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Tetronic®-based Composite Hydrogel scaffolds seeded with Rat-Bladder Smooth Muscle Cells for Urinary Bladder Tissue Engineering Applications

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Abstract

Natural hydrogels such as collagen offer desirable properties for tissue engineering, including cell adhesion sites, but their low mechanical strength is not suitable for bladder-tissue regeneration. In contrast, synthetic hydrogels such as PEG allow tuning of mechanical properties, but do not elicit protein adsorption or cell adhesion. For this reason, we explored the use of composite hydrogel blends composed of Tetronic (BASF) 1107-acrylate (T1107A) in combination with extracellular matrix (ECM) moieties collagen and hyaluronic acid seeded with bladder smooth muscle cells (BSMC). This composite hydrogel supported BSMC growth and distribution throughout the construct. When compared to the control (acellular) hydrogels, mechanical properties (peak stress, peak strain, and elastic modulus) of the cellular hydrogels were significantly greater. When compared to the 7-day time point after BSMC seeding, results of mechanical testing at the 14-day time point indicated a significant increase in both ultimate tensile stress (4.1 kPa to 11.6 kPa) and elastic modulus (11.8 kPa to 42.7 kPa) in cellular hydrogels. The time-dependent improvement in stiffness and strength of the cellular constructs can be attributed to the continuous collagen deposition and reconstruction by BSMC seeded in the matrix. The composite hydrogel provided a biocompatible scaffold for BSMC to thrive and strengthen the matrix; furthering this trend could lead to strengthening the construct to match the mechanical properties of the bladder.

Keywords

Composite hydrogel; Bladder smooth muscle cell; Collagen synthesis; Tetronic 1107 Acrylate; Tissue engineering

Introduction

Bladder augmentation has been conventionally performed using autologous natural tissue such as a portion of the intestine (enterocystoplasty) (1) to treat patients who lack sufficient bladder capacity or detrusor compliance.(2) However, enterocystoplasty can generate long-term complications, including mucus production by bowel epithelium, stone formation, malignancy and bacteriuria. (3,4) Other tissue grafts such as skeletal muscle flaps (5) and

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human amniotic membrane (6) have limitations including lithogenesis and immature smooth-muscle layer development. (5,6) Researchers have studied other tissue engineering approaches in bladder augmentation with cell-seeded collagen-PGA/PLGA composite scaffolds (7) small-intestine submucosa (SIS) (8),(9,10) and multilaminate matrices derived from silk fibroin. (11) However, these materials exhibited inadequate peak strain, (12) or extensibility (13) when compared to native bladder tissues. (14) For example, the collagen-PGA/PLGA scaffolds that were used in human clinical studies (15,16) would exhibit twice the stiffness (Young's modulus ~ 0.002 MPa) compared to native bladder tissue (Young's modulus ~ 0.001 MPa)(7) and hence, doesn't allow natural distension and contraction required for normal bladder function. Thus, mechanical mismatch is a major obstacle in designing a scaffold for bladder tissue engineering applications.

Hydrogels have been investigated as scaffold materials because they display low sliding resistance against other tissues and viscoelastic mechanical properties similar to the extracellular matrix (ECM).(17) The mechanical properties of hydrogels can be manipulated based on the cross links formed between polymer chains via chemical bonds and can be designed according to mechanical necessities. (17) The high water absorbance and liquid content (> 20 wt%) of hydrogels enables adequate nutrient and waste transport to support encapsulated cells. (18) Thus, synthetic and biologically-derived hydrogels are being explored as potential scaffold material for bladder tissue engineering applications. For example, using matrix metalloproteinase (MMP)-sensitive poly (ethylene glycol) (PEG) hydrogels (19), Adelow and colleagues demonstrated that human mesenchymal stem cells (MSC) seeded on these scaffolds differentiated into bladder smooth muscle-like cells after two weeks in culture (20). However, PEG-based scaffolds in general tend to be relatively weak for the mechanical necessities of a number of load-bearing organs including the urinary bladder (21) and have the drawback of post-polymerization swelling. Thus, alternative approaches are needed to improve the strength and stiffness of hydrogel-based bladder grafts.

