Novel Degradable Poly(ethylene glycol) Hydrogels for Controlled Release of Protein

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Abstract □ Hydrogels have become increasingly important in the biomedical field. This paper describes synthesis and characterization of two types of novel degradable poly(ethylene glycol) (PEG) hydrogels with potential utility as delivery carriers for bioactive drugs. The simplest gel is prepared by one-step polycondensation of difunctional PEG acid and branched PEG "polyol". The second type of the novel degradable PEG hydrogel was prepared in a two-step process, in which an ester-containing, amine-reactive PEG derivative was synthesized and then reacted with a branched PEG amine to form the gel. The two-step gels are formed in very mild conditions, and therefore fragile drugs such as proteins can be loaded during gel formation. Because most proteins have free amino groups in the sequence, these proteins can be covalently linked to the hydrogel network. This covalent attachment provides a new way to achieve long-term controlled release of proteins. These hydrogels have a wide range of degradation rates. Upon hydrolysis, these PEG hydrogels will degrade into low molecular weight PEG derivatives, which can be easily cleared by the body.

Introduction

Hydrogels are generally considered as biocompatible materials because of their high water content.[1] They have been used in a variety of biomaterial and biotechnology applications, such as tissue engineering, artificial organs, and drug delivery.[2] Recently, hydrogels have been extensively studied as drug carriers, especially for proteins. Hydrogels have been made from different polymers such as dextran,[3] gelatin,[4−6] amylose,[7−9] cellulose,[10] chitosan,[11] fibrin,[12] collagen,[13,14] alginate,[15,16] poly(vinyl alcohol),[17] poly(hydroxyethyl methacrylate),[18,19] poly(hydroxyethyl acrylate),[20] poly(acrylic acid) (Carbopol),[21] polyasparthydrazide,[22] and poly(ethylene glycol) (PEG).[23,24]

Most hydrogel-based drug delivery systems are designed as implants that release drug locally at a predetermined rate. Therefore, degradability of the gels is very important because surgical removal of the drug-depleted device is not required, and, drug release kinetics can be controlled. However, it is necessary that degradation products be biocompatible and nontoxic.

Poly(ethylene glycol) is a biocompatible, nontoxic, nonimmunogenic and water-soluble polymer of much use in biomaterials, biotechnology and medicine.[25] Two PEG−protein drugs, Adagen (Enzon) and Oncaspar (Enzon), have been approved by the FDA.[26] Therefore, PEG could be an ideal material for internal use in hydrogel-based drug-delivery devices. In fact, several PEG hydrogels have been studied as depot devices for drug delivery, and they have been prepared in several fundamentally different ways. The simplest route is to cross-link PEG by γ-irradiation[27] or electron-beam irradiation.[28] The hydrogels formed in this way are nondegradable.

Graham and co-workers[23,24,29] pioneered use of coupling reactions between a difunctional PEG, a triol, and a multifunctional isocyanate. This type of hydrogel was recently studied for release of proteins.[30,31] These hydrogels can also be made degradable by inclusion of copolymers containing ester linkages.[24] Suitably activated PEGs can be cross-linked by a variety of cross-linkers. Fortier and co-workers[32] have developed a hydrogel by cross-linking PEG *p*-nitrophenyl carbonate with bovine serum albumin (BSA). Similarly, Barrows and co-workers[33] have prepared a hydrogel by reaction between difunctional PEG succinimidyl succinate and human serum albumin (HSA).[33] Because of presence of an ester linkage in PEG succinate, the hydrogels are hydrolytically degradable. This hydrogel was developed as a tissue sealant and has not yet been utilized for drug delivery.

The degradable PEG hydrogels studied most were developed in Hubbell's laboratory.[34−36] These workers prepared PEG acrylates with a short segment of poly(lactide/glycolide) between the PEG and the acrylate group. Acrylate polymerization then provides a cross-linked network in which the poly(lactide/glycolide) provides degradability. Other degradable PEG hydrogels that take advantage of ester linkages include gels made from PEG−lysine acrylate[37] and PEG−poly(vinyl alcohol).[38,39] Hoffman[40] disclosed a novel hydrolytically degradable hydrogel prepared from PEG dialdehyde and poly(vinylamine) in which the Schiff base between the aldehyde and the amine provides degradability. Recently, West and Hubbell[41] reported enzymatically degradable PEG-based hydrogels made from photopolymerization of block copolymers of PEG and short oligopeptides that are further capped at each end with an acrylate group. Russell and co-workers[42] have formed a photodegradable PEG hydrogel by photochemical [2 + 2] coupling of vinyl PEGs. This reaction is reversible upon irradiation at a different wavelength.[42]

In our laboratory, we have developed two types of novel degradable PEG-based hydrogels. The first type of hydrogel was formed in a one-step polycondensation reaction between difunctional PEG acids and branched PEG polyols. The resulting ester linkages provide degradability. Upon hydrolysis of the ester linkages, these gels degrade into only PEG and PEG derivatives. The degradation rate can be controlled by slightly varying the molecular structure in the esters and the degree of branching in the cross-linker. The second type of hydrogel was formed under mild condition (aqueous solution and room temperature) via a two-step process in which an ester-containing active PEG derivative is first synthesized and then coupled to an amine cross-linker. In these hydrogels, protein drugs can be covalently attached to the hydrogel network via ester

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linkages. Thus, release of the protein drug would be controlled by hydrolysis of the ester linkage between the gel and the protein, by diffusion from the gel, and by degradation of the gel. There are several types of esters used in these hydrogels, which provide a range of degradation rates.

In this paper, we report the synthesis and characterization of these hydrogels as well as the potential of these hydrogels for long-term controlled release of protein drugs.

Experimental Section

Materials—Glycolic acid (GA), 3-hydroxybutyric acid (HBA), benzyl glycolate, thionyl chloride, and disperse yellow 9 were purchased from Aldrich (Milwaukee, WI). Carboxymethylated PEG 2000 PEG-CM, PEG-propionic acid 2000 (PA), branched PEGs (4-arm PEG 2000, 4-arm PEG 10,000, and 8-arm PEG 10,000), and *m*-PEG−silane (5 kD) were gifts from Shearwater Polymers, Inc. (Huntsville, AL). Fluorescein isothiocyanate-labeled BSA (FITC-BSA) and stannous octoate [Sn(Oct)2] were purchased from Sigma (St. Louis, MO). All other reagents were purchased from either Aldrich or Sigma and used without further purification.

