Synthesis and Characterization of Enzymatically Biodegradable PEG and Peptide-Based Hydrogels Prepared by Click Chemistry

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Herein we describe the synthesis and rheological characterization of a series of enzymatically sensitive PEG and peptide-based hydrogels by the Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction. The hydrogels were synthesized by a combination of alkyne-functionalized star-shaped PEG molecules (two 4-armed PEGs with M_w 10 and 20 kDa, respectively, and one 8-armed PEG of 20 kDa) and the protease-sensitive bis-azido peptide, N^{α}-(azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide (**6**) in the presence of $CuSO_4$ and sodium ascorbate in aqueous solution. The swelling ratio and the storage modulus (G') of the hydrogels could be tailored by several parameters, for example, the initial solid content of the hydrogel, the molecular weight of the PEG derivative, and by the architecture of the PEG molecule (4- versus 8-armed PEG derivative). The peptide sequence, D-Ala-Phe-Lys, was sensitive toward the proteases plasmin and trypsin to render the hydrogels biodegradable.

Introduction

Hydrogels are three-dimensional, hydrophilic polymeric networks capable of absorbing large amounts of water.[1] There has been a lively interest in hydrogels, because they can be used for a wide range of applications including drug delivery systems for the entrapment and release of pharmaceutically active proteins and as scaffolds for tissue engineering and repair.[2,3] Two major classes of hydrogels can be distinguished based on the nature of cross-linking that form the three-dimensional hydrophilic network.[4] In chemically cross-linked hydrogels, covalent bonds form the network, while in physically cross-linked hydrogels, noncovalent interactions like hydrogen bonding, stereocomplex formation, hydrophobic, and ionic interactions are responsible for network formation.[5] To render chemically cross-linked hydrogels biodegradable, hydrolysis sensitive moieties, like ester bonds, have to be incorporated either in the cross-linking entity or in the (polymeric) backbone of the hydrogel. A special class of biodegradable hydrogels comprise the enzymatically degradable hydrogels, which allow the degradation and a subsequent release of the entrapped bioactive compound to be controlled by cell-secreted and cell-activated enzymes.[6,7] A frequently used approach to obtain enzymatically degradable hydrogels is the incorporation of a short peptide sequence that can act as a substrate, which is specifically recognized and cleaved by endogenous proteases, like trypsin, plasmin, or a matrix metalloproteinase, as schematically shown in Figure 1.[7−12]

An attractive approach toward the synthesis of enzymatically degradable hydrogels that contain protease-sensitive peptide sequences is via the Cu(I)-catalyzed cycloaddition.[13−16] The Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction between azides and terminal alkynes was independently discovered in 2002 by the groups of Meldal[17,18] and Sharpless[19] and is compatible with most functional groups present in proteins and peptides, abolishing the need for protection groups. Furthermore, the Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction, also known as the most prominent example of "click chemistry",[20,21] can be performed in aqueous solution under mild reaction conditions in terms of pH and temperature, it has a high reaction rate, and generally results in a high yield of the desired product. These properties render the Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction highly suitable for the synthesis of hydrogels[22,23] based on unprotected bis-azido peptides and PEG-derivatized alkynes.

Previously, we have shown that the Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction is an effective tool for the synthesis of biodegradable peptide-based polymers.[24,25] In the present study we aimed to design a modular approach for the synthesis of tailorable biodegradable hydrogels. The Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction between a trypsin- and plasmin-sensitive bis-azido peptide and star-shaped alkyne-derivatized PEG moieties resulted in a series of hydrogels in which the chain length, chain architecture, and alkyne density of the PEG derivatives were found to be important factors in the design of the macroscopic properties of the hydrogels.

Experimental Section

Materials and Methods. Chemicals were obtained from commercial sources and used without further purification. The star-shaped 4- and 8-armed poly(ethylene glycol) derivatives (PEG_4-10k (**7**), PEG_4-20k (**8**), and PEG_8-20k (**11**)) were purchased, with a free hydroxyl moiety, from JenKem Technology U.S.A. Reactions were carried out at room temperature unless stated otherwise. Column chromatography was performed with Silica-P Flash silica gel (Silicycle). Retention factor values (R_f) were determined with thin layer chromatography (TLC) by using Merck silica gel 60 F-254 plates. Spots were visualized by UV-quenching, ninhydrin, TDM/Cl_2,[26] $KMnO_4$, or PPh_3/ninhydrin.[27]^{1}H NMR spectra (300 MHz) were recorded on a Varian Mercury plus

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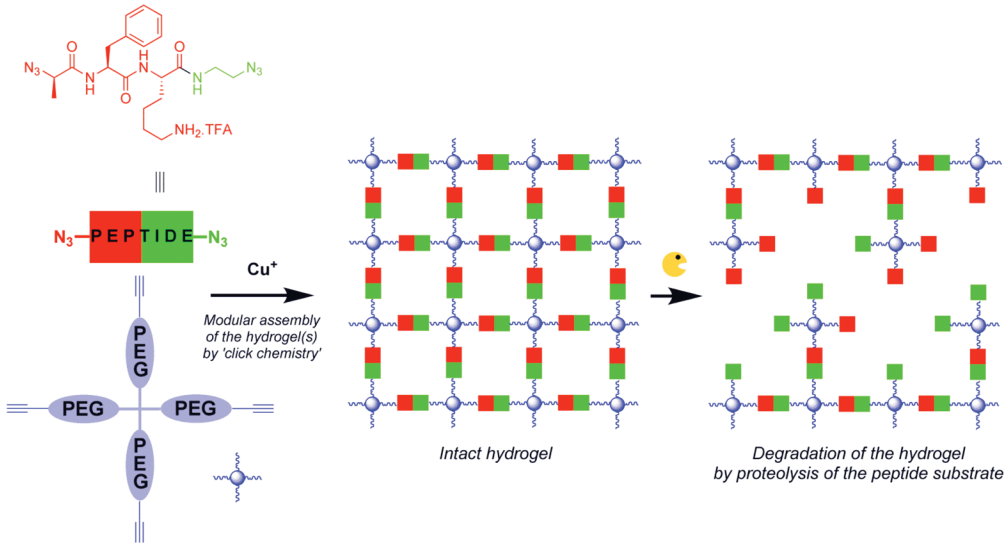


