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The effect of manipulation of silk scaffold fabrication parameters on matrix performance in a murine model of bladder augmentation

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Abstract

Autologous gastrointestinal segments are utilized as the primary option for bladder reconstructive procedures despite their inherent morbidity and significant complication rate. Multi-laminate biomaterials derived from *Bombyx mori* silk fibroin and prepared from a gel spinning process may serve as a superior alternative for bladder tissue engineering due to their robust mechanical properties, biocompatibility, and processing plasticity. In the present study, we sought to determine the impact of variations in winding (axial slew rate: 2 and 40 mm/sec) and post-winding (methanol and lyophilization) fabrication parameters on the *in vivo* performance of gel spun silk scaffolds in a murine model of bladder augmentation. Three silk matrix groups with distinct structural and mechanical properties were investigated following 10 weeks of implantation including our original prototype previously shown to support bladder regeneration, Group 1 (2mm/sec, methanol) as well as Group 2 (40mm/sec, methanol) and Group 3 (40mm/sec, lyophilization) configurations. Non surgical animals were assessed in parallel as controls. Quantification of residual scaffold area demonstrated that while Group 1 and 2 scaffolds were largely intact, processing parameters utilized for Group 3 led to significantly higher degrees of scaffold degradation in comparison to Group 1. 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 SM22 α) expression within the original defect site throughout all matrix groups similar to controls. Parallel evaluations demonstrated transitional urothelial formation with prominent uroplakin and p63 protein expression supported by Group 1 and 3 scaffolds, while Group 2 variants supported a thin, immature epithelium composed primarily of uroplakin-negative, p63-positive basal cells. Voided stain on paper analysis revealed similar voiding patterns between all matrix groups; however Group 2 animals displayed substantially lower voided volumes with increased frequency in comparison to controls. In addition, cystometric assessments revealed all matrix groups supported comparable degrees of bladder compliance similar to control levels. The results of this study demonstrate that selective alterations in winding and post-winding fabrication parameters can enhance the degradation rate of gel spun silk scaffolds *in vivo* while preserving their ability to support bladder tissue regeneration and function.

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Keywords

silk; bladder tissue engineering; smooth muscle cell; epithelium; urinary tract

Introduction

Functional or anatomical obstruction of the urinary tract is a frequent consequence of a multitude of congenital and acquired bladder disorders including posterior urethral valves, neurogenic bladder secondary to spina bifida or spinal cord injury, benign prostatic hyperplasia, bladder and cloacal exstrophy, severe voiding dysfunction, and transitional cell carcinoma [1]. In order to reduce the risk of incontinence and kidney damage from increased storage and voiding pressures associated with these pathologies, surgical correction often necessitates implantation of a biomaterial that can support bladder augmentation or organ substitution [2,3]. To date, gastrointestinal segments including the stomach, colon, and ileum represent the primary option for bladder reconstructive procedures [4]. However, severe complications, including chronic urinary tract infection, metabolic abnormalities, urinary stone formation, bowel dysfunction, perforation, and secondary malignancies, significantly hamper this approach [5,6].

Previous research into alternative biomaterials for bladder reconstruction has primarily focused on the deployment of biodegradable scaffolds composed of either natural or synthetic polymers to facilitate tissue regeneration [7]. Biomaterials provide a template for initial defect stabilization and also serve as a conduit for host tissue ingrowth. In order for complete bladder repair to occur, scaffolds must support reconstitution of both the smooth muscle compartment as well as the urothelium to restore organ contractile and barrier functions, respectively [8]. Collagen-based matrices derived from decellularized tissues including small intestinal submucosa (SIS) or bladder acellular matrix (BAM) have been shown to enhance bladder defect healing through the release of endogenous growth factors and extracellular matrix (ECM) cues in various animal models [9–11], however limitations in their mechanical integrity and biocompatibility often result in deleterious fibrosis [12], graft contracture [13], and calcification [14]. Synthetic polymers such as poly-glycolic acid (PGA) have the advantage that their structural and mechanical properties can be specifically tailored to match the target tissue of interest [15]. In addition, previous studies have demonstrated the ability of these matrices to support *de novo* smooth muscle and urothelial tissue formation in short term clinical trials [16]. However, PGA-based scaffolds are known to elicit chronic inflammatory responses *in vivo* [17] and therefore they have the potential to retard defect consolidation due to adverse foreign body reactions [18]. Since conventional scaffold formulations present substantial drawbacks for wide-scale deployment in clinical bladder tissue engineering strategies, there exists a significant need for new biomaterial configurations which can overcome these limitations and support the organ's ability to maintain low pressure storage of urine.

