Biodegradable Hydrogels for Time-Controlled Release of Tethered Peptides or Proteins

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Tethering drug substances to a gel network is an effective way of controlling the release kinetics of hydrogel-based drug delivery systems. Here, we report on in situ forming, biodegradable hydrogels that allow for the covalent attachment of peptides or proteins. Hydrogels were prepared by step-growth polymerization of branched poly(ethylene glycol). The gel strength ranged from 1075 to 2435 Pa; the degradation time varied between 24 and 120 h. Fluorescence recovery after photobleaching showed that fluorescently labeled bovine serum albumin (FITC-BSA) was successfully bound to the gel network during gel formation. Within 168 h, the mobility of the tethered molecules gradually increased due to polymer degradation. Using FITC-BSA and lysozyme as model proteins, we showed the potential of the developed hydrogels for time-controlled release. The obtained release profiles had a sigmoidal shape and matched the degradation profile very well; protein release was complete after 96 h.

Introduction

Over the past decades, hydrogels have been extensively used as matrices for controlled drug delivery and tissue engineering applications.[1–6] The popularity of these materials is based on their enormous chemical versatility, which allows for the design of a broad range of hydrogels with varying properties.[1–4] Furthermore, hydrogel-based materials generally show excellent biocompatibility because of their physicochemical similarity to the native extracellular matrix.[1–4] Finally, compared to other commonly used biomaterials, such as poly(lactic acid) or poly(lactide-*co*-glycolide), hydrophilic polymers are well suited for the entrapment of biomolecules due to their lack of hydrophobic interactions, which can affect the activity of these fragile species.[2,3]

Despite this multitude of advantageous characteristics, hydrogels also have several limitations. As a result of their high water content, most hydrogels usually restrict the mobility of encapsulated peptides or proteins only moderately. Consequently, a large amount of the incorporated molecules will be released within a few hours, which illustrates the necessity for more sophisticated strategies to prolong drug release.[1,2,5] Adjusting the cross-linking density is a common approach to reduce gel permeability and slow down the release kinetics. However, this approach is only effective in controlling the release of relatively large molecules, such as proteins with molecular weights of several thousand Daltons. Furthermore, increasing the cross-linking density often results in decreased hydrophilicity and hence decreased biocompatibility.[2] Tethering drug substances to the gel network via degradable anchor groups would be a further strategy to effectively control drug release. In these systems, the release kinetics is ideally controlled by the degradation of the anchor group while drug diffusivity only plays a secondary role.[1,2,5,7,8] However, the drug molecules often need to be chemically modified in order to allow for their covalent attachment, which makes these drug delivery systems highly complex.[9–11] Furthermore, drug conjugation may also decrease bioactivity, especially when peptides or proteins are the target therapeutics.[2] In addition, the anchor groups have to be carefully designed in order to avoid unwanted side-effects during degradation in vivo.[2]

In this paper, we report the preparation and characterization of in situ cross-linkable, biodegradable hydrogels for time-controlled release of peptides or proteins. For this purpose, branched poly(ethylene glycol) (PEG) was functionalized with aromatic succinimidyl carbonate groups that readily react with amino groups of other polymers, peptides, or proteins under formation of biodegradable carbamate bonds. Aromatic succinimidyl carbonates have been originally described as linking groups for the reversible PEGylation of proteins.[12,13] While extensively applied for the preparation of soluble protein prodrugs, this chemistry has never been used for the formation of hydrogels. For the preparation of covalently cross-linked hydrogels, PEG-succinimidyl carbonates were reacted with branched PEG-amines (Figure 1A). Based on previous studies,[14,15] the reactivity of primary amines toward succinimidyl carbonate groups was expected to be influenced by their chemical environment, resulting in different gel formation kinetics for PEG chains terminated with different amino groups (e.g., amino groups of alanine, 6-aminohexanoic acid, and lysine). These amino acid moieties were chosen to represent the different amino groups available in proteins (amino terminus and ε-amino groups of lysine residues). Moreover, the chemical environment within the N-substituent may also influence the degradation rate of carbamate bonds.[14,15] During gelation, dissolved peptides or proteins are expected to be immobilized within the gel network via pendant PEG chains (Figure 1). This will improve handling and flexibility of the drug delivery system, since any peptide or protein could be incorporated without requiring chemical modifications of these molecules. Similar to soluble prodrugs, the anchoring PEG chains are cleaved during gel degradation, and the native peptide or protein will be released into solution (Figure 1B).[12,13,16,17] Nondegradable gels (prepared from aliphatic PEG-succinimidyl carbonates and PEG-amines) served as a control group. The prepared hydrogels were characterized with respect to their mechanical properties, swelling capacities,

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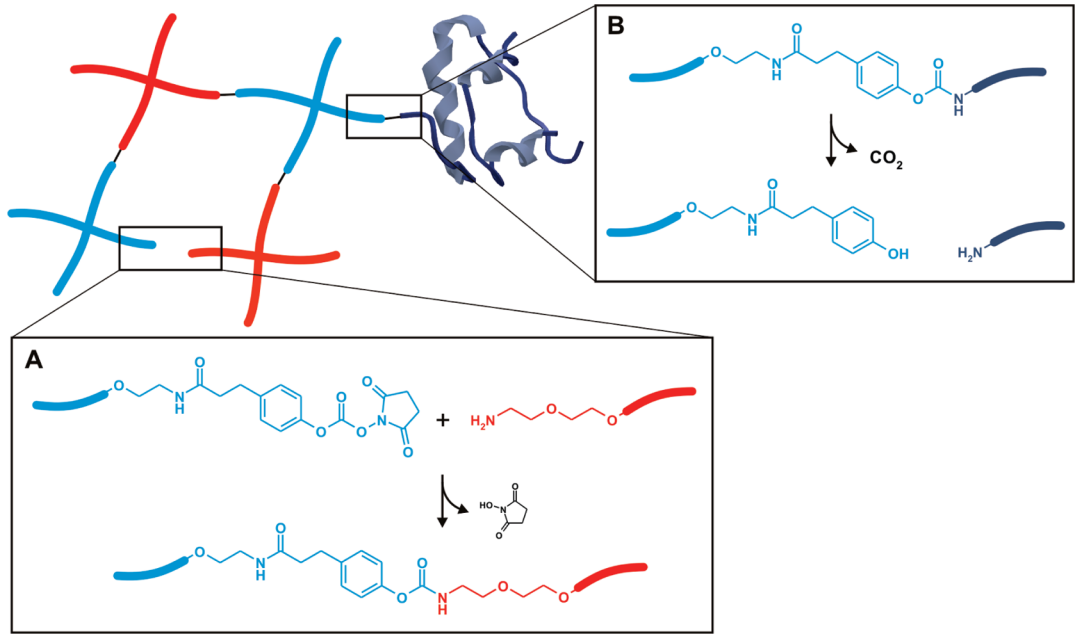

Figure 1. Schematic illustration of biodegradable hydrogels for time-controlled release of tethered peptides or proteins. For gel formation, branched PEG-succinimidyl carbonates are reacted with branched PEG-amines (A). The same chemistry allows for the immobilization of dissolved peptides or proteins via pendant PEG chains. During gel degradation, these anchoring PEG-chains are cleaved, and the unmodified peptide or protein will be released into solution (B).

and degradation rates. To prove their suitability as potential drug delivery systems, the gels were loaded with fluorescein isothiocyanate (FITC) labeled bovine serum albumin (BSA) and lysozyme as model proteins. The successful immobilization and subsequent liberation of FITC-BSA was investigated by fluorescence recovery after photobleaching (FRAP) experiments. And finally, the in vitro release kinetics of FITC-BSA and lysozyme were determined.

