Published in final edited form as:

Biomacromolecules. 2005 ; 6(1): 386–391. doi:10.1021/bm049508a.

Controlled Degradation and Mechanical Behavior of Photopolymerized Hyaluronic Acid Networks

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Abstract

Hyaluronic acid is a natural polysaccharide found abundantly throughout the body with many desirable properties for application as a biomaterial, including scaffolding for tissue engineering. In this work, hyaluronic acid with molecular weights ranging from 50 to 1100 kDa was modified with methacrylic anhydride and photopolymerized into networks with a wide range of physical properties. With macromer concentrations from 2 to 20 wt%, networks exhibited volumetric swelling ratios ranging from ~42 to 8, compressive moduli ranging from ~2 to over 100 kPa, and degradation times ranging from less than 1 day up to almost 38 days in the presence of 100 U/ml hyaluronidase. When 3T3-fibroblasts were photoencapsulated in the hydrogels, cells remained viable with low macromer concentrations, but decreased sequentially as the macromer concentration increased. Finally, auricular swine chondrocytes produced neocartilage when photoencapsulated in the hyaluronic acid networks. This work presents a next step towards the development of advanced in vivo curable biomaterials.

Keywords

cartilage; tissue engineering; hyaluronic acid; photopolymerization

Introduction

Tissue engineering is emerging as a viable treatment alternative to allogenic and alloplastic implants for cartilage damage. Numerous materials have been investigated as scaffolding for the delivery of chondrocytes to damaged cartilage including: collagen¹, fibrin^{2,3}, alginate^{4,5}, poly(α -hydroxy esters)^{6,7}, and hyaluronic acid⁸. Although this research has led to significant advances in cartilage tissue regeneration, novel scaffolds that optimize the amount and quality of neocartilage produced need to be developed.

Elisseff and coworkers⁹ were the first to report a photopolymerization process to suspend chondrocytes in hydrogel networks for tissue regeneration. Chondrocyte photoencapsulation is advantageous due to the ease of filling irregular shaped defects, which leads to good contact between the tissue engineered construct and surrounding native tissue. This technique allows for the non-invasive implantation of chondrocyte/polymer constructs by exposing prepolymer solutions containing cytocompatible photoinitiators to low intensity visible or ultraviolet light. These efforts have focused primarily on photopolymerizable hydrogels based on poly(ethylene

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glycol) (PEG). Bryant et al.¹⁰ introduced lactic acid units into the PEG networks to accelerate hydrogel degradation and enhance extracellular matrix distribution in the engineered tissue.

Hyaluronic acid (HA) is a linear polysaccharide of alternating D-glucuronic acid and N-acetyl-D-glucosamine, found in connective tissues such as cartilage. It degrades in the presence of hyaluronidases and free radicals and functions as a core molecule for the binding of keratin sulfate and chondroitin sulfate in forming aggrecans in cartilage¹¹. Although not completely understood, HA plays a role in cellular processes including cell proliferation, morphogenesis, inflammation, and wound repair. Several cell surface receptors for HA include CD44, ICAM-1, and RHAMM^{12,13}. In addition, HA is readily modified through both its carboxyl¹⁴ and hydroxyl^{15–18} groups. These properties make HA a desirable choice for the fabrication of chondrocyte carrier materials in cartilage regeneration, with hydrogel degradation controlled by a cell dictated enzymatic process instead of a hydrolytic one. Recently, Setton and coworkers¹⁹ illustrated the ability to encapsulate articular chondrocytes in one photopolymerizable HA network in vivo.

The overall objective of this study is to combine the benefits of a photocrosslinkable network with the desirable properties of HA for future application in cartilage tissue engineering. This manuscript systematically studies the effects of HA molecular weight, the degree of methacrylation and macromer concentration on the physical properties (e.g., swelling, mechanics, and degradation) of the resulting hydrogels. In addition, photoencapsulated cell viability and neocartilage tissue formation were investigated as a preliminary test for the potential use of photopolymerizable HA networks as chondrocyte carriers for cartilage regeneration.