Silk fibroin derived from *Bombyx mori* silkworm cocoons represents a unique natural fibrous protein which has been applied to a variety of tissue engineering applications due to its low immunogenicity [18,19], material stability and mechanical robustness [20,21], and biodegradability [22]. Gel spinning of silk fibroin protein solutions has been shown to generate multi-laminate matrices, organized from shear induced fibers, in which precise control over scaffold architecture, porosity, and mechanical properties can be achieved through variations in winding and post-winding fabrication parameters [23]. Recently, our group has demonstrated the efficacy of a prototype acellular gel spun silk matrix to support robust tissue regeneration in a rodent bladder augment model, resulting in organized urothelial and smooth muscle compartments as well as increased bladder capacity and

voided volumes, while maintaining organ compliance similar to unoperated controls [18]. Parallel comparisons between PGA and SIS scaffolds showed that the silk matrix provided significant advantages including superior tissue regeneration, reduced inflammatory reactions, and improved functional performance. However, a potential drawback of our prototype silk design was the relatively slow rate of degradation exhibited following 10 weeks of implantation wherein the bulk of the scaffold remained largely intact. These results were in contrast with conventional matrices which underwent substantial fragmentation and breakdown.

The rate of biomaterial degradation represents a key control point in the long-term success of scaffold configurations for bladder tissue engineering. Restoration of native tissue structure and function depends on optimal biomaterial degradation kinetics which must be permissive for integration of host tissue while still providing structural and mechanical support during initial defect consolidation. Rapid degradation may result in internal biomaterial collapse, hindering mass transfer and potentially leading to necrosis at the defect site [24], however slow scaffold resorption can increase the risk of urinary stone formation and impede integration of host tissue [25]. As a protein, silk fibroin is susceptible to biodegradation by proteolytic enzymes such as chymotrypsin, actinase, and carboxylase [26]. Analysis of silk fibroin-based foams *in vitro* and *in vivo* has provided evidence that the degradation kinetics of these scaffolds can be influenced by a number of factors including aqueous and organic processing solvents, concentration and molecular weight distribution of silk fibroin, extent of β -sheet crystallinity, pore size, and porosity [21,22]. In addition, researchers have indicated that rates of silk resorption *in vivo* are also dependent on the animal model, implantation site, as well as the presence of host immune system elements [27]. For bladder tissue engineering, it is important to understand how material processing variables can influence the performance of gel spun silk matrices in terms of degradation kinetics and restoration of organ function. Therefore in the present study, we sought to determine if selective alterations in winding and post-winding fabrication parameters can enhance the degradation rate of gel spun silk scaffolds *in vivo* while preserving their ability to support bladder tissue regeneration and function.