Experimental Section

Materials. Hexane, 3-(4-hydroxyphenyl)propionic acic, methylene chloride (DCM), and phthalimide were purchased from Acros Organics (Geel, Belgium). Boc-protected alanine (Boc-Ala-OH), Boc-6-aminohexanoic acid, and di-Boc protected lysine dicyclohexylammonium salt (Boc-Lys(Boc)-OH·DCHA) were obtained from Bachem GmbH (Weil am Rhein, Germany). Deuterated chloroform ($CDCl_3$) and deuterated dimethyl sulfoxide (DMSO-d_6) were obtained from Deutero GmbH (Kastellaun, Germany). Phosphate-buffered saline (PBS) was purchased from Invitrogen GmbH (Karlsruhe, Germany). Four-armed poly(ethylene glycol) (10 kDa molecular weight, 4armPEG10k-OH) was purchased from Nektar Therapeutics (Huntsville, AL). All SDS-PAGE reagents were obtained from Serva Electrophoresis GmbH (Heidelberg, Germany). Acetonitrile, Coomassie brilliant blue G-250, *N*,*N*′-dicyclohexylcarbodiimide (DCC), fluorescein isothiocyanate (FITC)-labeled bovine serum albumin (BSA), FITC-dextran (150 kDa molecular weight), *N*-hydroxysuccinimide (HOSu), diisopropyl azodicarboxylate, *N*,*N*′-diisopropylethylamine (DIPEA), lysozyme (from chicken egg white), methoxy poly(ethylene glycol) (2 kDa molecular weight, mPEG2k-OH) Sigmacote, and *N*,*N*′-discuccinimidyl carbonate (DSC) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Ethanol, ethyl acetate, and diethyl ether were of technical grade and used without further purification. All other chemicals were of analytical grade and purchased from Merck KGaA (Darmstadt, Germany). Deionized water was obtained using a Milli-Q water purification system from Millipore (Schwalbach, Germany).

Synthesis of PEG-Amines. Branched PEG-amines (10 kDa molecular weight, 4armPEG10k-NH_2) were synthesized as previously described.[18] First, phthalimido-PEGs were obtained by reaction of 4armPEG10k-OH, phthalimide, triphenylphosphine, and diisopropyl azodicarboxylate. The phthalimido groups were then converted into primary amines by hydrazinolysis. Methoxy PEG-amines (2 kDa molecular weight, mPEG2k-NH_2) were synthesized as described for 4armPEG10k-NH_2.

Synthesis of Aliphatic PEG-Succinimidyl Carbonates (4armPEG10k-SC). A total of 2.0 g of 4armPEG10k-OH (0.2 mmol) and 0.82 g of DSC (3.2 mmol) were dissolved in 20 mL of dried acetonitrile. Then, 520 μL of pyridine (6.4 mmol) were added, and the reaction mixture was stirred overnight at room temperature. The next day, the solvent was evaporated under reduced pressure, and 20 mL of methylene chloride (DCM) were added to the residue. The insoluble residue was filtered off, and the solution was concentrated under reduced pressure. The product was crystallized at 0 °C under vigorous stirring by dropwise addition of diethyl ether. For further purification, the crystallization step was repeated. The precipitate was collected by filtration, washed with cold diethyl ether, and dried under vacuum to yield 1.9 g (90%) of 4armPEG10k-SC (Figure 2A). ^{1}H NMR (4armPEG10k-SC, DMSO-d_6, 600 MHz): δ 2.80 ppm (s, 16H, $-C(O)CH_2CH_2C(O)-$), 3.30 ppm (s, 8H, R_3CCH_2O-), 3.50 ppm (s, $-OCH_2CH_2O-$), 3.69 ppm (t, 8H, $-OCH_2CH_2OC(O)O-$), 4.39 ppm (t, 8H, $-OCH_2CH_2OC(O)O-$).

Synthesis of Aromatic PEG-Succinimidyl Carbonates (4armPEG10k-dTyr-SC). In the first step, 0.67 g of 3-(4-hydroxyphenyl)propionic acid (4.0 mmol), 0.46 g of HOSu (4.0 mmol), and 0.83 g of DCC (4.0 mmol) were dissolved in 20 mL of dried 1,4-dioxane and stirred overnight at room temperature. The next day, the reaction mixture was passed through a glass filter funnel to remove precipitated *N*,*N*′-dicyclohexylurea (DCU), combined with a solution of 4armPEG10k-NH_2 (5.0 g, 0.5 mmol) and $NaHCO_3$ (0.19 g, 2.2 mmol) in water (20 mL), and stirred overnight at 50 °C. Afterward, the solvent was evaporated and the residue was dissolved in water. The raw product was then extracted with DCM. The combined organic phases were dried over anhydrous Na_2SO_4, and the solution was concentrated under reduced pressure. The intermediate was crystallized at 0 °C under vigorous stirring by dropwise addition of diethyl ether. The precipitate was collected by filtration, washed with cold diethyl ether, and dried under vacuum to yield 5.1 g (96%) of 4armPEG10k-dTyr. Thin layer

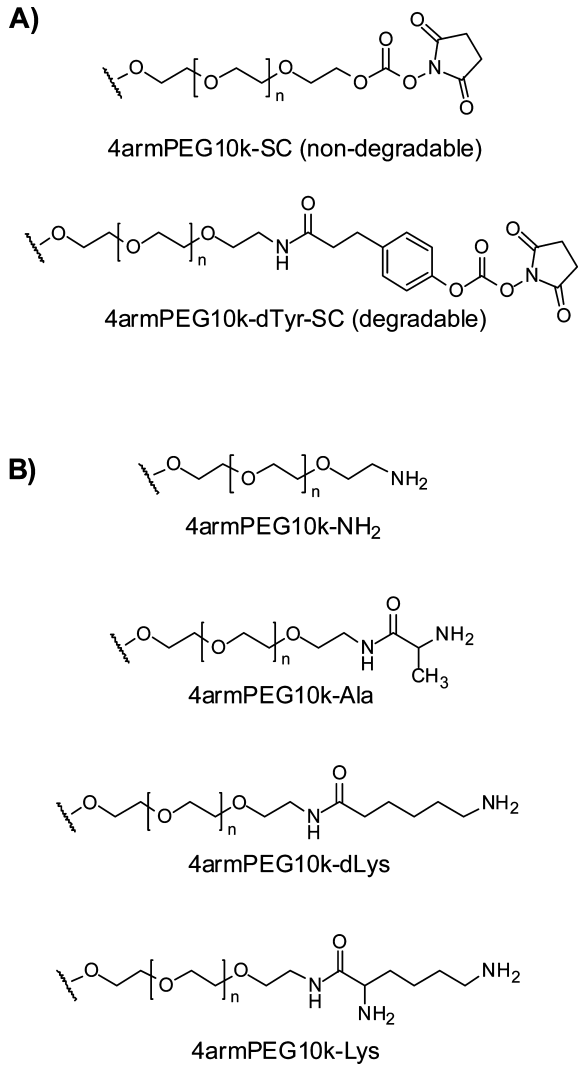

Figure 2. Polymers derived from branched poly(ethylene glycol). Amine-reactive PEG-succinimidyl carbonates (A) and amino acid-modified PEG-amines (B).