Figure 1. Schematic overview of the synthesis of enzymatically degradable PEG-peptide based hydrogels that were synthesized via the Cu(I)-catalyzed azide−alkyne cycloaddition reaction.

spectrometer and chemical shift values (δ) are given in ppm relative to TMS. ^{13}C NMR spectra (75 MHz) were recorded using the attached proton test (APT) pulse sequence and chemical shift values are given in ppm relative to $CDCl_3$ (77.0 ppm) or DMSO-d_6 (39.5 ppm). Fourier transform infrared spectra (FTIR) were measured on a Bio-Rad FTS-25 spectrophotometer. The retention time values (R_t) and purities of the synthetic peptide intermediates were evaluated by analytical RP-HPLC on a Shimadzu automated HPLC system equipped with a UV−vis detector operating at λ = 214 and 254 nm and by using an Alltech Prosphere C18 column (250 × 4.6 mm, 5 μm particle size, 300 Å pore size) at a flow rate of 1 mL/min using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95:5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 20 or 40 min.

Synthesis of N$^{\alpha}$-(Azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide (6). *tert-Butyl 2-Azidoethylcarbamate (***2***).* To a cooled (0 °C) solution of 2-bromoethylamine hydrobromide **1** (16.8 g, 82.7 mmol) and Boc_2O (19.8 g, 91 mmol) in CH_2Cl_2 (100 mL), Et_3N (12.6 mL, 91 mmol) was added dropwise, and the obtained reaction mixture was stirred for 1 h at 0 °C, followed by 16 h at room temperature. Then, the reaction mixture was diluted with CH_2Cl_2 (100 mL) and this solution was subsequently washed with 1 N $KHSO_4$ (3 × 100 mL) and brine (3 × 100 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo. The resulting *N*-(*tert*-butyloxycarbonyl)-2-bromoethylamine was obtained as a white solid in 87% yield (17.7 g). R_f = 0.16 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.45 (s, 9H, $(CH_3)_3$), 3.45 (m, 2H, CH_2Br), 3.52 (m, 2H, $NHCH_2$), 5.06 (broad s, 1H, NH). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 28.3, 32.6, 42.3, 79.7, 155.5. In the next step, a mixture of *N*-(*tert*-butyloxycarbonyl)-2-bromoethylamine (17.7 g, 79 mmol) and NaN_3 (10.3 g, 158 mmol) was dissolved in dry DMF (300 mL) and stirred for 16 h at room temperature. Then, the solvent was removed in vacuo and the residue was dissolved in CH_2Cl_2 (330 mL), and this solution was subsequently washed with 1 N $KHSO_4$ (3 × 100 mL) and brine (3 × 100 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo. The residue was coevaporated with toluene (3×) and $CHCl_3$ (3×) to remove any residual DMF, to give *tert*-butyl 2-azidoethylcarbamate (**2**) as a white solid in 87% yield (12.9 g). R_f = 0.95 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v). FTIR (KBr) ν: 2110 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.45 (s, 9H, $(CH_3)_3$), 3.31 (m, 2H, CH_2), 3.42 (m, 2H, CH_2), 4.98 (broad s, 1H, NH). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 28.2, 40.0, 51.1, 79.6, 155.7.

*Fmoc-Lys(Boc)-(2-azidoethyl)-amide (***3***).* Azide **2** (3.9 g, 20 mmol) was dissolved in TFA/CH_2Cl_2 1:1 v/v (80 mL), and the reaction mixture was stirred for 1 h at room temperature. After this period of stirring, the solvents were removed by evaporation and the residue was coevaporated with toluene (3×) and $CHCl_3$ (3×) to remove any residual TFA. The obtained residue was dissolved in CH_2Cl_2 (300 mL) and Fmoc-Lys(Boc)-OH (9.4 g, 20 mmol), BOP (8.8 g, 20 mmol), and DIPEA (11 mL, 63 mmol) were added, and the reaction mixture was stirred for 16 h at room temperature. Then, the solvent was removed in vacuo and the residue was dissolved in EtOAc (300 mL), and this solution was subsequently washed with 1 N $KHSO_4$ (3 × 100 mL), H_2O (1 × 100 mL), 5% $NaHCO_3$ (3 × 100 mL), H_2O (1 × 100 mL), and brine (3 × 100 mL). The EtOAc solution was dried (Na_2SO_4), filtered, and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/MeOH 96:4 v/v) to give Fmoc-Lys(Boc)-(2-azidoethyl)-amide **3** as a white solid in 97% yield (10.4 g). R_f = 0.78 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v). FTIR (KBr) ν: 2110 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.35−1.50 (m, 4H, $\gamma CH_2/\delta CH_2$ Lys), 1.43 (s, 9H, $(CH_3)_3$), 1.65 (m, 1H, βCH_2 Lys (1H)), 1.86 (m, 1H, βCH_2 Lys (1H)), 3.10 (m, 2H, εCH_2 Lys), 3.41 (m, 4H, $CH_2CH_2N_3$), 4.13 (m, 2H, αCH Lys (1H)/CH Fmoc (1H)), 4.40 (m, 2H, CH_2 Fmoc), 4.64 (broad s, 1H, NH urethane), 5.52 (broad s, 1H, NH urethane), 6.57 (broad s, 1H, NH amide), 7.26−7.78 (m, 8H, arom H Fmoc). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 22.5, 28.4, 29.5, 31.9, 38.9, 39.7, 47.1, 50.6, 54.8, 67.0, 79.2, 119.9, 125.0, 127.0, 127.7, 141.2, 143.7, 156.2, 172.1, 180.1.