One-Step Hydrogel Preparation—In an aluminum pan (1-in. diameter), 600 mg of dry difunctional PEG 2000 acid (0.6 mmol end groups) and one equivalent of dry branched PEG (e.g., 750 mg 8-arm PEG 10,000) were mixed with 15−20% stannous octoate and melted. After a thin film of the melt covered the pan surface uniformly, the pan was put into a vacuum oven and heated at 130 °C and 100 milliTorr for 6−24 h. A firm and transparent gel formed. After cooling in a stream of N_2, the gel became translucent. The gel was cut into thin disks. The shaped crude gels were then swollen in glacial acetic acid and washed three times with this solvent over a 2−3-day period. After washing, the gels were dried under reduced pressure. The tin content of the gel was determined by the inductively coupled plasma (ICP) method conducted at SouthEastern Analytical Services, Inc. (Huntsville, AL).

Two-Step Hydrogel Preparation—*A Two-Step Gel Made from Difunctional Double-Ester PEG*—Fifty milligrams of difunctional PEG-CM−HBA−*N*-hydroxysuccinimide (NHS) 2000 (vide infra) was dissolved in 0.25 mL of deionized water. To the solution was added 0.5 mL of a buffered solution with or without FITC-BSA (10 mg/mL) and 0.25 mL of 8-arm PEG−amine solution (250 mg/mL). After vigorous shaking, the solution was allowed to sit. The gel formed in a few minutes. A suitable pH range was 5.5 to 8.

A Two-Step Gel Made from Difunctional PEG-Succinimidyl Carbonate Containing an Ester in the Middle—Fifty milligrams of difunctional ester-containing PEG-succinimidyl carbonate 6800 (vide infra) was dissolved in 0.3 mL of deionized water. To the solution was added 0.3 mL of a buffered solution with or without FITC-BSA (10 mg/m) and 0.13 mL of 8-arm PEG−amine (250 mg/mL). After vigorous shaking, the solution was allowed to sit. The gel formed in a few minutes. A suitable pH range was 5.5 to 8.

Kinetics of the Ester Linkages—A solution of *m*-PEG−ester−PEG-*m* prepared as reported elsewhere[43] (1.0 wt %) and PEG 20,000 (0.2 wt %, as internal standard) was prepared in a desired buffer. The concentration of *m*-PEG−ester−PEG-*m* (*C*) and its hydrolysis products were monitored by high-performance liquid chromatography (HPLC)-gel permeation chromatography (GPC) (Ultrahydrogel 250 column, Waters). Assuming first-order kinetics, the hydrolytic half-life was calculated from the slope of the natural logarithm plot of *C* versus time.

Release Studies of the Model Drugs from the Gels—In the one-step gel, model drugs were first loaded into the hydrogels by placing a purified gel in a solution of the model drug. Depending on the drug molecular weight and its concentration, the soaking time ranged from a few days for *m*-PEG−dyes to a few weeks for FITC-BSA. All drug-loaded hydrogel disks were weighed and their diameters were measured before release studies were undertaken.

Automatic Method—The release study was conducted in a circulation system with an ultraviolet (UV) flow cell. The system is shown schematically in Figure 1. A drug-loaded hydrogel was immersed, at time $t = 0$, in phosphate buffer (0.1 M, pH 7.0). In protein-release measurements, the surface of the flask and the flow cell were coated (vide infra) with PEG to minimize nonspecific protein absorption. The buffer solution was maintained at 37 °C with gentle stirring. At the same time, the solution was continuously pumped into the flow cell of a Hewlett-Packard (8452A) UV spectrophotometer to monitor the release of the drug at its maximum absorption wavelength. The UV absorption was collected automatically at predetermined time intervals of 5 min. This method was mainly used for release experiments that lasted for <3 days.

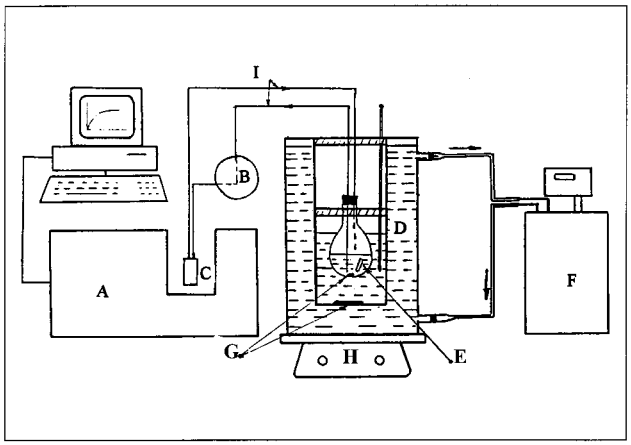


Figure 1—Schematic of the circulation system used in release study: (A) UV spectrophotometer; (B) peristaltic pump; (C) UV flow cuvette; (D) temperature control device; (E) a gel; (F) circulating water bath; (G) magnetic stir bars; (H) magnetic stir plate; (I) teflon tubing.

Manual Method—Release of proteins was studied mainly by the manual method. A hydrogel disk was immersed ($t = 0$) in phosphate buffer (0.1 M, pH 7.0) with the amount of the buffer >20 times the wet gel weight, and the buffer solution was maintained at 37 °C with a water bath and gently shaken. At a predetermined time, a small amount of buffer solution was removed for drug concentration determination and then returned after measurement.

Coating of the Glass Surface of the Circulation System with *m*-PEG−Silane[44]—The surface of the flask and the UV flow cell were treated with aqueous sodium hydroxide (1 wt %) at 90 °C for 10 min, aqueous hydrogen chloride (3 wt %) at 90 °C for 10 min, and hot H_2O_2 solution (30 wt %) for 1 h. The surface was then washed with a large amount of deionized water and dried in a vacuum oven at 150 °C overnight. After cooling to room temperature, the flask and the flow cell were filled with *m*-PEG−silane 5000 in dry toluene (2 wt %) and kept under N_2 overnight. The toluene solution was decanted and the surface was washed twice with dry toluene. The surface was then cured in a vacuum oven at 130 °C for 4 h.