chromatography (TLC) indicated that the product was free of 4armPEG10k-NH_2, 3-(4-hydroxyphenyl)propionic acid, and HOSu. ^{1}H NMR (4armPEG10k-dTyr, $CDCl_3$, 600 MHz): δ 2.43 ppm (t, 8H, $-C(O)CH_2CH_2-$), 2.87 ppm (t, 8H, $-C(O)CH_2CH_2-$), 3.41 ppm (s, 8H, R_3CCH_2O-), 3.64 ppm (s, $-OCH_2CH_2O-$), 6.13 ppm (t, 4H, $-NHC(O)-$), 6.76 ppm (d, 8H, $-C_6H_4OH$), 7.03 ppm (d, 8H, $-C_6H_4OH$).

The phenolic hydroxyl groups were then converted into amine-reactive succinimidyl carbonate groups. For a typical synthesis, 5.3 g of 4armPEG10k-dTyr (0.5 mmol) and 2.05 g of DSC (8.0 mmol) were dissolved in 50 mL of dried acetonitrile. Then, 1300 μL of pyridine (16.0 mmol) were added, and the reaction mixture was stirred overnight at room temperature. The next day, the solvent was evaporated under reduced pressure, and 50 mL of DCM were added to the residue. The insoluble residue was filtered off, and the solution was concentrated under reduced pressure. The product was crystallized at 0 °C under vigorous stirring by dropwise addition of diethyl ether. For further purification, the crystallization step was repeated. The precipitate was collected by filtration, washed with cold diethyl ether, and dried under vacuum to yield 5.2 g (93%) of 4armPEG10k-dTyr-SC (Figure 2A). ^{1}H NMR (4armPEG10k-dTyr-SC, $CDCl_3$, 600 MHz): δ 2.48 ppm (t, 8H, $-C(O)CH_2CH_2-$), 2.88 ppm (s, 16H, $-C(O)CH_2CH_2C(O)-$), 2.98 ppm (t, 8H, $-C(O)CH_2CH_2-$), 3.41 ppm (s, 8H, R_3CCH_2O-), 3.64 ppm (s, $-OCH_2CH_2O-$), 6.36 ppm (t, 4H, $-NHC(O)-$), 7.17 ppm (d, 8H, $-C_6H_4OSu$), 7.26 ppm (d, 8H, $-C_6H_4OSu$).

Synthesis of Methoxy PEG-Succinimidyl Carbonates (mPEG2k-dTyr-SC). mPEG2k-dTyr-SC was synthesized in a similar manner to that of 4armPEG10k-dTyr-SC with a 85% yield. ^{1}H NMR (mPEG2k-dTyr-SC, $CDCl_3$, 600 MHz): δ 2.48 ppm (t, 2H, $-C(O)CH_2CH_2-$), 2.88 ppm (s, 4H, $-C(O)CH_2CH_2C(O)-$), 2.98 ppm (t, 2H, $-C(O)CH_2CH_2-$), 3.38 ppm (s, 3H, H_3CO-), 3.64 ppm (s, $-OCH_2CH_2O-$), 6.43 ppm (t, 1H, $-NHC(O)-$), 7.18 ppm (d, 2H, $-C_6H_4OSu$), 7.26 ppm (d, 2H, $-C_6H_4OSu$).

Synthesis of Alanine-Modified PEG-Amines (4armPEG10k-Ala). In the coupling step, 1.51 g of Boc-Ala-OH (8.0 mmol) and 0.83 g of DCC (4.0 mmol) were dissolved in 20 mL of dried DCM. After stirring for 30 min at room temperature, the solution was passed through a glass filter funnel to remove precipitated DCU and combined with a solution of 4armPEG10k-NH_2 (5.0 g, 0.5 mmol) in dried DCM (50 mL). Then, 700 μL of DIPEA (4.0 mmol) were added, and the mixture was stirred for 1.5 h at room temperature. The solution was concentrated under reduced pressure and placed into an ice bath. The intermediate was crystallized at 0 °C under vigorous stirring by dropwise addition of diethyl ether. The precipitate was collected by filtration, washed with cold diethyl ether, and dried under vacuum to yield 5.1 g (97%) of 4armPEG10k-Ala-Boc. ^{1}H NMR (4armPEG10k-Ala-Boc, $CDCl_3$, 300 MHz): δ 1.36 ppm (d, 12H, $-CH(NHBoc)CH_3$), 1.44 ppm (s, 36H, $-OtBu$), 3.41 ppm (s, 8H, R_3CCH_2O-), 3.64 ppm (s, $-OCH_2CH_2O-$), 4.15 ppm (4H, $-CH(NHBoc)CH_3$), 5.20 ppm (4H, $-CH(NHBoc)CH_3$), 6.66 ppm (4H, $-NHC(O)-$).

To deprotect the amino acid moiety, 5.1 g of 4armPEG10k-Ala-Boc (0.5 mmol) were dissolved in 55 mL of methanolic HCl (prepared by dropping 5 mL of acetyl chloride into 50 mL of ice-cooled methanol). After stirring for 30 min at room temperature, the solvent was evaporated, and the residue was dissolved in 25 mL of water. The raw product was extracted with DCM. The combined organic phases were dried over anhydrous Na_2SO_4, and the solution was concentrated under reduced pressure. 4armPEG10k-Ala was crystallized at 0 °C under vigorous stirring by dropwise addition of diethyl ether. The precipitate was collected, washed with cold diethyl ether, and dried under vacuum to yield 4.7 g (96%) of the product (Figure 2B). ^{1}H NMR (4armPEG10k-Ala, $CDCl_3$, 600 MHz): δ 1.34 ppm (d, 12H, $-CH(NH_2)CH_3$), 3.41 ppm (s, 8H, R_3CCH_2O-), 3.64 ppm (s, $-OCH_2CH_2O-$), 7.55 ppm (t, 4H, $-NHC(O)-$).

Synthesis of 6-Aminohexanoic Acid-Modified PEG-Amines (4armPEG10k-dLys). 4armPEG10k-dLys (Figure 2B) was synthesized from 4armPEG10k-NH_2 and Boc-6-aminohexanoic acid with a 95% yield as described for 4armPEG10k-Ala. ^{1}H NMR (4armPEG10k-dLys, $CDCl_3$, 600 MHz): δ 1.36 ppm (m, 8H, $-CH_2CH_2CH_2CH_2CH_2NH_2$), 1.47 ppm (m, 8H, $-CH_2CH_2CH_2CH_2CH_2NH_2$), 1.66 ppm (m, 8H, $-CH_2CH_2CH_2CH_2CH_2NH_2$), 2.19 ppm (t, 8H, $-CH_2CH_2CH_2CH_2CH_2NH_2$), 2.70 ppm (t, 8H, $-CH_2CH_2CH_2CH_2CH_2NH_2$), 3.41 ppm (s, 8H, R_3CCH_2O-), 3.64 ppm (s, $-OCH_2CH_2O-$), 6.23 ppm (t, 4H, $-NHC(O)-$).

