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Shape Memory Silk Protein Sponges for Minimally Invasive Tissue Regeneration

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Abstract

Porous silk protein scaffolds were designed to display shape memory characteristics and volumetric recovery following compression. Two strategies were utilized to realize shape recovery: the addition of hygroscopic plasticizers like glycerol and tyrosine modifications with hydrophilic sulfonic acid chemistries. Silk sponges were evaluated for recovery following 80% compressive strain, total porosity and pore size distribution, secondary structure development, *in vivo* volume retention, cell infiltration, and inflammatory responses. Glycerol-modified sponges recovered up to 98.3% of their original dimensions following compression, while sulfonic acid / glycerol modified sponges swelled in water up to 71 times their compressed volume, well in excess of their original size. Longer silk extraction times (giving lower silk molecular weights) and higher concentrations of glycerol gave sponges with greater flexibility and shape fidelity, with no loss in modulus following compression. Sponges were over 95% porous, with secondary structure analysis indicating glycerol-induced β -sheet physical crosslinking. Tyrosine modifications with sulfonic acid interfered with β -sheet formation. Glycerol-modified sponges exhibited improved rates of cellular infiltration at subcutaneous implant sites with minimal immune response in mice. They also degraded more rapidly than unmodified sponges, a result posited to be cell-mediated. Overall, this work suggests that silk sponge systems may be useful for minimally invasive deployment in soft tissue augmentation procedures.

Graphical Abstract

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Keywords

Silk; glycerol; sponge; shape memory; minimally invasive

1. Introduction

Stimuli-responsive polymers are a subclass of “smart” materials that can recover from a deformed shape back to an original, pre-defined shape due to an external stimulus. Materials exhibiting this behavior are typically referred to as shape memory polymers. Often the external stimulus is heat, but electrical or magnetic fields, pH, light, pressure, water, ions, enzymes and sugars can also induce a change which may alter the mechanical, electrical and / or optical properties of the material, as well as its permeability and topographical features.^[1,2] For biomedical devices, the most physiologically accessible shape-recovery stimuli are temperature and water content.^[3] Shape memory materials often take the form of foams, fibers, meshes or films and are frequently used commercially as packing materials, vibration dampeners, pillows, mattresses and sensors.^[1,4] Their use in implantable biomedical devices, such as vascular stents,^[5,6] self-tying sutures,^[7] drug-eluting capsules^[8] and bioactuators^[9] have also been pursued.

One shape memory application yet to be fully explored involves injectable fillers for soft tissue regeneration. Volume loss due to injury, tumor resection or disease can impact several soft tissue types, including adipose, skin and muscle tissues. Implantable bulking materials which could fill the shape of the wound site and promote cellular ingrowth for tissue regeneration may improve patient recovery. Unfortunately, implantable materials often require complex, highly invasive deployment surgery, thus there is a need to find alternate insertion strategies. Minimally invasive alternatives would be ideal if not for the challenges of inserting bulky devices through narrow channels (needles, cannulae, etc.), combined with the need for biocompatible and bioresorbable characteristics in order to meet most tissue regeneration goals. Therefore, designing new bulking materials with shape memory characteristics may allow them to be compressed to fit through an insertion device and subsequently expanded by means of a physiological trigger back into the original shape while supporting tissue regeneration requirements.

For biomedical devices, implantable shape memory materials must be sufficiently tough and fatigue resistant for load bearing applications, exhibit a recovery force strong enough to expand in a confined space, possess controlled degradability and resorption, and be

biocompatible with the surrounding tissue.^[10] Several synthetic shape memory materials exist, such as polyurethane,^[11] silicone,^[12] poly(lactide),^[13] poly(caprolactone) and poly(glycolide).^[2,3] Synthetic materials generally have sufficient modulus and toughness when dry, but may experience loss of mechanical integrity when wet or exposed to the biological environment over time. Some of these materials also need to be designed to degrade through the use of water-soluble linkers. Finally, degradability of these materials is not always compatible with tissue regeneration, biocompatibility requirements and related issues; for example some degradation byproducts may cause immune or inflammatory responses, or the materials may not integrate well with tissue. Certain biologically-derived materials can also be processed to behave like SMPs. Natural materials often exhibit the benefits of being biocompatible and degradable by chemical and enzymatic processes in the body. As an example, shape memory genipin-crosslinked chitosan scaffolds that expand in the presence of water have been reported.^[14] Crosslinking with genipin allowed the chitosan network to undergo greater swelling in water compared to non-crosslinked chitosan (up to 400% by weight). Additionally, crosslinked chitosan showed 98% recovery after 60% strain deformation, though recovery took up to 15 minutes. Methacrylated alginate scaffolds were prepared via a cryogelation process which produced shape memory gels.^[15] Alginate gels recovered 92% of their original volume after 90% deformation, and could be injected through a needle for minimally invasive implantation. Furthermore, gels could be used as cell-delivery devices for therapeutic applications. Gels were implanted *in vivo*, recovered after 2 days and appeared biocompatible in mice with minimal degradation. Unfortunately, the bulk structure of the hydrogel generally does not provide adequate inlets for cells to migrate through and interact with the inner material structure, restricting degradation to surface erosion.^[16] Alternatively, porous materials may be designed via several techniques (porogen leaching, gas foaming, lyophilization) to possess interconnected networks of open cells, ideal for cellular infiltration. Penetration into the bulk material can allow cell-mediated degradation and deposition of new extracellular matrix at rates matching tissue development and regeneration; however, a previous attempt at designing injectable silk sponges highlighted their propensity to plastically deform under high stress.^[17]

In the present study, we utilized silk protein based sponges with open cellular structures and shape memory characteristics. Silk protein was identified as an attractive material for injectable fillers due to its biocompatibility and all-aqueous processing. Previous *in vitro* and *in vivo* studies have explored the use of silk hydrogels for tissue bulking, noting their capacity for mechanical tunability, cytocompatibility and biodegradability.^[18–20] This study aimed to test the compressive response of chemically modified silk sponges with the goal of designing porous materials with improved elasticity and shape memory. The formulations presented here show improved recovery and shape fidelity from simulated injection stresses and represent the next step in the evolution of minimally invasive silk-based devices.