*Fmoc-Phe-Lys(Boc)-(2-azidoethyl)-amide (***4***).* Compound **3** (10.4 g, 19.3 mmol) was dissolved in piperidine/THF 1:4 v/v (150 mL) and the reaction mixture was stirred for 2 h at room temperature before the volatiles were removed by evaporation. The residue was coevaporated with toluene (3×) and $CHCl_3$ (3×) and subsequently purified by column chromatography (CH_2Cl_2/MeOH 9:1 v/v) to give the intermediate H-Lys(Boc)-(2-azidoethyl)-amide as a colorless oil in 79% yield (4.8 g). R_f = 0.25 (DCM/MeOH 9:1 v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.37−1.61 (m, 5H, βCH_2 (1H)/$\gamma CH_2/\delta CH_2$ Lys), 1.44 (s, 9H, $(CH_3)_3$), 1.87 (m, 1H, βCH_2 Lys (1H)), 3.12 (m, 2H, εCH_2 Lys), 3.34−3.48 (m, 5H, αCH Lys (1H)/$CH_2CH_2N_3$), 4.61 (broad s, 1H, NH urethane), 7.70 (broad s, 1H, NH amide). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 22.8, 28.4, 29.9, 34.4, 38.6, 40.1, 50.8, 55.0, 79.1, 156.0, 175.3. In the next step, H-Lys(Boc)-(2-azidoethyl)-amide (4.8 g, 15.2 mmol), BOP (7.1 g, 15.2 mmol), and Fmoc-Phe-OH (5.9 g, 15.2 mmol) were dissolved in CH_2Cl_2 (250 mL), and to this solution, DIPEA (7.9 mL, 45 mmol) was added dropwise. The reaction mixture was stirred for 16 h at room temperature and the desired compound was removed by filtration and subsequently dissolved in a large volume of EtOAc (400 mL). This solution was washed with 1 N $KHSO_4$ (4 × 100 mL), H_2O (1 × 100 mL), 5% $NaHCO_3$ (3 × 100 mL), H_2O (1 × 100 mL), and brine (3 × 100 mL), and subsequently dried (Na_2SO_4), filtered, and evaporated to dryness to give Fmoc-Phe-Lys(Boc)-(2-azidoethyl)-amide **4** as an off-white solid in 80% yield (8.3 g). R_f = 0.85 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v). FTIR (KBr) ν: 2110 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz)

Table 1. Characterization of the Hydrogels

entry	hydrogel	G' (kPA)	swelling ratio (W_t/W_0)	solid content (%)	M_c[a] (g/mol)
1	2% PEG_4-10k	1.4 ± 0.3	n.d.	n.d.	34900 ± 4400
2	5% PEG_4-10k	12.7 ± 0.8	0.96 ± 0.06	5.2	9600 ± 600
3	7% PEG_4-10k	17.7 ± 0.8	n.d.	n.d.	9700 ± 400
4	10% PEG_4-10k	24.9 ± 2.3	1.50 ± 0.07	6.7	9900 ± 1100
5	15% PEG_4-10k	37.7 ± 4.2	1.87 ± 0.04	8.0	44400 ± 3700
6	2% PEG_4-20k	1.1 ± 0.1	n.d.	n.d.	26600 ± 2300
7	5% PEG_4-20k	4.6 ± 0.4	1.79 ± 0.04	2.8	23500 ± 1800
8	7% PEG_4-20k	7.3 ± 0.6	n.d.	n.d.	15900 ± 600
9	10% PEG_4-20k	15.5 ± 0.6	2.28 ± 0.10	4.4	14600 ± 300
10	15% PEG_4-20k	25.5 ± 0.6	2.92 ± 0.08	5.1	30500 ± 3400
11	2% PEG_8-20k	1.6 ± 0.2	n.d.	n.d.	8500 ± 200
12	5% PEG_8-20k	14.4 ± 0.3	0.83 ± 0.05	6.0	7300 ± 100
13	7% PEG_8-20k	23.7 ± 0.1	n.d.	n.d.	6200 ± 200
14	10% PEG_8-20k	39.8 ± 1.0	1.21 ± 0.03	8.2	7000 ± 400
15	15% PEG_8-20k	53.3 ± 3.2	1.64 ± 0.04	9.3	n.d.

[a] Theoretical value for M_c: PEG_4-10k, 5500 g/mol; PEG_4-20k, 10500 g/mol; PEG_8-20k, 5500 g/mol.

δ: 1.23−1.54 (m, 4H, $\gamma CH_2/\delta CH_2$ Lys), 1.41 (s, 9H, $(CH_3)_3$), 1.59 (m, 1H, βCH_2 Lys (1H)), 1.83 (m, 1H, βCH_2 Lys (1H)), 3.00 (m, 4H, βCH_2 Phe/εCH_2 Lys), 3.33 (m, 4H, $CH_2CH_2N_3$), 4.15 (m, 1H, αCH Lys), 4.28 (m, 1H, CH Fmoc), 4.40 (m, 3H, αCH Phe (1H)/CH_2 Fmoc (2H)), 4.73 (broad s, 1H, NH urethane), 5.64 (broad s, 1H, NH urethane), 6.80 (broad s, 2H, NH amide), 7.16−7.76 (m, 13H, arom H Fmoc (8H)/arom H Phe (5H)). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 22.5, 28.4, 29.3, 31.4, 38.1, 39.0, 47.0, 50.4, 53.1, 56.3, 67.3, 120.0, 124.9, 127.0, 127,7, 128.7, 129.2, 136.1, 141.2, 143.6, 156.2, 171.3, 171.5.