Synthesis of Lysine-Modified PEG-Amines (4armPEG10k-Lys). A total of 5.0 g of Boc-Lys(Boc)-OH·DCHA (9.5 mmol) was suspended in 50 mL of ethyl acetate. A solution of 10% citric acid in water was added until a clear mixture was obtained. After stirring for 30 min at 0 °C, the aqueous phase was separated and extracted with ethyl acetate. The combined organic phases were washed with water until neutral and dried over anhydrous Na_2SO_4. After evaporation of the solvent, Boc-Lys(Boc)-OH was dried under vacuum to yield 3.2 g (97%). 4armPEG10k-Lys was prepared from 4armPEG10k-NH_2 and Boc-Lys(Boc)-OH in a similar manner to that of 4armPEG10k-Ala with a 95% yield (Figure 2B). ^{1}H NMR (4armPEG10k-Lys, $CDCl_3$, 600 MHz): δ 1.32 − 1.54 ppm (m, 20H, $-CH_2CH_2CH_2CH_2NH_2$), 1.76−1.85 ppm (m, 4H, $-CH_2CH_2CH_2CH_2NH_2$), 2.69 ppm (t, 8H, $-CH_2CH_2CH_2CH_2NH_2$), 3.32 ppm (4H, $-NHC(O)CH(NH_2)-$), 3.39 ppm (s, 8H, R_3CCH_2O-), 3.61 ppm (s, $-OCH_2CH_2O-$), 7.54 ppm (4H, $-OCH_2CH_2NHC(O)-$).

Table 1. Composition of the Prepared Hydrogels[a]

group	polymer concentration	pH	amine component	PEG-succinimidyl carbonate
PEG-dLys	5%	8.0	4armPEG10k-dLys (25 mg)	4armPEG10k-dTyr-SC (25 mg)
PEG-NH_2	5%	7.4	4armPEG10k-NH_2 (25 mg)	4armPEG10k-dTyr-SC (25 mg)
PEG-Ala	5%	6.8	4armPEG10k-Ala (25 mg)	4armPEG10k-dTyr-SC (25 mg)
PEG-Lys	5%	6.4	4armPEG10k-Lys (17 mg)	4armPEG10k-dTyr-SC (33 mg)
control	5%	7.0	4armPEG10k-NH_2 (25 mg)	4armPEG10k-SC (25 mg)

[a] The amine component was dissolved in 1000 μl of 25 mM phosphate buffer and added to the PEG-succinimidyl carbonate. The stoichiometric ratio between succinimidyl carbonate and amino groups was balanced.

Preparation and Rheological Characterization of Hydrogels. Gelation kinetics and gel strength were studied by performing oscillatory shear experiments on a TA Instruments AR 2000 rheometer (TA Instruments, Eschborn, Germany) with parallel plate geometry. For the preparation of hydrogels, accurately weighed amounts of the amine component were dissolved in 1000 μL of 25 mM phosphate buffer (Table 1) and cooled to 5 °C. Immediately before starting the experiment, this polymer solution was added to 4armPEG10k-dTyr-SC (degradable gels) or 4armPEG10k-SC (nondegradable gels, Table 1). The stoichiometric ratio between succinimidyl carbonate and amino groups was balanced, and the overall polymer concentration was 5% (w/v) for all hydrogels. After vortexing, the precursor solution was poured onto the bottom plate of the rheometer, which was also cooled to 5 °C. The upper plate (20 mm in diameter) was then lowered to a gap size of 1000 μm, the temperature was raised to 25 °C, and the measurement was started. The evolution of storage (G') and loss moduli (G'') was recorded as a function of time at 1 Hz oscillatory frequency and a constant strain of 0.05. A solvent trap was used to prevent the evaporation of water. The crossover of G' and G'' was regarded as the gel point. After 90 min, the absolute value of the complex shear modulus ($G^* = G' + i \cdot G''$) was determined as a measure for the gel strength. All experiments were carried out in triplicate and the results are expressed as means ± standard deviations.

Equilibrium Swelling of Hydrogels and Determination of Network Parameters. For the swelling studies, accurately weighed amounts of the amine component were dissolved in 1000 μL of 25 mM phosphate buffer and added to the PEG-succinimidyl carbonate (Table 1). The stoichiometric ratio between succinimidyl carbonate and amino groups was balanced, and the overall polymer concentration was again 5% (w/v) for all gels. After vortexing, 250 μL of the precursor solution were cast into cylindrical glass molds (7 mm inner diameter) and allowed to gel for 2 h. The samples were then weighed in air and hexane before and after swelling for 24 h in 10 mL of PBS using a density determination kit (Mettler-Toledo, Giessen, Germany). To minimize hydrogel degradation, the samples were incubated at 5 °C. Based on Archimedes' principle, the gel volumes after cross-linking (V_{gc}) and after swelling (V_{gs}) were determined. The volume of the dry polymer (V_p) was calculated from the mass of the freeze-dried hydrogel and the density of the polymer in the dry state (taken as the density of PEG, 1.12 g·cm^{-3}). With these parameters, the polymer fraction of the gel after cross-linking, $\upsilon_{2c} = V_p/V_{gc}$, and in the swollen state, $\upsilon_{2s} = V_p/V_{gs}$, was calculated. The reciprocal of υ_{2s} is usually termed as the volumetric swelling ratio (Q).

The number of moles of elastically active chains in the hydrogel network, ν_e, was calculated using a modified version of the classical Flory−Rehner equation[19−21]

$$\nu_e = -\frac{V_p}{V_1 \upsilon_{2c}} \cdot \frac{[\ln(1-\upsilon_{2s}) + \upsilon_{2s} + \chi_1 \upsilon_{2s}^2]}{\left[\left(\frac{\upsilon_{2s}}{\upsilon_{2c}}\right)^{1/3} - \frac{2}{f}\left(\frac{\upsilon_{2s}}{\upsilon_{2c}}\right)\right]} \quad (1)$$

Here, χ_1 is the Flory−Huggins interaction parameter for PEG in water (taken as 0.43), V_1 is the molar volume of the solvent (18 cm^3·mol^{-1}), and f is the functionality of the cross-links (four in the case of four-armed PEG). Assuming a defect-free network, the average molecular weight between cross-links, $\bar{M}_c$, was calculated by $\bar{M}_c = m_p/\nu_e$, where m_p is the total mass of PEG in the hydrogel. With this parameter, the average network mesh size (ξ) was estimated by[22]

$$\xi = \upsilon_{2s}^{-1/3} l \left(\frac{2\bar{M}_c}{M_r}\right)^{1/2} C_n^{1/2} \quad (2)$$

where l is the bond length along the polymer backbone (taken as 0.146 nm), M_r is the molecular mass of the PEG repeating unit (44 g·mol^{-1}), and C_n is the Flory characteristic ratio (here taken as 4 for PEG).[23] All experiments were carried out in triplicate and the results are expressed as means ± standard deviations.