Tetronics (BASF) are four-armed polyethylene oxide-polypropylene oxide (PEO-PPO) block copolymers comprised of a hydrophobic PPO core domain surrounded by a hydrophilic PEO shell.(22) When compared to PEG hydrogels that are prepared only by covalent crosslinking, Tetronic-based hydrogels are prepared by a combination of covalent and noncovalent cross-linking, leading to improved physical properties. Previously, Sefton and colleagues characterized the semi-synthetic Tetronic T1107-collagen hydrogels (photo-polymerization of T1107-methacrylate in collagen-containing aqueous solutions) and demonstrated that the hydrogel scaffolds exhibited higher storage and loss moduli than pure collagen gels. (22) Although in this report human hepatoma HepG2 cells embedded in T1107-collagen were viable, (21) synthetic hydrogels with highly cross-linked networks tend to restrict proliferation and migration of encapsulated cells and delay matrix production in early stages of tissue remodeling.(23) To mitigate this, Kutty and colleagues explored addition of hyaluronic acid (HA) to PEG-based hydrogels and demonstrated improvement in spreading and cell proliferation of fibroblasts within the construct. (23) The addition of hyaluronidase inhibitors neomycin trisulfate and ascorbic acid-6-palmitate eliminated fibroblast-spreading within HA-containing semi-IPNs, confirming the importance of enzymatic HA degradation in creating localized imperfections leading to fibroblast

proliferation. These studies have demonstrated that combination of synthetic moieties such as T1107-methacrylate and PEG-bis-AP with ECM components such as collagen and HA improved overall strength and stiffness of the hydrogel construct and could serve scaffolds for tissue engineered applications. However, little is known about the effect of encapsulated cells on the mechanical properties of these hybrid synthetic-ECM hydrogel scaffolds.

The objective of the present study was to characterize a composite hydrogel scaffold as a matrix material for culture of BSMC in vitro. This is the first study to explore the use of hydrogel blends composed of Tetronic T1107-acrylate (T1107A) in combination with type I collagen and HA toward applications in urinary bladder tissue engineering. We examined cell morphology and ECM production by BSMC embedded in this composite hydrogel system and the time-course variation in the mechanical behavior of the cell-seeded composite hydrogel constructs.

Materials and Methods

Materials

Free samples of Tetronic T1107 (T1107, MW: 15 kDa, HLB: 18–23) and Irgacure 2959 were obtained from BASF corporation (USA). Acryloyl chloride, Celite 500 fine and 4-methoxyphenol, and hexanes were purchased from Sigma-Aldrich (St.Louis, MO, USA). Toluene (HPLC grade), ethyl ether (anhydrous, BHT stabilized), hexanes (HPLC grade) and anhydrous sodium sulfate were purchased from Fisher Scientific (NJ, USA). Dichloromethane (HPLC grade), triethylamine (TEA), sodium bicarbonate, calcium hydride and CDCl_3 were obtained from Acros Organics (NJ, USA). Dichloromethane was dried with calcium hydride and stored over molecular sieves (Grade 514, Type 4A). RPMI 1640 medium and trypsin-EDTA (0.05 %) were obtained from Gibco/Life Technologies (Canada). Fetal Bovine serum (FBS) was obtained from Hyclone (Logan, UT), Collagen type I from MP Biomedicals (Solon, OH, USA) and hyaluronic acid (HA) from Sigma-Aldrich (St.Louis, MO, USA). All chemicals were used as received.

Cell culture

BSMC were isolated from the bladders of adult Sprague-Dawley rats (Female, ~12 weeks old) following our established methods.(24) Prior to harvesting of the organs, the rats were euthanized in accordance with the policies of Clemson's Institutional Animal Care and Use Committee based on the Animal Welfare Act. Briefly, using the aseptic technique, the mucosal layer of the bladder was mechanically removed under a dissection microscope, and the rest of the tissue was digested in a solution containing RPMI 1640 medium, 0.1% collagenase (Type II, Worthington, NJ), and 0.2% trypsin-EDTA to dissociate cells from the extracellular matrix. The smooth muscle cells were collected by centrifugation of the tissue digest and then cultured in RPMI medium supplemented with 10% FBS and 1% Pencillin-Streptomycin (P/S) under standard cell-culture conditions (37 °C, humidified, 5% CO_2 /95% air). The smooth muscle phenotype of these cells was confirmed by immunostaining with mouse monoclonal antibodies for α -smooth muscle actin (1:500 dilution, Sigma-Aldrich, USA) and smooth muscle myosin heavy chain (SM1(1:400 dilution) & SM2(1:500 dilution); Hybridoma Banks, University of Iowa). Fluorescently labeled Alexa Fluor 488 was used as

