinations of these pH ranges yielded insoluble crosslinked materials. In contrast, elastin treated with EGDE in acidic solutions gave a water-soluble product that presumably contained few functional crosslinks. We therefore chose to concentrate on three approaches (i.e. single-step alkaline, single-step neutral and double-step alkaline/neutral; Fig. 2) for the remainder of the characterizations. The order of the pH treatments in the double-step process (i.e. alkaline/neutral versus neutral/alkaline) did not seem to affect the bulk properties of the crosslinked product; therefore, we focused only on the alkaline/neutral variation.

3.2. Amino acid analysis

A preliminary amino acid analysis (Table 1) of the unmodified and crosslinked α-elastin samples revealed two general findings. First, the amino acid composition of the crosslinked materials is not apparently different from the starting material, unmodified α-elastin. Second, the mass of elastin retained in the single-step neutral materials is significantly reduced in comparison to materials treated with at least one alkaline step; furthermore, because the masses of amino acids are barely at the level of detection, the values obtained for the single-step neutral materials may be inaccurate.

3.3. Determination of swelling ratio

Swelling ratios (Fig. 3) vary with the pH of the crosslinking treatment and were determined to be 9.4 ± 1.8, 25 ± 1.7, 9.6 ± 1.7, respectively, for single-step alkaline, single-step neutral and double-step alkaline/neutral crosslinking (average ± standard deviation, $n = 3$ for each case). The swelling ratio of the single-step neutral crosslinking was statistically greater than the swelling ratios of either the single-step alkaline or the double-step

Table 1
Amino acid analysis of hydrolyzed α-elastin materials

Amino acid	nmol measured (residues/1000)			
	Unmodified α-elastin	Single-step alkaline	Single-step neutral	Double-step alkaline/neutral
Hydroxyproline	0.29 (8)	0.54 (18)	ND	0.62 (14)
Desmosine	0.09 (3)	0.07 (2)	ND	0.14 (3)
Isodesmosine	0.07 (2)	0.03 (1)	ND	0.11 (2)
Glycine	10.16 (284)	8.45 (291)	0.34 (303)	13.33 (296)
Alanine	9.56 (267)	8.00 (276)	0.19 (169)	12.69 (282)
Valine	3.90 (108)	3.35 (116)	0.45 (399)	5.09 (113)
Proline	4.31 (120)	3.03 (105)	0.01 (8)	5.08 (113)
Leucine	2.11 (58)	1.34 (46)	0.05 (44)	2.50 (55)
Isoleucine	0.70 (19)	0.57 (19)	0.02 (19)	0.89 (20)
Phenylalanine	1.42 (39)	0.95 (33)	ND	1.25 (28)
Methionine	0	0	ND	0
Cysteine	0.21 (6)	ND	ND	ND
Aspartic acid	0.16 (4)	0.12 (4)	ND	0.19 (4)
Glutamic acid	0.77 (21)	0.67 (23)	0.07 (58)	1.09 (24)
Lysine	0.07 (2)	0.04 (1)	ND	0.05 (1)
Arginine	1.03 (28)	1.18 (41)	ND	1.17 (26)
Serine	0.21 (6)	0.18 (6)	ND	0.26 (6)
Threonine	0.34 (9)	0.29 (10)	ND	0.40 (9)
Tyrosine	0.55 (15)	0.23 (8)	ND	0.19 (4)
Sum	36.25 (1000)	29.33 (1000)	1.13 (1000)	45.53 (1000)
Total mass after normalization[a]	4.4 mg	6.2 mg	0.2 mg	7.7 mg

ND, not detected.
[a] nmol of amino acids × 85 ng/nmol and normalized for entire 10 mg sample.

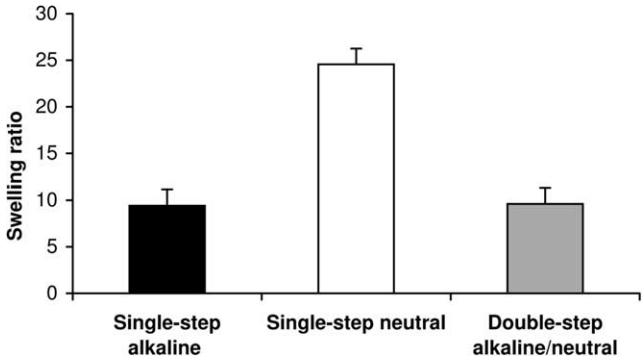

Fig. 3. Swelling ratios for crosslinked α-elastin biomaterials. The swelling ratio, calculated by dividing the swollen mass of the material by the dry mass, is related to the degree of crosslinking. Elastin crosslinked with ethylene glycol diglycidyl ether (EGDE) in either a single alkaline step or a double-step alkaline/neutral pH process show similar degrees of swelling. Elastin crosslinked with EGDE in a single neutral pH crosslinking step, however, had a significantly increased swelling ratio. These results indicate that the elastin materials treated with the single-step neutral process have comparatively less crosslinking than either of the materials treated with an alkaline pH step.

