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Injectable Silk Foams for Soft Tissue Regeneration

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Abstract

Soft tissue fillers are needed for restoration of a defect or augmentation of existing tissues. Autografts and lipotransfer have been under study for soft tissue reconstruction but yield inconsistent results, often with considerable resorption of the grafted tissue. A minimally invasive procedure would reduce scarring and recovery time as well as allow for the implant and/or grafted tissue to be placed closer to existing vasculature. Here, we demonstrate the feasibility of an injectable silk foam for soft tissue regeneration. Adipose derived stem cells survive and migrate through the foam over a 10 day period *in vitro*. The silk foams are also successfully injected into the subcutaneous space in a rat and over a 3 month period integrating with the surrounding native tissue. The injected foams are palpable and soft to the touch through the skin and returning to their original dimensions after pressure was applied and then released. The foams readily absorb

lipoaspirate making the foams useful as a scaffold or template for existing soft tissue filler technologies, useful either as a biomaterial alone or in combination with the lipoaspirate.

Keywords

Biomedical Application; Regenerative Medicine; Silk; Porous Scaffold; Soft Tissue; Biomedical Applications; Regenerative Medicine; Silk; Porous Scaffolds; Soft Tissue

1. Introduction

Soft tissue fillers are needed for soft tissue defects resulting from congenital abnormalities, trauma, or tumor resections and constitute about 4.8 million reconstructive surgery cases per year in the US alone.^[1,2] Autografts and lipotransfer have been studied for soft tissue fillers, however, their success is unpredictable, with 20-90% graft resorption within months.^[3-5] This leaves significant problems for the patient and thus the regeneration of soft tissue defects remains an unmet clinical need. Soft tissue fillers are also used in various cosmetic surgery applications. They can serve as injectable dermal fillers for reducing the appearance of wrinkles. This is a growing market, increasing 172% from 2000 to 2010, with the use of fat injections up 14% from 2009-2010 alone.^[2] Natural biomaterials, such as collagen or hyaluronic acid, have seen decreased usage as injectable dermal fillers when compared to synthetic biomaterials, such as polylactic acid and polymethyl-methacrylate, which are up 41% and 60%, respectively, from 2009-2010 and offer extended volume maintenance over the natural polymeric alternatives.^[2]

The major problem with autografts and lipotransfer as soft tissue fillers remains graft resorption and loss of volume maintenance, thought to be the result of avascular necrosis and/or degrading matrices. An injectable format for such systems would be a significant improvement over surgical intrusion as a way to minimize patient discomfort, reduce donor site morbidity if combined with fat grafting, and shorten recovery time. In addition, injecting in several but smaller volume amounts allows more of the injected material to be positioned closer to host vasculature, potentially leading to improved graft survival.

A useful biomaterial for such a scenario would be biocompatible, injectable, have controllable degradation rates and be tailorable to the variety of soft tissue needs, such as volume, porosity and mechanical properties. Silk, a protein biomaterial that has been used for decades for sutures,^[6,7] and recently FDA approved for use as a surgical mesh, can be processed into a variety of formats with control of degradation rate.^[8] We have previously shown that silk biomaterials can support soft tissue formation and maintenance, both *in vitro* and *in vivo*^[9-13] and silk porous sponges can be designed to degrade in periods of over 1 year *in vivo*.^[13,14]

Our previous work highlights our ability to design silk scaffolds that are robust and slowly degrading.^[13,14] However, the trade-off for having such robust scaffolds is the lack of deformation. In other words, those scaffolds are well-suited for implantation and large defects, but poorly suited for smaller, injectable filling needs. To address this unmet need, we sought to develop porous silk scaffolds using a foaming method and therefore to

distinguish this new format from our previous format (salt-leached sponge), we refer to this new format as silk foams. These foams are designed to be very deformable so as to fit through a needle, but then regain their shape immediately post injection. Further, since these foams would likely be used in small defects such as facial defects, a smooth and soft look from outside the injection site is desirable.

The objective of the present research was to study and develop an injectable silk format that allows for tissue ingrowth like the implantable porous sponges, yet be injectable and therefore useful for minimally invasive soft tissue filler applications. In this study, we evaluated the injectability and *in vitro* and *in vivo* biocompatibility of silk foams as a novel injectable biomaterials format for an application such as soft tissue regeneration.

