Synthesis and Characterization of Photopolymerized Multifunctional Hydrogels: Water-Soluble Poly(Vinyl Alcohol) and Chondroitin Sulfate Macromers for Chondrocyte Encapsulation

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ABSTRACT: To design a carrier for chondrocytes that emulates the critical aspects of native cartilage tissue, hydrogels were formulated from a synthetic component based on poly(vinyl alcohol) (PVA) and from a natural component, negatively charged chondroitin sulfate (ChSA, a main component of proteoglycans). The synthesis of photoreactive and crosslinkable macromers based on PVA and ChSA is described in detail. A range of macroscopic hydrogel properties was obtained by varying the macromer molecular weight, concentration, and functionality prior to photoinitiated polymerization. Depending on the formulation, the PVA homopolymer gels had compressive moduli (K) ranging from 5 to 1680 kPa with equilibrium mass swelling ratios (q) of 2.4 to 15. Similar variations in pure ChSA gels produced networks with K's from 10 to 2600 kPa and q's from 5.9 to 27.5. Copolymer networks containing both ChSA and PVA had increased mechanics and increased swelling as compared to the homopolymer gels. An additional benefit to incorporating ChSA was the creation of enzymatically degradable gels. By use of chondroitinase ABC, the degradation kinetics of various homo- and copolymer networks were investigated. Finally, preliminary histological results indicate that these copolymer gels can support chondrogenesis of photoencapsulated cells.

Introduction

In the tissue engineering field, a significant portion of research focuses on providing cells with an environment that promotes normal cellular functions and ultimately leads to the regeneration of new tissues. Current clinical approaches to treat damage or diseased tissues, however, typically rely on nonviable tissues such as cadaver grafts for ligament replacement or on synthetic devices such as mechanical heart valves. Living tissues are advantageous over nonfunctioning tissues because they are ostensibly integrated into the body more effectively. As a result the body can take over maintenance of the tissue, thereby lessening the need for multiple replacements over time due to wear. Perhaps one of the more debilitating diseases that affects millions of people is osteoarthritis,[1] where the cartilage present between joints deteriorates to such an extent that bone articulates on bone. Unfortunately, the body does not readily regenerate cartilage and repair generally requires "outside" intervention. The ultimate goal of the work presented here is the creation of a polymer matrix that can serve as a chondrocyte carrier, the cell type found in cartilage tissue, and support normal cell function. The focus of this research was on preparation of crosslinked homo- and copolymer hydrogel networks from naturally occurring and synthetically prepared multifunctional precursors as a first step to achieving this goal.

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Hydrogels have gained increasing interest over the years as cell transplantation vehicles for a variety of tissues.[2−9] They are highly swollen insoluble networks that can be used to entrap tissue-specific cells.[10,11] The high equilibrium swelling promotes nutrient diffusion into the gel and cellular waste out of the gel, while the insolubility can provide the structural integrity necessary for tissue regrowth. The hydrogel environment also allows chondrocytes to retain their native, rounded morphology,[2] a vital requirement for successful cartilage tissue engineering.

Another requirement is that the scaffold properties are similar to that of native cartilage, which is composed mainly of collagens and negatively charged proteoglycans.[12] Proteoglycans consist of a protein backbone with highly branched glycosaminoglycan side chains, made up primarily of chondroitin sulfate.[13] Together the collagen fibrils and the charged proteoglycans serve to give the tissue its tensile strength, load-bearing capabilities, and resilience. Taking these properties into consideration, we designed hydrogel scaffolds composed of a neutral synthetic component, poly(vinyl alcohol) (PVA), and incorporated a negatively charged biological polymer, chondroitin sulfate (ChSA). PVA was chosen because it is water-soluble, biocompatible,[14] and has an abundance of modifiable, pendant hydroxy groups. The ChSA component served two purposes: to emulate the native biochemical environment of cartilage and to introduce charge into the network, which can increase the water content and the stiffness of the gel. An additional advantage is that the presence of ChSA can help promote cartilage tissue formation through increased cell proliferation and stimulated proteoglycan secretion.[15,16]

To create the hydrogel scaffolds, the hydroxy groups present on both PVA and ChSA were modified with

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methacrylate groups, where the extent of modification was controlled via the reaction conditions employed. These multivinyl macromers were then photopolymerized to generate highly swollen crosslinked networks. The photopolymerization process is particularly advantageous for preparing a cell carrier system because the reaction conditions are mild: gelation occurs at physiological temperature and pH with temporal and spatial control. In addition, the chain polymerization mechanism allows for facile incorporation of a variety of components into any given network.[17]

Early reports on the design of photopolymerizable macromers[18−21] focused on the use of divinyl molecules for hydrogel formation. Using multivinyl macromers offers an additional route to tailor the network properties, especially with respect to the gel mass loss profiles that are not found in conventional divinyl systems. Not only can the overall formulation be adjusted to achieve the desired outcome, but the functionality as well.[22−24] Multifunctional macromers can be homopolymerized,[22,23,25,26] copolymerized with other multifunctional reagents (vide infra), or reacted with a difunctional reagent[24,27] or even with a monovinyl moiety to produce pendant molecules for a defined function, for example, to control cell interactions.[17,28] The plethora of possibilities offers an almost limitless number of options for creating an ideal network for a given application.

In this work, we strive to demonstrate that PVA and ChSA can easily be modified with methacrylate groups and then photopolymerized to produce crosslinked hydrogels with a variety of properties. The PVA and ChSA homopolymer gels were characterized with respect to their water uptake and compressive moduli as a function of the gel formulation. Copolymerization of the two macromers was also undertaken to investigate the effects on the above properties. The copolymerization also produces a unique scaffold for chondrocyte encapsulation; the integrity of the gel is maintained over time via incorporation of the PVA, but the cells are able to remodel their surroundings through degradation of the ChSA portions. Since previous work has shown that degradation is beneficial to new cartilage formation,[5] chondrocytes were encapsulated in the PVA/ChSA copolymer gels, which were then analyzed histologically to examine chondrocyte morphology and matrix production at early time points.

Materials and Methods

Materials. Poly(vinyl alcohol) (PVA) hydrolyzed to 98% with molecular weights of 13−23K according to the manufacturer (Aldrich) and 78K according to the manufacturer (Polysciences) were used without further purification. 2-Isocyanatoethyl methacrylate (Aldrich, 98%), methacrylic anhydride (Aldrich, 94%), and 2-hydroxy-1-[4-(hydroxyethyl)phenyl]-2-methyl-1-propane (I2959, Ciba Specialty Chemicals) were used as received. Chondroitin sulfate A (ChSA) was purchased from Sigma and used without further purification. Chondroitinase ABC (protease free) was purchased from Seikagaku America as a lyophilized powder and dissolved in phosphate-buffered saline (PBS, pH 7.4) prior to use. Solvents were used as received.

