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# Cleavable carbamate linkers for controlled protein delivery from hydrogels

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ABSTRACT

The reversible attachment of proteins to polymers is one potential strategy to control protein release from hydrogels. In this study, we report the reversible attachment of lysozyme to poly(ethylene glycol) (PEG) by degradable carbamate linkers. Phenyl groups with different substituents were used to control the rate of carbamate hydrolysis and the resulting protein release. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed modification with 1–3 PEG chains per lysozyme molecule. Protein PEGylation and PEG chain elimination occurred without changes in secondary protein structure, as demonstrated by circular dichroism spectroscopy. The lytic activity of lysozyme was restored to 73.4 ± 1.7%–92.5 ± 1.2% during PEG chain elimination. Attached PEG chains were eliminated within 24 h to 28 days, depending on the used linker molecule. When formulated into hydrogels, a maximum of about 60% of the initial dose was released within 7 days to 21 days. Linker elimination occurs 'traceless', so that the protein is released in its native, unmodified form. Altogether, we believe that tethering proteins by degradable carbamate linkers is a promising strategy to control their release from hydrogels.



## 1. Introduction

Even though the significance of therapeutic proteins has increased enormously since the development of recombinant insulin in 1979, several shortcomings still outweigh potential therapeutic benefits in many cases [1,2]. First and foremost, it is often difficult to reach effective protein concentrations at the target tissue due to degradation in the blood stream, unspecific binding to off-target sites or difficulties in crossing endothelial barriers [3]. The necessary injections and the short application intervals often compromise the patient compliance and increase the health care costs. Furthermore, many proteins do not target specific cell types, which limits their use as therapeutics. For example, growth factors may be highly selective *in vitro*, but show a significant number of systemic side effects *in vivo* [3]. One promising approach to solve these problems would be the local release of proteins from injectable hydrogels that serve as a drug carrier and control the release kinetics of their payload. Among the different strategies to control protein release, the reversible binding of proteins to the hydrogel carrier seems most promising; the release rate would then be controlled by the degradation kinetics of the used linker molecules and not only by the fast diffusion of the incorporated protein [2–4].

This approach has already been successfully applied to control the release of small molecules from hydrogels by using hydrolytically [5,6] or enzymatically cleavable linkers [7]. Similar techniques have been proposed for sustained protein release. Bovine serum albumin was covalently bound to poly(ethylene glycol) (PEG) hydrogels containing labile ester bonds and slowly released while the hydrogels degraded [8]. In another approach, vascular endothelial growth factor was immobilized in enzymatically degradable PEG hydrogels, which would allow cell-demanded protein release [9,10]. Biotinylated insulin-like growth factor 1 was tethered to biotinylated peptide nanofibers by using streptavidin as a cross-linker and proved to be successful in the treatment of myocardial infarction [11]. Reduction-sensitive linkers were investigated as an alternative way for temporary protein immobilization. Upon incubation with glutathione, the immobilized lysozyme was released from dextran hydrogels to the same extent as native lysozyme [12]. And recently, a very interesting concept has been proposed that relies on the formation of thermally reversible covalent bonds. Cell adhesion peptides with furan functionalities were covalently bound to maleimide moieties of the hydrogel and released by retro-Diels–Alder reactions [13]. A common disadvantage of these approaches is the use of linker molecules that remain bound to the proteins even after their release from the hydrogel. These remaining 'tags' may compromise protein bioactivity or induce immune responses [14–16].

Our concept, in contrast, relies on the use of aromatic succinimidyl carbonates that react with protein amino groups under formation of degradable carbamates. These linker molecules have been originally developed for the reversible PEGylation of proteins and decompose

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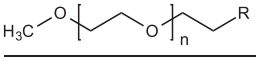
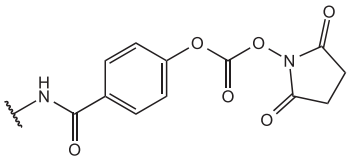
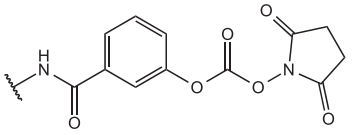
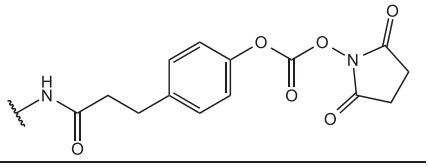
without leaving any 'tags' on the protein [14,17]. In an earlier publication, we reported the synthesis of branched PEG-succinimidyl carbonates that react with branched PEG-amines to form hydrolytically degradable hydrogels. Proteins present in the reaction mixture were reversibly tethered to the hydrogel network and released during gel degradation [18]. A possible drawback of this approach is that protein release is controlled by both the decomposition of the linker molecules and the degradation of the hydrogel carrier. In this paper, we entirely focus on the carbamate linkers and investigate the influence of their chemical structure on the release kinetics of tethered proteins. Three different linker molecules were studied; lysozyme was used as a model protein. Linear methoxy poly(ethylene glycol) (mPEG) was used to study lysozyme PEGylation and PEG chain elimination kinetics. Non-degradable hydrogels were prepared by cross-linking branched PEG molecules using the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC). For lysozyme immobilization, branched carbamate linkers with two different types of reactive groups were synthesized and subsequently used for hydrogel preparation. Release studies were performed to demonstrate the feasibility of our approach.

