Photo-Cross-Linking of Type I Collagen Gels in the Presence of Smooth Muscle Cells: Mechanical Properties, Cell Viability, and Function

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The effectiveness of photomediated cross-linking of type I collagen gels in the presence of rat aortic smooth muscle cells (RASMC) as a method to enhance gel mechanical properties while retaining native collagen triple helical structure and maintaining high cell viability was investigated. Collagen was chemically modified to incorporate an acrylate moiety. Collagen methacrylamide was cast into gels in the presence of a photoinitiator along with RASMC. The gels were cross-linked using visible light irradiation. Neither acrylate modification nor the cross-linking reaction altered collagen triple helical content. The cross-linking reaction, however, moved the denaturation temperature beyond the physiologic range. A twelve-fold increase in shear modulus was observed after cross-linking. Cell viability in the range of 70% ($n = 4$, $p > 0.05$) was observed in the photo-cross-linked gels. Moreover the cells were able to contract the cross-linked gel in a manner commensurate with that observed for natural type I collagen. Methacrylate-mediated photo-cross-linking is a facile route to improve mechanical properties of collagen gels in the presence of cells while maintaining high cell viability. This enhances the potential for type I collagen gels to be used as scaffolds for tissue engineering.

Introduction

The failure of the current generation of commercially available synthetic vascular prostheses is related to maladaptive biological reactions at both the blood–material and tissue–material interfaces. In response to these problems, alternative strategies have endeavored to design an arterial prosthesis through the mimicry of some or all of the characteristics of the arterial wall. For example, several investigators have sought to develop a functional endothelial monolayer on the luminal surface of a synthetic vascular prosthesis by the transplantation of autologous or allogeneic endothelial cells onto the prosthetic surface prior to graft implantation.[1–4] Likewise, alternative approaches have focused on the development of synthetic prostheses that contain angiogenic factors in order to regenerate an endothelial lining after graft implantation. Although promising results have been reported with both strategies, vascular grafts that are composed, either in whole or in part, from synthetic polymeric materials remain at risk for bacterial colonization and subsequent graft infection and, in addition, are capable of promoting a low-level, chronic inflammatory response that may contribute to the development of neointimal hyperplasia.[5] Moreover, a "compliance mismatch" that characteristi-

cally exists between high modulus prosthetic grafts and the host artery may also lead to neointimal hyperplasia and late graft failure.[6,7] Thus, the inherent limitations of a biohybrid prosthesis that consists, in part, of nonbiological elements have motivated investigators to develop arterial constructs that are comprised exclusively of biological components.

In 1986, Weinberg and Bell[8] generated an arterial construct consisting of a cell populated collagen gel. Although cell-mediated reorganization of the surrounding collagen matrix enhanced the mechanical integrity of the construct, this model did not display adequate mechanical properties necessary for in vivo applications. In response to this limitation, other investigators have sought to improve the mechanical characteristics of tissue engineered media/adventitial equivalents by the introduction of techniques that promote cell-assisted matrix protein assembly or endogenous collagen cross-linking. The latter approach has been pursued primarily by the supplementation of culture media with ascorbic acid or ribose.[9–13] Despite encouraging results, months of incubation are required before any of these constructs are suitable for implantation. To accelerate the time period for the maturation of a mechanically robust media equivalent, we postulated that the introduction of photochemically cross-linkable moieties into native collagen might facilitate rapid and controllable matrix protein cross-linking. We report, herein, visible light mediated photo-cross-linking of smooth muscle cell containing collagen matrixes as a mechanism to improve the mechanical properties of tissue engineered media equivalents.

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Experimental Section

Materials. Methacrylic anhydride, eosin Y (EY, 5 wt % in water), triethanolamine (TEA), and 1-vinyl-2-pyrrolidinone (VP) were obtained from Aldrich (Milwaukee, WI) and used as received. A standard photoinitiator solution was prepared according to compositions published elsewhere.[14] The solution composition was 10 mM EY, 225 mM TEA, and 37 mM VP. Solutions were stored in radio-opaque containers after preparation and prepared each time prior to use. Dialysis was conducted using Spectra/pro membrane (MWCO 6000–8000) obtained from VWR Scientific (West Chester, PA). Antibodies against smooth muscle α-actin (SMA), vimentin, and smooth muscle-specific-myosin were obtained from Sigma Chemicals (St. Louis, MI). Collagenase type IA was likewise obtained from Sigma and used as received.