Materials and Methods

Macromer Synthesis and Polymerization

Methacrylated hyaluronic acid (MeHA) was synthesized by the addition of methacrylic anhydride (Sigma, ~20-fold excess) to a solution of 1 wt% HA (Lifecore, MW = 50kDa, 350kDa, 1100kDa) in deionized water adjusted to a pH of 8 with 5 N NaOH (Aldrich) and reacted on ice for 24 hours. This synthesis was previously described¹⁸ and the structure is shown in Figure 1. For purification, the macromer solution was dialyzed (MW cutoff 5–8kDa) against deionized water for at least 48 hours and the final product was obtained by lyophilization. Poly(ethylene glycol) dimethacrylate (PEGDM) was synthesized by the addition of methacryloyl chloride (Sigma) and triethylamine (Aldrich) to PEG (Polysciences, hydroxyl end groups, MW = 4kDa) dissolved in methylene chloride (Aldrich) on ice, as previously described²⁰. The final product was purified by repeated dissolution in methylene chloride and precipitation in diethyl ether (Aldrich). ¹NMR (Varian Unity-300) was used to determine the final functionality and purity of the MeHA (in D₂O) and PEGDM (in CDCl₃) macromers. Hydrogels were fabricated by dissolving the MeHA and PEGDM macromers at various concentrations in phosphate buffered saline (PBS) containing 0.05 wt% 2-methyl-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959, I2959), pipetting between glass slides with a 1 mm spacer, and polymerizing with the addition of ~4 mW/cm² ultraviolet light for 10 minutes using a longwave ultraviolet lamp (Model 100AP, Blak-Ray).

Hydrogel Characterization

After polymerization and swelling in PBS for 48 hours to equilibrium, hydrogel disks were weighed (wet weight) and then dried (dry weight) to determine the volumetric swelling ratio (Q_v), which is reported as the ratio of the wet weight to the dry weight ($n = 3$ per composition) and assuming a density of 1.23 g/cm³ for the MeHA macromers¹⁶. The compressive modulus of the various swollen hydrogels was determined on an Instron 5542 mechanical tester using

a parallel plate apparatus and loading of 10% of the initial thickness per minute ($\sim 200 \mu\text{m}/\text{min}$). Samples for mechanical testing ($n = 5$ per composition) were cylindrical ($\sim 2 \text{ mm}$ height, $\sim 7 \text{ mm}$ diameter) and were compressed until failure or until 60% of the initial thickness was reached. The modulus was determined as the slope of the stress versus strain curve at low strains ($<20\%$).

For degradation analysis, polymer disks (1 mm thickness, 9 mm diameter) were punched from hydrogel slabs using stainless steel bores. Samples ($n = 3$ per composition) were degraded in solutions of either 10 or 100 U hyaluronidase (Sigma) per ml of PBS (replaced every 48 hours throughout the study and stored frozen until analysis) at 37°C on an orbital shaker. The time for complete degradation of the hydrogel disks is reported. The amount of uronic acid (a degradation component of HA) released during degradation was measured using a previously established carbazole reaction technique²¹. Briefly, 100 μl of the degradation solution was added to a concentrated sulfuric acid/sodium tetraborate decahydrate (Sigma) solution and heated to 100°C for 10 minutes. After adding 100 μl of 0.125% carbazole (Sigma) in absolute ethanol and heating to 100°C for 15 minutes, the solution absorbance at 530 nm was measured. The amount of uronic acid was determined using solutions of known concentrations of the 50 kDa HA as a standard. The percent of total uronic acid released at each time point is reported. Degradation products were also analyzed with ^1NMR to determine if any unreacted or partially reacted monomer was present.

