Photocrosslinked Hyaluronic Acid Hydrogels: Natural, Biodegradable Tissue Engineering Scaffolds

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Abstract: Ideally, rationally designed tissue engineering scaffolds promote natural wound healing and regeneration. Therefore, we sought to synthesize a biomimetic hydrogel specifically designed to promote tissue repair and chose hyaluronic acid (HA; also called hyaluronan) as our initial material. Hyaluronic acid is a naturally occurring polymer associated with various cellular processes involved in wound healing, such as angiogenesis. Hyaluronic acid also presents unique advantages: it is easy to produce and modify, hydrophilic and nonadhesive, and naturally biodegradable. We prepared a range of glycidyl methacrylate-HA (GMHA) conjugates, which were subsequently photopolymerized to form crosslinked GMHA hydrogels. A range of hydrogel degradation rates was achieved as well as a corresponding, modest range of material properties (e.g., swelling, mesh size). Increased amounts of conjugated methacrylate groups corresponded with increased crosslink densities and decreased degradation rates and yet had an insignificant effect on human aortic endothelial cell cytocompatibility and proliferation. Rat subcutaneous implants of the GMHA hydrogels showed good biocompatibility, little inflammatory response, and similar levels of vascularization at the implant edge compared with those of fibrin positive controls. Therefore, these novel GMHA hydrogels are suitable for modification with adhesive peptide sequences (e.g., RGD) and use in a variety of wound-healing applications. © 2003 Wiley Periodicals, Inc. Biotechnol Bioeng 82: 578–589, 2003.

Keywords: biomimetic; degradable; hyaluronic acid; hydrogel; photopolymerization; tissue engineering

INTRODUCTION

Future biomaterials applications will rely on scaffolds and drug delivery devices that interact with living systems in a specifically designed manner. A number of recent review articles have stated that such materials will be “biomaterials that heal”: that is, biomimetic materials that are designed to promote natural healing and regeneration (Hubbell, 1999; Ratner, 2002; Stocum, 1998). Therefore, several criteria are required: (1) the design of the biomaterial must incorporate the natural wound healing biology; (2) nonspecific adsorption of proteins should be inhibited; and (3) the biomaterial surface should present biomimetic molecules like those that are present in a wound (Ratner, 2002).

To this end, numerous researchers have created and tested in vivo scaffolds composed of polysaccharides (Cadee et al., 2000; Draget et al., 1997; Suh and Matthew, 2000; Weisser et al., 2001), proteins (Lee et al., 2001; Pandit et al., 1998) and synthetic molecules (Cruise et al., 1999; West and Hubbell, 1996; Woerly et al., 1998). These materials have found various degrees of success, but few have met all the requirements described above for “healable biomaterials”. Additionally, candidate materials should be nonimmunogenic and have controlled biodegradability, biocompatible polymerization chemistry, and versatile modification strategies (e.g., multiple different reaction sites) (Anseth and Burdick, 2002; Ratner, 2002; Stocum, 1998). One naturally derived polymer, hyaluronic acid (HA; Fig. 1a), inherently meets many of the above requirements and when crosslinked via photopolymerization, has the potential to provide an ideal wound healing scaffold.

Hyaluronic acid is a naturally derived, nonimmunogenic, nonadhesive glycosaminoglycan that plays a prominent role in various wound-healing processes, as it is naturally angiogenic when degraded to small fragments (Chen and Abatangelo, 1999). Hyaluronic acid promotes early inflam-
information, which is critical for initiating wound healing, but then moderates later stages of this process, allowing matrix stabilization and reduction of long-term inflammation (Chen and Abatangelo, 1999; Table I). Furthermore, immunohistochemical analysis reveals that the environments around the migrating and proliferating fetal cells and adult tissues involved in regeneration and wound healing are enriched with HA (Chen and Abatangelo, 1999). As a mimic of the embryonic environment, exogenous HA supplementation promotes faster and more extensive regeneration in adult injuries (Chen and Abatangelo, 1999). This cumulative evidence suggests that HA is an ideal candidate material for modulating wound healing.

Several physiochemical aspects of HA are advantageous for biomaterial fabrication and application. For example, HA can be easily and controllably produced in large quantities through microbial fermentation, enabling the scale-up of HA-derived products (Lapcik et al., 1998) and avoiding the risk of animal-derived pathogens. Nascent HA is highly hydrophilic and thus inherently nonadhesive to proteins and cells (Nehls and Hayen, 2000), valuable properties for preventing scar tissue (West, 1998) and capsule formation (Ratner, 2002).

Hyaluronic acid is enzymatically degraded by hyaluronidase and is completely resorbable through multiple metabolic pathways (Band, 1998). Although HA is quickly broken down in vivo, crosslinking individual HA polymer chains together decreases their degradation rates (Fig. 1b, c). Many strategies exist for crosslinking HA (Band, 1998), and of these, we chose photopolymerization—a technique that provides advantages such as increased spatial and temporal control over crosslinking and biocompatibility with in situ polymerization (Anseth and Burdick, 2002; Pathak et al., 1992).

In this article, we sought to create unique HA hydrogels designed to support wound healing. We characterized several hydrogel physiochemical parameters, including swelling ratio, stiffness, and in vitro enzymatic degradation. Furthermore, human aortic endothelial cell studies and rat subcutaneous implants were used to analyze the possible deleterious effects of the synthesis and polymerization reactions. We controllably synthesized GMHA hydrogels to yield biocompatible materials that generally retain the desirable wound-healing properties of nascent HA.