2. Results

2.1. Development and injectability of silk foams

The custom-designed injection gun injector (**Figure 1A**) was simple to use, easy to sterilize and could be handled during the *in vivo* process. The freeze-processing technique described can produce silk foams (**Figure 1B**) that can be successfully injected from the injection gun when guided with the ramrod. Foam stiffness and porosity can be modified based on silk concentration; stiffness increases with concentration, while porosity decreases (1% foam pore diameter 500 microns, 3%: 400 microns, 6%: 300 microns (**Figure S1**). The foam was compressed during injection but regained its original dimensions after deployment and in subsequent work there was no significant change in mechanical properties after the process. Expansion ratios have been demonstrated up to 2.5x (not shown). Elastic modulus and yield stress increased as silk concentration increased (**Table 1**). Elastic modulus was found to be 21.91 ± 3.62 kPa and 101.90 ± 5.00 kPa for 3% and 6% silk foams, respectively. Yield stress was 3.71 ± 0.85 kPa and 14.78 ± 1.44 kPa for 3% and 6% silk foams, respectively.

2.2. Cells adhere and migrate through foam

For clinical feasibility, it was important that cells are able to attach and migrate throughout the foam over time. Adipose derived stem cells (ASCs) were seeded on the 3% silk foams and maintained for 10 days. During foam generation layers of pores had formed (**Figure 2A**). To ensure that these layers did not form a barrier to cell migration into the foams we imaged the foams midway through and the face opposite to the seeded surface. After 10 days in culture, the cells had migrated through the foams (**Figure 2A**). Cells were seen throughout the foam except at the bottom surface (opposite the seeding surface). Cells lined the pores of the foam, but did not fill up the pores at this time point (**Figure 2B**). We assessed the foams for DNA content at 1 and 10 days post-seeding. There was a significant decrease in cell number from day 1 to day 10 ($p < 0.0359$) commonly seen in 3D cultures as cells that have not attached well are washed away during media changes (**Figure 2C**).^[16] *In vitro* degradation of foams in collagenase was significant ($p < 0.001$) at days 30, 60 and 90 for silk foams 1 and 3% w/v, while degradation was significant ($p < 0.001$) after at days 60 and 90 for 6% silk foams (**Figure 2D**). Foams in nonspecific protease (protease XIV) had degraded completely by day 30 for 3 and 6% silk foams, and day 7 for 1% silk foams. Significant degradation ($p < 0.001$) occurred rapidly in protease XIV, occurring after 1 day in

1 and 3% silk foams, and day 14 for 6% silk foams (Figure 2D). Silk foams in a buffer control had minor but significant degradation at day 90 (1% silk foam $p<0.001$, 3% silk foam $p<0.006$, 6% silk foam $p<0.0047$) (Figure 2D).

2.3. In vivo injections

Foams were successfully injected and all rats healed well with no adverse effects seen as a result of the injections. Importantly, the foams did not migrate away from the site of injection. The appearance of the foams from outside the skin was smooth and soft when palpated. By day 14, the puncture site showed no negative signs such as scarring or infection (Figure 3A). The foams were visible and palpable through the skin, and soft to the touch, out to 90 days or the end of the study. No obvious differences were seen between the different w/v% silk foams. At 14, 30, 60 and 90 days the rats were sacrificed and the foams were explanted. By day 14 the foams had vascularity leading to them and were clearly visible (Figure 3B). At 90 days, the vascularity leading to the foams had decreased as did their visibility (Figure 3B) as they began to degrade. At day 14, foam structure was the most visible in the 3 and 6% foams, while tissue had begun to infiltrate the 1% foam (Figure 3C). After 90 days all foams had significant tissue infiltration (Figure 3C).

In vivo degradation rates were not affected by initial silk foam concentrations (Figure 3D). After 90 days *in vivo*, 1% silk foams had $44 \pm 14\%$ remaining, 3% silk foams had $40 \pm 14\%$ remaining and 6% silk foams had $55 \pm 16\%$ remaining (Figure 3D). Foams degraded significantly after 60 days implantation ($p<0.03$) but no further increase in degradation was seen between 60 and 90 days (Figure 3D).