Preparation of Difunctional Double-Ester PEG—*PEG Ester Acids* (*CM−R−COOH and PA−R−COOH*)—*Method 1*—PEG 2000 acid (10 g, 10 mmol end-group, PEG-CM or PEG-PA) was azeotropically dried with 60 mL of toluene under N_2. After 2 h, the solution was cooled to room temperature, and thionyl chloride solution (20 mL, 40 mmol) in CH_2Cl_2 was injected. The solution was stirred at room-temperature overnight. The solvent was condensed using a rotary evaporator, and the residual syrup was dried under reduced pressure for ~4 h over P_2O_5 powder. Hydroxy acid (GA or HBA, 27 mmol) was azeotropically dried with 140 mL of 1,4-dioxane, and the distillation was stopped when ~70 mL of solution remained. The solution was slowly cooled to room temperature under N_2. The glycolic acid−dioxane solution was then added to the PEG acyl chloride. After the PEG was dissolved, 6 mL of dry triethylamine was injected to the system (precipitate formed immediately), and the solution was stirred overnight. The salt was filtered off and the filtrate was condensed on a rotary evaporator at 55 °C and dried under reduced pressure. The crude product was then dissolved in 100 mL of distilled water and the pH of the solution was adjusted to 3.0. The aqueous phase was extracted three times with a total of 200 mL of methylene chloride. The organic phase was dried over sodium sulfate, filtered to remove salt, condensed on a rotary evaporator, and precipitated into 1000 mL of ethyl ether. The precipitate was collected by filtration and dried under reduced pressure to yield 8.5−9.2 g (85−

92%). ^{1}H NMR (DMSO-d_6): PEG-GA: δ 3.5 (br m, PEG), 4.3–4.6 (s, PEGCOOCH_2COOH), 2.59 [t, PEGOCH$_2$CH_2COO (PA)], 4.19 [s, PEGOCH_2COO (CM)]; PEG-HBA: δ 3.5 (br m, PEG), 2.54 (d, PEGCOOCH(CH_3)CH$_2$COOH), 5.1 (h, PEGCOOCH(CH$_3$)CH_2-COOH), 1.2 (d, PEGCOOCH(CH_3)CH$_2$COOH), 2.54 [t, PEG-OCH$_2$CH_2COO (PA)], 4.055 [s, PEGOCH_2COO (CM)].

Method 2—*m*-PEG 5000 acid (8 g, 1.6 mmol, PEG-CM or PEG-PA), benzyl glycolate (GA-BZ), or 3-hydroxybenzylbutyrate (HBA-BZ, vide infra; 2.24 mmol), 1-hydroxybenzotriazole (HOBT, 1.6 mmol), 4-(dimethylamino)pyridine (DMAP, 2 mmol), and dicyclohexylcarbodiimide (DCC, 2.4 mmol) were dissolved in 80 mL of dry methylene chloride. The solution was stirred under nitrogen overnight, and the solvent was removed by rotary evaporation. The resulting syrup was redissolved in 30 mL of dry toluene, and the insoluble solid was filtered off. Then the solution was precipitated into 500 mL of dry ethyl ether. The product was reprecipitated with ether, then collected by filtration and dried under reduced pressure to yield 7.8 g (98%). ^{1}H NMR(DMSO-d_6): PA-GA-BZ: δ 3.5 (br m, PEG), 4.74 (s, PEGCOOCH_2COOBZ), 2.62 (t, PEGOCH$_2$CH_2COOGA), 5.17 (GACOOCH$_2$C$_6$H$_5$), 7.37 (GA-COOCH$_2$C$_6$H$_5$); CM-HBA-BZ: δ 3.5 (br m, PEG), 2.72 (m, PEGCOOCH(CH$_3$)CH_2COOBZ), 5.22 (h, PEGCOOCH(CH$_3$)CH$_2$-COOBZ), 1.23 (d, PEGCOOCH(CH_3)CH$_2$COOH), 4.0 (q, PEGOCH_2-COOHBA), 5.09 (s, HBACOOCH_2C$_6$H$_5$), 7.36 (br s, HBACOO-CH$_2$C$_6$$H_5$).

A solution of PEG acid–hydroxyacid benzyl ester obtained in the previous step (5 g, 1 mmol) in 1,4-dioxane (50 mL) was hydrogenated with H_2 (2 atm pressure) and 1.5 g of Pd/C (10%) overnight. The catalyst was removed by filtration, and the solvent was condensed by rotary evaporation. The residual syrup was added to 200 mL of ethyl ether. The product was collected by filtration and dried under reduced pressure to yield 4 g (80%).

PEG Ester–NHS Esters (*Double Ester, CM–R–COO–NHS and PA–R–COO–NHS*)—The PEG ester acid (3.3 g, ~3.3 mmol) and 420 mg NHS (3.5 mmol) were dissolved in 50 mL of methylene chloride. Then, DCC (800 mg, 3.8 mmol) in 5 mL of dry methylene chloride was added. The solution was stirred under nitrogen overnight and the solvent was removed by rotary evaporation. The resulting syrup was redissolved in 10 mL of dry toluene and the insoluble solid was filtered off. The solution was then precipitated into 100 mL of dry ethyl ether. The precipitate was collected by filtration and dried under reduced pressure to yield 3.1 g (94%). ^{1}H NMR (DMSO-d_6): PEG-GA-NHS: δ 3.5 (br m, PEG), 5.15–5.21 (s, PEGCOOCH_2COONHS), 2.67 (t, PEGOCH$_2$CH_2COO (PA)), 4.27 (s, PEGOCH_2COO ppm (CM)), 2.82 (s, NHS, 4H); PEG-HBA-NHS: δ 3.5 (br m, PEG), 3.0–3.2 (m, COOCH(CH$_3$)CH_2COONHS), 5.26 (h, COOCH(CH$_3$)CH$_2$COONHS), 1.3 (d, COOCH(CH_3)CH$_2$-COONHS), 2.54 (t, OCH$_2$CH_2COO(PA)), 4.1 (s, OCH$_2$COO (CM)), 2.81 (s, NHS).

PEG Ester–Glycolic Acid–p-Nitrophenyl ester (*CM-GA-NP or PA-GA-NP*)—PEG ester–glycolic acid 2000 (1.5 g, ~1.5 mmol), *p*-nitrophenol (420 mg, 3 mmol), and DMAP (195 mg, 1.575 mmol) were dissolved in 90 mL of dry methylene chloride. To it was added DCC (360 mg, 1.725 mmol) in 5 mL of dry methylene chloride. The solution was stirred under N_2 overnight, and the solvent was removed by rotary evaporation. The resulting syrup was redissolved in 30 mL of dry toluene, and the insoluble solid was filtered off. Then the solution was precipitated into 300 mL of dry ethyl ether. The product was reprecipitated with ether, then collected by filtration and dried under reduced pressure to yield 1.425 g (95%). ^{1}H NMR (DMSO-d_6): δ 3.5 (br m, PEG), 5.01 (s, PEGCOOCH_2COONP), 2.69 (t, PEGOCH$_2$CH_2COO(PA)), 8.35 & 7.48 (d & d, Ha & Hb in NP, 4H).

