Methacrylated Glycol Chitosan as a Photopolymerizable Biomaterial

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Received June 21, 2007; Revised Manuscript Received September 13, 2007

Glycol chitosan is a derivative of chitosan that is soluble at neutral pH and possesses potentially useful biological properties. With the goal of obtaining biocompatible hydrogels for use as tissue engineering scaffolds or drug delivery depots, glycol chitosan was converted to a photopolymerizable prepolymer through graft methacrylation using glycidyl methacrylate in aqueous media at pH 9. *N*-Methacrylation was verified by both ^{1}H NMR and ^{13}C NMR. The degree of *N*-methacrylation, measured via ^{1}H NMR, was easily varied from 1.5% to approximately 25% by varying the molar ratio of glycidyl methacrylate to glycol chitosan and the reaction time. Using a chondrocyte cell line, the *N*-methacrylated glycol chitosan was found to be noncytotoxic up to a concentration of 1 mg/mL. The prepolymer was cross-linked in solution using UV light and Irgacure 2959 photoinitiator under various conditions to yield gels of low sol content (~5%), high equilibrium water content (85–95%), and thicknesses of up to 6 mm. Cross-polarization magic-angle spinning ^{13}C solid state NMR verified the complete conversion of the double bonds in the gel. Chondrocytes seeded directly onto the gel surface, populated the entirety of the gel and remained viable for up to one week. The hydrogels degraded slowly in vitro in the presence of lysozyme at a rate that increased as the cross-link density of the gels decreased.

Introduction

Photopolymerization to form hydrogels has attracted considerable interest in the field of tissue engineering and drug delivery. The reason for this interest is that gel constructs have similar water contents to the extracellular matrix and thus allow for efficient nutrient transport, which is important for maintaining cell viability, as well as contributing to biocompatibility by reducing mechanical irritation to the surrounding tissue. In tissue engineering applications, photopolymerization can be used for the rapid entrapment of cells with minimal cell death and to prepare scaffolds in a variety of geometries, and judicious choice of the photopolymerizable macromonomer can allow for effective cell attachment and proliferation.[1–4] In recent years, there has been increasing interest in developing photopolymerized materials for protein drug delivery applications.[5–14] For both tissue engineering and drug delivery, advantages of photopolymerizable gels are that gelation can be performed at physiologic temperature and in situ with minimal heat generation.

As glycosaminoglycans comprise a significant portion of, and play important roles within, the extracellular matrix, they are obvious choices for the preparation of photopolymerizable hydrogel constructs. A number of different approaches have been taken to prepared photopolymerizable glycosaminoglycans. For example, cinnamate[15] and methacrylate[2–4,16] functional groups have been grafted to both hyaluronate and chondroitin sulfate, while hyaluronate has been styrenated.[17] However, these polysaccharides are generally expensive and so alternative materials have been investigated.

Chitosan is a nonphysiologic glycosaminoglycan that has been demonstrated to be biocompatible,[18–20] to accelerate wound healing,[21,22] has antimicrobial properties,[23–26] and supports the growth and function of osteoblasts and chondrocytes.[27,28] It is therefore a potentially useful biomaterial for use in the preparation of photopolymerizable hydrogels as tissue engineering constructs and drug delivery matrices. Photopolymerizable chitosan derivatives have been prepared previously through styrenation[17] and methacrylation using reactive aldehyde intermediates.[29] The styrene grafting approach resulted in only a 12% gel yield when the reaction was done with low molecular weight chitosan, while methacrylation via reactive aldehydes required the synthesis of methacrylated vanillin or hydroxybenzaldehydes. Further, the degradation of chitosan in vivo is slow, with degradation times in excess of 20 weeks depending on its degree of deacetylation,[30,31] which has been attributed to its low solubility at physiologic pH and its crystallinity.[20] For tissue engineering applications, it is often desirable to encapsulate cells directly in the hydrogel matrix, and this would not be possible with chitosan-only gels due to their low water content at pH 7.4. Thus, a useful property of a chitosan-based photopolymerizable prepolymer would be solubility in aqueous solution at neutral pH.

A water soluble photopolymerizable chitosan has previously been prepared by grafting 4-azidobenzoic acid to available free amine groups of lactose modified chitosan.[32] A possible disadvantage of this approach is that exposure to UV irradiation converts the azide group to nitrene groups that are highly reactive with amino groups. Nonspecific interactions of these nitrenes with amino groups during photopolymerization may result in protein denaturation and grafting of the protein to the chitosan itself. This is likely the reason for the observed incomplete bovine serum albumin and fibroblast growth factor-2 release from matrices in which the modified chitosan was photopolymerized in the presence of these proteins in solution.[33]

In this paper we explore the potential of methacrylated glycol chitosan as an alternative photopolymerizable chitosan. Glycol chitosan (Figure 1) is soluble over a wide range of pH and, in the few reports to date, has been found to be cytocompatible.

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10.1021/bm700691e CCC: $37.00
Published on Web 11/22/2007

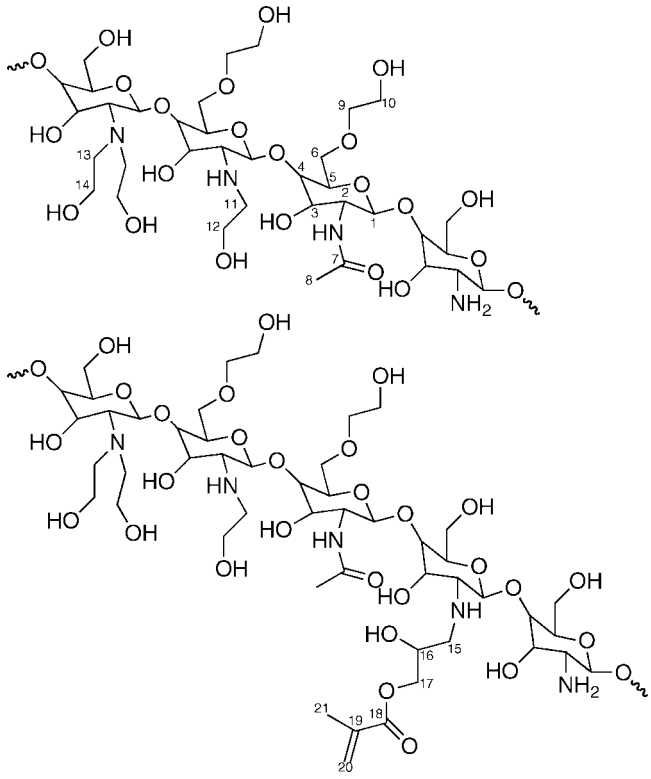

Figure 1. Structure of glycol chitosan (top) and *N*-methacrylated glycol chitosan (bottom).

