Heterogeneous-Phase Reaction of Glycidyl Methacrylate and Chondroitin Sulfate: Mechanism of Ring-Opening−Transesterification Competition

Qiang Li,† Dong-an Wang,† and Jennifer H. Elisseeff*

Department of Biomedical Engineering, Johns Hopkins University, Baltimore, Maryland 21218

Received July 24, 2002
Revised Manuscript Received November 5, 2002

Introduction

The methacrylate-conjugation of chondroitin sulfate (CS) with glycidyl methacrylate (GMA) is a novel strategy for producing photo-cross-linkable polysaccharides that can generate biodegradable hydrogels by in situ injection and light-induced polymerization. Since CS is one of the major components of native cartilage, responsible for the tissue's compressive strength and also capable of absorbing a large volume of water,[1,2] we hypothesize that CS-based hydrogels may be useful for a number of biomedical applications**.** A "similar" reaction of dextran with GMA in a homogeneous-phase system (DMSO) was studied and reported by Hennink et al.[3,4] An unexpected transesterification, instead of epoxide ring-opening, dominated the reaction. However, in our study concerning the different solubility/miscibility of the water-soluble CS and the water-insoluble GMA, a slow heterogeneous-phase reaction of CS with GMA was designed and performed in aqueous medium regardless of GMAs potential side-reaction with water. By monitoring the methacrylate-conjugation with NMR, a different reaction mechanism was discovered. Two reactions, including a rapid reversible transesterification and a slow irreversible ring-opening conjugation, took place simultaneously. As the reaction proceeds, there is a decline in the amount of transesterification products, while the concentration of ring-opening products gradually increased with time. These reactions are illustrated in Scheme 1.

Experimental Section

Materials. Chondroitin sulfate A sodium salt (CS; type A 70%, balanced with type C; from bovine trachea) was obtained from SIGMA. Glycidyl methacrylate (GMA, 98%) was purchased from Polysciences Inc., PA. Poly(ethylene oxide) diacrylate (PEODA, 100%, M_n 3400) was purchased from Shearwater Inc., AL.

GMA−CS Reaction. Eight groups of GMA−CS reactions were carried out. For each reaction, CS (10 g, ∼20 mmol of disaccharide repeating unit) was dissolved in 100 mL of phosphate buffered saline (PBS, pH 7.4), followed by addition of GMA (3 g, ∼20 mmol) while vigorously stirring at room temperature for 1−15 days. The GMA−CS products from six time points of the reactions were respectively collected by acetone precipitation at days 1, 3, 5, 7, 10, and 15. The purification was performed by anhydrous acetone extraction twice to remove all the compounds that failed to covalently graft onto CS chains. The purified products were lyophilized for 48 h. The spontaneous pH variation in the 15 days' free reaction system was monitored and recorded daily.[5] Two parallel GMA−CS reactions were carried out under similar conditions, but the system pH value of each was strictly controlled to be constant at 8.5 and 3.0, respectively, with NaOH and HCl. The final yield of each reaction was 7−8 g.

NMR Methods. NMR spectra were recorded with a Unity Plus 500 MHz spectrometer (Varian Associates Inc., Palo Alto, CA). For ^{1}H NMR in deuterium-d_2 (D_2O, SIGMA), 50 mg of CS−GMA was dissolved in 1.0 mL of D_2O, and the ^{2}HOH peak at 4.8 ppm was used as the reference. For ^{13}C NMR in deuterium-d_2, the pulse length was 7 μs, the acquisition time was 1.300 s, and repetitions were 80 000 times at 50 °C.

Photo-Cross-Linking and Hydrogel Swelling Ratio. The 1:1 (w/w) mixture of GMA−CS and PEODA (containing 10% GMA−CS, w/v) was prepared in water. Under UV irradiation at ∼8 mW/cm^2, the macromers were photopolymerized for 30 min by the initiation with Darocur 2959 (0.05% w/w, cytocompatible UV photoinitiator, 2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone,[6] Ciba-Geigy). For making each hydrogel construct, 75 μL of macromer solution was added to a cylindrical hold. The photo-cross-linked hydrogels were equilibrated in PBS at 37 °C for 18 h. The hydrogels' equilibrated swelling ratio, Q, is calculated by $Q \approx W_{wet}/W_{dry}$, where W_{wet} represents the hydrogel's wet weight and W_{dry} represents the dry weight measured after lyophilization for 24 h.