The explanted foams were sectioned along the cross-sectional face pictured in Figure 3C. The cells and tissue had completely infiltrated the foams (Figure 4A). The foams had more tissue present with lower silk concentrations (Figure 4A). The silk pore walls were present through 90 days indicating retention of intact structure to this point. Masson's Trichrome staining showed a mix of collagen with mostly cells (Figure 4B). By 90 days, it was difficult to discern any differences between the various study groups. Vessel density did not vary significantly across silk foam concentrations or over time (Figure 4C). However, greater ranges of vessel numbers were seen at day 14 as compared to day 90 (Figure 4C). SEM images showed that the foams maintained open pore structures at 14 days (Figure S1). Less matrix deposition was visible with increased silk concentration, similar to the histological results. By 90 days, the silk foam structure was only visible in the 6% silk foam group (Figure S1). Tissue and cells had completely remodeled the 1% and 3% silk foam groups by 90 days, making it more difficult to distinguish the silk structure. Macrophages (M0, CD68+) were present within the foam independent of silk concentration (Figure 5A). At 14 days, both M1 (CD80+, pro-inflammatory) and M2 (CD163+, tissue-healing) polarized macrophages were seen (Figure 5A). There was no difference in the number of M0, M1 or M2 macrophages detected at day 14 (Figure 5B). However at 90 days, there was a greater presence all phenotypes, M0, M1 and M2 polarized macrophages. M1 positive cells were found preferentially at the silk interface while M2 positive cells were found more throughout the tissue (Figure 5A). The fold increase in macrophage presence from day 14 to 90 for M0 macrophages was 1.5, 2.6 for M1 and 3.5 for M2.

3. Discussion

The goal of this work was to determine the *in vivo* utility of injectable silk foams for an application such as soft tissue regeneration. For regenerative medicine applications, the foams must be easy to handle, able to be sterilized, storable and able to be seeded with a biological component such as ASCs and lipoaspirate. Our previous study with implantable silk sponges yielded fully regenerated tissue over an 18 month period in a rat model.^[13] The silk sponges implanted without any biological additions (e.g., cells, lipoaspirate), yielded well-organized connective-like tissue in this *in vivo* study. However, when pre-seeded with lipoaspirate or pre-cultured ASCs, the tissue that formed was observed to be adipose-tissue-like based on histological appearance of mature adipocytes and staining for lipid droplets by Oil Red O. The silk sponge degraded at a rate slow enough to allow for cells to repopulate the silk matrix template. In the present study, we aimed to develop a similar system to the implanted silk sponges, but for use in minimally invasive techniques. Development of a minimally invasive system allows smaller volumes of soft tissue fillers to be injected and for the grafted tissue to be closer to the surrounding vasculature; providing improved graft survival and integration with less necrosis. Yoshimura et al, have used this injection method in women for cosmetic breast augmentation but without a biomaterial carrier.^[17] In their study, 270 mL of stem cell enriched lipoaspirate was injected and only about 100-200 mL of the initial volume remained after 2 months. The inclusion of an injectable matrix with controllable degradation rates, such as the silk matrices in the present study, could act as a template for regeneration while the body remodels around the matrix. For such a goal, it is important for the biomaterial matrix to degrade on a timeframe that matches that of the regeneration process in order to maintain structure and volume.

The study presented here demonstrates the successful use of injectable foams *in vivo*. To ensure an easy, stable and reproducible delivery method, we developed an injection gun in which we can attach a catheter containing a silk foam. The injection gun contains a drawrod to guide a ramrod behind the foam, gently injecting it with little force. This eliminated the need for a carrier solution which could potentially clog the needle. After 90 days, about 50% of the foam volume remained in each group (Figure 3D). In order to be effective for soft tissue regeneration, the degradation rate must be commensurate with soft tissue regeneration *in vivo*. Conventional methods for processing silk biomaterial scaffolds, such as by methanol treatment or autoclaving, decrease the rate of degradation by increasing crystallinity, and thus were employed here. Future iterations of these foams may include increasing silk concentration to further decrease the degradation rate, as we have previously shown with implanted silk sponges.^[14] The pore size may also need to be modified to account for the decrease in injectability when the percent silk in the formulation is increased. While the 6% silk could be injected, this was more difficult to achieve than with the 1% and 3% silk foams. Pore size can be altered by controlling freezing temperature, and/or cycling between freezing and partially thawed states. These silk foams exhibited mechanical properties within the range of similarly processed foams. Foams comprised of a synthetic biomaterial, such as poly (L-lactic acid) were found to be more stiff, with an elastic modulus of 5 MPa.^[18] While natural biomaterials such as collagen-glycosaminoglycan foams were much less stiff with elastic modulus of 208 Pa.^[19] However, the elastic modulus of the 3% silk

foam was found to be an order of magnitude greater than human adipose tissue, while the 6% silk foam was found to be 2 orders of magnitude greater than human adipose tissue.^[20,21] Although these properties do not match the tissue mechanics initially, it would be likely that as the silk foam degrades it will begin to approach the native tissue properties and therefore the range of mechanical properties of these silk foams are desirable.^[22]

