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Characterization of glycidyl methacrylate – Crosslinked hyaluronan hydrogel scaffolds incorporating elastogenic hyaluronan oligomers

S. Ibrahim^a, C.R. Kothapalli^b, Q.K. Kang^{a,c}, and A. Ramamurthi^{a,d,e,*}

^aDepartment of Bioengineering, Clemson University, Clemson, SC 29634, USA

^bDepartment of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

^cDepartment of Orthopedic Surgery, Medical University of South Carolina, Charleston, SC 29425, USA

^dDepartment of Regenerative Medicine and Cell Biology, Medical University of South Carolina, Charleston, SC 29425, USA

^eDepartment of Biomedical Engineering, The Cleveland Clinic, Cleveland, OH 44195, USA

Abstract

Prior studies on two-dimensional cell cultures suggest that hyaluronic acid (HA) stimulates cell-mediated regeneration of extracellular matrix structures, specifically those containing elastin, though such biologic effects are dependent on HA fragment size. Towards being able to regenerate three-dimensional (3-D) elastic tissue constructs, the present paper studies photo-crosslinked hydrogels containing glycidyl methacrylate (GM)-derivatized bio-inert high molecular weight(HMW)HA (1×10^6 Da) and a bioactive HA oligomer mixture (HA-o: MW ~0.75 kDa). The mechanical (rheology, degradation) and physical (apparent crosslinking density, swelling ratio) properties of the gels varied as a function of incorporated HA oligomer content; however, overall, the mechanics of these hydrogels were too weak for vascular applications as stand-alone materials. Upon *in vivo* subcutaneous implantation, only a few inflammatory cells were evident around GM–HA gels, however their number increased as HA-o content within the gels increased, and the collagen I distribution was uniform. Smooth muscle cells (SMC) were encapsulated into GM hydrogels, and calcein acetoxymethyl detection revealed that the cells were able to endure twofold the level of UV exposure used to crosslink the gels. After 21 days of culture, SMC elastin production, measured by immunofluorescence quantification, showed HA-o to increase cellular deposition of elastic matrix twofold relative to HA-o-free GM–HAgels. These results demonstrate that cell response to HA/HA-o is not altered by their methacrylation and photo-crosslinking into a hydrogel, and that HA-o incorporation into cell-encapsulating hydrogel scaffolds can be useful for

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*Corresponding author at: Department of Biomedical Engineering, The Cleveland Clinic, Cleveland, OH 44195, USA. Tel.: +1 216 444 4326; fax: +1 216 444 9198. ramamua@ccf.org, aramamu@clemson.edu (A. Ramamurthi).

Appendix A Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 4, 5, 7, 8, 9 and 10 are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2010.08.006.

enhancing their production of elastic matrix structures in a 3-D space, important for regenerating elastic tissues.

Keywords

Hyaluronan oligomers; Hydrogel; Elastic matrix regeneration; Tissue engineering

1. Introduction

In recent years, the field of tissue engineering has gradually transitioned from using synthetic cell scaffolds to using scaffolds based on naturally occurring extracellular matrix (ECM) components. These natural materials have been increasingly realized to provide cells' biological and biomechanical cues to guide their proliferation, differentiation and functionality in a physiologically relevant manner. In addition, they have some remarkable advantages over synthetic materials, including selective cell adhesion, mechanical properties similar to those exhibited by native tissues, and biodegradability, which allows complete resorption of the matrix after tissue regeneration is complete. Among ECM molecules that are increasingly studied in the context of regenerative materials is hyaluronic acid (HA), a glycosaminoglycan commonly found in connective tissues (e.g., skin) and composed of repeating disaccharide units of *N*-acetyl- β -glucosamine and β -glucuronic acid [1]. Most cells have the ability to synthesize HA at some point during their cell cycle, signifying that the molecule has a vital function in several fundamental biological processes [2].

In recent years, HA has been recognized as a potentially effective biomaterial for soft tissue regeneration, since: (a) it is a critical regulator of many biological phenomena, including embryonic development, tissue organization, wound healing and angiogenesis; (b) it is highly biocompatible and does not elicit a foreign-body response upon cross-transplantation, owing to the preserved structural homology of HA across species [2]; and (c) for the same reason, it can be derived from many sources, including bacterial fermentation, and synthesized in large quantities for clinical use [3]. Further, although HA is enzymatically degraded *in vivo* by hyaluronidases [4], and completely resorbed via several metabolic pathways, it can be chemically derivatized and/or crosslinked into stable hydrogels or scaffolds [5,6]. Although the modes of interaction between HA and the human body are still incompletely understood, the favorable characteristics outlined above have recently prompted an investigation into its utility as a scaffolding biomaterial for tissue-engineering applications, such as cartilage [7] and dermal [8] repair and regeneration. In this line of thinking, the authors are currently investigating the potential use of HA as a biomaterial to modulate vascular regeneration *in vivo*. Since HA forms a significant (4–7% w/w) component of vascular ECM [9], it is hypothesized that HA-derived biomaterials will mimic the *in vivo* environment and provide healthy biomechanical and biochemical signals to cultured vascular cells.

One of the outstanding challenges in faithful regeneration of vascular and other such elastic tissues is in being able to regenerate elastic matrix structures. Elastin is a critical structural protein in the medial layer of blood vessels, which facilitates their elastic recoil and provides

the resilience necessary for cyclic distension and contraction [10,11]. It is secreted as a soluble protein precursor (tropoelastin) by smooth muscle cells (SMC), recruited onto a microfibrillar template (fibrillin), and crosslinked by desmosine mediated by lysyl oxidase (LOX) [12]. In addition to providing tissue elasticity, elastin also mechano-transduces SMC behavior (e.g., proliferation) through binding to cell surface elastin–laminin receptors [13] to regulate SMC activity [14], particularly during vascular morphogenesis [15]. Thus, disruption of elastin due to inflammatory diseases [14,16], direct mechanical injury [17] or its malformation in congenital and inherited conditions can encourage SMC hyperproliferation and medial thickening, leading to reduced arterial compliance and hypertension [18]. However, a major impediment to tissue engineering elastic vascular (and other) tissue replacements is the extremely poor elastin regenerative capacity of adult cell types.

