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Modular elastic patches – mechanical and biological effects

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Abstract

A modular approach to engineering crosslinked elastic biomaterials is presented for fine tuning of material mechanical and biological properties. The three components, soluble elastin, hyaluronic acid and silk fibroin, contribute with different features to the overall properties of the final material system. The elastic biomaterial is chemically crosslinked via interaction between primary amine groups naturally present on the two proteins, silk and elastin, or chemically introduced on hyaluronan and N-succinimide functionalities of the crosslinker. The materials obtained by crosslinking the three components in different ratios have Young's moduli ranging from ~700 kPa to 100 kPa, strain to failure between ~ 65-15 % and ultimate tensile strengths of ~ 50 to 20 kPa. The biological effects and enzymatic degradation rates of the different composites are also different based on material composition. These findings further underline the strength of modular, multi-component systems in creating a range of biomaterials, targeted tissue engineering and regenerative medicine applications, with application-tailored mechanical and biological properties.

Keywords

elastin; hyaluronic acid; silk; composites; biomaterials; elastic

1. Introduction

The central paradigm of tissue engineering relies on a well orchestrated interplay between biomaterials, cells, and signaling molecules, with a goal to mimic the complexity found in nature. Often these approaches fall short of this intent, due to many factors including cost, low shelf-life and scale-up, and quality control issues ^{1, 2}. One path to help address these problems could be offered by the use of a minimalist, modular biosystem approach ³, that recapitulates the key factors needed for successful tissue or organ regeneration and that when applied to the site of injury would coordinate and permit autologous factors to complete the regenerative process. The individuality of each tissue or organ and each regenerative application underscores that a “one formulation fits all” approach to the problem is unrealistic ⁴.

Under physiological condition, cells are surrounded by a complex network of proteins and glycosaminoglycans (GAGs) that constitute the extracellular environment (ECM) ⁵. Besides its structural role, the ECM coordinates complex signaling events that direct cell fate and is in turn constantly altered by cells in order to serve their developmental needs. The ECM is variable in composition and mechanical compliance across tissues, but common, key components can be identified. For example, tissues that are subjected to variations in pressure or tension, such as the bladder, lungs, blood vessels, elastic cartilage or skin have high elastin content⁶. Elastin is produced primarily by fibroblasts or smooth muscle cells as

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soluble tropoelastin⁷. This precursor then assembles in the extracellular space into elastic fibers and sheets that are stabilized through enzymatic intermolecular crosslinking of lysine residues. Elastin is strongly hydrophobic with a high content of glycine, alanine, proline and valine⁸. It confers tissues with both resiliency and flexibility⁹, but also plays a key role in cellular processes such as morphogenesis¹⁰ and proliferation¹¹.

Another ubiquitous ECM component is hyaluronic acid or hyaluronan (HA), a glycosaminoglycan (GAG). HA is an unbranched, polyanionic polymer of disaccharide units composed of glucuronic acid and N-acetyl glucosamine¹². Similar to elastin, in addition to its structural role, HA is involved in various processes such as the maintenance of tissue water homeostasis¹³, modulation of cell motility and differentiation^{14, 15}, morphogenesis and embryogenesis¹⁶, and inflammation¹⁷. Both of these ECM components have been previously used for biomaterial engineering and in various tissue regeneration applications^{8, 18}. While HA-based materials are already on the market or at various regulatory stages², elastin-based scaffolds face hurdles of insolubility, biocompatibility, bioavailability and calcification. The latter issue was shown to be solvable or at least diminished by the addition of GAGs, specifically HA¹⁹. The first two issues were addressed by developing elastin-like peptides^{20, 21} or by expressing recombinant human tropoelastin²²; both approaches were reported to lead to well-tolerated and readily available biomaterials.