Synthesis of 3-Hydroxybenzylbutyrate—3-Hydroxybutyric acid sodium salt (2 g, 15.8 mmol) and benzylbromide (16 mmol) were dissolved in 40 mL of acetonitrile. The solution was refluxing over 2 days under N_2. The solid in the solution was removed by filtration, and the solvent was condensed under reduced pressure. The product was obtained by chromatography of a silica gel with R_f of ~0.45 when 50% hexane and 50% ethyl acetate were used as eluent; yield, 2.72 g (88%). ^{1}H NMR (DMSO-d_6): δ 1.09 (d, HOCH(CH_3)CH$_2$COOCH$_2$C$_6$H$_5$), 2.40 (d, HOCH(CH$_3$)CH_2COO-CH$_2$C$_6$H$_5$), 4.03 (h, HOCH(CH$_3$)CH$_2$COOCH$_2$C$_6$H$_5$), 4.79 (d, *H*OCH(CH$_3$)CH$_2$COOCH$_2$C$_6$H$_5$), 5.09 (s, HOCH(CH$_3$)CH$_2$COO-CH_2C$_6$H$_5$) and 7.36 (br s, HOCH(CH$_3$)CH$_2$COOCH$_2$C$_6$$H_5$).

Determination of Hydrolysis Half-Lives of PEG Active Ester—Measurements were conducted using a HP8452a UV–vis spectrophotometer. In a typical experiment, 1 mg of PEG active ester was dissolved in 3.0 mL of buffer solution and shaken promptly to obtain solution as soon as possible. Then, the solution was transferred into a UV cuvette and the absorbance at 260 nm for NHS ester or at 402 nm for *p*-nitrophenyl ester was followed as a function of time. The hydrolysis half-life was determined from the first-order kinetic plot (natural logarithm of final absorbance minus absorbance at time t versus time).

Preparation of Difunctional PEG Ester-Containing Active Carbonate—*Benzyloxy–PEG–tert-Butyl Carboxymethyl Ester* (*C$_6$H$_5$CH$_2$O–PEG–OCH$_2$COOC(CH$_3$)$_3$*—Benzyloxy-PEG 3400 (40 g, 11.7 mmol end groups) was azeotropically dried with 250 mL of toluene under N_2. After 2 h, the solution was cooled to room temperature. Potassium *tert*-butoxide (2.8 g, 23.5 mmol) in 90 mL of *tert*-butyl alcohol and 90 mL toluene was added to the aforementioned PEG solution. The mixture was stirred for 2 h at room temperature. *tert*-Butyl bromoacetate (4 mL, 26.3 mmol) was added, and the solution was stirred under N_2 at room-temperature overnight. The solution was filtered, condensed under reduced pressure, and precipitated into 300 mL of ether. The product was collected by filtration and dried under reduced pressure at room temperature.

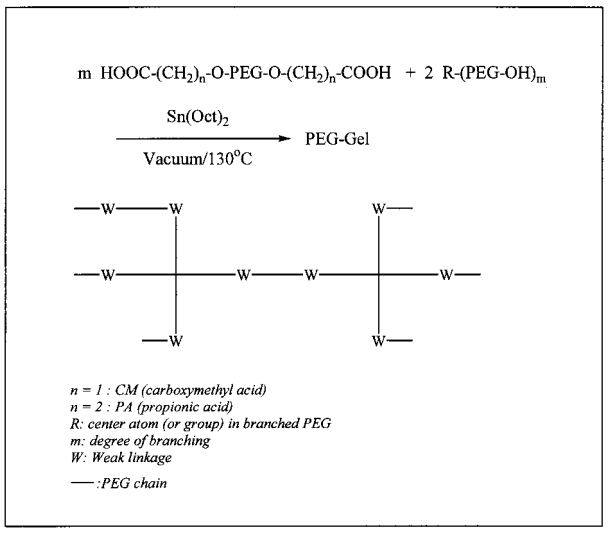
α-*Benzloxy–ω-carboxymethyl PEG* [(*C$_6$H$_5$CH$_2$O–PEG–OCH$_2$-COOH*]—Benzyloxy–PEG–*tert*-butyl carboxymethyl ester (20 g) was dissolved in distilled water and the pH was adjusted to 12.0 with 1 N NaOH solution. The solution was kept at pH 12.0 for the first 2 h by continuously adding NaOH solution (1 N) and then stirred overnight. The pH of the solution was adjusted to 2.5 by addition of 1 N HCl aqueous solution. The solution was extracted three times with methylene chloride. The combined organic phase was dried over anhydrous sodium sulfate, filtered to remove salt, condensed by rotary evaporation, and then precipitated into 250 mL of ether. The product was collected by filtration and dried under reduced pressure at room temperature to yield 18 g (90%). ^{1}H NMR (DMSO-d_6): δ 3.5 (br m, PEG), 4.01 (s, PEGOCH$_2$COOH), 4.49 (s, C$_6$H$_5$CH$_2$OPEG), 7.33 (br s, C$_6$H$_5$CH$_2$OPEG).

Benzyloxy–PEG-CM–PEG-benzyloxy (*C$_6$H$_5$CH$_2$O–PEG–OCH$_2$-COO–PEG-OCH$_2$C$_6$H$_5$*)—In a 100-mL round-bottomed flask, benzyloxy–PEG carboxymethyl acid 3400 (3.4 g, 1 mmol) in toluene was azeotropically distilled for 2 h and then cooled to room temperature. A solution of thionyl chloride (2 M, 4 mL, 8 mmol, Aldrich) in methylene chloride was injected and the mixture was stirred under N_2 overnight. The solvent was condensed by rotary evaporation and the syrup was dried under reduced pressure for ~4 h over P_2O_5 powder. To the residue was added 5 mL of anhydrous methylene chloride and azeotropically dried benzyloxy–PEG 3400 (2.55 g, 0.75 mmol) in toluene (20 mL). After benzyloxy–PEG acyl chloride was dissolved, freshly distilled triethylamine (0.6 mL) was added. The mixture was stirred overnight. The triethylamine salt was removed by filtration and the product collected by precipitation with ethyl ether. It was further purified by dissolving in water and extracting with methylene chloride. HPLC-GPC) of the product showed that 100% of benzyloxy-PEG had been converted into the PEG ester and ~15 wt % benzyloxy–PEG acid remained.

The mixture was then purified by ion-exchange chromatography (DEAE sepharose fast flow, Pharmacia) to remove the benzyloxy–PEG acid. This purification yielded 100% pure α-benzyloxy-ω-benzyloxy–PEG ester 6800 was obtained (4.1 g, 80%).