Results and Discussion

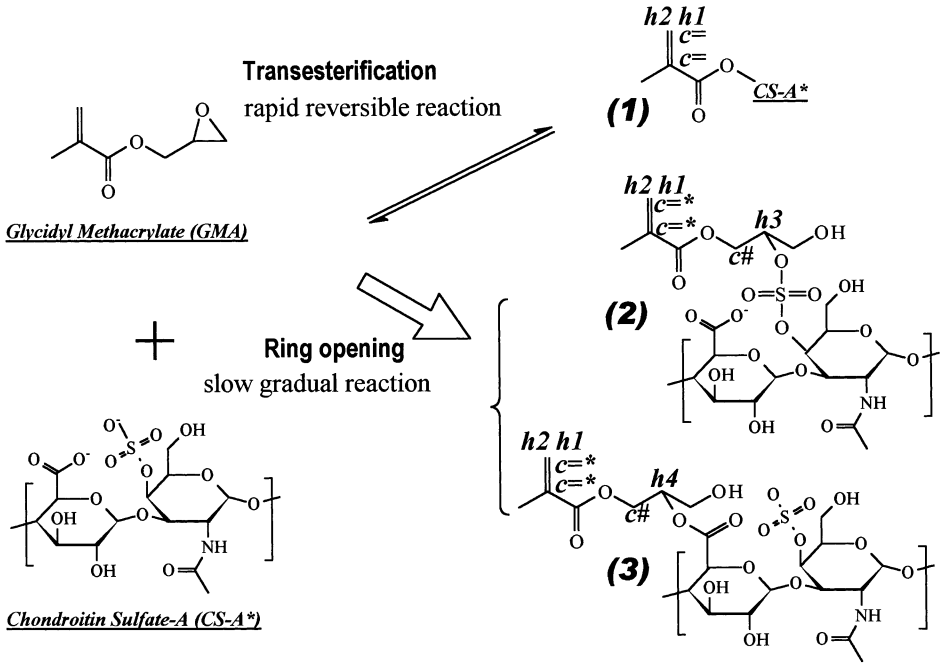
Free Reaction System. The NMR spectra of GMA-CS are provided in Figures 1 and 2. The assignments of the relevant NMR peaks are demonstrated by the corresponding hydrogen/carbon labels in Scheme 1. From Figure 1, two ^{1}H NMR peaks (marked h1 and h2) representing the two vinyl protons at $\delta_{vinyl-H}$ 6.03 and 5.61 ppm were utilized to determine the presence of methacrylate groups on CS. After 1 day's reaction, the introduction of h1 and h2 in the ^{1}H NMR indicated the initial methacrylate-conjugation of CS, which was also confirmed by ^{13}C NMR (Figure 2) with vinyl-carbon peaks (labeled C═) present at $\delta_{C=}$ 136 and 128 ppm. Due to the lack of spectrometric evidences for glyceryl spacers' presence, and also due to the considerable stability of the electronegative glycidol leaving groups as reported by Hennink et al.,[4] by day 1 the transesterification, instead of epoxide ring-opening, dominated the methacrylate-conjugation of CS via reversible cleaving of the glycidol groups. The corresponding product was compound **1**, indicated in Scheme 1. However, from days 3−15, the glyceryl methine protons at δ_{CH} 5.52 ppm (connected to sulfate, labeled h3) and 5.20 ppm (connected to carboxyl ester, labeled h4)[7] began to emerge on ^{1}H NMR with intensities increasing as the reaction proceeded. Simultaneously, on the ^{13}C NMR spectrum of product−day 15, the vinyl-carbon peaks were split into four: the original two peaks (peak C═) were enhanced, and two new peaks (labeled C═*) were present at $\delta_{C=*}$ 121 and 143 ppm. These additional peaks also implied the presence of the glyceryl spacer. Further evidence by ^{13}C NMR was the emergence of the peak at $\delta_{C\#}$ 63 ppm (labeled C#) that represented the methylene carbon on the glyceryl spacer connected to the methacrylate group.[7] Undoubtedly, the glyceryl moieties originated from the ring-opening reaction on

† Equal contribution.

* Author for correspondence: Jennifer H. Elisseeff, Ph.D., Department of Biomedical Engineering, Johns Hopkins University, 3400 North Charles Street/Clark Hall 106, Baltimore, MD 21218, U.S.A. Telephone: (410) 516-4915. Fax: (410) 516-8152. E-mail: jhe@bme.jhu.edu.

10.1021/ma021190w CCC: $25.00
Published on Web 03/13/2003

Scheme 1. Schematic Illustration for the Free Reaction of Glycidyl Methacrylate with Chondroitin Sulfate: Competition of Ring-Opening and Transesterification[a]



[a] The proton/carbon labels, h* and c*, are assigned for ^{1}H and ^{13}C NMR analysis provided in Figures 1 and 3C.

GMA's glycidyl residue. The two ring-opening products are indicated in Scheme 1 as compounds **2** and **3**.