**MATERIALS AND METHODS**

**Synthesis and Crosslinking of GMHA Conjugates**

We first added photopolymerizable methacrylate groups to HA to yield glycidyl methacrylate-HA (GMHA) conjugates. Briefly, we prepared a series of GMHA polymers by treating a 1% w/v solution of fermentation-derived HA (∼2 × 10^6 molecular weight; Clear Solutions Biotech, Inc., Stony Brook, NY) in distilled water with a 6-, 10-, or 20-fold molar excess of glycidyl methacrylate (GM; Fig. 1d) in the presence of excess triethylamine and tetrabutyl ammonium bromide overnight at room temperature, followed by a 1-h incubation at 60°C. For example, GMHA synthesized with a sixfold molar excess GM was made as follows: 1.0 g HA was dissolved in 100 mL distilled water; 2.2 mL triethylamine, 2.2 mL GM, and 2.2 g tetrabutyl ammonium bromide were added separately and thoroughly mixed before the next component was added. After the reaction, the solution was precipitated in acetone (20 times the volume of the reaction solution) and dissolved in distilled water twice to remove excess reactants. The GMHA solution was lyophilized and stored desiccated at 4°C. 1H-NMR spectroscopy was used to verify the methacrylation reaction on HA. GMHA conjugates were dissolved in D_2O and the spectra were recorded using a Varian Inova-500 spectrophotometer.

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**Table I.** Hyaluronic acid (HA) and the wound healing process.

<table>
<thead>
<tr>
<th>Wound-healing phase</th>
<th>Contributing role of HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td>• Activation of macrophages and neutrophils</td>
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<tr>
<td></td>
<td>• Production of proinflammatory cytokines</td>
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<tr>
<td></td>
<td>• Moderation of inflammation through free radical scavenging, antioxidant properties, inhibition of inflammatory proteinases</td>
</tr>
<tr>
<td>Granulation</td>
<td>• Cellular differentiation, proliferation, and migration</td>
</tr>
<tr>
<td>Remodeling</td>
<td>• Angiogenesis</td>
</tr>
<tr>
<td></td>
<td>• Reduced scar formation</td>
</tr>
</tbody>
</table>

*Adapted from Chen and Abatangelo (1999).
Next, we synthesized the photocrosslinked hydrogels by exposing GMHA (0.5–2.0% w/v in phosphate-buffered saline) to UV light (365 nm, ~22 mW/cm², 0.5–5 min exposure) in the presence of the photoinitiator Irgacure 2959 (0.01–3% w/v; Ciba Specialty Chemicals, Basel, Switzerland) and N-vinyl pyrrolidinone (0.03–12% v/v).

**Swelling Experiments and Flory-Rehner Calculations**

To analyze the GMHA hydrogel structure (e.g., relative degrees of crosslinking), we performed a number of swelling experiments. The hydrogel swelling ratio based on mass \( Q_v \) was calculated by dividing the gel mass after swelling \( (M_s) \) by the dry gel mass \( (M_d) \). A Perkin-Elmer (Wellesley, MA) thermogravimetric analyzer (TGA) was used to measure \( M_s \) and \( M_d \). The hydrogels were preswollen in phosphate-buffered saline (PBS) overnight, cut into small pieces (10–20 mg), and placed in the TGA weighing pan. The initial swollen mass was recorded as \( M_s \), and the sample was heated slowly to 90–100°C or until a constant mass was achieved using a purging procedure with dry nitrogen gas. The final mass obtained was recorded as \( M_d \).

We then used Flory-Rehner calculations to determine the crosslink density and mesh size of the GMHA hydrogels. The average molecular weight between crosslinks, \( M_c \), was calculated using a simplification of the Flory-Rehner equation (Flory, 1953; Metters et al., 1999):

\[
Q_v^{5/3} = \frac{\chi V_1}{V_1 \left( \frac{1}{2} - \chi \right)} \quad (1)
\]

where \( Q_v \) is the volumetric swelling ratio, \( V_1 \) is the specific volume of the dry polymer, \( \bar{V} \) is the average molecular weight between crosslinks, \( V_1 \) is the molar volume of the solvent (18 mol/cm³ for water), and \( \chi \) is the Flory polymer-solvent interaction parameter.

\( Q_v \) was determined from the degree of mass swelling, \( Q_M \) (Marsano et al., 2000):

\[
Q_v = 1 + \frac{\rho_p}{\rho_s} (Q_M - 1) \quad (2)
\]

where \( \rho_p \) is the density of the dry polymer (1.229 g/cm³) and \( \rho_p \) is the density of the solvent (1 g/cm³ for water). \( Q_M \) and \( \rho_p \) values were determined experimentally and used to calculate \( Q_v \).

The value of \( \chi \) for HA was estimated to be 0.473, based on several assumptions. First, it was assumed that \( \chi \) for HA is comparable to that for dextran, a well-studied polysaccharide, because HA and dextran have similar chemical structures. In addition, \( \chi \) estimates for HA that were based on an analysis similar to those analyses published by Gekko (Cleland and Wang, 1970; Gekko, 1981) gave values within 2% of the value of \( \chi \) for dextran. Finally, differences between soluble, unmodified polysaccharides and crosslinked polymers were assumed to be negligible.