Photoencapsulated Cell Viability

As an initial assessment of photoencapsulated cell viability, 3T3-fibroblasts (ATCC, passage 5) were encapsulated by suspending in 50 μl ($\sim 5 \text{ mm}$ diameter, $\sim 2 \text{ mm}$ height) of macromer solution (containing 0.05 wt% I2959) and polymerizing with the addition of $\sim 4 \text{ mW}/\text{cm}^2$ ultraviolet light for 10 minutes using a longwave ultraviolet lamp (Model 100AP, Blak-Ray). These conditions were previously determined to be cytocompatible for the photoencapsulating chondrocytes²². The viability of photoencapsulated fibroblasts (40×10^6 cells/ml) was assessed immediately after encapsulation and after 1 week of in vitro culture (DMEM, 10% fetal bovine serum, Invitrogen) using a commercially available MTT viability assay (ATCC, 30–1010K). For this assay, 100 μl of the provided MTT reagent (tetrazolium salt solution) was added directly to the wells containing the constructs ($n = 3$ per macromer solution) and placed in an incubator at 37°C for 4 hours. The purple formazan produced by active mitochondria was solubilized by construct homogenization in 1 ml of the provided detergent solution and orbital shaking for 2 hours. The absorbance of these solutions was then read at 570 nm (Molecular Devices SpectraMax 384).

Chondrocyte Isolation and Photoencapsulation

Swine aged 3 to 6 months were euthanized using an overdose of Pentobarbital (100 mg/kg IV) and cartilage tissue was harvested in a sterile fashion. The harvested auricular cartilage was cut into $\sim 1 \text{ mm}^3$ pieces, washed in PBS, and digested for 18 hours at 37°C in a sterile 0.1% collagenase (Worthington) solution in Ham's F-12 medium. After digesting, the solution was passed through a 100 μm filter to remove undigested cartilage and centrifuged. The chondrocytes were washed twice with PBS, counted using a hemacytometer, and the viability was determined using trypan blue exclusion prior to encapsulation. Chondrocytes were photoencapsulated in the various hydrogel networks (40×10^6 chondrocytes/ml) by suspension in the desired macromer solution, pipetting into a sterile mold (50 μl volume), and polymerization as described above.

In Vivo Tissue Formation

Hydrogel constructs containing photoencapsulated chondrocytes were placed into subcutaneous pockets in the dorsum of nude mice (4 implants per mouse). Male nude mice (5

to 6 weeks old, ~25 g, nu/nu, Massachusetts General Hospital) were used for all studies. After 4, 6, and 8 weeks, mice were euthanized and constructs were harvested for histological or biochemical analysis. For histological analysis, constructs were fixed in 10% formalin for 24 hours, embedded in paraffin, and processed using standard histological staining procedures. The histological sections were stained with Safranin O to visualize glycosaminoglycans (GAG). For biochemical analysis, constructs ($n = 5$) were digested in a papain solution (125 $\mu\text{g/ml}$ papain type III, 10 mM l-cysteine, 100 mM phosphate, and 10 mM EDTA at pH 6.3) for 15 hours at 60°C. Total DNA content was determined using a bisbenzimidazol fluorescent dye (Hoechst 33258) method²³ added at a concentration of 0.2 mg/ml in 0.01 M Tris, 1 mM EDTA and 0.1 M NaCl with chondrocyte number determined using a conversion factor of 7.7 pg of DNA per chondrocyte. Total GAG content was determined using the dimethylmethylene blue dye method²⁴ and normalized to chondrocyte number. All reagents for biochemical analysis were obtained from Aldrich unless stated otherwise.

Statistical Analysis

Statistical analysis was performed using a Student's t-test (only to compare two individual samples) with a minimum confidence level of 0.05 for statistical significance. All values are reported as the mean and standard error of the mean.