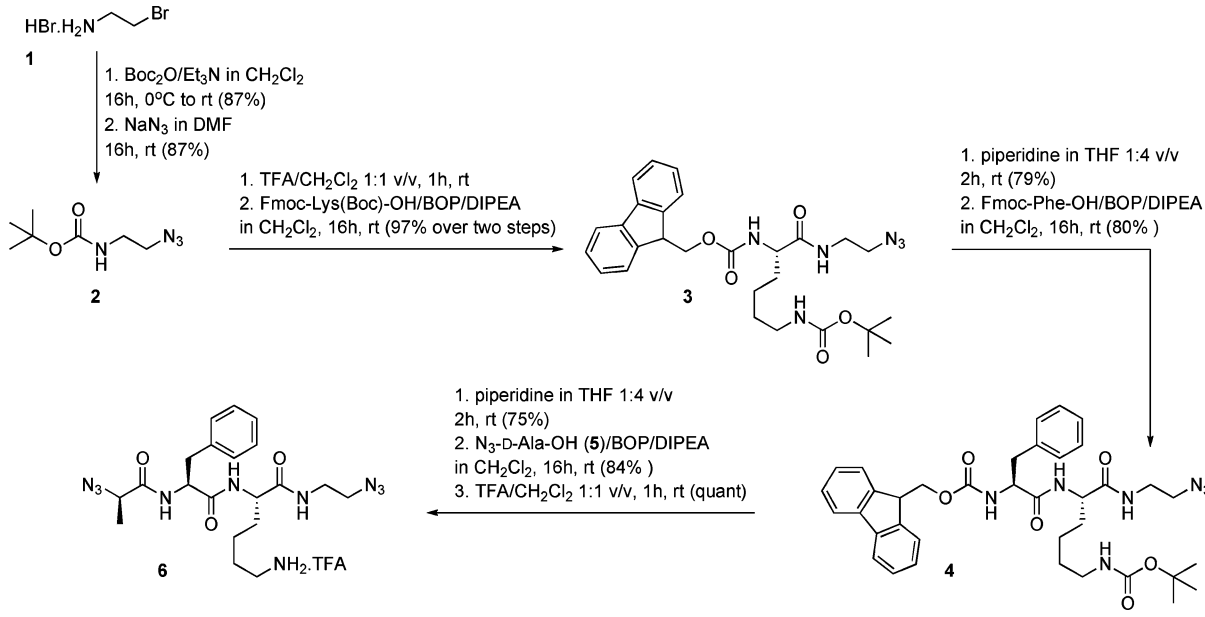
*N_3-D-Ala-OH (**5**).* N_3-D-Ala-OH was synthesized according to a procedure described by Lundquist and Pelletier,[28] which was based on the method developed by Wong and co-workers.[29,30] Azide **5** was obtained as a yellowish oil in quantitative yield. R_f = 0.59 ($CHCl_3$/MeOH/AcOH 95:20:3 v/v/v). FTIR (KBr) ν: 2110 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.55 (d, 3H, βCH_3), 4.03 (m, 1H, αCH). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 16.6, 57.0, 177.4.

*N^{α}-(Azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide (**6**).* Protected dipeptide **4** (8.3 g, 12.2 mmol) was dissolved in piperidine/THF 1:4 v/v (200 mL), and the reaction mixture was stirred for 2 h at room temperature before the volatiles were removed by evaporation. The residue was coevaporated with toluene (3×) and $CHCl_3$ (3×) and subsequently purified by column chromatography (CH_2Cl_2/MeOH 9:1 v/v) to give the desired H-Phe-Lys(Boc)-(2-azidoethyl)-amide as a yellow oil in 75% yield (4.2 g). R_f = 0.42 (CH_2Cl_2/MeOH 95:5 v/v). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.25−1.68 (m, 5H, βCH_2 (1H)/γCH_2/δCH_2 Lys), 1.44 (s, 9H, $(CH_3)_3$), 1.83 (m, 1H, βCH_2 (1H) Lys), 2.71−2.79 (dd (J_{ax} = 9.1, J_{ab} = 13.8 Hz), 1H, βCH_2 Phe (1H)), 3.09 (m, 2H, εCH_2 Lys), 3.21−3.27 (dd (J_{ax} = 4.0, J_{ab} = 13.6 Hz), 1H, βCH_2 Phe (1H)), 3.42 (m, 4H, $CH_2CH_2N_3$), 3.65 (m, 1H, αCH Phe), 4.34 (m, 1H, αCH Lys) 4.59 (broad s, 1H, NH urethane), 6.73 (broad s, 1H, NH amide), 7.20−7.36 (m, 5H, arom H Phe), 7.75 (broad d, NH amide). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 22.6, 28.3, 29.4, 31.7, 38.9, 40.0, 40.7, 50.4, 52.5, 56.1, 78.9, 126.8, 128.6, 129.2, 137.4, 156.0, 171.9, 175.0. In the next step, N_3-D-Ala-OH (1.2 g, 9 mmol), H-Phe-Lys(Boc)-(2-azidoethyl)-amide (4.2 g, 9 mmol), and BOP (4.3 g, 9 mmol) were dissolved in CH_2Cl_2 (250 mL), and to this solution, DIPEA (5 mL, 27 mmol) was added dropwise. The reaction mixture was stirred for 16 h at room temperature. Then, the solvent was removed in vacuo and the residue was dissolved in EtOAc (250 mL). This solution was subsequently washed with 1 N $KHSO_4$ (4 × 100 mL), H_2O (1 × 100 mL), 5% $NaHCO_3$ (3 × 100 mL), H_2O (1 × 100 mL), and brine (3 × 100 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2/MeOH 97:3 v/v) to give the protected tripeptide, N_3-D-Ala-Phe-Lys(Boc)-(2-azidoethyl)-amide, as a white solid in 84% yield (4.2 g). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.26−1.64 (m, 7H, βCH_2 (1H)/γCH_2/δCH_2 Lys/βCH_3 Ala), 1.44 (s, 9H, $(CH_3)_3$), 1.84 (m, 1H, βCH_2 Lys (1H)), 3.08 (m, 4H, βCH_2 Phe/εCH_2 Lys), 3.41 (m, 4H, $CH_2CH_2N_3$), 4.00 (m, 1H, αCH Ala), 4.41 (m, 1H, αCH Lys), 4.76 (m, 2H, αCH Phe (1H)/NH urethane (1H)), 6.80 (broad s, 1H, NH amide), 6.89 (broad s, 1H, NH amide), 7.17−7.77 (m, 6H, arom H Phe (5H)/NH amide (1H)). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 17.0, 22.7, 28.4, 29.5, 32.2, 38.5, 39.0, 40.1, 53.0, 54.1, 58.2, 79.0, 127.0, 128.5, 129.3, 131.1, 136.1, 156.1, 170.6, 170.9, 171.8. In the final step, N_3-D-Ala-Phe-Lys(Boc)-(2-azidoethyl)-amide (4.2 g, 7.5 mmol) was dissolved in TFA/CH_2Cl_2 1:1 v/v (60 mL) and the reaction mixture was stirred for 1 h at room temperature. Then, the solvents were removed by evaporation and the residue was coevaporated with toluene (3×) and chloroform (3×) to remove any residual TFA, and finally lyophilized from H_2O to yield quantitatively N^{α}-(azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide **6** as a white solid (3.7 g). R_t = 18.47 min (C18). FTIR (KBr) ν: 2110 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 1.33−1.47 (m, 2H, γCH_2 Lys), 1.38 (d (J = 7.2 Hz), 3H, βCH_3 Ala), 1.61−1.70 (m, 3H, βCH_2 (1H)/δCH_2 Lys), 1.82 (m, 1H, βCH_2 Lys (1H)), 2.93−3.00 (m, 3H, βCH_2 Phe (1H)/εCH_2 Lys (2H)), 3.11−3.18 (dd (J_{ax} = 6.6, J_{ab} = 13.8 Hz), 1H, βCH_2 Phe (1H)), 3.36 (m, 4H, $CH_2CH_2N_3$), 3.93 (m, 1H, αCH Ala), 4.35 (m, 1H, αCH Lys), 4.62 (m, 1H, αCH Phe), 7.19−7.33 (m, 6H, arom H Phe (5H)/NH amide (1H)), 7.52 (d, 1H, NH amide), 7.91 (d, 1H, NH amide), 7.98 (broad s, 3H, NH_3). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 16.7, 21.9, 26.4, 31.0, 37.7, 38.9, 39.3, 50.2, 53.0, 54.7, 58.1, 127.0, 128.5, 129.1, 136.0, 171.3, 171.7, 172.1. ESI-LCMS: Calcd for $C_{20}H_{30}N_{10}O_3$, 458.25; found m/z $[M + H]^+$, 459.20, $[M + Na]^+$ 481.24, $[2 M + H]^+$ 917.40.