Materials and Methods

Biomaterials

Silk tubes were formed using a previously described gel spinning technique [23]. Briefly, aqueous silk fibroin solutions were prepared from *Bombyx mori* silkworm cocoons using previously described procedures [21]. Tubes were then produced by spinning concentrated silk solutions [25–40% (w/v)] onto a rotating (200 rpm) and axially reciprocating mandrel (6 mm in diameter) using a custom gel spinning platform and program. Three groups of tubes representing a range of winding and post-winding conditions were created with each consisting of a total of approximately 0.5 ml of silk solution applied to the mandrel with 4 cm in length via a 25–30 gauge needle. Group 1 scaffolds, previously demonstrated to support murine bladder augmentation [18], were spun with an axial slew rate (ASR) of 2 mm/sec followed by treatment with methanol to induce transformation from amorphous liquid to the β -form silk fibroin conformation characterized by anti-parallel β -sheets [28]. Group 2 and 3 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. Group 2 matrices were treated with methanol as described for Group 1, while Group 3 tubes were subjected to lyophilization. The tubes were then removed from the mandrel after briefly soaking in a surfactant solution and subjected to analytical analysis described below. Prior to surgical procedures, matrices were bisected along their central axis, sterilized in 70% ethanol, and rinsed in phosphate buffered saline (PBS) overnight.

Scanning electron microscopy (SEM)

Structural analysis was performed on each silk group to assess winding patterns and porosity generated using various winding and post-winding processing techniques. Tube samples were sputter coated with gold using a Polaron SC502 Sputter Coater (Fisons, VG Microtech, East Sussex, England) and imaged using a JEOL JSM-840 Scanning Microscope (JEOL Ltd., Tokyo, Japan).

Mechanical Testing

Uniaxial tensile tests were performed on an Instron 3366 testing frame (Norwood, MA) equipped with a 100 N capacity load cell and Biopuls pneumatic clamps. Silk tubes (N=4 per group) were hydrated in PBS for at least 24 h to reach a swelling equilibrium prior to testing. Each silk tube was clamped without cut. The sectional area of the silk tubes was calculated by measuring the average thickness and width of collapsed tubes in wet state and utilized to convert load data to tensile stress values. 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, tensile strength and % elongation to failure were calculated from stress/strain plots. We calculated the initial elastic modulus by using a least-squares (LS) fitting between 0.02 N load and 5% strain past this initial load point. Ultimate tensile strength (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.

Murine Bladder Augmentation

Biomaterial groups were evaluated in a bladder augmentation model utilizing an immunocompetent mouse strain (CD1, 6 weeks, 22–24g, Jackson Laboratories, Bar Harbor, ME) following IACUC approved protocols as previously described [18]. 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 where the tissue construct was incorporated was marked with 7-0 polypropylene (Prolene) sutures in a square (1 cm²) configuration. These sutures were used as holding sutures for the anastomosis of the biomaterials. A 1 cm² bladder defect was then created with fine scissors by a longitudinal incision immediately inside of the holding sutures. Biomaterials were then integrated into the bladder defect using 8-0 polyglactin (Vicryl) continuous suture. A watertight seal was confirmed by filling the bladder with sterile saline via instillation through a 30 gauge hypodermic needle. Matrix groups were assessed independently for 10 weeks of implantation with animals subsequently subjected to voided stain on paper (VSOP), cystometric, and/or histological analyses (Table 1). Non surgical animals of equal size, weight, and age were also assessed in parallel as controls.

VSOP Analysis

Conscious voiding function was studied utilizing a VSOP technique previously described [29]. VSOP was used to analyze voided volume, voiding frequency, and voiding pattern. Mice were housed individually in metabolic cages for three hours between 2:00 pm and 5:00 pm with food pellets and water readily available. Cellulose filter paper (Whatman International Ltd, Grade 1) cut to 25 cm diameter was placed underneath the mesh floor of the cages to catch urinary voids. Voiding characteristics were analyzed using an ultraviolet bioimaging system (Chemigenius², Syngene, Cambridge, UK) with N=3–14 voids per animal. In order to calculate VSOP parameters, a calibration curve was generated using

defined volumes (25–300 μ l) of artificial urine (Ward's Natural Science, Rochester, NY) and stained area on filter paper.