Degradation of Hydrogels. The gel samples were prepared as described above. After gelation, the initial weight of each hydrogel was determined. To study the degradation of the gel networks, the samples were immersed in 10 mL of PBS (containing 0.025% sodium azide) and incubated at 37 °C in a shaking water bath (approximately 50 rpm). Every day, the samples were poured out over 24 mm Netwell Inserts (Corning GmbH, Kaiserslautern, Germany) with a 500 μm mesh size polyester membrane and weighed. Degradation was assumed to be complete when the sample passed through the Netwell Insert and no remaining material could be detected.

Mobility of Incorporated Macromolecules Determined by Fluorescence Recovery after Photobleaching (FRAP). To investigate the mobility of incorporated macromolecules with and without amino groups, the hydrogels were loaded with FITC-BSA (66 kDa molecular weight) and FITC-dextran (150 kDa molecular weight). The samples were prepared by dissolving 12.5 mg of 4armPEG10k-NH_2 in 450 μL of 25 mM phosphate buffer, pH 7.4. Subsequently, 50 μL of the FITC-BSA or FITC-dextran stock solution (prepared in the same buffer, c = 10 mg/mL) were added. The mixture was then added to 12.5 mg of 4armPEG10k-dTyr-SC (degradable gels) or 12.5 mg of 4armPEG10k-SC (nondegradable control gels), vigorously stirred, and cast into Lab-Tek II Chambered Coverglasses (Thermo Fisher Scientific, Langenselbold, Germany). After 2 h of gelation, the samples were positioned on the microscope stage.

The FRAP experiments were performed on a Zeiss Axiovert 200 M microscope coupled to a LSM 510 META scanning device (Carl Zeiss MicroImaging GmbH, Jena, Germany). A Plan-Neofluar 20× objective lens with a numerical aperture of 0.50 was used. All bleaching experiments were performed using the 488 nm-line of a 30 mW Ar-ion laser operating at 50% output power. After the region of interest has been brought into focus, a time series of digital images with a resolution of 512 × 128 pixel was recorded using a highly attenuated laser beam (0.5% transmission). The interval between two consecutive images was 2 s. After acquisition of five prebleach images, a uniform disk with a diameter of 36 μm was bleached at maximum laser intensity (100% transmission). Immediately after bleaching, the laser intensity was switched back to the previous value (0.5% transmission), and a series of 75 images was acquired to measure the recovery of fluorescence. To extract the experimental recovery curve from the image stack, the mean fluorescence intensities inside the bleached region, $I_{frap}(t)$, and inside a reference region were calculated for each time point using the NIH software ImageJ. In the next step, $I_{frap}(t)$ was normalized

to the prebleach intensity, corrected for any bleaching effects that might have occurred during image acquisition and normalized to the full scale.

To follow changes in the macromolecular mobility during gel degradation, the samples were covered with 500 μL of PBS (containing 0.025% sodium azide) and incubated at 37 °C. At predetermined time points (t = 48, 96, and 168 h) the FRAP experiments were repeated as described above.

Reversible PEGylation of Lysozyme and Characterization by Gel Electrophoresis. A total of 250 μL of a lysozyme stock solution (prepared in 25 mM phosphate buffer, pH 7.4 at 50 mg/mL) was added to 56 mg of mPEG2k-dTyr-SC and incubated for 2 h at room temperature. The molar ratio of succinimidyl carbonate to protein amino groups was 4:1. To demonstrate the elimination of attached PEG chains, 100 μL of this solution were diluted with 900 μL of 50 mM borate buffer, pH 9.0, and incubated for 24 h at 50 °C. Nondenaturating SDS-polyacrylamide gel electrophoresis (PAGE) was used to distinguish PEG-lysozyme conjugates with varying degrees of substitution. The electrophoresis was conducted using a polyacrylamide gel of 14% cross-linking; gels were stained by a colloidal Coomassie brilliant blue G-250 dispersion.

Release of FITC-BSA and Lysozyme. The hydrogels were loaded with FITC-BSA (66 kDa molecular weight) and lysozyme (14 kDa molecular weight) to evaluate their suitability as potential drug delivery systems. These two proteins were chosen because of their different mass and different number of amino groups and serve as model compounds for therapeutic peptides or proteins. To prepare the gel samples, 25 mg of 4armPEG10k-NH_2 were dissolved in 900 μL of 25 mM phosphate buffer, pH 7.4, and mixed with 100 μL of the respective protein stock solution (prepared in the same buffer, c = 10 mg/mL). This mixture was then added to 25 mg of 4armPEG10k-dTyr-SC and vigorously vortexed. In each case, 250 μL of these precursor solutions were cast into cylindrical glass molds (7 mm inner diameter) and allowed to gel for 2 h. The protein loading was 250 μg per gel. Afterward, the gels were removed from the glass molds, immersed in 10 mL of PBS (containing 0.025% sodium azide), and maintained at 37 °C in a shaking water bath (approximately 50 rpm). All vials were treated with Sigmacote to prevent the adsorption of proteins. At specified time intervals, 500 μL of the release medium were collected and replaced with fresh buffer. Blank hydrogels without protein and protein solutions (250 μg in 10 mL of PBS) served as control groups.

The released protein amounts were determined as described by Bradford.[24] In brief, 100 μL of sampled release buffer were pipetted into a microtiter plate and incubated with 100 μL of Bradford reagent for 10 min. The protein content was quantified by measuring the absorption at 595 nm using a Shimadzu CS-9301PC 96-well plate reader (Shimadzu GmbH, Duisburg, Germany). Calibration curves were obtained from known concentrations of FITC-BSA and lysozyme. All experiments were carried out in triplicate and the results are expressed as means ± standard deviations.

Statistical Analysis. The results from mechanical testing, swelling studies, and FRAP experiments were compared using single-factor analysis of variance (ANOVA) and Tukey's multiple comparison test; $p < 0.05$ was regarded as statistically significant. Statistical analysis was performed using SigmaStat 3.0 software (Systat Software, San Jose, CA).

Results and Discussion

The aim of the present study was to prepare in situ forming hydrogels that allow for the time-controlled release of incorporated proteins. For this purpose, branched PEG-succinimidyl carbonates were synthesized (Figure 2A). These polymers react with primary amino groups of other polymers, peptides, or proteins under formation of carbamate bonds. In case of proteins, succinimidyl carbonates typically react with N-terminal amino acids and ε-amino groups of lysine residues.[25] Because bond formation and cleavage may vary depending on the chemical environment of the reacting amino groups, the polymers used for gel formation were further modified with alanine (mimicking the amino terminus of proteins), 6-aminohexanoic acid, and lysine (Figure 2B). All syntheses were straightforward, and the desired products were obtained with high yields. End-groups were almost fully converted, as indicated by ^{1}H NMR spectra.