Instrumentation. All ^{1}H NMR spectra were recorded at room temperature on an INOVA 600 spectrometer (Varian, Palo Alto, CA) operating at a ^{1}H resonance frequency of 600 MHz. Thirty-two scans were acquired for signal-to-noise averaging, and a recycle delay of 30 s was used to ensure quantitative spectra. In all cases D_2O was used as the internal standard, and a concentration of 10 mg/mL was employed.

CD spectroscopy was used to determine the extent of collagen triple helical preservation. All CD spectra were recorded on a JASCO 720 spectropolarimeter (Jasco Inc., Easton, MD) at a scan rate of 200 nm/min and a collagen concentration of 1 mg/mL in 10 mM HCl.

All solid-state NMR experiments were conducted at room temperature on a Bruker DSX 300 spectrometer (Bruker, Billerica, MA) operating at a ^{1}H resonance frequency of 300 MHz in a Bruker double resonance magic angle spinning probehead. Standard cross polarization (CP) pulse sequence was employed under conditions of magic angle spinning (MAS). A spinning speed of 5 kHz was employed. A total supression of spinning sidebands (TOSS) sequence was used in conjunction with CP to provide a spectrum free of spinning sidebands.[15] A 4.5-μs [1]H 90° pulse, a 1-ms contact time, a 9-μs [13]C 180° pulse, and a 3-s recycle delay were employed with 5000–16 000 scans accumulated for signal averaging.

Rheological data were measured on an ARES III rheometer (Rheometric Scientific Inc., Piscataway, NJ) in the parallel plate mode with a plate diameter of 25 mm. The gap in the parallel plate setup was adjusted to be between 300 and 500 μm depending upon the sample volume. Gel samples were placed between the parallel plates at the desired temperature with 30 min provided for temperature equilibration. A strain amplitude of 10% and a frequency of 10 rad/s were employed.

Isolation of Rat Aortic Smooth Muscle Cells. Rat aortic smooth muscle cells (RASM) were obtained from Sprague–Dawley rats (250 g, 6–8 weeks old), as described in detail elsewhere. Primary cell lines (passages 4–8) were characterized by immunostaining cells with antibodies raised against smooth muscle α-actin (SMA), vimentin, and smooth muscle-specific-myosin. Cultures that exhibited >90% positive cells for myosin and SMA were used for smooth muscle cell studies. RASMs cultured in DMEM supplemented with ʟ-glutamine, penicillin-streptomycin, and 10% FBS.

Preparation of Monomeric Collagen. Acid-soluble collagen was derived from tail tendons obtained from Sprague–Dawley rats weighing between 250 and 350 g using a protocol similar to that described by Silver and Trelstad.[16] Briefly, tendon fibers were extracted from rat tails using a wire stripper, immersed in 10 mM HCl (pH 2.0; 10 fibers per 100 mL), and stirred for 4 h at room temperature. The soluble component was separated from the insoluble portion by centrifugation at 30 000 g at 4 °C for 30 min with sequential filtration through 20, 5, 0.65, and 0.45 μm filters (Millipore Corp., Bedford, MA). NaCl was added to the filtrate so as to obtain a salt concentration of 0.7 M. The mixture was then stirred for 1 h,; and the precipitate collected after a 1 h centrifugation at 30 000 g and 4 °C. The pellet was allowed to dissolve overnight in 10 mM HCl (pH 2.0) and dialyzed against 20 mM phosphate buffer (disodium hydrogen phosphate at pH 7.4) for at least 8 h at room temperature. A second dialysis was then performed against a 20 mM phosphate buffer solution for at least 4 h at 4 °C. The dialyzate was centrifuged at 30 000 g at 4 °C for 1 h, and the pellet was then dialyzed overnight against HCl (pH 2.0) to obtain collagen in solution that was stored at 4 °C. Lyophilized collagen was obtained by dialyzing the collagen solution overnight against distilled, deionized water, followed by lyophilization.