In the above context, sequential studies conducted by the authors' group [19,20] have shown HA oligomer mixtures to enhance elastin matrix deposition by adult SMC dramatically, while HMW (>1 MDa) HA appears only to prompt elastin matrix deposition and fiber formation physically, possibly by causing physical coacervation of elastin precursors by opposite-charge interactions, to facilitate their crosslinking [21]. It was also demonstrated later that immobilization of the HA oligomers (HA-o) onto two-dimensional (2-D) cellular substrates (i.e., glass), without involvement of a chemical crosslinker, does not alter the above-mentioned stimulation of ECM production [22]. These results reinforced the hypothesis that HA-o, when chemically derivatized, retain their innate biological signaling characteristics, and will thus retain their utility when incorporated into biomaterials. In creating such HA-based biomaterials capable of eliciting desired matrix regenerative responses by embedded vascular cells, the authors' approach is to create "bioactivated" gels containing relatively bio-inert long-chain HA, imperative to maintain mechanical integrity and potentially provide a high degree of biocompatibility, and cell-interactive HA oligomers. However, it is unknown as to (a) whether cells embedded within such three-dimensional (3-D) scaffolds would continue to interact with each other and the surrounding HA in a manner that replicates their ability to be stimulated elastogenically with HA-o in 2-D culture [23], (b) how cellular response to HA oligomers/HA would be influenced by the presence and density of chemical crosslinkers, and (c) how HA oligomer content within the crosslinked scaffolds would impact the desired matrix regenerative responses, such as gel handling, mechanics and biocompatibility. The present work thus investigates these aspects within SMC-encapsulating HA hydrogels, crosslinked with glycidyl methacrylate (GM), a photocrosslinker.

2. Materials and methods

2.1. Preparation of HA oligomer mixtures

HMW HA (MW 1.5×10^6 Da; Sigma, St Louis, MO) was enzymatically digested to produce a mixture of HA oligomers (HA-o) as per the protocol reported earlier [22]. Briefly, HMW HA (5 mg mL⁻¹) was digested with bovine testicular hyaluronidase (0.45 mg mL⁻¹; 439 U mg⁻¹; Sigma) in a solution of digest buffer (150 mM NaCl, 100 mM CH₃COONa, 1 mM Na₂-EDTA, pH 5.0) at 37 °C for 18 h. The enzyme activity was terminated by boiling the

resulting digestate for 2 min and cooling on ice. Following centrifugation (2800 rpm, 10 min) to separate the enzyme from the mixture, the supernatant was dialyzed in water (12 h) using dialysis cassettes (Pierce Biotechnology Inc.) with a MW cut-off of 2 kDa, and then freeze-dried overnight to generate lyophilized HA oligomers. The composition of final purified lyophilized mixture was analyzed by fluorophore-assisted carbohydrate electrophoresis, as described previously [19]. After electrophoresis, the gels were illuminated with UV-B light ($\lambda = 365$ nm) in a FluorChem 8900 (Alpha Innotech, San Leandro, CA) and band intensities were quantified and compared with an HA oligomer ladder (10–20-mer; 1915–3811 Da) and commercially procured HA 2-mer (397 Da) and HA 6-mer (1156 Da; Associates of Cape Cod, East Falmouth, MA) to determine the size range of HA fragments within the digest. Analysis of band intensities indicated that the enzymatic digest contained predominantly HA 4-mers (MW 756 Da; 75% w/w), and minor fractions of larger oligomers ($13.9 \pm 3.6\%$ w/w HA 6-mers and $8.0 \pm 1.6\%$ w/w of HA 12-mers) [19].

2.2. Hydrogel formulation

HA was derivatized with glycidyl methacrylate (GM–HA) using a previously described procedure (Fig. 1) [24]. Briefly, HMW HA and HA-o (10 mg ml^{-1}) were separately dissolved in DI water, then mixed with triethylamine (3.6% v/v; Sigma; a chemical catalyst), GM (3.6% v/v; Sigma; a crosslinker), and tetrabutyl ammonium bromide (3.6% w/v; Sigma; a phase-transfer catalyst). The batch reaction was allowed to continue overnight (18 h, 23 °C) and was finally completed by incubation of the mixture for 1 h at 60 °C. The aqueous product (GM–HA) was recovered/purified by precipitation in acetone, re-dissolved in DI water (three cycles), and then precipitated in acetone (Sigma). The GM–HA precipitate was finally freeze-dried, and stored at stored at -20 °C. To create hydrogels, GM–HA (i.e., derivatized HMW HA with 0%, 5%, 10% or 20% of derivatized HA-o) was dissolved in PBS (45 mg ml^{-1}) and mixed with Irgacure 2959® (0.01% w/v; Ciba, Basel, Switzerland; a photo-initiator). Thorough mixing of the viscous solution that resulted was achieved by repeated transfer of the mixture between two sterile syringes (Beckton Dickinson, Franklin Lakes, NJ) through a three-way stopcock (Kimble Kontes, Vineland, NJ). The mixture was then centrifuged for 5 min at 1000g to remove air bubbles and aliquoted into cylindrical molds (for rheology, compression: 2 cm^2 , 0.5 mL; for all other mechanical analytical techniques: 0.79 cm^2 , 0.2 mL; for cell culture: 0.32 cm^2 , 0.1 mL). The aliquoted solution was then exposed to UV light ($\lambda = 365$ nm, $\sim 22 \text{ mW cm}^{-2}$) using a Black-Ray model B 100 AP UV lamp (UVP Technologies, Upland, CA) for 1.11 min mg^{-1} HA (2 cm^2 mold: 25 min; 0.79 cm^2 mold: 10 min; 0.32 cm^2 mold: 5 min). The dimensions (height and diameter) of the swollen, cylindrical hydrogels, which were crosslinked in the 0.32 cm^2 mold, were measured using a digital caliper (Fisher, Pittsburgh, PA).

2.3. Fourier transform IR spectroscopy of hydrogels

Fourier transform IR–attenuated total reflectance (FTIR–ATR) was performed to determine the chemical alterations induced by the initial incorporation of GM onto the HA backbone and its UV-induced transition into a crosslinked GM–HA hydrogel. Freeze-dried samples of crosslinked GM–HA were analyzed on a Varian 660-IR FTIR spectrometer (Varian, Palo Alto, CA) and compared with uncrosslinked GM–HA and the underivatized HA mixture ($n = 3$). The spectra were recorded in a frequency range between 400 and 4000 cm^{-1} , with a

spectral resolution of 4 cm^{-1} . A spectral library was used to identify the peaks and the corresponding bond vibrations.