The quantitative analysis was based on ^{1}H NMR data. The substitution efficiency of methacrylate-conjugation is defined as the amount of methacryloyl groups per one chondroitin sulfate disaccharide repeating unit, and there are altogether 12 methine/methylene protons on the disaccharide backbone. Thus, on each ^{1}H NMR spectrum, the overall integral of the peaks representing those protons (δ_{CH} 3.00−4.57 ppm) was normalized as 12, from which the kinetic curves of GMA−CS substitution efficiency are presented in Figure 3. According to the discussion above, both the transesterification product **1** and the ring-opening products **2** and **3** contributed to the integral of the vinyl-proton peaks h1 and h2 ([h1] and [h2]). Simultaneously, the ring-opening products **2** and **3** also have their characteristic glyceryl methine peaks, respectively h3 and h4, with integral values [h3] and [h4]. As the peaks h1−4 are all representing single protons, the total substitution efficiency can be calculated as ([h1] + [h2])/2 {column **1** + **2** + **3** in Figure 3A}, in which the contribution of transesterification is ([h1] + [h2])/2 − ([h3] + [h4]) {curve **1** in Figure 3A} and the contribution of ring-opening is ([h3] + [h4]) {curve **2** + **3** in Figure 3A}. Figure 3A indicates that the transesterification is a kinetically rapid and thermodynamically reversible procedure. Within approximately 1 day, the reaction balance was reached and ∼50% methacrylate-conjugation of the total 15 days' substitution efficiency was achieved. While the ring-opening procedure is kinetically slower, the behavior of it in the first day was not obvious and the contribution for methacrylate-conjugation was minimal. However, the ring-opening procedure is based on a thermodynamically irreversible mechanism. The reaction proceeds linearly and independently from other mechanisms. After the transesterification balance was achieved, the ring-opening behavior was gradually revealed, which was continuously and irreversibly consuming the common reactant GMA and made the transesterification balance keep shifting in the reverse direction. Therefore, the yield of transesterification was decreasing while the ring-opening products gradually accumulated. At day 5, the contributions for total methacrylate-conjugation by the two reactions became equal. By day 15, the ring-opening reaction contributed to more than 80% of the total substitution efficiency, but the products of transesterification were reduced to less than 20%. Apparently, the product of transesterification, compound **1**, was gradually "transformed" into ring-opening resultants, compound **2** or **3**. The distribution of the two ring-opening products **2** and **3** is demonstrated kinetically in Figure 3B, which proceeded synchronously and linearly with a constant [h3]/[h4] ratio {curve **2**/**3** in Figure 3B} of ∼0.7. The mechanism described above can also be confirmed by the variation of the h1 and h2 ^{1}H NMR peak split. Figure 1 indicates that the largest h1 and h2 peak split took place on the spectra of days 5 and 7; accordingly, Figure 3A indicates that at days 5 and 7 the amounts of transesterification product and ring-opening products are relatively closest to each other. This agrees with the principle that the largest components' distribution makes the largest peak split.

Mechanistic Analysis with pH-Controlled Reactions. The ^{1}H NMR spectra of the products from pH-controlled reactions with constant pH 3.0 and 8.5 are provided in Figure 4A and B. The final reaction system at constant pH 8.5 was homogeneous and clear, which implied that all the water-insoluble GMA had been consumed. On the ^{1}H NMR spectrum for the constant pH 8.5 system (Figure 4B), the vinyl-proton peaks h1−2 and the glyceryl methine proton peaks h3−4 (respectively from products **2** and **3**, marked in Schemes 1 and 2) were similarly very weak. However, three new (groups of) peaks were found to be significant at 5.50, 5.18, and 2.5−2.7 ppm, which were respectively marked as h5, h6, and h7. Peaks h5 and h6 also represented the glyceryl methine proton connected to sulfate and carboxyl ester, but without the presence of methacrylate ester at the β-site (demonstrated in Scheme 2).[7] That's