2. Results

2.1. Diazonium Modified Silk Sponges

Modification by diazonium coupling was confirmed by spectrophotometric analysis (Supplemental Figure S1).^[21] Modification was detected by observing a decrease in the

tyrosine absorption peak at 280 nm compared to the silk-only control, as well as an increase in the azobenzene absorption with peaks at 325 and 390 nm in the modified silk. The theoretical yield of modified tyrosine residues was calculated as 58.6% based on the available tyrosine residues per silk protein and the added diazonium salt concentration. Actual tyrosine modification per molecule of silk protein was estimated using Beer's Law.^[21,22] Modified tyrosine concentration was calculated from the absorbance at 325 nm using an extinction coefficient of $22,000 \text{ M}^{-1} \text{ cm}^{-1}$.^[22] The percentage was estimated by comparing this value to the molarity of silk protein. The yield of azo-modified tyrosine was 60%. The modified diazonium-silk stock was diluted with native silk solution to achieve solutions with lower ratios of modified tyrosine.

2.2. Compressive Mechanics and Recovery of Modified Silk Sponges

2.2.1. Glycerol Only Modified Sponges—Representative stress-strain profiles of 10 minute extracted silk sponges modified with either glycerol (40% by mass), methanol post-treatment or both are shown in Figure 1A. In general, the combination of high glycerol content (20% w/w or greater) and methanol post-treatment increased the stiffness of the silk sponges compared to silk alone (methanol treated) or untreated silk-glycerol (no methanol treatment). Similarly, changes in hysteresis (reported as the ratio of the area under the curve of the loading cycle vs. that of the unloading cycle) were observed, with methanol treated silk-only sponges exhibiting greater hysteresis during compression compared to both untreated and methanol treated silk-glycerol sponges (Figure 1B).

The compressive modulus of the sponges ranged from approximately 7.5 kPa to 45.9 kPa after hydration in PBS (Figure 1C), a range which is suitable for reconstruction of a variety of soft tissues.^[23] In sponges post-treated with methanol, increasing the glycerol content correlates to an increased compressive modulus. Without a methanol wash, only the silk sponges containing high glycerol content (30% w/w or greater) were considered for testing since low glycerol content was not sufficient to cause physical crosslinking and insolubility in the material. Silk-glycerol sponges without methanol post-treatment were significantly softer than their methanol treated counterparts.

Silk extraction time alone did not have an impact on sponge stiffness. At low glycerol content (0 and 10% w/w) there was no significant difference in sponge compressive modulus. In general, changes in glycerol content had the greatest impact on 10 and 30 minute extracted silk sponges. The 10 mE silk sponges increased in modulus as glycerol content increased, with significant changes at 30 and 40% w/w compared to control sponges. The 30 mE sponges gave a biphasic response, with a peak compressive modulus at 20% w/w glycerol content. There was no significant difference in the modulus of 60 mE sponges compared to silk-only controls below 30% w/w glycerol. Without methanol treatment, 10 mE and 60 mE sponges were not significantly stiffer than their silk-only controls.

Shape fidelity, observed by comparing the compressive modulus and the sample height before ("pre") and after ("post") the application of 80% compressive strain, was impacted by both protein molecular weight and glycerol content (Figures 1C,D). In methanol post-treated samples, 10 minute extracted silk showed the largest reduction in modulus following compression, while there was no significant difference in the modulus of 60 mE sponges

(Supplemental Table 1). Silk-glycerol sponges (30% and 40% w/w) without methanol post-treatment displayed no significant changes in modulus as result of compression, regardless of extraction time. Shape recovery generally improved with an increase in glycerol concentration. The 10 mE sponges with 40% w/w glycerol and no methanol treatment exhibited the greatest recovery (recovery of $98\pm1\%$ of initial height, vs. $78\pm3\%$ recovery in 10 mE control sponges; Supplemental Video SV1). In 10 mE sponges, both control sponges and sponges containing 40% w/w glycerol showed similar fatigue resistance in the form of minimal changes in compressive moduli over 1000 cycles (Supplemental Figure S2).

2.2.2. Diazonium Modified Sponges—The 10% diazonium modified silk sponges (10 minute extraction) with methanol post-treatment were stiffer compared to unmodified silk-only sponges (12.9 ± 0.7 kPa vs. 6.7 ± 0.6 kPa; Figure 2A). As the calculated content of diazonium modified tyrosine residues increased to 30% and 60%, compressive modulus was reduced to 11.0 ± 0.8 and 5.8 ± 0.1 kPa, respectively. Addition of 30% w/w glycerol to 10% and 30% diazonium modified sponges (without methanol post-treatment) reduced the compressive modulus to 9.0 ± 1.7 and 9.2 ± 0.02 kPa, respectively. Silk-glycerol sponges with 60% diazonium modification were not able to maintain their shape, resulting in a gel-like consistency and making compressive modulus measurements impossible.

The application of 80% compressive strain significantly reduced the compressive modulus of post-compressed material for all diazonium modified sponges, with and without glycerol, except for the 60% SAA (“sulfonic acid azosilk”) modified sponges without glycerol. The 10% and 30% diazonium modified sponges with glycerol experienced the largest reductions in compressive modulus following compression, indicating low resistance to large deformations. The peak compressive stress at 80% compressive strain was also recorded and used as a predictor of injection force, as greater peak compressive stresses would likely result in higher injection forces. In general, the addition of 30% w/w glycerol increased the mean peak compressive stresses of all sponges compared to their 0% glycerol counterparts. As extent of diazonium modification increased, peak compressive stress decreased, following the compressive modulus trend.

2.3. Swelling Ratios

Swelling was quantified by measuring the volume of sponges before and after high axial compression. Sponges derived from 10 mE silk fibroin were subjected to 95% compressive strain, or 5% of their original height and dried by wicking away residual and unbound water. Hydration-induced swelling was calculated by measuring the axial and radial dimensions of samples before and after immersion in 1X PBS, thereby measuring media absorption (Figure 3). Volumetric measurements were preferred over mass measurements because of difficulty removing excess water in highly absorptive formulations before weighing. Alternatively, sample dimensions were easily assessed while samples were immersed in fluid. Additionally, this test complements the recovery tests (which focused solely on sample height) because it takes into account the total volume, an important parameter for injectability. After compression, the silk-only (control) sponges swelled to $2.9\times$ their compressed volume upon immersion, exhibiting incomplete recovery to their original volume. In comparison, untreated silk sponges with 30% w/w glycerol swelled to $5.9\times$ of

their compressed volume upon immersion. In general, diazonium modification increased the hydration-induced volumetric expansion of silk sponges after compression. Diazonium sponges with 10%, 30% and 60% tyrosine modification showed 7.7×, 9.5×, 16.9× hydration-induced volumetric expansion from a compressed state, respectively. By adding 30% w/w glycerol, the hydration-induced volumetric expansion of these diazonium modified sponges changed to 5.4×, 14.2× and 71.4×, respectively, however, there was no statistical significance in the difference of hydration-induced volumetric expansion of 10% and 30% diazonium modified sponges with vs. without glycerol.