Ester-Containing PEG diol (*HO-PEG–OCH$_2$COO*–PEG-OH)—A solution of benzyloxy–PEG-CM–PEG–benzyloxy 6800 prepared in the previous step (2 g, 0.59 mmol) in 1,4-dioxane (20 mL) was hydrogenated with H_2 (2 atm pressure) and 1 g of Pd/C (10%) overnight. The catalyst was removed by filtration, and the solvent was condensed by rotary evaporation. The residual syrup was added into 100 mL of ethyl ether. The product was collected by filtration and dried under reduced pressure to yield 1.5 g (75%). ^{1}H NMR(DMSO-d_6): δ 3.5 (br m, PEG), 4.14 (s, PEGOCH_2-COOPEG), 4.18 (t, PEGOCH$_2$COOCH_2CH$_2$OPEG).

Difunctional Ester-Containing PEG-Succimidyl Carbonate (*NHS–OOCO–PEG–OCH$_2$COO–PEG–OCOONHS*)—Ester-containing PEG diol 6800 (2 g, 0.29 mmol) was azeotropically distilled with 100 mL acetonitrile and then slowly cooled to room temperature. To the resulting solution were added DSC (310 milligram, 0.58 mmol) and pyridine (0.15 mL), and the solution was stirred at room-temperature overnight. The solvent was removed under reduced pressure. The resulting solid was dissolved in 35 mL of dry methylene chloride, and the insoluble solid was removed by filtration. The filtrate was washed with pH 4.5 sodium chloride



Scheme 1

saturated acetate buffer. The organic phase was dried over anhydrous sodium sulfate, condensed by rotary evaporation, and precipitated into ethyl ether. The product was collected by filtration and dried under reduced pressure to yield 1.8 g (90%). ^{1}H NMR (DMSO-d_6): δ 3.5 (br m, PEG), 4.14 (s, PEGOCH$_2$-COOPEG), 4.18 (t, PEGOCH$_2$−COOCH$_2$CH$_2$OPEG-), 4.45 (t, −PEGOCH$_2$CH$_2$OCONHS), 2.81 (s, NHS).

Results and Discussion

1. Synthesis and Characterization—*One-Step PEG Hydrogels*—The one-step PEG hydrogels were prepared in a condensation reaction as shown in Scheme 1. The R group in this Scheme is a branched core moiety, generally a polyol that is ethoxylated to form a branched PEG (e.g., for 4-arm PEG, this core moiety is simply C, and the polyol that is ethoxylated is pentaerythritol). The PEG carboxylic acids of Scheme 1 were prepared using linear PEG (MW 2000 to 20 000) and the branched PEG alcohols were 4-arm or 8-arm (MW 2000 to 15 000). For the one-step gel, the weak linkage W in the Scheme is the ester linkage −O−(CH$_2$)n−CO$_2$−CH$_2$CH$_2$O−, where n = 1 (carboxymethylated PEG) and n = 2 (PEG propionic acid). Stannous octoate (Sn(Oct)$_2$) was chosen as a Lewis acid catalyst for hydrogel formation. This compound has been documented as having low toxicity[45,46] and is widely used in synthesis of biomaterials of polylactide and polyglycolide.[47] Also, stannous octoate is not covalently attached to the polymer chains[47] and thus can be removed when necessary. Indeed, all the one-step hydrogels were washed in glacial acetic acid to remove the catalyst. The tin content in the dry gel dropped from 0.78 wt % before purification to 0.006 wt % after purification according to ICP measurement.

The gels were characterized in terms of gel conversion, swelling degree, and water content. All gels made by this method have a high gel conversion with >92% of PEG reactants incorporated into the gel. Also these gels have high water content with the equilibrium state of being >80% water. A slight temperature dependence of equilibrium swelling was also observed.

Hydrolysis half-lives of the ester linkages were determined by the GPC method, and are listed in Table 1. The data are collected from hydrolysis of water-soluble ester-containing PEG derivatrives.[48]

As can be seen from Table 1, there is a large difference between hydrolysis rates of the PA ester and the CM ester. At the same temperature and pH, the CM ester hydrolyzes 7−10 times faster than the PA ester. The pH dependence

Table 1—Hydrolysis Half-Lives (Days, ±10%) of the Esters in 0.1 M Phosphate Buffer (pH 7.0)

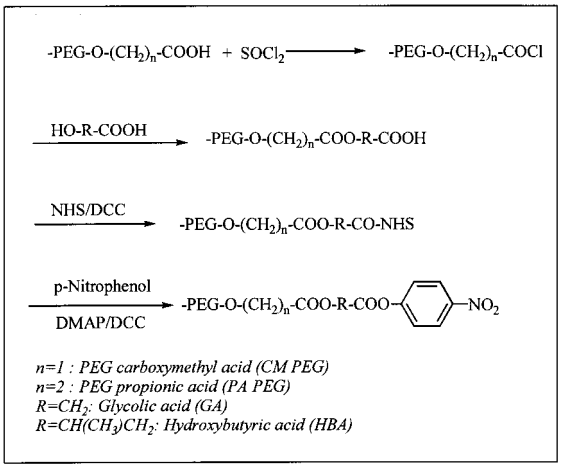
	PA ester linkage[a]			CM ester linkage[b]		
p*Ka* of the acid	4.45 ± 0.1			3.67 ± 0.05		
pH	5.5	7.0	8.1	5.5	7.0	8.1
room temp. (22–23 °C)	>500	250	37	>150	30	5
37 °C	—	43	—	—	4	—
50 °C	—	15	—	—	1.5	—

[a] PA is propionic acid. [b] CM is carboxymethylated PEG.

Table 2—Degradation Half-Lives of Esters and Dissolution Times of One-Step Hydrogels (pH 7.0, 37 °C)

		complete dissolution of the gel (days)	
gel type	$t_{1/2}$[c] (days)	4-arm PEG	8-arm PEG
PA[a]	43	25–30	60–70
CM[b]	4	2–3	5–7

[a] Ester linkage made from PEG propionic acid (PA). [b] Ester linkage made from carboxymethylated PEG (CM). [c] Hydrolysis half-lives of corresponding ester linkage measured in water-soluble compounds.



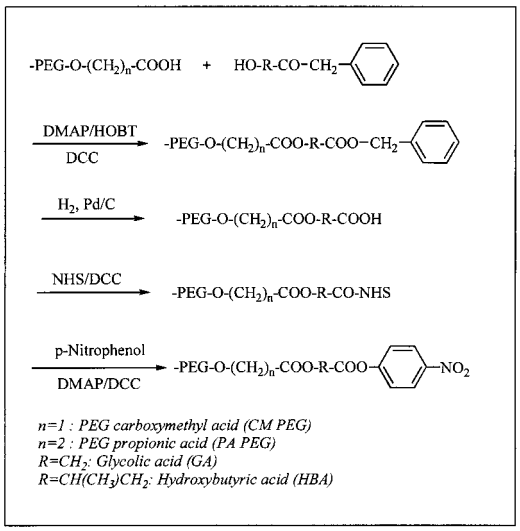
Scheme 2

of hydrolysis is also substantial; gels are most stable at pH 5.5, and hydrolysis rate increases with pH >5.5.

