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ARTICLE

Hydrolytically Stable Bioactive Synthetic Glycopeptide Homo- and Copolymers by Combination of NCA Polymerization and Click Reaction

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ABSTRACT: The synthesis of poly(DL-propargylglycine) and poly(γ-benzyl-L-glutamate-*co*-DL-propargylglycine) was performed by NCA polymerization at 0 °C to yield well-defined polypeptides with polydispersity indices below 1.3. FTIR results confirm β-sheet and α-helical conformation of the homopolymer and copolymer, respectively. The subsequent glycosylation was achieved by Huisgen [3 + 2] cylcoaddition ("click" reaction) with azide-functional galactose. FTIR, NMR, SEC, and MALDI−ToF analyses verify the successful glycosylation and suggest a high efficiency of the click reaction. The homoglycopeptide was found to be water-soluble and to form aggregates in water above a critical concentration of 0.079 mg/mL. Selective lectin binding experiments confirmed that the glycopeptides can be used in biorecognotion applications. Moreover, the selective hydrolysis of the benzyl ester groups in the copolymer was achieved without loss of the galactose.

Introduction

Carbohydrates are important in many complex biological processes as diverse as signal transmission, fertilization, inflammation, protein folding and many more. In particular, glycoproteins are seen to play a key role in these processes and in the past decade many efforts were directed toward their synthesis.[1] Well-defined artificial glycoconjugates are an interesting alternative to glycoproteins (biomimetic analogues). Glycopolymers, i.e., sugar-conjugated synthetic macromolecules, are nowadays synthetically well accessible and often show biological activity useful in biomedical applications. In particular the advances in controlled radical polymerization techniques and highly efficient coupling reactions have accelerated the development of synthetic glycopolymers.[2−8] While the majority of the reported glycopolymers are acrylate-based, it is desirable to develop systems with a higher resemblance to natural glycopeptides by employing synthetic polypeptides. Useful in that respect are recently developed techniques for the ring-opening polymerization of amino acid *N*-carboxyanhydrides (NCA) allowing a high level of control over the polypeptide structures.[9−15] Availability of a versatile synthetic protocol to glycopeptides derived from NCAs would give access to a promising class of biomimetic analogues and open new application areas owing to their structural similarity to natural peptides. However, up to now only a very limited number of examples of synthetic polypeptide−sugar conjugates were reported. This is primarily due to the inability to readily synthesize glycopolypeptides in a controlled manner. For example, the polymerization of sugar-functional NCA has been described, but the monomer synthesis is challenging and requires the use of protected sugars.[16,17] The polymer analogous glycosylation of polypeptides offers a possible alternative. It is widely applied for the glycosylation of individual amino acids in natural proteins by reaction of functional and in some cases activated sugars with an asparagine (N-linkage) or with a serine or threonine (O-linkage) of the polypeptide.[1] While both strategies make use of the functionalities of the natural amino acids in the polypeptide and could also be applied for the glycosylation of synthetic polypeptides, they have two major drawbacks. First, they would require protection of the amino acids before NCA synthesis, and second, the nature of the glycosidic linkage between the saccharide and the peptides makes it prone to chemical and enzymatic hydrolysis.

"Click"-type reactions address both drawbacks and are ideally suited for peptide glycosylylation due to their high efficiency.[18,19] Reactions such as the Huisgen [3 + 2] cycloaddition between organic azides and acetylenes have already proven to be highly versatile for the glycosylation of synthetic polyacrylates.[8] However, reports on the application of this reaction in peptide modification and more specifically in the synthesis of glycopeptides are still rare. Directed mutagenesis was, for example, used to replace a natural amino acid in a defined position of the amino acid sequence of peptides with a non-natural azide or alkyne functional amino acid. Selective glycosylation of these individual amino acids was then achieved by click reaction with the corresponding alkyne or azide sugar.[20−22] For synthetic NCA-derived polypeptides Lecommandoux described the synthesis of amphiphilic block copolymers including the coupling of oligosaccharides by Huisgen cycloaddition to polypeptide end-groups.[23−26] A few recent publications report on the side-chain modification of NCA-derived polypeptides. Thiol−ene coupling was used by us for the modification of a cystein containing copolypeptide[27] and Schlaad applied the same technique to couple thiol functional sugars to poly(DL-allylglycine).[28] Hammond first described azide−alkyne "click" reactions for the modification of NCA-derived polypeptides.[29] Poly(ethylene glycol) (PEG) azide was coupled to an alkyne functional homopolypeptide synthesized from γ-propargyl-L-glutamate NCA. Very recently Xuesi Chen reported the application of "click" reactions to synthesize a mannose functional polypeptide using the same NCA[30] and Donghui Zhang applied the same technique to click alkyne functional sugar to an azide functional polypeptide.[31] The latter was obtained by a multistep synthesis and a final azide-halogen exchange on poly(γ-3-chloropropanyl-L-glutamate). All azide−alkyne systems