Acrylation of Collagen. Collagen methacrylamide was prepared by the reaction of type I rat tail collagen with methacrylic anhydride in a manner adapted from a methodology reported by van Den Bulcke et al. for derivatizing gelatin.[17] Briefly, type I rat tail collagen was dissolved in 10 mM HCl (0.25 wt %) and vigorously stirred at 4 °C for 24 h. After complete dissolution, 0.2 M Na_2HPO_4 buffer was added to achieve a pH of 7.5. Methacrylic anhydride (MA) was added in varying molar ratios to target collagen residues, lysine, and hydroxylysine, to obtain a desired degree of derivatization. After an 8-h reaction period, the mixture was dialyzed against 10 mM HCL (150 volume excess) for 48 h at 4 °C with frequent changes in dialyzate. ^{1}H NMR was used to monitor the incorporation of acrylate groups.

Collagen Gel Formation and Cross-Linking. Cells containing collagen gels were prepared by techniques described previously.[18–21] Briefly, 1 mL of FBS and 30 μL of photoinitiator solution were added to 5 mL of 2× DMEM (glucose 1.0 gm/L without phenol red) at 4 °C. A total of 2.5×10^6 rat aortic smooth muscle cells in 2 mL of DMEM without phenol red and 5 mL of 0.4 wt % collagen in 10 mM HCl were then added, gently mixed, and the solution placed on ice. Alkalinization was achieved by adding 0.5 mL of 0.1 N NaOH and the cell containing collagen solution was immediately plated into multiwell plates. Solutions were allowed to gel during a 30-min period, after which DMEM media (w/o phenol red) was added and the gels irradiated with visible light at 50 mW/cm² for either 30 or 60 min using a DynaLume quartz halogen illuminator equipped with a heat shield. The photo-cross-linking reaction was monitored by solid-state NMR.

Determination of Extractable Component of Collagen Gels. After 1 h of irradiation both the control gel and the cross-linked gel were then frozen at −80 °C for 2 h and
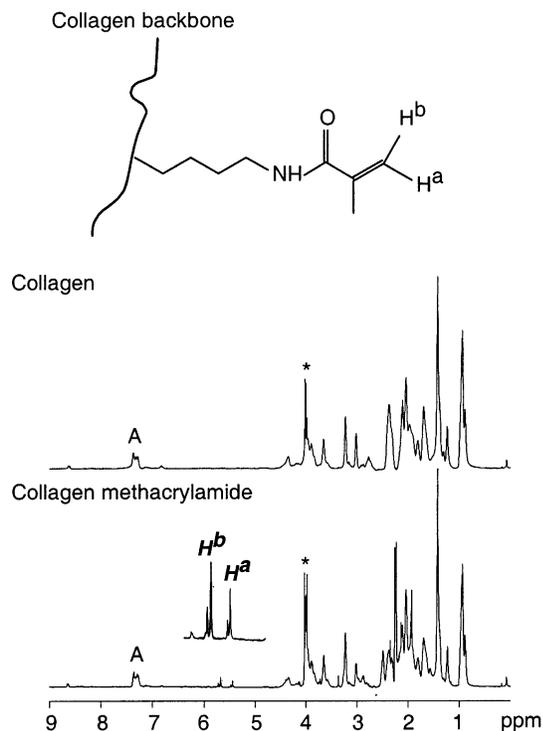
Figure 1. ^{1}H NMR spectra of collagen and collagen methacrylamide recorded in D20 (*) at room temperature. The inset shows the expanded region between 5 and 6 ppm. The peaks H^a (5.3 ppm) and H^b at 5.6 ppm show the incorporation of double bonds into collagen. A: Peaks due to the aromatic residues. Degree of functionalization can be estimated from the integrated intensities of A, H^a, and H^b.

then lyophilized. The dry weight of the gels was recorded. The gels were then rehydrated in 4 mL of 10 mM HCl (pH 2) at room temperature. Samples were incubated at 4 °C for 24 h, after which the supernatant was aspirated and the remaining gel lyophilized, reweighed, and the percent acid soluble extractables calculated. Similarly, second and third extractions were also performed.