the secondary antibody (Invitrogen, 1:200 dilution). The immunostaining and imaging revealed that the isolated rat bladder SMC clearly expressed both α -smooth muscle actin and SM-MHC (SM1 and SM2) up to six passages. Therefore, only cells up to six passages were used in these experiments.

Synthesis of T-1107 acrylate

T1107-acrylate (T1107A) was prepared by reaction of T1107 terminal hydroxyl groups with acryloyl chloride. (25) Briefly, T1107 (30g; 2 nmol) was dehydrated by azeotropic distillation for 2 h with toluene, which was then removed by rotary evaporation (Buchi Rotavapor®, Switzerland). After being cooled to room temperature, the dried T1107 was dissolved in 270 ml dehydrated dichloromethane and mixed with TEA (1.694 ml; 8 mmol). Acryloyl chloride (1.494 ml; 9 mmol) in 30 ml of dry dichloromethane was added dropwise to this mixture, and the reaction was allowed to continue at 4°C for 24 h. The reactant was filtered through Celite to remove TEA-HCl salt and then concentrated by rotary evaporation to reduce the solvent to one-tenth of its initial volume. The residue obtained was precipitated in 250 ml of cold ethyl ether and 250 ml of hexane, recovered by filtration and dried under vacuum for a few hours. The product was redissolved in 300 ml of dichloromethane and washed with 30 ml of 10% w/v sodium bicarbonate solution until the pH of the solution was neutral. This was followed by water washes (30 ml each) until the pH of the water was neutral and drying with anhydrous sodium sulfate. After the solution was concentrated by rotary evaporation, the residue was precipitated and washed three times with cold ethyl ether (−20 °C). The final product was recovered by filtration, dried for 48 h in a vacuum desiccator and stored at 4°C until use. (25) The NMR (Bruker Avance III 300, USA) spectra obtained on the final product was used to determine its acrylation efficiency.

Preparation of composite hydrogel

The T1107A (117.5 mg/ml) powder was added to collagen type I solution (6 mg/ml in 0.02 N acetic acid) and mixed overnight at 4 °C. The collagen-T1107A solution (319 μ l) and photo-initiator Irgacure 2959 (I-2959; 7.5 μ l, 1:10 dilution in ethanol) were incubated at 4°C for 30 min with periodic vortexing. The centrifuge tubes containing the hydrogel solution were then brought to room temperature inside the biosafety cabinet; subsequently, hyaluronic acid (HA) (MW=1.5–1.8 MDa; 75–150 μ l; 1:100 dilution in H₂O) was added to this solution using a 1000 μ l pipette. BSMC (4×10^6 /ml) in 3 $\times$ RPMI +10% FBS solution were then mixed into the solution to form the final hydrogel solution (0.75mL), composed of 5% T1107A, 0.25% collagen type I, 0.1–0.2% HA, 0.1% I-2959 and $\sim 3 \times 10^6$ cells. The total volume was balanced using 3 $\times$ RPMI + 10% FBS solution for different concentrations of HA, and the BSMC were omitted in acellular hydrogels. The gel specimens were cast in custom-made Teflon molds (3cm $\times$ 1cm $\times$ 0.5cm) with biovyon (Porvair plc, Norfolk, UK) wafers on the ends as anchors (24) and polymerized under UV light for 12 min. The SMC-seeded composite hydrogel constructs were cultured in RPMI medium supplemented with 10% FBS and 1% penicillin-streptomycin for up to 14 days.

