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The Performance of Silk Scaffolds in a Rat Model of Augmentation Cystoplasty

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Abstract

The diverse processing plasticity of silk-based biomaterials offers a versatile platform for understanding the impact of structural and mechanical matrix properties on bladder regenerative processes. Three distinct groups of 3-D matrices were fabricated from aqueous solutions of *Bombyx mori* silk fibroin either by a gel spinning technique (GS1 and GS2 groups) or a solvent-casting/salt-leaching method in combination with silk film casting (FF group). SEM analyses revealed that GS1 matrices consisted of smooth, compact multi-laminates of parallel-oriented silk fibers while GS2 scaffolds were composed of porous (pore size range, 5–50µm) lamellar-like sheets buttressed by a dense outer layer. Bi-layer FF scaffolds were comprised of porous foams (pore size, ~400 µm) fused on their external face with a homogenous, nonporous silk film. Silk groups and small intestinal submucosa (SIS) matrices were evaluated in a rat model of augmentation cystoplasty for 10 weeks of implantation and compared to cystotomy controls. *Gross* tissue evaluations revealed the presence of intra-luminal stones in all experimental groups. The incidence and size of urinary calculi was the highest in animals implanted with gel spun silk matrices and SIS with frequencies 57% and stone diameters of 3–4mm. In contrast, rats augmented with FF scaffolds displayed substantially lower rates (20%) and stone size (2mm), similar to the levels observed in controls (13%, 2mm). Histological (hematoxylin and eosin, Masson's trichrome) and immunohistochemical (IHC) analyses showed comparable extents of smooth muscle regeneration and contractile protein (α-smooth muscle actin and SM22α) expression within defect sites supported by all matrix groups similar to controls. Parallel evaluations demonstrated the formation of a transitional, multi-layered urothelium with prominent uroplakin and p63 protein expression in all experimental groups. *De novo* innervation and vascularization processes were evident in all regenerated tissues indicated by Fox3-positive neuronal cells and vessels lined with CD31 expressing endothelial cells. In comparison to other

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biomaterial groups, cystometric analyses at 10 weeks post-op revealed that animals implanted with the FF matrix configuration displayed superior urodynamic characteristics including compliance, functional capacity, as well as spontaneous non voiding contractions consistent with control levels. Our data demonstrate that variations in scaffold processing techniques can influence the *in vivo* functional performance of silk matrices in bladder reconstructive procedures.

Keywords

silk; bladder tissue engineering; bladder; wound healing

1. Introduction

Congenital and acquired urinary tract pathologies such as neurogenic bladder, bladder exstrophy, and posterior urethral valves routinely require enterocystoplasty in order to reduce urinary storage and voiding pressures and mitigate the risk of renal damage and incontinence [1,2]. Although the use of autologous gastrointestinal segments represents the gold standard of care for bladder reconstruction, this strategy is associated with significant complications including chronic urinary tract infection, metabolic abnormalities, and secondary malignancies [3,4]. Biomaterials including acellular bladder matrix, small intestinal submucosa (SIS), and poly-glycolic acid (PGA), either alone or seeded with primary or progenitor bladder cell sources, have been previously explored as alternatives for bladder defect repair in a variety of animal models [5–8] as well as short-term clinical studies [9,10]. Despite the ability of these matrices to support bladder tissue regeneration, restoration of organ function is often hindered by suboptimal scaffold properties which frequently lead to long-term graft failure due to implant contracture, graft rupture, and/or fibrosis [10–14]. Therefore, there is a major clinical need to develop scaffold configurations which can overcome these limitations and thus serve as viable options for bladder tissue engineering.