Using these foam fabrication methods we could design foams of a volume of about 1500 cm³, making them amenable to large scale production. For these studies we fabricated these larger foams and cut foams to the desired size. Although not demonstrated here, it was possible to extrude larger diameter foams using wider needles. However for these studies we did not want to exceed the 14G needle sized used, with the maximum size foam possible being 6 mm in diameter.

The *in vitro* preliminary cell study established that ASCs migrated through the foams. The cells lined the walls of the pores. The silk foams were also shown to quickly adsorb the liquid fraction of lipoaspirate (not shown) making this system attractive for follow-on lipo-injection type applications. Lipo-injections are commonly done clinically, however, we do not know if the addition of silk will impact lipoaspirate tissue survival. Lipoaspirate is a mix of mature adipocytes, adipocyte pre-cursors, proteins, and growth factors and it is estimated that about 30% of the adipocytes rupture during lipoaspirate processing.^[23] Future studies should use these methods to determine cell viability before and after injection with the silk matrix.

The *in vivo* study in a subcutaneous rat model demonstrated the feasibility of using silk foams in a minimally invasive context. Macroscopically, the foams integrated well over time and were seen with blood vessels feeding into the foams. Microscopically, a limited number of small blood vessels were seen in H&E sections, suggesting that the large vessels seen macroscopically had not induced a significant vascularization within the silk foam. In our previous study, with implantable silk porous sponges, mature vasculature was detected only after 6 months of implantation.^[13] While our objective in this study was to show proof-of-concept for such an injectable system, expanding the study to include longer time points would be necessary to determine the efficacy for stable, vascularized regenerative applications. Macrophages (M0, CD68+) were present in histological sections in all groups. Macrophages can be pro-inflammatory (M1, CD80+) or a tissue remodeling phenotype (M2, CD163+).^[24,25] To better understand the type of tissue remodeling, we investigated macrophage polarization phenotypes. There was an increase in the number of all phenotypes from 14 to 90 days, suggesting interplay between chronic inflammation and tissue remodeling. Interestingly, the fold increase in M0 positive cells was 1.5 times greater at day 90 than at day 14, 2.6 for M1 and 3.5 for M2. Not surprisingly, M1 polarized macrophages tended to be concentrated near the silk biomaterial and are known to secrete proteinases that degrade matrix. While M2 polarized macrophages, known to secrete factors to induce vascularization and promote tissue remodeling, were found more dispersed within the new tissue within the foam. Taken together, this macrophage profile is encouraging, with a balance of all phenotypes but a greater increase in the tissue remodeling phenotype. It should be mentioned that these markers along the macrophage lineage have some overlap in positive staining for CD68, CD80 and CD163.^[17] In future studies, macrophage staining

could be corroborated with cytokine and proteinase expression. The addition of a cellular component also affects polarization and as such a follow-up study including the effect of lipoaspirate seeding on macrophage polarization, and therefore, remodeling would be of interest.^[24,25]

Mechanical properties of these foams did not appear to change upon examination post-explantation. The foams remained robust and regained their shape after deformation. However, we were unable to perform bulk mechanical testing on these foams after in vivo implantation given the size restrictions of the needle and therefore foams used. To perform reliable bulk mechanical testing as with our initial testing we would have needed a final foam diameter of ~10 mm. Given the degradation rates seen ~50% in these studies, we would have needed to inject a foam of ~20 mm.

4. Conclusion

This study provides a foundation for using silk foams as injectable matrices for clinical applications such as augmenting soft tissue regeneration, as fat grafting alone has a history of yielding inconsistent results. Previously, we have shown that slowly degrading silk sponges can act as a template for tissue regeneration. In this study, we aimed to translate that system into a minimally invasive system, reducing scarring, decreasing recovery time and decreased donor site morbidity. The injectable foams offer a new option for filling soft tissue defects, with material, mechanical and biological results consistent with tissue regeneration.