Functionalization of Star-Shaped PEG Derivatives with Alkyne Moieties. In a typical procedure, PEG derivative **7** (PEG_4-10k, 10.2 g, 1.02 mmol) was dissolved in dry THF (150 mL), and to this solution, NaH (420 mg as a 60%-suspension in mineral oil, 10.5 mmol, 2.5 equiv per arm) was added and the reaction mixture was stirred for 15 min at room temperature. Then, propargyl bromide (600 μL as an 80% solution in toluene, 5.4 mmol, 1.3 equiv per arm) was added, and the resulting reaction mixture was stirred for 16 h at room temperature. The solvent was removed in vacuo, and the residue was dissolved in H_2O (150 mL) and dialyzed against demi-H_2O (MWCO 3500 Da). After lyophilization, the alkyne-functionalized PEG derivative **9** was obtained as a white solid in 88% yield (8.8 g). ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 2.44 (broad s, 4H, (∼O-CH_2-C≡CH)$_4$), 3.64 (broad s, ∼904H, (∼O-(CH_2-CH_2∼)$_{57}$)$_4$), 3.88 (m, 8H, C-(CH_2-O∼)$_4$), 4.20 (broad s, 8H, (∼O-CH_2-C≡CH)$_4$).

Preparation of the Hydrogels. In a typical gelation experiment (10% gel, 10 kDa 4-arm PEG, entry 4, Table 1), a stock solution was prepared that contained PEG derivative **9** (1.35 g, 0.13 mmol ≈ 0.52 mmol alkyne), N^{α}-(azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide **6** (120 mg, 0.26 mmol ≈ 0.52 mmol azide), and sodium ascorbate (135 mg, 0.68 mmol), and this mixture was dissolved in H_2O (4.5 mL), which resulted in an alkyne/azide molar ratio of 1. A sample of this stock solution (220 μL) was transferred into a syringe and diluted with H_2O (270 μL). Subsequently, an aliquot (63 μL) of an aqueous solution of $CuSO_4$ (100 mg (0.40 mmol) $CuSO_4 \cdot 5H_2O$ in 10 mL of H_2O) was added, and the reaction mixture was vortexed. A gel was formed within several minutes.

Scheme 1. Synthesis of the Bis-azido Peptide, N$^{\alpha}$-(Azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide (**6**)



Rheological Characterization. Rheological characterization of the hydrogels was performed on an AR G-2 Rheometer (TA Instruments) equipped with a 2° steel cone geometry with a diameter of 40 mm and a solvent trap. After the addition of the $CuSO_4$ solution to the aqueous mixture of PEG/peptide/sodium ascorbate, an aliquot (600 μL) of the reaction mixture was immediately placed between the plates of the rheometer. Rheological gel characteristics were monitored by oscillatory time sweep experiments. During the time sweep experiments, the G' (shear storage modulus) and G'' (loss modulus) were measured in the oscillation mode with a controlled strain of 0.1% at a frequency of 1 Hz at 20 °C for a period of 20 min.

Swelling Behavior of the Hydrogels. In a typical swelling experiment the gels (dried with a tissue) were weighed (W_0), placed into 100 mM PBS buffer (10 mL, pH 7.4, containing 0.3 mg/mL NaN_3), and incubated at 37 °C. To determine the swelling ratio (defined as W_t/W_0), the gels were removed from the buffer solution at regular time intervals, dried with a tissue, and weighed (W_t). The buffer solution was refreshed every 24 h.

Degradation Experiments. The gels were incubated at room temperature in 0.1 M EDTA solution for 24 h, followed by several washings with PBS buffer mainly to remove residual copper ions, which resulted in colorless/opaque hydrogels. Subsequently, the gels were incubated at 37 °C in 100 mM PBS buffer (5 mL, pH 7.4, 0.3 mg/mL NaN_3), and after 24 h, the gels were dried with a tissue, weighed (W_0), and subsequently incubated in 100 mM PBS buffer (5 mL, pH 7.4, 0.3 mg/mL NaN_3) containing either plasmin (2.6 μM) or trypsin (0.8 μM). At regular time intervals, the gels were removed from the buffer solution, dried with a tissue, and weighed (W_t). The enzyme-containing buffer solution was refreshed every 24 h. To study the degradation of the peptide, bis-azido peptide **6** (3 mg) was dissolved in PBS buffer (1 mL, pH 7.4), and to this solution an aqueous solution of either trypsin (at a final concentration of 0.8 μM) or plasmin (at a final concentration of 2.6 μM) was added. The peptide/enzyme mixtures were incubated at 37 °C. At regular time intervals a sample (100 μL) was drawn and quenched with ice-cold 1 M NaOAc buffer (200 μL, pH 3.8) and stored at 4 °C prior to HPLC analysis.