2.4. Apparent crosslinking density of hydrogels

The structural integrity of GM–HA is primarily maintained by crosslinks between the GM moieties, which serve to link the HA strands to one another and limit their freedom of motion. The incorporation of GM–HA-o may reduce the effectiveness of these crosslinks, diminishing the overall mechanical properties of the gel. In addition, it is certainly possible that this derivatization of GM–HA is not 100% efficient, and that UV-induced crosslinking of GM is not complete. Accordingly, it was deemed necessary to define a new parameter, termed “apparent crosslinking”, which in essence represents the effective crosslinking within each formulated gel. Uniaxial compression testing was performed to estimate the apparent crosslinking density within the gels. Cylindrical gel samples (8-mm diameter) were punched out of a larger gel using a corneal trephine (BRI, Malden, MA) and compressed without constraining the edges (unconfined compression testing), on a DMA Q800 (TA Instruments, New Castle, DE). The gels were subjected to an initial force of 0.05 N and were then compressed at a rate of 20% strain min^{-1} ($n = 8$ per formulation). All tests were performed in air, though the gels were kept well hydrated in PBS while being compressed. Stress–strain curves were developed according to Eq. (1), developed by Flory [25], and the initial slope of the curve (0–30% strain) was used to estimate the apparent crosslinking density of the gel:

$$\sigma = RT \left(\frac{v_e}{V_o} \right) \phi_{2,x}^{2/3} \phi_{2,s}^{1/3} \left(\alpha - \frac{1}{\alpha^2} \right) \quad (1)$$

where σ is the uniaxial compressive stress (Pa), R is the universal gas constant ($\text{J} (\text{mol K})^{-1}$), T is the temperature (K), $\phi_{2,x}$ is the polymer volume fraction post-crosslinking, $\phi_{2,s}$ is the polymer volume fraction swollen, v_e/V_o is the apparent crosslinking density (mol cm^{-3}), and α is the compressed fraction.

2.5. Rheological properties of hydrogels

To characterize further the impact of crosslinking and oligomer incorporation on hydrogel mechanics, rheological oscillatory shear stress experiments were performed. The gel stiffness was experimentally determined by measuring the storage (G') and loss (G'') moduli. An AR G2 rheometer (TA Instruments, New Castle, DE) was used in the parallel plate geometry, with a 25-mm plate and constant normal force of 0.2 N. A deformation angle of 1 mrad was maintained throughout each frequency sweep of 0.08–10 Hz ($n = 4$ per sample).

2.6. Swelling ratio of hydrogels

Swelling tests were performed to study the effects of HA oligomer content on the bulk hydrodynamic properties of the GM–HA gels. Fully hydrated gels were blotted to remove surface liquid. The weights of the swollen gel samples were recorded using a sensitive balance (OH AUS, Pine Brook, NJ). The gels were then freeze-dried and weighed again. The swelling ratio was calculated by the following formula ($n = 4$ per formulation):

$$\text{Swelling_ratio} = \frac{W_s}{W_d} \quad (2)$$

where W_s is the swollen mass of the gel (mg), and W_d is the dry mass of the gel (mg). Gel mass rather than volume was used to estimate swelling, since the dried gels are irregular, highly porous and collapsed, which renders estimation of their volume very difficult.

2.7. Scanning electron microscopy imaging of hydrogels

SEM imaging was performed to determine how the incorporation of GM–HA-o and its content altered the interior pore structure of the GM–HA hydrogels. The fully hydrated gels were dehydrated with acetone, vacuum-dried, snap-fractured and the interior surface gold-coated for 4 min using a SPI-Module Sputter Coater (Structure Probe, Inc., West Chester, PA) and imaged by SEM (Jeol 100-JSM 5410 LV, Pleasanton, CA) at 150× magnification ($n = 3$).

2.8. In vitro degradation of hydrogels

In vitro enzymatic degradation of the hydrogels was measured as a function of time by incubating the GM–HA gels in a solution of testicular hyaluronidase, and then monitoring the dry mass of the samples that remained undegraded at various time points. Since the intention of these experiments was only to investigate the impact of HA-oligomer incorporation on gel stability as determined from relative degradation rates of the different gel formulations, and not to measure the overall degradation time of the gels in a physiological environment, enzymatic degradation tests were intentionally conducted at a non-physiological pH of 5, where the enzyme activity is maximal. The gels were initially soaked in digest buffer (pH 5.0) overnight to reach swelling equilibrium. Gels were then incubated in a solution of bovine testicular hyaluronidase (Sigma), prepared in digest buffer (2 mL of 50 U mL⁻¹), for 6 h at 37 °C with mild mixing on a platform shaker. At each assay time point (0, 2, 4, 6 and 8 h), gels ($n = 3$) were recovered, lyophilized, weighed and then replaced in a fresh aliquot of the enzyme solution and incubated until the next assay time point. The enzyme solution was replaced after each analysis time point. The HA mass degradation profile was fitted according to first-order degradation kinetics using non-linear regression to estimate the gel degradation rate constant (k).

$$C(t) = C_o e^{-kt} \quad (3)$$

where $C(t)$ is the dry mass (mg) of the gel at time t , C_o is the initial dry mass of the gel (mg), k is the degradation rate (h⁻¹), and t is time (h).

2.9. In vivo biocompatibility of hydrogels

The biocompatibility of the hydrogels was determined by their subcutaneous implantation in rats. All animal work reported in this study were approved by the IACUC at the Medical University of South Carolina and conformed to NIH guidelines for care and humane use of laboratory animals. Prior to implantation, the hydrogels were sterilized in 95% v/v ethanol (Sigma) for 2 h and then re-hydrated in sterile 1× PBS overnight. Sprague–Dawley rats

(~250 g) were anesthetized (0.01 mL g⁻¹ intramuscular injection of 4% w/v chloral hydrate; Sigma), shaved, and a 5-cm incision made in the skin along the spine. Blunt dissection was used to form a pocket between the skin and muscle. The muscle surface was then cleared of fascia. GM–HA hydrogels of different formulations and matrigel (Control; Sigma) were placed directly into these pockets (5 implants/animal; $n = 7$). One additional rat was surgically manipulated to create three subcutaneous pockets, but these were not implanted with any biomaterials (sham treatment controls).

After implantation, the surgical incision was closed with 4-0 silk suture with an FS-2 cutting needle (Ethicon, Piscataway, NJ).