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reported so far rely on glutamic acid esters thereby introducing a potentially hydrolytically unstable ester bond between the carbohydrate and the polypeptide. This could have disadvantages in a biological environment as enzymatic ester cleavage could result in reduced activity. It has been suggested that the use of non-natural amino acid with the amino acid side chain connected to the sugar unit via an isosteric linkage may lead to a chemically and metabolically more stable analogue while retaining biological activity.[32] Moreover, ester linkages potentially limit the possibilities to combine this approach with common deprotection techniques applied in synthetic polypeptide chemistry and thus the possibility to design more complex polypeptides. We therefore investigated the applicability of the commercial non-natural alkyne functional amino acid DL-propargylglycine for the synthesis of glycopeptides. In this paper we report on the synthesis and characterization of homo- and copolymers with γ-benzyl-L-glutamate and their glycosylation by Huisgen "click" reactions. We show that a high degree of conjugation can be achieved by this method and investigate the influence of the glycosylation on the peptide conformation. Furthermore, we provide first evidence for the bioactivity of the glycopeptides and their stability under selective ester deprotection conditions.

Experimental Procedures

Materials. All chemicals were purchased from Sigma-Aldrich and used as received unless otherwise noted. γ-Benzyl-L-glutamate and DL-propargylglycine were supplied by Bachem. Diethyl ether was purchased from VWR. Anhydrous DMF, DMSO, ethyl acetate, THF, and methanol were used directly from the bottle under an inert and dry atmosphere. *Ricinus communis* (castor bean) Agglutinin RCA_{120} (10 mg/mL in buffered aqueous solution) and Concanavalin A (Con A, Type IV, lyophilized powder) from *Canavalia ensiformis* (Jack bean) were purchased from Aldrich and used as received. 0.01 M Phosphate-buffered saline (PBS) at pH 7.4 was prepared by dissolving one tablet of PBS (Sigma-Aldrich) into 200 mL of distilled water. γ-benzyl-L-glutamate NCA was synthesized following a literature procedure.[33] 1-β-Azido-2,3,4,6-tetraacetyl-D-galactose was synthesized following a literature procedure.[34] The spectroscopic data were in agreement with literature data.