Tissue engineering biomaterials are commonly based on native components that would provide biocompatibility, but that are also rapidly degradable under the action of specific enzymes. For the present study we considered a biomaterial design that would be versatile, could potentially allow for longer *in vivo* residence times and would generate material systems with adjustable mechanical and biological properties. To this end, elastin and HA were chosen as key components. Silk fibroin was selected as the third component since, based on its properties, it could offer biocompatibility and mechanical strength to the modular composites, while prolonging the overall *in vivo* degradation times. Silk fibroin is a structural protein produced by insects such as spiders or silk-moths/worms. The sequence of the silk protein from *Bombyx mori* shares some common features with elastin, with the bulk of the protein organized into alanine and glycine-rich hydrophobic blocks and large side chain amino acids clustered in chain-end hydrophilic blocks²³. The hydrophilic and hydrophobic blocks are further organized into amorphous and crystalline regions²⁴. The latter can organize into crystalline beta-sheets via intra- and inter- molecular hydrogen bonding and hydrophobic interactions endowing silks with impressive mechanical properties, as silks are known as the toughest natural fibers with strengths up to 4.8 GPa and elasticity up to 35%²⁵. In the present study we investigated the modulation of the mechanical and biological parameters of these multi-component systems.

2. Materials and methods

Elastin (elastin soluble, ES12) was from Elastin Products Company, Owensville, MO. Hyaluronic acid (HA, MW = 1.1 MDa, polydispersity 1.2) was a generous gift from Genzyme, Cambridge, MA. Bis (sulfosuccinimidyl) suberate (BS3), sulfosuccinimidyl-7-amino-4-methylcoumarin-3-acetate (AMCA) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Thermo Fisher Scientific, Rockford, IL. Polyethylene oxide (PEO) and ethylene diamine were from Sigma-Aldrich, St. Louis, MO. Cell culture reagents and live/dead assay kit were purchased from Invitrogen, Carlsbad, CA. Elastase (high purity porcine pancreatic, EC134) was from Elastin Products Company, Owensville, MO, hyaluronidase (type I-S from bovine testes) and protease XIV were from Sigma-Aldrich, St. Louis, MO.

2.1. Synthesis of amino-hyaluronan (H₂N-HA)

HA was dissolved in water to a concentration of 0.5% w/v solution. To this, ethylene diamine was added drop wise under magnetic stirring, in ~10-fold molar excess to HA disaccharide unit. EDC, 4-fold molar excess to disaccharide unit, was then added to the reaction mixture and the solution pH was adjusted to 4.75–6 with 6N HCl. The solution pH was monitored for 30 min and adjusted as needed to keep in the aforementioned range. The reaction was completed overnight then dialyzed against water, using MWCO 3,500 cassettes (Thermo Fisher Scientific, Rockford, IL) with 6 changes of water over 72 h. Subsequently, the solution was lyophilized. The reaction product was verified by ¹H-NMR (300 MHz, D₂O) – chemical shifts corresponding to the substituent at δ 1.04 (-NH-CH₂-CH₂-NH₂), 2.73 (-NH-CH₂-CH₂-NH₂) and 3.00 (-NH-CH₂-CH₂-NH₂). The number of primary amines was quantified by derivatization with sulfo-NHS-AMCA according to manufacturer's protocol. By using Lambert-Beer equation ($A = C \cdot \epsilon$, where A is absorbance at 346 nm, c is amine concentration and ϵ is the molar extinction coefficient, 19,000 M⁻¹ cm⁻¹ for sulfo-NHS-AMCA) and was found to be in the 12% range (primary amines per one hundred disaccharide units). The reaction was performed on two different H₂N-HA batches and the results were averaged.

2.2. Preparation of silk solution

Silk fibroin was obtained as previously described²⁶. Briefly, *Bombyx mori* cocoons were cleaned and cut into small pieces. In a subsequent degumming process, sericin was removed by boiling the cocoons for 1 h in 0.02 M Na₂CO₃ solution. The silk fibroin was then dissolved in 9M LiBr at 60°C for 1 h to a concentration of 20% w/v then it was dialyzed against water (MWCO 3,500) for 72 h.