For example, Carreño-Gómez and Duncan saw only mild concentration-independent (up to 2.5 mg·mL^{-1}) levels of toxicity of glycol chitosan toward a murine melanoma cell line. They also observed reduced hemolysis in a 24 h incubation with red blood cells compared against other chitosan forms.[34] Furthermore, we have recently found that glycol chitosan is not only noncytotoxic, but stimulates chondrocyte growth at low concentrations.[35] The glycol chitosan was made photopolymerizable by methacrylation through the reaction of glycol chitosan with glycidyl methacrylate. Glycidyl methacrylate has been used to modify chitosan[36] and oligochitosan;[37] however, methacrylated chitosan was not soluble at physiologic pH, while significant water solubility of methacrylated oligochitosan was obtained only for tetramers. Furthermore, these materials were not investigated as photopolymerizable prepolymers.

Methacrylated glycol chitosan was prepared at different molar ratios of glycidyl methacrylate to free amine/glycol chitosan residue. The cytotoxicity of the methacrylated glycol chitosan was assessed using an immortalized chondrocyte cell line. Hydrogels prepared from the methacrylated glycol chitosan were characterized in terms of their sol content as influenced by degree of methacrylation, UV intensity, time of irradiation, and gel depth. The equilibrium swelling capacity of low-sol–gels was measured in water, and their mechanical properties were determined using indentation. Finally, the cytotoxicity of hydrogels prepared from this prepolymer was assessed using the same chondrocyte cell line.

Materials and Methods

Materials. Glycol chitosan, sodium nitrate, sodium hydroxide, glycidyl methacrylate, lysozyme, and 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) were all obtained from Sigma-Aldrich, Ltd., Canada. Deuterium oxide was purchased from Cambridge Isotope Laboratories, Inc. All reagents were used as received. Water used was of type I purity, obtained from a Millipore Milli-Q Plus ultrapure water system.

Glycol Chitosan Purification. Glycol chitosan (1 g) was dissolved in water (75 mL) and filtered to remove insoluble impurities. The filtrate was then dialysed against water with molecular weight cutoff 50 kDa dialysis tubing for 8 h. Both the membranes and the media were replaced at 4 h. The purified high molecular weight glycol chitosan was frozen at −20 °C for a minimum of 8 h and lyophilized for at least 48 h.

Glycol Chitosan Methacrylation via Glycidyl Methacrylate. Glycol chitosan was dissolved in 20 mL of water to give a 1.9 w/v% solution, which was adjusted with 1 M NaOH to give solutions of pH 9.0. Various volumes of glycidyl methacrylate were added to the glycol chitosan solution to provide initial molar ratios of glycidyl methacrylate: free amine per mol glycol chitosan residue (see calculation method below) ranging from 0.14 to 0.70 and allowed to react for the given times, up to 48 h. The reaction media was then neutralized and dialysed with a molecular weight cutoff of 1 kDa dialysis tubing against water for 4 h. The membranes and media were replaced at 2 h. The methacrylated glycol chitosan solution was lyophilized to yield a crystalline white powder.

Photocrosslinking of Methacrylated Glycol Chitosan. A concentrated solution of methacrylated glycol chitosan in deionized water (6 w/v%) containing the photoinitiator I2959 (1 w/v%) was poured into a cylindrical Teflon mold (7.5 mm diameter × 6 mm). I2959 was used in this study, as it has been demonstrated to be the least cytotoxic to various cells.[38] The solution was then exposed to long-wave ultraviolet light (320 to 480 nm, EXFO Lite, EFOS Corporation, Mississauga, Canada) at intensities ranging from 25 to 102 mW/cm^2 for up to 9 min to yield the methacrylated glycol chitosan hydrogels.

Nuclear Magnetic Resonance Spectroscopy. ^{1}H NMR spectra were conducted with a Bruker Avance-600 Ultrashield spectrometer equipped with a 5 mm TBI S3 probe with Z gradient and variable temperature capability. Samples were prepared at 20 mg/mL in deuterium oxide, preheated at 60 °C for 6 h, then adjusted to pH > 10 with 1 M sodium hydroxide (30 μL) prior to running. Samples were allowed to equilibrate for 10 min at 90 °C within the spectrometer prior to shimming to ensure a homogeneous sample temperature. All chemical shifts were referenced to the HOD peak as a primary reference and to tetramethylsilane (TMS) as a secondary reference. Spectral data was collected in Bruker's XWIN-NMR software. ^{13}C NMR was performed at a concentration of 55 mg/mL at 80 °C. Cross-polarization magic angle spinning solid state ^{13}C NMR was performed on photocrosslinked gels dried in vacuo and ground using a mortar and pestle. The samples were run on a Bruker 600 MHz Avance spectrometer with a spinning rate of 14050 Hz, contact time of 2 ms, and a relaxation delay of 2 s, using a broadband CP-MAS probe.

Gel Permeation Chromatography with Light Scattering. GPC with light scattering data were obtained with a Waters 1525 Binary HPLC pump and a Precision Detectors Enterprise^{MDP} PD2100 series equipped with refractive index and light scattering detectors with angles of 15 and 90°. GPC was achieved using Waters Ultrahydrogel 2000, 250, and 120 columns connected in series. Samples, dissolved in a 0.8 M Na_2NO_3 solution, were filtered (0.45 μm) and injected (100 μL) with a Waters 717plus autosampler onto the column at 0.8 mL/min and 25 °C at a concentration of 10 mg/mL. All data were obtained and processed in Precision Detectors' Precision Aquire32 and Discovery32 software programs using an absolute refractive index of 1.3255 mL/g determined on a Wyatt Optilab rEX and a refractive index increment (dn/dc) value of 0.115 mL/g.

Sol Determination. Immediately after photocrosslinking, the gels were frozen in liquid nitrogen. The frozen gels were lyophilized, and their dry mass recorded. The gels were then swollen three times in type I purified water at 37 °C, with the water replaced every 45 min. The gels were again frozen in liquid nitrogen, lyophilized, and the final mass recorded. The sol content was calculated as

$$\text{sol} = \frac{m_{\text{dry,ini}} - m_{\text{dry,fin}}}{m_{\text{dry,ini}}} \quad (1)$$

where $m_{\text{dry,ini}}$ and $m_{\text{dry,fin}}$ represent the mass of the dried gel before and after swelling, respectively.

Equilibrium Water Content. Sol-removed gels were swollen in pH 7.4 phosphate-buffered saline for 96 h. The wet gels were collected, surface moisture was removed by blotting, and the mass of each gel measured. The equilibrium water content (EWC) was calculated as

$$\text{EWC} = \frac{m_{\text{wet}} - m_{\text{dry,ini}}}{m_{\text{wet}}} \quad (2)$$

Young's Modulus. The Young's modulus of different gels were measured via indentation as described in Jin and Lewis,[39] using a TA XT plus texture analyzer from Texture Technologies Corp., New York. Flat-ended indenters of 3 and 7 mm diameter were used, and gels were photocrosslinked in a cylindrical mold, covered with a glass coverslip to ensure a flat surface. The sol content of the gels was first removed, and the gels were swollen to equilibrium in pH 7.4 phosphate-buffered saline. Using two different-sized indenters allows for the measurement of the Poisson ratio, ν, via

$$\frac{(p/\omega)_1}{(p/\omega)_2} = \frac{a_1}{a_2}\frac{\kappa_1(a_1/h_1, \upsilon_1)}{\kappa_2(a_2/h_2, \upsilon_2)} \quad (3)$$

in which a is the radius of a given indenter, p is the indenting force, ω is the indenting depth, the subscripts refer to a given indenter, and κ is a correction factor that accounts for the finite layer effect, obtained from Hayes et al.[40] In eq 3, ν is the only unknown. The slope of the resulting force-indentation depth curves was calculated for indentation using both indenters to provide (p/ω). Solving for ν then allows for the calculation of Young's modulus, E, using

$$\frac{p}{\omega} = \frac{2aE\kappa}{(1-\nu^2)} \quad (4)$$

Three measurements were made per gel, and three gels were prepared for each degree of substitution examined.