Cell Viability Assay. Cell viability was determined 1 day after photo-cross-linking by direct cell counting. Media was aspirated followed by addition of 1 mL of solution of collagenase at 0.04 wt % in PBS to each well. After 1 h, dissolved gels were centrifuged for 10 min at 2400 rpm. The cell pellet was resuspended in PBS, and cell counting was performed. Live cells were identified by trypan blue exclusion.

Gel Contraction Assay. Gels cast in multiwell plates were teased away from the plate wall 1 day after plating. Total gel area was measured 0, 1, 5, and 9 days after plating with media changes every 2 days.

Statistical Analysis. All data are presented as mean $\pm$ standard deviation. Differences among groups were evaluated using single factor ANOVA. A value of $p < 0.05$ was considered significant.

Results

Derivatization of Collagen with Photo-Cross-Linkable Acrylamide Groups. The ^{1}H NMR spectra of collagen and collagen-methacrylamide was recorded in D$_2$O at room temperature (Figure 1). The spectrum of collagen-methacryl-
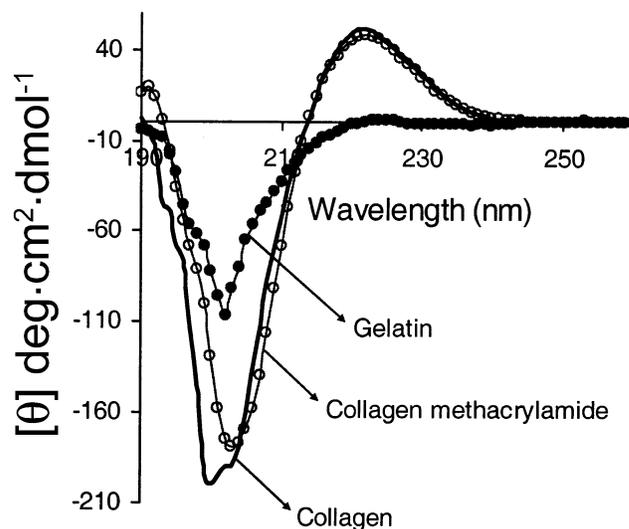

Figure 2. CD spectra of collagen (—), collagen methacrylamide (○), and gelatin (●). The spectra are identical with respect to the 221-nm peak indicating complete retention of triple helical structure.

amide confirms incorporation of the acrylamide double bonds at 5.3 and 5.6 ppm. Conveniently, the degree of functionalization (DOF), which is defined as the ratio of the number of amino groups functionalized with methacrylamide groups to the total number of amino groups present in collagen prior to the reaction, can be determined by comparing the integrated intensity of the aromatic region, representing the concentration of collagen, with the intensity of the double bond region. The DOF depends on the molar reactant ratio of methacrylic anhydride to collagen amino residues. Accordingly, the DOF varied from 39% to 86% by changing the feed ratio from 0.5 to 5 (data not shown).

An important consideration during the chemical modification of collagen is maintaining the integrity of the triple helical structure, which is a critical determinant of collagen stability and mechanical properties. Both CD spectroscopy and differential scanning calorimetry (DSC) in solution can be used to determine the extent of triple helical preservation. The representative CD spectra of collagen, collagen methacrylamide, and gelatin in 10 mM HCl at room temperature are illustrated in Figure 2. The CD spectra of both collagen and collagen methacrylamide show the characteristic peak due to the triple helix at 221 nm, which is absent from the gelatin CD spectrum. Moreover the 221-nm peak intensities are approximately the same indicating almost complete triple helical retention after the acrylation reaction.