Quantification of mass swelling ratio of composite hydrogel

Acellular hydrogel specimens were equilibrated in PBS for 24 h to remove unpolymerized monomers and lyophilized. After dry weights (w_d) were recorded, the specimens were soaked in distilled water and allowed to swell for 24 h. Subsequently the wet weights (w_s) were recorded, and mass swelling ratio, q , was calculated as the ratio of wet to dry weight (w_s/w_d).

Examination of BSMC viability and morphology

BSMC viability inside the composite hydrogels was qualitatively determined with a live/dead viability/cytotoxicity kit (molecular probes, NY, USA). After 4, 7 and 14 days in culture, the composite hydrogels were incubated with 2 μ M Calcein-AM to stain the living cells and 4 μ M Ethidium homodimer-1 to stain the dead cells. After 30 min, the constructs were visualized using a laser confocal microscopy (C1Si Confocal; Nikon Ti Eclipse).

To qualitatively determine cell morphology, the BSMC encapsulated in the hydrogel were fixed in paraformaldehyde (1:50 dilution in PBS, P6148, Sigma) at room temperature for 15 min, and the excess aldehyde in the constructs was quenched with 0.1M glycine (T9284, Sigma) for 5 min at the end of the prescribed time periods. The cells were permeabilized with 0.1% triton-X 100 (T9284, Sigma) for 1 min and incubated with rhodamine-phalloidin (1:100 dilution in PBS, R415, Molecular Probes/Invitrogen) for 15 min. This was followed by nuclear staining with DAPI (1:100 dilution, D-1306, Molecular probes, Eugene, OR) for 5 min. The hydrogels were then subjected to triplicate PBS washes for 5 min, and the specimens were imaged using laser confocal microscopy (C1Si Confocal; Nikon Ti Eclipse).

Mechanical characterization of composite hydrogel

The mechanical characterization of the composite hydrogels was performed by uniaxial tensile testing using MTS Synergie 100 to quantify peak stress, elastic modulus and peak stretch of the samples. The dog bone-shape hydrogel constructs were subjected to tension under hydrated conditions (PBS at 37°C) at a rate of 5mm/min until rupture. Average Lagrangian peak stress values were calculated from the load applied to the specimens over continuous time-points divided by the original cross-sectional area of the hydrogel. The stress-stretch relationship was analyzed where stress (τ) was plotted against stretch (λ) (where λ is the stretch ratio of deformed to reference lengths). The elastic modulus (kPa) of the hydrogels was calculated from the linear regions of their respective stress-strain curves. Peak strain was also calculated.

Quantification of collagen synthesis by BSMC

After 7 and 14 days of culture, hydroxyproline assay was performed according to the published method to quantify the collagen synthesized by BSMC (hydroxyproline represents 12% of collagen (w/w)). (26) Acellular hydrogel samples were used as control. Briefly, all hydrogel samples were flash frozen in liquid nitrogen and lyophilized using a freeze dry system (Freezone 4.5, Labconco, USA). The lyophilized samples were then hydrolyzed in 4N NaOH at 120 °C for 4 hours and neutralized with citric acid (1.4N). Series concentrations (0–50%) of hydroxyproline (100 μ g/ml) dilutions were prepared as standards.

Each of the samples and standards was mixed with 1 ml of chloramine-T and incubated at room temperature for 20 min. This was followed by addition of p-dimethylaminobenzaldehyde (PDMAB) and additional incubation at 65°C for 15 min. The light absorbance of samples was measured at $\lambda=550$ nm in triplicate using a universal microplate spectrophotometer (μ Quant, Biotek Instruments Inc., USA).

Statistical analysis

All numerical data were analyzed using the single-factor Analysis of Variance (ANOVA), followed by a post-hoc test when statistical significance was detected. All statistical analyses were performed using JMP ANOVA statistical software package (VENDOR). Four samples were used for uniaxial mechanical testing and three samples were used in the hydroxyproline assay.