At 3 weeks, the hydrogels and the adherent tissue capsules were explanted from the subcutaneous pockets, fixed in 4% w/v paraformaldehyde (Sigma) and soaked for 1-h intervals in 30% w/v sucrose (Sigma), 1:1 v/v mixture of 30% w/v sucrose: optimal cutting temperature solution (OCT; Sakura, Torrance, CA) compound, and finally in pure OCT. The explants were then embedded within OCT, frozen on dry ice, and stored at -80°C . Prior to sectioning, the frozen blocks were cooled to -20°C (overnight) and cryosectioned perpendicular to the skin and muscle surfaces. The 8- μm -thick sections were transferred onto HistoBond® glass slides (VWR, West Chester, PA) and stained with haematoxylin and eosin (Sigma-Aldrich) to detect inflammatory cell infiltration around and within the implant. The tissue sections were imaged on a DMIRB microscope equipped with a JVC TK-C1380 color camera (Leica, Allendale, NJ). In addition, immunofluorescence methods were used to detect the collagen, and hence the presence of a fibrous capsule within the tissue mass surrounding the implant. The 15- μm -thick tissue sections were initially quenched with 1% v/v phosphomolybdic acid to suppress auto-fluorescence due to collagen, and then incubated with a primary antibody for collagen I for 1 h (rabbit vs rat collagen I; 1:100 v/v in PBS; Chemicon International, Temecula, CA), and then tagged with a FITC-conjugated secondary antibody (donkey vs rabbit IgG; 1:500 v/v in PBS; Chemicon International) for 1 h. Draq 5 (1:2000 in PBS, 10 min; Biostatus, Leicestershire, UK) was used to label the cell nuclei fluorescently and visualize the cell density in the region surrounding the implant. Fluorescently labeled tissue sections were imaged on a TCS SP2 AOBS confocal microscope (Leica) at successive 5- μm depths, and the individual z -stack of images collapsed on top of one another to create an overlay.

2.10. Cell culture

Rat aortic smooth muscle cells (RASMC) were isolated from the aortae of Sprague–Dawley rats using methods previously described by Oakes et al. [26]. These primary cells were then proliferated to confluence and then passaged to expand the culture. Late-passage cells (P6–8), which are even more elastogenically challenged relative to primary adult RASMC, were used in this study. To encapsulate cells, the uncrosslinked GM–HA mixture was sterilized by syringe filtration, added to the wells of a sterile mold (0.32 cm², 0.1 ml) and gently mixed with the RASMC (3×10^6 cells ml⁻¹) using a micropipette, while avoiding air bubbles. The GM–HA was then mixed with a photo-initiator (Irgacure) and crosslinked by exposure to UV (5 min) and the resulting hydrogels containing encapsulated SMC were cultured in DMEM: F12 (VWR Scientific, West Chester, PA) containing 10% v/v FBS

(VWR Scientific) and 1% v/v penicillin–streptomycin (VWR Scientific). Spent medium was replaced thrice weekly over the duration of culture (21 days).

2.11. Fluorescence detection of cell viability

Calcein acetoxymethyl ester (Calcein AM; VWR) was used to label live SMC fluorescently within GM–HA and estimate their viability as a function of the duration of exposure to UV light for the purpose of photocrosslinking GM–HA. Briefly, SMC were suspended in an GM–HA-o-free mixture of GM–HA and Irgacure®, within a 0.32-cm² area mold (gel volume = 0.1 mL), the mixtures were crosslinked by exposure to UV over 3, 5, 7 and 10 min, and the SMC were cultured for 4 h prior to viability testing. At this time, the SMC were incubated with calcein AM (8×10^{-3} mg mL⁻¹) in Hanks' Buffered Salt Solution (HBSS; VWR; 45 min) and fixed in 4% w/v paraformaldehyde (Sigma). Draq 5 (1:2000 v/v in PBS, 10 min; Biostatus, Leicestershire, UK) was also used to label the cell nuclei fluorescently. The gels were viewed using a TCS SP2 AOBS confocal microscope (Leica, Bannockburn, IL) and imaged at successive *x–y* sections 8 µm apart, across the thickness of the gel (80 µm; *n* = 4 regions/section). This vertical (*z*) stack of images was then overlaid into a single image. For each gel containing the greatest amount of GM–HA-o, the percentage viability of encapsulated SMC was estimated from *n* = 4 regions/section by the exclusion of Draq 5-labeled cells not co-labeling with calcein AM.

2.12. Immunofluorescence labeling of elastin

Elastin production by SMC cultured within GM–HA hydrogels of different formulations were visualized by immunofluorescence, and the cumulative amounts in each case were quantified by calculating the volumetric mean fluorescence intensity (MFI) of their respective *z*-stack overlay images, collected over the thickness of the gels, and calculated on a per cell basis. The encapsulated SMC were cultured for 3 weeks to allow for sufficient matrix deposition, and then transversely cryo-sectioned into sections 15 µm thick. The sections were transferred onto HistoBond® glass slides (VWR), quenched with 1% w/v phosphomolybdic acid (Sigma) to suppress collagen-autofluorescence, incubated with a primary antibody against elastin for 1 h (rabbit vs rat elastin; 1:100 v/v in PBS; Elastin Products Company, Inc., Owensville, MO), and the elastin was then visualized by treatment with a FITC-conjugated secondary antibody (donkey vs rabbit IgG; 1:500 v/v in PBS; Chemicon International) for 1 h. Draq 5 (1:2000 in PBS, 10 min; Biostatus) was used to label the cell nuclei fluorescently. The labeled gels were imaged on a TCS SP2 AOBS confocal microscope (Leica) using the *z*-axis function to image sections 5 µm apart along the thickness of the gels at a constant gain and offset for FITC (600, 0.9, respectively). The stack of images along the *z*-axis were then overlaid into a single image (*n* = 9) prior to quantifying MFI per cell using Image J software.

3. Results

3.1. Crosslinked HA hydrogels

Variably composed mixtures of GM-HMW HA and GM–HA-o were photo-crosslinked, to result in a total of four different gel formulations. Briefly, these formulations contained 0%, 5%, 10%, 20% w/w GM–HA-o, respectively, besides GM-HMW HA. Table 1 shows the

measured dimensions of the hydrated hydrogels following crosslinking within the 0.32 cm² mold. Fully hydrated hydrogels showed marginal increases in their height (*h*) and diameter (*d*) with increases in GM–HA-o content.