CD spectroscopy obtained as a function of temperature was also used to characterize collagen thermal stability (Figure 3). Changes in the denaturation temperature may indicate subtle changes in the tertiary structure not otherwise indicated by a spectrum conducted at room temperature. Indeed, a small decrease in denaturation temperature from 39 °C for native collagen to 36 °C for methacrylamide-modified was noted. These values correlate well with those computed from DSC (data not shown). Thus, the intrinsic structure of monomeric collagen is largely preserved after reaction with methacrylic anhydride.

Visible Light Mediated Photo-Cross-Linking of Collagen. Cross-linking of the methacrylated collagen gels after
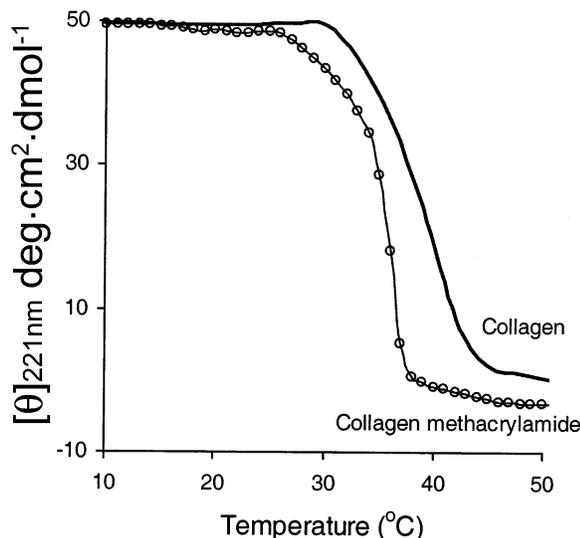
Figure 3. Thermal denaturation profiles of collagen (—) and collagen methacrylamide (○) determined by observing the 221-nm peak as a function of temperature. Collagen denaturation temperature is lowered by about 3 °C upon methacrylation.

Figure 4. ^{13}C CP/MAS/TOSS spectra of collagen methacrylamide and cross-linked collagen methacrylamide recorded at room temperature (23 °C). Contact time = 1 ms, and spinning speed ($\omega_r/2\pi$) = 5 kHz. Disappearance of the peaks C^a and C^b in the spectrum for the cross-linked material indicate cross-linking after exposure to visible light.

irradiation with visible light was examined by solid-state ^{13}C NMR, analysis of gel extractables, and rheological measurements. Collagen methacrylamide gels were subjected to visible light irradiation at 50 mW/cm² for 60 min, after which gels were frozen at −80 °C for 2 h and lyophilized. It bears emphasis that a cross-link can *only* form through the reaction of two methacrylamide groups with the consumption of the methacrylamide double bond. The solid-state NMR spectrum for the cross-linked sample confirms the disappearance of the double bonds, which is consistent with methacrylamide group cross-linking (Figure 4).

To assess the relative degree of intra- vs interchain cross-linking, the acid soluble or extractable fraction of the gel was determined. In principle, the extractable component should be negligible if all collagen species were fully cross-linked by intermolecular reactions. As seen from Figure 5, the cross-linked sample retained nearly 63% of its initial mass, whereas the non-crosslinked sample was completely solubilized. Thus, upon irradiation, collagen methacrylamide forms a cross-linked collagen network predominantly through interchain cross-links.

Rheology. Dynamic mechanical spectra for native collagen and cross-linked collagen methacrylamide gels under shear are shown in Figure 6A. The spectra are characterized by a storage modulus (G′), which essentially remains unchanged in the frequency range investigated, and a loss modulus (G″) that is an order of magnitude lower than the storage modulus. The spectra are typical of viscoelastic materials. The storage modulus for the cross-linked collagen gel (162 kPa) is an order of magnitude higher than that of unmodified collagen gel (13.5 kPa). Thus, mechanically stronger gels are obtained after chemical cross-linking of collagen.