Cell Culture. The immortalized human chondrocyte cell line C-28/I2 was kindly supplied by Dr. Mary Goldring, Harvard Medical Institutes, Beth Israel Deaconess Medical Center. Growth media for cell culture was purchased from Invitrogen, Inc., Canada. Fetal bovine serum (FBS) was Canadian-sourced Hyclone characterized FBS. Unless otherwise stated, culture reagents were from Sigma-Aldrich/Fluka Biochemika and were of high purity grade, cell-culture tested. All media was sterile-filtered (<0.22 μm) prior to use and pH-adjusted to 7.40. Cells were seeded in 75 cm^2 tissue culture polystyrene (TCPS) flasks at a density of 1.0×10^6 cells/flask. Growth media was a 50:50 mixture of Ham's F-12/DMEM. The media was supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 4500 mg/mL D-glucose, 12 mM *N*-2-hydroxyethylpiperazine *N*′-2-ethane sulfonic acid (HEPES), and 50 μg/mL ascorbic acid. Cells were maintained in a humidified atmosphere under 5% CO_2 at 37 °C. Growth media was aspirated and replaced with fresh media every other day. When cells had reached near-confluence, they were removed from culture surfaces by addition of 0.05% trypsin-EDTA. Cell solutions were washed/centrifuged twice with fresh media to remove metabolic waste and any dead cells. The cells (P2-P3) were passaged twice following thawing from frozen concentrate and maintained chondrocytic phenotype during culture as assessed by visual morphology.

Cytotoxicity Assays. In vitro cytotoxicity of the noncross-linked methacrylated glycol chitosan in the absence of I2959 was assessed through effect on cell proliferation in monolayer cultures of chondrocytes. Sterilization of all materials in their dry state was achieved by UV exposure (~40 $\mu W/cm^2$) for 30 min and thereafter maintained under aseptic conditions. This UV exposure was confirmed to be insufficient to react methacrylate double bonds, as assessed by ^{1}H NMR and GPC. For these experiments, the C-28/I2 cells were seeded in 96-well culture plates at a density of 1.5×10^4 cells/well. A 100 μL cell solution was chosen based upon protocols stated in the assay instructions. A sample size of seven was used for all cytotoxicity data to overcome the large variability associated with sample solubility and cell density. As a positive control, an equivalent number of wells were seeded with cells, and the conversion of the tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate; Roche Diagnostics, Canada) to formazan in the absence of any external material was measured. The negative control was latex rubber. Lyophilized samples were dissolved in test media at varying concentrations (0.1, 0.5, 1.0 mg/mL) prior to placement inside the wells. Cells were cultured to near-confluence, and the growth media was aspirated, followed by the addition of test solutions. Following a 22 h incubation period, 10 μL of WST-1 reagent was added, and the plates were again incubated for an additional two hours. All readings were conducted on a μQuant microplate reader (BioTek Instruments, Inc.). Results of the absorbance values were corrected by subtracting the background reading of the media and WST-1 reagent alone in the corresponding trial and were then normalized against the positive control.

To assess the behavior of cells on and within gelled methacrylated glycol chitosan, a cell attachment/migration study was used. Gel discs were prepared by photocrosslinking a 6 w/v% methacrylated glycol chitosan (mGC) solution in a Teflon mold at 50 mW/cm^2 for 5 min in a laminar flow hood to maintain sterile conditions. The gel/Teflon mold combination was seated inside the wells of a six-well TCPS plate. The C-28/I2 chondrocytes (P3) were seeded on the Teflon surface of the mold, in uniform density, spaced out around the circumference, at a density of 1.5×10^4 cells/well. The idea behind this procedure was that cells would not adhere to the Teflon surface and would, therefore, have to attach to the chitosan gel or would remain in solution and be aspirated with media changes. Any cells that survived would begin to grow on the surface of the chitosan. The cells were grown on/within the gels for one week, with a change of growth media every other day. After seven days, the combined gel/Teflon constructs were removed from the incubator and washed successively with PBS. Cell viability and location within the gels was assessed using a LIVE/DEAD assay. The LIVE/DEAD dye solutions were incubated with the gel constructs for 30–45 min, as per assay guidelines. The media was removed, and the labeled gels were observed under a confocal microscope. Imaging was accomplished using an excitation wavelength of 488 nm and a PL Fluotar 10×/0.30 optical dry objective lens. Images were taken at a depth of 2/3 of the total depth of the gel.

In Vitro Degradation. Hydrogels (7.5 mm D × 4 mm H) were prepared through photocrosslinking 5, 9, and 14% degree of substitution *N*-methacrylate glycol chitosan at 100 mW/cm^2 light intensity for 5 min. The gels were rinsed with distilled water three times then incubated in a mixture of 4 mg/mL lysozyme from chicken egg white and 0.1% (w/v) sodium azide. The lysozyme mixture solution was changed every week. Every month, three gel samples were frozen, lyophilized, and weighed to obtain the final weight after degradation.

Results and Discussion

The structure of glycol chitosan, as determined from ^{1}H NMR,[35] is shown in Figure 1, along with that of methacrylated glycol chitosan. Glycol chitosan has both hydroxyl and amine groups that will undergo a nucleophilic substitution reaction with glycidyl methacrylate. The most favorable nucleophile is the primary amine,[41] as the reaction of the epoxide with the hydroxyl groups requires high pH conditions (typically 11–12) and typically low degrees of substitution are achieved.[42]

^{1}H NMR of methacrylated glycol chitosan is provided in Figure 2. Methacrylation is achieved, as evidenced by peaks arising at 5.85 ppm and 6.2 ppm due to protons on the vinyl carbon and a peak at 2.45 ppm due to the methyl group on the methacrylate. The same groups in unreacted glycidyl methacrylate appear at 6.3, 6.7, and 2.55 ppm, respectively. *N*-Methacrylation can be discerned in the spectrum, as a reducti