Gel-permeation chromatography was used to determine $\bar{M}_n$ and the polydispersity index of the starting polymers. The GPC was equipped with a Waters 515 pump, a Waters U6K manual injector, polymer standards serviced supreme columns (linear, 30 Å, 100 Å, and 1000 Å), and a Waters 2410 refractive index detector. Samples were run in an aqueous buffer (0.1 $NaNO_3$) at 1 mL/min and 35 °C. PVA was compared against linear PEG standards and ChSA was compared against dextran standards, both with ethylene glycol as an internal standard.

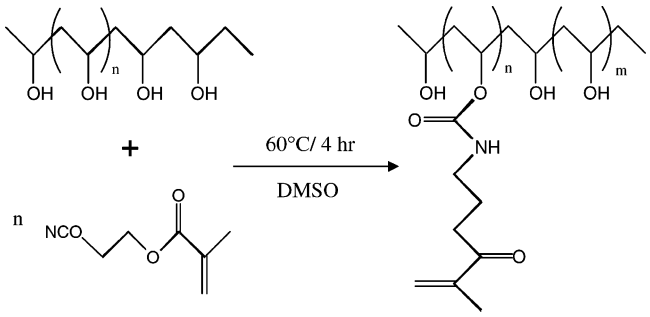

Figure 1. Synthesis of poly(vinyl alcohol) macromer.

Synthesis of the Poly(Vinyl Alcohol) Macromer. PVA (5 g) was dissolved in 25 mL of distilled dimethyl sulfoxide (DMSO) at 60 °C at a concentration of 20% (w/v). The solution was purged with nitrogen for 30 min. 2-Isocyanatoethyl methacrylate (2-ICEMA) (e.g., 160 μL) was added dropwise to the PVA solution in stoichiometric amounts to obtain a range of methacrylate substitutions (e.g., 1 mol %). A very small amount of 2,6-di-*tert*-butyl-1,4-methylphenol was added to the reaction to inhibit the methacrylates from polymerizing during the macromer synthesis. The reaction was maintained at 60 °C for 4 h, after which time the product was precipitated into toluene. The reaction scheme is detailed in Figure 1. For the cell encapsulation experiments, a previously developed synthesis for the methacrylation of PVA, which is easily purified for cytocompatibility, was used.[22,29] The degree of substitution was <1 mol % as determined by ^{1}H NMR (details given below).

The degree of substitution of methacrylate side groups to the PVA macromer was determined via ^{1}H NMR analysis (Varian VXR-300S and Varian Inova-500) with D_2O as the solvent. The area under the integrals for the vinyl resonances (δ = 5.7, δ = 6.1, d) was compared to that for the PVA backbone protons (-CH_2- and -CH-) at δ = 1.4−1.8 and 3.8−4.1 ppm, respectively, to calculate the molar percent of substitution.

Synthesis of the Chondroitin Sulfate Macromer. ChSA (5 g) was dissolved in 20 mL of deionized water (dH_2O) at a concentration of 25% (w/v). The solution was heated to 60 °C and the pH was adjusted to ∼10 with aqueous sodium hydroxide. Methacrylic anhydride was added dropwise to the ChSA solution, and the mixture was stirred vigorously for 24 h at 60 °C (see Figure 2). The pH was maintained at ∼10 throughout the reaction. The reaction was monitored at 24 and 48 h by ^{1}H NMR (details given below), which indicated that no further substitution occurred with longer reaction times and suggested that the high pH did not result in hydrolysis of the bound methacrylate groups. Varying amounts of methacrylic anhydride were used to obtain a range of methacrylate substitutions ranging from <1 to 25 mol % and are given in Table 1. The methacrylated ChSA macromer was precipitated into cold methanol. Prior to cell encapsulation, the ChSA macromer was further purified by dialysis followed by lyophilization to produce the solid product.

The degree of substitution of methacrylate side groups to the ChSA macromer was determined via ^{1}H NMR analysis with D_2O as the solvent. The area under the integral for the vinyl resonances was compared to that for the acetyl group [^{1}H NMR (D_2O): δ = 1.6−1.8 (two overlapping s, from acetyl methyl group and methyl group on vinyl bond), 3−4.8 (br m, ChSA sugar backbone), 5.5−6 (br d, protons on vinyl bond)].

Hydrogel Preparation. Macromers (0.1 g) were dissolved in dH_2O at a concentration of 5−30% (w/w). The UV photoinitiator used in this study, I2959 (stock solution of 0.006 mg/mL dH_2O), was added to the macromer solution at a concentration of 0.05% (w/w) [e.g., 83 μL of initiator stock solution was added to a solution containing 0.1 g of macromer and 917 mL of dH_2O to give a final macromer concentration of 10% (w/w)]. To minimize oxygen inhibition during polymerization, the solution was poured in a Teflon mold (1 mm thick) with a

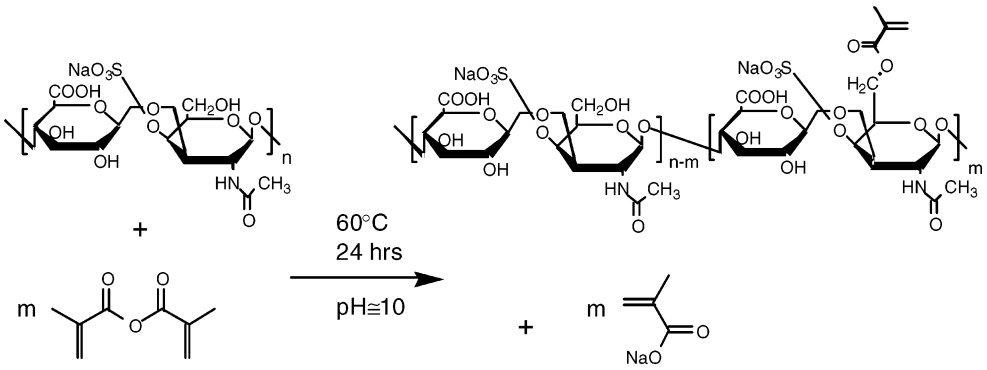

Figure 2. Synthesis of chondroitin sulfate (ChSA) macromer.