Figure 6B shows the temperature dependence of G′. Both samples show a dramatic decrease in the modulus beyond the denaturation point (loss of triple helix). It is evident from the figure that this drop in modulus occurs at a higher temperature for the cross-linked gel (45 °C) when compared to the un-cross-linked gel (39 °C). Thus, cross-linking
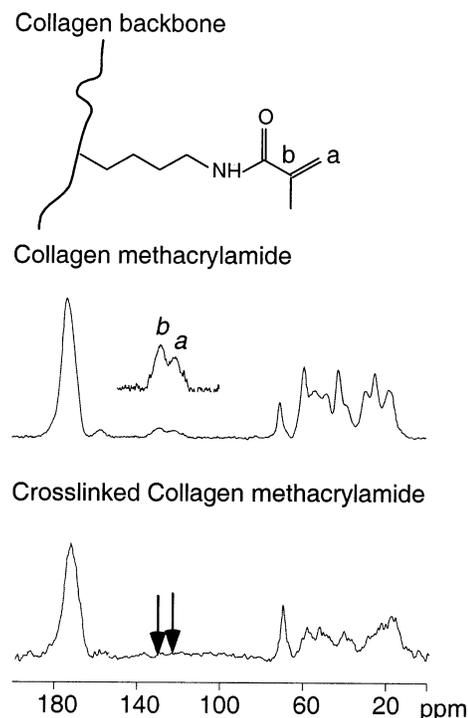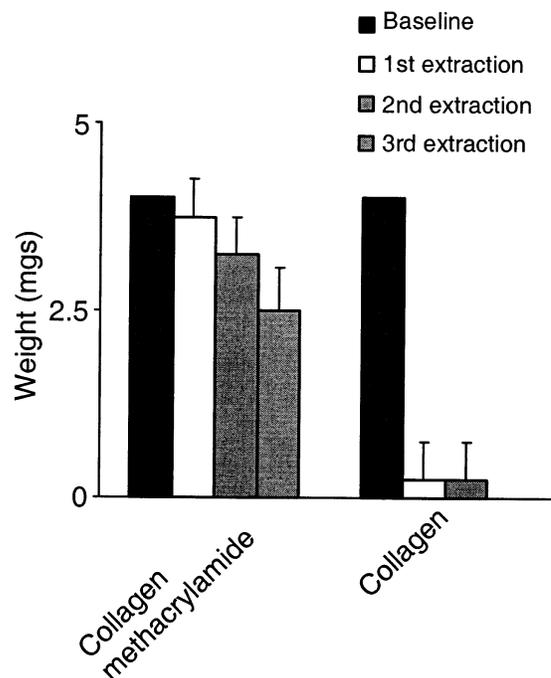

Figure 5. Gel extraction data for underivatized collagen and collagen methacrylamide gels subjected to visible light irradiation for 60 min (50 mW/cm²). Three successive gels extractions at pH 2 (in 10 mM HCl) were then performed. Data presented as mean ± standard error ($n = 4$).

increases denaturation temperature of the gel well beyond the physiologic range.