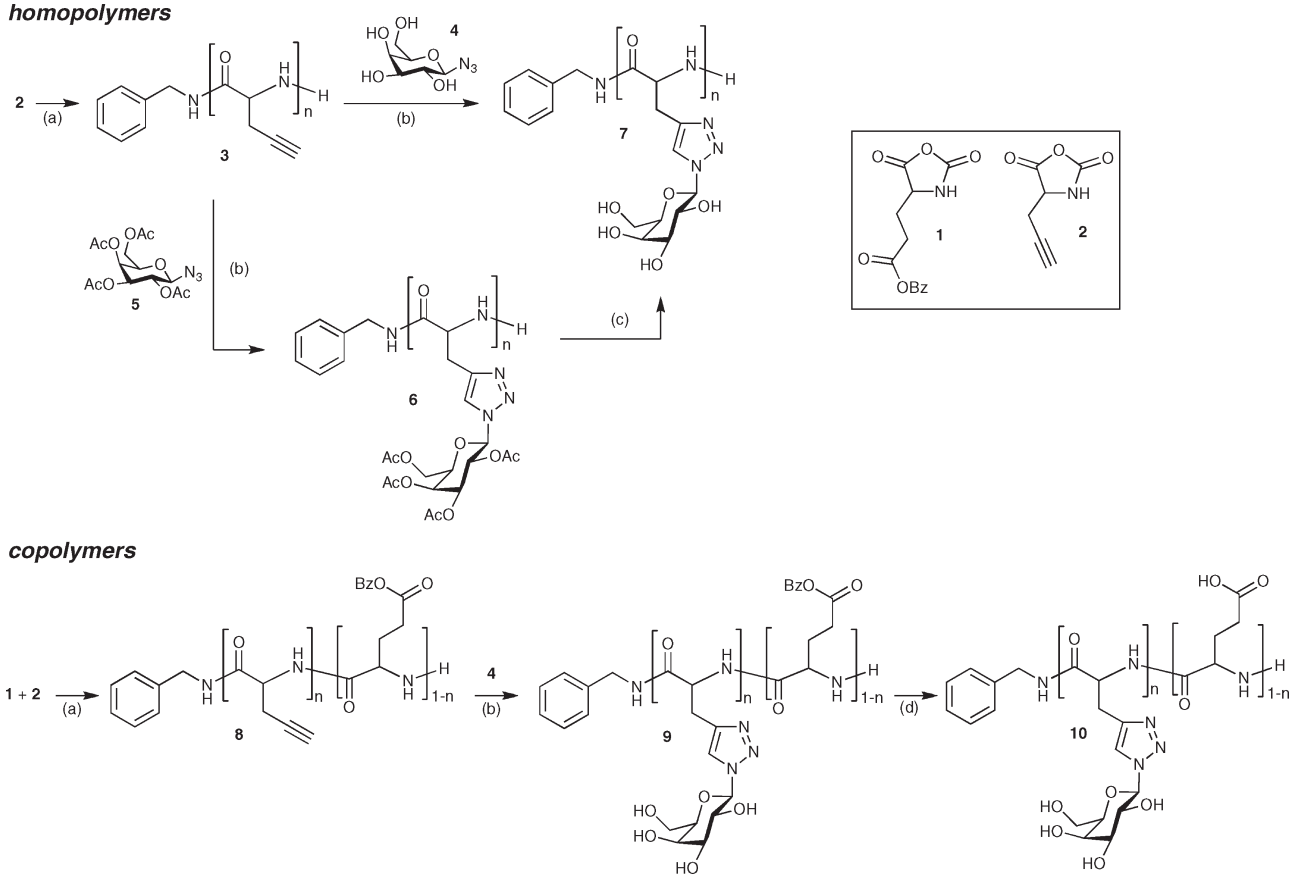
Methods. ^{1}H and ^{13}C NMR spectra were recorded at room temperature with a Bruker Avance 400 (400 MHz) and a Bruker Avance Ultrashield 600 (600 MHz). DMSO-d_6, $CDCl_3$, acetone-d_6, and D_2O were used as solvents and signals were referred to the signal of residual protonated solvent signals. TMS was used as an internal standard for DMSO-d_6 and $CDCl_3$. ATR-FTIR spectra were collected on a Perkin-Elmer Spectrum 100 in the spectral region of 650−4000 cm^{-1} and were obtained from 4 scans with a resolution of 2 cm^{-1}. A background measurement was taken before the sample was loaded onto the ATR unit for measurements. SEC analysis using Hexafluoroisopropanol (HFIP, Biosolve, AR-S from supplier or redistilled) as eluent was carried out using a Shimadzu LC-10AD pump (flow rate 0.8 mL/min) and a WATERS 2414 with a differential refractive index detector (at 35 °C) calibrated with poly(methyl methacrylate) (range 1000 to 2000000 g/mol). Two PSS PFG-lin-XL (7 μm, 8 × 300 mm) columns at 40 °C were used. Injections were done by a Spark Holland MIDAS injector using a 50 μL injection volume. Before SEC analysis was performed, the samples were filtered through a 0.2 μm PTFE filter (13 mm, PP housing, Alltech). Matrix assisted laser desorption/ionitiation−time of flight−mass spectroscopy (MALDI−ToF) analysis was carried out on a Voyager DE-STR from Applied Biosystems (laser frequency 20 Hz, 337 nm and a voltage of 25 kV). The matrix material used was *trans*-2-[3-(4-*tert*-Butylphenyl)-2-methyl-2-propenylidene] malononitrile (DCTB; 40 mg/mL). Potassium trifluoroacetic acid (KTFA) was added as cationic ionization agent (5 mg/mL). The polymer sample was dissolved in HFIP (1 mg/mL), to which the matrix material and the ionization agent were added (5:1:5), and the mixture was placed on the target plate. Samples were precipitated from the reaction medium in diethyl ether, filtered and placed in a freezer before measuring. Melting points were measured by Differential scanning calorimetry (DSC) on a TA DSC Q200 calorimetry in nitrogen at a heating rate of 10 °C/min and a cooling rate of 5 °C/min in the range from −20 to +200 °C. TEM images were obtained using a JEOL 2100 TEM scan instrument (at an accelerating voltage of 200 kV) for samples deposited on carbon-coated (400 mesh) copper grids. The preparation of samples for TEM analysis involved depositing a drop (15 μL) of the glycopeptide solution, which was dissolved in DI water onto the grids and allowing water to evaporate prior to imaging. The turbidity assay of the lectin with different concentrations of glycopeptides was monitored at 450 nm in Varian Cary 50 by UV quartz cuvette. The dynamic light scattering (DLS) experiments of glycopeptides in DI water solution were performed at 25 °C on a Zetasizer Nano ZS particle analyzer (Malvern Instruments, Worcestershire UK) using a detection angle of 173° and a 4 mW He−Ne laser operating at a wavelength of 633 nm. Emission spectra for the critical aggregation concentration were recorded on a Varian Cary Eclipse fluorescence spectrophotometer at an excitation wavelength of 340 nm using a 1 cm optical path length quartz cuvette. The spectra were averaged from triplicate recorded spectra. CD-spectroscopy was performed on a Jasco J-815 spectrometer with 0.0045 mM solution of the peptide in demineralized water.