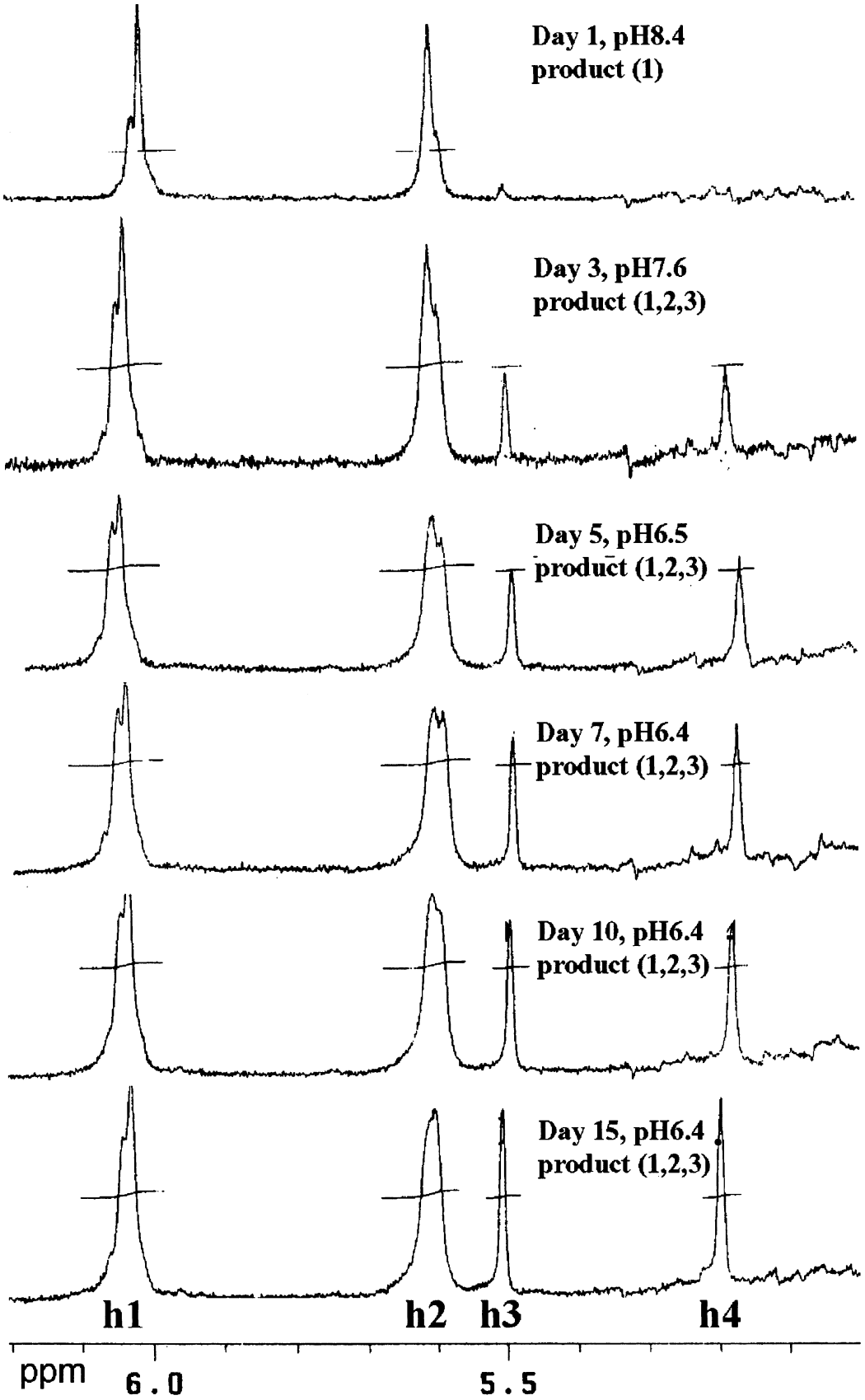


Figure 1. [1]H NMR spectra indicating the free methacrylate-conjugation of chondroitin sulfate. Curves from the top to the bottom, respectively, represent CS−GMA days 1, 3, 5, 7, 10, and 15. The assignments of the NMR peaks and the components of the testing samples are respectively demonstrated by the proton/carbon labels and the product labels marked in Schemes 1 and 2.

why the chemical shifts of h5 and h6 were respectively a little lower than those of h3 and h4. The h7 peak was the characteristic peak of the epoxide methylene proton on a glycidyl residue.[7] The weakening of peaks h3 and h4 provided indirect evidence for the cleavage of the methacrylate group from products **2** and **3**; while the