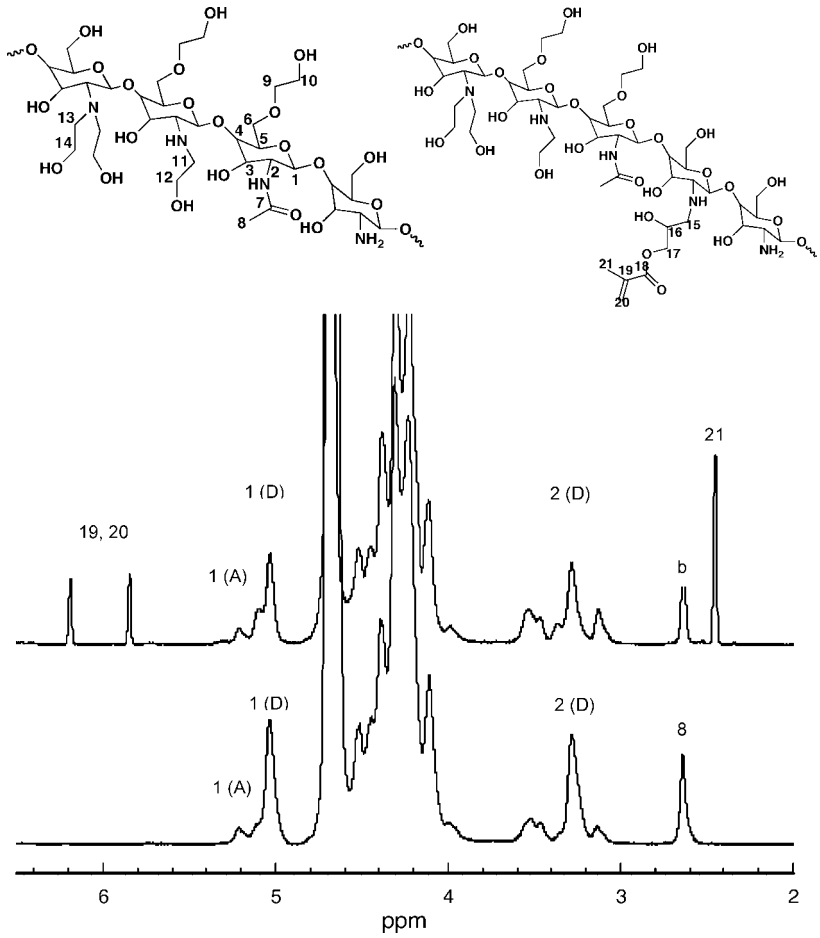

Figure 2. ^{1}H NMR spectra of unmodified and methacrylated glycol chitosan. Peak assignments correspond to ^{1}H designations in Figure 1. The (D) refers to deacetylated residues, while the (A) refers to acetylated residues.

in peak area at 3.25 ppm (H-2 proton for the deacetylated residues) and the appearance of a small peak centered at 3.35 ppm corresponding to H-2 for the *N*-methacrylated residue. There is also a concurrent reduction in the H-1 peak for the deacetylated residue (5.05 ppm) and the appearance of a new peak at 5.1 ppm corresponding to H-1 for the *N*-methacrylated residue. The *N*-methacrylation of glycol chitosan was confirmed using ^{13}C NMR, as shown in Figure 3. Peaks corresponding to the vinyl carbons appear at 128 and 137 ppm, for the methyl carbon on the methacrylate at 18 ppm, and the carbonyl carbon of the methacrylate at 171 ppm.

The reaction conditions were investigated by monitoring the degree of substitution with time and as a function of molar excess of glycidyl methacrylate to reactive amine/residue. The degree of substitution (DOS) of methacrylate groups onto the glycol chitosan backbone was calculated as

$$\mathrm{DOS} = \frac{(I_{5.8} + I_{6.2})/2}{I_{5.05} + I_{5.2}} \times 100\% \qquad (5)$$

wherein I represents the integration of the peak corresponding to the subscript ^{1}H NMR ppm. This DOS is effectively the number of grafted methacrylate groups per 100 residues. The moles of reactive amine per mol residue was calculated as

$$\mathrm{NH_2/residue} = \frac{I_{3.25}}{I_{5.05} + I_{5.2}} \qquad (6)$$

The reaction proceeds relatively quickly initially, then slows with time (Figure 4A), reaching a maximum conversion by 48 h, although little increase in DOS is obtained by running the reaction past 24 h. These results are consistent with those of Abo-Shosha and Ibrahim who examined the reaction of cellulose-poly(glycidy1 methacrylate) with methylamine in water under basic conditions[43] and are attributed to the competing reaction of the hydrolysis of the epoxide ring. The DOS is readily controlled by the molar excess of glycidyl methacrylate to reactive amine/residue (GMA/NH_2); DOS increases linearly as the initial GMA/NH_2 ratio increases (Figure 4B), which is again consistent with the results of Abo-Shosha and Ibrahim.[43]

The possibility of a Michael-type addition reaction occurring between the unprotonated amine and the already grafted vinyl group was also considered. This reaction has been shown to proceed slowly at room temperature in aqueous media and was not expected to be significant.[44] As Table 1 shows, the increase in molecular weight of glycol chitosan upon methacrylation can be readily attributed to the degree of substitution, and the polydispersity index does not increase significantly. Thus, it is not likely that considerable Michael-type addition reactions occurred during the methacrylation reaction time.

The cytotoxicity of the methacrylated glycol chitosan was assessed using an immortalized chondrocyte cell line (Figure 5). Chondrocytes were chosen because of the recent interest in the use of chitosan containing matrices for articular cartilage tissue engineering.[45–49] Cytotoxicity was assessed as a function of concentration and degree of methacrylation. The response is reported in comparison to the control, which was chondrocyte growth in culture medium alone.