Synthesis of DL-Propargylglycine NCA (**2**). DL-Propargylglycine **1** (2.5 g, 22.1 mmol) and α-pinene (14.88 g, 109 mmol) were dissolved in 60 mL of anhydrous THF in a three-neck round-bottom flask. The reaction mixture was heated to 50 °C under nitrogen and then triphosgene (4.92 g, 16.6 mmol) in 20 mL of THF was added dropwise over a period of 1 h. The reaction was continued for 4 h until the mixture became gradually clear. The mixture was concentrated under reduced pressure and the NCA precipitated by addition of 100 mL of *n*-heptane. The mixture was then placed in a freezer overnight. After filtration, the crude product was dissolved in dry THF, and recrystallized twice by addition of *n*-heptane. The obtained solid was washed with *n*-heptane, yielding off-white crystals in 75% yield. ^{1}H NMR (400 MHz, acetone-d_6, δ, ppm): 2.62 (t, J = 2.5 Hz, 1H, ≡CH,), 2.84 (dd, J = 4.5, 2.5 Hz, 2H, $-CH_2-C\equiv$), 4.75 (t, J = 4.5 Hz, 1H, CH), 8.04 (s, 1H, NH), ^{13}C NMR (400 MHz, acetone-d_6, δ, ppm): 22.26 (-$CH_2-C\equiv$), 57.30 (*C*H), 73.64 (≡*C*H), 78.23 ($-C\equiv$CH), 152.71 (−O(*C*O)NH−), 170.62 (−O(*C*O)CH). FTIR (neat, cm^{-1}): 3363, 3247, 1854, 1771, 1286, 1195, 1111, 1089, 934, 893, 777, 756, 723, 698, 668. Mp: 114 °C.

Synthesis of Poly(D,L-propargylglycine) (**3**). The DL-propargylglycine NCA (800 mg, 5.76 mmol) and anhydrous LiBr (261 mg, 3 mmol) were dissolved in 28 mL of dry DMF in a Schlenk tube. A solution of benzylamine (31.08 mg, 0.288 mmol) in 2 mL of dry DMF was added after the NCA and LiBr were totally dissolved. The reaction was maintained for 5 days at 0 °C under an inert atmosphere. The reaction mixture was precipitated into an excess diethyl ether, filtered and dried under vacuum to yield a pale yellow solid. Yield: 84%. M_n: 2250 g/mol, PDI: 1.16.

Synthesis of 1-Azido-1-deoxy-β-D-galactopyranoside (*1-Azido-β-galactose*) (**5**). The synthesis was carried out following a slightly modified literature procedure.[35] 1-β-Azido-2,3,4,6-tetraacetyl-D-galactose (373 mg, 1 mmol) was dissolved in 5 mL of anhydrous methanol in a Schlenk tube. To this solution a catalytic amount of anhydrous potassium carbonate (6 mg, 0.04 mmol) was added and the reaction mixture was vigorously stirred at room temperature under a nitrogen atmosphere for 3 h. Amberlite IR-120 ion-exchange resin was washed with methanol and then added to and stirred with the reaction mixture for 1 h. The resin was then filtered off under gravity and the resulting solution was concentrated to dryness in vacuum to yield a white powder (184 mg, 90%). Mp 150 °C. ^{1}H NMR (400 MHz, D_2O, δ, ppm): 3.49 (dd,

Scheme 1. Synthesis of Glycopeptides[a]



[a] Key: (a) LiBr, DMF, benzylamine, 0 °C; (b) $Cu(PPh_3)_3Br$, Et_3N, DMSO, 30 °C; (c) MeONa, DCM/MeOH, room temperature; (d) TFA, HBr, room temperature.

J_{2-1} = 8.7 Hz, J_{2-3} = 9.8 Hz, 1H, H-2), 3.66 (dd, J_{3-2} = 9.8 Hz, J_{3-4} = 3.3 Hz, 1H, H-3), 3.72−3.78 (m, 3H, H-5, H-6a′, H-6b′), 3.94 (d, J_{4-3} = 3.3 Hz, 1H, H-4), 4.64 (d, J_{1-2} = 8.7 Hz, 1H, H-1). ^{13}C NMR (400 MHz, D_2O, δ, ppm): 63.44 (C-6), 71.00 (C-4), 72.81 (C-3), 75.13 (C-5), 79.70 (C-2), 93.05 (C-1).

Glycosylation of Poly(D,L-propargylglycine) Using Protected Galactose (***6***). The poly(DL-propargylglycine) (100 mg, ca. 0.938 mmol of alkyne units), 1-β-azido-2,3,4,6-tetraacetyl-D-galactose (526 mg, 1.407 mmol, 1.5 equiv) and triethylamine (68 μL, 0.492 mmol, 0.5 equiv) were dissolved in 5 mL of anhydrous DMSO in a Schlenk tube. The mixture was stirred and degassed by bubbling with nitrogen for 30 min. $(PPh_3)_3CuBr$ (88 mg, 0.094 mmol, 0.1 equiv) was then added and nitrogen was bubbled through the resulting solution for another 30 min. Then the Schlenk tube was placed in an oil bath at 30 °C for 72 h under nitrogen atmosphere. The reaction solution was precipitated into a large excess of diethyl ether. The filtrated solid was dissolved in THF and passed through a short neutral aluminum oxide column eluting with THF (2×). The polymer was recovered by precipitation in diethyl ether and dried under high vacuum. Yield: 40%. M_n: 6450 g/mol, PDI: 1.25.