Silk fibroin-based biomaterials encompass a unique array of properties including high structural strength and elasticity [15], diverse processing plasticity [16], and tunable biodegradability [17], which make them well suited for the consolidation of hollow organ defects. In particular, recent reports from our laboratory have demonstrated the efficacy of silk scaffolds for supporting bladder tissue repair in a murine model of augmentation cystoplasty [14,18]. These studies revealed that silk matrices produced from a gel spinning process displayed significant advantages for bladder reconstruction in comparison to conventional PGA and SIS scaffolds such as improved functional performance, reduced inflammatory reactions, and enhanced tissue regeneration [14]. Through variations in winding and post-winding processing parameters, the gel spinning process allows for precise control over structural and mechanical properties of silk biomaterials [18–20] permitting the design of scaffold configurations which can initially reinforce defect sites, but gradually undergo degradation to allow for host tissue replacement [18]. Indeed, we have shown that selective post-winding processing parameters such as lyophilization can generate an internal interconnected porous architecture within gel spun silk scaffolds leading to elevated rates of *in vivo* matrix degradation and increased extents of host bladder tissue integration in comparison to their non porous counterparts [18]. These reports highlight the potential of silk scaffolds to serve as robust, flexible platforms for bladder tissue regeneration.

In this study, we evaluated the performance of silk biomaterials in a rat model of augmentation cystoplasty. The ability of various 3-D scaffold configurations produced from different processing techniques, including gel spinning [19] or solvent-casting/salt-leaching [15], were compared for their potential to support bladder tissue repair and functional

voiding responses. We hypothesized that NaCl-leaching of aqueous-based silk fibroin solutions may offer particular advantages over gel spinning for the construction of biomaterials for bladder tissue engineering. Previous studies have shown that the combination of large scaffold pore sizes ($>400\mu\text{m}$) coupled with low initial concentrations of silk fibroin solutions (6% wt/vol) utilized during scaffold processing can enhance the degree of matrix degradation as well as cell infiltration and tissue ingrowth [17,21]. Optimization of these parameters is essential for efficient scaffold remodeling and regeneration of native tissue structure and function. By manipulating the NaCl crystal diameter, the solvent-casting/salt-leaching method allows for greater scalability of scaffold pore size ($470\text{--}940\mu\text{m}$) [15] in respect to lyophilization of gel spun matrices which routinely generates pore diameters below $200\mu\text{m}$ [18–20]. In addition, lower concentrations of silk fibroin solutions are utilized for the formation of NaCl-leached scaffolds ($<10\%$ wt/vol) [15] in comparison to the gel spun spinning technique which requires solutions containing 20–30% wt/vol for scaffold fabrication [14,18–20]. A systematic analysis of the impact of processing methods on the ability of silk scaffolds to support bladder tissue regeneration is a crucial step in the development of biomaterial configurations for clinical organ repair.

2. Materials and Methods

2.1. Biomaterials

Aqueous silk fibroin solutions were prepared from *Bombyx mori* silkworm cocoons using previously described procedures [15]. Three distinct groups of silk-based matrices were fabricated from these solutions by either a gel spinning technique [19] or a solvent-casting/salt-leaching method [15] in combination with silk film casting. Gel spun scaffolds were produced by spinning concentrated silk solutions [25–30% (wt/vol), 0.5 ml/scaffold] onto a rotating (200 rpm) and axially reciprocating mandrel (6 mm in diameter) using a custom gel spinning platform and program. Two groups of matrices previously shown to support murine bladder augmentation, but with different structural and mechanical properties, were generated with various winding and post-winding conditions using this method [14,18]. GS1 matrices were spun with an axial slew rate (ASR) of 2 mm/sec followed by treatment with methanol. GS2 scaffolds were composed of ~ 0.4 ml of silk solution spun at an ASR of 40 mm/sec followed by ~ 0.1 ml spun at 2 mm/sec in order to consolidate gaps between the resultant silk fibers. This matrix group was then subjected to lyophilization and subsequent methanol treatment. The FF scaffold group was composed of 3-D porous silk foams which were annealed to silk films on their top external face. Briefly, a silk fibroin solution (8% wt/vol) was poured into a rectangular casting vessel and dried in a laminar flow hood for 48h to achieve formation of a silk film. A 6% wt/vol silk fibroin solution was then mixed with sieved granular NaCl ($500\text{--}600\mu\text{m}$, average crystal size) and layered on to the surface of the silk film. The resultant solution was allowed to cast for 48h and NaCl was subsequently removed by washing the scaffold for 72h in distilled water with regular volume changes. Fusion of the silk film with the bulk foam matrix occurred to generate a bi-layer scaffold configuration. Silk matrix groups were then sterilized in 70% ethanol, rinsed in phosphate buffered saline (PBS), and subjected to analytical or surgical procedures described below. Small intestinal submucosa (SIS) (Cook, Bloomington, IN) scaffolds were evaluated in parallel as standard points of comparison since this biomaterial has been previously deployed in bladder augmentation approaches in both animal [5] and human models [10].