The effective crosslink density, \( v_c \), was calculated as follows (Huglin et al., 1986):

\[
v_c = \frac{\rho_p}{M_c} \quad (3)
\]

The swollen hydrogel mesh size, \( \xi \), was determined with the following equation (de Jong et al., 2001; Lowman and Peppas, 1999):

\[
\xi = Q_v^{1/3} \sqrt{\frac{r_o^2}{2n}} \quad (4)
\]

where \( \sqrt{r_o^2} \) is the root-mean square distance between crosslinks and depends on the molecular weight between crosslinks. For HA, the following root-mean-square end-to-end distance value was previously reported (Cleland, 1970):

\[
\left( \frac{r_o^2}{2n} \right)^{1/2} = 2.4 \text{ nm} \quad (5)
\]

where \( n \) is the number of disaccharide repeat units for HA with a given molecular weight. For HA with the molecular weight \( (M_n) \) \( 2 \times 10^6 \), \( n \) is 5305, and therefore,

\[
\sqrt{r_o^2} = 0.1748 \sqrt{M_n} \text{ (nm)} \quad (6)
\]

A combination of Eqs. (4) and (6) and a substitution of \( M_c \) for \( M_n \) gives

\[
\xi = 0.1748 \sqrt{M_c} Q_v^{1/3} \text{ (nm)} \quad (7)
\]

Because approximations were made in the Flory-Rehner calculations, the values determined (e.g., \( M_c \), \( v_c \), \( \xi \)) were considered approximations. However, these values were useful for making order-of-magnitude comparisons of the GMHA chemistries in biologically relevant features, such as mesh size.

**Rheological Experiments**

To further characterize the crosslinking of the GMHA hydrogels, we performed rheological oscillatory shear stress experiments. Degree of crosslinking relates to gel stiffness, which is represented by the experimentally determined complex modulus (G*). A Paar Physica (Ashland, VA) MRC 300 modular compact rheometer was used in the parallel plate geometry, with a 25-mm plate and a gap size of 1 mm. Constant values of deformation, 0.1–10 mrad, were maintained throughout each frequency sweep of 1–10 Hz. G* was obtained at 3 Hz oscillation and 1 mrad deformation for each sample and was used to compare the relative mechanical stiffness of the hydrogels.

**In Vitro Degradation by Hyaluronidase**

In vitro enzymatic degradation of the hydrogels was measured as a function of time by incubating the gels in hyaluronidase and monitoring the mass of the hydrogel. First, the gels were synthesized in 8-mm diameter, 2-mm deep
rubber perfusion chamber molds (Sigma-Aldrich, St. Louis, MO). The hydrogels were carefully transferred to 24-well plates with Teflon-coated cover glass forceps and were soaked in citrate buffer (0.15 M NaCl, 0.15 M NaHPO₄, 7H₂O, 0.03 M citric acid, pH 5.3) overnight to reach swelling equilibrium. The gels were removed from the citrate buffer, excess liquid was blotted from the surface with filter paper, and the gel masses were determined. Bovine testicular hyaluronidase (Sigma-Aldrich) in citrate buffer (0.5 mL of 5, 50, or 500 u/mL) was added to each gel and then incubated for various times at 37°C with mild mixing on a platform shaker. Every 1–2 h, the solution was removed, the gels were blotted, and the masses were carefully determined. The gels were then returned to the 24-well plate and replenished with fresh hyaluronidase solution for the remainder of the degradation study. Gels with little crosslinking (and therefore easily damaged) were used only for a single time point. Crosslinked GMHA hydrogels in citrate buffer were used as a negative control.

HAEC Cytocompatibility Studies

We investigated the cytocompatibility of human aortic endothelial cells (HAECs) with crosslinked and uncrosslinked 11% methacrylated GMHA hydrogels using an indirect contact method adapted from Trudel and Massia (2002). Transwell inserts (Costar, 6.5 mm, 0.4-μm pore size polyester membrane filter; Corning Costar Corp., Acton, MA) were used to hold the hydrogel in indirect contact (1 mm separation distance) with the HAECs, which were seeded in the wells of the 24-well plates. The HAECs (Clonetics, Walkersville, MD) were obtained at passage 3, used at passages 6–8, and maintained in Clonetics’ endothelial basal media-2: modified MCDB-131 supplemented with growth factors (EGM-2). All HA and GMHA solutions were sterilized by filtration through a syringe filter (0.8/0.2 μm Supor Acrodisc PF pre-filter) syringe filter, Pall Gelman, Ann Arbor, MI).

First, we compared the cytocompatibilities of HA and uncrosslinked GMHA. The HAECs were seeded in a 24-well plate at a density of 6250 cells in 1 mL EGM-2 per well. After the cells had adhered (a minimum of 30 min after seeding), the wells were dosed with 0.1 mL of 1% GMHA in PBS, 1% HA in PBS or PBS alone (EGM-2 positive control). The final dilutions were approximately 0.1% GMHA and 0.1% HA. After 24 h incubation, the cell viabilities were measured using the CellTiter 96 nonradioactive cell proliferation assay. At least three wells were exposed to each treatment, and the experiment was performed 3 times.

Next, the cytocompatibility effects of the crosslinked GMHA hydrogels were examined. A 0.1 mL GMHA solution with 1% Irgacure 2959 and 0.3% N-vinyl pyrrolidinone was dispensed for each Transwell insert and exposed to UV light for 1 min in a laminar flow hood. The gels were washed in EGM-2 (twice for 1 min and once for 5 min). Solutions of 1% GMHA with either 1% Irgacure 2959 or 0.3% N-vinyl pyrrolidinone were also tested to examine the effects of each component individually. After dilution by the medium, the final dilutions were 0.1% GMHA (crosslinked or uncrosslinked), 0.1% Irgacure 2959, and 0.03% N-vinyl pyrrolidinone. Finally, the experiment was repeated with crosslinked GMHA hydrogels made with 10-fold less Irgacure 2959 and N-vinyl pyrrolidinone.