The deacetylation of glycopeptides clicked with protected galactose was carried out following a slightly modified literature procedure.[4] A 75 mg sample of acetylated glycopeptide was dissolved in 6 mL of CH_2Cl_2/CH_3OH mixture (v/v = 2:1) in a Schlenk tube. The solution was degassed with nitrogen for 15 min and 11 mg sodium methoxide was added. After few seconds, the reaction solution became very turbid and the mixture was stirred under N_2 atmosphere at room temperature overnight to allow maximum deacetylation of the glycopeptide. The mixture was dried under reduced pressure. Yield: 99%.

Glycosylation of Poly(D,L-propargylglycine) Using Unprotected Galactose (***7***). The poly(DL-propargylglycine) **3** (100 mg, 0.938 mmol of clickable alkyne units), 1-Azido-1-deoxy-β-D-galactopyranoside **4** (289 mg, 1.407 mmol, 1.5 equiv) and triethylamine (68 μL, 0.492 mmol, 0.5 equiv) were dissolved in 5 mL of anhydrous DMSO in a Schlenk tube. The mixture was stirred and degassed by bubbling nitrogen for 30 min. $(PPh_3)_3CuBr$ (88 mg, 0.0938 mmol, 0.1 equiv) was then added and nitrogen was bubbled through the resulting solution for another 30 min. Then the Schlenk tube was placed in an oil bath at 30 °C for 72 h under nitrogen atmosphere. The solution was precipitated into THF twice to remove the catalyst, and then the obtained solid was redissolved in DMSO and precipitated twice in methanol. The polymer was filtered and dried in a vacuum oven. Yield 55%. M_n: 4150 g/mol, PDI: 1.13.

Synthesis of Poly(γ-benzyl-L-glutamate-co-DL-propargylglycine) (***8***). γ-Benzyl-L-glutamate NCA **1** (1.01 g, 3.82 mmol), DL-propargylglycine NCA **2** (267 mg, 1.91 mmol), and anhydrous LiBr (261 mg, 3 mmol) were dissolved in 28 mL of anhydrous DMF. A solution of benzylamine (20.44 mg, 0.191 mmol) in 2 mL of dry DMF was added after both NCAs were dissolved. The reaction was maintained for 5 days at 0 °C under an inert atmosphere. The reaction mixture was precipitated into an excess diethyl ether, filtered and dried under vacuum as a pale yellow solid. Yield: 80%. M_n: 5800 g/mol, PDI: 1.15.

Glycosylation of Poly(γ-benzyl-L-glutamate-co-DL-propargylglycine) (***9***). Poly(γ-benzyl-L-glutamate-*co*-DL-propargylglycine) (400 mg, ca. 0.735 mmol of alkyne units), 1-Azido-1-deoxy-β-D-galactopyranoside (226 mg, 1.102 mmol, 1.5 equiv to alkyne) and triethylamine (51 μL, 0.367 mmol, 0.5 equiv) were dissolved in 8 mL of anhydrous DMSO in the Schlenk tube. The mixture was stirred and degassed by bubbling nitrogen for 30 min. $(PPh_3)_3CuBr$

(68 mg, 0.0735 mmol, 0.1 equiv) was then added and nitrogen was bubbled through the resulting solution for another 30 min. Then the Schlenk tube was placed in an oil bath at 30 °C for 72 h under nitrogen atmosphere. The amphiphilic nature of the copolymer after glycosylation makes the identification of a proper precipitation solvent difficult. After the reaction, the polymer solution was added dropwise to an excess diethyl ether, the obtained polymer redissolved in DMSO and precipitated twice in a 1:2 THF/methanol mixture. The polymer was centrifuged and dried in vacuum oven. Yield: 30%. M_n: 7050 g/mol, PDI: 1.21.

Benzyl Ester Hydrolysis of Glycosylated Poly(γ-benzyl-L-glutamate-co-DL-propargylglycine) (***10***). The hydrolysis was done following a modified literature procedure.[36] The glycosylated copolymer (100 mg) was dissolved in 2.0 mL of trifluoroacetic acid (TFA). A 6-fold excess with respect to γ-benzyl-L-glutamate of a 33% of HBr in acetic acid (0.3 mL) was added. After 16 h, the mixture was added dropwise into diethyl ether. The precipitates were redissolved in DMF, precipitated twice in diethyl ether, and dialyzed in water for 3 days. The polymer was filtered and dried under reduced pressure. Yield 60%.

Critical Aggregation Concentration. The critical aggregation concentration of the glycopeptide was determined following a literature procedure.[37] A stock solution of the glycopeptide with a concentration of 1.0 mg/mL was prepared by dissolving the glycopeptides in DI water. This stock solution was further diluted to yield a series of solutions with concentrations varying from 1.0 to 0.001 mg/mL. A defined amount of the fluorescence probe *N*-phenyl-1-naphthalamine (PNA) in acetone was added to each of the solutions in a 20 mL volumetric flask and then acetone was evaporated overnight. The concentration of PNA in the final solution was 2.0×10^{-6} mol/L. In total, the samples were kept for 48 h to equilibrate the PNA and aggregates before the fluorescence spectra were measured.