2.2. Scanning electron microscopy (SEM)

Structural analysis of matrix groups was performed in order to assess differences in scaffold morphology generated by various fabrication techniques. Matrix samples were sputter coated with platinum/palladium and imaged with a Zeiss EVO10 Field Emission Scanning Electron Microscope (Carl Zeiss AG, Germany) at 3 kV.

2.3. Mechanical testing

Uniaxial tensile tests were performed as previously described [18] on an Instron 3366 testing frame (Norwood, MA) equipped with a 100 N capacity load cell and Biopuls pneumatic clamps. Matrix groups (N=3–4 per group) were hydrated in PBS for at least 24 h to reach a swelling equilibrium prior to testing. Test samples were submerged in a temperature-controlled testing container (Biopuls) filled with PBS (37°C). A displacement control mode with a crosshead displacement rate of 5 mm/min was used, and the gauge length was 15 mm. The initial elastic modulus (EM), ultimate tensile strength (UTS) and % elongation to failure were calculated from stress/strain plots. EM was calculated by using a least-squares (LS) fitting between 0.02 N load and 5% strain past this initial load point. UTS was determined as the highest stress value attained during the test and the % elongation to failure was the last data point before a >10% decrease in the load.

2.4. Rat bladder augmentation

Biomaterial groups were evaluated in a bladder augmentation model using adult female immunocompetent Sprague Dawley rats (6 weeks old, Charles River Laboratories, Wilmington, MA) following IACUC approved protocols as previously described [22]. Briefly, animals were anesthetized using isoflurane inhalation and then shaved to expose the surgical site. A low midline laparotomy incision was then made and the underlying tissue (rectus muscle and peritoneum) was dissected free to expose the bladder. The anterior portion (immediately distal to the dome) of the bladder was marked with 7-0 polypropylene (Prolene) sutures in a square configuration. A longitudinal cystotomy incision was then made in the anterior bladder wall in the middle of these holding sutures using fine scissors to create a bladder defect. A square piece of biomaterial (7×7mm²) was then anastomosed to this site using a 7-0 vicryl continuous suture. In addition, a control group of animals receiving a cystotomy alone were treated similarly. A watertight seal was confirmed by filling the bladder with sterile saline via instillation through a 30 gauge hypodermic needle. Matrix and control groups were assessed independently for 10 weeks of implantation with animals subsequently subjected to cystometric, histological and immunohistochemical analyses described below.