2.4. Secondary Structure Analysis

2.4.1. Glycerol Only Modified Sponges—Secondary structure was measured to correlate changes in protein structure with differences in bulk sponge mechanical properties (Figure 4, Supplemental Table 2). FT-IR spectra were recorded on materials with and without glycerol, before and after methanol post-treatment to detect differences in crystallinity as a result of each modification. Extraction time did not play a significant role in determining protein secondary structure. In silk-glycerol sponges before methanol treatment, an increase in β -sheet content begins to occur at 15% w/w glycerol and higher. At 20% w/w glycerol and below, it was observed that sponges were either partially or fully soluble in aqueous media, and therefore not used for mechanical analysis without a methanol post-treatment step. The increased β -sheet content in silk materials with high glycerol concentration has been reported in several other studies.^[24–26] In tandem, increasing glycerol concentration to 20% w/w and higher in sponges prior to methanol treatment triggered a decrease in the β -turn, random coil and α -helix content. After methanol treatment, all sponges containing 15% w/w glycerol or less exhibited a significant increase in β -sheet content and significant decreases in random coil and α -helix content. Sponges containing 20% w/w glycerol exhibited a slight decrease in β -sheet content after methanol treatment and slight increases in the content of the other three structural elements. Additionally, the peaks from 800–1150 cm^{-1} have previously been associated with absorption bands from glycerol.^[27] Reduced peak intensity in this region suggests that glycerol has been removed from the material after methanol washing.

2.4.2. Diazonium Modified Sponges—The secondary structural elements of diazonium modified silk sponges were compared between 10 mM 30% w/w glycerol sponge and 10 mM untreated 0% w/w glycerol sponge groups (Figure 5). As seen previously, the addition of 30% w/w glycerol to an unmodified silk sponge results in a significant increase in β -sheet content in comparison to control silk-only sponges ($50.5\% \pm 0.3\%$ vs. $35.6 \pm 1.0\%$). However, with the addition of diazonium modification on the tyrosine residues, we observed a large decrease in β -sheet content within the range of the levels of tyrosine modification tested. In general, β -sheet content decreased in silk-glycerol sponges as diazonium content increased. Additionally, there were significant increases in α -helix and random coil content and significant decreases in β -turn content in diazonium modified silk-glycerol sponges vs. silk-only sponges.

2.5. Pore Morphology and Porosity

To facilitate proper tissue integration, porous materials must contain interconnected, open cell structures large enough to accommodate cellular infiltration. Recognizing that ice crystal formation could impact structure, attempts were nevertheless made to preserve a pore morphology similar to that found in the hydrated state by swelling sponges in deionized water and freeze-drying them once more prior to SEM analysis. Figure 6A shows the macroscopic appearance of unmodified vs. diazonium modified silk sponges, while Figure 6B shows the microscopic pore morphology of cross-sectioned sponges (10 mE silk, with and without glycerol) with different concentrations of diazonium modification. Regardless of the presence of glycerol, we observed that increasing the concentration of tyrosine modification produced pores with enlarged diameters. Furthermore, the addition of glycerol yielded pores with a more rounded morphology, while silk sponges without glycerol typically exhibited jagged or lamellar pores.

Mercury intrusion porosimetry was also used to characterize the pore volume and size distribution of modified silk sponges (Supplemental Table 3). Extraction time impacted pore size distribution and morphology, with longer extraction times resulting in greater polydispersity (Figure 6C). The volume median pore diameters were 13 μm , 18 μm , and 22 μm for 10 mE, 30 mE and 60 mE, respectively. With the addition of 30% w/w glycerol, the pore size distribution functions narrowed for all extraction times, with pore diameters predominantly in the 5–50 μm range (Figure 6D). The volume median pore diameters were 16 μm , 17 μm , and 22 μm for 10 mE, 30 mE and 60 mE, respectively. By modifying silk with hydrophilic sulfanilic acid groups, we observed a large increase in the pore diameters after hydration (Figure 6E). For silk sponges with 10%, 30% and 60% diazonium modification, the volume median pore diameters were 62 μm , 60 μm , and 86 μm , respectively, while the addition of 30% w/w glycerol gave volume median pore diameters of 72 μm , 58 μm , and 108 μm , respectively. All samples were highly porous, with 30 mE, 30% w/w glycerol samples having the lowest porosity at 88.5% void space, and 10 mE, 10% diazonium sponges having the highest porosity at 97.2% void space (volume of void space versus total material volume).

2.6. In vivo degradation

Silk-only (control) and silk-glycerol sponges were subcutaneously implanted into mice to monitor inflammatory response and cellular infiltration into the bulk sponge structure. Sponges were sterilized via a wet autoclave cycle and implanted after being fully-hydrated in 1X PBS. Histological analysis of recovered materials after 2, 4, 8 and 12 weeks showed that there was minimal immune response in both groups, but extensive cell infiltration and material degradation in silk-glycerol sponges compared to control groups (Figure 7). By 12 weeks, H&E staining showed that cells were mostly relegated to the perimeter of the silk-only control sponges. In contrast, cells were able to infiltrate deeper into the core of the silk-glycerol sponges. By measuring the diameter of the excised sponges, we observed initial swelling of the silk-only control over 8 weeks, while silk-glycerol sponges had already begun to degrade by 2 weeks. By measuring the dimensions of explants after surgical recovery, $69.9\% \pm 3.0\%$ of the initial silk-glycerol sponge volume remained after 12 weeks, compared to $93.1\% \pm 17.0\%$ initial volume of the silk-only controls.