Results

Synthesis of T-1107 acrylate

Schematic representation of T1107A synthesis is shown in Figure 1. The percent acrylation efficiency was calculated based on the ratio of the integrals of the PEG backbone ($\delta=3.5$ – 3.7) and acrylic peaks ($\delta=6.15$ – 6.4) (Figure 2) as previously shown in studies done by Cho et al. (27) A yield of 22.5 g of T1107A was obtained from the original 30g of T1107 used in the synthesis and T1107A having more than 85% acrylation efficiency was used in the experiments. Efficient removal of unreacted acryloyl chloride was confirmed by the ratio of acrylate to activated PEG terminal ($\delta=4.3$) methylene peaks, which closely approximated the theoretical value, 3:2.

Mass swelling ratio and mechanical characterization of composite hydrogel scaffold

The ratios of swollen mass to dry mass of acellular composite hydrogel specimens (Figure 3) were similar for the three different concentrations of HA (0.1, 0.15 and 0.2%) used in the hydrogels (Figure 4A). In contrast, the results of tensile testing indicated that higher the concentration of HA, greater the elastic modulus (defined as the slope of the linear region of the stress/stretch curve) of the composite hydrogels (Figure 4B). The formulations containing 0.15 and 0.20% HA exhibited significantly ($P<0.01$) greater elastic modulus (stiffness) than the specimens with 0.10% HA content (Figure 4B). Based on this result, we incorporated 0.2% HA in our composite hydrogels for the rest of our study.

BSMC Cell Viability and Morphology

The live/dead study indicated the presence of viable cells in the scaffold at a magnification of 100 $\times$ after 4, 7 and 14 days of culture (Figure 5). The Rhodamine-phalloidin staining and confocal imaging revealed distribution of BSMC up to a depth of 1.13 mm of the construct, which after 14 days in culture, revealed an increased distribution for a depth of 1.26 mm along with increased BSMC migration towards the surface of the hydrogel scaffolds (Figure 6A and 6B). When the constructs were viewed at the 600 $\times$ magnification at the 14 day time-point, the individual BSMC demonstrated a spread cell morphology (Figure 6C).

Effects of cell-seeding on mechanical properties of composite hydrogel

When compared to acellular gels (Peak Stress: 4.1 ± 1.2 kPa, Elastic modulus (stiffness): 11.8 ± 1.1 kPa, Peak Stretch: 1.21 ± 1.23) hydrogels seeded with BSMC (4×10^6 cells /mL) exhibited greater values for peak stress, peak stretch, and elastic modulus (Figure 7). Among the BSMC-seeded hydrogels, specimens cultured for 14 days exhibited significantly ($P < 0.05$) greater values of peak stress (11.6 ± 2.2 kPa) and elastic modulus (42.7 ± 4.0 kPa) compared to specimens cultured for 7 days (Peak stress: 5.2 ± 0.6 kPa, Elastic modulus: 19.3 ± 2.8 kPa). When compared to cell-seeded hydrogel specimens cultured 7 days (1.23 ± 0.04), specimens cultured 14 days (1.39 ± 0.12) exhibited peak stretch values with a strong trend of higher value; this was not statistically different due to large variability (Figure 7B).

Hydroxyproline assay

The results of the hydroxyproline assay indicated that compared to acellular samples (35.1 ± 3.1 μ g collagen/mg sample mass), the 7-day (48.5 ± 9.6 μ g collagen/mg sample mass) and 14-day cellular constructs (61.7 ± 5.9 μ g collagen/mg sample mass) contained significantly ($p < 0.05$) greater amounts of collagen (Figure 8).