Murine Cystometric Analysis

Murine cystometric analyses were performed utilizing an open abdominal technique. Mice were anesthetized with intraperitoneal ketamine. A 1cm lower-midline incision was made and the bladder identified. The bladder was accessed with a 27G needle attached to a pressure transducer (ADInstruments, Colorado Springs, CO) and a Harvard 22 syringe pump (Harvard Apparatus, Holliston, MA). The pressure transducer was calibrated with a 40cm water column. Room temperature saline was infused at a rate of 15 μ l/min. Data was collected with a Bridge Amp and a Power Lab 4/30 (ADInstruments, Colorado Springs, CO) and analyzed with Lab Chart 6 (ADInstruments). After establishment of a regular voiding pattern, bladder compliance was determined by dividing the change in bladder volume by change in bladder pressure during the filling phase. A total of 4 voiding cycles were analyzed per mouse to determine compliance.

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 μ m) were cut and then stained with hematoxylin and eosin (H&E) or Masson's trichrome (MTS) as previously described [30]. For immunohistochemical (IHC) analysis, contractile smooth muscle markers such as α -smooth muscle actin (α -SMA) and SM22 α as well as urothelial-associated proteins, uroplakins (UP) and p63 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] followed by incubation with species-matched Cy3-conjugated secondary antibodies (Millipore, Billerica, MA). Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI), and specimens were visualized using a Nikon Eclipse TE2000-U fluorescence microscope (Nikon Instruments Inc., Melville, NY) and representative images were acquired using SPOTTM Imaging Solutions software (version 4.6, Diagnostic Instruments, Inc., Sterling Heights, MI). Residual scaffold area was determined from photomicrographs of H&E-stained sections wherein the relative pixel area of scaffold remnants was quantified using NIH ImageJ software.

Statistical Analysis

Data for all quantitative measurements were analyzed by ANOVA with post-hoc Bonferroni testing using commercially available statistical software (StatPlus:mac 2008, AnalystSoft, Vancouver, BC). Statistically significant values were defined as $p < 0.05$.

Results and Discussion

SEM analysis of each silk group demonstrated that alterations in winding and post-winding fabrication parameters led to selective differences in matrix architecture (Figure 1A) which were consistent with observations from other published reports [23]. Previous studies have shown that the winding angle, defined as the angle of the spun silk to the horizontal plane of the mandrel, is directly proportional to the ASR used during liquid silk spinning while

constant rotational speed is maintained [23]. Group 1 scaffolds were created with an ASR of 2 mm/sec which led to deposition of silk fibers in an orientation normal to the mandrel axis which could be seen in both surface and cross-sectional views. An increase in ASR to 40 mm/sec during Group 2 matrix construction was observed to generate an internal criss-cross pattern of silk fibers in relation to the mandrel axis; however, deposition of the final silk layers at an ASR of 2 mm/sec led to similar surface features as encountered in Group 1. Post-winding methanol treatment employed during fabrication of Group 1 and 2 scaffolds was shown to conserve the morphology of the initial winding patterns as well as induce stratified cross-sectional architectures between the deposited silk layers. In contrast, lyophilization of Group 3 scaffolds, which were generated with similar winding parameters as Group 2, produced porous lamellar-like structures throughout the tubular cross-section. In addition, initial winding morphology was obscured in Group 3 matrices following the freeze-drying process and an increase in surface roughness was also apparent in comparison to all other groups.

Mechanical properties of each matrix group were assessed using tensile testing to determine the relationship between winding and post-winding fabrication parameters on elastic modulus, ultimate tensile strength, and elongation to failure (Figure 1B). In some cases, increases in ASR were found to directly correlate with elastic moduli as observed in comparisons between Groups 1 and 2; in particular, this parameter was significantly enhanced from 2.7 ± 0.35 MPa (Group 1) to 4.0 ± 0.81 MPa (Group 2). This trend was dependent on methanol post-winding treatment since lyophilization utilized in Group 3 fabrication generated matrices with a mean elastic modulus of 2.7 ± 0.6 MPa, similar to Group 1. These results suggest that the criss-cross pattern of silk fibers generated in the Group 2 matrices by the 40 mm/sec ASR and preserved by methanol treatment functioned to reinforce the overall stiffness of the construct potentially by aligning the silk fibers more parallel to the direction of tensile loading. Indeed, similar phenomena have been reported for electrospun polyurethane elastomers in which elastic moduli were found to be a function of the degree of matrix fiber alignment in respect to the loading axis [31].