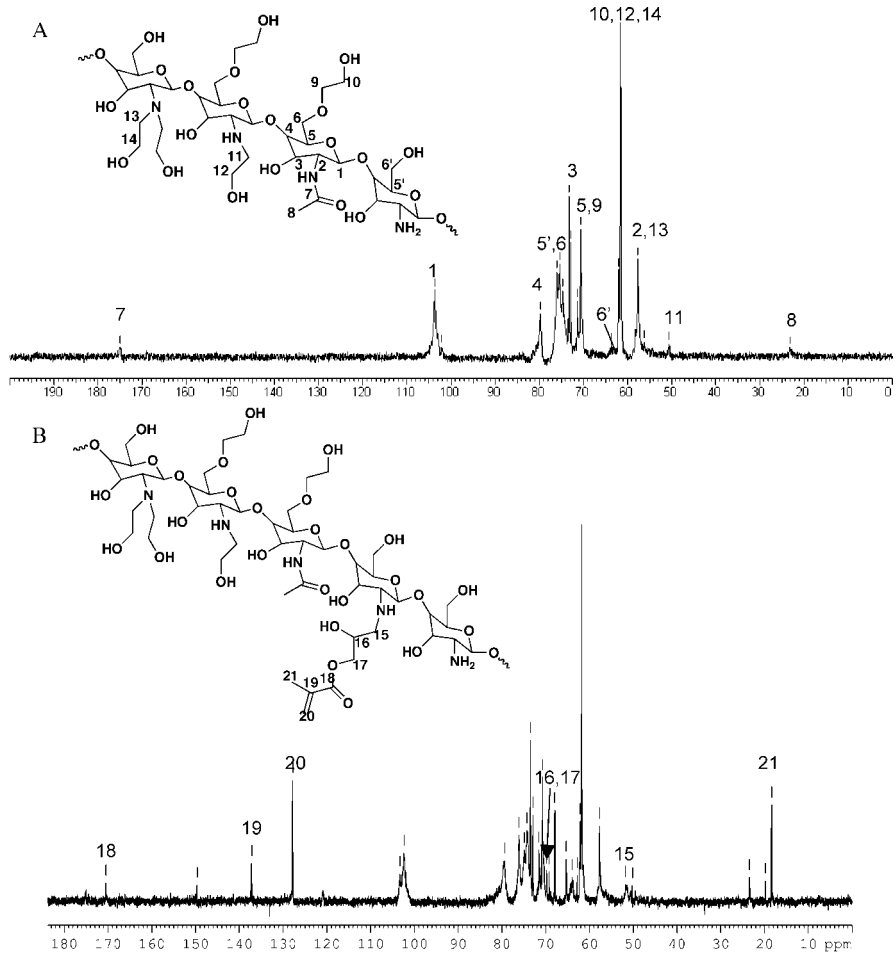

Figure 3. ^{13}C NMR spectra of glycol chitosan (A) and methacrylated glycol chitosan (B). Peak assignments correspond to designations in Figure 1.

As reported elsewhere,[35] at low concentrations, glycol chitosan exhibits a stimulatory effect on chondrocyte growth. These results are in contrast to those reported by Carreño-Gómez and Duncan, who reported mild cytotoxicity of glycol chitosan toward murine melanoma cells (approximately constant 75% viability relative to cell medium only, for a concentration range from 0.1 to 2.5 mg·mL^{-1}).[34] Clearly, different cell lines respond to glycol chitosan in different fashions. There was no difference in chondrocyte response ($p > 0.05$) with respect to degree of methacrylate substitution. Chitosan has been demonstrated by Howling et al. to also stimulate fibroblast growth. The mechanism through which this occurs is postulated to be an indirect effect wherein the chitosan interacts with heparin and growth factors present in the media, as stimulation of proliferation was not observed when the concentration of serum in the media was reduced. A number of means through which the interaction of chitosan with the serum factors could stimulate growth were postulated, including protection from enzymatic degradation, providing a slow release mechanism as the complex breaks down or by localizing the bound factor near the cells and the cell receptors. It is possible methacrylated glycol chitosan has the same effect, as it is likely still positively charged. The actual mechanism is unknown; however, the present findings indicate the potential of using methacrylated glycol chitosan as a scaffold for regenerating articular cartilage.

An important parameter in the manufacture of hydrogel constructs for tissue engineering using a photocrosslinkable material is the sol content of the network formed. A low sol content is indicative of efficient cross-linking. The influence of prepolymer solution depth, irradiation time, and irradiation intensity on sol content were examined, and the results are given in Figure 6. As would be expected, prepolymer solutions of greater depth require longer times to reach minimal sol contents at a given irradiation time and light intensity (Figure 6A). The 6 mm deep prepolymer solutions required 5 min at 102 mW/cm^2 intensity to reach a sol content of 6 ± 2.7%, whereas 4 mm deep prepolymer solutions produced gels with similar sol contents (6.8 ± 2.6%) after only 1 min at 102 mW/cm^2 intensity. For the 4 mm deep prepolymer solutions, there was little change in sol content with longer gelation times, with 5 min at 102 mW/cm^2 resulting in gels with a sol content of 3.3 ± 1.3%. For the 6 mm deep prepolymer solutions, there was no significant change in sol content after 5 min of irradiation at 102 mW/cm^2. Figure 6B shows that there is little influence of DOS on gel sol content at a given photocrosslinking condition, as there is no significant difference between sol contents of gels prepared with 5% DOS versus 21% DOS. Furthermore, sol contents decreased as irradiation intensity increased for a 5 min irradiation time, up to an irradiation intensity of 73 mW/cm^2, after which intensity had no noticeable effect. However, gels with low sol contents (8.8 ± 1.3%) were obtained at the lowest intensity accurately measurable of 25 mW/cm^2. The low sol contents indicate that the photocrosslinking conditions were effective at producing networks.

The hydrogels contained significant amounts of water, as reflected in their equilibrium water content (Figure 7). The equilibrium water content was higher in gels prepared from prepolymers of lower DOS (Figure 7A), with little effect of

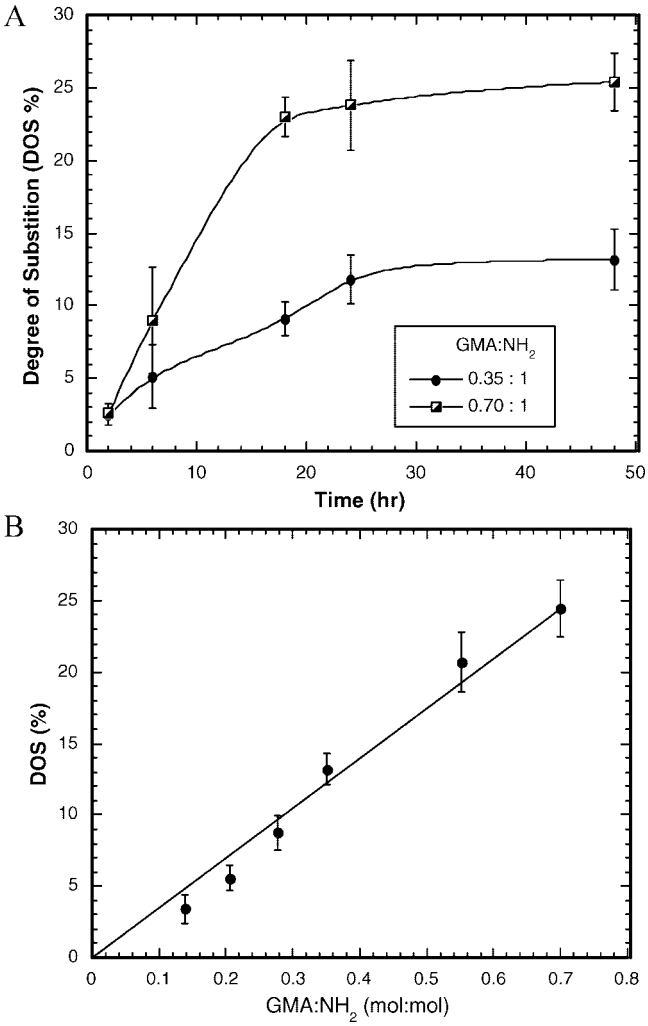

Figure 4. Influence of reaction conditions on degree of substitution. (A) Reaction time and (B) initial molar excess GMA (DOS at 48 h).