Discussion

The long-term goal of the present study is to develop a functional 3D tissue construct for bladder augmentation. In pursuit of this goal, we prepared a composite hydrogel using a blend of Tetronic T1107A, type I collagen, and HA cross-linked to form a semi-IPN scaffold for encapsulation of BSMC. First, to evaluate the impact of HA inclusion on mass swelling ratio and elastic modulus (stiffness) of our composite hydrogel, different concentrations of HA (0.1%, 0.15%, 0.20%) were mixed into the acellular hydrogel constructs. Results show that while the mass swelling ratios of hydrogels with different concentration of HA were nearly identical, the elastic moduli increased with increasing concentration of HA (Figure 4). These results are in agreement with the report by Kutty et al., which demonstrated that in PEG-bis-AP/HA semi-IPNs, increasing concentration of HA from 0.06% to 0.18% caused a minimal increase in volumetric swelling ratio and a significant increase in elastic modulus relative to controls without HA. (23) The lack of any change in the mass swelling ratio indicates that the inclusion of HA in our composite scaffold did not affect the density of the construct's crosslinks and consequently, did not affect swelling. These results are in contrast with a previous study, which demonstrated that in collagen-HA semi-interpenetrating network hydrogels, inclusion of increasing concentrations of HA led to a corresponding decrease in the swelling ratio. (28) This was because the HA chains in the collagen-HA semi-interpenetrating network hydrogels were cross-linked into the composition, causing stiffer gels. (29) In the present study, the HA molecules were not cross-linked, and they merely entangled with the main Tetronic network inside the composite hydrogels. Thus, increasing HA weight percentage did not cause increased crosslinking and decreased swelling. However, HA solid content, due to its high molecular weight relative to collagen (23), molecularly reinforced the hydrogel (29) and had a significant impact on elastic modulus (stiffness) of the composite scaffold (Figure 4). Previous studies have shown that the elastic modulus of alginate gels is affected by the molecular weight of polysaccharides encapsulated in it. (30) HA, one of the longest types of

glycosaminoglycans (GAG), led to increased elastic modulus (stiffness) in the composite hydrogel with increasing concentration. This was demonstrated by Kim et al., who showed that higher weight percentage of HA led to increased elastic modulus in MMP-sensitive HA-based hydrogels.(31)

In the present study, the BSMC encapsulated in composite hydrogel displayed high cell viability at various time points (Figure 5). Moreover, the confocal imaging demonstrated that uniform distribution of BSMC was visible in the scaffold initially at 4 days which improved after 14 days (Figure 6). These results are in agreement with previous studies that during a similar culture period, smooth muscle cells seeded in collagen-based hydrogels tended to migrate towards the surface of the constructs. (32,33). The increased BSMC distribution may be due to facilitation of cell spreading and migration by the degradation of HA and localized increases in mesh size of the scaffold, as previously reported with fibroblasts encapsulated in HA-containing PEG-based semi-IPN hydrogels. (23). Though BSMC at 4 and 7 days exhibited the round morphology of the synthetic phenotype of smooth muscle cells (Figure 5A and B), the BSMC at the 14day time-point exhibited a more spread morphology (Figure 5C and 6C) indicating a possible shift toward contractile phenotype. Contractile and synthetic phenotypes of SMCs represent two opposite ends of the phenotypic spectrum with a continuum of varying phenotypic intermediates.(24) The typical characteristics of synthetic phenotype of smooth muscle cells include high proliferation, rounder morphology and production of excessive amounts of ECM compared to quiescent contractile phenotype, which typically demonstrates an elongated morphology.

In mechanical testing, the hydrogel scaffolds increased in strength and elastic modulus (stiffness) when seeded with BSMC and cultured for up to 14 days. Compared to the acellular hydrogels, the mechanical properties (peak stress, peak strain and elastic modulus) of the cellular hydrogels were significantly greater. The average peak stress and elastic modulus of the BSMC-seeded composite hydrogels were significantly ($p < 0.05$) higher 14 days after cell seeding than specimens tested 7 days after BSMC seeding. We hypothesized that the increase in strength and elastic modulus (stiffness) of the cellular constructs was due to de novo ECM synthesis by BSMC, and we quantified the amount of collagen within the cell-seeded composite hydrogel specimens using hydroxyproline assay. (34) Collagen (especially types 1 and 3) is the major protein of the urinary bladder ECM and contains various cell-adhesion domains important for maintaining native phenotype and cellular activity. (35) Since the 7-day and 14-day cellular constructs contained significantly ($p < 0.05$) greater amounts of collagen per specimen mass than the acellular composite hydrogel scaffolds (Figure 5), we concluded that BSMC deposited the newly synthesized collagen, which led to the increase in strength and stiffness of the gel constructs (Figure 4). Although the increased collagen content suggested correlative increases in mechanical strength, the ultimate tensile stress of the BSMC-seeded composite hydrogels in the present study (11.6 ± 2.2 kPa) did not display the level achieved in other studies (32) or an estimated peak physiological stress of the native bladder (~ 100 kPa).(36) This may be because the two-week end-point used in the present study was too short (32) or chemical stimulation was needed to support crosslinking of newly synthesized collagen fibrils that contributes to the stiffening of the overall construct.(37) For example, Berglund et al. reported that the ultimate tensile stress of endothelial cell-seeded collagen hybrid scaffolds was more than