A modest elevation in UTS was also observed in Group 2 and 3 matrices which exhibited mean values of 0.53 ± 0.05 MPa and 0.54 ± 0.09 MPa, respectively in comparison to 0.42 ± 0.06 MPa displayed by Group 1 constructs. The ability of higher ASR to enhance the UTS of these two scaffold groups independent of post-winding treatments may be associated with the increased number of silk layers deposited during gel spinning. The greater number of interfaces between adjacent layers would presumably generate higher degrees of adhesive forces subsequently increasing overall matrix structural strength in comparison to Group 1. This conclusion is in line with previous studies which have demonstrated that shearing and elongation forces between laminates of synthetic co-polymer composites increase as a function of layer number [32]. Elongation to failure assessments demonstrated that Group 3 scaffolds displayed significantly higher values at $51 \pm 6\%$ in comparison to Groups 1 and 2 which exhibited $28 \pm 3\%$ and $25 \pm 4\%$, respectively. This increase appears to be a consequence of both the ASR and post-winding fabrication parameters used during Group 3 construction which overall produced tougher, but more flexible scaffolds by lowering the degree of stiffness in comparison to Group 2 while increasing the UTS in relation to Group 1 values.

Animals augmented with each of the scaffold groups had an uneventful post-operative period with no mortality over the course of the 10 week implantation period. At the time of planned euthanasia, no signs of bladder calculi, mucus, or hydronephrosis were observed within any of the implant groups following macroscopic examination. These results are consistent with our previous study in which bladder reconstruction performed with Group 1 scaffolds led to an 82% survival rate between 3–10 weeks of implantation. In contrast,

animals augmented with PGA or SIS exhibited higher degrees of mortality due to scaffold perforation and subsequent urinary ascites with 71% and 66% survival rates observed, respectively [18]. Similar degrees of animal survival between Groups 1–3 suggest that despite variations in the structural and mechanical features of the implants, each possessed sufficient integrity to support defect stabilization. Since rupture of augmented bladders represents a significant life threatening complication following enterocystoplasty [33], biomaterial configurations which can minimize this risk, such as gel spun silk matrices described in this study, may provide potential benefits for clinical organ repair.

Gross histological examination of H&E-stained whole bladder sections revealed that in all experimental groups there was prominent ingrowth of connective tissue from the border of the native bladder wall, that spanned the original defect site by 10 weeks post-op (Figure 2A). In all cases, the majority of the residual silk scaffold was located within the lumen; however while the bulk of the matrices in Groups 1 and 2 remained largely intact, Group 3 scaffolds underwent substantial breakdown and were highly fragmented. Quantification of residual scaffold area demonstrated a modest reduction between Group 1 and 2 scaffolds, however significantly lower levels were observed in Group 3 matrices in comparison to Group 1 (Figure 2B). These results suggest that the combination of specific ASR and post-winding fabrication conditions utilized for Group 3 led to an enhanced degree of matrix degradation. This effect is presumably related to the highly porous structure of this scaffold configuration allowing for more efficient exposure to proteolytic enzymes and subsequent polymer hydrolysis due to increases in overall surface area in comparison to the more compact architecture of the other matrix groups. The ability of gel spinning fabrication parameters to alter the degradation rates of silk matrices *in vivo* represents a significant control point in the design of clinically relevant biomaterials for bladder augmentation since scaffolds should initially reinforce the defect site, but gradually dissipate and be replaced by host tissue formation.