Table 1. Change in Number Average Molecular Weight (M_n) and Polydispersity (PI) of Glycol Chitosan upon Methacrylation

M_n initial (kDa)	PI initial	% DOS	M_n after (kDa)	PI after
96.3	2.2	10.2	106.9	2.1
96.3	2.2	25.4	116.4	2.1

irradiation intensity at a fixed irradiation time of 5 min. Gels prepared from greater prepolymer solution heights had higher equilibrium water contents (EWC) at given cross-linking conditions (Figure 7B). For example, at cross-linking conditions of 102 mW/cm^2 and 3 min, 4 mm thick gels had an EWC of 88.4 ± 1.1%, while 6 mm thick gels had an EWC of 94.8 ± 0.7%. As the sol had been removed prior to swelling to equilibrium, the difference in EWC is likely due to a difference in cross-link density within the lower regions of the 6 mm thick gels. Free-radical generation decreases with penetration depth due to radiation absorbance, and so, the lower regions of the 6 mm thick gels had a lower cross-link density. Thus, the gels should be prepared with thickness less than 6 mm to avoid a heterogeneous structure.

Interestingly, as the irradiation time increased beyond 5 min for an irradiation intensity of 102 mW/cm^2, the EWC increased (Figure 7B) for both 4 mm and 6 mm thick gels, although the sol contents for both thicknesses remained essentially constant (Figure 6A). This result indicates that the cross-link density

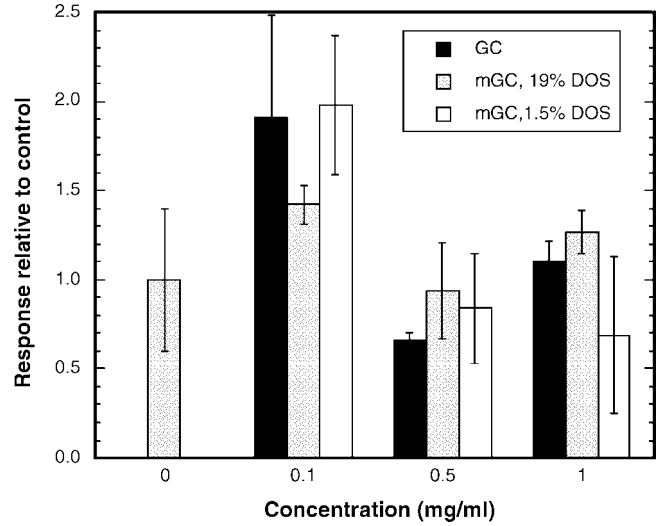

Figure 5. Cytotoxicity evaluation of glycol chitosan (GC) and methacrylated glycol chitosan (mGC) as a function of concentration and DOS using immortalized chondrocytes ($N = 7$). The data are given as sample response relative to the positive control (media alone).

decreases as radiation time increases beyond 5 min, while a network is still effectively formed. This result is attributed to free-radical cleavage of the chitosan backbone through photoinduced production of radicals that can no longer be consumed by carbon-carbon double bonds. This reasoning arises from the fact that chitosan and glycol chitosan can be readily depolymerized through free radical attack of the glycosidic bond using potassium persulfate free-radical generation.[35,50]

As one possible use of the photocrosslinked glycol chitosan gels under investigation is as a scaffold for regenerating articular cartilage, it was important to know the Young's modulus of the gels. It is generally accepted that to produce de novo tissue of appropriate biological and physical properties, the scaffold used should have mechanical properties that are consistent with the mechanical properties of the tissue. Indentation testing is commonly used to measure the mechanical properties of articular cartilage,[39] and so, it was applied to the photocrosslinked glycol chitosan gels. Representative force-indentation depth curves for the two different-sized indenters and 4 mm thick gels are given in Figure 8, while the Young's moduli calculated from such curves as a function of DOS are given in Figure 9. The analysis used assumes that the gel is an isotropic, homogeneous elastic. The Poisson ratio measured for the gels remained essentially constant at between 0.12–0.21. This value is lower than the often assumed value of 0.5 for hydrogels, but is expected, given the large EWC of >90% of the gels measured. This value also compares favorably with those obtained by Goldsmith et al. for hydrogels composed of poly(*N*-vinylpyrrolidone-comethyl methacrylic acid) with cellulose acetate (0–0.30), which had an EWC of 50%.[51] The calculated instantaneous Young's modulus, E, increases as the DOS of the prepolymer used to prepare the gel increases, but the increase in E diminishes in extent beyond a DOS of 7% when the variability in the measurement is taken into account. The maximum E value measured was 0.44 MPa. This value is much smaller than that of human tibial articular cartilage, which has been reported to be 1–10 MPa.[52] Thus, the mechanical properties of this gel may need to be further increased if it is to be used as a scaffold for regeneration of articular cartilage, and it does not appear that this can be achieved through increasing the DOS of the glycol chitosan prepolymer. The possibility of cocrosslinking with another polymer is currently being pursued.

CDV

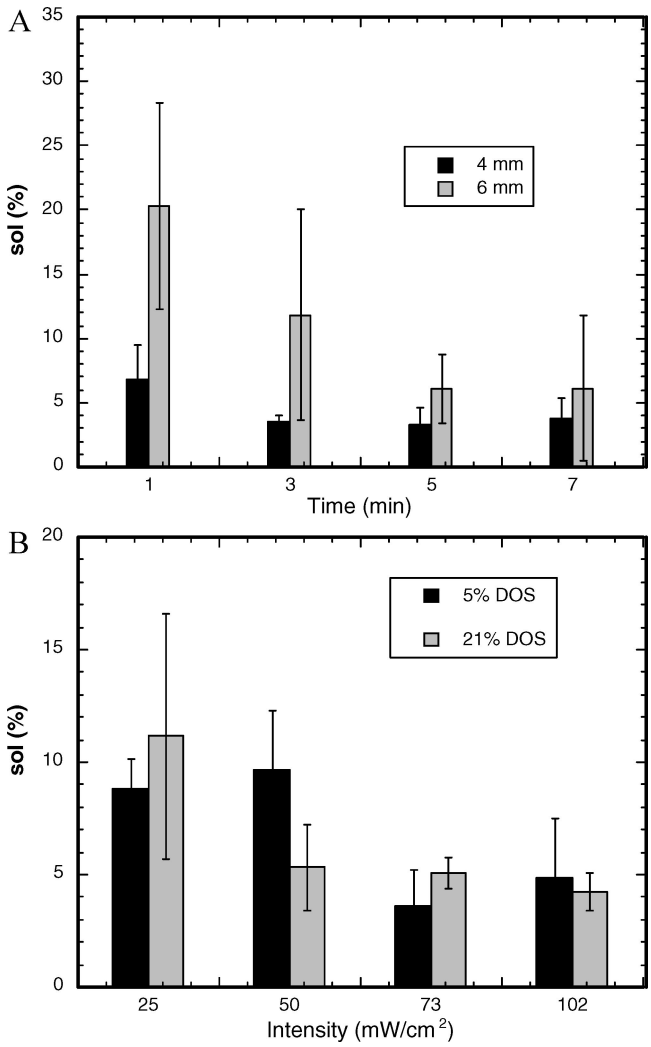

Figure 6. Influence of (A) irradiation time and prepolymer solution depth (21% DOS, 102 mW/cm^2) and (B) irradiation intensity and degree of methacrylate substitution (DOS) on sol content (6 w/v% solution, 4 mm depth) of gels obtained after 5 min of irradiation time.