3. Discussion

The goal of this study was to investigate biodegradable sponges that exhibit high swelling and shape recovery after compression for use with injectable delivery systems and soft tissue filler applications. As shown in previous work,^[17,28,29] porous silk scaffolds are mechanically robust, tunable and capable of long-term *in vivo* volume retention. Unfortunately, these silk scaffolds are also relatively brittle and tend to permanently deform under high stress. While ideal for large scale defects and soft tissue reconstruction requiring invasive surgical techniques, these 3-dimensional silk scaffolds would require substantial reformulation to achieve the mechanical elasticity required for minimally invasive deployment strategies and small volume defect remediation. Previous approaches to design porous silk materials with shape memory characteristics have focused on chemical modification with poly(*N*-isopropylacrylamide) (PNIPAAm).^[30,31] These materials can swell and compress in response to changes in temperature. This is ideal for injectable applications where triggered material compression is desired during injection and expansion after implantation; however, there are concerns regarding the safety of synthetic and potentially toxic additives such as PNIPAAm in biological systems, as well as their lack of biodegradability. In the present study, we sought to use non-toxic, biologically safe components and chemical modification strategies to design materials that exhibited triggered expansion in response to hydration while also retaining full degradability over time. These materials could theoretically be compressed and injected in an unsaturated state, then expand in hydrated media to their original volume and open-cell structure to accommodate tissue bulking, cell infiltration and remodeling of the surrounding extracellular matrix.

These new sponge designs predominantly focus on merging two separate strategies: blending of silk protein with hygroscopic polyol additives (such as glycerol), and chemical modification of silk with hydrophilic chemistries (such as 4-sulfanilic acid or poly-lysine). Several polyol plasticizers have been reported in recent studies as additives to make silk materials with elastic properties.^[24–26] Preliminary studies have shown that certain polyols, specifically glycerol, have the intrinsic ability to induce physical crosslinking in silk, thus producing blended silk materials with high crystallinity and insolubility. While the current work has focused primarily on glycerol, little is understood regarding the silk-glycerol interactions that result in materials with high flexibility and shape memory properties. In silk-glycerol sponges, two main trends are observed: an increase in post-compression recovery and an increase in the compressive modulus of sponges correlating to increases in glycerol concentration. In Figure 1D, compressive recovery, especially for sponges derived from silk with low extraction times, steadily increases with glycerol content in methanol post-treated materials. In sponges without methanol post-treatment, high glycerol content (40% w/w) showed reduced recovery for 60 mE sponges, no change in 30 mE sponges, and further improvement in 10 mE sponges. It is clear that extraction time plays a role in recovery of untreated silk-glycerol sponges. Assessments of secondary structure by FTIR show an increase in crystallinity (β -sheet content) in high glycerol containing sponges, which likely contributes to mechanical robustness and shape recovery. However, the differences in secondary structure across extraction times are minimal within each group. Previous reports also indicate that silk sponges derived from low molecular weight silks are

prone to collapse under physical loads, a phenomenon attributed to insufficient molecular entanglement.^[32] Low chain entanglement may be the reason sponges based on silk with longer extraction times show reduced recovery after compression.

The increased compressive modulus of silk-glycerol sponges may be explained by the solution glass transition temperatures (solution T_g s) observed with differential scanning calorimetry (Supplemental Figure S3). Glycerol acts as a plastizing agent, lowering the T_g of silk-glycerol solutions. Our freeze-drying protocol primarily dries at -20°C , which exceeds the glass transition temperature of a silk-glycerol (10% w/w) solution. This may result in collapse of the protein cake as crystallized water is sublimed, potentially causing densification. Silk-glycerol sponges that were not methanol post-treated likely swelled in water, and the structural collapse was irrelevant. However, methanol treated sponges may have further crosslinked in this densified state, thus locking in a structure that was more dense than the silk-only control sponges, possibly explaining the increased mechanical stiffness observed in Figure 1A for silk-glycerol sponges post-treated in methanol. It was expected that silk-glycerol materials would have lower elastic moduli compared to silk-only controls;^[25,26] however, the increase in β -sheet formation as a result of glycerol presence may increase stiffness. Future work should study the mechanics of sponges formed at temperatures below the solution T_g , which may recapitulate the trends observed in other work assuming that the T_g of fully dried materials is greater than ambient temperature.

Chemical modification of silk protein with hydrophilic groups has been previously reported.^[21,33] These studies have assessed diazonium modification of silk materials for biocompatibility, drug delivery and protein structure. The work presented here focuses on the yet unexplored material properties of diazonium modified silk protein sponges. As predicted, the addition of hydrophilic chemistries improved the uptake of aqueous media into the silk scaffold, and the inhibition of β -sheet formation allowed for improved relaxation of the protein matrix, allowing volumetric swelling up to $80\times$ its compressed state in certain formulations. Surprisingly, despite the significant reduction in β -sheet content, all diazonium sponges (both methanol and glycerol treated) were mostly insoluble. A more rigorous characterization of the materials is required to determine if sponges are partially soluble and to assess where physical crosslinking is still occurring. Furthermore, all recovery/expansion tests in the current work were done in unconfined conditions. Future analysis should test sponge recovery in a confined state, mimicking implantation. It is unlikely that a 60% SAA / 30% w/w glycerol silk sponges would possess the mechanical robustness ability to store sufficient mechanical energy to expand under confined conditions; however, sponges with lower modification may retain enough structure to exert an expansion force.

Pore morphology, interconnectivity and total volume were important characteristics in the development of our sponges. While highly dependent on cell type, matrices with small pores ($<100\text{ }\mu\text{m}$ diameter) may not accommodate cellular infiltration as pores are too constrained, particularly for larger cells like adipocytes, while excessively large diameter pores may prevent adequate cell attachment.^[32,34] In addition, total porosity and pore interconnectivity both play important roles in uptake of media and cell infiltration, which could impact volumetric swelling, material degradation and cell-mediated remodeling rate. Therefore, we

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electd to use a controlled freeze-drying process which is known to produce scaffolds with high porosity and interconnectivity. Pore shape can be altered during lyophilization by controlling the freezing temperature and ramp rate during cooling, which could effectively cause directional freezing from the lyophilizer tray at rapid freezing rates.^[35] Since the goal was to create mechanically isotropic materials, we chose to use a slow freezing rate in a thermally conductive molding material to improve heat transfer and limit uni-directional ice crystal formation and elongated pores as seen in other studies.^[36] This method produced pores with a highly rounded morphology, as shown in Figure 6B.