HAEC Proliferation Response to Degraded GMHA

Degraded HA promotes angiogenesis and endothelial cell proliferation (West and Kumar, 1989). However, this response can be altered by the addition of methacrylate groups to HA. Therefore, the proliferation response of HAECs was determined in the presence of degraded HA and degraded GMHA in 96-well plates. Hyaluronic acid and GMHA (5, 7, 11% methacrylation) were degraded in 50 u/mL hyaluronidase in citrate buffer for 4 h at 37°C, and then the hyaluronidase was inactivated by incubation at 56°C for 30 min. The HA and GMHA fragments were diluted to 1.5 μg/mL (this concentration of purified HA fragments increases the proliferation of bovine microvascular endothelial cells (West and Kumar, 1989)) in “starvation medium” (Clonetics’ endothelial basal media-2: modified MCDB-131 (EBM-2) with 1% heat-inactivated fetal bovine serum at pH 7.4).

The HAECs were seeded at 10,000 cells per well in EGM-2 and allowed to adhere for 2 h. The EGM-2 was removed and the cells were incubated overnight in the starvation medium. Next, this medium was replaced with the HA or GMHA fragment solutions and incubated for 48 h. Additional wells were also treated with EGM-2 (the positive control) or the starvation medium with heat-inactivated hyaluronidase (the negative control). The cell viabilities were determined using Promega’s CellTiter 96 nonradioactive cell proliferation assay. At least three wells were exposed to each treatment, and the experiment was performed 3 times.

GMHA Implants

We investigated the response of the endothelial cells to the GMHA hydrogels in subcutaneous implants in rats. We used “tissue engineered construct” (TEC, Fig. 2a, b) devices to identify the location of the hydrogel implant and to simplify the harvesting procedure (Brey et al., 2002; King et al., 2002). Sterile GMHA solutions (7% methacrylation, 1% GMHA, 1% Irgacure 2959, 0.3% N-vinyl pyrrolidinone in PBS; 0.3 mL per TEC) were dispensed in the TECs and exposed to UV light for 1 min. Human fibrin (Tissel fibrin sealant, Baxter Healthcare Corp., Glendale, CA) was used in the TECs as a positive control for vascularization (Dvorak et al., 1987). Agarose (1.5% w/v in water; Sigma-Aldrich) was used as a negative control. Four ≈ 250 g male
Lewis rats (Harlan, Indianapolis, IN) were anesthetized (0.1 mL/g intramuscular injection of 100 mg/mL ketamine, 20 mg/mL xylazine, 0.4 mg/mL atropine), shaved, and two 2.5-cm incisions were made in the skin along each animal’s spine (see Fig. 2c). Blunt dissection was used to form a pocket between the skin and muscle, and the muscle surface was cleared of fascia. The TECs were sutured to the muscle using 5.0 prolene polypropylene sutures (Ethicon, Inc., Somerville, NJ), with the hydrogel in direct contact with the muscle surface (Fig. 2b). After implantation of four TECs per rat (one fibrin positive control, one agarose negative control, and two HA gels), the skin was closed with 9-mm surgical staples.

At 2 weeks, the TECs and enclosed tissue were explanted, snap-frozen in Tissue-Tek optimal cutting temperature (OCT) compound (Sakura Finetek USA, Torrance, CA), and cryosectioned perpendicular to the muscle surface (in 6-μm sections). The sections were stained with the CD31 endothelial cell marker and counterstained for Gill’s #3 hematoxylin (Sigma-Aldrich), according to the methods described in Brey et al. (2002). Briefly, the sections were stained with a mouse antimouse CD31 primary antibody (Serotec, Inc., Raleigh, NC) and a goat antirat IgG secondary antibody conjugated with horseradish peroxidase (Serotec, Inc.). CD31-positive cells were visualized using DAB, a 3,3-diaminobenzidinetetrahydrochloride substrate (Research Genetics, Huntsville, AL). The sections were imaged under brightfield using an Olympus IX-70 inverted microscope (Melville, NY). Images were captured with a Hamamatsu C-5810 color CCD camera (Hamamatsu Corp., Bridgewater, NJ) connected to a Macintosh PowerMac computer, running IPLab image analysis software (Scanalytics, Fairfax, VA). The area of CD31-positive cells was calculated as a percentage of total area viewed per image (Brey et al., 2002). The area of DAB stain as well as the total area of the image was quantified using NIH Image software (http://rsb.info.nih.gov/nih-image).

**Statistical Analysis**

We performed Student’s *t*-tests to determine the statistical significance of the differences between results. A significance level of *p* < 0.05 was used as the cutoff (i.e., *p* values are reported only for cases in which *p* < 0.05).

**RESULTS**

**GMHA Synthesis and Hydrogel Formation**

While the exact reaction mechanism was not confirmed in these studies, it is likely that glycidyl methacrylate reacts with a nucleophilic site on HA, possibly by a transesterification reaction similar to the method described for the glycidyl methacrylate modification of dextran (van Dijk-Wolthuis et al., 1997). 1H-NMR spectroscopy confirmed the GMHA reaction, showing acrylate peaks at ~5.6 and ~6.1 ppm and the HA methyl peak at 1.9 ppm (Fig. 3). The spectrum for HA (Fig. 3a) is consistent with published results (Bulpitt and Aeschlimann, 1999); the GMHA spectrum (Fig. 3b) is characteristic of the results from multiple reactions (*n* = 5).