Table 1. ChSA Reactants and Products

volume of methacrylic anhydride[b] (mL)	degree of methacrylate substitution[a] (mol %)	
	theoretical[c]	experimental
20	> 100	25
5	96	8
2	38	< 1

[a] Estimated by ^{1}H NMR. [b] Twenty milliliters of 25% (w/w) ChSA was added to each reaction. [c] Based on substitution at the primary or secondary alcohols.

punched hole (5−8 mm in diameter) that was placed between two glass slides. The solution was exposed to 365 nm light at an intensity of ~10 mW/cm^2 for 10 min. The same conditions were employed for cell encapsulation experiments, as cytocompatiblity was previously demonstrated.[29]

Material Characterization: Polymerization. The polymerization of the ChSA macromer was monitored by magic-angle spinning ^{1}H NMR to measure the conversion of the double bonds as a function of exposure time. Briefly, a 10% (w/w) solution of 25% methacrylated ChSA was prepared in D_2O with 1% (w/w) I2959 as the photoinitiator. The solution was placed into a 4 mm ghx-Nano probe with a 40 μL capacity and exposed to a Black Ray UV light source (λ = 365 nm, I_0 = 10 mW/cm^2), and the optimized proton spectra were recorded on a Varion Inova at 400 mHz after each increase in exposure time. The samples were spun at 3000 Hz to minimize the line broadening and increase the accuracy of the measurements. Conversion was determined on the basis of the ratio of the area under the vinyl resonances to that for the acetyl group.

Hydrogel Swelling and Mechanics. Hydrogel disks (5 mm diameter, 1 mm thick) prepared from the PVA and ChSA macromers were swelled in PBS at 37 °C for at least 48 h to reach equilibrium. After equilibrium swelling was reached, the disks were weighed and then lyophilized for 24 h. After complete drying, the dry polymer mass was measured. The equilibrium mass swelling ratio, *q*, was calculated from the ratio of the equilibrium swollen mass to the polymer dry mass. A dynamic mechanical analyzer (DMA-7, Perkin-Elmer) was used to measure the compressive modulus of elasticity of the swollen gels in unconfined compression at a rate of 10−300 mN/min at room temperature. A sample size of 3−5 was used.

Enzymatic Degradation. Hydrogel disks (8 mm diameter, 1 mm thick) were prepared from 10% (w/w) solutions composed of PVA alone, a 50/50 (w/w) mixture of PVA and ChSA, and ChSA alone, with 0.05% (w/w) I2959 as the photoinitiator (% methacrylation: PVA = 5, ChSA = 15). The disks were preweighed, placed in 4 mL glass vials, exposed to chondroitinase ABC (2 mL of 6.6 × 10^{-3} unit mL^{-1}), and then kept in an orbital shaker incubator thermostated at 37 °C. At timed intervals, samples were removed in triplicate, and both wet and dry weights were recorded to determine swelling and mass loss as a function of degradation time. The enzyme solutions were replaced daily to maintain maximum activity.

Chondrocyte Studies: Cell Encapsulation. Chondrocytes were isolated from the femoral−patellar groove of a young calf from Research 87 (Marlboro, MA) as described elsewhere.[30] The PVA−MA macromer (0.1 g) was dissolved in dH_2O to a final concentration of 20% (w/w) and then filter-sterilized by use of a 0.22 μm filter. The ChSA−MA macromer (0.1 g) was dissolved in deionized water at a concentration of 5% (w/w), filter-sterilized by use of a 0.22 μm filter, lyophilized for 2 days, and then dissolved in filter-sterilized (0.22 μm filter) dH_2O to a final concentration of 20% (w/w). At this macromer concentration, dH_2O was found to maintain the highest level of cell viability postencapsulation (unpublished observations). Hydrogels were formulated with 20% (w/w) ChSA−MA (<1 mol % MA) to produce a pure chondroitin sulfate gel and with 10% (w/w) ChSA−MA (<1 mol % MA) and 10% (w/w) PVA−MA (<1 mol % MA) to make a copolymer gel. Since the low crosslinking density produces highly swollen gels, an adhesive peptide sequence was incorporated into the gel at low concentrations to encourage retention of the chondrocytes within the gels. By use of a previously described procedure, RGD was incorporated into the network by conjugating it to a monoacrylated poly(ethylene glycol) chain[31] (MW = 3400) and adding it to the macromer solution at a concentration of 2.3 mM prior to polymerization. The isolated chondrocytes were mixed with the macromer/initiator/RGD solution at a concentration of 75 million cells/mL of macromer solution. The cell−macromer solution was placed in a disk mold (5 mm diameter, 2 mm thck) and exposed to 365 nm light at an intensity of ~10 mW/cm^2 for 10 min with 0.05% (w/w) I2959 as the photoinitiator. The cell−hydrogel constructs were cultured in vitro on an orbital shaker in an incubator thermostated at 37 °C for 3 days in Dulbecco's modified Eagle medium (Gibco) supplemented with 1% penicillin−streptomycin (Gibco), 0.5 μg/mL fungizone (Gibco), 0.01 M MEM nonessential amino acids (Gibco), 10 mM Hepes, 0.04 mM L-proline, and 10% fetal bovine serum.

Histological Analysis. After 3 days, two cell−hydrogel constructs of pure ChSA and PVA/ChSA were fixed in 10% formalin overnight and embedded in paraffin. Following standard histological procedures, sections 8 μm thick were stained with hematoxylin and safranin O, which stain nuclei black and negatively charged proteoglycans red, respectively.

Statistical Analysis. Statistical analysis was performed by use of an unpaired Student's *t*-test with a confidence level of 0.05. All values in this paper are reported as the mean with a standard deviation.

Results and Discussion

Macromonomer Preparation. Upon addition of a vinyl moiety to a molecule, polymer formation through chain polymerization becomes a possibility. When multiple vinyl groups are added per molecule, crosslinked network formation is possible. The latter is our focus, with particular emphasis on hydrogels formed from high molecular weight, water-soluble multivinyl macromolecules. In the presence of a photoinitiator and a light source, the vinyl groups react to generate an insoluble, but water-swellable, crosslinked network via a photopolymerization process. The crosslinker core, or pendant moiety, is unaffected by the polymerization pro-

cess, which allows for incorporation of various chemistries into it, like hydrolytically degradable units,[18] protease-sensitive sequences,[32,33] naturally occurring molecules,[25,34−36] and cell adhesive oligopeptide sequences.[17,31,37−41] Furthermore, the final network structure and chemistry can readily be altered by changing the initial macromolecule functionality and/or through copolymerization of multiple molecules to impart the desired properties. We chose this general strategy to synthesize hydrogels with properties that are appropriate for a chondrocyte carrier and support cartilage tissue evolution.