Carbohydrate−Lectin Binding Recognition. The lectin recognition activity of the glycopeptide solution was analyzed by the change of the turbidity at 450 nm at room temperature. A 2 mg/mL sample of RCA_{120} lectin was first prepared in 0.01 M phosphate buffered saline (PBS) at pH 7.4. Then 600 μL of lectin solution was transferred into a cuvette and a baseline measured. A solution of 60 μL of glycopeptide with different concentrations in PBS buffer solution was added into the cuvette containing the lectin solution. The solution in the cuvette was gently mixed using a pipet and immediately the absorbance at 450 nm was recorded every 5 min. As a control lectin Con A was used under the same experimental conditions as well as PBS buffer solution without any glycopeptide.

Results and Discussion

γ-Benzyl-L-glutamate and DL-propargylglycine were converted into the corresponding NCAs **1** and **2** by reaction with triphosgene. The homo polymerization of **2** was carried out with benzyl amine as an initiator in DMF at 0 °C to prevent side-reactions and maintain structural control (Scheme 1).[38] The only previous report on the synthesis and polymerization of NCA **2** was by Schlögel and Pelousek in 1960, who observed a low solubility of the poly(DL-propargylglycine) in most solvents.[39] Indeed, under the applied polymerization conditions a fast gelling of the reaction medium was observed and only very low molecular weight oligopeptides were obtained. This is most likely caused by intermolecular hydrogen bonding (β-sheets), which limits the polymer growth. Addition of LiBr to the polymerization medium improved the polymerization results and poly(DL-propargylglycine) **3** with a number-average molecular weight (M_n) of 2250 g/mol and a low polydispersity index (PDI) of 1.16 was obtained (Table 1, Figure 1). In agreement with the report of Schlögel and Pelousek, the solubility of this polypeptide is very poor in most common solvents. MALDI−ToF analysis confirms the low polydispersity and the structural homogeneity of the material, evident from the

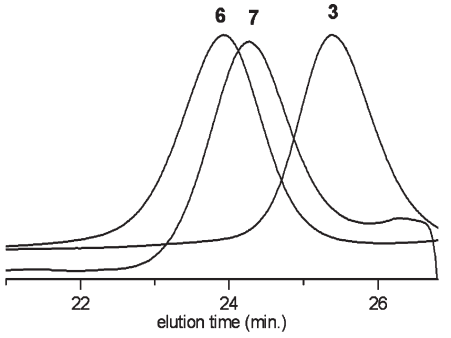


Figure 1. SEC traces (HFIP, PMMA standards) of poly(DL-propargylglycine) (**3**; M_n, 2250 g/mol; PDI, 1.16) and the glycosylated poly(DL-propargylglycine) by "click" reaction with protected (**6**; M_n, 6450 g/mol; PDI, 1.25) and unprotected galactose (**7**; M_n, 4150 g/mol; PDI, 1.13).

Table 1. Molecular Weights and Polydispersity Indices (PDI) of Polypeptides before and after Click Reaction

sample	M_n(GPC) (g/mol)	PDI
3	2250	1.16
6	6450	1.25
7	4150	1.13
8	5800	1.15
9	7050	1.21

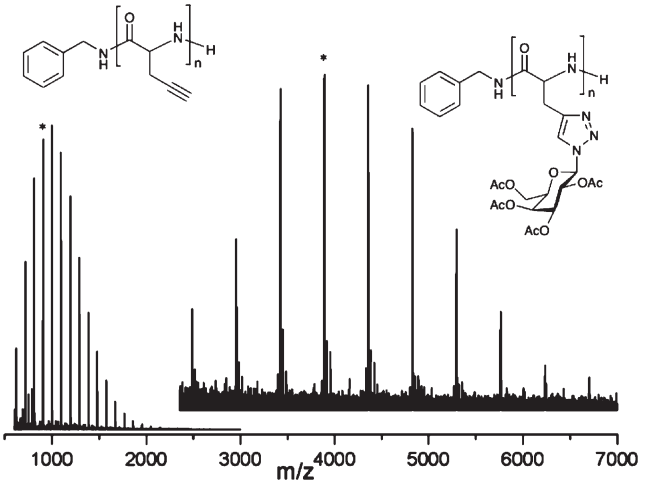


Figure 2. MALDI−ToF−MS spectra of poly(DL-propargylglycine) (**3**) and the glycosylated poly(DL-propargylglycine) by "click" reaction with protected galactose (**6**) (small peaks represent copper adducts). The asterisk denotes the polymers with $n = 8 + K^+$: m/z 905.91 (**3**) and m/z 3892.26 (**6**).