In a comparison of the two spectrum, there are notable differences in the peak heights and shapes. We expect that these differences could be due to methacrylation, and possibly, degradation of the polymer chains during the modi-
fication reaction. (A detailed investigation of the GMHA structure, however, is not within the scope of this study.) Nevertheless, without knowledge of the exact structure, it is possible to approximate percent methacrylation, which is calculated from the relative integrations of the methacrylate protons and HA’s methyl protons (Smeds et al., 1999). Batches of GMHA made with 6-, 10-, and 20-fold excess GM were found to have approximately 5, 7, and 11% methacrylation, respectively. The $^1$H-NMR spectra for the un-crosslinked GMHA on their own are not entirely conclusive; thus, further characterizations (e.g., correlate the swelling ratio with percent methacrylation) were conducted to confirm the importance of the varying degrees of methacrylation.

The hydrogels were made by exposing GMHA to UV-light in the presence of the photoinitiator Irgacure 2959 and N-vinyl pyrrolidinone (a reaction accelerant and comonomer). The GMHA liquid crosslinked into materials ranging from viscous liquids to brittle solids, depending on the reaction conditions. Increasing the percent methacrylation or increasing the concentrations of GMHA, Irgacure 2959, or N-vinyl pyrrolidinone, yielded gels with increased firmness. Table II displays the minimum concentrations of Irgacure 2959 and N-vinyl pyrrolidinone required for each of the

Figure 3. $^1$H-NMR spectroscopy of HA and GMHA. (a) Native HA ($2 \times 10^6$ molecular weight); (b) GMHA synthesized with 10-fold molar excess GM, yielding about 7% methacrylation on native HA. Methyl peaks at = 1.9 ppm (denoted by *) are found for native HA and GMHA. Expanded view of – 5.4 to – 6.4 ppm display the appearance of (↓) small peaks in (b), indicative of the presence of GM.
chemistries to form hydrogels in 1 or 5 min UV exposure. These minimal concentrations were investigated to confirm that gels could be synthesized under cytocompatible conditions, as discussed later.

**GMHA Hydrogel Characterization**

Three complementary techniques were used to assess the crosslinking characteristics of the GMHA hydrogels: swelling ratio determination, Flory-Rehner calculations, and rheology. The swelling ratio, which varies inversely with the amount of methacrylate groups present on GMHA (Fig. 4), is used in the Flory-Rehner theory to calculate the molecular weights between crosslinks, effective crosslink densities, and mesh sizes (Table III). For 5, 7, and 11% methacrylation, the swelling ratios were determined to be 52.5 ± 1.6, 50.1 ± 4.6, and 42.5 ± 0.3, respectively (n = 3 for each case). GMHA with 11% methacrylation had a statistically lower swelling ratio than GMHA with 5% (p < 0.001) or 7% (p < 0.05) methacrylation. Generally, as percent methacrylation on GMHA increased, the gels showed decreasing swelling ratios, indicating increased levels of crosslinking. However, because a small range of percent methacrylation values was tested, a more complex relationship between methacrylation and the resulting physical properties is not proposed.

The trend of increased crosslinking with increased methacrylation was also apparent in the Flory-Rehner calculations: increased methacrylation corresponded to decreased molecular weights between crosslinks (5%: 8.45 × 10^5 g/mol, 7%: 7.82 × 10^5 g/mol, 11%: 5.92 × 10^5 g/mol), as well as increased effective crosslink density (5%: 1.45 × 10^10 mol/cm³, 7%: 1.57 × 10^-6 mol/cm³, 11%: 2.07 × 10^-6 mol/cm³) and decreased mesh size (5%: 644 nm, 7%: 619 nm, 11%: 539 nm). Rheological measurement of the complex modulus (G*, Table III) further verified this trend: values of G*, while not statistically significant, increased slightly as the percent methacrylation increased (5%: 109.4 ± 16.3 Pa, 7%: 131.3 ± 6.4 Pa, 11%: 154.5 ± 28.2 Pa), again suggesting increased levels of crosslinking.

**In Vitro Degradation of GMHA Hydrogels**

In vitro degradation rates of the GMHA hydrogels were found by incubating hydrogels in three concentrations of hyaluronidase (5, 50, 500 u/mL in citrate buffer) and determining the gel masses over time. Enzyme concentrations were chosen to approximate endogenous levels (hyaluronidase concentration in human serum is 2.6 u/mL (Delpech et al., 1987)), as well as concentrations 10- and 100-fold greater to ensure complete gel degradation before hyaluronidase inactivation (hyaluronidase remains active up to 5 h at 37°C) (Tung et al., 1994).