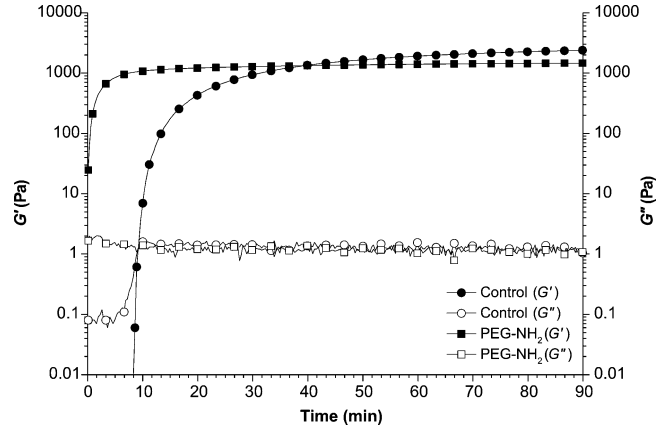

Figure 3. Typical rheograms of degradable (■/□) and nondegradable hydrogels (●/○). Both gels were prepared from 4armPEG10k-NH_2 at pH 7.4 (degradable gel) and pH 7.0 (nondegradable gel). The closed symbols (■/●) represent G', and the open symbols (□/○) represent G''.

Table 2. Gel Strength ($|G^*|$), Volumetric Swelling Ratio (Q), Molecular Weight between Cross-Links ($\bar{M}_c$), and Average Network Mesh Size (ξ) of the Prepared Hydrogels[a]

group	$\|G^*\|$ (Pa)	Q	$\bar{M}_c$ (g·mol^{-1})	ξ (nm)
PEG-dLys	1075 ± 68	36.8 ± 0.5	10477 ± 202	21.2 ± 0.3
PEG-NH_2	1486 ± 132	34.7 ± 0.1	9300 ± 82	19.6 ± 0.1
PEG-Ala	2137 ± 55	32.2 ± 0.3	7905 ± 123	17.6 ± 0.2
PEG-Lys	2435 ± 105[b]	30.9 ± 0.3[b]		
control	2429 ± 73[b]	30.6 ± 0.8[b]	6946 ± 122	16.3 ± 0.2

[a] Data are presented as means ± standard deviations (n = 3). The differences in the mean values are statistically significant ($p < 0.05$). [b] Statistically not significant ($p > 0.05$).

Physicochemical Characterization of Hydrogels. Hydrogels were prepared by step-growth polymerization of branched, amino acid-modified PEG-amines with branched PEG-succinimidyl carbonates. As it was expected, gelation kinetics and gel strength were strongly dependent on the polymers used for gel preparation. In preliminary tests, the buffer pH value was therefore adjusted for each gel type (Table 1). When the buffer pH value was too low, polymerization was slow and the gel strength remained comparatively low. On the other hand, if the pH value was too high, the gels solidified immediately and could no longer be placed on the rheometer. Although the pH values used for gel preparation (pH 6.4 to 8.0) differed in part from the physiological value, the prepared hydrogels would still be applicable without causing tissue damage.[26]

Nondegradable hydrogels were prepared from 4armPEG10k-SC and 4armPEG10k-NH_2 at pH 7.0. At the beginning of the experiment, the sample behaved like a free-flowing liquid ($G'' > G'$); gelation occurred after 9.2 ± 0.4 min. During the course of the experiment, cross-linking further proceeded as indicated by the steadily increasing value of G' (Figure 3). After 90 min, the value of G' exceeded that of G'' by several orders of magnitude ($G' \gg G''$); the gel strength was 2429 ± 73 Pa (Table 2). These data agreed very well with those reported previously for gels prepared from branched PEG-succinimidyl propionates and PEG-amines.[18] For the preparation of biodegradable gels, 4armPEG10k-NH_2 was polymerized with 4armPEG10k-dTyr-

SC at pH 7.4. These samples solidified in less than 1 min and the gel strength was 1486 ± 132 Pa (Table 2). Compared to 4armPEG10k-SC, the reactivity of 4armPEG10k-dTyr-SC seemed to be considerably increased (Figure 3). This could explain both the accelerated polymerization as well as the lower strength of these gels. With increasing reactivity of succinimidyl carbonate groups, their susceptibility to hydrolysis will simultaneously increase.[13] This will result in lower cross-linking densities and, hence, reduced gel strengths. In the case of 4armPEG10k-dLys, the buffer pH value was increased to pH 8.0. This was required because of the high pK_a value of the 6-aminohexanoic acid moiety (10.6 ± 0.1). The prepared hydrogels solidified after 1.1 ± 0.2 min and the absolute value of G^* was 1075 ± 68 Pa (Table 2). However, when 4armPEG10k-Ala and 4armPEG10k-Lys were used for gel preparation, the buffer pH value was decreased to pH 6.8 and pH 6.4, respectively. Otherwise, gelation occurred within a few seconds and the samples could not be placed on the rheometer. The high reactivity of 4armPEG10k-Ala was attributed to the comparatively low pK_a value of the alanine moiety (8.2 ± 0.3). And in case of 4armPEG10k-Lys, gelation was most likely facilitated by the increased number of amino groups per macromer (8 vs 4). The stiffness was 2137 ± 55 Pa for gels prepared from 4armPEG10k-Ala and 2435 ± 105 Pa for those prepared from 4armPEG10k-Lys (Table 2).

When compared to each other, the gels prepared from 4armPEG10k-dLys had the lowest cross-linking density of all degradable hydrogels (estimated by G^*), whereas those made from 4armPEG10k-Lys showed the highest value. This corresponded well with the results of equilibrium swelling studies. The volumetric swelling ratio Q was inversely related to gel strength and was highest for hydrogels prepared from 4armPEG10k-dLys and lowest for those prepared from 4armPEG10k-Lys (Table 2). Using eqs 1 and 2, these data also allowed for the calculation of the average network mesh size (ξ). The gels with the lowest cross-linking density showed the highest value of ξ (21.2 ± 0.3 nm); hydrogels with higher stiffness and hence higher cross-linking density exhibited lower values of ξ (Table 2). For gels prepared from 4armPEG10k-Lys, the average network mesh size could not be calculated, as these gels contained macromers of two different functionalities and eq 1 can only be applied for gels containing one single type of cross-links.[19–21]

Degradation of Hydrogels. Samples were incubated in PBS at 37 °C and weighed at predetermined time points to study hydrogel degradation. As expected, the gels of the control group did not degrade within the observation period. During the first 24 h, the wet weight of these samples first increased due to swelling. However, after the nondegradable gels were swollen to equilibrium, their wet weight remained constant (Figure 4). In these networks, the individual macromers are linked together by aliphatic carbamate bonds, which are stable under the experimental conditions.[14] In degradable gels, however, the polymer chains were held together by aromatic carbamate groups. These carbamates hydrolyze in neutral and basic solutions by an E1cB elimination reaction involving the intermediate formation of an unstable isocyanate.[14,15] The isocyanate readily reacts with water and disintegrates into a primary amine and carbon dioxide (see Figure 1 for comparison).[14,15]

During the initial phase of the study, the wet weight of the degradable samples first increased (Figure 4). The hydrolysis of carbamate groups obviously enlarged the average network mesh size of these hydrogels, which resulted in an increased swelling capacity. After a critical amount of bonds had been cleaved, the wet weight decreased and the gels dissolved slowly. Interestingly, the two gels prepared from 4armPEG10k-Lys and 4armPEG10k-Ala disintegrated within the first 48 h. This was not expected, as these hydrogels had the highest cross-linking density of all biodegradable gels. In contrast to that, the hydrogels containing 4armPEG10k-NH_2 and 4armPEG10k-dLys were stable over 72 and 96 h, respectively, and dissolved within the following 24 h (Figure 4).