5. Experimental Section

Materials

Bombyx mori silkworm cocoons were purchased from Tajima Shoji Co. (Yokohama, Japan). DiI, cell labeling dye and cell culture reagents were purchased from Life Technologies (Grand Island, NY). Histological solvents were purchased from Fisher Scientific (Pittsburgh, PA) and histological reagents and Masson's Trichrome Stains Kit were purchased from Sigma-Aldrich (St. Louis, MO).

Silk foam process

Silk solution was prepared as we have published.^[26] Briefly, cocoons were chopped and placed in boiling NaCO₂ (0.02M) for 30 minutes to remove sericin, and then washed 3 times in ultrapure water. The resulting silk fibroin fibers were left to dry overnight. The dry silk was solubilized in LiBr (9.3 M) in 20% w/v at 60°C for 4 hours. The silk solution was then dialyzed in ultrapure water in a 3,500 molecular weight cutoff membrane for 2 days with a total of 6 water changes, to remove the LiBr. Silk solutions of 7-8% were obtained and diluted with ultrapure water to yield the final solutions for this study. A silk foam sheet was created by using a freezer processing technique. Silk solution (1, 3, 6% w/v), was poured into a plastic Petri dish. The dishes were then stored in an EdgeStar Model FP430 thermoelectric cooler maintained at -8 to -10°C for 3 days. At this temperature, free water in the silk solution freezes before water bound to silk fibroin chains, causing local increase in fibroin concentration and assembly. The resulting material appears gel-like. The material

was then transferred to a VirTis Genesis (Model 25L Genesis SQ Super XL-70) (SP Industries, Warminster, PA) Lyophilizer for 3 days to sublimate free water from the gel-like solid. The result of lyophilization was a silk foam containing a consistent interconnected fine-pore structure. The foam was soaked in 70% methanol for 1 hour to induce beta sheet formation. While 10% shrinkage of the foam occurred, the result is a fairly stiff foam when dry and a tough, compliant foam when fully hydrated. Foams were autoclaved for sterilization prior to seeding and implantation.

Injection gun design

A Hauptner syringe (Neogen's Ideal Instruments, Lexington, KY) was custom-modified to implant silk foams subcutaneously for a rat implantation study. The pistol-style syringe has a spring-loaded handle that forces an injector drawrod into the syringe body by a pre-set distance (using an injection stroke adjuster). Since this device was to be used for injecting foams and not solutions or gels, a foam ramrod was manufactured to fit through the end of the syringe, where the catheter adaptor is located. In this case, a tapered catheter was designed to inject foam into a body. The taper allows a foam sample to be pre-positioned in the barrel before attaching the catheter to the syringe and allows the foam to be gradually compressed during the process of injection. The rat study protocol involved the initial formation of a small hole in the rat skin using a 14 gauge needle positioned within the catheter. The foam ramrod is inserted into the syringe. The needle/catheter is used to place the catheter in the desired position. The silk foam is positioned in the barrel of the catheter using tweezers. The injection handle is then repeatedly squeezed, providing a ratcheting action that slowly injects the foam into the animal.

Mechanical Testing

An Instron 3366 testing frame with a 10 N load cell (Instron, Norwood, MA) was used for all unconfined compression mechanical tests. Samples were cut using an 8 mm diameter biopsy punch and hydrated in phosphate-buffered saline (PBS, 0.1 M) for at least 1 h to reach swelling equilibrium prior to testing. The heights of the samples were 4 mm. During testing, samples were submerged in a temperature-controlled testing container (Biopuls) filled with PBS solution at room temperature. All testing was performed using a displacement control method at a rate of 1 mm/min and strain was automatically zeroed at a 5 mN tare load. Testing was carried out from 0 to 80% strain. Elastic moduli were calculated by fitting a linear regression to a small section of the elastic region of the stress/strain curve. Yield stresses were calculated using an offset yield approach. A line was drawn parallel to the modulus line but offset by 0.5%. The point of intersection between this line and the stress/strain curve was taken to be the yield stress. Three samples were tested for each experimental condition and the results are reported as mean plus standard deviation.