The PVA and ChSA macromers were prepared by reaction of the polymer hydroxyl groups with methacrylate moieties. Two routes were utilized. For the PVA macromer synthesis, 2-isocyanatoethyl methacrylate (2-ICEMA) was reacted in varying amounts with the PVA in anhydrous DMSO at 60 °C (see Figure 1). This reaction condition afforded a homogeneous reaction under relatively mild conditions, something that other functionalization routes[18,22,42] do not necessarily offer. The percent substitution ranged from 1 to 4 mol % as determined by ^{1}H NMR and was dependent on the concentration of 2-ICEMA added. The incorporation efficiency of 2-ICEMA into the PVA was determined to be 80−100%.

Glycidyl methacrylate has been used to modify ChSA with methacrylate side groups; however, this reaction requires extended reaction times of 15 days to reach 8% substitution.[35] Here we describe an alternative reaction scheme that enables well-controlled and high degrees of substitutions within 24 h. Since the solubility of highly charged ChSA is limited in organic solvents, the methacrylation scheme was carried out in an aqueous environment with methacrylic anhydride[34] (MAh) to impart the desired functionality. Figure 2 details the synthetic scheme. To compensate for the tendency of MAh to react with water to form methacrylic acid, an excess of MAh was used in all cases; the volume of the reactants and the resulting degrees of methacrylation are given in Table 1. In all cases, the actual degree of methacrylation was ≤10% of the expected substitution based on the total volume of MAh added, similar to what other researchers have found.[43] Nonetheless, the degree of methacrylation was readily controllable and reproducible (as shown for the macromer with 25% substitution).

A representative ^{1}H NMR spectrum for the ChSA macromer is given in Figure 3. Within each repeat unit on the ChSA, there exist three hydroxyl groups that can potentially be modified. In a heteronuclear shift correlation through multiple bond connectivities (HMBC) experiment, which analyzes the relationship between the protons and carbons in the structure, there did not appear to be a preferred substitution site. The methacrylate groups were randomly distributed among the hydroxy groups contained in the sugar repeat units.

The macromers, multivinyl PVA−MA and ChSA−MA, were then photopolymerized to create hydrogel networks with tunable properties, achieved through systematic alteration of the overall macromer formulation.

Photopolymerizations. In highly crosslinked networks, double-bond conversion may be limited by several factors, including inaccessibility to the reactive groups and the increasing viscosity associated with increasing conversion over time. Incomplete conversion

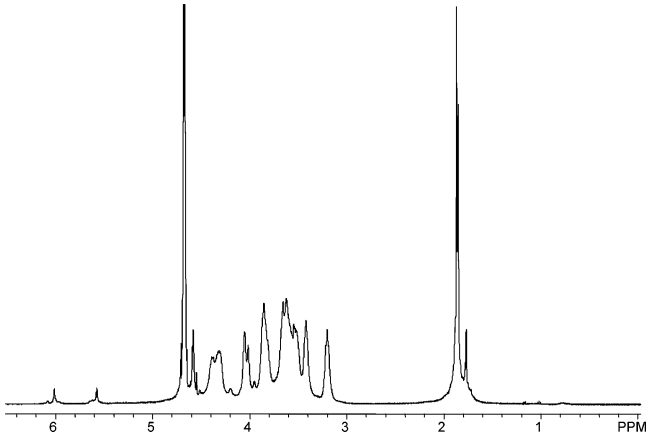

Figure 3. ^{1}H NMR of the chondroitin sulfate (ChSA) macromer. The success of methacrylate substitution is verified by the presence of the vinyl resonances at 5.7 and 6.1 ppm.

may affect the overall mechanical properties, the biocompatibility, and even the degradation and mass loss rates associated with a given network. While one might argue that the solution polymerization of high molecular weight, multivinyl monomers should provide for increased mobility and high conversions, these systems have not been well characterized. For these reasons, we chose to investigate the kinetics of the ChSA−MA photopolymerization to confirm that we achieved high conversions during our reactions.

To probe the kinetics of the reaction, near-infrared spectroscopy is often employed in our lab to monitor the conversion of double bonds in aqueous solution;[22] close to 100% conversion is reached in the photopolymerization of multivinyl PVA molecules, even at high macromer concentrations [20−50% (w/w)]. Unfortunately, application of this technique was not successful for monitoring the ChSA photopolymerization, due to the complex nature of the ChSA structure, as well as to the dilute environment [90% (w/w) water]. As an alternative method, a magic-angle spinning (MAS) ^{1}H NMR experiment was carried out. MAS is advantageous because the high rate of spin and the angle at which the sample is maintained both serve to decrease the line broadening that naturally occurs in highly restrained systems and significantly enhance the resolution, providing more accurate integral values. A 10% (w/w) solution of ChSA−MA (25% MA) was exposed to ∼10 mW/ cm^2 of 365 nm UV light for increasing amounts of time. Periodically, the ^{1}H NMR spectrum was recorded to determine the total double-bond conversion as a function of time. While the polymerization was not monitored continuously, a typical multifunctional macromer polymerization profile was observed, with regions of autoacceleration and autodeceleration present. Figure 4 shows that after 10 s of exposure, ∼60% of the total double bonds were reacted, and by 700 s, ∼100% conversion was achieved (i.e., no vinyl resonances were detected). This information suggests that in this system, double-bond conversion is complete or nearly complete and should not affect the overall properties of our networks. Moreover, relatively mild reaction conditions lead to network formation on a clinically acceptable time scale.

Network Physical Properties. In designing hydrogels for tissue engineering cartilage, two properties that are especially important are the volumetric swelling ratio (Q), which is a measure of the amount of water the gel imbibes, and the compressive modulus (K).

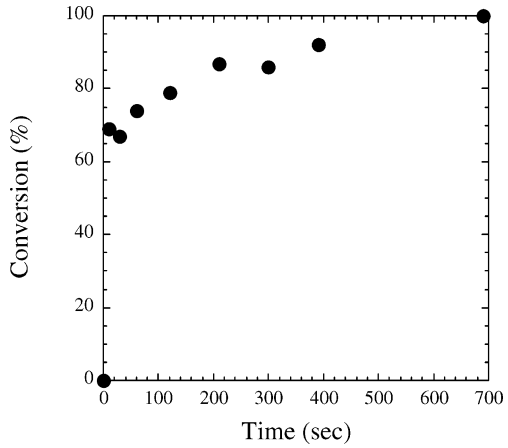

Figure 4. Plot of conversion (percent) vs exposure time (seconds) for the photopolymerization of ChSA [10% (w/w), 25% MA] containing 1% I2959 exposed to 10 mW/cm^2 of 365 nm light as monitored by solid-state MAS NMR in D_2O.