For each experimental condition, H&E and MTS analyses revealed that the regenerated bladder wall consisted of a robust smooth muscle layer (pink: H&E; red: MTS) organized into bundles dispersed throughout the periphery of the consolidated tissue (Figure 3). No evidence of severe fibrotic remodeling was noted in this compartment in any of the implant groups. In addition, IHC assessments demonstrated that the reconstituted smooth muscle compartments of all scaffold groups stained positive for α -SMA and SM22 α contractile protein expression similar to the extents seen in non surgical controls; a feature indicative of smooth muscle maturation. Although the exact mechanisms of bladder smooth muscle regeneration supported by the silk scaffolds are currently unknown, previous studies have demonstrated that this process is linked to epithelial interactions, trans-differentiation of fibroblasts into SMCs, or dedifferentiation and migration of peripheral SMCs from the host bladder wall into the defect site [34–36].

An ECM-rich lamina propria populated with fibroblastic cells was evident in the regenerated tissues supported by all matrix configurations following 10 weeks post-op. Within these compartments, histological features indicative of a minimally acute inflammatory reaction were noted in all group implantation sites characterized by the presence of disperse eosinophil granulocytes (H&E analysis, Figure 4). In addition, a mild chronic inflammatory reaction was also apparent in each augmented group consisting of mononuclear cell aggregates organized in a follicular pattern (H&E analysis, Figure 4). Although, our previous study did not detect evidence of chronic inflammatory processes in Group 1 scaffolds [18], the current data may be a reflection of subtle variations in the composition of silk cocoon batches or the efficiency of the alkaline degumming process which may have led to higher levels of residual immunogenic components (i.e. sericin) in the resultant gel spun matrices described in this report. Nevertheless, foreign body reactions characterized by the

presence of multi-nuclear giant cells, which have the potential to lead to implant failure [37], were not observed in any of the regenerated tissues described here. Since conventional augment biomaterials such as those derived from PGA have been shown to elicit foreign body responses within the bladder [18], gel spun silk matrices may offer advantages for clinical applications due to their improved biocompatibility.

Following bladder reconstruction, reconstitution of the native urothelium at biomaterial implantation sites represents a critical event in the restoration of organ barrier function. Normal murine bladder urothelium consists of a three-layered transitional epithelium which is axially subdivided into basal, intermediate, and superficial cell layers [38]. After the onset of urothelial injury, basal cells have been reported to undergo initial proliferation and subsequent migration across the defect site generating a thin layer of re-epithelialization [38]. This stage is followed by hyperplasia of the basal cell layer which gradually differentiates towards the lumen into a pre-maturation zone of intermediate cells, ultimately giving rise to the terminally differentiated superficial cells [38]. During this maturation process, p63-positive basal cells [39] induce expression of UP as they progress toward an intermediate phenotype [38]. Generation of the superficial cell layer occurs with the loss of p63 expression and enhanced production of UP [38,39] which assemble into heterodimers and form asymmetrical unit membranes essential for the maintenance of the urothelial permeability barrier [40]. Urothelial regeneration is complete following normalization of basal cell proliferation and restoration of normal layer stratification [38].