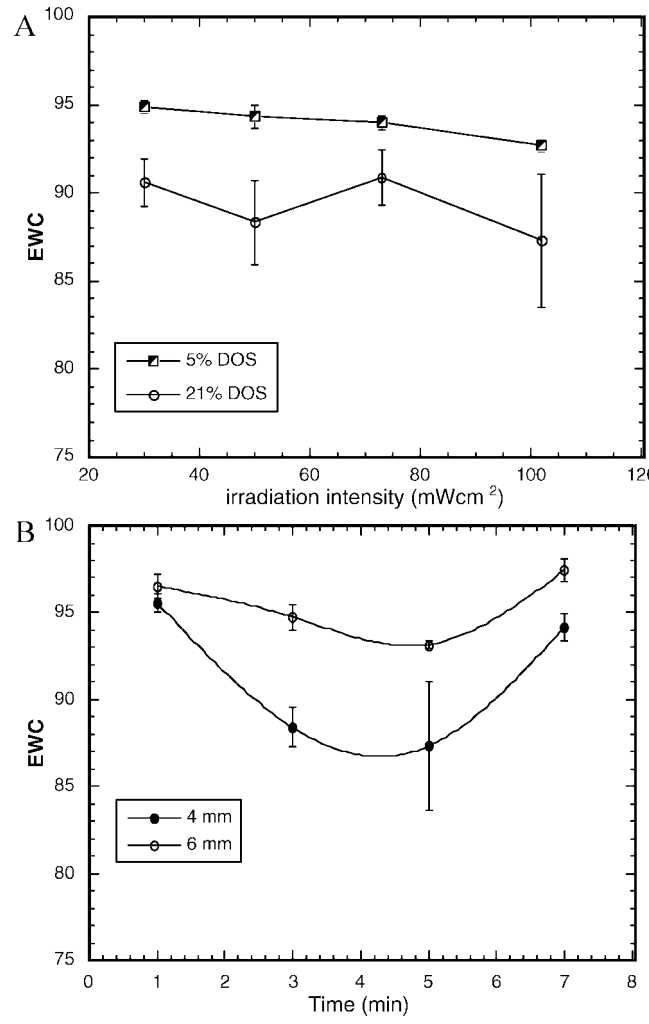

Figure 7. Equilibrium water content of the hydrogels. (A) Influence of degree of substitution (DOS) and irradiation intensity given a 5 min irradation time. The gels were prepared with a 4 mm thickness. (B) Influence of radiation time at 102 mW/cm^2 and thickness for samples prepared with 21% DOS.

Solid state ^{13}C NMR on the sol-removed gels was done to determine the degree of double-bond conversion during photocrosslinking. The spectrum obtained from a gel prepared using 21% DOS methacrylated glycol chitosan, and a photocrosslinking time of 5 min is given in Figure 10. There are no peaks present between 120 and 140 ppm that would correspond to carbon–carbon double bonds, while all other ^{13}C peaks noted in Figure 4 are present, albeit broadened. It can, therefore, be concluded that, within the network, there are no unconsumed double bonds that could potentially lead to cytotoxicity.

The cytotoxicity of the hydrogel formed from the methacrylated glycol chitosan prepolymer (DOS 5%, 25 mW/cm^2, 5 min irradiation) was assessed using an immortalized chondrocyte cell line. The chondrocytes were seeded on top of the gels, grown in static culture for 7 days with media exchanged every two days, and their viability assessed using a LIVE/DEAD assay. In this assay, living cells fluoresce green and dead cells fluoresce red. Using a confocal laser scanning microscope, images were obtained at a depth of 2/3 from the top surface. Images were taken along the diameter. The purpose of the assay was to ascertain whether the chondrocytes would remain viable after being seeded on the cell (i.e., to determine whether extracted fractions of the gel were cytotoxic) and to ascertain

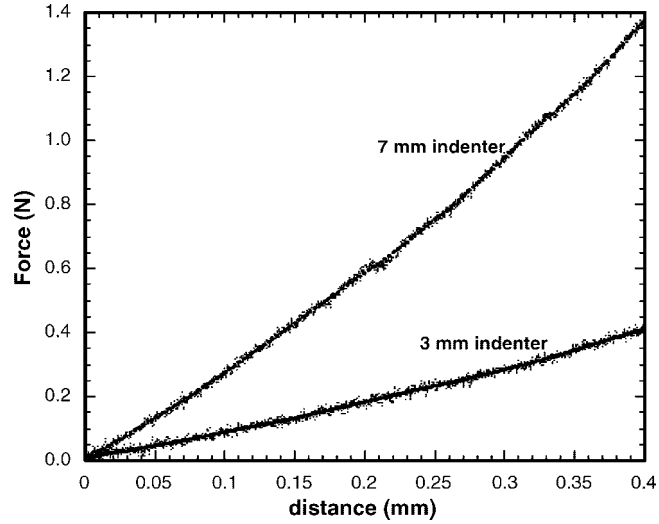

Figure 8. Representative force–indentation distance curves for gels prepared with 7% DOS methacrylated glycol chitosan.

whether the cells would remain at the surface or move into the gel. The results, shown in Figure 11, indicate that the gel was noncytotoxic, as the chondrocytes remained viable over the 7 day time span. Nearly all of the chondrocytes seen in the

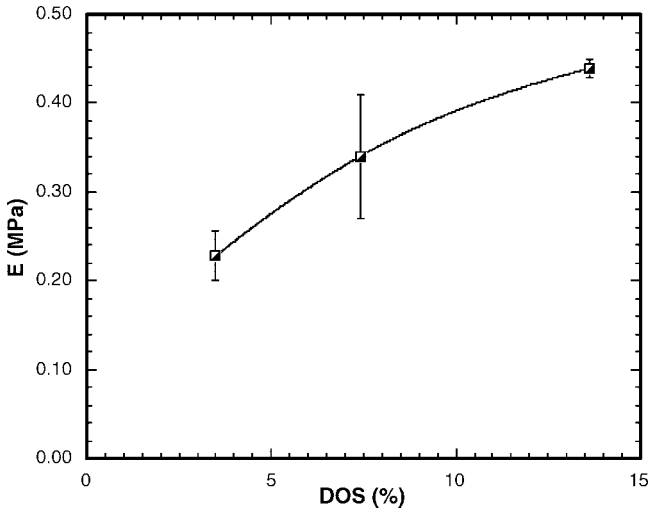

Figure 9. Young's modulus (*E*) versus methacrylated glycol chitosan prepolymer degree of methacrylation (DOS).