Table 2. Macroscopic Gel Properties of PVA Hydrogels

expt	% PVA−MA[a]	$\bar{M}_n$	PDI[b]	degree of methacrylate substitution[c] (mol %)	q[d]	K[e] (kPa)
1	5	11 800	2.07	4	15.0 ± 0.8	5 ± 1
2	10	11 800	2.07	4	7.2 ± 0.3	37 ± 2
3	20	11 800	2.07	4	3.2 ± 0.1	680 ± 60
4	30	11 800	2.07	4	2.4 ± 0.0	1680 ± 70
5	20	11 800	2.07	1	7.1 ± 0.3	45 ± 1.5
6	20	60 900	2.93	1	7.7 ± 0.06	105 ± 4

[a] Initial percentage (w/w) of PVA−MA present in aqueous solution prior to polymerization. [b] Polydispersity index. [c] Estimated by ^{1}H NMR. [d] Equilibrium mass swelling ratio (q) = equilibrium swollen mass/polymer dry mass. [e] Compressive modulus; experimental values are given as mean ± SD.

Generally in uncharged and highly swollen gels, a more highly crosslinked network swells less, that is, has smaller Q values ($Q \sim n_{xl}^{-3/5}$, where n_{xl} = number of effective crosslinks[44]) but has a higher compressive modulus, that is, larger K values ($K \sim n_{xl}^{6/5}$).[44] To be useful as a cartilage tissue engineering matrix, there must be a balance within the network between the Q values, which should be high to maintain cell viability and nutrient transfer, and the gel stiffness, which should also be high to provide mechanical integrity, particularly for a load-bearing application. We attempted to solve this paradox by first manipulating the properties of the crosslinked homopolymers PVA−MA and ChSA−MA networks to achieve the desired hydrogel characteristics; then we investigated combining the two macromers into copolymer gels as a way to further tailor the network properties.

A range of network crosslinking densities can be obtained from synthetic macromers by varying several different parameters, including the macromer concentration prior to polymerization, the percent chain functionality (i.e., the degree of methacrylation) and the overall macromer molecular weight. Two PVA macromers were used in this study, one with a low molecular weight, $\bar{M}_n$, of 11 800 g/mol and a broad distribution with a polydispersity index of 2.1, and one with a higher molecular weight, $\bar{M}_n$, of 60 900 and a broader distribution with a polydispersity of 2.9. The functionality was varied from 1 to 5 mol % (vide supra). The properties of the resulting rubbery PVA gels are summarized in Table 2. We chose to measure q, the mass equilibrium swelling ratio, which is the difference between the wet and dry polymer weights, because of its simplicity and accuracy. When the densities of the polymer and solvent approach 1 and ideal mixing is assumed, q provides a useful approximation of Q. For a given $\bar{M}_n$ (11 800 g/mol) and functionality (5% MA), increasing the macromer concentration from 5% to 30% (w/w) prior to the photopolymerization resulted in a decrease in the q value from 15 to 2.4, an 84% decrease in the water content, but the K value increased approximately 340 times, from 5 to 1680 kPa (Table 2, experiments 1 and 4). Increasing the degree of methacrylation on the low $\bar{M}_n$ PVA−MA macromer from 1% to 5% resulted in a 55% decrease in q, from 7.1 to 3.2, but generated a 15-fold increase in the compressive modulus, from 45 to 680 kPa (Table 2, experiments 5 and 3). For a given macromer concentration [20% (w/w)], increasing $\bar{M}_n$ from 11 800 to 60 900 g/mol results in a 3.3-fold decrease in double-bond concentration. Consequently, q increased slightly from 7.1 to 7.7, reflecting the decrease in crosslinking density with increased $\bar{M}_n$, but interestingly, the compressive modulus also increased (Table 2, experiments 5 and 6). This effect is likely due to increased chain entanglements in the polymer solution prior to polymerization (a phenomenon that is enhanced as $\bar{M}_n$ increases), which can act as physical crosslinks, leading to increased K values. It is nonidealities such as these that cause deviation from the scaling laws discussed above. Additionally, in hydrogels derived from the solution polymerization of high molecular weight macromers, where the concentration of double bonds is quite low, imperfections in the network structure (e.g., cycles) may also alter the straightforward relationship between the crosslinking density and the physical properties of the networks that the scaling laws predict.[22] Nevertheless, the expected trends generally held: as the crosslinking density increased, the swelling decreased and the compressive modulus increased. These experiments also demonstrate that a range of gel properties can be achieved through simple manipulations of the PVA−MA macromer chemistry and/or photopolymerization conditions.

Table 3. Macroscopic Gel Properties of Chondroitin Sulfate[a] Hydrogels

expt	% CS−MA[b]	degree of methacrylate substitution[c] (mol %)	q[d]	K[e] (kPa)
1	5	25	30.0 ± 0.8	16 ± 2
2	10	25	17.1 ± 1.2	160 ± 40
3	20	25	9.0 ± 1.0	900 ± 30
4	30	25	5.9 ± 0.4	2600 ± 400
5	10	8	21.6 ± 1.1	54 ± 5
6	20	8	9.5 ± 0.4	630 ± 40
7	30	8	9.1 ± 0.9	1200 ± 76
8	30	<1	27.5 ± 5.3	10 ± 3

[a] $\bar{M}_n$ and PDI of the starting material are 48 700 Da and 1.49, respectively. [b] Initial percentage (w/w) of CS−MA present in aqueous solution prior to polymerization. [c] Estimated by NMR. [d] Equilibrium mass swelling ratio (q) = equilibrium swollen mass/polymer dry mass. [e] Compressive modulus; experimental values are given as mean ± SD.

Similar experiments were undertaken to investigate the properties of the hydrogels formed from the ChSA−MA macromers. The $\bar{M}_n$ of the ChSA starting material was 48 700 g/mol with a polydispersity index of 1.49. The final macroscopic gel properties are summarized in Table 3. In these charged gels, q ranged from 5.9 to 30 and K ranged from 16 to 2600 kPa when the percent methacrylation was kept constant at 25% (Table 3, experiments 1−4). As found for the PVA−MA gels,