Adipose Derived Stem Cell (ASC) Isolation

Adult human subcutaneous adipose tissue was obtained from abdominoplasties under Tufts University Institutional Review Board (Tufts University IRB Protocol #0906007). No patient identifiers were collected. The specimens were kept at room temperature in saline and used within the same day. The adipose tissue was separated from the skin by blunt dissection and chopped. Chopped adipose tissue was placed into 50 mL conical tubes and

minced well with scissors. The tissues were washed in equal volumes warmed PBS, until essentially free of blood. An equal volume of collagenase I (1 mg/mL) in bovine serum albumin in PBS (1% w/v) was added to the tissue and placed under gentle agitation at 37°C for 1 hour. The tissue samples were centrifuged at 300 x g for 10 minutes at room temperature. The supernatant containing the tissue was removed and the pellet re-suspended in PBS and centrifuged at the same settings to remove the collagenase solution. The pellet was re-suspended in growth media and plated so that the initial tissue volume (70 g) was plated per T225 cm² tissue culture flask. ASCs were expanded and cultured as previously described.^[13,15, 27] DNA content was assessed by PicoGreen assay kit (Life Technologies, Grand Island, NY) and used according to manufacturer's protocol.

Cell Culture and 3D Culture

Cells were expanded first in growth media comprised of DMEM/F12, fetal bovine serum (FBS, 10%), penicillin streptomycin fungizone (PSF, 1%). A preliminary *in vitro* study with cells at passage 4 was performed to ensure cells were able to attach, migrate, and survive on the foams. The foams (8 mm x 8 mm, diameter x height) were soaked for 1 hour in growth media, then ASCs (250,000 cells) were seeded in three sequential 20 µL aliquots. The cells were labeled with a vital cell membrane tracking dye, DiI, prior to seeding. The seeded foams were placed in an incubator for 2 hours before media was added to the well. Cells and cultures were fed 2 times per week, and maintained in 37°C, humidified incubators for 10 days until imaged.

In vivo implantation

Animals were cared for in compliance with Tufts University Institutional Animal Care and Use Committee (IACUC) in accordance with the Office of Laboratory Animal Welfare (OLAW) at the National Institutes of Health (NIH). Female Sprague-Dawley rats, 9 weeks old were purchased from Charles River Breeding Labs, and were allowed to acclimate for 1 week prior to implantation. The animals were first anesthetized with isoflurane (1-4%) and were maintained with 1% (or to effect) isoflurane in oxygen, and kept on a heating pad through the entire procedure. After induction of anesthesia, injection site was wiped down with alcohol. The skin was pinched into a tent, to form a space for the catheter. The catheter and needle was fed into the tented space, and the needle retracted. The foam (5 mm x 2 mm, diameter x height) was placed in the catheter, the injection gun was attached to the catheter and then the handle squeezed until the foam was injected subcutaneously. The catheter was removed and the animals were allowed to awaken and had free access to food and water. Six injections were made per animal, 3 along each side of the dorsal midline.

In vivo degradation

In vivo degradation was calculated as previously described.^[13]

Histology

Explanted constructs were immediately placed into 4% formalin for 24 hours. Formalin fixed samples were put through a series of dehydration solvents and finally paraffin using an automated tissue processor. Samples were embedded in paraffin, cut in 10 micron sections,

and let to adhere on glass slides. The sections were rehydrated and stained with Hematoxylin and Eosin (H&E) for general morphology and organization and, Masson's Trichrome for tissue remodeling. For immunohistochemical staining, the sections underwent antigen retrieval under heated, low pH conditions. Primary antibodies for rat CD68, CD80, CD163 were purchased from AbD Serotec (Raleigh, NC). Antibody diluent was purchased from Cell Signaling Technologies (Danvers, MA). The secondary antibodies, ABC (avidin, biotin complex) kit, DAB substrate, hematoxylin counterstain and, antigen retrieval solution were purchased from Vector Labs (Burlingame, CA). Non-specific binding was avoided by incubation with normal blocking serum. After the excess serum was removed, the sections were incubated for 30 minutes with rat anti-mouse CD68 (diluted 1:100 in antibody diluent), rat anti-mouse CD80 (1:100), and rat anti-mouse CD163 (1:100). Sections were washed in PBS and, then incubated with a secondary antibody for 30 minutes. The sections were washed in PBS and then incubated with VECTASTAIN Elite ABC reagent for 30 minutes, washed in PBS, incubated with ImmPACT DAB enzyme substrate for 5 minutes and washed in water. The sections were then counterstained with hematoxylin and mounted.