3.2. FTIR spectroscopy

FTIR-ATR spectra of lyophilized, crosslinked GM–HA hydrogels containing various concentrations of GM–HA-o (0%, 5%, 10%, 20% w/w), non-photocrosslinked HA and HA-o, and non-photocrosslinked GM–HA and GM–HA-o, respectively, were measured. Owing to the similarity within each tested group of samples, Fig. 2 shows only a representative spectrum obtained for one of the formulations at each successive step, leading to crosslinking of GM–HA into a hydrogel. The spectrum of uncrosslinked HA (A) agreed well with that provided by the American Society for Testing of Materials. Upon adding GM to the HA backbone, the emergence of absorption bands at 936.12 and 1492.06 cm⁻¹ (B) indicated the presence of the methacrylate carbon-to-carbon double bonds [27] and the successful derivatization of HA and HA-o with GM. UV exposure resulted in bonding between methacrylate groups, the radical breakdown of their carbon-to-carbon double bonds and the disappearance of the absorption bands at 936.12 and 1492.06 cm⁻¹ within crosslinked GM–HA (C).

3.3. Degree of crosslinking

The apparent crosslinking density and swelling ratio of the different GM–HA hydrogel formulations were used to estimate the degree of crosslinking within them. The data in Table 2 and Fig. 3 indicate that the apparent crosslinking density and swelling ratio, respectively, are dependent on the presence of HA-o content. Though the incorporation of HA-o into hydrogels increased their swelling capacity over gels composed of only GM-HMW HA, the extent of swelling did not appear to correlate particularly with the HA-o content ($p > 0.5$). However, the measured apparent crosslinking density of hydrogels was found to correlate inversely with HA-o content ($p = 0.038$). However, the measured crosslinking density was much lower than the calculated theoretical value. Assuming that 100% of the incorporated HA had been derivatized by GM into GM–HA, and 100% of the derivatized GM–HA was crosslinked by UV to yield crosslinked GM–HA gels, the apparent crosslinking density was calculated to be roughly 1–2% of that calculated theoretically.

3.4. Hydrogel strength and resistance to degradation

Rheological characterization quantified the viscous and elastic responses of GM–HA with varying concentrations of HA-o, thus providing information on their stiffness. In general, regardless of their formulation, both the storage moduli (G') and loss moduli (G'') of the gels were independent of frequency, with the G' values constantly higher than G'' (Fig. 4). The addition of HA-o within the GM–HA gels decreased both G' and G'' , indicating reduced hydrogel stiffness. To investigate the biodegradability of the GM–HA hydrogels, the sensitivity of the gels to a super-physiological concentration of bovine testicular hyaluronidase was tested. The weaker (less stiff) GM–HA gels containing greater concentrations of HA-o also exhibited steeper degradation profiles (Fig. 5) reflective of greater gel degradation rates (Table 3).

3.5. Hydrogel interior morphology

SEM micrographs of the interior structure of dehydrated GM–HA hydrogels containing different amounts of HA-o are shown in Fig. 6. In the absence of incorporated oligomers, the process of dehydration resulted in collapse of the HA strands within the GM–HA gels, to create a solid and minimally porous mass. However, as HA-o oligomers were increasingly incorporated within the hydrogels, the interior structure of GM–HA was rendered increasingly porous.

3.6. Biocompatibility of GM–HA hydrogels

In all cases, H&E staining revealed reduced cellularity around the defect created by the HA-o-free GM–HA implants (Fig. 7A–D), compared with that around matrigel controls (Fig. 7E); the cellularity of the tissue further out from the defect appeared to increase with increasing HA-o content (Fig. 7A–D). Most of these cells were flattened and aligned with the collagen fibers, suggestive of fibroblast morphology. However, the cluster of cells immediately around the gels were not particularly flattened, and were seen in greater number around GM–HA gels containing a higher content of HA-o, suggesting that they might be inflammatory cells. Thus, GM–HA gels containing a greater content of HA-o possibly elicit enhanced inflammatory responses relative to gels that contain a lower content of HA-o. A significant amount of tissue infiltration (see arrows in Fig. 7F–I) was observed within GM–HA gels, similar to that observed within matrigel controls (Fig. 7J). However, HA-o content did not appear particularly to influence tissue infiltration into the gels. Immunofluorescence labeling (Fig. 8) confirmed the histological observations (above) as to greater cellularity (blue) of tissue capsules around GM–HA gels containing greater HA-o content, and for all gels, greatest density of cells in the regions immediately surrounding the gel defects. However, collagen matrix (green channel) in the tissue surrounding the implant was less dense immediately adjacent to the defect compared with regions further out.

3.7. SMC viability

Fluorescence micrographs (Fig. 9) showed that the majority of Draq-5-labeled SMC (blue) encapsulated within GM–HA gels also stained for calcein AM (green channel), confirming their viability, irrespective of the duration of UV-exposure required for gel crosslinking ($0.67\text{--}2.22\text{ min mg}^{-1}$, equivalent to 3–10 min for hydrogels crosslinked in 0.32 cm^2 molds). At the time of labeling (4 h after encapsulation), the SMC continued to retain a rounded phenotype.

3.8. Elastin production by encapsulated SMC in long-term culture

Incorporation of HA-o into GM–HA gels stimulated elastic matrix (green channel) synthesis by encapsulated SMC (blue) relative to those cells cultured within HA-o-free gels (Fig. 10). At 3 weeks of culture, cell nuclei exhibited an elongated appearance, suggesting that the SMC had acquired an elongated/spread morphology quite different from that exhibited at early culture time points (e.g., 4 h; compare Figs. 9 and 10). A comparison of the volumetric fluorescence intensities due to elastin, between the gel formulations, confirmed this observation ($p < 0.001$ for all HA-o concentrations vs 0% w/w HA-o), and additionally showed elastin production to be uninfluenced by HA-o content (Fig. 11).