2.5. Cystometric analyses

Bladder urodynamics were evaluated in all rodents using conscious unrestrained cystometry at 10 weeks post implantation as previously described [22]. A suprapubic catheter was surgically inserted in the bladder prior to this. After induction with isoflurane anesthesia, the animals were prepped and draped in a sterile fashion. A dorsal midline incision was made in between the scapulae of the rats. A laparotomy was created using a ventral midline incision. Polyethylene-50 tubing with a flared tip was tunneled from the dorsal incision into the peritoneal cavity. A purse-string 6-0 prolene stitch was used to secure the flared tip of the polyethylene-50 tubing into the dome of the bladder. The exteriorized polyethylene-50 tubing on the dorsal aspect was attached to a luer lock adapter and secured to the skin with a 3-0 silk suture. Cystometry was conducted 1–3 d after suprapubic catheter placement. The suprapubic catheter was attached to a physiological pressure transducer (model MLT844, ADInstruments, Colorado Springs, CO) to allow measurement of intravesical pressure, while the bladder was continuously infused with sterile PBS at 100 µl/min. Post void residual volume was measured by aspirating the suprapubic catheter at the conclusion of cystometry. After establishment of a regular voiding pattern, multiple other variables were extrapolated from the cystometric tracings, such as compliance, voided volume, peak voiding pressure, intercontraction interval and spontaneous non voiding contractions (SNVC). A total of 6 animals per group with 4 voids per animal were analyzed to determine urodynamic parameters.

2.6. Histological and immunohistochemical analyses

Following 10 weeks of implantation, animals were euthanized by CO₂ asphyxiation and bladders were excised for standard histological processing. Briefly, organs were fixed in 10% neutral-buffered formalin, dehydrated in graded alcohols, and then embedded in paraffin in an axial orientation to capture the entire circumferential surface of the bladder within each section. Correct orientation (anterior vs posterior) within the paraffin block was determined by suture placement on the specimen. Sections (10 µm) were cut and then stained with hematoxylin and eosin (H&E) or Masson's trichrome (MTS) as previously described [18]. For immunohistochemical (IHC) analysis, contractile smooth muscle markers such as α -smooth muscle actin (α -SMA) and SM22 α ; urothelial-associated proteins, uroplakins (UP) and p63; neuronal and endothelial markers, Fox3 and CD31, respectively were detected using the following primary antibodies: anti- α -SMA [Sigma-Aldrich, St. Louis, MO, cat.# A2457, 1:200 dilution], anti-SM22 α [Abcam, Cambridge, MA, cat.# ab14106, 1:200 dilution], anti-pan-UP [rabbit antisera raised against total bovine UP extracts, 1:100 dilution], anti-p63 [Santa Cruz Biotechnology, Santa Cruz, CA, cat.# sc-8431, 1:200 dilution], anti-Fox3 [Abcam, cat.# ab104225, 1:200 dilution], anti-CD31 [Abcam, cat.# ab228364, 1:100 dilution]. Sections were then incubated with species-matched Cy3-conjugated secondary antibodies (Millipore, Billerica, MA) and nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI). Specimens were visualized using an Axioplan-2 microscope (Carl Zeiss MicroImaging, Thornwood, NY) and representative images were acquired using Axiovision software (version 4.8).

2.7. Statistical analysis

Urodynamic measurements were analyzed by generalized estimating equations with post-hoc Bonferroni testing using commercially available statistical software (SAS9.3 software, www.sas.com). Statistically significant values were defined as $p < 0.05$. Urodynamic parameters are displayed as means $\pm$ standard deviation.

3. Results and Discussion

SEM analyses of silk matrix groups revealed that scaffold processing techniques as well as distinct fabrication parameters led to selective differences in overall biomaterial structural architecture (Figure 1A). As observed in previous studies [14, 18, 20], GS1 scaffolds consisted of compact multi-laminates of parallel-oriented silk fibers while GS2 matrices were composed of porous (pore size range, 5–50µm) lamellar-like sheets buttressed by a dense outer layer. In contrast, FF scaffolds consisted of a bi-layer structure with compartments dictated by the mode of fabrication. The solvent-cast/NaCl-leached layer comprised the bulk of the matrix and resembled a foam configuration with large pores (pore size, ~400µm) interconnected by a network of smaller pores dispersed along their periphery. This layer was fused on the external face with a homogeneous, nonporous silk film (200µm thick) generated by the annealing of dehydrated silk solutions during matrix casting. Spontaneous pore occlusion of the solvent-cast/NaCl-leached layer was also observed in the bulk matrix along the plane adjacent to the casting vessels in scaffolds produced in the absence of silk films (data not shown); however continuity of this feature was heterogeneous along the surface area as well as highly variable between matrix replicates. Tensile testing of FF scaffolds prior to implantation (Figure 1B) demonstrated a lower degree of UTS and EM compared to the previously published properties of both gel spun matrix configurations [18]. SIS scaffolds exhibited substantially higher UTS and EM in comparison to all silk groups. Elongation to failure measurements revealed that the FF group was ~4 fold more elastic than any other matrix configuration tested suggesting that although these scaffolds had lower respective UTS they could achieve larger degrees of deformation; a potential advantage for the consolidation of highly distensible bladder defects.