In vitro Degradation Study

Sterile foams were placed in micro-centrifuge tubes with 1 mL of PBS, 1 mg/mL collagenase or 1 mg/mL proteinase XIV solutions supplemented with 1% PSF. Tubes were placed in 37°C on a rotisserie shaker. Buffer or enzyme solutions were replaced every 2 days. Foams were weighed initially dry and after incubation with buffer or enzyme solution after allowing to dry overnight. Degradation was calculated by taking the absolute value of the difference of the initial weight from the final weight over the initial weight and multiplying by 100%.

Image Analysis

Vessel density was calculated by counting the number of vessels present in a 300 mm² field of view in 10x H&E sections. This measurement was repeated for 4 independent fields of view. To quantify macrophage polarization, a grid was generated atop a 20x immunohistological section of M0, M1 or M2 stained sample using ImageJ's (NIH) plug-in "Grid". Cells stained for the immune markers were counted as "positive" and were counted in random grid regions (100x100 microns) with at least 200 cells counted total per group. Data are displayed as mean number of positive cells per 100 x 100 micron gridded area.

Statistical Analysis

Samples for quantifiable analyses were n= 4-6 with each biological replicate having technical duplicates. Results are shown as means $\pm$ SD. All statistical analyses were performed with JMP Pro software package (SAS, Cary, NC).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematization of silk foam injection method using ramrod guided injection gun (A). Macroscopic image of foam cross-section, scale bar 5 cm (B-left). Foam cut to desired size for injection (5mm diameter x 2 mm height, scale bar 5 mm (B-right). Foams were easily injected *in vivo* with custom designed injection gun, scale bar 5 cm (C).

Figure 2.

Foams support cell migration after 10 days *in vitro*. Cells migrate through foam layers (A). Foams contain layers as a result of the processing conditions. Confocal images of fluorescently labeled cells (fluorescent-left panel; brightfield-right panel) on silk foam. Side surface perpendicular to seeding surface was imaged. Cells line the foam pores (B). Confocal images of fluorescently labeled cells (fluorescent-left panel; brightfield-right panel) on silk foam. Surface that was seeded was also imaged. Cells survived on foams *in vitro*. An initial decrease is seen post-seeding (* $p=0.0359$) (C). Silk foam degradation profile *in vitro*. Silk foam degrades completely in presence of non-specific protease (protease XIV), but not fully in collagenase. Negligible degradation is seen in buffer control (D).

Figure 3.

Foams integrate well with surrounding tissue. Skin is well healed at injection site after injection and foams are palpable through skin (A). Foams after 14 (left) and 90 (right) days *in vivo* (B). At 14 days, vascularity was clearly leading to foams, but had decreased macroscopically after 90 days. Cross-sections of foams after implantation, scale bar – 2mm (C). Integration with surrounding tissue increased with time. *In vivo* degradation profile demonstrates foams degraded significantly from days 14, 30 to days 60 and 90 (* $p < 0.03$) (D).

Figure 4.

Morphology and organization of tissue after implantation. Representative H&E images of foams after 14 and 90 days *in vivo* (A). Arrows point to silk foam. Scale bar-100 microns. Representative Masson's Trichrome images of foams after 14 and 90 days *in vivo* (B). Arrows point to areas of silk foam. Scale bar-100 microns. Vessel density did not vary significantly over time or with silk foam percentage (C).

Figure 5.

Representative immunohistochemical images of macrophage (M0, CD68+), pro-inflammatory phenotype (M1, CD80+) and tissue-healing phenotype (M2, CD163+) after 14 and 90 days *in vivo* on 3% silk foams (A). All phenotypes increased over time (M0 # $p < 0.03$, M1 * $p < 0.001$, M2 * $p < 0.001$) (B).

Table 1

Mechanical properties of silk foams. Mechanical properties increased with increasing silk concentration. Values expressed as mean $\pm$ standard deviation, n/d - signal not detectable above noise (1.23 ± 0.15 kPa) with 10 N load cell. (n=3).

Silk Foam (wt %)	Elastic Modulus (kPa)	Yield Stress (kPa)
1%	n/d	n/d
3%	21.91 ± 3.62	3.71 ± 0.85
6%	101.90 ± 5.00	14.78 ± 1.44