In the current study, histological (H&E and MTS) and IHC assessments revealed substantial differences in the ability of the silk groups to support urothelial formation and maturation along the defect sites (Figure 5). Similar to non surgical controls, Group 1 scaffolds supported a three layer transitional urothelium exemplified by the presence of p63-positive basal and intermediate cell compartments which were lined by luminal p63-negative superficial cells. Robust pan-UP protein expression was also noted in this group in both superficial and intermediate cell layers to a degree comparable to non augmented bladders. These results were in contrast with the regenerated tissues supported by Group 2 matrices which were covered with a one layer thick epithelium composed primarily of basal cells characterized by p63 expression, but with undetectable production of UP proteins. These data indicate that Group 2 scaffolds promoted only early stages of urothelial regeneration at 10 weeks post-op, since induction of UP and formation of intermediate and superficial cells had not yet occurred. Within Group 3 scaffold implantation sites, a transitional urothelium was observed bordering the reconstituted lamina propria which exhibited hyperplastic basal/intermediate cell compartments consisting of 4–6 layers of p63-positive populations capped by a p63-negative superficial cell layer. In addition, strong pan-UP protein expression, similar to Group 1, was demonstrated in the superficial and proximal intermediate cell populations. Taken together, these results suggest that Group 3 scaffolds promoted higher degrees of urothelial maturation at the defect site in comparison to Group 2 matrices, but this process was overall less developed than observed in Group 1 since normalization of basal/intermediate cell proliferation and restoration of native layer stratification was incomplete. Future studies will center on kinetic evaluations of the regeneration process supported by the different matrix groups in order to better understand both the initial and long-term effects of scaffold processing parameters on implant performance.

The function of reconstructed bladders was assessed by VSOP analysis at 10 weeks post-op. VSOP calibration measurements showed there was a linear correlation between liquid volume and stained area on the filter paper within the range of 25–300 μ l (Figure 6A, $y = 0.106x + 2.44$, $R^2 = 0.9752$). Mice implanted with each scaffold configuration displayed voiding patterns similar to non surgical controls wherein micturition primarily occurred around the periphery of the filter paper (Figure 6B). As expected, both voiding frequency

and voided volumes were elevated in Groups 1 and 3 compared to control levels, consistent with increases in functional bladder capacity (Figure 6C, D). Group 2 animals displayed the highest voiding frequency in comparison to all other groups; however, they also demonstrated reduced voided volumes relative to the unoperated controls (Figure 6C, D). Previously, a mouse model of cyclophosphamide-induced cystitis has demonstrated a positive correlation between the disruption of the urothelial permeability layer and alterations in voiding behavior such as elevated voiding frequency and reduced functional bladder capacity [29]. Since histological and IHC analyses revealed an incomplete state of urothelial regeneration in Group 2 subjects, characterized by the absence of intermediate and superficial cell layers as well as prominent UP expression, these features may be responsible for the observed alterations in functional voiding characteristics.

Cystometric evaluations of bladder compliance revealed a high degree of intra-group variability in all experimental conditions, however no statistically significant differences were observed between the matrix groups and non surgical controls (Figure 7). Bladder compliance represents the ability of the organ to expand while maintaining safe, low urine storage pressures. Poor compliance is often a result of pathological bladder remodeling [18] which can lead to renal damage or failure [41]. The ability of the different silk configurations to maintain similar degrees of bladder compliance relative to unoperated controls is consistent with our macroscopic and histological observations, which showed no evidence of hydronephrosis as well as comparable extents of smooth muscle maturation and absence of severe fibrotic remodeling.

Conclusions

The results presented in this study provide the evidence that the *in vivo* performance of gel spun silk matrices in bladder reconstructive procedures can be manipulated by altering their structural and mechanical properties through selective variations in scaffold fabrication conditions. Our data demonstrate that modulation of winding and post-winding processing parameters between the silk groups examined was capable of tuning the degree of scaffold degradation, extent of urothelial regeneration, and voiding characteristics in augmented animals. In comparison to our original prototype matrix (Group 1), Group 3 scaffolds underwent greater degrees of degradation while supporting similar levels of smooth muscle formation and preserving bladder compliance and voiding functions. In addition, Group 3 matrices showed substantial improvements over Group 2 in their ability to support urothelial maturation; however, longer term studies are needed to ascertain if normalization of urothelial stratification can be achieved similar to Group 1. In summary, the silk gel spinning process provides a robust platform for the design of matrices for bladder augmentation wherein optimization of tissue regeneration and restoration of organ function are dependent on scaffold fabrication parameters.