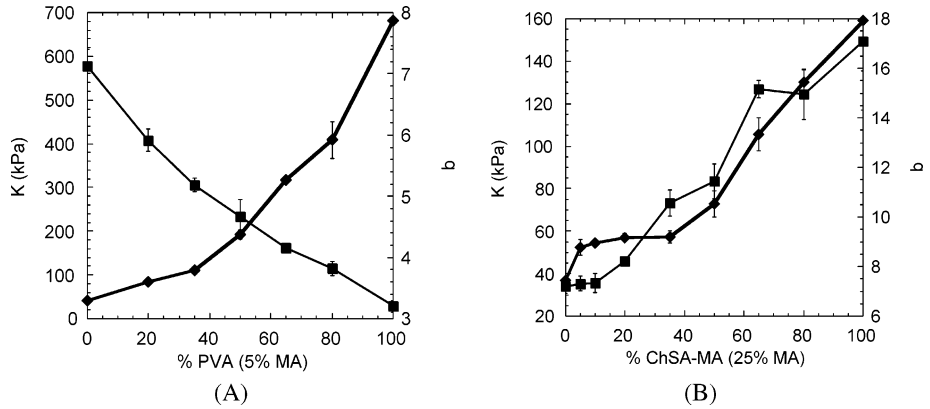

Figure 5. Plots of the equilibrium swelling ratio (q, ■) and compressive modulus (K, ◆) as a function of hydrogel composition for (A) PVA copolymer hydrogels with PVA−MA (1% MA) and PVA−MA (5% MA) macromers [total macromer concentration = 10% (w/w)] and (B) PVA/ChSA copolymer hydrogels with PVA−MA (5% MA) and ChSA−MA (25% MA) macromers [total macromer concentration = 20% (w/w)].

increasing the macromer concentration or percent methacrylation increased the gel crosslinking density, which decreased the overall water content but increased the compressive modulus (Table 3, experiments 3 and 4 or 5 and 6, respectively). In comparing the gels formed from the two different macromers, it becomes obvious that the chemistry plays a significant role in the final properties. For example, a gel formulated from PVA−MA with $K = 680$ kPa, similar to that of native cartilage (where $K = 500-1000$[45]), had a q of 3.2 (Table 2, experiment 3). In contrast, a gel formulated from ChSA−MA with a similar K (630 kPa) had a q of 9.5 (Table 3, experiment 6); that is, the ChSA−MA gel took up 6 times more water than the PVA−MA gel, making it more like the native tissue where $q \sim 5$.[45] This is particularly noteworthy when one considers that both macromers contained approximately the same percentage of MA and the gels were both formulated from 20% (w/w) solutions. These differences may be ascribed to the negatively charged sulfate groups within each repeat unit on the parent ChSA molecule, which can cause an increase in the water content and the gel compressive modulus due to electrostatic repulsive forces at physiological pH.[46] On the basis of these findings, we chose to investigate copolymerizations as a way to further tailor the properties of our networks.

Copolymer Gels. Control over the gel properties is key to developing cellular scaffolds that exhibit properties closest to those of native tissues. While gels prepared from single macromers are often utilized, further manipulation of the gel properties can be accomplished through the simple mixing of two different macromers in solution prior to polymerization. For example, PVA−MA macromers with 1% and 5% substitution were copolymerized in varying amounts, with the resulting K and q values illustrated in Figure 5A. As the content of 5% PVA−MA in the feed increased, q decreased while K increased, which corresponds to the increasing crosslinking density. While not a focus of this paper, this trend also reflects the differences in the network connectivity as a function of the double-bond concentration, which, in degradable networks, can result in substantial changes in the dynamic swelling and mass loss profiles.[47]

In an effort to develop a scaffold that emulates native cartilage and has properties that approach those of the tissue, gels were also formulated by copolymerizing PVA−MA (11 800 $\bar{M}_n$, 5% MA) and ChSA−MA (25% MA) macromers to examine the influence of introducing negative charge into the networks. The influence of varying the ratio of ChSA−MA to PVA−MA on the gel properties is shown in Figure 5B [a 10% (w/w) macromer solution was used in all the experiments]. Interestingly, as the ChSA−MA content in the gel increased, both q and K increased, a behavior that is generally not observed in neutral hydrogel systems. It appears that the presence of the charged ChSA−MA alters the PVA−MA gel properties such that an increase in the compressive modulus is obtained without sacrificing the water content, something that it is not possible to achieve with the homopolymer PVA−MA hydrogels. This exciting result led to further investigation of these gels, with particular emphasis on the degradability and biocompatibilty of the copolymer networks.

Degradation Profiles and Cell Encapsulation Studies. In native cartilage, the tissue is continually remodeled by the cells through secretion of proteoglycans. It has been suggested that the enzyme chondroitinase plays a role in this process in both healthy and injured joints,[48] so to assess whether the modified ChSA−MA gels were susceptible to cellular remodeling, crosslinked gels were placed in a solution containing the enzyme chondroitinase ABC and the mass loss was measured as a function of degradation time. While cells will secrete low levels of chondroitinase, here we exongenously delivered a relatively high concentration of the enzyme to test the degradability of the crosslinked gels. Homopolymer ChSA−MA (25% MA) gels and 50/50 (w/w) copolymer gels of ChSA−MA (25% MA) and PVA−MA (5% MA) were evaluated; their mass loss profiles are shown in Figure 6.

In the presence of chondroitinase ABC (6.6×10^{-3} unit/mL), the homopolymer ChSA−MA gels degraded completely in 20 h (Figure 6, ●) while the copolymer gel reached an equilibrium percent mass loss in ~10 h (Figure 6, ■). Control gels kept in the absence of the enzyme did not degrade (data not shown). Only partial degradation was observed in the copolymer gels, as expected on the basis of the presence of nondegradable PVA, yet the overall mass loss was only ~35%. This result is lower than predicted from the initial macromer composition; however, the copolymer networks are actually composed of 48% ChSA, 42% PVA, and 10% kinetic chains, as calculated from the average molecular weights of the macromers. We expect the nondegradable PVA and kinetic chains, which are attached to both the ChSA

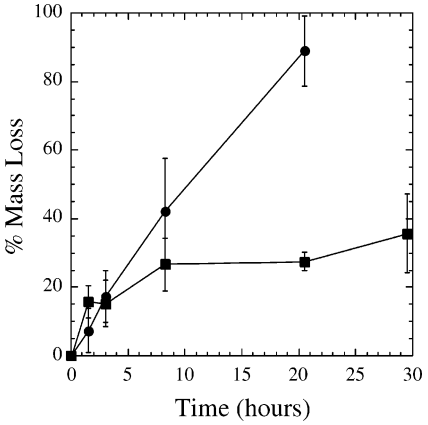

Figure 6. Plot of percent mass loss vs time for the enzymatic degradation of gels composed of ChSA (25% MA, ●) and ChSA/PVA [50% ChSA (25% MA)/50% PVA (5% MA), ■] by chondroitinase ABC.

and the PVA chains, to remain in the network after enzymatic treatment, accounting for approximately 52% of the mass. Furthermore, the ChSA located near the crosslinks may not be accessible to the enzyme as a result of steric hindrance, preventing complete degradation of the ChSA. Our results suggest that ~18% of the ChSA remains within the gel. Regardless, these results demonstrate that modification and subsequent photopolymerization of the parent ChSA molecule does not adversely affect the susceptibility of the ChSA to enzymatic degradation, a characteristic that is highly desirable for tissue engineering applications. Furthermore, it is possible to incorporate hydrolytically degradable segments into the PVA crosslink to render a completely degradable hydrogel.[47]