presence of peaks exclusively corresponding to propargylglycine repeating units and benzyl amide and amine end-groups, respectively (Figure 2). Inspection of the ^{1}H- and ^{13}C NMR further confirms the structure of the poly(DL-propargylglycine) (Figure 3 and Figure 4). Most characteristic for this polymer are the carbon peaks at 22 ppm (*f*) 73 ppm (*h*) and 80 ppm (*g*) and the proton peak at 2.6 ppm (*d*) and 2.8 ppm (*e*) from the alkyne moiety. From the integrated peak area ratio of the benzyl amine *a* at 7.27 ppm and the combined peaks *b* and *c* (4.2−4.6 ppm) in the ^{1}H NMR spectrum a molecular weight of 1912 g/mol can be calculated, which is slightly lower than the SEC molecular weight (PMMA standards). Besides the amide II band at 1513 cm^{-1}, IR spectroscopy reveals the presence of two amide I bands at 1630 (strong) and 1700 (weak) cm^{-1} characteristic of a β-sheet conformation (Figure 5).[40,41] This is in agreement with an investigation by Akaike who found that polyvaline containing various ratios of D- and L-amino acids all had IR-spectra and X-ray diffraction patters that were consistent with a

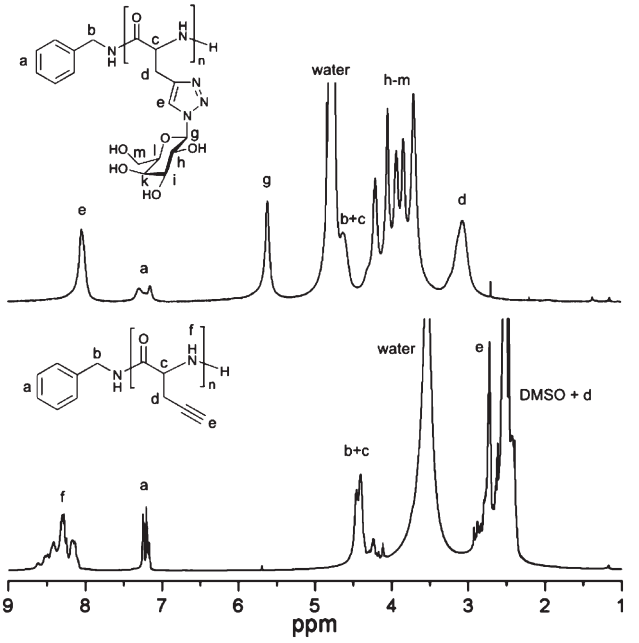


Figure 3. ^{1}H NMR spectra of poly(DL-propargylglycine) **3** in DMSO-d_6 and glycosylated polypeptide **7** after "click" reaction with unprotected galactose **4** in D_2O.

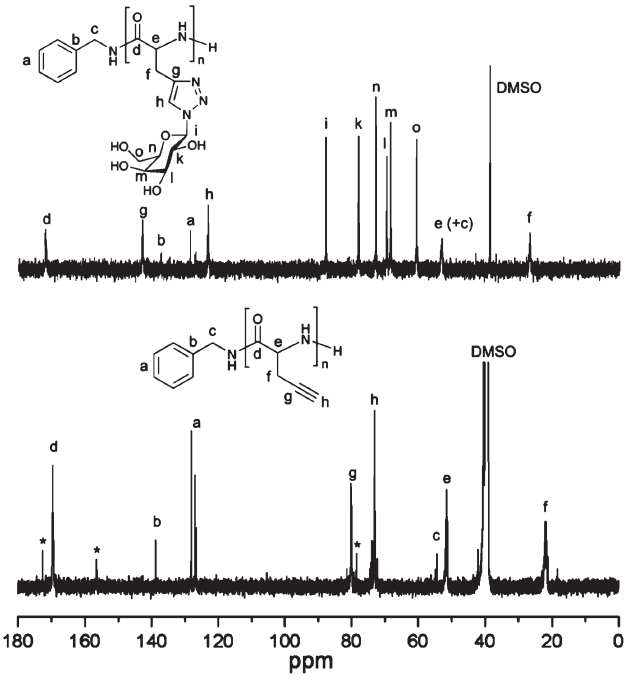


Figure 4. ^{13}C NMR spectra of poly(D,L-propargylglycine) **3** in DMSO-d_6 and glycosylated polypeptide **7** after "click" reaction with unprotected galactose **4** in D_2O (asterisk denotes residual NCA signals).

β-sheet configuration.[42] The only noticeable effect of the D-enantiomer was an increased spacing between the sheets. Because of the low solubility of **3** in suitable solvents it was not possible to confirm the conformation by circular dichroism (CD) spectroscopy.