4. Discussion

In the past, the relative bio-inertness of natural polysaccharides such as HA prompted their extensive study as biocompatible materials and bio-interfaces for clinical use [28,29]. As a vital component of tissue ECM, HA not only contributes to sequestration [30] and prolonged release of cytokines/growth factors useful for directing cell behavior, but also helps as a drug delivery vehicle [31]. HA and its fragmented forms (e.g., oligomers) also play a prominent regulatory role in wound healing *in vivo*, and thus could be potentially useful for fabricating cellular scaffolding biomaterials for enabling tissue regeneration *in vitro* or *in vivo*. Many unique properties of HA, including its viscoelasticity and lack of immunogenicity, and multiple functional groups (e.g., -OH, -COOH) available for chemical derivatization, have made it an appealing cell scaffold platform for tissue-engineering applications [32–34].

In order to create HA scaffolds capable of surviving prolonged periods of implantation, the HA must be converted into a stable, biocompatible, insoluble biomaterial with good handling properties and mechanics appropriate to the site of implantation. The crosslinking scheme described in this study involves incorporation of photopolymerizable methacrylate groups on the HA chain and presents several advantages over derivatization methods, although both reactions occur via anionic functional groups of HA (-COOH, -OH, -CH₃COONH). In a photo-polymerization technique, reaction solutions can be completely mixed prior to UV exposure, so that rapid, controllable and minimally invasive crosslinking can occur [35,36]. Furthermore, since photo-polymerization can be performed under physiological conditions, it potentially permits co-polymerization of biomolecules which retain their native activity thereafter, and incorporation of cells that would remain viable. *In vivo*, cells are exposed to a 3-D architectural microenvironment wherein the behavior is regulated by their interactions with other cells, ECM and growth factors. HA hydrogels resemble the physical characteristics of the ECM [37] and can thus be used to encapsulate cells to provide a more physiological microenvironment to cultured cells.

Investigation of size-specific effects of HA on matrix regeneration outcomes, particularly of hard-to-regenerate elastic fibers by adult SMC, has been a major focus of research by the present authors. Previous studies have suggested that HA hydrogels containing only long-chain HA (MW > 1 × 10⁶ Da) interact poorly with cells, which is detrimental to their use as cellular scaffolds for tissue regeneration [38,39]. In addition, long-chain HA has been implicated in cell-excluding mechanisms, whereas HA oligomers (<20-mers) are recognized to be more bioactive [40]. It was shown previously that elastogenesis and elastin matrix synthesis by SMC was up-regulated by exogenous supplements of an HA digest containing a broad range of HA oligomers. This digest (HA-o) was found to contain 13.9 ± 3.6% HA 6-mers and 8.0 ± 1.6% HA 12-mers and 75.0 ± 0.4% w/w HA 4-mer content [19]. This upregulatory phenomenon was maintained when the oligomers were presented to SMC as a surface-immobilized substrate, wherein HA was chemically derivatized, but not crosslinked. In addition, the present authors reported successful synthesis and crosslinking of elastin by SMC seeded on top of bioactivated HA hydrogels containing a surface mixture of bio-inert HMW HA and shorter bioactive HA fragments and oligomers [21] generated by UV-induced scission of the HMW HA. However, since the effects of UV light can be difficult to

control and potentially cause random ionizations which can be structurally disruptive, a better approach is to create “bioactivated” gels containing bio-inert long-chain HA, necessary to maintain mechanical integrity and potentially provide a high degree of biocompatibility, and smaller, more cell-interactive HA oligomers of defined sizes, which would specifically evoke the desired cell responses. Therefore, this study sought to explore the concept of incorporating bioactive HA oligomers into photo-crosslinked mixtures of GM-derivatized long-chain HA, to create standalone biomaterials for use as vascular regenerative scaffolds capable of directing encapsulated SMC to synthesize mimics of native elastic matrix structures.

The synthesis of GM–HA relied on the reaction of GM with HA in the presence of a catalyst, triethylamine. It has been suggested that during this derivatization, two reactions occur simultaneously at pH 7.4. The first is a reversible trans-esterification which occurs through the primary hydroxyl group, and the second is an irreversible ring-opening conjugation which occurs through the carboxylic acid group toward the highest substituted carbon of the epoxide [41]. However, as the reaction proceeds, there is a decline in the amount of trans-esterification products and a parallel increase in the concentration of ring-opening products. This suggests that the reaction of HA with GM requires a long reaction time (5–10 days) to promote ring-opening vs generation of trans-esterification products. Owing to the relatively short reaction time (18 h) used in this study, the authors believe that the GM–HA produced primarily included the trans-esterification product (Fig. 1). In addition, the initial derivatization of HA by GM was conducted in a solution that was highly viscous due to the presence of HMW HA, and was therefore less-conducive to reaction between GM and HA. This explains the fairly low (1–2%) amount of crosslinking induced within GM–HA gels (apparent crosslinking density). Another likely reason for poor crosslinking may be due to the derivatization of the vast majority of HA/HA-o with one GM group, which could be considered as crosslinking inhibitors. Modification of HA or HA-o with two methacrylate groups, if at all possible, may be a future strategy to promote formation of crosslinked networks of HA or HA-o within the gels to enhance their mechanics and handling properties.

FTIR analysis cannot determine the primary mechanism of the reaction between HA and GM, but was able to show the carbon double bond of the methacrylate group (absorption bands 936 and 1492 cm^{-1}), which was indicative of successful derivatization of HA with GM. The main difference registered between the FTIR spectra performed before and after UV irradiation was the disappearance of the absorption bands (936 and 1492 cm^{-1}) corresponding to the carbon double bonds in the irradiated polymer. The disappearance of these bands suggests that the crosslinking reaction was complete following UV irradiation of HA for 1.11 min mg^{-1} HA. After UV irradiation, the IR spectrographs of GM–HA were indistinguishable from HA, implying that GM–HA retained the basic structure of HA and hence its function.

Contrary to the hypothesis that GM crosslinking of HA would decelerate and possibly inhibit its natural breakdown, GM–HA was found to be susceptible to degradation. Therefore, the impact of incorporated oligomer content on the rate of degradation of GM–HA gels when exposed to a super-physiological concentration of testicular hyaluronidase *in*

vitro was investigated. The degradation rates of these gels were influenced by the hydrogel swelling capacity (and thereby enzyme concentration within the gels). Hydrogels containing a higher HA-o content exhibited a slightly higher capacity for hydration, and thus, higher swelling ratios, which allowed greater entry of enzymes from the bulk solution into the gel interior.