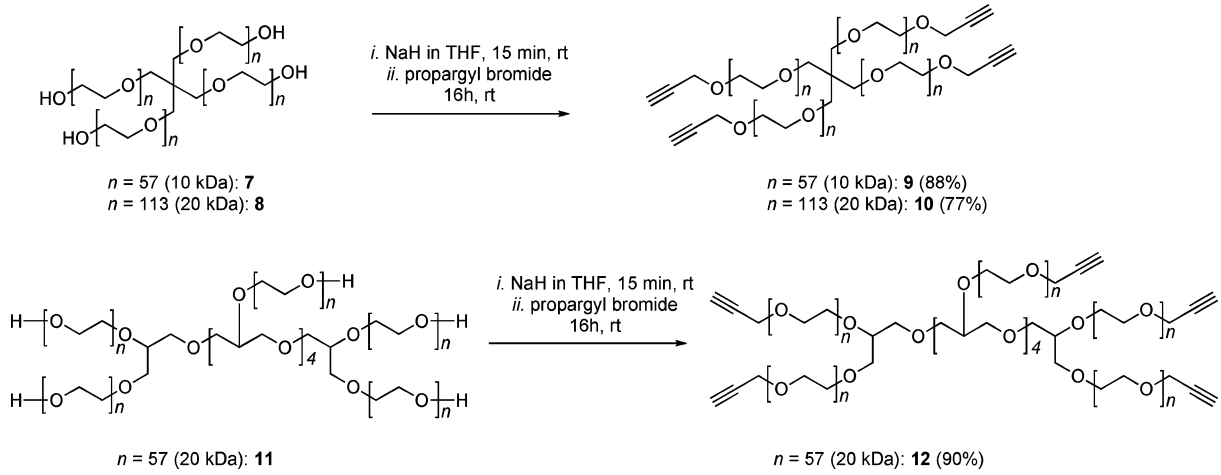
Results and Discussion

Synthesis and Characterization of the Hydrogel Building Blocks. The synthesis of bis-azido peptide **6**, N$^{\alpha}$-(azido)-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide, was performed as shown in Scheme 1. This tripeptide was chosen since it can be hydrolyzed by trypsin as well as plasmin.[31,32] The synthesis started with the protection of the amino group with a Boc functionality and a subsequent substitution of the bromine with an azide moiety. Azide **2** was obtained in 76% overall yield based on 2-bromoethylamine hydrobromide **1** as starting compound. After Boc-removal by treatment with TFA, the resulting amine was coupled to Fmoc-Lys(Boc)-OH in the presence of BOP/DIPEA as coupling reagents to give compound **3** in 97% yield over two steps. In the next step, the Fmoc group was removed by piperidine in THF and after flash chromatography the intermediate amine was directly used in the coupling reaction with Fmoc-Phe-OH in the presence of BOP/DIPEA. The fully protected dipeptide **4** was obtained as an off-white solid in 80% yield. Then, treatment with piperidine/THF resulted in the removal of the Fmoc group and the α-amine was coupled to N_3-D-Ala-OH (**5**) with BOP/DIPEA to give the protected tripeptide in 63% yield over two steps. Azide **5** was prepared by a Cu(II)-catalyzed diazotransfer with triflic azide based on a procedure as described by Lundquist and Pelletier[28] according to the method of Wong and co-workers.[29,30] Finally, the protected tripeptide was treated with TFA to remove the Boc group and the bis-azido tripeptide **6** was obtained as a white solid in quantitative yield. The purity of this bis-azido building block **6** was analyzed by TLC and HPLC analysis and the identity was characterized by ^{1}H, ^{13}C, FTIR, and ESI-LCMS.

The functionalization of the star-shaped PEG derivatives **7**, **8**, and **11** was performed as depicted in Scheme 2. The PEG alcohols were treated with NaH and subsequently reacted with propargyl bromide. The alkynes **9**, **10**, and **12** were purified by dialysis and the degree of substitution was determined by ^{1}H NMR spectroscopy, which showed close to quantitative substitution based on the intensities of the methylene protons of the PEG chains and the propargyl moieties.

Preparation and Characterization of the Hydrogels. The hydrogels were prepared by mixing an equimolar amount of bis-azido peptide **6** with a PEG-alkyne derivative (**9**, **10**, or **12**) in H_2O in the presence of sodium ascorbate. To initiate gel formation an aqueous solution of $CuSO_4$ was added. In general terms, gel formation was rapid and complete within 5 min at room temperature, as judged by rheology measurements since the storage modulus (G') did not increase after this time point

Scheme 2. Synthesis of the Alkyne-Functionalized Star-Shaped PEG Derivatives, PEG_4-10k (**9**), PEG_4-10k (**10**), and PEG_8-20k (**12**)



(Figure 2). The dried hydrogels were analyzed by FTIR and the characteristic signal of the azide moiety (at ν 2110 cm^{-1}) was absent, indicating that the azides were quantitatively transformed into triazoles by reacting with alkynes in the presence of Cu(I) as the catalyst.

The rate of hydrogel formation was followed by rheological measurements. It turned out that in less than 5 min after the addition of $CuSO_4$, a fully elastic hydrogel was formed characterized by a storage modulus (G') of 25 kPa and tan δ < 0.01, as shown in Figure 2. Furthermore, increasing the solid content of the hydrogels resulted not only in an increased rate of hydrogel formation due to an increased concentration of the mutually reactive azide/alkyne functionalities, but also in an increased storage modulus of the hydrogel because a higher solid content resulted in a higher cross-link density, as shown in Figure 3 and Table 1.

The storage modulus of the hydrogel was also dependent on the molecular weight and the number of arms of the star-shaped PEG-alkyne derivatives. Within a series of equal solid content (e.g., 10%), increasing the molecular weight from PEG_4-10k to PEG_4-20k resulted in a decrease of the storage modulus from 24.9 ± 2.3 to 15.5 ± 0.6 kPa (entries 4 and 9, Table 1). This difference can be explained by the fact that the molar concentration of the alkyne groups in PEG_4-20k is half as in PEG_4-10k and the lower concentration of reactive groups will result in a lower cross-link density and, thus, lower storage modulus. On the other hand, at equal polymer concentration, increasing the number of arms from PEG_4-20k to PEG_8-20k increased the storage modulus from 15.5 ± 0.6 to 39.8 ± 1.0 kPa (entries 9 and 14, Table 1). Although the PEG-arms of PEG_8-20k and PEG_4-10k have the same length and consequently both hydrogels have the same average molecular weight between adjacent cross-links (M_c), the storage modulus is the highest in case of PEG_8-20k due to an increased cross-link density (entries 4, 9, and 14, Table 1).[33]