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Pore characteristics were measured by mercury intrusion porosimetry (MIP). While many previous studies have relied on scanning electron microscopy or histochemical staining to analyze pore geometry of polymer sponges, these methods can be subjective as material cross-sectioning can cause damage to the microstructure and may not result in pores with well-defined boundaries. MIP alternatively measures the porosity of an intact material throughout its entire volume, so long as pores are open and the volume is accessible to mercury intrusion. Unfortunately, the main limitation with several of these techniques is that the materials must be measured dry, while implanted materials would normally be fully hydrated. Dried sponges would be predicted to have smaller pore diameters compared to the same sponges in their hydrated state, assuming sponges expand when hydrated. Future studies analyzing porous scaffold morphology should include an analytical technical that can measure hydrated scaffolds with high resolution, such as micro-computed tomography or environmental scanning electron microscopy.

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Nevertheless, we observed that the protein extraction time impacted the heterogeneity of the pore network, with longer extraction time resulting in greater polydispersity of pore shapes. This outcome may have to do with the viscosity of the solution, since shorter extraction times produces more viscous silk solutions, and high viscosity limits ice crystal formation and spreading. The addition of 30% w/w glycerol narrows the pore size distribution, limiting pore sizes to the 5–55 μm range. The addition of glycerol has been shown to increase silk solution viscosity which may limit ice crystal growth in high extraction silk solutions;^[24] however, another explanation may be that glycerol can sequester water, enhancing protein-water separation and causing high density and low density protein regions during freeze-drying. Finally, the addition of silk with diazonium-modified tyrosine residues greatly increased the pore size in silk scaffolds. We presume that the hydrophilic chemistry and reduced β -sheet content allowed enhanced uptake of media and relaxation of the protein matrix to accommodate high swelling. As shown in Figure 6B, scanning electron microscopy reveals that the internal morphology of tyrosine modified silk sponges becomes more fibrillar with the addition of glycerol. The increased surface area likely contributes to the 60% SAA / 30% w/w glycerol sponge's ability to uptake fluid. However, the material displays an exceptionally low stiffness and would be unsuitable as a bulking agent or tissue filler. Future studies should be aimed at developing a model correlating mechanics to the relative density of the sponge material. This may help anticipate physical outcomes based on the extent of swelling and predict which formulations will yield the desired mechanics in a hydrated environment.

In all cases, pore morphology was measured in materials that had not previously undergone mechanical compression. Future studies should investigate the impact of high levels of compressive strain on the internal structure of these porous materials. In this work, we assumed that volume recovery implied preservation of the initial matrix geometry; however, this may not be the case. High levels of compression can cause cell walls to collapse, crack or irreversibly deform. Shape fidelity, as assessed by comparing mechanical compression before and after the application of 80% compressive strain, is a useful metric for estimating pore damage, since the mechanical properties of a sponge are in large part related to cell wall integrity and the relative density of the material. Glycerol containing sponges without methanol post-treatment presented no significant difference between pre- and post-compressive moduli independent of extraction time, indicating that glycerol may help to preserve the mechanical integrity of the sponge by increasing cell wall flexibility and / or preventing permanent deformation or fracture. In addition to further studies concerning shape recovery, future work to determine fatigue resistance from a large number of small-strain deformation cycles (similar to the mechanical stresses an implant may experience in the body) will be examined, particularly with focus on nanoscale pore surface morphology (i.e. formation of cracks indicating ductile failure or brittleness over time).

In vivo evaluation revealed that silk-glycerol sponges experience a significant increase in degradation rate compared to silk-only controls when implanted subcutaneously (Figure 7). This is consistent with a recently published *in vitro* degradation study showing increased degradation rate of silk-glycerol sponges in protease solution compared to silk-only controls.^[25] To be clear, sponge degradation was quantified by measuring sample diameter using calipers, not by measuring the area of un-infiltrated space. Sponge diameter and cell infiltration depth were two separate attributes, as we often observed residual silk among the fibrous tissue infiltrate. The increased cellular infiltration into silk-glycerol sponges, however, likely explains the rapid degradation, as silk material breakdown was largely cell mediated. In addition, sponge diameter did not appear to decrease during these experiments; indeed, by histological examination, pore volume appears to be roughly comparable at all time points. If sponges were being compressed *in vivo*, we would likely observe densification of the bulk material. Additionally, we noted the absence of multinucleated giant cells at all time points indicating minimal tissue inflammation.^[37,38]

It is unclear why cells are able to better infiltrate into silk-glycerol sponges. Glycerol is a derivative of fat and oils intrinsic to the body. Residual glycerol present within the silk sponges may be recognized by cells, prompting migration into what is otherwise a foreign material. Previous work has shown that nano-scale pores exist in silk-glycerol scaffolds after the glycerol has been removed by washing.^[25] These pores increase surface area and roughness, and may ultimately assist in cell binding and traction. Alternatively, secondary structure changes in the protein may alter how the body views and interacts with the scaffold.

Lastly, the material mechanics, pore morphology and *in vivo* degradation rate were all examined at a single concentration of silk protein (3% w/v). Higher protein concentrations would likely increase elastic modulus for all sponges, making the materials suitable for repairing a wider range of soft tissues, as well as extend volume retention *in vivo*; however,

it is unclear how the recovery and shape memory characteristics would compare to the current sponges. 3% w/v protein concentration was originally chosen to mimic the modulus of adipose tissue based on work,^[32] though many tissue bulking procedures may require longer volume retention than the current silk-glycerol sponges are capable of in order to support adequate tissue regeneration. For applications requiring short term bulking (3–6 months), 3% w/v silk sponges with 30% w/w glycerol are ideal candidates for rapid tissue integration and recapitulation of native soft tissue mechanics.

4. Conclusions

The approach discussed here improves upon the current silk sponge technology by providing a material which can undergo rapid compressive recovery triggered by the presence of aqueous media. The use of diazonium chemical modifications and glycerol additives give silk protein sponges the ability to undergo significant hydration-induced swelling, making the materials useful in filling void space or wounds in the body and enhancing cellular infiltration for tissue remodeling. These modified silk sponges could be used as soft tissue fillers for skin defects, aesthetic enhancements (breast, thigh, butt, etc.) or as resorbable grafts for facial disfigurement, with volume retention tunable based on sponge density and degree of crosslinking. Additionally, the elastomeric properties of these silk sponges would make them viable candidates for minimally invasive implantation strategies in drug delivery, tissue regeneration or wound clotting.