The porous foam compartment of the FF scaffold configuration independent of the annealed silk film was first analyzed for its potential to support bladder defect consolidation within the rat model of augmentation cystoplasty. Following initial surgical implantation, ex vivo assessments of defect closure demonstrated prominent fluid leaks within center of the scaffold coupled with a failure of the implant to support bladder distension during saline instillation. The inability of silk foams to restore the integrity of defect sites is presumably due to the interconnected porous nature of this scaffold type which was insufficient to mimic the barrier function of the native bladder wall following a rise in intravesical pressure. In contrast, FF scaffolds consisting of silk films annealed to the exterior face of the porous silk foams were observed to support organ integrity and distension following surgical integration similar to the performance of gel spun silk scaffold configurations and SIS. These results demonstrate that the annealed silk film compartment is essential for the bi-layer FF group to maintain initial defect consolidation within this model system.

Over the course of the 10 week implantation period, survival rates of the augmented animals in 3 out of 4 scaffold groups were similar to cystotomy controls prior to scheduled euthanasia. Bladder reconstruction with GS1 and FF groups displayed survival rates of 100% (10/10 for GS1 and 8/8 for FF) while animals implanted with SIS exhibited an 88% survival rate (8/9); both values were comparable to the 90% rate observed following cystotomy alone. In contrast, animals implanted with GS2 scaffolds displayed a reduced rate of survival at 60% (9/15). All spontaneous animal deaths in each group occurred within the first post-operative week and post-mortem analyses revealed urinary ascites as the probable cause in all cases due to scaffold perforation. Previous reports deploying GS2 matrices for murine bladder augmentation demonstrated a 100% survival rate (6/6) [18], however the greater tendency for animal death from scaffold urine leaks observed in this study is presumably related to the increase in scaffold area used between the rat and murine models. The lyophilization process utilized during GS2 construction is prone to generating microfractures in the nonporous outer layer of this scaffold configuration (Figure 1A) [18] thereby increasing the potential for the integrity of the matrix to become compromised with increased surface area.

At 10 weeks post-op, gross tissue evaluations of the lower urinary tract revealed no signs of bladder mucus or hydronephrosis in any of the sacrificed animals, however the presence of luminal bladder stones was evident in all experimental groups examined (Figure 2A, B). These data are consistent with other previous studies which have shown that the formation of bladder calculi is a common occurrence in rat models of bladder reconstruction with biodegradable acellular materials [23–25]. The incidence of urinary calculi was the highest in the gel spun silk cohorts with animals augmented with GS1 and GS2 matrices exhibiting a frequency of 75% and 71%, respectively. SIS implants elicited similar extents of stone formation by comparison with a frequency of 57%. In contrast, rats subjected to cystotomy alone or FF scaffold implantation demonstrated the lowest respective incidence of stone formation at 13% and 20%. In addition, stone diameters in these groups were also found to be substantially lower (2mm) in comparison to all other experimental conditions (3–4mm). These results show that the frequency and extent of bladder stone formation within the rat augmentation cystoplasty model is dependent on biomaterial composition (silk versus SIS) as well as the processing method utilized for silk scaffold construction.