procedures (which were not significantly different from each other). In sum, these results suggest that alkaline treatments result in more crosslinks than a similar treatment at neutral pH.

3.4. In vitro degradation by elastase

Degradation rates (Fig. 4) were calculated from the initial linear slope of material per cent weight loss versus time plots (representative raw data for double-step alkaline/neutral crosslinking shown in Fig. 4a). The degradation rates (% weight loss/h) of the double-step alkaline/neutral treated α-elastin materials were increased in the presence of greater elastase concentrations (Fig. 4b). Specifically, the degradation rate increased linearly with logarithmic increases in elastase activity; the greatest degradation rate occurred in 1.0 u/ml elastase (19 ± 4.1%/h), followed by 0.1 u/ml (8.2 ± 2.7%/h) and then 0.01 u/ml (0.7 ± 0.58%/h) (average ± standard deviation, $n = 3$ for each case). The degradation rate in 0.01 u/ml elastase was statistically different than the degradation rates in either of the higher enzyme concentrations (which were not significantly different from each other).

The degradation rates of the elastin materials resulting from the three crosslinking approaches were compared at a single elastase concentration of 0.1 u/ml (Fig. 4c). The degradation rate of the single-step neutral crosslinking (50 ± 2.9%/h) was significantly greater than the swelling ratios of either the single-step alkaline (15 ± 1.4%/h) or the double-step (8.2 ± 2.7%/h) procedures (which were not significantly different from each other). Therefore, materials exposed to an alkaline crosslinking step had significantly reduced degradation