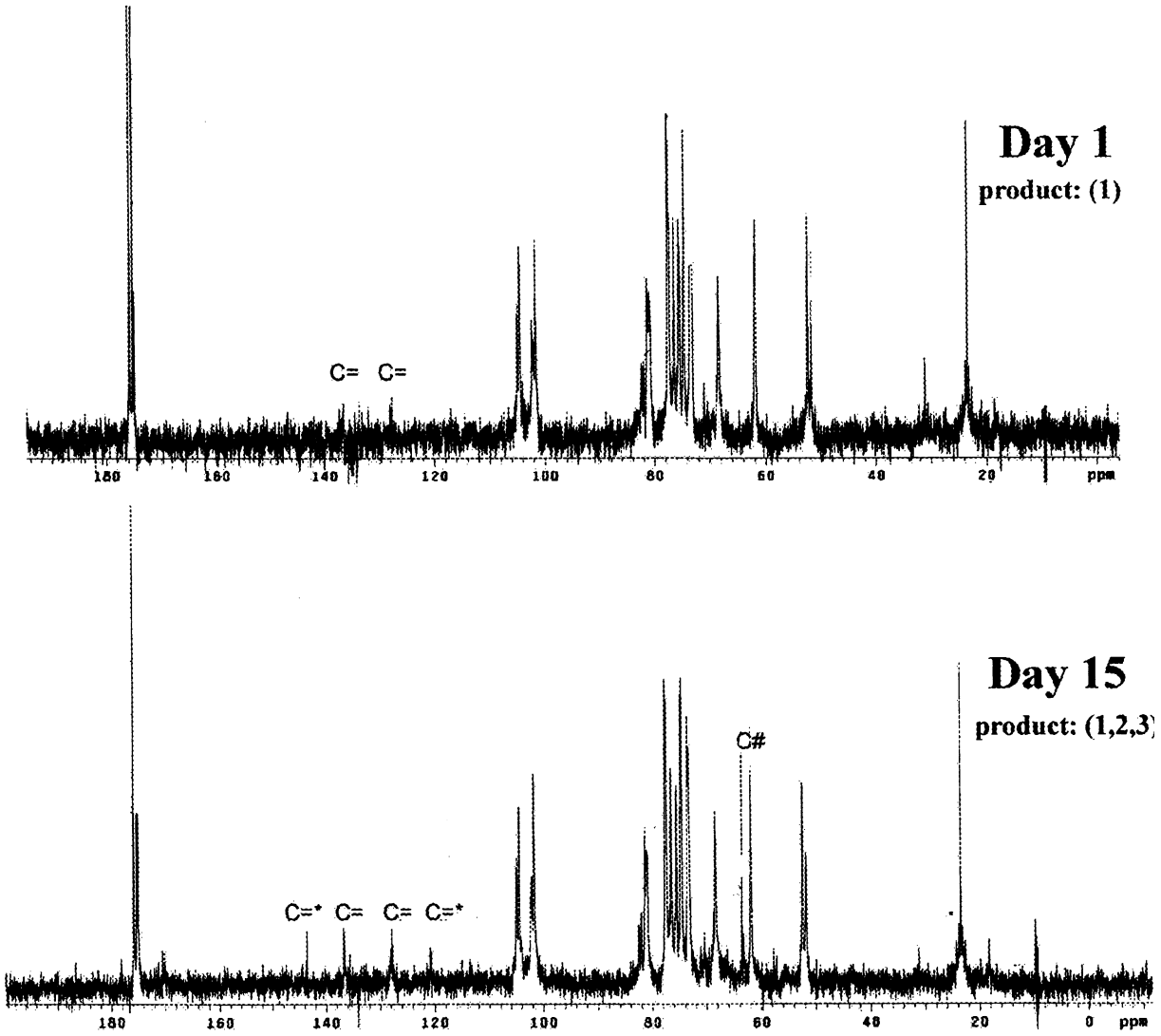


Figure 2. ^{13}C NMR spectra indicating the free methacrylate-conjugation of chondroitin sulfate: CS−GMA day 1 (upper panel) and day 15 (lower panel). The assignments of the NMR peaks and the components of the testing samples are respectively demonstrated by the proton/carbon labels and the product labels marked in Schemes 1 and 2.

weakening of peaks h1 and h2 directly indicated the methacrylate group's cleavage from products **2**, **3**, and **1**. These spectrometric data revealed the serious hydrolysis (marked as procedures IV and V in Scheme 2) of products **1**, **2**, and **3** in basic circumstances during the 15 days' reaction. The hydrolysis products of **2** and **3** were respectively **2′** and **3′**, marked in Scheme 2. From these results, the GMA−CS reactions at constant pH 8.5 could be demonstrated as in Scheme 2. In addition to the slow and irreversible GMA−epoxide ring-opening reaction, the basic conditions simultaneously enabled the reactions of hydrolysis and reversible transesterification. When GMA and CS were mixed together under basic conditions, the reactions of GMA−epoxide ring-opening by the CS sulfate or carboxyl nucleophilic attack (marked as procedure III in Scheme 2), GMA−CS transesterification (marked as procedure II in Scheme 2), and GMA self-hydrolysis (marked as procedure I in Scheme 2) started simultaneously. Procedure I resulted in methacrylic acid (MA acid) and glycidyl alcohol (G alcohol). Procedure II resulted in product **1** and G alcohol, but **1** was further hydrolyzed into CS, MA acid, and G alcohol (marked as procedure IV in Scheme 2), which made the whole serial reaction continuously move forward. Procedure III was slow and gradually produced **2** and **3**, but **2** and **3** were eventually hydrolyzed into **2′** and **3′** (marked as procedure V in Scheme 2); subsequently, another reversible transesterification occurred between the G alcohol (produced by procedures I and IV) and **2′** or **3′** to produce **2″** or **3″**—the glycidyl CS (marked as procedure VI in Scheme 2). Peak h7 just came from **2″** and **3″**. As the reaction proceeded, eventually the products **1**, **2**, and **3** were largely hydrolyzed, while the products **2′**, **3′**, **2″**, and **3″** were accumulated in the system. As for the constant pH 3.0 system, Figure 4A only indicated the presence of the two vinyl protons (h1 and h2) grafted on the CS backbone, without any signals of sulfate- or carboxyl-neighboring methine protons. Under the controlled acidic conditions, since both methacrylate hydrolysis and GMA−CS transesterification declined, and the nucleophilic capacity of the CS's pendent sulfate/carboxyl groups was also largely weakened, the NMR result implied that the GMA−CS conjugation proceeded mainly via the GMA−epoxide ring-opening reaction due to the saccharide-hydroxyls' nucleophilic attack. The reaction is illustrated in Scheme 2 as procedure VII, and the resultant is marked as product **4**.

In the free reaction system, the variation of the system pH value was monitored as the reaction pro-