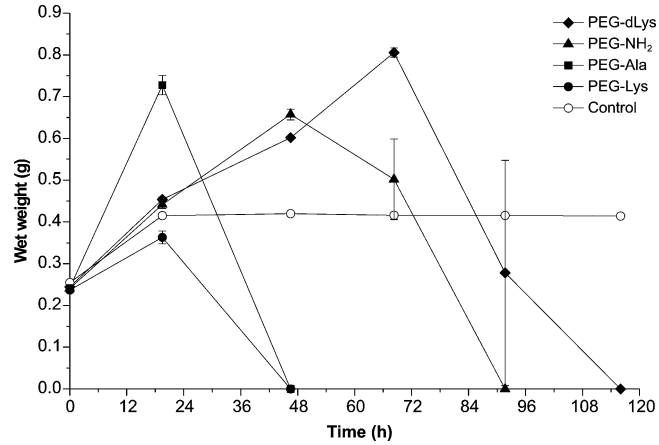

Figure 4. Degradation of hydrogels. Gels prepared from 4armPEG10k-Lys (●) and 4armPEG10k-Ala (■) degraded within 48 h. Hydrogels made from 4armPEG10k-NH_2 (▲) and 4armPEG10k-dLys (♦) disintegrated over 4 and 5 days, respectively. The nondegradable gels (○) remained stable over the observation period. The experiments were carried out in triplicate and the results are presented as means ± standard deviations.

Obviously, the nature of the amino groups not only influenced gelation kinetics and gel strength but also affected degradation rate. It is known from the literature that the reactivity of phenyl carbamates increases with increasing polar effects within the *N*-substituent. Phenyl carbamates of amino acid amides or dipeptides, for example, were hydrolyzed within a few hours. Phenyl *N*-ethylcarbamates, however, showed a half-life of several days.[15] The same effects could account for the degradation profiles of the different hydrogels. In case of 4armPEG10k-Lys and 4armPEG10k-Ala, the amino group is in close proximity to an amide linkage, which makes the resulting carbamates more susceptible to hydrolysis. In contrast to that, the chemical structures of 4armPEG10k-NH_2 and 4armPEG10k-dLys resemble those of phenyl *N*-ethylcarbamates, which can explain the better hydrolytic resistance of the corresponding hydrogels.

As a compromise between both, gel strength and degradation time, hydrogels prepared from 4armPEG10k-NH_2 were chosen for all further experiments. In future studies it may be also possible to prolong the degradation time by introducing strongly electropositive carboxylic acid moieties into the *N*-substituent, because these groups were reported to increase the hydrolytic resistance of phenyl carbamates.[15]

Mobility of Incorporated Macromolecules. To show their suitability as a drug delivery system, the developed hydrogels were loaded with FITC-BSA (66 kDa molecular weight) and FITC-dextrans (150 kDa molecular weight). The hydrodynamic diameters of these two macromolecules were 7.2[27] and 16.6 nm,[28] respectively. When comparing these values with the average network mesh sizes of nondegradable (16.3 nm) and degradable gels (prepared from 4armPEG10k-NH_2, 19.6 nm), the diffusivity of both macromolecules should be restricted only to a minor extent. The diffusivity of the incorporated FITC-

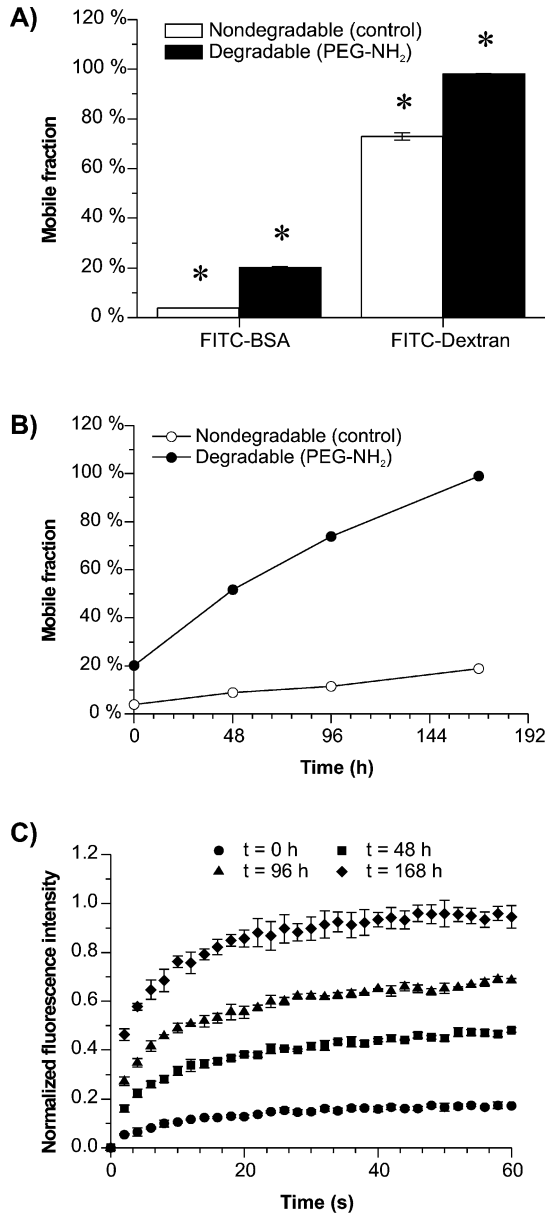

Figure 5. Mobile fractions of FITC-dextran and FITC-BSA in nondegradable (□) and degradable (■) hydrogels directly after cross-linking (A). *Indicates statistical significance ($p < 0.05$). Mobile fractions of FITC-BSA in nondegradable (○) and degradable (●) hydrogels over time (B). Mobility of FITC-BSA in degradable hydrogels (C). The recovery profiles were acquired 0 (●), 48 (■), 96 (▲), and 168 h (♦) after cross-linking.

dextran served as benchmark and allowed for the evaluation of the successful immobilization of FITC-BSA.

Directly after cross-linking, 73% of the FITC-dextran molecules incorporated into nondegradable hydrogels were mobile. In the case of degradable gels, even 98% of the incorporated FTIC-dextrans were mobile (Figure 5A). Due to the lack of amino groups, FITC-dextrans cannot be bound to the gel network by reaction with PEG-succinimidyl carbonates. The obtained results further indicate that incorporated macromolecules will not be immobilized within the gel network by physical entrapment. In FITC-BSA-loaded hydrogels, however, the situation was different. Directly after hydrogel preparation, most of the incorporated protein molecules were immobilized within the gel network. The mobile fractions were 4% in nondegradable gels and 20% in degradable hydrogels (Figure 5A). Because the hydrodynamic diameter of FITC-BSA was well below the average network mesh size of the prepared gels, it was concluded that the protein molecules were successfully immobilized by covalent attachment to the hydrogel backbone. This demonstrates the general potential for tethering therapeutic peptides or proteins to the gel backbone by simply dissolving them together with the gel-forming polymers. Hydrogel cross-linking and drug loading can be performed simultaneously without the need for additional synthetic steps.