2.3. Preparation of elastic patches

Patch components were dissolved in 1X PBS, pH 7.4 at room temperature to concentrations and formulations listed in Table 1. BS3 in 1XPBS, pH 7.4 at room temperature was added to a final concentration of 23 mM. The mixture was immediately pipetted into polydimethylsiloxane (PDMS) molds depending on the application, and then placed at 37°C for 12 h to facilitate coacervation and crosslinking. Crosslinked patches were washed and stored in 1XPBS, pH 7.4 at 4°C until use.

2.4. Cytocompatibility assay

Primary human mesenteric artery cells (passage 5–7) obtained via explantation as previously described²⁷ were seeded at a density of 2.5×10⁴ cells/ml onto various elastic patch formulations and cultured for 5 days. Cell viability was assessed by using a live/dead viability/cytotoxicity kit. Images were collected with a fluorescent microscope equipped with a dual red/green filter (Leica Microsystems, Wetzlar, Germany) at 100X magnification.

2.5. Determination of smooth muscle cell- specific marker expression

Primary human mesenteric artery cells (passage 5–7) obtained via explantation as previously described²⁷ were seeded at a density of 2.5×10⁴ cells/ml onto various elastic patch formulations and cultured for 5 days. Cells were then lysed, reverse transcribed then evaluated for calponin alpha-smooth muscle cell actin (contractile phenotype markers) vimentin and tropomyosin (synthetic phenotype markers) expression.

2.6. Enzymatic degradation studies

Elastic patches were prepared as previously described and cast into rectangular molds. Samples were then cut into four equal parts and kept in 1X PBS, pH 7.4 overnight for equilibration. Next day, samples were blotted; the weight of each square was recorded and

quadruplicate samples were placed in elastase (5 U/ml) solution in 1X PBS, pH 7.4, hyaluronidase (5 U/ml) solution in 1X PBS, pH 7.4, protease XIV (5 U/ml) solution in 1X PBS, pH 7.4 or a mixture of all enzymes (all at 5 U/ml) solution in 1X PBS, pH 7.4. Samples were then placed in a 37°C incubator with a shaker, at 100 rpm. The weight variations of each sample were recorded daily, by removing, blotting and weighing each square and re-placing it in fresh enzyme solution, under aforementioned conditions.

2.7. Mechanical testing

Mechanical properties of elastic patches were determined under wet conditions, as previously described²². Tensile tests were performed on an Instron 3366 testing frame equipped with a 10N capacity load cell and Biopuls™ testing system, including submersible pneumatic clamps. Gel samples were cast into PDMS molds to the size appropriate for clamping and gage length necessary for video extensometry. Samples were soaked in 1X PBS, pH 7.4 for >1 h. Subsequently, samples were loaded into the tester and allowed to soak at 37 °C for 2 min. For testing, a strain control rate of 1% s⁻¹ was specified, based on the initial clamp-to-clamp length (nominal length ~45mm, nominal elongation rate 450µm/sec). Load and elongation data was captured at 10Hz. Video Extensometer strain data was recorded at the same rate, based on two fiducial painted markers placed at a nominal distance of ~1cm on the surface of the thinnest portion of each film. The original cross sectional area determined by measuring the film thickness using a thickness gage (Mode No. 7309, Mitutoyo Corp.), and multiplying by the specimen gage width (4.68 mm). The nominal tensile stress and strain were graphed, based on the original cross-sectional area and length, respectively, and the elastic modulus, strain to failure, and ultimate tensile strength (UTS) determined. UTS was determined as the highest stress value attained during the test. The elastic modulus was calculated by using a least-squares' (LS) fitting between the range 0 and 5% strain after an initial spike but before catastrophic failure. This was deemed sufficient to objectively capture the linear portion of the stress/strain curve for all samples tested. The strain to failure was determined as the last data point before a >10% decrease in load.

2.7. Statistical analysis

Values, represented as mean ± standard deviation (S.D.) were compared using Student's *t*-test (2-tailed, type 3) with *p*<0.5 considered statistically significant and *p*<0.05 considered highly significant.