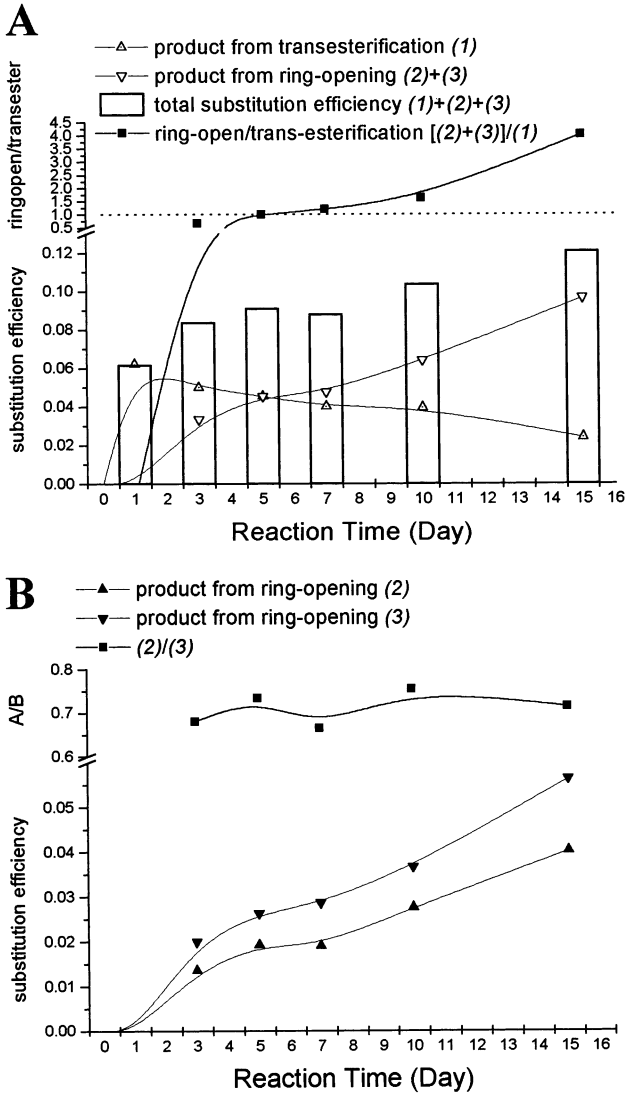


Figure 3. Kinetic curves for substitution efficiency of the free methacrylate-conjugation: (A) total substitution efficiency and contributions by ring-opening and transesterification; (B) individual contributions by the two ring-opening products (**2** and **3**) (marked in Schemes 1 and 2).

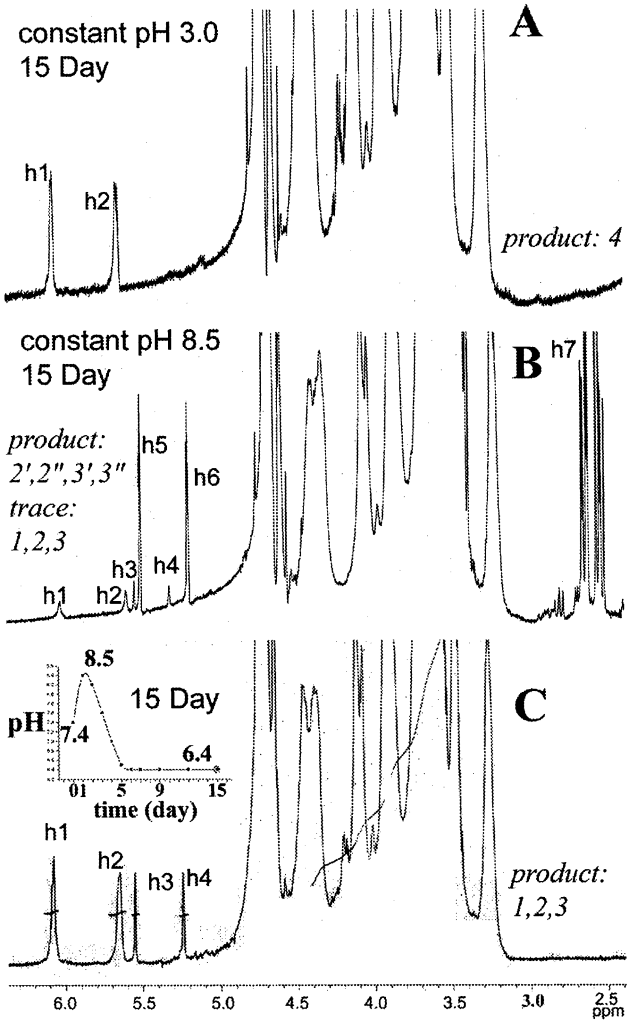
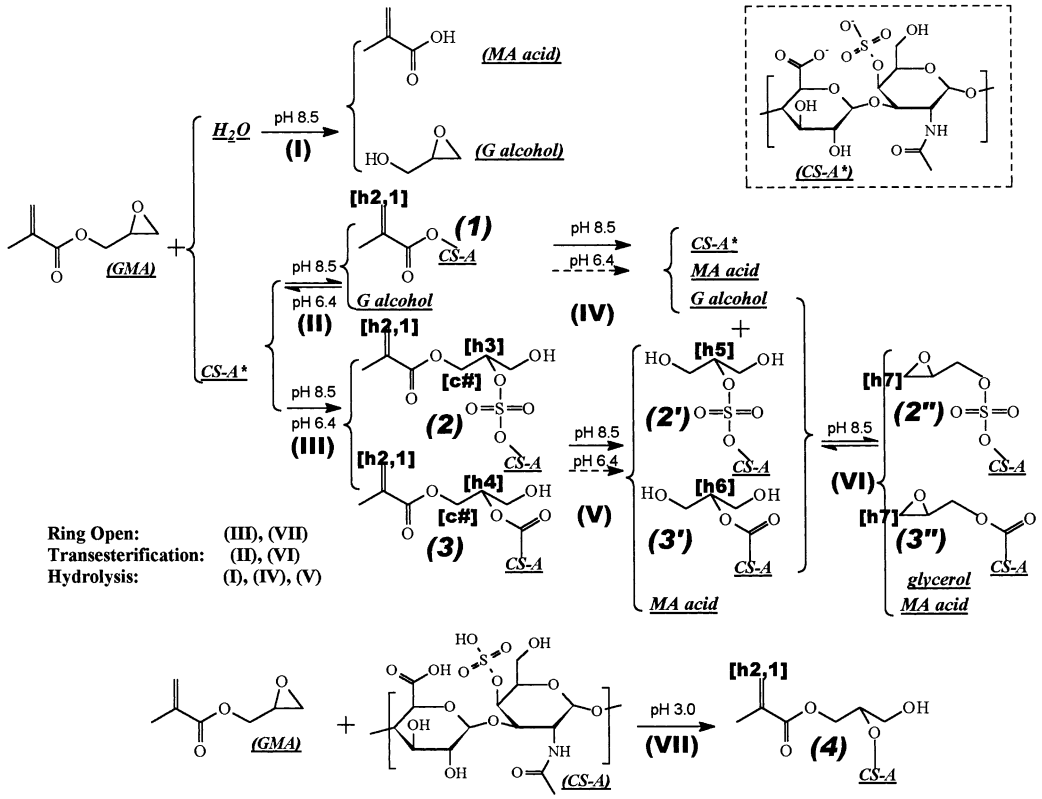