5. Materials & Methods

5.1. Preparation of Silk Solutions

Silk fibroin solution was prepared as previously reported.^[39] Briefly, silk fibroin protein was extracted from *Bombyx mori* cocoons by boiling in a 0.02 M sodium carbonate solution for 10, 30 or 60 minutes (hereafter referred to as 10 mE (“minutes extracted”), 30 mE, and 60 mE, respectively) to remove sericin. The extracted silk fibroin was washed and dried for 12 hours in a chemical hood before being dissolved in 9.3 M LiBr solution at 60°C for 4 hours, yielding a 20% w/v solution. This solution was dialyzed against distilled water using Pierce Slide-a-Lyzer cassettes, MWCO 3500 Da (Rockford, IL) for 3 days to remove LiBr. The solution was centrifuged (14,000G, 20 minute cycle) to remove aggregates that formed during purification. The final concentration of aqueous silk fibroin (hereafter referred to as silk) was ~6–8% w/v. This concentration was diluted with deionized water to 3% w/v for all experiments and stored at 2–5°C until use. For diazonium coupling modifications only, silk solution was additionally dialyzed against borate buffer (100 mM borate, 150 mM sodium chloride, pH 9) (Buph borate buffer packs; Pierce, Woburn MA) for 24 hours.

5.2. Preparation of Modified Silk Blends

Two methods were employed to fabricate silk sponges with enhanced hydrophilicity and shape memory characteristics. All formulations are summarized in Table 1. First, aqueous silk protein was blended with specific polyol additives, such as glycerol (Sigma-Aldrich, St. Louis, MO), as described previously.^[26] Briefly, glycerol solution at 700 mg/mL was added to silk solutions at various weight:weight (w/w) ratios: 1:99, 5:95, 10:90, 15:85, 20:80,

30:70 and 40:60 weight glycerol:weight silk. Solutions were homogenized via gentle inversion for 1–2 minutes until phase separation was no longer visible.

The second method of silk modification was by a diazonium coupling reaction to modify tyrosine residues with sulfanilic acid.^[21] Briefly, chilled solutions of sulfanilic acid (30 mL in water, 0.065 M) and p-toluenesulfonic acid (7.5 mL, 1.76 M) were vortexed and kept in an ice bath for 10 minutes. Next, sodium nitrite (7.5 mL, 0.8 M) was added to the solution to prepare the diazonium salt, which was vortexed briefly and kept on ice for an additional 30 minutes. Diazonium salt solution was then added to 10 mM silk solution in borate buffer to achieve a theoretical yield of ~60% tyrosine modification. Modification was confirmed and quantified by spectrophotometric analysis. The concentration of azo groups was derived from the max absorbance at 325 nm using an extinction coefficient of 22,000 M⁻¹ cm⁻¹.^[22] This value was divided by the total moles of tyrosine present in the silk amino chain, assuming 277 tyrosine residues per molecule. The calculated modification (57% tyrosine modified) matched the theoretical modification (58.1% tyrosine modified, determined based on the moles of tyrosine present and moles of diazonium added during the reaction). Diazonium modified silk solution was diluted in unmodified 10 mM silk solution to achieve blends with 10% and 30% modification of tyrosine residues. Solutions were diluted to 3% w/v silk protein and stored at 2–5°C for no longer than 24 hours before use. In the figures, diazonium modified sponges are referred to as SAA (“sulfonic acid azosilk”).

5.3. Freeze-Dry Processing and Post-Treatment

A controlled lyophilization method was used for the consistent generation of porous networks within silk matrices. Modified silk blends were frozen in a custom designed aluminum mold (Supplemental Figure S4). The aluminum mold contained 380 wells (12 mm Ø; 20 mm depth) for making large batches of sponges. 1.5 mL aliquots of silk solution were added to each well before being transferred to a VirTis Genesis 25L Super XL Freeze Dryer (SP Scientific, Stone Ridge, NY). Samples were frozen using a modification of a previously published protocol.^[40] Briefly, samples were cooled from ambient temperature and held at 5°C for 1 hour, then cooled to –45°C at 0.05°C/min and held for 12 hours. Primary drying was performed at –20°C at 50 mT vacuum until the Pirani gauge pressure registered lower than the capacitance manometer pressure.^[41] Secondary drying was performed at 4°C for 10 hours.

5.4. Post-Processing

Upon completion of lyophilization, sponges containing 0–15% (w/w) glycerol were immediately immersed in 90% (v/v) methanol for 12 hours, then washed and stored in deionized water until testing. Sponges containing 20% (w/w) or more glycerol were post-processed in one of two ways: 1) methanol treatment for 12 hours and storage in deionized water, or 2) storage in deionized water with no methanol treatment. For each group, a subset of samples was removed from deionized water and lyophilized a second time. These samples were stored in closed tubes with desiccant packets at room temperature for image analysis and porosity characterization.

5.5. Analytical Techniques

5.5.1. Compressive Modulus, Recovery, Swelling and Fatigue—All mechanical properties of silk sponges were assessed via unconfined compression testing using a TA Instruments RSA3 Dynamic Mechanical Analyzer (TA Instruments, New Castle, DE) hydrated in a 1X PBS bath. Prior to testing, sponges were cut into a uniform cylindrical size (8 mm Ø; 5 mm height) and rehydrated in 1X PBS under a vacuum of ~60 kPa for 30 minutes to ensure proper hydration throughout the material. Samples were then kept in PBS for 12 hours at room temperature and pressure. After rehydration, the sponges were loaded between stainless steel parallel plates within an immersion bath containing 1X PBS. All samples were analyzed with an initial preload of 0.5 grams to ensure proper contact between the upper plate and the sample. A custom macro was used to measure (in sequence) the elastic modulus, hysteresis and shape recovery of the sponges before and after the application of 80% axial strain (20% of initial height) to simulate a demanding physical stress, such as injection. Before each test, sponges were pre-conditioned via four cycles to 40% axial strain at a 1 mm/min ramp rate and allowed to recover in PBS for 30 minutes. After pre-conditioning, the initial height of the sample was recorded with the applied 0.5 gram preload.