3. Results

Elastic patches were obtained by chemically crosslinking elastin, elastin with HA or with silk, and elastin with HA and silk. Primary amine groups needed for chemical crosslinking were introduced on hyaluronic acid (HA) by coupling carboxyl functionalities present on each disaccharide subunit of the GAG with ethylene diamine via carbodiimide mediated chemistry (Fig. 1A). The reaction product was confirmed by ¹H-NMR, showing the appearance of new peaks in the 1–3 ppm region, specific to the attached ethylene diamine (Fig. 1B). The presence of primary amines on the molecule was further confirmed and their number quantified by using a water soluble fluorophore, sulfo-NHS-AMCA. For this, the N-succinimide ester of the dye reacts with primary amines present on the target molecule, yielding a blue fluorescent compound with excitation/emission in the 345–350/440–460 nm range (Fig. 2). For H₂N-HA, the primary amine content was determined from 346 nm absorbance of the dye-H₂N-HA complex and was found to be ~12% (primary amines per hundred of disaccharide units).

The cytocompatibility of the materials was assessed by culturing primary cells on representative samples. Since the bio- and cyto-compatibility of HA^{28–30} and silk fibroin^{31–33} have been previously documented, possible cytotoxic effects would have been attributable to soluble elastin, the crosslinker, or the reaction side products. Primary human mesenteric artery smooth muscle cells were used and all formulations sustained high cellular viability (less than 5% cell death observed microscopically) and attachment. However, cells elicited different behaviors based on the substrate formulation (Fig. 3). Thus, on elastin only (E), cells appeared isolated and their morphology was mostly rounded. On EH, cells appeared to be organized in networks and this pattern was observed to various extents in all HA containing samples. In contrast, on ES, cells appeared to have proliferated in clusters while maintaining a rounded morphology²⁶. Cells cultured on EHS1 elicited a morphology that resembled that observed on E and EH. EHS2 induced morphologies similar to ES while cells on EHS3 had similar morphology to both EH and ES-cultured cells. Smooth muscle cells can transition between contractile and synthetic phenotypes; therefore, to further investigate the substrate formulation effect on smooth muscle cells, the gene expression profiles of both contractile and synthetic markers was investigated. The presence of HA in the substrates appeared to significantly increase actin expression in the cells ($p < 0.07$ for EH and $p < 0.5$ for EHS3), while addition of silk did not alter gene levels considerably (Fig. 4A). Calponin levels were increased on EH patches but decreased on ES when compared to the levels observed on E. EHS formulations induced lower (EHS1 and EHS2) ($p < 0.5$) or comparable (EHS3) levels of calponin in the smooth muscle cells (Fig. 4A). For the synthetic markers, the presence of silk appeared to drastically decrease expression levels, for both genes, in a concentration dependent manner ($p < 0.5$) (Fig. 4B). The HA samples indicated similar gene expression profiles to those observed on E (Fig. 4B).

Next, the enzymatic degradation profile of the various formulations was addressed, to evaluate the effect of the moduli on enzymatic susceptibility (Fig. 5). EHS2, indicated as EHS on the figure, was chosen as representative for the tri-modular system, in order to reduce the number of handled samples. Interestingly, elastase rapidly degraded all samples, except EH within 24 hours. Similar degradation rates were recorded for digestion with protease XIV. In the presence of hyaluronidase, degradation was noticeable only after a 3 day lag time in which the dominant phenomenon was sample swelling. Nevertheless, by day 5 all samples were degraded either totally or to an extent that did not permit further handling (ES). In the presence of the enzyme cocktail, degradation occurred gradually, the least susceptible to degradation being the HA containing samples. Control samples, incubated in $1\times$ PBS, pH 7.4 under identical conditions, swelled comparably for the first 3 days. By day 4, samples lost their integrity and this prevented further evaluation (results not shown).

The elastic patches obtained by crosslinking were yellow-brownish, similar in color to the elastin starting material and by manual stretching appeared to have various degrees of elasticity. The silk containing samples were clear in appearance while the addition of H₂N-HA yielded slightly opaque samples (Fig. 6A inset).