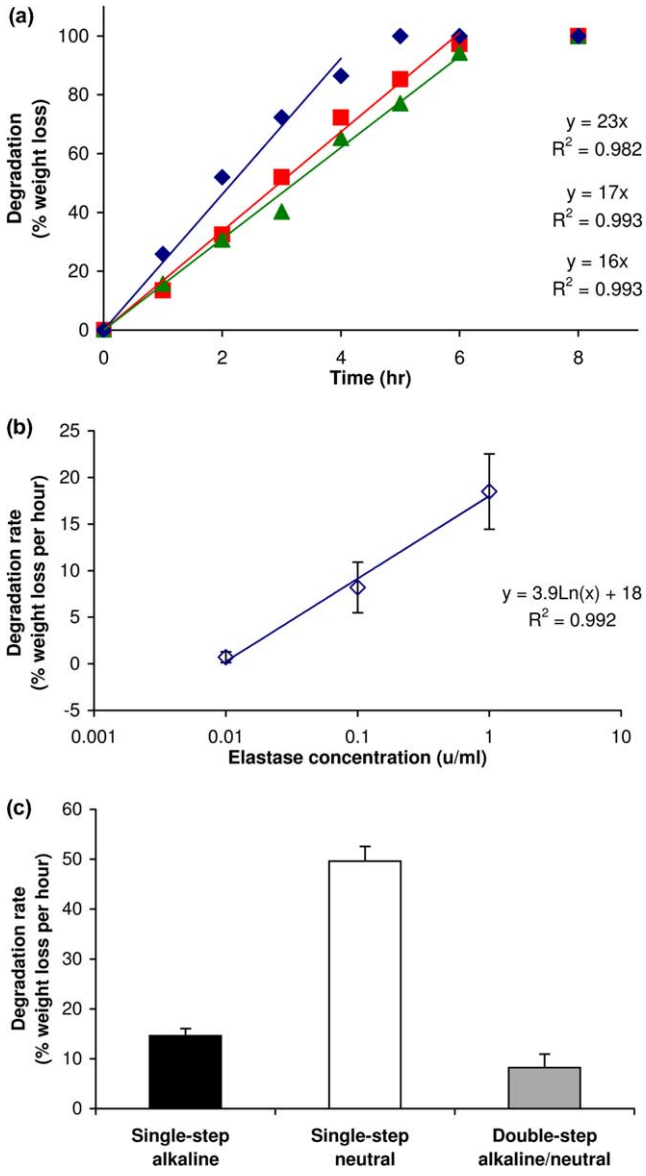

Fig. 4. In vitro degradation of crosslinked α-elastin. (a) Representative initial linear degradation rates of elastin crosslinked with the double-step alkaline/neutral process were calculated from the slope of percent weight loss versus degradation time in 1.0 u/ml elastase. (b) Degradation rates were calculated for the double-step crosslinked elastin in 0.01, 0.1 or 1.0 u/ml elastase. Each data point represents the average ± standard deviation. (c) Degradation rates in 0.1 u/ml elastase were also determined for the single-step crosslinking procedures at either alkaline or neutral pH.

rates compared to the neutral crosslinked elastin, again indicating a correlation between the reaction pH and the degree of crosslinking. No significant change in mass was observed in the negative-control elastin materials, which were incubated in buffer alone.

3.5. Determination of elastic modulus

Like the swelling ratio and degradation rate results, elastic modulus (Fig. 5) varies with the pH of the

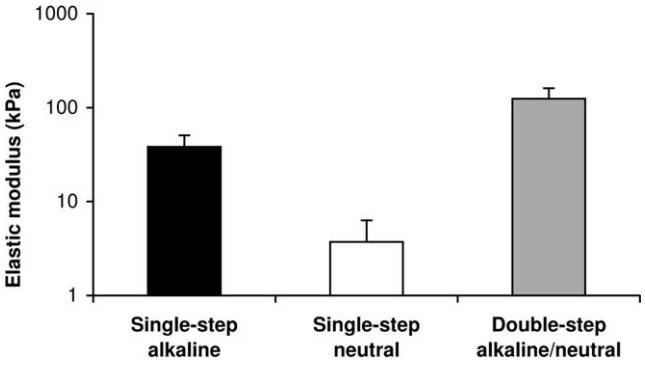

Fig. 5. Elastic modulus of the crosslinked α-elastin. The compressive elastic modulus of the elastin materials was determined using a custom-built microindentation system [37]. Each bar represents average + standard deviation and all pair-wise comparisons between averages are significant. Single-step alkaline, $n = 3$; single-step neutral, $n = 4$; double-step alkaline/neutral, $n = 4$.

crosslinking treatment and were determined to be 38 ± 12 kPa, 4.0 ± 2.6 kPa and 120 ± 37 kPa, respectively, for single-step alkaline, single-step neutral and double-step alkaline/neutral crosslinking (average ± standard deviation, $n = 3$–4 for each case, all pair-wise comparisons between averages are significant). Though there was a small degree of heterogeneity within each sample, the elastic modulus did not vary significantly over these areas. Heterogeneity was most pronounced within the single-step neutral treated materials, suggesting that alkaline treatments result in more complete and homogeneous crosslinking than the neutral pH treatment alone.

3.6. Attachment and proliferation of VSMC on crosslinked α-elastin

The attachment and proliferation of VSMC (Fig. 6a) on double-step alkaline/neutral crosslinked α-elastin was determined at 1, 4 and 8 d. The data are represented as a per cent increase in cell number from the time of cell seeding. After 1 day, the per cent increase in VSMC adhering to the elastin materials (99.6 ± 23%) is not significantly different from the per cent increase adhering to TCPS (103 ± 23%). The cell numbers on both materials are increased at day 4 (elastin: 260 ± 98%; TCPS: 520 ± 240%). At 8 d, there is a significant ($p = 0.004$) increase in the number of cells adhering to the TCPS (2100 ± 210%) compared to crosslinked elastin (540 ± 210%). The number of cells adhering to TCPS at 8 d is distinct from the number adhering at either 1 d or 4 d. All other differences between time-points within individual treatments are not significant.