The ability of these hydrogels to function as a scaffold for cartilage tissue formation was investigated by photoencapsulating chondrocytes in a copolymer gel and examining their morphology and matrix production at early time points. In native cartilage, chondrocytes are surrounded by an extracellular matrix that consists of both negatively charged proteoglycans, like ChSA, and collagen fibrils, which the chondrocytes adhere to through the cellular adhesive oligopeptide sequence RGD.[49] Since the chosen hydrogels (100% ChSA−MA and 50/50 ChSA−MA/PVA−MA) are loosely crosslinked and highly swollen ($q \sim 21$ for the pure ChSA−MA gels), an acrylate-functionalized RGD sequence[31] was also incorporated into the gels not only to serve as an anchor point for the chondrocytes but also to allow the cells to function normally.[49] The gels were cultured for 3 days and examined histologically by staining for proteoglycans.

Histological micrographs of chondrocytes photoencapsulated in a ChSA gel and a copolymer gel are shown in Figure 7, panels A and B, respectively. Since the safranin O stains newly synthesized proteoglycans as well as the ChSA in the gel, no quantitative results can be obtained with respect to extracellular matrix production; however, the chondrocytes appeared healthy and rounded and formed their characteristic lacunae. Additionally, staining was observed within the lacunae, suggesting that the chondrocytes are indeed producing proteoglycans since no macromer-derived ChSA would be present in that region of the cell. Studies are underway to examine chondrocyte metabolism in these gels over longer culture times and to tune the gel chemistry for optimal cartilage growth.

Conclusions

Hydrogel matrices that were designed to mimic the native environment of cartilage were created for tissue engineering applications. Specifically, photopolymerized gels containing a synthetic PVA component as well as a naturally occurring glycosaminoglycan, ChSA, were prepared and the ability of the gels to support normal chondrocyte function was investigated. After modification with photopolymerizable vinyl moieties, the physical properties of the crosslinked gels were easily manipulated through changes in the percent methacrylation and the percent macromer in solution prior to polymerization and via copolymerization of the two macromers. The gels formulated from the PVA−MA macromer, the ChSA−MA macromer, or the comacromers had compressive moduli that ranged over 3 orders of magnitude from 5 to 2600 kPa. Incorporating the negatively charged ChSA into the neutral PVA gels altered the properties such that the gels had high equilibrium swelling values, as well as high compressive moduli. Furthermore, the addition of ChSA produced gels that were degradable in the presence of chondroitinase ABC.

This study demonstrates the flexibility that photopolymerizations offer for generating cell carriers from a range of macromer chemistries and provides a route to allow the same scaffold to serve multiple functions, for example, as a mechanical support as well as a cellularly recognizable environment. The results also show that the macroscopic properties of the gels can be manipulated by simple variations to the scaffold composition without needing to develop new chemistries. Finally,

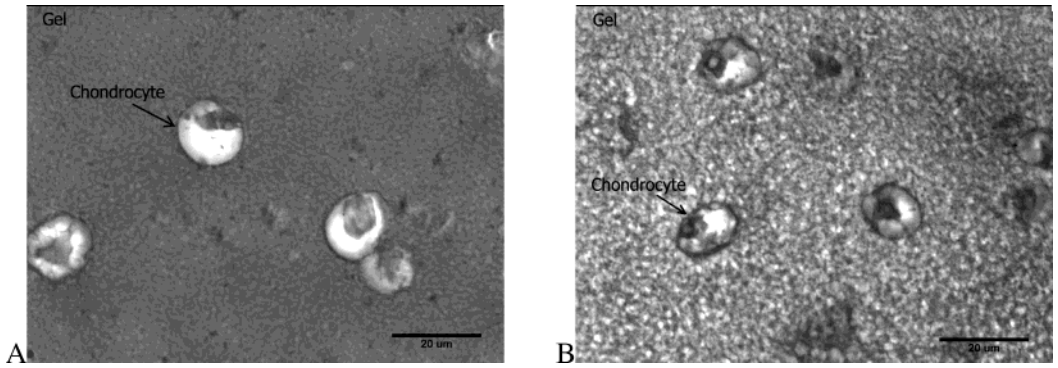

Figure 7. Histological analysis of chondrocytes encapsulated in photopolymerized hydrogels. Gels were composed of (A) ChSA and (B) ChSA/PVA (50%/50%) and cultured for 3 days. The histological sections were stained with safranin O, which stains proteoglycans and ChSA red (shown in gray scale). Nuclei were stained black with hematoxylin.

preliminary results show that these hydrogel scaffolds, composed of a synthetic component and a native glycosaminoglycan found in cartilage, can be used to photoencapsulate chondrocytes and, with the inclusion of adhesion peptides, support natural cell function as observed morphologically.