Histological examination of whole bladder sections (H&E and MTS) demonstrated that each scaffold group supported robust degrees of connective tissue ingrowth which traversed from the periphery of the native bladder wall to the interior of the original defect site (Figure 3). Residual GS1 and GS2 matrices were localized to the bladder lumen and were primarily intact exhibiting minimal extents of degradation. These results are consistent with the gross tissue observations wherein scaffold remnants from both groups were found encapsulated

within luminal bladder stones. In contrast to our previous study [18], the rate of *in vivo* scaffold degradation was not substantially elevated by the porous architecture of GS2 matrices in comparison to the non porous GS1 group. An increase in GS2 area utilized in the rat ($7 \times 7 \text{ mm}^2$) versus the murine ($4 \times 4 \text{ mm}^2$) model may have allowed for enhanced structural stability and therefore less fragmentation of the bulk matrix following 10 weeks of implantation. Indeed, similar relationships between degradation profiles and scale-up of scaffold dimensions have been reported for a variety of biomaterial formulations [26].

The degradation pattern of the FF scaffolds was found to be dependent on the structural architecture of the bi-layer matrix compartments with the porous region exhibiting extensive degrees of fragmentation within the bladder wall while the non porous silk films remained largely intact. Higher initial levels of silk fibroin utilized for film construction in respect to the porous compartment may have also contributed to the observed differences in degradation profile. In addition, the enhanced rate of degradation demonstrated by the porous regions of the FF scaffolds in comparison to the GS2 matrices was presumably related to the increase in pore size as well as the lower content of silk fibroin which would have allowed for more efficient exposure of the matrix interior to proteolytic enzymes and subsequent polymer hydrolysis [17,18]. Analysis of the degradation profile of collagenous SIS scaffolds revealed more extensive fragmentation in respect to all silk groups with minimal degrees of residual matrix detected within the bladder lumen. Taken together, these data demonstrate that the silk scaffolds investigated in this study are more structurally stable than SIS matrices *in vivo*, however alterations in the rate of silk scaffold degradation can be achieved through different scaffold processing techniques with the solvent-casting/salt-leaching method encouraging increased degrees of matrix degradation in comparison to gel spinning protocols.

For each biomaterial group, H&E and MTS analyses demonstrated the presence of robust smooth muscle bundles (H&E: pink; MTS: red) localized throughout the periphery of the regenerated bladder wall (Figure 3). IHC assessments revealed that the reconstituted smooth muscle layers of all scaffold groups stained positive for both α -SMA and SM22 α contractile protein expression to similar extents as those observed with the cystotomy controls indicating prominent smooth muscle maturation (Figure 4). No evidence of severe fibrotic events was detected in these regions in any of the experimental groups examined. In addition, an ECM-rich lamina propria populated with fibroblastic populations was also evident in the regenerated tissues supported by all matrix configurations. In comparison to cystotomy controls, histological features within the lamina propria indicative of minimally acute inflammatory reactions were noted at each implantation site characterized by the presence of disperse eosinophil granulocytes, however evidence of substantial chronic inflammatory events was not observed in response to any experimental condition.

Histological evaluations also demonstrated that each implant group supported the formation of a multi-layer urothelium covering the entire luminal surface of the original defect site (Figure 5). The transitional nature of the urothelium was confirmed in all regenerated tissues by IHC analysis wherein p63-positive basal and intermediate cell layers were lined with luminal p63-negative superficial cells. Varying degrees of hyperplasia were observed in the basal and intermediate cell compartments supported by all implant groups in comparison to cystotomy controls. This feature may reflect incomplete urothelial maturation since normalization of basal/intermediate cell proliferation is required during wound healing for native tissue stratification to be achieved [18,27]. However across all matrix groups, robust pan-UP protein expression was noted in both regenerated superficial and intermediate cell layers to levels similar to those observed in control tissues. Expression and assembly of UP proteins into heterodimers which form asymmetrical unit membranes is essential for maintaining the integrity of the urothelial permeability barrier [28].