The average molecular weight between adjacent cross-links (M_c) was calculated from the plateau modulus (G_0), using eq 1, which was derived from the rubber elasticity theory,[34−36] and have been listed in Table 1.

$$G_0 = \rho RT/M_c \quad (1)$$

In this equation, ρ represents the concentration of the polymer solution (g/m^3), R represents the gas constant, and T represents the absolute temperature. The theoretical M_c values represent two times the sum of the molecular weight of each PEG-arm and the bis-azido peptide (PEG_4-10k, 5.5 kDa; PEG_4-20k, 10.5 kDa; PEG_8-20k, 5.5 kDa). The calculated M_c values were found to be higher than the theoretical values, which can be explained by imperfections in the hydrogel networks, like loop formation and the presence of loose ends.[37] It should further be mentioned that eq 1 is only valid for ideal networks. The most pronounced differences in calculated and theoretical M_c values were found in hydrogels with the lowest solid content, an indication that at

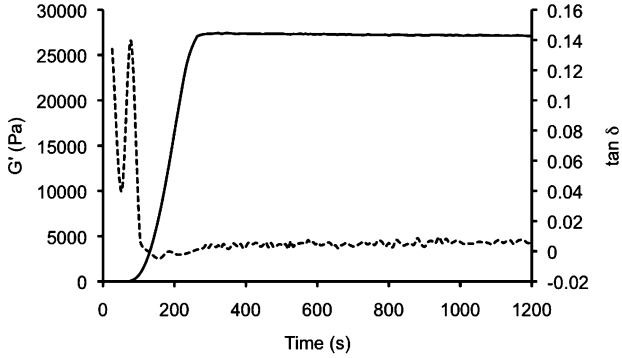


Figure 2. Kinetics of hydrogel formation (10% PEG_4-10k, entry 4, Table 1) in H_2O (G' (solid line) and tan δ (dotted line)).

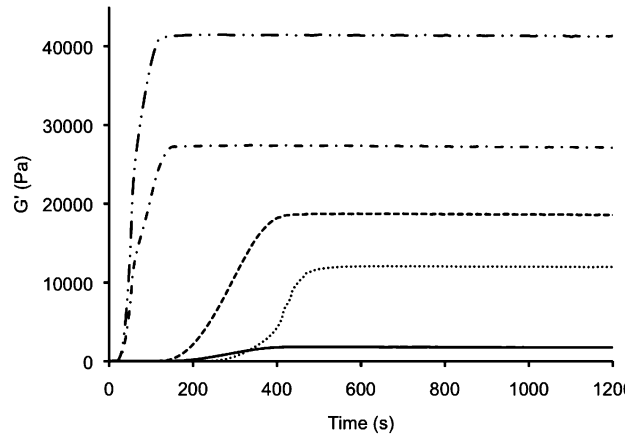


Figure 3. Kinetics of hydrogel formation as function of solid content. Reaction conditions: PEG_4-10k **9**, bis-azido peptide **6** (molar ratio 1:1), sodium ascorbate, and $CuSO_4$ in H_2O at 20 °C. Solid content: 2% (solid line), 5% (round dot), 7% (square dot), 10% (dash-dot), and 15% (long-dash dot-dot).

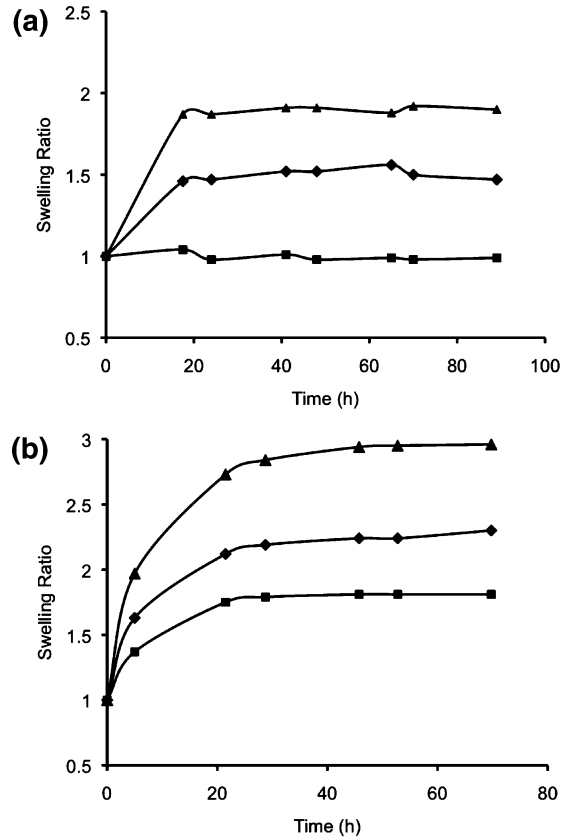


Figure 4. Swelling ratios (W_t/W_0) of hydrogels based on PEG_4-10k (a), and PEG_4-20k (b) in PBS buffer (pH 7.4 at 37 °C). Solid content: 5% (filled square), 10% (filled diamond), and 15% (filled triangle). The data are shown as average ± SD and $n = 3$.