The doubling time (Fig. 6b) of VSMC on the crosslinked elastin materials (73 ± 9.9 h) was reduced compared to that on TCPS (41 ± 0.60 h) (factor of ~56%, $p = 0.005$). Together, these data indicate that double-

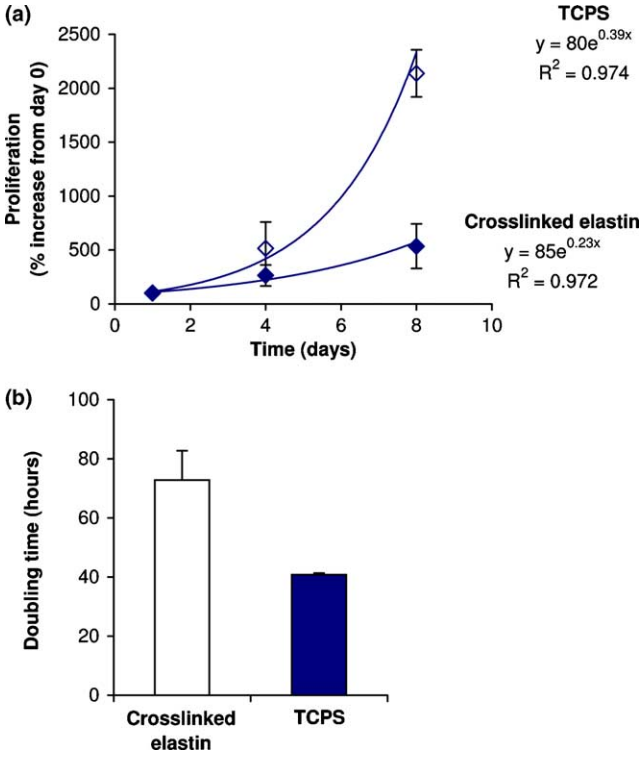

Fig. 6. VSMC cultured on double-step crosslinked α-elastin films show a significantly decreased proliferation rate compared to cells plated on tissue culture polystyrene (TCPS). (a) At 24 h, the number of cells adhering to the crosslinked elastin is indistinguishable from the number adhering to TCPS. However, at 8 days, differences in cell number are significant ($p = 0.004$). Together, these results suggest that elastin biomaterials support a similar level of initial attachment, but a decreased proliferation rate compared to TCPS. (b) The doubling time of VSMC cultured on the crosslinked elastin was slower than that on TCPS ($p = 0.005$). Each data point represents the average and standard error of the mean for three separate experiments (total $n = 9$). Cells were plated on day 0.

step crosslinked α-elastin supports similar levels of initial cell adhesion, but a decreased proliferation rate compared to TCPS. Data is the average and standard error of the mean for three separate experiments (total $n = 9$).

4. Discussion

The specific goals of this work were to develop elastin-based biomaterials that are (1) easy to synthesize and (2) maintain the biological and material properties of native elastin. To create these materials, we chose a pH-dependent diepoxy crosslinking scheme (Figs. 1b, Fig. 2). We selected the reaction conditions to potentially target three types of functional groups: the carboxyls present on aspartic acid, glutamic acid and the C-terminus of each fragment; the amines of lysine and the N-termini; and the hydroxyls present on serine, threonine and tyrosine.

Given α-elastin's amino acid composition, one may make predictions as to the relative amounts of crosslinking possible under each pH condition. Alpha-elastin contains ~3% hydroxyl residues, <1% amine residues, and ~3% carboxyl residues (Table 1 and Partridge et al. [25]). Because there are relatively more hydroxyl residues than amine residues, one may expect that an alkaline treatment (targeting hydroxyls) will result in a higher degree of crosslinking than a neutral treatment (targeting amines). As discussed below, we have validated this hypothesis via characterization of physical material properties that are modulated by crosslink density. Furthermore, we predicted that the acidic treatment would form hydrolytically degradable ester bonds with α-elastin's carboxyl groups. Not surprisingly then, we subsequently found that acidic crosslinking conditions did not stabilize the product against dissolution in water.

To better understand the composition of the crosslinked materials, we carried out preliminary amino acid analyses of the α-elastin materials (Table 1). Unfortunately, it is difficult to precisely determine the sites or extents of crosslinking due to the heterogeneous population of fragments and their associated terminal amino acids. Furthermore, the putative linkages were likely hydrolyzed during the harsh acid hydrolysis step in the amino acid analysis procedure. Nonetheless, two results of the amino acid analysis are noteworthy. First, there was a substantial decrease in the mass of elastin retained in the single-step neutral crosslinked materials compared to the starting mass of α-elastin. In other words, due to the relatively low levels of crosslinking obtained from the neutral treatment, a fraction of the elastin fragments were not incorporated into the insoluble product and likely were washed out in subsequent steps. The second finding was that there was no apparent difference in the composition of unmodified α-elastin versus alkaline-treated α-elastin materials. Therefore, the majority of the α-elastin fragments were retained in the insoluble alkaline-treated products. We expect that this finding will be significant when comparing the biological properties of these materials (to be determined in future work) to others' studies of α-elastin in soluble [29], insoluble [31] or crosslinked [30] forms.