IHC analyses revealed evidence of *de novo* vascularization and innervation processes in the regenerated tissues supported by each implant group (Figure 6). Vessels containing prominent CD31 positive endothelial cells were present throughout the original defect sites while neuronal lineages displaying Fox3 peri-nuclear protein expression were found localized to the sub-urothelial region of the regenerated bladder walls similar to cystotomy controls. These results demonstrate that silk scaffold configurations described in this study are capable of supporting regeneration of innervated, vascularized smooth muscle and urothelial tissues to levels comparable to conventional SIS matrices in a rat model of bladder defect repair.

The functionality of reconstructed bladders was assessed by conscious unrestrained cystometry following 10 weeks of biomaterial implantation (Figure 7). Voided volume was used as a surrogate marker for bladder capacity given that post-void residual volumes were similar in each experimental group (data not shown) [29]. Rats augmented with GS1 and FF scaffolds had significantly higher mean voided volumes compared to cystotomy controls and in contrast to the other scaffold groups, GS2 and SIS. In accordance with larger voided volumes, animals with GS1 and FF implants also had significantly longer intercontraction intervals compared to control levels, indicating greater functional bladder capacity since more time was required for bladder filling between voiding cycles. SNVC were used as a measure of detrusor overactivity as previously described [30]. Rats implanted with SIS, GS1 and GS2 matrices had significantly higher numbers of SNVC compared to the cystotomy group. In contrast, animals augmented with FF scaffolds displayed SNVC levels similar to controls. The difference in SNVC is likely due to the increase in frequency and size of intraluminal calculi observed in the SIS, GS1, and GS2 in respect to control and FF groups, since bladder stones are known to elicit organ irritation resulting in detrusor overactivity and urge incontinence [31]. Compliance is a measure of the ability of the urinary bladder to store large volumes of urine at low intravesical pressures. Significant gains in bladder compliance were observed in rats implanted with FF scaffolds in comparison to cystotomy controls and in contrast to all other scaffold configurations. Peak intravesical pressures, however were similar between all experimental groups. These results are consistent with the ability of FF scaffolds to support bladder tissue regeneration with increased functional capacity relative to controls and distensible mechanical properties.

4. Conclusions

The results presented in this study detail the feasibility of silk-based biomaterials to support regeneration of innervated, vascularized smooth muscle and urothelial tissues in a rat model of augmentation cystoplasty. Our data demonstrate that variations in scaffold processing techniques (gel spinning and solvent-casting/salt-leaching) and their associated fabrication parameters can influence the *in vivo* functional performance of silk matrices in bladder reconstructive procedures by manipulating their structural and mechanical properties. In comparison to other biomaterial groups, bladders implanted with the bi-layer FF matrix configuration, generated by the fusion of solvent-cast/NaCl-leached silk foams with silk films, displayed substantial reductions in the frequency and extent of urinary calculi as well as superior urodynamic characteristics including compliance, functional capacity, as well as SNVC similar to controls. In addition, the potential of the FF scaffold fabrication process to allow for scale-up of matrix dimensions (surface area and thickness) by orders of magnitude through adjustments in the size of the casting molds is a desirable property since implants can be produced to accommodate a range of defect sizes relevant to clinical translation. In summary, silk biomaterials, in particular the FF matrix configuration, offer promising avenues for functional tissue engineering of urinary tract defects.

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B

<u>Groups</u>	<u>UTS (MPa)</u>	<u>EM (MPa)</u>	<u>ETF (%)</u>
Silk (GS1)	0.42 ± 0.06*	2.7 ± 0.35*	28 ± 3*
Silk (GS2)	0.54 ± 0.09*	2.7 ± 0.6*	51 ± 6*
Silk (FF)	0.29 ± .04	1.7 ± 0.16	197 ± 45
SIS	17.1 ± 3.8	5.8 ± 0.33	48 ± 4

Figure 1.