Degradation rates (Fig. 5) were calculated from the initial linear slope of gel mass vs. time plots (data not shown). At 500 u/mL hyaluronidase, the degradation rates of 5% (99.4 ± 8.9 %/h, n = 6) and 7% (105.1 ± 3.6 %/h, n = 6) methacrylated GMHA were not statistically different, indicating that 500 u/mL is far in excess of the concentration required for controlled degradation. Comparisons between hydrogel chemistries were also carried out at 10- to 100-fold lower concentrations of hyaluronidase. At 5 u/mL hyaluronidase, significant differences were measured in the degradation rates: the degradation of 5% methacrylated GMHA gels (10.6 ± 2.0 %/h, n = 6) was significantly faster than that of 7% methacrylated GMHA gels (4.6 ± 0.8 %/h, n = 5; p < 0.001), and the degradation of 7% was faster than that of 11% methacrylated GMHA gels (0.7 ± 0.3 %/h, n = 4; p < 0.0001). Similarly, at 50 u/mL hyaluronidase, the degradation rate of 5% methacrylated GMHA gels (42.3 ± 7.3 %/h, n = 6) was significantly greater than that of 7% gels (25.7 ± 6.0 %/h, n = 6; p < 0.001), and the degradation rate of 7% methacrylated GMHA gels was greater than that of 11% gels (17.9 ± 3.2 %/h, n = 6; p < 0.001). Therefore, considering the 5- and 50-u/mL hyaluronidase results, increasing methacrylation on GMHA resulted in a decreased degradation rate, again indicating a correlation between methacrylation and the degree of crosslinking. No significant change in mass was observed in the negative-control GMHA hydrogels, which were incubated in citrate buffer alone.

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**Table II.** Minimum concentrations of Irgacure 2959 and N-vinyl pyrrolidinone for hydrogel formation.a

<table>
<thead>
<tr>
<th>Methacrylation</th>
<th>1-min UV exposure</th>
<th>5-min UV exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>% Irgacure</td>
<td>% VP</td>
</tr>
<tr>
<td>5</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>7</td>
<td>0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>11</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*aIrgacure 2959 is a photoinitiator; N-vinyl pyrrolidinone served as a reaction accelerant and co-monomer.

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**Figure 4.** Swelling ratios for GMHA hydrogels. The hydrogel swelling ratio, calculated by dividing the swollen gel mass by the dry gel mass, is an indicator of the degree of crosslinking in the gel. The swelling ratio for 11% methacrylated GMHA hydrogels was significantly lower than that for 5% methacrylated (p < 0.001) and 7% methacrylated hydrogels (p < 0.05). Each data point represents the average ± SD (n = 3).
Table III. Physical properties of GMHA crosslinked hydrogels.

<table>
<thead>
<tr>
<th>% Methacrylation</th>
<th>$Q_{\alpha}^{a}$</th>
<th>$Q_{v}$</th>
<th>$M_{w}$ (g/mol)</th>
<th>$\nu_{c}$ (mol/cm$^3$)</th>
<th>$\xi$ (nm)</th>
<th>$G^{\prime}$ (Pa)$^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>52.5 ± 1.6</td>
<td>64.3</td>
<td>8.45 x 10$^3$</td>
<td>1.45 x 10$^6$</td>
<td>644</td>
<td>109.4 ± 16.3</td>
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<td>7</td>
<td>50.1 ± 0.4</td>
<td>61.4</td>
<td>7.82 x 10$^3$</td>
<td>1.57 x 10$^6$</td>
<td>619</td>
<td>131.3 ± 6.4</td>
</tr>
<tr>
<td>11</td>
<td>42.5 ± 0.3</td>
<td>51.9</td>
<td>5.92 x 10$^3$</td>
<td>2.07 x 10$^6$</td>
<td>539</td>
<td>154.5 ± 28.2</td>
</tr>
</tbody>
</table>

Note. Abbreviations: glycidyl methacrylate-hyaluronic acid conjugates (GMHA), mass swelling ratio ($Q_{\alpha}$), volumetric swelling ratio ($Q_{v}$), average molecular weight between crosslinks ($M_{w}$), effective crosslink density ($\nu_{c}$), mesh size ($\xi$), complex modulus ($G^{\prime}$).

$Q_{\alpha}$ for 11% methacrylated GMHA is significantly lower than for 5% and 7% methacrylated GMHA; n = 3.

$^{a}n = 2$ Values for $Q_{\alpha}$ and $G^{\prime}$ are mean ± standard deviation.

HAEC Cytocompatibility Response to GMHA Hydrogels

In vitro HAEC cytocompatibility was measured in response to GMHA solutions and crosslinked hydrogels. Cell viabilities were measured after 24 h and normalized vs. EGM-2. The concentrations for HA, GMHA, Irgacure 2959, and N-vinyl pyrrolidinone are reported in this section as effective concentrations after dilution by the medium. As shown in Figure 6a, no statistically significant difference was found between the response to 0.1% GMHA in solution (95.92 ± 5.31, n = 6) and EGM-2 (100.00 ± 3.37, n = 13). This indicates that the GM conjugation chemistry did not have a harsh cytotoxic effect and that the acetone precipitation step after the GM modification reaction adequately rinsed away excess GM from the product. Interestingly, 0.1% GMHA had a small but statistically significant increase in viability when compared to 0.1% HA (87.83 ± 4.93, n = 6).

The cytocompatibility effects of crosslinked GMHA were also investigated, as shown in Figure 6b. When compared to 0.1% GMHA in solution, a significant decrease in viability was measured for crosslinked gels (final concentration following dilution of 0.1% Irgacure 2959 and 0.03% N-vinyl pyrrolidinone; 78.42 ± 4.58; p < 0.001). Treatments at a final dilution of 0.1% GMHA with either 0.1% Irgacure 2959 or 0.03% N-vinyl pyrrolidinone were also tested. When compared to 0.1% GMHA in solution, no decrease in viability was measured for 0.03% N-vinyl pyrrolidinone (100.44 ± 7.66). However, a statistically significant decrease was measured for 0.1% Irgacure 2959 (61.16 ± 5.77, p < 0.0001). These results indicate that Irgacure 2959 may play a role in the decreased cytocompatibility of GMHA hydrogels synthesized under these conditions.