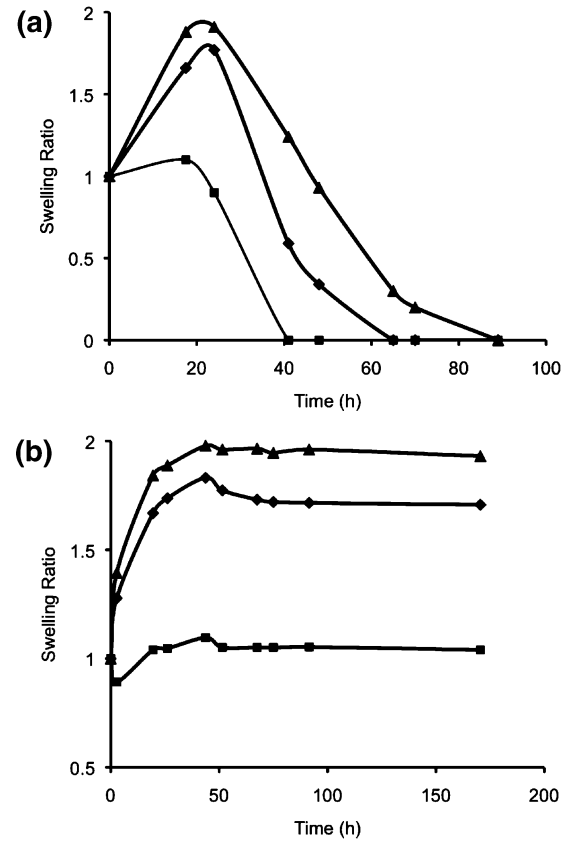


Figure 5. The swelling ratios (W_t/W_0) of hydrogels based on PEG_4-10k in the presence of trypsin at 0.8 μM (a), and PEG_4-10k (b) in the presence plasmin at 2.6 μM of PBS buffer (pH 7.4 at 37 °C). Solid content: 5% (filled square), 10% (filled diamond), and 15% (filled triangle). The data are shown as average ± SD and $n = 3$.

a low alkyne/azide concentration the intramolecular loop formation was preferred over the intermolecular cross-link reaction. Another indication of loop-formation rather than the presence of loose ends was found on basis of FTIR analysis, because the characteristic signal of the azide moiety (ν 2110 cm^{-1}) was clearly absent.

The swelling behavior (expressed as swelling ratio: W_t/W_0) of the hydrogels was evaluated by incubating the gels in PBS buffer (100 mM, pH 7.4 at 37 °C) during a particular time period. In the case of the hydrogel prepared with PEG_4-10k, the maximal swelling ratio was reached after 20 h of incubation (Figure 4a, Table 1). Interestingly, the hydrogel with 5% solid content did not increase in weight, while the hydrogels with 10 and 15% solid content swelled to 1.5 and 1.8 times their initial weight, respectively (Figure 4a). Apparently, the hydrogel with 5% solid content is dimensionally stable, which could be explained by the fact that the PEG-chains were already stretched, which made the hydrogel unable to absorb an additional amount of water. The swelling behavior of hydrogels based on PEG_4-20k is shown in Figure 4b and Table 1. From the literature it is known that the degree of equilibrium swelling of a polymeric hydrogel is inversely proportional to the mechanical strength.[33,38] The swelling ratios of the hydrogels based on PEG_4-20k were found to be in agreement with the literature, because the highest swelling ratios were found with these hydrogels presently studied even in case of the gel with 5% solid content was found to absorb almost 80% of its intitial water content (Figure 4b and Table 1, entry 7).

Enzymatic Degradation of the Hydrogels. The tripeptide D-alanyl-phenylalanyl-lysine (D-Ala-Phe-Lys) is known from the literature as a selective substrate for the serine proteases trypsin and plasmin.[28] Plasmin plays an important role in fibrinolysis and wound healing, however, it has also a critical role in tumor cell invasiveness and metastasis[39,40] and is present at significantly elevated concentrations in tumor tissue.[41] The elevated concentration of plasmin (and other proteolytic enzymes) at the site of the tumor has been used for triggered drug release from enzymatically degradable dendrimers.[42] Therefore, the tripeptide D-Ala-Phe-Lys was converted into a bis-azido derivative, azido-D-alanyl-phenylalanyl-lysyl-(2-azidoethyl)-amide **6**, which was suitable for a Cu(I)-catalyzed 1,3-dipolar cycloaddition with alkyne-functionalized star-shaped PEG derivatives for the formation of enzymatically degradable peptide-hydrogels. The bis-azido peptide **6** was digested in a PBS buffer (100 mM, pH 7.4 at 37 °C) by trypsin (at 0.8 μM) and plasmin (at 2.6 μM) with half-life times $t_{1/2}$ of approximately 60 min and 60 h, respectively, an indication that the N- and C-terminal modification were of minimal influence on the hydrolysis rate.

A series of three hydrogels based on PEG_4-10k with a solid content of, respectively, 5, 10, and 15%, were prepared and incubated with either trypsin (at 0.8 μM) or plasmin (at 2.6 μM) in a PBS buffer (100 mM, pH 7.4 at 37 °C). In the case of incubation with trypsin, the swelling ratio increased during the first 20 h to obtain a maximum, after which the swelling ratio rapidly decreased until the hydrogels were completely degraded after 40−80 h (Figure 5a). In line with the expectations, because the hydrogels with the lowest solid content and thus with the lowest cross-linking density, were degraded most rapidly.

Another series of hydrogels (PEG_4-10k, solid content 5, 10, and 15%, respectively) was incubated with plasmin. Contrary to our expectations, plasmin was not able to degrade the

hydrogels, because no significant reduction in swelling ratio was observed, even after 200 h of incubation (Figure 5b). Additional washing steps with an EDTA solution as a metal chelator to remove any residual copper, followed by PBS buffer, to remove any residual sodium ascorbate, were performed because both compounds were found to be inhibitors of plasmin,[43] prior the incubation with plasmin were unsuccessful. Also a 10-fold increase of plasmin concentration (26 μM) did not result in a measurable degradation of the hydrogels, irrespective of the solid content or cross-linking density of the hydrogels. Because plasmin has a higher molecular weight than trypsin (75.4 vs 23.8 kDa), its size may prevent access to the hydrogel, which might be an explanation for the hydrolytic stability of the hydrogels against plasmin. Another explanation could be that the tripeptide substrate is sterically inaccessible for plasmin or that it does not fit in the active site of plasmin due to its conversion into a bis-triazole embedded in a polymeric network.

Conclusion

We have shown that the Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction can be used for the convenient synthesis hydrogels based on a bis-azido peptide and alkyne-functionalized star-shaped PEG derivatives. The storage modulus of the hydrogels could be tailored by variation of the solid content of the hydrogel, by variation of the molecular weight and three-dimensional architecture of the PEG derivatives, or by variation of the azide/alkyne molar ratio. The bis-azido peptide could be hydrolyzed by both trypsin and plasmin, while the hydrogels were only sensitive to degradation by trypsin and completely degraded after 40−80 h incubation at physiological conditions, depending on their cross-linking density.

Supporting Information Available. Copies of ^{1}H NMR spectra of compounds **6**, **9**, **10**, and **12**, ^{13}C NMR spectrum and HPLC and LCMS analysis data of compound **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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