The physical characterization of the elastin materials revealed that crosslinking pH could be used to modulate the swelling ratio (Fig. 3), degradation rate, (Fig. 4), and elastic modulus (Fig. 5). Alpha-elastin materials with at least one alkaline crosslinking step (i.e. single-step alkaline and double-step alkaline/neutral) were associated with physical properties indicative of higher extents of crosslinking compared with single-step neutral crosslinking method.

We found that the elastic moduli of the crosslinked α-elastin materials were similar to those of recombinant elastin-like polypeptide materials (Table 2). We also found the crosslinked α-elastin materials to be fragile, which is comparable to other elastin-based materials (reviewed by Bellingham and Keeley [41]). Because the moduli of the α-elastin materials are below that of native elastin, we envision using these materials as provisional matrices for in vitro remodeling [1,5,8,9] and the incorporation of new exogenous or cell-secreted elastin [12], collagen [1,7], or other matrix components [42]. All of the crosslinked samples we investigated can serve as provisional matrices because they are all degradable by elastase. This is in contrast to many of the recombinant elastin-like polypeptide materials that do not contain elastase-degradable sites [17–20,22,23].

Table 2
Comparison of the elastic modulus of native elastin, crosslinked α-elastin materials and recombinant elastin-like materials reported in the literature

Elastin type	Crosslinking	Elastic modulus (kPa)	Reference
Native elastin		100–1200, commonly ~400	[2]
		300–600	[44]
Elastin-derived fragments			
α-elastin	Crosslinked with a diepoxy linker	4–120	See Fig. 5
Recombinant			
Tropoelastin	Crosslinked with a bifunctional amine-reactant linker	220–280	[14]
Elastin-like polypeptides (poly[VPGXG][a])	Crosslinked with a trifunctional amine-reactant linker	1.6–15[b]	[20]
Elastin-derived polypeptides (various crosslinking and hydrophobic domains)	Crosslinked with a chemical analog of lysyl oxidase (copper sulfate, PQQ[c])	250	[21]
Elastin-derived polypeptides (poly[VPGVG]$_4$[VPGKG])	Photocrosslinked (acrylate-modified polypeptides)	450 (wet)	[23]
Elastin- and fibronectin-derived polypeptides (includes poly[VPGIG])	Crosslinked with glutaraldehyde	99–321	[19]
	Crosslinked by diisocyanate	~50–1100	[18]

[a] X was chosen to be lysine every 7 or 17 pentapeptides, otherwise X was valine.
[b] Complex modulus.
[c] PQQ, pyrroloquinoline quinine.

Ultimately, the control of VSMC phenotype will be critical to the success of cell-based vascular graft biomaterials [1]. Our preliminary investigation of VSMC adhesion and proliferation indicate that bovine aortic VSMCs adhere relatively well, but proliferate slower on crosslinked α-elastin in comparison to the TCPS controls. These results are an indication that α-elastin biomaterials may promote the contractile VSMC phenotype. These results are not surprising, as investigations of aortic VSMC derived from porcine [29,30,32] and rabbit [31] sources have found equivalent results on other forms of α-elastin. Though we cannot rule out the possibility that the decrease in VSMC proliferation rate was due to the toxicity of unreacted EGDE, it is unlikely as (a) epoxy crosslinkers are associated with minimal toxicity effects [26], and (b) all of the samples were quenched with glycine.

5. Conclusions and future directions

In general, we found the results from our analysis of crosslinking, material properties, and cellular response to diepoxy-crosslinked α-elastin to be encouraging. We expect that these stable, compliant elastin-like materials will play an important role in the development of future vascular graft materials. Ongoing work in our laboratory focuses on more detailed investigations of VSMC response to the crosslinked α-elastin by examining the molecular markers of the VSMC contractile phenotype. We also note the fundamental importance of aligned microstructure (e.g. collagen fibers) [2,3], microfibrils [43] and other elastic fiber-associated molecules [42] to the broader mechanical function of vascular tissues; therefore, future work also focuses on creating composite 'tissue-like' materials that interact dynamically with their living cellular components.

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