Figure 4. ^{1}H NMR spectra indicating the methacrylate-conjugation of chondroitin sulfate. Products from 15 days' reaction at constant pH 3.0 (A); at constant pH 8.5 (B); and from the free reaction system with the pH−time profile of the 15 days' reaction system (C). The assignments of the NMR peaks and the components of the testing samples are respectively demonstrated by the proton/carbon labels and the product labels marked in Schemes 1 and 2.

ceeded. The pH−time profile is exhibited in Figure 4C. The solvent for the free reaction was PBS with an initial pH of 7.4. Following the addition of GMA, the pH value rapidly increased to a maximum of 8.5 within 1 day, and then, due to the production of MA acid by the methacrylate hydrolysis, the pH value began to decrease**.** After 3 days, the system reached acidic conditions. After the day 5, the system pH value stabilized at 6.4. The pH remained constant because the accumulation of MA acid caused the system to become increasingly acidic, while the trend of hydrolysis reduced this effect. Finally, the hydrolysis was self-declined. According to the pH−time profile, the reaction proceeded in basic conditions only for 2−3 days, while in the following 10 days the reaction proceeded in a weak acidic system. Therefore, the "pH 8.5-reaction-mechanism" described above had directed the system behavior for only 2 days. Within these 2 days, besides a large number of unreated GMA−CS materials and some GMA hydrolysis products, mainly product **1** from the fastest GMA−CS transesterification (procedure II), which had not hydrolyzed (procedure IV), remained in the system. Since the ring-opening procedure III was much slower than the transesterification II, plus the hydrolysis V, little product **2** and **3** and minute amounts of products **2′**, **3′**, **2″**, and **3″** were produced in the system, as indicated in Figure 1, day 1. Following the system pH decrease and stabilization at 6.4, the hydrolysis (I, IV, and V) declined, and the products **1**, **2**, and **3** were largely liberated from hydrolysis consumption, by which the accumulation of the ring-opening products **2** and **3** was enabled. However, the production of transesterification (II) product **1** was getting negative because of the pH-resistance for the forward reaction, as well as the backward shift of the reaction balance due to the continuous consumption of GMA and CS (as the common reactants of both GMA−CS transesterification and GMA−epoxide ring-opening) by the ring-opening reactions III. The latter factor was discussed in the prior section of this paper. All the reaction pathways described above are illustrated in Scheme 2, and the overall kinetics of the GMA−CS conjugation in the free reaction system was also presented in the prior section.

GMA−CS Gelation. The results of photopolymerization indicated that only when the substitution efficiency of the CS methacrylate-conjugation reached 0.1

Scheme 2. Schematic Illustration for the pH-Controlled Reaction of Glycidyl Methacrylate with Chondroitin Sulfate: Completed Reaction Pathways[a]



[a] The proton/carbon labels, h* and c*, are assigned for ^{1}H and ^{13}C NMR analysis provided in Figures 1 and 3. The relevant products or midproducts are marked with italic and bold arabic numerals. The reaction procedures are marked with bold roman numerals.

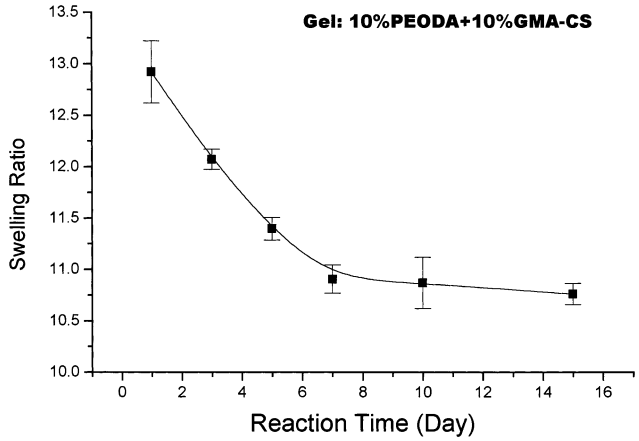


Figure 5. Kinetic curve for the swelling ratio of the 10% GMA−CS (free reaction product) co-gel with 10% PEODA.