Cell Viability. Cell viability following photopolymerization was assessed using trypan blue exclusion assay (Figure 7). Cell viability in excess of 85% was observed in gels cast
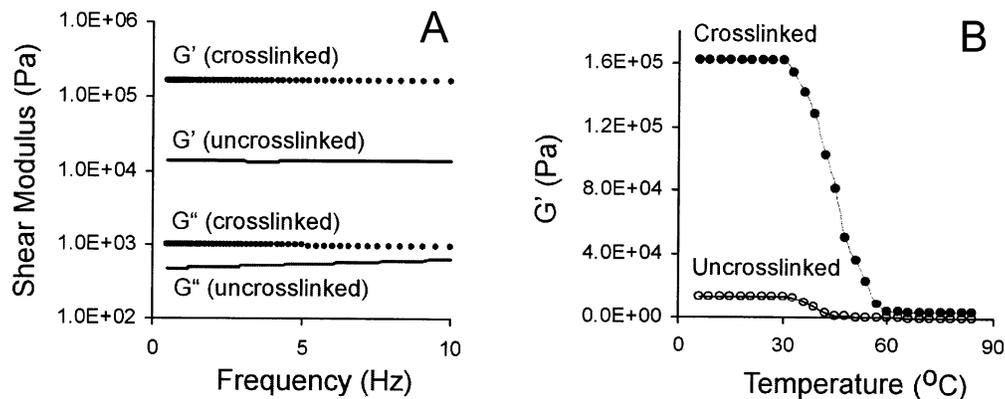
Figure 6. (A) Shear storage (G′) and loss moduli (G″) of cross-linked methacrylamide collagen and underivatized collagen gels as a function of frequency. Cross-linking provides an 8-fold improvement in the storage modulus. (B) G′ of cross-linked and un-cross-linked collagen gels as a function of temperature. Cross-linking increases the denaturation point of collagen gel to 45 °C as compared to 39 °C for the non-crosslinked version, as determined by obtaining the derivative of the curve.
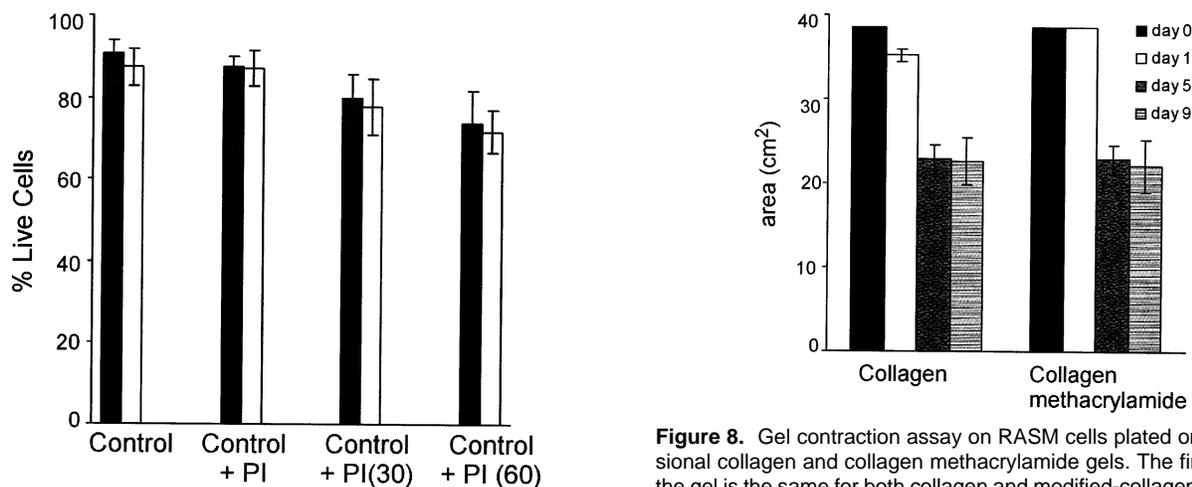

Figure 7. Cell viability (% live cells) for RASM cells plated on 3-dimensional collagen (■) and collagen methacrylamide (□) gels. PI, photoinitiator; PI(30), gel with PI irradiated for 30 min; and PI-(60), gel with PI irradiated for 60 min. The cell viability is unaffected by collagen modification. However, some cellular injury is observed under visible light irradiation. 70+% viability is maintained even after photcross-linking ($n = 4$ and $p > 0.05$).

Figure 8. Gel contraction assay on RASM cells plated on 3-dimensional collagen and collagen methacrylamide gels. The final area of the gel is the same for both collagen and modified-collagen indicating that SMCs maintain their activity after photo-cross-linking ($n = 3$ and $p > 0.05$).

from both collagen and collagen methacrylamide prior to cross-linking. Thus, chemical modification of collagen does not alter cell viability in the gel. Moreover, loss of cell viability was not observed in the presence of the photoiniator alone in the absence of exposure to visible light. Irradiation in the presence of initiator, however, did induce a degree of cell death presumably due to the formation of free radicals. Although neither the duration of irradiation nor the type of collagen influenced cell viability, after 60 min of photo-cross-linking approximately 71% of cells were viable. Thus, visible light photo-cross-linking provides a relatively noncytotoxic method to cross-link collagen in the presence of cells.