**Table 1**
Linear poly(ethylene glycol) derivatives (molecular weight 5 kDa).



| Compound | R |
|---|---|
| **1** | $NH_2$ |
| **2a** |  |
| **2b** |  |
| **2c** |  |

## 2. Materials and methods

### 2.1. Materials

3-Hydroxybenzoic acid, 3-(4-hydroxyphenyl)propionic acid, phthalimide and toluene were purchased from Acros Organics (Geel, Belgium). Tetramethylethylenediamine (TEMED) was purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). 2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS) was obtained from GE Healthcare Europe GmbH (Freiburg, Germany). Deuterated Chloroform ($CDCl_3$) was purchased from Deutero GmbH (Kastellaun, Germany). Phosphate-buffered saline (PBS) was purchased from Invitrogen GmbH (Karlsruhe, Germany). Four-armed poly(ethylene glycol) with a molecular weight of 10 kDa was purchased from JenKem Technology (Allen, TX, USA). Iodine was purchased from Riedel-de Haën AG (Seelze, Germany). Acrylamide/bisacrylamide solution (37.5:1) and sodium dodecylsulfate were obtained from Serva Electrophoresis GmbH (Heidelberg, Germany). Acetonitril, Coomassie brilliant blue G-250, *N,N′*-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (HOSu), diisopropyl azodicarboxylate, *N,N′*-discuccinimidyl carbonate (DSC), fluorescamine, 4-hydroxybenzoic acid, lysozyme (from chicken egg white), magnesium sulfate, Micrococcus lysodeikticus, sodium tetraborate decahydrate, 4-pentynoic acid, methoxy poly(ethylene glycol) with a molecular weight of 5 kDa and tetrahydrofuran were purchased from Sigma-Aldrich (Taufkirchen, Germany). Acetone, methylene chloride (DCM), diethyl ether and ethanol were from CSC Jäcklechemie (Nürnberg, Germany). All other chemicals were purchased from Merck KGaA (Darmstadt, Germany). Deionized water was obtained by using a Milli-Q water purification system from Millipore (Schwalbach, Germany).

### 2.2. General procedures

$^1$H-NMR spectra were recorded in $CDCl_3$ at room temperature on a Bruker Avance 300 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany).

The number of free amino groups was determined as described by Udenfriend et al. [19]. The sample was dissolved in 50 mM borate buffer, pH 8.5 and diluted to a concentration of 0.2 μmol/mL. Then, 100 μL of the sample were mixed with 1300 μL of borate buffer and 600 μL of a fluorescamine solution in acetone (0.3 mg/mL). The fluorescence intensity ($\lambda_{ex}$ = 390 nm, $\lambda_{em}$ = 480 nm) was measured on a PerkinElmer LS 55 Fluorescence spectrometer (Perkin-Elmer, Wiesbaden, Germany). Compound **1** (Table 1) was used to prepare a calibration curve and the number of free amino groups was calculated.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was conducted by using polyacrylamide gels of 14% cross-linking. The gels were loaded with 26.25 μg of protein per lane; native lysozyme was used as a reference. The running buffer consisted of 25 mM TRIS, 192 mM glycine and 0.1% SDS. The system was programmed to apply a constant voltage of 120 V and a decreasing current starting at 68 mA over 1.5 h. Afterward, the gels were stained with Coomassie brilliant blue G-250 and scanned. The conjugated PEG was detected by placing the gels into barium chloride and iodine solutions as described by Natarajan et al. [20]. For quantification, the band areas and densities of the Coomassie brilliant blue-stained gels were measured by using the NIH software ImageJ [21].

For circular dichroism (CD) spectroscopy, protein samples were diluted to a concentration of approx. 0.25 mg/mL; the exact concentration was determined spectrophotometrically at 280 nm. CD spectra were recorded on a Jasco J-815 CD spectrometer (Jasco Germany GmbH, Groß-Umstadt, Germany) from 260 to 190 nm at room temperature using cuvettes with a path length of 0.5 mm. Secondary structure fractions were quantified as described by Böhm et al. [22].

### 2.3. Synthesis of compounds *1* and *3*

Compounds **1** (Table 1) and **3** (Table 2) were synthesized in 95% and 93% yield as described by Brandl et al. [23].

### 2.4. Synthesis of compound *2a*

For the synthesis of compound **2a** (Table 1), a previously published procedure was modified as follows [18]. First, 0.11 g of 4-hydroxybenzoic acid (0.8 mmol), 0.09 g of HOSu (0.8 mmol) and 0.17 g of DCC (0.8 mmol) were dissolved in 10 mL of 1,4-dioxane and stirred for 4–6 h at room temperature. The *N,N′*-dicyclohexylurea (DCU) byproduct was filtered off; 2.0 g of compound **1** (0.40 mmol) and 0.04 g (0.48 mmol) of $NaHCO_3$ were then dissolved in 10 mL of water and mixed with the filtrate. The reaction mixture was stirred overnight at 50 °C. The next day, the solvent was evaporated and the residue was taken up in water. The raw product was extracted with DCM and the combined organic phases were dried over anhydrous $MgSO_4$. The solution was concentrated under reduced pressure and the product was crystallized at 0 °C by dropwise addition of diethyl ether. The precipitate was washed with cold diethyl ether and dried under vacuum to yield 1.9 g (93%). $^1$H-NMR ($CDCl_3$,