100kPa at the end of 23 days. (38) Tranquillo and colleagues demonstrated that addition of 1 or 5 ng/ml of TGF- β 1 and 2 μ g/ml of insulin led to a seven-fold increase in collagen concentration and cross-linking with a three-fold increase in ultimate tensile strength in fibrin gel-based constructs seeded with neonatal vascular smooth muscle cells. (39) Thus, to promote cross-linking of the newly synthesized collagen it may be necessary to culture the composite hydrogels for longer time periods of 3–5 weeks and/or in the presence of soluble chemical compounds. (39)(40)

Conclusion

Mechanical mismatch has been a major obstacle in designing a scaffold for bladder tissue engineering applications. The composite hydrogel system in our current study provided a viable environment for bladder smooth muscle cells to survive and reconstruct the scaffold, thereby improving the construct's overall strength and stiffness. Culturing the construct for longer time periods after BSMC seeding and addition of growth factors to the composite hydrogel system would further aid in accurately mimicking the mechanical properties of the bladder wall.

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Figure 1. Schematic representation of synthesis of T1107A

The formation of acrylate groups on all four arms of T1107, a block copolymer of (a) Polyethylene oxide (PEO) and (b) polypropylene oxide (PPO), is described.

Figure 2. Representative ¹H-NMR spectrum of acrylated T1107

Analysis of the NMR data based on the integrals of the PEG backbone ($\delta=3.5\text{--}3.7$) and acrylic peaks ($\delta=6.15\text{--}6.4$) revealed an acrylation efficiency of 92 %.

Figure 3. Representative image of the acellular composite hydrogel

The composite hydrogel was obtained by subjecting the gel composition to UV radiation for 12 minutes between BioVYON wafers which assist as grips during uniaxial tensile testing.

Figure 4. Effect of HA content on the mass swelling ratio and elastic modulus of the composite acellular hydrogels

While swelling ratio (A) was unaffected, the elastic modulus (B) increased significantly as concentration of HA in the composite hydrogels increased. Values are mean $\pm$ SEM, * $p < 0.01$, $n = 3$ per group.

Figure 5. Live/Dead staining of BSMC in the composite hydrogel

High viability of BSMC was confirmed by the presence of live (green) cells and the absence of dead (red) cells in the scaffold after 4 (A), 7 (B) and 14 (C) days of cell seeding. In addition, BSMC exhibited spread cell morphology at day 14 (C) (Magnification=100×).

Figure 6. Confocal imaging of BSMC in the composite hydrogels

The results demonstrate BSMC are well distributed at day 4 (A: magnification = 40 $\times$), and increased cell migration towards the surface at day 14 (B: magnification = 40 $\times$). The spread individual bladder smooth muscle cells are also seen at the 14-day culture period (C: magnification = 600 $\times$).

Figure 7. Mechanical properties of the composite cellular hydrogels

Specimens were subjected to uniaxial tension at the rate of 5mm/min until failure and average peak stress (A), peak stretch (B) and elastic modulus (C) were calculated from the stress-stretch relationship (D) of composite cellular hydrogels at the 7 and 14 day time point. The lines across the bar graphs represent the corresponding values for acellular composite hydrogels (0.2% HA). Values are mean $\pm$ SEM; analyzed by ANOVA, * $p < 0.05$, ** $p < 0.01$, $n = 4$.

Figure 8. Collagen concentration in the composite hydrogels

Results of hydroxyproline assay revealed that cellular specimens contained significantly greater amounts of collagen after 7 and 14 days of culture compared to the acellular specimens. Values are mean $\pm$ SEM; * $p < 0.01$, $n = 3$.