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References and Notes

(1) National Institute of Arthritis and Musculoskeletal and Skin Diseases. *Handout on Health: Osteoarthritis*; NIH Publication 02-4617; National Institutes of Health: Bethesda, MD, July 2002.
(2) Cao, Y.; Rodriguez, A.; Vacanti, M.; Ibarra, C.; Arevalo, C.; Vacanti, C. *J. Biomater. Sci., Polym. Ed.* **1998**, *9*, 474−8.
(3) Ting, V.; Sims, C. D.; Brecht, L. E.; McCarthy, J. G.; Kasabian, A. K.; Connelly, P. R.; Elisseeff, J.; Gittes, G. K.; Longaker, M. T. *Ann. Plastic Surg.* **1998**, *40*, 413−21.
(4) Elisseeff, J.; McIntosh, W.; Anseth, K. S.; Riley, S.; Ragan, P.; Langer, R. *J. Biomed. Mater. Res.* **2000**, *51*, 164−71.
(5) Bryant, S. J.; Anseth, K. S. *J. Biomed. Mater. Res.* **2002**, *59*, 63−72.
(6) Alsberg, E.; Franceschi, R. T.; Mooney, D. J. *J. Dental Res.* **2000**, *79*, 94.
(7) Akhouayri, O.; Lafage-Proust, M. H.; Rattner, A.; Laroche, N.; Caillot-Augusseau, A.; Alexandre, C.; Vico, L. *J. Cell. Biochem.* **2000**, *76*, 217−30.
(8) Awad, H. A.; Bulter, D. L.; Boivin, G. P.; Smith, F. N. L.; Malaviya, P.; Huibregtse, B.; Caplan, A. I. *Tissue Eng.* **1999**, *5*, 267−77.
(9) Woerly, S. *Neurosurg. Rev.* **2000**, *23*, 59−77.
(10) Hubbell, J. A.; Hill-West, J. L.; Pathak, C. P.; Sawhney, A. S. *Proc. Int. Symp. Controlled Release Bioact. Mater.* **1993**, *20*, 137−8.
(11) Peppas, N. A.; Bures, P.; Leobandung, W.; Ichikawa, H. *Eur. J. Pharm. Biopharm.* **2000**, *50*, 27−46.
(12) Buckwalter, J. A. In *Encyclopedia of Human Biology*; Dulbecco, R., Ed.; Academic Press: San Diego, CA, 1997; Vol. 2, pp 431−45.
(13) Jackson, R. L.; Busch, S. J.; Cardin, A. D. *Physiol. Rev.* **1991**, *71*, 481−539.
(14) Peppas, N. A., Ed. *Hydrogels in Medicine and Pharmacy*; CRC Press: Boca Raton, FL, 1986; Vol. 1.
(15) Sechriest, V. F.; Miao, Y. J.; Niyibizi, C.; Westerhausen-Larson, Z.; Matthew, H. W.; Evans, C. H.; Fu, F. H.; Suh, J. K. *J. Biomed. Mater. Res.* **1999**, *49*, 534−41.
(16) van Susante, J. L. C.; Pieper, J.; Buma, P.; van Kuppevelt, T. H.; van Beuningen, H.; van der Kraan, P. M.; Veerkamp, J. H.; van den Berg, W. B.; Veth, R. P. H. *Biomaterials* **2001**, *22*, 2359−69.
(17) Burdick, J. A.; Anseth, K. S. *Biomaterials* **2002**, *23*, 4315−23.
(18) Sawhney, A. S.; Pathak, C. P.; Hubbell, J. A. *Macromolecules* **1993**, *26*, 581−7.
(19) Hill-West, J. L.; Chowdhury, S. M.; Sawhney, A. S.; Pathak, C. P.; Dunn, R. C.; Hubbell, J. A. *Obstet. Gynecol.* **1994**, *83*, 59−64.
(20) Elisseeff, J.; Anseth, K. S.; Sims, C. D.; McIntosh, W.; Randolph, M.; Langer, R. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 3104−7.
(21) Metters, A. T.; Anseth, K. S.; Bowman, C. N. *Biomed. Sci. Instrum.* **1999**, *35*, 33−8.
(22) Martens, P. J.; Anseth, K. S. *Polymer* **2000**, *41*, 7715−22.
(23) Nuttelman, C. R.; Henry, S. M.; Anseth, K. S. *Biomaterials* **2001**, *23*, 3617−26.
(24) Anseth, K. S.; Metters, A. T.; Bryant, S. J.; Martens, P. J.; Elisseeff, J. H.; Bowman, C. N. *J. Controlled Release* **2002**, *78*, 199−209.
(25) van Dijk-Wolthius, W. N. E.; Frassen, O.; Talsma, H.; van Steenbergen, M. J.; Kettenes-van den Bosch, J. J.; Hennink, W. E. *Macromolecules* **1995**, *28*, 6317−22.
(26) Franssen, O.; van Ooijen, R. D.; de Boer, D.; Maes, R. A. A.; Herron, J. N.; Hennink, W. E. *Macromolecules* **1997**, *30*, 7408−13.
(27) Martens, P. J.; Bryant, S. J.; Anseth, K. S. *Biomacromolecules* **2003**, *4*, 283−92.
(28) Nuttleman, C. R.; Mortisen, D. J.; Henry, S. M.; Anseth, K. S. *J. Biomed. Mater. Res.* **2001**, *57*, 217−23.
(29) Bryant, S. J.; Nuttleman, C. R.; Anseth, K. S. *J. Biomater. Sci., Polym. Ed.* **2000**, *11*, 439−57.
(30) Freed, L.; Vunjak-Novakovic, G. In *The Biomedical Engineering Handbook*; Bronzind, J., Ed.; CRC Press: Boca Raton, FL, 1995; pp 1788−1806.
(31) Hern, D. L.; Hubbell, J. A. *J. Biomed. Mater. Res.* **1998**, *39*, 266−76.
(32) West, J. L.; Hubbell, J. A. *Macromolecules* **1999**, *32*, 241−4.
(33) Kim, S.; Healy, K. E. *Biomacromolecules* **2003**, *4*, 1214−23.
(34) Smeds, K. A.; Grinstaff, M. W. *J. Biomed. Mater. Res.* **2001**, *54*, 115−21.
(35) Li, Q.; Williams, C. G.; Sun, D. D. N.; Wang, J.; Leong, K.; Elisseeff, J. H. *J. Biomed. Mater. Res.* **2004**, *68A*, 28−33.
(36) Leach, J. B.; Bivens, K. A.; Patrick, C. W., Jr.; Schmidt, C. E. *Biotechnol. Bioeng.* **2003**, *82*, 578−89.
(37) Jo, S.; Shin, H.; Mikos, A. G. *Biomacromolecules* **2001**, *2*, 255−61.
(38) Mann, B. K.; West, J. L. *J. Biomed. Mater. Res.* **2002**, *60*, 86−93.
(39) Schmedlen, R. H.; Masters, K. S.; West, J. L. *Biomaterials* **2002**, *23*, 4325−32.
(40) Gobin, A. S.; West, J. L. *FASEB J.* **2002**, *16*, 751−3.
(41) Gobin, A. S.; West, J. L. *J. Biomed. Mater. Res.* **2003**, *67A*, 255−9.
(42) Kim, S. H.; Chu, C. C. *J. Biomed. Mater. Res.* **1999**, *49*, 517−27.
(43) Wang, L.-F.; Shen, S.-S.; Lu, S. C. *Carbohydr. Polym.* **2003**, *52*, 389−96.
(44) Flory, P. J. *Principles of Polymer Chemistry*; Cornell University Press: Ithaca, NY, 1953.
(45) Armstrong, C. G.; Mow, V. C. *J. Bone Joint Surg. Am.* **1982**, *64*, 88−94.
(46) Khare, A. R.; Peppas, N. A. *Biomaterials* **1995**, *16*, 559−67.
(47) Martens, P. J.; Holland, T.; Anseth, K. S. *Polymer* **2002**, *43*, 6093−100.
(48) Lindhorst, E.; Vail, T. P.; Guilak, F.; Wang, H.; Setton, L. A.; Vilim, V.; Kraus, V. B. *J. Orthop. Res.* **2000**, *18*, 269−80.
(49) Durr, J.; Goodman, S.; Potocnik, A.; von der Mark, H. *Exp. Cell Res.* **1993**, *207*, 235−44.

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