Results and Discussion

Network Synthesis

HA networks were fabricated from MeHA precursors with varying molecular weights and methacrylations and with different MeHA concentrations to determine the range of properties possible for these potentially useful biomaterials. As illustrated in Figure 1, the MeHA macromers undergo a free radical polymerization with the addition of light and an initiator to form a crosslinked hydrogel that consists of HA and kinetic chains of poly(methacrylic acid). The various MeHA solutions investigated in this work are summarized in Table 1. Although the MeHA macromers were synthesized using the same techniques and with the same concentrations of HA and methacrylic anhydride, a slightly higher methacrylation was obtained with the 50 kDa HA. This is potentially explained due to decreased viscosity during this reaction compared to the 350 and 1100 kDa HA, increasing the mobility of various species during the reaction. The macromer concentrations investigated were chosen as the highest concentrations of MeHA that could still be pipetted into molds or for suspending cells. For instance, this was only possible up to 2 wt% of the 1100 kDa MeHA macromer, but was possible for a 10-fold higher amount of the 50 kDa macromer, allowing for a wider range of network properties.

Network Swelling, Mechanics, and Degradation

The equilibrium volumetric swelling ratio of the various HA networks are shown in Figure 2. As expected, a decrease in Q_V is seen with an increase in the concentration of macromer in the precursor solutions. For example, Q_V is ~41 for networks fabricated from 2 wt% of the 50 kDa macromer, but decreases to ~8 when the macromer concentration is increased 10-fold to 20 wt %. The same trend is seen for the 350 kDa macromer. For each of the molecular weights, there is a statistically significant ($p > 0.05$) decrease in Q_V with an increase in macromer concentration, but there were no statistical differences between the different MeHA molecular weights when the same concentration of macromer was used for network formation. Using Flory-Rehner calculations^{16,25}, the network mesh size and the crosslinking density, which are important when explaining mechanics and degradation, are directly correlated to Q_V .

Stress versus strain curves for several of the investigated networks are shown in Figure 3. The general slope is linear at low strains (<20%) and then increases with an increase in strain.

Overall, the modulus (i.e., slope of stress versus strain curve at low strain) correlates well with the network crosslinking density (i.e., swelling). As the macromer concentration increases for each of the MeHA molecular weights, a statistically significant increase in the modulus is seen. For instance, networks fabricated from 2 wt% of the 50 kDa macromer had a modulus of only ~12 kPa, but increased substantially to ~100 kPa when the macromer concentration was increased to 20 wt%.

The overall time for complete degradation of the networks in a solution of 100 U hyaluronidase/ml of PBS is shown in Figure 4. The hyaluronidase degradation of HA results in the cleavage of internal beta-N-acetyl-D-glucosaminidic linkages, which yields fragments with N-acetylglucosamine at the reducing terminus and glucuronic acid at the non-reducing end. In general, the swollen networks decreased in size throughout the degradation and exposure to the hyaluronidase. This behavior was previously seen for other crosslinked hyaluronic acid hydrogels²⁶. This is potentially due to both an increase in erosion at the surface of the gels due to diffusion restrictions of the enzyme into the interior of the gel and an attraction of the positive amine groups produced during degradation and the negatively charged carboxylic acid groups of the HA. Again, there was good correlation between degradation time and the hydrogel crosslinking density. For example, an increase in macromer concentration extended the time for complete degradation in a dose-dependant fashion. Also, no measurable double bonds were found during NMR analysis of the degradation products, indicating that the radical polymerization reaches near 100% conversion with the initiation conditions used (i.e., 10 minutes, 10 mW/cm², 0.05wt% I2959). It should be noted that these results only give information regarding relative degradation times and do not represent actual times for in vivo degradation, which is dependent on the actual local enzyme concentration.

The amount of uronic acid in the degradation solutions is shown in Figure 5 and plotted as the overall percentage of uronic acid detected with degradation time. For networks formed with the 50 kDa MeHA macromer, an increase in the macromer concentration (i.e., crosslinking density) extended the time for complete uronic acid release. For the 5 wt% HA network, ~40% of uronic acid is detected within two days of degradation and then a near linear release of uronic acid is observed until complete degradation. For the 10 wt% HA network, ~50% of the uronic acid is detected in the first 5 days of degradation, yet degradation extends to almost 20 days. A burst is observed at the end of degradation, due to the rapid solubilization of kinetic chains and HA when the network becomes loosely crosslinked. The rapid uronic acid release at short degradation periods could be due to the release of HA with low methacrylation, as a distribution of methacrylations is expected throughout the MeHA macromers. As seen in Figure 5B, the overall time and rate of hydrogel degradation is faster with a higher enzyme concentration (100 versus 10 U/ml).