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Figure 1. Structural and mechanical analyses of scaffold groups

[A] Photomicrographs of representative SEM images demonstrating top and cross-sectional views of silk matrix configurations constructed with different winding [axial slew rates (ASR), 2 and 40 mm/sec] and post-winding (PW) [methanol and lyophilization] treatments. Group 1: ASR = 2 mm/sec, PW = methanol; Group 2: ASR = 40 mm/sec, PW = methanol; Group 3: ASR = 40 mm/sec, PW = lyophilization. Surface coating with ASR = 2 mm/sec for Groups 2 and 3. Arrows denote perpendicular alignment of fibers relative to mandrel (red); internal cross hatching of fibers relative to mandrel (yellow); dense lamellar structure (white); enhanced porosity (green). [B] Evaluation of elastic modulus, ultimate tensile strength (UTS), and % elongation to failure in matrix groups defined in [A]. Means $\pm$ standard deviation per data point. (*) = $p < 0.05$, significantly different from all other groups.

Figure 2. Global histological comparisons of the extent of tissue regeneration and in vivo degradation in silk variants

[A] Photomicrographs of total bladders (H&E-stained sections) augmented with each scaffold group over the course of the 10 week implantation period. (*) denotes scaffold fragments. Brackets represent area of tissue regeneration. Scale bar = 2.5 mm. [B] Analysis of residual scaffold area present in each experimental group listed in [A]. Means $\pm$ standard deviation per data point. (γ) = $p < 0.05$, significantly different from Group 1.

Figure 3. Histological and immunohistochemical evaluations of the extent of smooth muscle regeneration following biomaterial incorporation

[1st, 2nd rows] Photomicrographs of magnified regenerated tissue area in bladders augmented with each scaffold group following 10 weeks post-op and non surgical controls subjected to H&E and MTS analyses. Scale bar = 200 μ m. For all panels (SM) denotes smooth muscle bundles. [3rd, 4th 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 4. Inflammatory responses elicited by scaffold groups

Photomicrographs of magnified regenerated tissue area in bladders augmented with each scaffold group following 10 weeks post-op subjected to H&E analyses. [1st row], minimal acute inflammatory reactions denoted by the presence of disperse eosinophil granulocytes (orange arrows). [2nd row], mild chronic inflammatory reactions characterized by aggregates of mobilized mononuclear cells (*). For 1st row, scale bar = 200 μ m. For 2nd row, scale bar = 800 μ m.

Figure 5. Histological and immunohistochemical assessments of the degree of urothelial regeneration supported by scaffold groups

[1st, 2nd rows] Photomicrographs of magnified regenerated tissue area in bladders augmented with each scaffold group following 10 weeks post-op and non surgical controls subjected to H&E and MTS analyses. For all panels (UE) denotes urothelial compartment and (*) denotes scaffold fragments. [3rd, 4th 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 1–2 and 4, scale bar = 200 μ m. For 3rd row, scale bar = 400 μ m.

Figure 6. Voided stain on paper analysis of animals implanted with various scaffold groups [A] Linear correlation between liquid volume and stained area on filter paper within the range of 25–300 μ l, ($y = 0.125x + 0.898$, $R^2 = 0.9979$). [B] Photomicrographs of UV-enhanced voiding patterns on filter paper representative of each matrix group and non surgical controls. Scale bar = 9.5 cm. Comparison of voiding frequencies [C] and voided volume [D] between scaffold groups and control animals. For [C, D], data are expressed as means $\pm$ standard deviation.

Figure 7. Cystometric analysis of compliance in bladders augmented with different scaffold groups

Scatter plot comparisons of compliance values in each surgical group expressed as a % normalized to median values displayed by untreated controls. (+) denotes group replicate values with (–) representing median levels.

Table 1

Experimental design and analyses

Summary of animal numbers and type of analyses for each implant group.

Groups	Total number of animals analyzed	Histology	VSOP	Urodynamics
Control	6	6	6	5
1	10	4	7	6
2	5	5	5	4
3	6	4	6	4