Sponge samples were subjected to the following sequence of tests: 1) load/unload to 20% strain (for pre-compressed modulus; referred as “pre”); 2) load/unload to 80% axial strain (high compression cycle); 3) load/unload to 20% axial strain (for post-compressed modulus; referred to as “post”). The rate of displacement was kept constant at 1 mm/min for all tests. The compressive modulus was calculated by the slope of the linear elastic region of each stress-strain curve in the range of 1–5% axial strain.

Recovery was calculated by comparing the height of the sponge (with applied 0.5 gram preload) pre vs. post 80% axial strain. Hysteresis was measured as the ratio of the area under the curve between the loading and unloading regions of each strain sweep. For fatigue measurements, storage modulus was monitored during cyclic loading at frequency of 0.5 Hz at either 1, 5 or 10% axial strain for 1000 cycles. Swelling ratios were calculated by measuring the axial and radial dimensions of sponges at a chosen maximal compression (5% initial height; 95% axial strain) and after dehydration by wicking away residual water. Samples were then rehydrated in 1X PBS for 30 seconds, and the sample volume was re-measured and compared to the compressed volume.

5.5.2. Mercury Intrusion Porosimetry—Mercury intrusion porosimetry measurements were conducted to evaluate the porosity and pore size distribution of modified silk sponges after hydration and subsequent lyophilization to dry the material while preserving the hydrated shape. Cylindrical sponge samples (variable size based on degree of swelling) were analyzed via a Quantachrome PoreMaster mercury intrusion porosimeter (Quantachrome Instruments, Boynton Beach, FL). For each formulation, five samples were massed and loaded together into a single glass sample cell with a 0.5 cc stem volume. A low pressure intrusion cycle (pressure range: ~0.7–50 psi, or 4.8–340 kPa, corresponding to a pore size range of ~300–10 µm) was performed to evaluate pore sizes within the micrometer range. Results are reported as a histogram of pore size (in µm) vs. a pore size distribution function,

F_V , where $F_V = -[dV/d\log(D)]$, V is the cumulative pore volume, and D is the pore diameter.

5.5.3. Fourier Transform-Infrared (FT-IR)—FT-IR and Fourier self-deconvolution (FSD) were used to analyze and quantify protein secondary structure in the silk sponges. Sponges were measured using a JASCO FTIR 6200 spectrometer (JASCO, Tokyo, Japan) combined with a MIRacle™ attenuated total reflection (ATR) germanium crystal. Background and spectral scans were measured from 4000–600 cm^{-1} at a resolution of 2 cm^{-1} for 32 scans per sample. Sponge samples were measured either as processed (immediately after lyophilization) or after hydration. If hydrated, sponges were dried in an air flow hood for 12 hours and kept under desiccant until analysis to reduce interference from water. Secondary structure was quantified within the amide I region (1590–1710 cm^{-1}) by FSD and peak fitting using Opus 5.0 software (Bruker, Billerica, MA). Each raw IR spectrum underwent baselining, 9-point smoothing (Savitzky-Golay method) and deconvolution using a Lorentzian line shape with half-bandwidth of 25–26 cm^{-1} and noise reduction factor of 0.3. Apodization was performed via a Blackman-Harris function. The deconvoluted amide I region was curve fit with 11 Gaussian line shape profiles as previously described.^[42] Peaks were assigned to specific secondary structural elements based on previous work,^[43] and relative contributions of each element were calculated using the ratio of the area under the peak to the sum of the areas of all fitted amide I peaks.

5.5.4. Differential Scanning Calorimetry—Silk solutions containing varying concentrations of glycerol (0–10% w/w) were diluted to 4% w/v silk protein and analyzed using a TA Instruments G100 differential scanning calorimeter (New Castle, DE). 10 μL of sample was placed in a hermetic aluminum pan, covered with a lid and sealed using a sample press to reduce evaporation during heating. Temperature-modulated measurements were performed on samples cooled to -45°C and heated at a rate of $2.0^\circ\text{C}/\text{min}$ to 20°C , with a modulation period of 60 s and temperature modulation amplitude of $\pm 0.318^\circ\text{C}$. The glass transition temperature of the solution was derived from the location of the inflection point in the associated step observed in the data.

5.5.5. Scanning Electron Microscopy (SEM)—SEM was used to evaluate the pore morphology of modified silk sponges. Samples were imaged using a Zeiss EVO MA10 scanning electron microscope (Carl Zeiss AG, Germany). To assess pore shape and size in a hydrated environment, cylindrical samples were imaged after hydration and subsequent re-lyophilization to prevent shrinkage or collapse during drying. Samples were cut along the horizontal axis, mounted onto copper tape, and sputtered with gold before imaging.

5.5.6. In Vivo Subcutaneous Implantation—All animal studies were conducted under protocols approved by and in compliance with regulations specified by the Tufts University Institutional Animal Care and Use Committee (IACUC) and the National Institute for Health's Office of Laboratory Animal Welfare (OLAW). Balb-C female mice at 6–8 weeks of age were purchased from Charles River Labs (Wilmington, MA) and allowed to acclimate for 1 week prior to implantation studies. Animals were anesthetized via inhalation of isoflurane at 3% for induction and maintained at 2% during surgical procedures. All animals

were kept on heating pads throughout anesthesia. Silk sponges were sterilized in a wet autoclave cycle for 25 minutes and equilibrated in sterile 1X PBS for 24 hours prior to surgery. Mice were randomly assigned four time points (2, 4, 8 and 12 weeks) with two animals per time point. Each mouse received two types of silk sponge samples (5 mm Ø; 3 mm height): 3% w/v silk-only and 3% w/v silk containing 30% w/w glycerol (N=4 per group per time point). Sponges were implanted subcutaneously in the scapular and dorsal inguinal regions. At each time point, animals were euthanized and silk sponges were excised along with surrounding tissue for histological examination. The sponge diameter for each sample was measured using calipers, after which the samples were fixed and stored in formalin.

5.5.7. Histochemistry—Excised tissues were fixed in 10% neutral buffered formalin and embedded in paraffin wax following dehydration by xylene and graded ethanol baths. Samples were sectioned, mounted onto glass slides and stained with hematoxylin and eosin (H&E; Sigma-Aldrich, St. Louis, MO) to visualize cell infiltration. Samples were imaged using a Zeiss Axiovert 40 CFL light microscope with a 10X objective lens (Carl Zeiss, Germany).