Gel Contraction. As a measure of smooth muscle cell function, gel contraction was measured in gels composed of non-cross-linked native collagen, as well as cross-linked collagen methacrylamide (60 min; Figure 8). Photo-cross-linked gels exhibited a small delay in gel shrinkage compared to native non-crosslinked collagen gels, but within 5 days, both gels had contracted to approximately 43% of their initial area.

Discussion

Weinberg and Bell[22] were the first to generate an arterial construct consisting of a cell populated collagen gel. Significantly, cell-mediated reorganization of the surrounding collagen matrix enhanced the mechanical integrity of the construct. However, even when reinforced with a Dacron mesh, this model did not display sufficient tensile strength necessary for in vivo applications. In contrast, recent investigations have demonstrated that a mechanically robust multilayered arterial equivalent can be generated from cell driven assembly of secreted endogenous matrix proteins. For example, L'Heureux and colleagues[23,24] observed that, following prolonged culture of mesenchymal cells in medium containing ascorbic acid, cellular sheets composed of smooth muscle cells or fibroblasts could be rolled around a mandrel in sequential layers to construct a tubular vessel. Rupture strengths exceeding 2000 mmHg were observed. Likewise, Campbell et al.[25] have reported a novel strategy of making vascular grafts within the recipients own peritoneal cavity. Nonetheless, a requirement for months of incubation before vessels are suitable for implantation remains a significant limitation of these approaches. As a consequence, efforts continue to be directed at improving the mechanical characteristics of collagen gel-based constructs, either by increas-

ing collagen concentration, reducing endogenous proteolytic enzyme activity, or introducing techniques that promote cell-assisted matrix protein assembly and cross-linking.[9–13] Both endogenous glycation mediated cross-linking processes and methods to control cell alignment, including magnetic alignment and mechanical conditioning, have been investigated.[20,26] All of these approaches have had only a modest impact in improving construct mechanical properties.

In this study, visible light mediated photo-cross-linking was investigated with an aim of improving the mechanical properties of collagen gels. Collagen was derivatized, through lysine and hydroxylysine residues, with methacrylamide moieties. The addition of this group, while lowering the denaturation temperature, left the triple helical content unaltered as demonstrated by CD spectrsocopy, which is a critical requirement for optimization of initial construct mechanical properties. Specifically, collagen's tensile strength, its stability in a biological environment, and its capacity to present specific ligands for cell surface receptors are properties that are in large measure dependent on the integrity of collagen's characteristic triple-helical conformation.[27,28–31] For example, the energy requirement for collagen degradation by collagenase I (MMP-1) is reduced by a factor of 2 if native fibrillar (multimeric) collagen is in a monomeric form but by a factor of 10 if the triple helix is denatured.[32] Thus, the unique coiled-coil triple helix is the dominant structural feature, which dictates collagen stability and its mechanical properties. Consequently, the generation of a robust load-bearing network with appropriate mechanical integrity and strength mandates maximal preservation of the collagen triple helix.

Photo-cross-linking proved to be an effective approach for enhancing collagen network mechanical properties. Indeed, the shear modulus of the cross-linked system was an order of magnitude greater than that of the un-cross-linked gel. Moreover, cell viability and function was maintained even after photo-cross-linking. Nonetheless, despite cross-linking, we observed a modest level of unincorporated acid-soluble collagen and a shear modulus that lies approximately an order of magnitude below that of a native blood vessel. Thus, further optimization of this system will be required in order to generate a media equivalent that is suitable for in vivo applications. In this regard, approaches directed at increasing collagen concentration and the degree of collagen derivatization are currently under investigation.

In summary, this report is the first to demonstrate that collagen can be derivatized with a cross-linkable moiety with preservation of the triple helix. As a consequence, we have been able to achieve both temporal and spatial control over collagen cross-linking in the presence of absence of cells. Significantly, this strategy establishes a foundation for enhancing the mechanical and enzymatic stability of collagen-based materials for a variety of applications in tissue engineering and drug delivery.

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