Cell Encapsulation and Viability

3T3-fibroblasts were encapsulated in the various networks and their viability was determined both immediately after encapsulation and after 1 week of in vitro culture. These results are shown in Figure 6. Immediately after polymerization, a range of fibroblast viability is noted, with a decrease in viability as the macromer concentration increased. This could potentially be attributed to an increase in the radical concentration during encapsulation due to an increase in the reactive group concentration (i.e., methacrylates) in the precursor solutions with higher macromer concentrations. The reduction in MeHA molecular weight did not seem to affect the viability of the encapsulated cells. After 1 week of culture, a further decrease in viability is seen with many of the macromer solutions, especially with the more highly crosslinked ones. For the 50 kDa macromer, a statistically significant decrease in viability is found with each increase in macromer concentration. The high crosslinking density can decrease the ability for nutrients and wastes to be exchanged between the encapsulated cells and surrounding culture

media and lead to compromised cell viability. In general, the highest and sustained viability was observed in both the 1100 kDa and 50 kDa macromers at 2 wt%. Fluorescent Live/Dead staining (results not shown) indicated that >95% of the encapsulated cells in these gels remained viable. Overall, these results indicate that although the higher macromer concentrations led to desirable mechanical properties, their application as cell carriers is limited due to low viability of photoencapsulated cells. It should be noted that photopolymerizable HA networks could find use in many biological applications including drug delivery, fabrication of microfluidic devices, biomaterials surface modifications, and for tissue engineering as porous scaffolding.

Neocartilage Formation

Primary auricular swine chondrocytes were photoencapsulated in one of the HA hydrogels (2wt% 350kDa MeHA) that allowed high viability of photoencapsulated fibroblasts to assess the potential of these HA hydrogels for cartilage regeneration. PEGDM, which has been extensively investigated as a photocrosslinkable carrier for chondrocytes,^{27,28} was used as a control. Even after culture in the dorsum of nude mice for only 4 weeks, all of the constructs became more opaque as neocartilage tissue filled the networks. When auricular chondrocytes were encapsulated, the constructs resembled native cartilage with a white, shiny appearance. Representative stains for glycosaminoglycans (GAG) are shown in Figure 7. Although light background staining is observed for control HA networks without cells (results not shown), a more intense staining was observed when neocartilage was produced in the gels. When MeHA was used for network formation the GAG is evenly distributed throughout the tissue, whereas the GAG is confined primarily to the pericellular regions for the networks formed from PEGDM. This behavior is anticipated since the PEG networks can actually prevent the distribution of large extracellular matrix molecules since the networks are nondegradable. Thus, the cell controlled degradation of the HA networks may be crucial in optimizing cartilaginous tissue formation and distribution.

When quantified (Figure 7), changes in GAG production (reported as ng chondroitin sulfate per encapsulated chondrocyte) is seen with culture time. After 12 weeks, GAG values represent ~75% of those found for native articular cartilage from the same swine used for chondrocyte isolation (results not shown). There is little difference between the overall amount of GAG produced by chondrocytes in the MeHA versus PEGDM hydrogels, but the histology suggests there is a significant difference in the distribution of this tissue. As a control, the GAG content was determined for all hydrogels immediately after chondrocyte photoencapsulation and almost no GAG is detected, indicating very little interference by the HA hydrogels in this assay. Overall, these *in vivo* results indicate that further work should be pursued to investigate these HA hydrogels as chondrocyte carriers in cartilage tissue engineering.