Structural and mechanical analyses of scaffold groups. **[A]** Photomicrographs of representative SEM images demonstrating top and cross-sectional views of matrix configurations. Inset: Bottom FF scaffold view. Scale bar = 400 µm. **[B]** Evaluation of ultimate tensile strength (UTS), elastic modulus (EM), and % elongation to failure (ETF) in matrix groups defined in **[A]**. Means ± standard deviation per data point. (*) represents data previously reported [18].

A

Groups	Stone Frequency	Stone Diameter (mm)
Cystotomy	1/8 (13%)	2
SIS	4/7 (57%)	4±2
Silk (GS1)	6/8 (75%)	4±1
Silk (GS2)	5/7 (71%)	3±3
Silk (FF)	2/10 (20%)	2±1

Figure 2.

Characterization of urinary stone incidence and size in regenerated bladders. **[A]** Quantification of stone frequency and diameter in bladders augmented with each matrix group and cystotomy controls. **[B]** Photomicrographs of urinary stones in regenerated bladders and controls. Arrows denote stones. Scale bar= 2.5 mm.

Figure 3.

Global histological comparisons of the extent of tissue regeneration and in vivo degradation of matrix groups. [1st and 2nd rows] Photomicrographs of total bladders (H&E and MTS-stained sections) augmented with each scaffold group over the course of the 10 week implantation period as well as cystotomy controls. (*) denotes scaffold fragments. Brackets represent area of tissue regeneration. Scale bar = 2.5mm. [3rd row] magnified H&E-stained regenerated area bracketed in row 1. SM=smooth muscle bundles; LP=lamina propria; UE=urothelium. Scale bars= 1 mm.

Figure 4.

Histological and immunohistochemical (IHC) evaluations of the extent of smooth muscle regeneration following biomaterial incorporation. [**1st row**] Photomicrographs of magnified regenerated tissue area in bladders augmented with each scaffold group following 10 weeks post-op and cystotomy controls subjected to H&E analysis. Scale bar = 200 μ m. For all panels (SM) denotes smooth muscle bundles. [**2nd and 3rd rows**] Photomicrographs of contractile protein (α -SMA and SM22 α) expression analyzed by IHC (red, Cy3) in samples described in row 1. DAPI nuclear counterstain (blue). Scale bar = 400 μ m.

Figure 5.

Histological and immunohistochemical (IHC) assessments of the degree of urothelial regeneration supported by scaffold groups. [1st row] Photomicrographs of magnified regenerated tissue area in bladders augmented with each scaffold group following 10 weeks post-op and cystotomy controls subjected to H&E analysis. For all panels (UE) denotes urothelial compartment. [2nd and 3rd rows] Photomicrographs of pan-uroplakin and p63 protein expression analyzed by IHC (red, Cy3) in samples described in row 1. DAPI nuclear counterstain (blue). Arrows denote p63-positive basal and/or intermediate urothelial cells (yellow); p63-negative superficial urothelial cells (white). For rows 1, scale bar = 200 μ m. For rows 2 and 3, scale bar = 400 μ m.

Figure 6. Immunohistochemical assessments of the degree of innervation and vascularization supported by scaffold groups. Photomicrographs of Fox3 and CD31 protein expression analyzed by IHC (red, Cy3) in magnified regenerated tissue area in bladders augmented with each scaffold group following 10 weeks post-op and cystotomy controls. Arrows denote neurons. V denotes vessels. DAPI nuclear counterstain (blue). Scale bar = 400 μ m.

Figure 7.

Cystometric analysis and quantification of urodynamic parameters in augmented bladders following 10 weeks post-implantation. [A] Representative cystometric tracings of voiding cycles displayed by cystotomy controls, GS1, GS2, and FF augmented bladders. (*) denotes individual voids. Scale bar = 5 min. [B] Comparisons of urodynamic parameters between experimental groups displayed in [A]. (*)=p<0.05 in comparison to cystotomy controls.