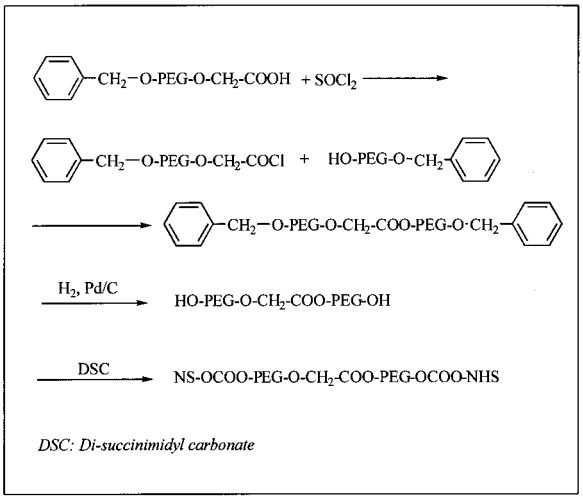
Degradation of the gels was investigated by following weight loss with time. Two distinct time periods were observed during degradation. One can be referred to as the "stable period" during which the gel maintains its shape and mass loss is slow. The other is the "unstable period" during which the gel starts to lose shape, becomes a viscous "slime", and mass loss is much faster. Table 2 gives the degradation times of the gels. Note that the degree of branching in the PEG alcohol used in preparing the gel (4-arm or 8-arm) also influences the degradation process.

Two-Step PEG Hydrogels—In preparing a two-step hydrogel, an ester-containing amine-reactive PEG is first synthesized and then coupled to an amine cross-linker. Two types of ester-containing amine-reactive PEGs were synthesized. The first type was prepared from difunctional "double-ester" PEGs, as shown in Scheme 2.

In this synthetic route (Scheme 2), PEG has only 90% of end groups converted into double ester as shown by NMR analysis. To improve the yield of the desired compound, another method was developed, as shown in Scheme 3. In this way, the double ester PEG is >99% pure according to NMR analysis.



Scheme 3



Scheme 4

As shown in the Schemes, two PEG carboxylic acids (PA and CM) and two natural metabolites (GA and HBA) make four combinations with four hydrolysis rates. The hydrolysis kinetics were determined by GPC and were reported previously.[43,48] If a protein is attached to this type of PEG compound, hydrolysis of the ester linkage leaves a small hydroxy "tag" attached to the protein.

$$\text{PEG}-\text{COO}-\text{R}-\text{COO}-\text{NHS} + \text{NH}_2-\text{protein} \rightarrow \text{PEG}-\text{COO}-\text{R}-\text{CONH}-\text{protein}$$

$$\text{PEG}-\text{COO}-\text{R}-\text{CONH}-\text{protein} \rightarrow \text{PEG-COOH} + \text{HO}-\text{R}-\text{CONH}-\text{protein}$$

The second type of ester-containing amine-reactive PEG was synthesized as illustrated in Scheme 4. This compound leaves a PEG chain attached to the protein after hydrolysis, compared with the small molecule "tag" in the previous case.

$$\text{PEG}-\text{COO}-\text{PEG}-\text{OCOO}-\text{NHS} + \text{NH}_2-\text{protein} \rightarrow \text{PEG}-\text{COO}-\text{PEG}-\text{OCONH}-\text{protein}$$

$$\text{PEG}-\text{COO}-\text{PEG}-\text{OCONH}-\text{protein} \rightarrow \text{PEG}-\text{COOH} + \text{HO}-\text{PEG-OCONH}-\text{protein}$$

The two-step hydrogels were then prepared from these PEG derivatives and multi-arm PEG amines in aqueous solution (pH 5.5–8) at room temperature.

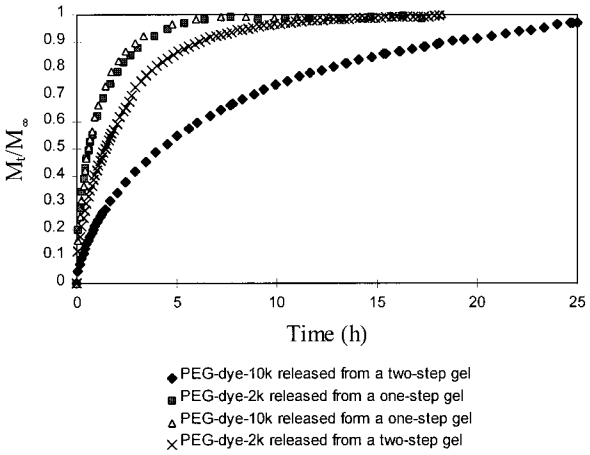


Figure 2—Release of *m*-PEG–dyes from PEG gels made from difunctional PEG 2k and 4-arm PEG 2k (±5%).

In all of the gel precursors, organic solvent residues are <100 ppm in concentration according to GC–MS analysis. The gels can be lyophylized and stored at low temperature, and then be used after reconstitution.

2. Release Studies—Release of model drugs from the hydrogels was studied. The first type of model drug was an *m*-PEG–dye, which was synthesized in our laboratory.[43] The *m*-PEG–dyes were loaded into the one-step hydrogels by a diffusion process, whereas the two-step hydrogels formed in the presence of *m*-PEG–dye. The release experiment was conducted in a circulation system at 37 °C in pH 7.0 buffer. The components of the circulation system are shown in Figure 1.

Release profiles of *m*-PEG–dyes from these hydrogels are shown in Figure 2. Compared with those from the one-step gels, the release of *m*-PEG–dyes is slower from the two-step gels. Presumably this result is due to the fact that for the two-step gels, dye is loaded throughout the gel, not just in a layer near the outer surface, as is the case with the diffusion-loaded, one-step gel.

The *m*-PEG–dyes have no active functional groups and are not covalently attached to the hydrogel networks. Because gel degradation is much slower than the release process (hours days), the release of *m*-PEG–dyes should be controlled only by diffusion. The diffusional release mechanism was supported by a linear relationship between the amount of drug released and the square root of time for the first 60% of drug to release.[43] From diffusion theory, larger molecules have smaller diffusion coefficients and diffuse more slowly. This phenomenon can be seen in Figure 2, where *m*-PEG–dyes of higher molecular weight are released more slowly. This phenomenon is more obvious in the two-step gel than in the one-step gel, probably because of the more uniform distribution of the drugs throughout the two-step gels.