The mechanical testing of the different patches indicated that the material parameters were dependent on the individual compositions. Thus, the elastic moduli of the two and three-component composites could be varied between approximately 100–230 kPa, strain to failure between ~ 15–40% and ultimate tensile strengths in the 30 kPa range (Fig. 3). We assumed that the majority of the stress-strain response for each system was elastic due to observations in routine handling and previous reports²². As such, the pure elastin samples were more elastic than all the composite blends investigated in the present study, as seen by the results on strain to failure (Fig. 3C). It was also observed that additions of silk or H₂N-HA to elastin decreased the stiffness of the patches. Silk containing samples had statistically lower elastic moduli ($p < 0.5$ for ES and $p < 0.05$ for EHS1) when compared to the EH

samples (Fig. 3B). Similarly, UTS and strain to failure metrics revealed different trends among the blend groups studied, to a much lower extent than seen for the elastic moduli ($p < 0.5$) (Fig. 3A). The elastic moduli of the two and three-component samples for all tests were considerably different from those of the reference elastin sample ($p < 0.07$) (Fig 3B).

4. Discussion

Modular materials with different mechanical properties were obtained via covalent crosslinking of elastin, HA and silk fibroin. The three individual components used in this study were chosen based on their particular properties with potential applications in mind. Elastin has the ability provide materials with desired elastic properties and control over the adherence of specific cell types and cellular functions³⁴, hyaluronan confers a suitable substrate for chemistry while – providing biocompatibility and hydrophilicity³. Moreover, hyaluronan plays a key role in processes such as cardiogenesis and cardiomyocyte differentiation^{35, 36} and wound healing³⁷, all valuable features in application-targeted tissue engineering products. Silk was chosen for its ability to add another level of control over the mechanical and biological features of the composites, while presenting mechanical strength and *in vivo* durability.³⁸

Numerous reports exist in the literature underlining the effects of substrate stiffness on cellular behavior and ultimately on successful tissue engineering^{29, 39, 40}. Modular systems were previously shown to provide a platform for the generation of biomaterials with tailored mechanical and biological properties according to applications³. Here, we show a simple way to achieve biocompatible crosslinked, modular biomaterials based on elastin, hyaluronan and silk. To our knowledge, this is the first report where these three components are chemically connected to offer a new suite of materials. These novel composites were compatible with primary human smooth muscle cells while the cellular phenotypes were dependent on the substrate formulation. Cells on elastin appeared isolated with minimally stretched morphologies. In contrast, the presence of HA in samples induced a more stretched morphology and appeared to promote cellular network formation, specifically for the EH material variant. Silk addition caused cells to cluster in spheroid-like formations. Interestingly, cells cultured on EHS1 elicited morphologies resembling those on E rather than ES. In contrast, EHS2 and EHS 3 formulations induced mixed morphologies, similar to those observed on both EH and ES. The different moduli ratios also altered gene expression profiles as determined by RT-PCR. HA appeared to induce increased actin production, while silk did not have a significant effect. In contrast, calponin levels were modulated by both HA and silk. The synthetic markers tested were affected only by the presence of silk but not by HA. These findings highlight once again the importance of substrate components, especially in the context of cell priming for specific tissue engineering applications.

The enzymatic stability of the patches was found to be dependent on both the degrading enzyme and substrate formulation. Elastase and protease XIV has similar affinity for substrates with EH formulations being slightly more resilient to degradation. Hyaluronidase-induced degradation occurred slowly and for the first 3 days of the assay, water uptake (swelling) was the dominant process. It is possible that a high degree of hydration is required for this enzyme to reach its substrate. In the presence of all three enzymes, samples degraded at relatively comparable rates. Remarkably, our data indicates that silk is highly susceptible to elastase degradation. Although believed that there was no mammalian enzyme with silk specificity, this observation indicates that pancreatic elastase is capable of recognizing and efficiently degrading silk. This result actually makes sense in the context of the high amino acid sequence similarities between the two proteins (both have high “GAG” sequence frequency) and the pancreatic elastase specificity (serine protease that hydrolyzes peptide bonds adjacent to the carboxyl group of alanines). The optical properties of EH and

ES samples (opaque versus clear) were also significantly different. This aspect would also be essential as it could be application-tailored (i.e. corneal grafts versus skin graft) and might be of practical value for future experimental designs. The Young's modulus and strain-to-fail mechanical parameters of the modular systems were determined to be different than the elastin-only system. Some differences were observed between two and three component formulations, indicating that the addition of another modulus to a two-component system had the potential to alter its properties. However, no significant differences were noted for UTS values indicating that the presence of elastin dictates the extent of stretching in these materials. Overall, both HA and silk appear to decrease the stiffness of the patches (Young's moduli) and the addition of silk seems to additionally "soften" materials, but both components also decrease material resilience (strain-to-fail values).