Next, hydrogels with 10-fold less Irgacure 2959 and N-vinyl pyrrolidinone were tested. As shown in Figure 6c, no statistically significant decrease in viability (compared to GMHA in solution) was measured for hydrogels made with 0.03% N-vinyl pyrrolidinone and 0.1% Irgacure 2959 (concentrations following dilution of 0.003% and 0.01%, respectively; 94.19 ± 4.45). Similar results were found for 0.1% GMHA solutions with 0.003% N-vinyl pyrrolidinone (109.14 ± 8.26) and 0.01% Irgacure 2959 (96.90 ± 10.03).

Our findings were similar to those involving the cytotoxicity effects of Irgacure 2959 (also called Darocur 2959) on fibroblasts (Bryant et al., 2000). At 48 h, Bryant et al. found that fibroblasts treated with 0.01% Darocur 2959 had a survival rate similar to those without treatment and that treatment with 0.1% Darocur 2959 yielded a slight decrease in viability. Although there were marked differences in the experimental procedures (e.g., cell type used), our study verified the general trend of increased cytotoxicity associated with increased Irgacure 2959 concentrations.

HAEC Proliferation Response to GMHA Hydrogels

The HAEC proliferation response to degraded HA and GMHA is reported in Figure 7. Since we wanted to examine GMHA’s ability to promote endothelial cell proliferation relative to unmodified HA, GMHA was not crosslinked, and Irgacure 2959 and N-vinyl pyrrolidinone were not included in the proliferation experiments. We measured relative cell viabilities using methods similar to those in the cytocompatibility studies except that the absorbance values were normalized to the negative control: heat-inactivated hyaluronidase in starvation medium. Values for HA and all

![Figure 5](image-url). In vitro GMHA hydrogel degradation. Degradation rates of GMHA hydrogels with ( ), 5, ( ), 7, and ( ) 11% methacrylation in the presence of 5, 50, and 500 u/mL hyaluronidase. GMHA hydrogels with increased methacrylation show decreased degradation rates. All comparisons between methacrylation conjugates and hyaluronidase concentrations were significantly different (p < 0.01), except the comparison of 5% and 7% methacrylated GMHA at 500 u/mL hyaluronidase. Each data point represents the average ± SD (n > 4).
GMHA fragments tested were statistically similar to each other and statistically greater than that for the hyaluronidase negative control, indicating that there was an increased proliferation response to HA and that there was no significant loss of activity due to the conjugation chemistry. However, proliferation rates in the presence of HA and GMHA fragments did not increase to the levels of the fully supplemented EGM-2 positive control \((p < 0.001\) in all cases), probably because the HA and GMHA fragments were diluted in the starvation medium and did not contain all of the appropriate factors required for optimal growth. Yet HA and all GMHA conjugates showed HAEC proliferation rates higher than that of the negative control, in general agreement with published studies of HA fragments (West and Kumar, 1989).

**GMHA Implants**

To investigate the in vivo endothelial cell response to the crosslinked GMHA, we implanted hydrogels of 7% methacrylated GMHA subcutaneously in rats. After 2 weeks, the TECs were removed, and the tissue contained in the construct was harvested, cryosectioned, and stained with CD31-DAB and hematoxylin. Agarose (Fig. 8a) was not infiltrated by cells and thus served as a suitable negative control. Cellular migration into the gel interstices was detected in the GMHA implants (Fig. 8b), indicating that cells were attaching to and degrading the edge of the GMHA hydrogels.
Inflammation, which was anticipated because of the biological activity of HA (Chen and Abatangelo, 1999) was present but minimal. Although the GMHA hydrogels were not completely degraded at 2 weeks, the fibrin hydrogels (Fig. 8c) were entirely degraded. The tissues in the fibrin TECs were well infiltrated by cells and highly vascularized. The percent endothelial cells at the edge of the implant measured for GMHA hydrogels (7.1 ± 0.1, n = 4) was statistically indistinguishable from that for fibrin (6.6 ± 1.7, n = 9), indicating that both materials promoted similar levels of interactions with endothelial cells.

DISCUSSION

Hyaluronic acid presents unique advantages for synthesizing healable scaffolds because of its prominent role in wound healing, as well as its natural biodegradability, ease of production and modification, and hydrophilic and non-adhesive character. Furthermore, while most natural and synthetic biomaterials possess relatively few functional side groups, HA has three reaction groups: hydroxyl, carboxyl, and acetamido. These reaction sites lend versatility to HA for modification with chemically crosslinkable groups and bioactive moieties. The aim of the research presented here was to take advantage of these distinctive properties to create a novel tissue-engineering scaffold.

A variety of chemical reactions exist for HA modification (e.g., esterification, nitration, acetylation) and polymerization (based on formaldehyde, epoxide, polycyclic anhydride, vinyl sulfone, or carbodiimide) (Band, 1998). Bulpitt and Aeschlimann (1999) developed an in situ polymerizable HA for articular cartilage applications by synthesizing HA-aldehyde or HA-amine derivatives and crosslinking the derivatives with a variety of commercially available crosslinkers, such as bifunctional esters, and glutaraldehyde. Although this method produces HA hydrogels with a variety of properties and degradation rates, there are disadvantages: the modification procedure involves many synthesis and purification steps, and the crosslinking reaction occurs immediately upon mixing the solutions of modified HA and crosslinker. Because HA forms highly viscous solutions, we have found that the crosslinking chemistries that occur upon mixing are hard to control and yield inconsistent gels.