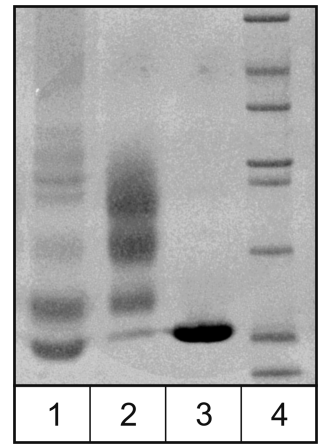

Figure 6. Elimination of PEG chains from PEGylated lysozyme. Lane 1, PEGylated lysozyme after hydrolysis for 24 h at pH 9.0 and 50 °C; lane 2, modified lysozyme directly after PEGylation; lane 3, native lysozyme; lane 4, molecular weight marker (6.5, 14.2, 20, 24, 29, 36, 45, and 66 kDa).

To follow the mobility of FITC-BSA over time, the gel samples were covered with PBS. In nondegradable hydrogels, protein mobility did not change substantially during the observation period. After 168 h of incubation, approximately 20% of the incorporated protein molecules seemed to be mobile (Figure 5B). This increase can be attributed to hydrolysis of aliphatic carbamate groups, slow degradation of the protein or release of the fluorescence label. In degradable hydrogels, however, FITC-BSA mobility gradually increased (Figure 5B,C). After 168 h, when the hydrogel was completely degraded, all protein molecules were mobile. The lower degradation rate most likely results from differences in the experimental setup (see Figure 4 for comparison). The FRAP experiments indicate that the developed hydrogels would allow for the time-controlled release of therapeutic peptides or proteins. As a result of the degradation mechanism, encapsulated peptides or proteins are expected to be released in the unaltered state. This would be an advantage over previous drug delivery systems,[8] as it preserves bioactivity and lowers the risk of unwanted immune reactions.[29,30]

Elimination of PEG Chains from PEGylated Lysozyme. To demonstrate the complete reversibility of protein tethering, lysozyme was PEGylated with a monofunctional derivative of 4armPEG10k-dTyr-SC (PEGylation using mPEG2k-dTyr-SC). Directly after PEGylation, most protein molecules were carrying 3−5 PEG chains, as indicated by SDS-PAGE (Figure 6, lane 2). However, after incubation for 24 h at pH 9.0 and 50 °C, native lysozyme as well as slightly PEGylated protein molecules could be detected (Figure 6, lane 1). Together with the proposed mechanism of carbamate degradation,[14,15] these findings clearly demonstrate the complete reversibility of protein modifications using aromatic PEG-succinimidyl carbonates. During gel degradation, the tethered protein molecules will be detached from their anchoring PEG chains in a similar manner; released

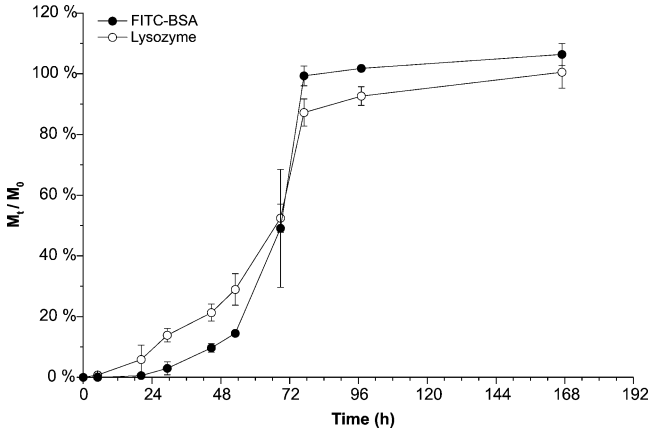

Figure 7. Release of FITC-BSA (●) and lysozyme (○) from biodegradable hydrogels. Data are presented as means ± standard deviations ($n = 3$).

peptides or proteins will not carry substantial amounts of residual polymer, which is an advantage over previously reported approaches.[8]

Release of FITC-BSA and Lysozyme. In the final experiment, the release of FITC-BSA and lysozyme was quantified. As expected from degradation studies and FRAP experiments, almost no FITC-BSA was released during the first 24 h (Figure 7). With the onset of gel degradation, however, more and more protein was released into solution. The obtained release profile had a sigmoidal shape and matched the degradation profile very well. After 96 h, the release of FITC-BSA was completed. In FRAP experiments, only 75% of the incorporated protein molecules were mobile after the same time period. These differences are most likely due to the different amounts of PBS used for FRAP and release experiments (500 μL vs 10 mL of PBS). Compared to FITC-dextrans, which were released from similar gels almost completely within 24 h,[18] the covalent attachment considerably prolonged the release of incorporated protein molecules. In addition to FITC-BSA, the hydrogels were also loaded with lysozyme. In general, the resulting release profile was similar to that of FITC-BSA (Figure 7). During the initial phase, however, the release of lysozyme was significantly higher. After 54 h, approximately 30% of the total amount of lysozyme was released into solution. In the case of FITC-BSA, however, only 15% of the incorporated protein molecules were released at the same time point. Furthermore, the release profile of lysozyme was almost linear during the first 54 h.

These variations are explained by the different characteristics of the encapsulated proteins. In the case of lysozyme, one protein molecule can be bound to the gel network by a maximum of seven amino groups (amino terminus and ε-amino groups of lysine residues). Bovine serum albumin, in contrast, is bearing 60 amino group (amino terminus and ε-amino groups of lysine residues) that can theoretically react with the hydrogel backbone. The probability that one protein molecule is detached from the polymer network is therefore much higher for lysozyme than for FITC-BSA. Furthermore, the lower mass of lysozyme (14 kDa vs 66 kDa) results in an increased diffusivity within the hydrogel network. This effect will be particularly pronounced during the initial phase of protein release because the average network mesh size increases during hydrogel degradation.

Altogether, the prepared hydrogels proved to be suitable for the time-controlled release of incorporated peptides or proteins. The obtained release profiles will depend on both the encapsulated macromolecules and the polymers used for gel formation. In future experiments, different polymers (e.g., fast and slow degrading macromers) could be combined to adjust the resultant release profiles. In the end, this might allow for a constant release of therapeutic peptides or proteins over a time period of several days.

Conclusion

We successfully synthesized different derivatives of poly(ethylene glycol) that allow for the preparation of in situ forming hydrogels. Gel strength and degradability could be tailored by altering the polymer end-groups. Because cross-linking is performed in situ, the developed hydrogels could be easily delivered by injection. During the gelation process, dissolved proteins were covalently bound to the polymer backbone, as shown by FRAP experiments. In the same way, therapeutic peptides or proteins could be tethered to the hydrogel network without the need for chemical modifications of these molecules. The nonradical cross-linking approach is, thereby, favorable to the stability of these fragile molecules. During hydrogel degradation, the incorporated proteins were released into solution. Release kinetics will depend on both the incorporated proteins and the polymers used for gel formation. The chosen linker group disintegrates without leaving any polymer residues attached to the protein, which is an advantage over previous drug delivery systems. Altogether, the developed hydrogels proved to be suitable for the time-controlled release of incorporated molecules. Further modifications of the described polymers might result in long-lasting hydrogels that would allow for the sustained release of therapeutic peptides or proteins over a time period of several days up to a few weeks.

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