Conclusions

This work presents a systematic study of the ability to fabricate photopolymerizable HA networks with a wide range of properties. Specifically, alterations in the HA molecular weight allowed high macromer concentrations to be incorporated in the precursor solution, leading to networks with high crosslinking densities. Although networks with a compressive modulus over 100 kPa were formed, the viability of fibroblasts in these networks was compromised potentially due to restrictions in nutrient transport through the network and a high radical concentration during polymerization. However, in the less crosslinked networks, viability was sustained and swine chondrocytes produced neocartilage tissue when implanted subcutaneously in nude mice. The variability in macromer molecular weight at concentrations that promoted cell viability will allow for a wide variety of precursor solution viscosities, which will be important for clinical and non-invasive implantation of these gels.

Acknowledgements

Support for this research was provided through NIH from grants K22 DE-015761-01 and 5R01 DE-13023-05. The authors thank Melody Craff for her assistance in chondrocyte isolation and construct implantation.

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Figure 1.

Top: chemical structure of methacrylated hyaluronic acid (MeHA). Bottom: general schematic of the free radical polymerization of MeHA to form crosslinked hydrogel networks. This process involves the formation of radicals from the exposure of the initiator to light, which propagate through the vinyl groups of the MeHA to form kinetic chains (shown as dashed lines). These networks eventually degrade by enzymatic cleavage of the HA backbone.

Figure 2. Equilibrium volumetric swelling ratio (Q_v) for photocrosslinked HA networks with variations in macromer molecular weight and concentration. The swelling ratios are statistically different (denoted by * between two bars) between the different macromer concentrations for each molecular weight MeHA.

Figure 3. Mechanical properties of HA hydrogels. A. Representative stress versus strain plots of hydrogels fabricated from 10 (solid) and 5 (dotted) wt% macromers (50 kDa MeHA). B. Compressive modulus for various HA networks at equilibrium swelling. The compressive moduli are statistically different (denoted by * between two bars) between the different macromer concentrations for each molecular weight MeHA.

Figure 4. Time for complete degradation of HA hydrogels in 100 U hyaluronidase/ml of PBS, where the hyaluronidase was replenished every other day throughout degradation.

Figure 5.

Uronic acid measured during the degradation of HA hydrogels. A. Cumulative percentage of uronic acid detected for HA hydrogels formed from 2 (●), 5 (■), and 10 (▲) wt% of the 50 kDa MeHA and degraded in 100 U hyaluronidase/ml. B. Cumulative percentage of uronic acid detected for HA hydrogels formed from 5 wt% 350 kDa MeHA and degraded in both 100 (●) and 10 (■) U hyaluronidase/ml.

Figure 6.

Viability of photoencapsulated 3T3-fibroblasts in the HA hydrogels. Absorbance (indicative of encapsulated cell mitochondrial activity and viability) for the various HA networks after 1 day (black) and 1 week (white) of in vitro culture. The MTT solution was diluted 4-fold for all samples to obtain absorbance values in the linear range. The absorbance is statistically different (denoted by * between two bars) between the different macromer concentrations for the 50 kDa MeHA at the 1 week time point.

Figure 7. Neocartilage formation by swine chondrocytes photoencapsulated in HA and PEG hydrogels. A. Histological sections (stained for glycosaminoglycans, bar = 100 μ m) of auricular chondrocytes photoencapsulated in 2% 350kDa MeHA (left) and 10% PEGDM (right) hydrogels 6 weeks after implantation in nude mice. B. Glycosaminoglycan content (reported as ng chondroitin sulfate/chondrocyte) immediately after photoencapsulation (black) and after 4 (grey) and 8 (white) weeks of auricular chondrocyte culture in nude mice for 2% 350 kDa MeHA and 10 wt% PEGDM.

Table 1

Summary of photopolymerizable MeHA macromer solutions investigated.

MW of Macromer (kDa)	Methacrylation (%)	Macromer in Precursor Solution (wt%)
1100	6	2
350	7	2
		5
50	12	2
		5
		10
		20