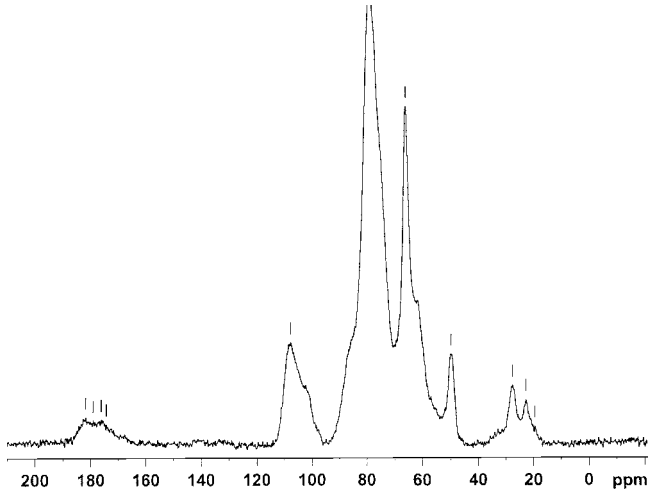

Figure 10. CP-MAS ^{13}C NMR of photocrosslinked gel prepared with 21% DOS.

confocal images were viable and had distributed throughout the chitosan gel, likely by diffusion and gravitational effects. Some cellular attachment was observed; otherwise, the cells would have diffused outward to the base of the gel and laid down upon the tissue culture polystyrene beneath. This migration study proved that chondrocyte cells could be seeded onto the chitosan gel of the bilayer construct and would be sustainable to develop extracellular matrix for the purpose of tissue engineering.

Chitosan is known to be degraded in vitro by lysozyme,[53] which is ubiquitous in vivo. The rate of degradation of chitosan increases as the degree of deacetylation decreases and as the water solubility of chitosan derivatives increases.,[55] However, there have been no reports to date on the in vitro degradation of glycol chitosan via lysozyme. To determine the influence of cross-link density of the photocrosslinked hydrogels on in vitro degradation rate, hydrogels prepared from varying %DOS were incubated in PBS containing 4 mg/mL lysozyme at 37 °C. The results are shown in Figure 12.

All the hydrogels lost mass during the degradation period, with the extent and rate of mass loss increasing as the cross-link density decreased, that is, as % DOS decreased. A lower cross-link density allows for more ready access of the enzyme to the polymer backbone as the molecular weight between cross-links increases, as the active binding site of lysozyme binds 6 sugar rings.[54] Moreover, the penetration of the enzyme into the hydrogel bulk would increase as the cross-link density decreased. Both of these effects would increase the rate of degradation. The degradation rates under these conditions were slow, with the greatest mass loss achieved only approximately 30% after 5 months. In vivo degradation is likely to be very different, as the gels will be attacked by other enzymes[53] as well as oxidative species[54] and will experience mechanical stress. Nevertheless, the results of this in vitro study provide an idea of the expected influence of cross-link density on the degradation rate.

Conclusions

Glycol chitosan can be reacted with glycidyl methacrylate to yield a water-soluble, photopolymerizable prepolymer that can be used to prepare hydrogels of low sol content. The degree of methacrylation can be readily manipulated by adjusting the molar excess of glycidyl methacrylate to glycol chitosan reactive amine ratio and the reaction time. The methacrylated glycol chitosan possesses the same noncytotoxicity as nonmethacrylated glycol chitosan toward an immortalized chondrocyte cell line. Moreover, the gels prepared through photocrosslinking this prepolymer did not possess unreacted carbon–carbon double bonds and supported the growth of immortalized chondrocytes over a period of one week. The hydrogels degraded slowly in the presence of lysozyme at a rate that increased as the cross-

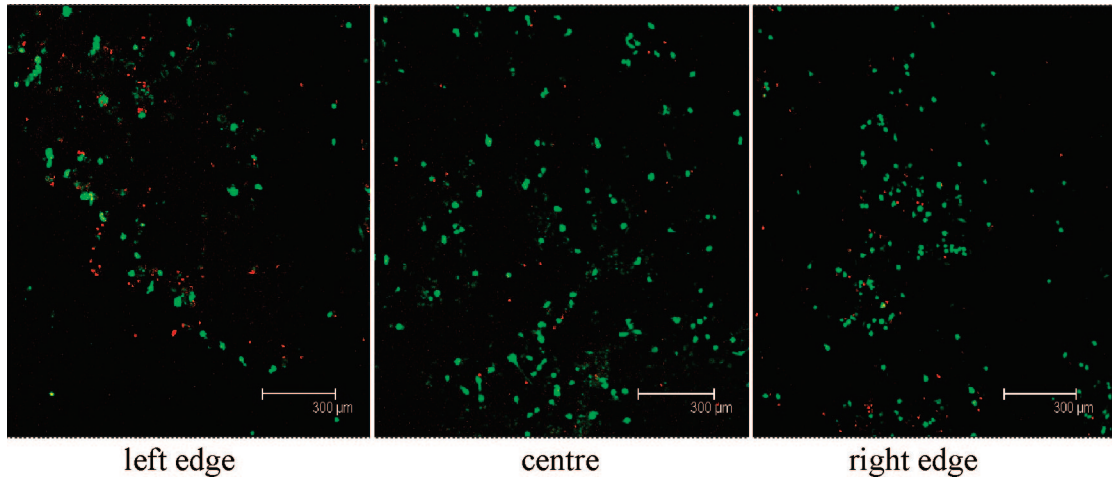

Figure 11. Chondrocyte viability within the *N*-methacrylate glycol chitosan hydrogels at 7 days, as assessed using a LIVE/DEAD assay. Images were taken along the diameter at 2/3 depth from the surface of the gels.

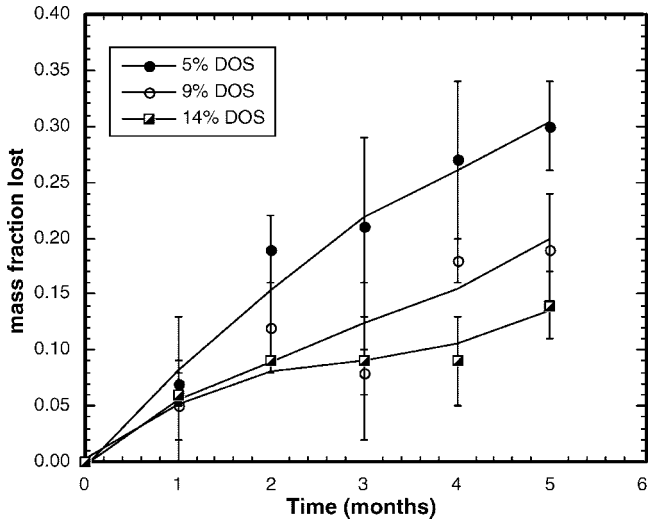

Figure 12. In vitro degradation of hydrogels prepared using different DOS methacrylated glycol chitosan in PBS containing 4 mg/mL lysozyme at 37 °C.

link density of the gels decreased. Methacrylated glycol chitosan thus appears to be a promising biomaterial for use in tissue engineering and drug delivery.

Acknowledgment. Funding for this project was provided through grants from the Natural Sciences and Engineering Research Council of Canada (Collaborative Health Research Projects) and the Advanced Foods and Materials Network.

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BM700691E