The FITC-BSA was used as a protein model drug in a release study. It was loaded into the two-step hydrogels in the gel formation step. As shown in Figure 3, the release profile of FITC-BSA is approximately linear until the gel dissolved, at which point the remaining protein is released in a "burst". This type of release is totally in contrast to the diffusional release of *m*-PEG–dyes, which was shown in Figure 2. Because of the presence of amino groups, the protein is incorporated covalently into the degradable gel matrix. Therefore, its release is controlled by hydrolysis of the ester linkage in the tether that attaches the protein to the hydrogel, by degradation of the matrix, and by diffusion of unattached proteins from inside to outside of the hydrogel.

Although the initial "burst", which is commonly seen in diffusional release, disappears in this hydrolysis-controlled

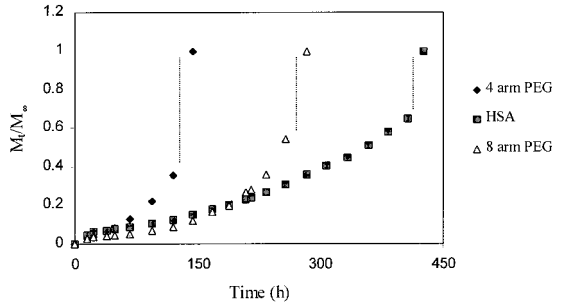


Figure 3—Release of FITC-BSA from two-step gels with different number of cross-linking (±10%). The vertical dashed lines indicate the point of gel dissolution. HAS is human serum albumin as a cross-linker.

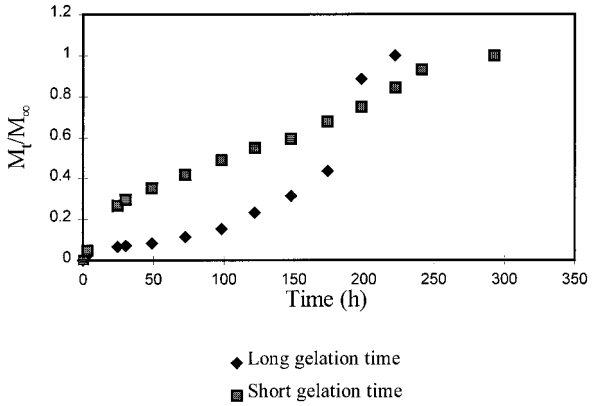


Figure 4—Release of FITC-BSA from CM-HBA gels with different gel formation times with 8-arm PEG amine 10k as cross-linker (±10%).

process, there was also an undesirable late "burst" caused by gel dissolution. If the drug can be released before gel dissolution, the late "burst" will be avoided.

Several factors can be adjusted to remove the burst release. As shown in Figure 3, increased gel degradation life helps to minimize the late burst. Reducing the degree of attachment of protein to the network is also effective. As shown in Figure 4, speeding up the time for gel formation results in fewer covalent attachments of the FITC-BSA and thus a faster release. A small portion of initial diffusional release is observed in this case. Thus it appears that if the gelation condition is appropriate, release of protein can approach zero-order kinetics.

In a two-step hydrogel, if all the protein is attached to the hydrogel network and if diffusion is much faster than the hydrolysis process, the total release profile should follow the kinetics of degradation of the tether as well as of the gels. Consequently, the release profile will be independent of gel thickness, but dependent on temperature and pH in vitro. Because drug delivery devices are of interest at physiological conditions (pH ~7 and 37 °C), release studies were conducted only at 37 °C and pH 7. In Figure 5 are shown the release profiles of FITC-BSA from two different thickness of the same two-step PEG hydrogels made from difunctional CM-HBA double-ester and 8-arm PEG. Because gel size is not a factor, it is possible with two-step hydrogel to achieve controlled release of proteins in an injectable microgel formulation. This subject will be the focus of future work.

3. Mathematical Discussion of Protein Release from the Two-Step Hydrogels—Because of the amino groups in a protein, in situ loading of protein drugs in the two-step hydrogels results in covalent attachment of protein drugs to the hydrogel network. These proteins are attached to the hydrogel through hydrolytically degradable PEG tethers, and therefore they could be released from the

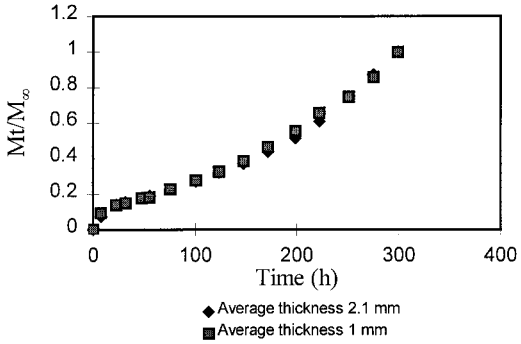


Figure 5—Release of FITC-BSA from different thickness gels (±10%).

gel by hydrolysis of the weak linkage in the tether. As shown in Figure 4, covalent attachment to the gel significantly alters release kinetics from a diffusion-controlled case, and thus this approach could be used to control release of proteins. In general, the release profile is affected by the number of attachments of protein drug, gel degradation time, and diffusion rate. The maximum number of attachment sites depends on the number of accessible reactive groups on the drug. For each drug, the number of attachments is achieved in a random fashion. However, the distribution of drug as a function of number of attachments per drug follows statistical law.

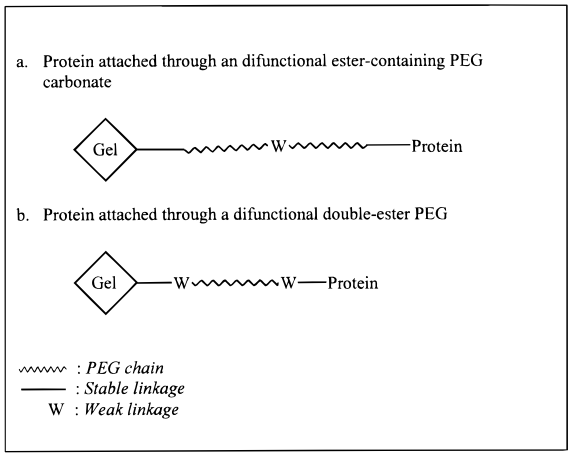
Once a protein is attached to the hydrogel, it must be released first by degradation of the linkage inside the gel and then by a diffusion process to the outside of the gel. A few papers have studied the effect of linkage cleavage on drug release.[49−52] Pitt and co-workers[53,54] mathematically analyzed drug release from a polymeric system that combined drug cleavage and diffusion. In their work, the drug was attached to the polymer through only one labile linkage, so that drug liberation followed first-order kinetics. Mathematically, Pitt and co-workers[53] analyzed the diffusion of a drug formed by first-order cleavage of drug-polymer bonds in a polymer film with the following equation:

$$\frac{\partial C(x,t)}{\partial t} = \frac{\partial}{\partial x}\left(D\,\frac{\partial C(x,t)}{\partial x}\right) + kC_0^{\mathrm{b}}\mathrm{e}^{-kt} \qquad (1)$$

where $C(x,t)$ is local concentration of free drug inside the film, D is the diffusion coefficient of the free drug, C_0^{b} is the uniform initial concentration of bound drug, k is the rate constant of the first-order hydrolysis, t is time, and x is the distance from one surface. It is important to note that bound drugs and free drug inside the film are considered as two different species.

Equation 1 describes the case in which there is only one hydrolytic weak linkage along each tether, and the bonded protein is attached through only one tether, as shown in Scheme 5a. This type of gel is formed in two-step hydrogels prepared with ester-containing PEG carbonates (the second type of ester-containing amine-reactive compound).

If two degradable linkages are present in one tether, such as in a difunctional double-ester PEG that has two esters in each PEG chain, breaking any one of them will lead to release of the protein (Scheme 5b). Note that breaking the linkage near the protein leads to release of protein with a small tag on it, whereas breaking the linkage at the far end results in protein attached with a degradable PEG chain. These two kinds of released proteins have different molecular weights and thus should have different diffusion coefficients. However, for large proteins and small PEGs, the difference in diffusion may be negligible. In this case, the diffusion of a unattached drug formed by cleavage of drug−polymer bonds in a gel film follows eq 2, which differs



Scheme 5

from eq 1 simply by multiplying k by two.

$$\frac{\partial C(x,t)}{\partial t} = \frac{\partial}{\partial x}\left(D\frac{\partial C(x,t)}{\partial x}\right) + 2kC_0 e^{-2kt} \qquad (2)$$

In a two-step gel−protein system, some proteins are attached to the hydrogel network by more than two tethers. In this case, the diffusion of an unattached protein formed by cleavage of drug−polymer bonds in such a gel film can be described as in eq 3.

$$\frac{\partial C(x,t)}{\partial t} = \frac{\partial}{\partial x}\left(D\frac{\partial C(x,t)}{\partial x}\right) + \sum_{n=1} nmkC_0^{bn} e^{-mkt}(1 - e^{-mkt})^{n-1} \qquad (3)$$

where C_0^{bn} is the initial concentration of protein bonded with n PEG tethers, and m is the number of weak linkages along each tether. In the two-step gels, m is either 1 or 2.

Solution of eq 3 can be complicated. However, if diffusion of protein is much faster than hydrolysis, the release profile can be simplified into eq 4. In obtaining eq 4, it was assumed that the amount of drug generated from cleavage of the polymer−drug bonds is negligible during the release of initial free drugs, and it was assumed that diffusivity of the drug and gel thickness do not change during the initial diffusion period.

$$M_t = M_0^0\left(1 - \frac{8}{\pi^2}\sum_{i=0}^{\infty}\frac{1}{(2i+1)^2}\exp\left(-\frac{(2i+1)^2\pi^2 Dt}{L^2}\right) + \sum_{n=1} M_0^{bn}(1 - e^{-mkt})^n \qquad (4)$$

Here, M_0^0 is the initial amount of free protein and M_0^{bn} is the initial amount of protein attached by n PEG tethers.

If the initial concentration of different types of proteins is known, the release profile can be simulated according to eq 4. Figure 6 shows the simulation results of release profiles of proteins with different numbers of attachments from a double-ester gel, in which diffusion is a much faster process than hydrolysis of weak linkages and degradation of gel.

If gel degradation is taken into consideration, the release profile needs to be reevaluated. For example, if the gel starts to dissolve from 200 to 280 h, curve A will become more like the experimental result shown in Figure 4 (short gelation time). Similarly, curve B will become very close to the release profile of the long gelation time of Figure 4 if the gel dissolves sharply from 150 to 200 h.

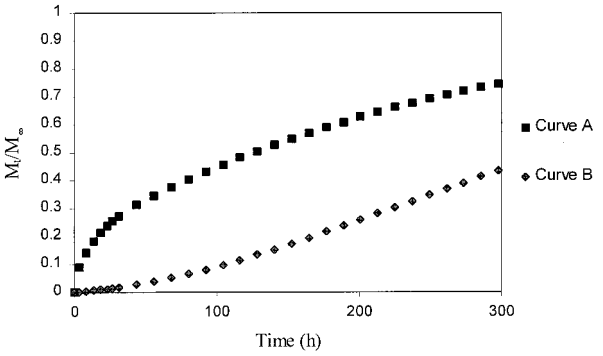


Figure 6—Computer simulation of release profile of proteins from a two-step hydrogel. Curve A: assuming 20% free protein, 70% single-PEG attached protein, and 10% double-PEG attached protein; Curve B: assuming no free protein, 10% single-PEG attached protein, 60% double-PEG attached protein, and 40% triple-PEG attached protein.

By means of computer simulation, release profiles of FITC-BSA that was loaded in situ into the two-step gels can be theoretically evaluated. However, it is still difficult to precisely predict the release of the proteins without doing release experiments. The big hurdle in prediction is to obtain the precise distribution of proteins as a function of the number of attachments.

Conclusion and Future Work

A series of degradable PEG hydrogels have been developed with excellent potential to serve as drug delivery carriers. These gels are hydrolytically degradable, and the degradation products are nontoxic PEG derivatives. The first type of hydrogel is prepared by simple one-step polycondensation of difunctional PEG acid and branched PEG polyol. The second type is prepared in a two-step process. In the first step, amine-reactive, ester-containing PEG derivatives were synthesized. These derivatives react with branched PEG amines to form a gel in the second step. These hydrogels can release physically entrapped drugs in a sustained manner by a diffusion mechanism. In addition, the two-step gels can covalently link protein drugs to the gel network, and therefore release of the drug is controlled by degradation of the linkages as well as by diffusion. Mathematical analysis shows that covalent attachment provides control of protein release. Because release of attached proteins is independent of dimension, two-step gels can be formulated into injectable microgels, which will be studied in the future. Also, the stability of proteins in terms of conformational integrity and bioactivity during both incorporation and release will be investigated.

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