Use of photopolymerizable methacrylate groups presents several advantages over other crosslinking methods. Specifically, photopolymerization avoids the disadvantages stemming from reactions that occur immediately upon solution mixing and those that depend on pH, ionic strength, or temperature. With photopolymerization, the solutions can be completely mixed before exposure to the appropriate wavelength of light, yielding a rapid, controllable, and minimally invasive crosslinking method (Anseth and Burdick, 2002; Pathak et al., 1992). Furthermore, photopolymerization can be performed under physiological conditions, and it allows for either the copolymerization of bioactive molecules or the incorporation of cells. Many photopolymerization chemistries are known, but they have primarily been investigated with nondegradable synthetic polymers (Anseth and Burdick, 2002).

Several methods have been introduced for synthesizing methacrylate-modified HA, one using methacrylic anhydride (Smeds et al., 1999) and others with GM (Jin et al., 2001; Trudel and Massia, 2002). We have found that the GM modification consistently yields methacrylate-modified HA, and that the addition of triethylamine and tetrabutyl ammonium bromide, a phase-transfer catalyst, permits a

![Figure 8.](image)
faster reaction of aqueous HA with the nonaqueous GM. We have also used acetone precipitation to rinse out the unused reactants from GMHA, thereby avoiding the lengthy dialysis procedures used in other methods (Jin et al., 2001; Trudel and Massia, 2002). HAEC cytocompatibility and proliferation studies (discussed later) suggest that the excess glycidyl methacrylate, a known cytotoxic chemical, is adequately rinsed from the GMHA conjugates by the acetone precipitation step.

In our study, physical characterization of the GMHA hydrogels revealed that increasing levels of methacrylation could be used to fine-tune the swelling ratio (Fig. 4), crosslink density, mesh size and complex modulus (Table III), and degradation rate (Fig. 5) within a modest range. However, we feel that manipulating any of the system variables (e.g., percent methacrylation and choice and concentration of photopolymerization reactants) will yield gels with a broader range of properties and increased cytocompatibility. For example, the range of degradation rates achieved in vitro could be broadened by variations in the crosslink density, as described above, or by the incorporation of hyaluronidase in the polymerization solution (yielding faster degradation rates).

The cytocompatibility of GMHA hydrogels is critical to the ultimate success of wound healing. We have found no statistically significant decrease in the in vitro HAEC cytocompatibility associated with the GMHA conjugates in solution (Fig. 6). Treatments containing N-vinyl pyrrolidinone and GMHA in solution yielded cytocompatibilities statistically similar to that of EGM-2. However, we did note significant decreases in cell viability associated with GMHA in the presence of higher concentrations of Irgacure 2959, similar to published work with fibroblasts (Bryant et al., 2000). Nonetheless, compared with results of a recent in vitro study of smooth muscle cell cytotoxicity in response to photopolymerizable HA (Trudel and Massia, 2002), the GMHA hydrogels presented here were much more cytocompatible.

Once the range of cytocompatible concentrations of Irgacure 2959 and N-vinyl pyrrolidinone were determined, we sought to reconfirm that hydrogels can indeed be synthesized under such conditions (Table II). It should be noted that in the cytotoxicity studies, Irgacure 2959 and N-vinylpyrrolidinone are diluted 10-fold by the cell culture medium. Therefore, the gels presented in Figure 6c were made with 0.1% Irgacure 2959 and 0.03% N-vinyl pyrrolidinone. The minimum amount of Irgacure 2959 and N-vinyl pyrrolidinone required for hydrogel formation was 0.01% and 0.03%, respectively (for 11% methacylated GMHA, the same percentage used in the cytocompatibility studies). In other words, for the case of 11% methacylated GMHA, the concentrations found to be cytocompatible were equal to or greater than the concentrations required for hydrogel formation.

To use crosslinked GMHA as a healable biomaterial, HA’s intrinsic biological activity and role in wound healing must be retained after the methacrylate modification reaction. In the in vitro HAEC proliferation assay that we used to investigate the biological activities of HA and GMHA fragments, the proliferation rate was enhanced by 20% in the HA fragments over the negative control (Fig. 7); furthermore, no statistical difference was found in the native HA and the various GMHA methacrylation chemistries. Although this was the only in vitro method used to gauge GMHA’s retention of HA’s biological activity, it was a promising start for follow-up studies exploring the effects of methacrylation modifications on biologically active polymers.

In the rat subcutaneous implants used to determine the in vivo cellular response to the GMHA hydrogels (Fig. 8), a small inflammatory response was associated with the GMHA implants; this response, however, is expected with high concentrations of HA and is vital for wound-healing initiation. Also, endothelial cells infiltrated the GMHA gels, and comparable levels of these CD31-positive cells were present in both the fibrin-positive controls and the GMHA hydrogels. Future work in this area includes further modification of GMHA to promote controlled endothelial cell infiltration; for example, binding HA’s carboxyl groups to the amino termini of adhesive peptide sequences, extracellular matrix molecules or growth factors via 1-ethyl-(dimethylaminopropyl) carbodiimide-mediated reactions such as those reported for peptide-alginate composites (Rowley et al., 1999).

In conclusion, we report a simple method for synthesizing glycidyl methacrylate-HA conjugates. The resulting GMHA polymers were cytocompatible, photopolymerizable, and biodegradable, and they retained HA’s intrinsic biological activity in promoting endothelial cell proliferation. Furthermore, the GMHA hydrogels were comparable to fibrin in the extent to which the materials promoted interactions with endothelial cells in rat subcutaneous implants. GMHA hydrogels are, we believe, promising candidates for tissue engineering constructs that support wound healing.

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