Rheological analysis provided a quantitative evaluation of the viscous and elastic responses of GM-HA. Both the storage modulus (G') and the loss modulus (G'') were independent of frequency, and G' values were always higher than G'' , which is typical of a “strong hydrogel”, whose response to oscillating frequency more closely resembles a solid than a liquid. The G' and G'' values of GM-HA gels that did not incorporate any HA-o oligomers were similar to those exhibited by other commercially available HA hydrogel products for soft tissue augmentation; the G' and G'' of Hylaform (a dermal filler) are 185 Pa and 21 Pa, respectively, at 3.0 Hz [42]. However, vascular regenerative applications demand far more resilient biomaterials than does skin, owing to the dynamic and strenuous nature of blood vessels. A completely esterified form of HA (HYAFF), which has shown potential as a regenerative vascular grafting material (but not yet shown to be conducive to elastogenesis of SMC), possesses G' and G'' values on the order of 420 kPa and 17 kPa, respectively, at 1 Hz [6,43]. The high strength of this and other successful vascular grafting materials indicates that the GM-HA hydrogels as presently formulated would not by themselves be suitable for use as vascular scaffolding materials, but rather must be composited with other existing natural or synthetic graft materials, at least from the standpoint of surviving the forces experienced in a vascular environment. Future work will thus be directed at increasing the derivatization time of HA with GM to enhance ring-opening and, thereby, crosslinking efficiency and mechanical strength.

SEM images revealed that dehydration itself caused the gels to shrink, drawing the HMW HA strands together to appear as a solid non-porous material, in the absence of HA oligomers. The addition of HA-o resulted in a more porous gel containing dense, uniform-sized pores. HA oligomers also decreased the apparent crosslinking density of GM-HA gels and enhanced their swelling capacity, resulting in a loose polymer matrix with greater porosity. Although dehydration reduced the degree of porosity of the GM-HA gels from that of the hydrated state, the increase in porosity of gels with a greater HA-o content was still maintained, which probably enabled encapsulated cells to adhere, spread and migrate within these gels, and also the surrounding tissue to infiltrate within. Numerous recent studies have explored SEM imaging as a tool for characterizing the pore size and porosity of hydrogels made completely or partially of HA [44,45]. In these studies, it was observed that the pore size was in the order of a few microns in homogeneous crosslinked gels, with the sample preparation technique being similar to that employed in the present study. Thus, the present authors believe that the SEM images truly reflect the hydrogel characteristics such as pore size and porosity, and are not an artifact of the sample preparation process.

The subcutaneous implantation studies suggested that GM involved in the crosslinking of these hydrogels was not toxic to cell survival, an observation others have also made [46]. Around gels containing no HA-o, only a few inflammatory cells were seen at the implant-tissue interface. At that location, these cells were not associated with collagen, and did not

appear flattened like fibroblasts, and so were hypothesized to be inflammatory cells. Thus, based on the sparse density of these cells, it is deduced that GM–HA hydrogels elicit a minimal inflammatory response. The uniformly thin collagen layer around the implants also suggests the lack of exaggerated fibrous capsule formation. The addition of HA-o into GM–HA hydrogels stimulated a mild increase in the density of non-collagen capsule-associated inflammatory cells at the gel–tissue interface relative to GM–HA-o free gels, suggesting that HA-o incorporation at these doses enhances an inflammatory response, which appears modest in absolute terms, however. It is hypothesized that this immunological response leading to inflammatory reaction could possibly be due to gel breakdown and release of GM–HA fragments, either by hydrolytic or enzymatic (hyaluronidases in surrounding tissue) activity. Since the GM–HA gels exhibited lower apparent crosslinking density and higher degradation rates with increasing content of HA-o, probably an outcome of increased swelling capacity (and thereby greater entry and content of hyaluronidases from bulk solution into the gel interior), gels containing greater amounts of GM–HA-o could be more susceptible to enzymatic degradation, which also explains the presence of more inflammatory cells around these gels *in vivo*. In addition, future use of derivatized, purified HA-o, which was shown previously to have identical elastogenic properties [19] can potentially attenuate the marginal inflammatory response to released HA fragments. In addition, tissue infiltration into the defect site was observed for all gel formulations, possibly due to the low crosslinking density (lowered stiffness), which enables cells to infiltrate readily.

The goal of this work was to incorporate HA oligomers into photo-crosslinked GM–HA gels containing predominantly HMW HA, in order to provide greater bioactivity, and specifically enhance elastogenesis of encapsulated SMC. Calcein AM staining was used to elucidate the effects of photo-polymerization on SMC encapsulation within hydrogel [47], and it was found that the SMC were able to endure UV exposure times which were twice the dosage deemed necessary for gelation via crosslinking (2.22 vs 1.11 min - mg⁻¹ HA), and remained viable in the presence of 0.01% w/v I2959 photo-initiator. After 3 weeks of culture of encapsulated SMC, elastin was detected within all the GM–HA gels, and the presence of HA-o doubled elastin matrix deposition. One possible explanation for elastin detection in HA-o free gels could be that the enzymes released by SMC might have degraded long-chain HA to lower fragments, which in turn stimulated SMCS to produce elastin. Nevertheless, this is the first study reporting successful incorporation of oligomers into crosslinked hydrogels made of long-chain HA molecules (MW > 1 M Da) for eliciting enhanced elastogenic responses from adult SMC. One mechanism by which HA oligomers might have impacted cellular events is through interaction with cell surface receptors such as CD44, which in turn transduce intracellular signals [48]. The intracellular domain of CD44 interacts with cytoskeletal proteins and regulates signaling, thereby providing a direct link between extracellular HA and the cell cytoskeleton [49]. Binding of HA oligomers to CD44 has been shown to activate intracellular signaling pathways which significantly alter cell proliferation and motility [50] and may also promote elastin production. Long-chain HA has been reported to play a prominent role not only in the synthesis and organization of microfibrils (a precursor for elastic fiber deposition) [51–53], but also in the synthesis [54], organization and stabilization [55] of elastin by SMC. In addition, HA has been suggested to play an

indirect role in elastogenesis through its intimate binding of versican, which in turn interacts with microfibrillar proteins (fibulin-1, 2) and elastin-associated proteins to form higher-order macromolecular structures important for elastic fiber assembly [51–53]. Recent studies provide evidence that some GAGs (e.g., HA) coacervate soluble tropoelastin molecules on their highly anionic surfaces, to facilitate LOX-mediated crosslinking into an insoluble matrix [56], and stabilize elastin fibers against degradation by elastases. In conclusion, these studies support the authors' observations that both HMW HA and HA oligomers may play significant roles in the production of elastin within GM–HA.