Finally, the data presented underlines once again the potential of modular biomaterials composed of simple, application-dictated constituents. The elastic moduli of various tissues fall in the following range: human myocardium: ~350 kPa⁴¹, skin ~120 kPa⁴², blood vessels ~530 kPa⁴³. By comparison, the materials described here appear to have mechanical parameters that span the same range as these native tissues. The choice of the individual components in such modular materials would be ultimately dictated by the end-applications, but the adaptable properties of such materials could make them suitable for a wide variety of tissue engineering and regenerative applications such as cardiac patch development and cardiac reconstruction, vascular graft, skin grafts, surgical applications such as stents and wound healing devices.

5. Conclusions

We show the design, mechanical and biological characterization of crosslinked, modular multi-component elastic biomaterials. As illustrated by our results compared to the single component systems, the stiffness, strength and elasticity of the materials could be modulated simply by varying the nature, concentration or ratio of the components. Moreover, the gene expression patterns in primary human smooth muscle cells can also be dictated by the substrate formulation and ultimately its mechanical properties. Overall, this work highlights once more the strength and versatility of the modular approach and underscores the importance of choosing the components for the purpose of fine-tuning the mechanical and potentially, biological properties of biomaterials, as well as the array of potential applications.

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Figure 1. Amino-hyaluronan ($\text{H}_2\text{N-HA}$). A. Synthetic scheme for $\text{H}_2\text{N-HA}$. B. Comparison of ^1H -NMR spectra of hyaluronan and $\text{H}_2\text{N-HA}$, confirming successful synthesis (peak at ~ 1 ppm is a residual signal present in the commercially available ethylene diamine).

Figure 2.
Quantification of primary amines in H₂N-HA.

Figure 3.
Cytocompatibility of elastic materials with primary human mesenteric artery smooth muscle cells after 5 days in culture.

A.

B.

Figure 4.

A. Contractile and B. synthetic smooth muscle cell marker expression profiles in mesenteric artery primary smooth muscle cells cultured on for 5 days various elastic patches ($n = 3$). Black statistical significance markers are for actin and vimentin, respectively. Grey statistical significance markers are for calponin and tropomyosin, respectively. Statistical significance: * $p < 0.07$, ** $p < 0.3$.

Figure 5. Enzymatic degradation profiles of elastic patches in the presence of elastase (5U/ml), hyaluronidase (5 U/ml), protease XIV (5 U/ml) and a mix of all three (each at 5 U/ml) (n = 4).

Figure 6.

Mechanical parameters of elastic patches (panel A inset – appearance of E, ES and EH samples prepared for mechanical testing). Differences in parameters were considered highly significant if * $p < 0.07$ and statistically significant if ** $p < 0.5$ and ($n = 4$ for EH and EHS1 samples and $n = 6$ for all other samples).

* Because of difficulties with handling that affected samples integrity, no reliable mechanical data could be collected for the EHS2 samples.

Table 1

Elastin-hyaluronan and elastin-hyaluronan-silk gels composition

Sample	Sample composition	Final concentrations (% w/v)		
		Elastin (E)	Hyaluronan (H)	Silk (S)
E	Elastin	25	0	0
EH	Elastin/Hyaluronan	25	2.3	0
ES	Elastin/Silk	25	0	5.8
EHS1	Elastin/Hyaluronan/Silk	25	0.7	5.8
EHS2	Elastin/Hyaluronan/Silk	25	1.5	3.8
EHS3	Elastin/Hyaluronan/Silk	25	2.3	1.7