5.6. Statistics

Data is expressed as mean $\pm$ one standard deviation. For mechanical data (N=3 per group) and secondary structure analysis (N=3 per group), 1-way ANOVA and Tukey post-hoc analysis for $p < 0.05$ were used to determine significance of experimental groups compared to the silk-only controls. The significance of differences in mechanical properties of “pre” and “post” compressed sponges (N=3 per group, dependent sampling) was determined by paired t-test with statistical significance accepted at $p < 0.05$. The significance of changes in *in vivo* degradation was measured via unpaired t-test between silk-only controls and silk-glycerol samples (N=4 per group per time point) at each time point for $p < 0.05$. Where given, * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Mechanical compression analysis for silk-glycerol sponges. **A)** Stress-strain curves for 10 mE silk sponges; silk-only with methanol wash post-treatment, silk with 40% w/w glycerol with no post-treatment, and silk with 40% w/w glycerol with methanol wash post-treatment. **B)** Evaluation of hysteresis as defined by the ratio of the area under the curve for the loading cycle vs. that of the unloading cycle. Smaller values indicate less hysteresis during compression cycles. **C)** Compressive moduli of silk sponges with varying extraction times and glycerol concentrations. **D)** Height recovery of sponges after the application of 80%

compressive strain. In C and D, left panel sponges were post-treated with a methanol wash, right panel sponges were not post-treated. All data are means with error bars of $\pm$ one standard deviation for N=3 per group. In panel C, the significance of differences in mechanical properties of “pre” and “post” compressed sponges (dependent sampling) was determined by paired t-test. In panel D, 1-way ANOVA and Tukey post-hoc analysis were used to determine significance of differences in recovery of the experimental groups compared to the silk-only controls. Statistical significance was accepted at $p < 0.05$ and indicated with an asterisk (*). Additional information in Supplemental Table 1.

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Figure 2.

Mechanical compression analysis for diazonium modified silk sponges. **A)** Compressive moduli of 10 mE silk sponges with varying concentrations of modified tyrosine residues and glycerol. **B)** Peak compressive stress at 80% compressive strain. Data presented as means with error bars of one standard deviation. In panel A, the significance of differences in the mechanical properties of “pre” and “post” compressed sponges (N=3 per group, dependent sampling) was determined by paired t-test with statistical significance accepted at $p < 0.05$, indicated with an asterisk (*). SAA = sulfonic acid azosilk. Additional information in Supplemental Table 1.

Figure 3.

Volumetric expansion of silk sponges in 1X PBS after the application of 95% compressive strain. The combination of tyrosine modification by sulfanilic acid (% SAA) with glycerol addition increases hydration-induced volumetric expansion of compressed materials compared to silk-only controls. At high levels of diazonium modification, the swollen sponges lose their rigidity, assuming an amorphous shape and displaying a gel-like consistency. The inset shows all groups excluding 60% SAA, 30% w/w glycerol. Data presented as means with error bars of one standard deviation. SAA = sulfonic acid azosilk; inset: SO = silk only; G = 30% w/w glycerol; S = % sulfonic acid azosilk.

Figure 4.

Secondary structure of silk-glycerol sponges by FT-IR analysis. Sponges containing varying amounts of glycerol were analyzed for secondary protein structure before and after post-treatment in methanol. Blend ratios: (a) Silk-only; (b) 1% w/w glycerol; (c) 5% w/w glycerol; (d) 10% w/w glycerol; (e) 15% w/w glycerol; (f) 20% w/w glycerol; (g) 30% w/w glycerol; (h) 40% w/w glycerol. Amide I region peak fit data presented as means with error bars of $\pm$ one standard deviation. Statistical analysis presented in Supplemental Table 2. Significance of differences between experimental groups and silk-only controls determined by 1-way ANOVA and Tukey post-hoc analysis (N=3 per group) for $p < 0.05$.

Figure 5.

Secondary structure of diazonium modified silk sponges by FT-IR analysis. Modification of tyrosine residues with hydrophilic chemistries resulted in a significant reduction in β -sheet content. Formulations: a) silk only; b) 30% w/w glycerol; c) 10% SAA; d) 10% SAA, 30% w/w glycerol; e) 30% SAA; f) 30% SAA, 30% w/w glycerol; f) 60% SAA; g) 60% SAA, 30% w/w glycerol. Amide I region peak fit data presented as means with error bars of $\pm$ one standard deviation. Significance of differences between 0% glycerol and 30% glycerol sponges (N=3 per group, independent sampling) determined by unpaired t-test with statistical significance accepted at $p < 0.05$, indicated with an asterisk (*). SAA = sulfonic acid azosilk.

Figure 6.

Pore morphology of modified silk sponges. **A)** Macroscopic presentation of unmodified silk-only sponge (left) and diazonium modified sponge (right). **B)** Scanning electron microscopy showing the inner material morphology for sponges with and without glycerol and tyrosine modification. Glycerol imparts a rounded pore shape, while increasing diazonium modification elicits formation of fibrillar structures. Scale bars are 100 μm for main images, 200 μm for insets. **C and D)** Pore size distributions by mercury intrusion porosimetry. The addition of 30% w/w glycerol narrows the distribution and reduces the porosity for sponges

derived from silk extracted for longer times. **E and F)** Pore size distributions for diazonium modified sponges. The swelling resulting from tyrosine modification causes a shift towards larger pore diameters. SAA = sulfonic acid azosilk; Gly = glycerol.

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Figure 7.

In vivo subcutaneous implantation of silk-glycerol sponges in mouse models. Histological examination shows enhanced cell infiltration in glycerol containing sponges vs. silk-only controls, where cell infiltration is limited to the perimeter of the material. Macroscopic evaluation of sponge volume reveals that glycerol addition increases the *in vivo* degradation rate. Dotted circles are to allow visual comparison of silk only (white) vs. 30% w/w glycerol (red) groups at 2 and 12 week time points. The significance of changes in the extent of *in vivo* degradation was measured via unpaired t-test between silk-only controls and silk-glycerol samples (N=4 per group per time point) at each time point for $p < 0.05$, indicated with an asterisk (*).