The ultimate test of the utility of a cellular scaffold to regenerating functional tissue constructs lies in its ability to coax cells to synthesize and organize matrix structures which structurally and functionally resemble native ECM, and thus replicate the mechanics of native tissues. This study did not investigate whether the deposition of elastin had any effect on the mechanical properties of the gel. To do this, two vital criteria will need to be met. The first is greatly to enhance the inherently poor elastic matrix yield (i.e., ratio of amounts of matrix elastin to total elastin (soluble precursor + matrix)) of adult RASMC in culture (typically ~10%), to be able to obtain sufficient amounts of elastic matrix which would actually result in measurable changes to gel mechanics, and the second is to improve elastic fiber deposition over disorganized clumps of amorphous elastin, since it is the actual fibers that impact material mechanics. Previous publications have reported on biomolecular factors that greatly enhance elastic matrix yield and fiber formation. The authors fully intend to provide these to cells within the GM–HA gels in a future study and evaluate changes to gel mechanics arising from new elastic matrix deposition, and thence evaluate the utility of these constructs as vascular tissue replacements.

5. Conclusions

In this study, hydrogels composed of HMW HA and its oligomers, derivatized with UV-crosslinkable GM, were successfully developed as potential biomaterials for regeneration of the elastin-rich tunica media layer of blood vessels. The study is significant from three perspectives. First, while numerous studies have used HA hydrogels for vascular tissue-engineering applications by crosslinking long-chain HA molecules, introducing HA oligomers into gels made of long-chain HA to promote the bioactivity and specific matrix regenerative outcomes within these otherwise bio-inert gels is a unique approach. Second, this is the first time that the dose-dependent elastogenic effect of HA oligomers on adult SMC has been demonstrated in a 3-D microenvironment. The study outcomes will thus guide future efforts to incorporate HA oligomers into tissue engineering scaffolds for the purpose of enhancing elastic matrix regeneration, which is one of the foremost unsolved challenges that deter progress in tissue engineering of elastic soft tissue replacements.

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Fig. 1.

Crosslinking chemistry of GM HA. GM-HA hydrogels were prepared by a two-step process. HA was initially incubated with GM, a chemical catalyst (TEA) and phase-transfer catalyst (TBAB) in a batch reaction combining the glycidyl units of GM and hydroxyl units of HA to create the uncrosslinked GM-HA product. A GM-HA solution was then combined with a photoinitiator (I2959) and treated with UV to stimulate the radical reaction of the methacrylate units of GM-HA.

Fig. 2. FTIR-ATR spectra of (A) HA, (B) uncrosslinked GM-HA and (C) crosslinked GM-HA. The initial addition of GM to HA resulted in an absorption band at 936.12 cm⁻¹ identifying the carbon-to-carbon double bond of GM methacrylate. This absorption band disappeared upon UV exposure, signifying the successful crosslinking between the methacrylate groups.

Fig. 3. Swelling ratios of GM-HA gels. The incorporation of HA-o within the GM-HA hydrogel mildly increased their swelling capacity. These increases were, however, not statistically significant except at high HA-o content. *Denotes $p < 0.05$ in comparison with 0%.

Fig. 4. Viscoelastic properties of GM-HA. The storage moduli (G') in all cases was greater than the loss moduli (G''). The addition of HA-o reduced the storage moduli, suggesting lowered gel stiffness of the hydrogels.

Fig. 5.

Degradation of GM-HA *in vitro*. Increasing the concentration of HA-o within the gels reduced their resistance to degradation by testicular hyaluronidase and enhanced the degradation rate. GM-HA hydrogels containing oligomers were completely degraded after 6 h exposure to the super-physiological concentration of the enzyme.

Fig. 6.
Interior morphology of GM-HA. The addition of HA oligomers into GM-HA gels (A, 0%; B, 5%; C, 10%; D, 20%) resulted in enhanced porosity of the gels.

Fig. 7.

H&E staining of GM-HA. Microscopic images show that fewer inflammatory cells (see arrows) surrounded the GM-HA implants (A–D) than the matrigel control (E), but their density appeared to increase slightly with HA oligomer concentration. Macroscopic images depict a significant amount of tissue infiltration (see arrows) within GM-HA (F–I) implants similar to that of the control (J), and did not appear to be affected by HA oligomer content.

Fig. 8. Immunofluorescence analysis collagen I surrounding GM-HA implants. The cellularity (blue) in the region immediately surrounding implants increased slightly with a greater HA oligomer content, and the distribution of collagen I (green) surrounding the defect region was fairly homogeneous.

Fig. 9. SMC survival of UV crosslinking. Most of the embedded cells survived all exposure times of UV radiation, indicated by the overlaying of nuclei (blue) and live cell (green).

Fig. 10.

SMC elastin production within GM-HA. The elastin content (green) within GM-HA gels containing HA oligomers was higher than the hydrogels without HA oligomers. The nuclei of the embedded SMC appeared elongated and, therefore, may have attained a natural spread morphology.

Fig. 11.

Quantification of volumetric fluorescence intensities due to elastin synthesized by SMC cultured within GM-HA. The fluorescence intensity due to elastin was enhanced by the addition of HA oligomers into the GM-HA gel. The amount of elastin synthesized, however, was independent of the concentration of HA oligomers within the GM-HA gels.

*Denotes $p < 0.05$ in comparison with 0%.

Table 1

Size of hydrated GM–HA hydrogels.

Dimension	HA-o w/w (%)			
	0	5	10	20
h^* (cm)	0.945	0.957	0.998	1.039
d^* (cm)	0.965	0.978	1.019	1.061

* Height (h) and diameter (d) of cylindrical gels.

Table 2

Apparent crosslinking density of GM-HA.

HA-o w/w (%)	ν_e (mol cm ⁻³ × 10 ⁶)	
	Theoretical	Measured
0	1378.52	2.66 ± 0.24
5	1325.72	2.14 ± 0.15
10	1171.18	1.31 ± 0.81
20	1035.80	0.88 ± 0.05

Table 3Degradation rate of GM–HA *in vitro*.

HA-o w/w (%)	<i>k</i> (h ⁻¹)	<i>p</i> Value
0	0.0103	<0.00001
5	0.014	<0.00001
10	0.0136	0.0042
20	0.0221	0.0016