(1 methacryloyl group per 10 CS disaccharide residues), like that of CS−GMA days 10 and 15, could tangible hydrogels be generated solely by the single component of CS−GMA with the conditions of 8 mW/cm^2 UV irradiation, 10 min, and 20% (w/w) macromer. To kinetically study each macromer's performance for cross-linking, the comparable co-gels of CS−GMA/PEODA[8,9] were prepared as described above, with which the swelling ratios were respectively determined, and the corresponding kinetic curve was plotted as Figure 5. The trend of swelling ratio is in accord with the total substitution efficiency of methacrylate-conjugation: the gel made of more methacrylated CS−GMA macromer possesses the lower swelling ratio because of the higher density of methacryloyl cross-linking sites. This again confirms the increase of the total substitution efficiency exhibited in Figure 3A.

Conclusions

In comparison with the reaction conditions reported by Hennink et al.,[4] those of our study have two major differences: a heterogeneous-phase mixture and an aqueous medium. The involvement of a protonated polar solvent (as water might be the only good solvent for CS) provided the thermodynamic promotion to the ring-opening mechanism. The heterogeneous-phase conditions due to the opposite solubility/miscibility of the two reactants in solvents would mainly affect the kinetic behaviors of the reaction—entirely slow the progress of the reactions, by which the gap of different reaction rates was magnified for easier detection, especially during the initial period of the free reaction system. Among the three main reactions in the free reaction system, the reversible GMA−CS transesterification was rapid and pH-dependent; the methacrylate hydrolysis would decrease the system pH and self-decline; the irreversible ring-opening of GMA−epoxide by attack of CS's nucleophilic pendent groups was slow and minimally affected by the mild pH variation. Therefore, the progress of the free reaction system was investigated as the following. When large amounts of GMA were added into a neutral aqueous solution of CS, all of the three reactions described above occurred. The GMA−CS transesterification (II) proceeded rapidly and reached the balance in 1 day or so. The corresponding products **1** and G alcohol were produced. The system pH value increased to 8.5. Subsequently, the system pH began to decrease due to the slower methacrylate hydrolysis

(I, IV, and V) producing methacrylic acid, which gradually terminated the GMA−CS transesterification, and also declined later on when the system pH value dropped to 6.4. The slowest ring-opening reaction (III) was relatively independent of the system pH variation; however, in the initial days the ring-opening products (**2** and **3**) were largely hydrolyzed, since the hydrolysis procedure (V) was faster at that time. The real accumulation of the ring-opening products did not take place until the hydrolysis began to slow. The later long-term acidic conditions continuously minimized the GMA−CS transesterification and methacrylate hydrolysis, but they had little effect on the ring-opening reaction. Thus, the accumulation of the ring-opening products was enabled. Simultaneously, as the common reactants of both ring-opening reaction and transesterification, GMA and CS began to be primarily consumed by the ring-opening procedure, which finally led the pH-resisted transesterification to reverse.

Acknowledgment. We acknowledge Mr. Charles Long of Department of Chemistry, Johns Hopkins University, for the use and direction of NMR facilities.

References and Notes

(1) Mow, V. C.; Ratcliffe, A.; Poole, A. R. *Biomaterials* **1992**, *13*, 67−97.

(2) Schnaper, H. W.; Kleinman, H. K. *Pediatr. Nephrol.* **1993**, *7*, 96−104.

(3) van Dijk-Wolthuis, W. N. E.; Franssen, O.; Talsma, H.; van Steenbergen, M. J.; Kettenes-van den Bosch, J. J.; Hennink, W. E. *Macromolecules* **1995**, *28*, 6317−6322.

(4) van Dijk-Wolthuis, W. N. E.; Kettenes-van den Bosch, J. J.; van der Kerk-van Hoof, A.; Hennink, W. E. *Macromolecules* **1997**, *30*, 3411−3413.

(5) van Dijk-Wolthuis, W. N. E.; van Steenbergen, M. J.; Underberg, W. J. M.; Hennink, W. E. *J. Pharm. Sci.* **1997**, *86*, 413−417.

(6) Bryant, S. J.; Nuttelmen, C. R.; Anseth, K. S. *J. Biomater. Sci. Polym. Ed.* **2000**, *11*, 439.

(7) Varian Associates. *NMR spectra catalog* 1962−1963.

(8) Elisseeff, J.; Anseth, K.; Sims, D.; McIntosh, W.; Randolph, M.; Langer, R. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 3104−7.

(9) Elisseeff, J.; McIntosh, W.; Anseth, K.; Riley, S.; Ragan, P.; Langer, R. *J Biomed. Mater. Res.* **2000**, *51*, 164−71.

MA021190W