The copolymerization of **2** with γ-benzyl-L-glutamate NCA **1** at a monomer feed ratio of 1:2 was carried out under similar conditions as the homopolymerization. Owing to the slightly higher solubility of the copolymer a higher molecular weight of 5800 g/mol (PDI: 1.15) was obtained. ^{1}H NMR spectroscopy confirms the presence of both monomers in the copolymer in a ratio

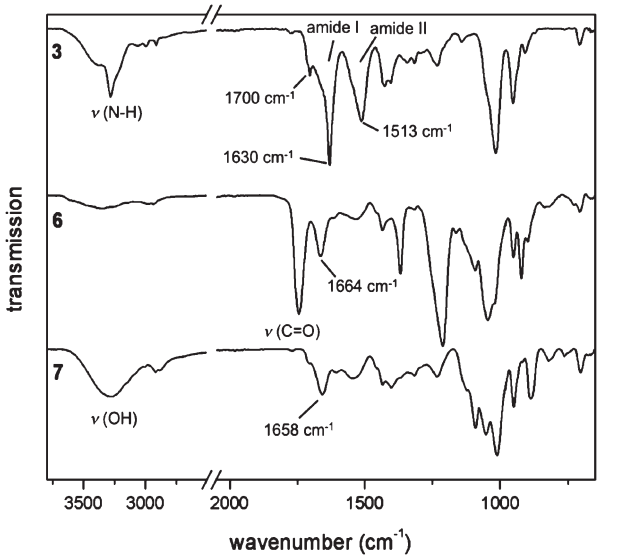


Figure 5. FTIR spectra of poly(DL-propargylglycine) (**3**) and the glycosylated poly(DL-propargylglycine) by "click" reaction with protected (**6**) and unprotected galactose (**7**).

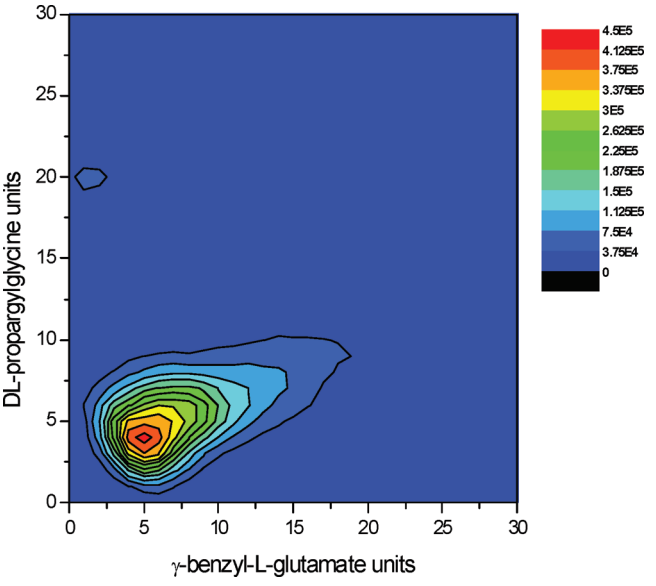


Figure 6. MALDI−ToF−MS contour plot of copolymer **8**.

corresponding to the monomer feed ratio (Supporting Information). Moreover, the MALDI−ToF spectrum shows the complex signal pattern of a copolymer (Supporting Information). Using a software based MALDI−ToF deconvolution method developed in our group, the spectrum was converted into composition contour plots.[43−45] The shape of the contour plot allows conclusions to be drawn concerning the molecular distribution of the comonomers in the chain. The contour plot of copolymer **8** exhibits a directional coefficient and a single distribution with a maximum at five γ-benzyl-L-glutamate and three propagylglycine units (Figure 6). This is characteristic for a random copolymer and in good agreement with the monomer feed ratio. Interestingly, the copolymer adopts an α-helical conformation as evident from the positions of the IR amide bands at 1655 and 1543 cm^{-1} (Figure 7) and the CD-spectrum (Supporting Information). It can be speculated that the strong helix-forming ability of the γ-benzyl-L-glutamate units is the driving force for the helical conformation but without further data the role of both amino acids in the secondary structure cannot be determined.

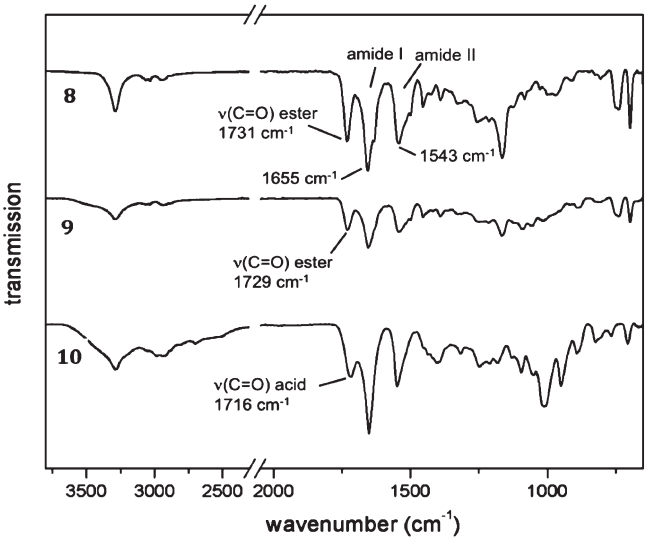


Figure 7. FTIR spectra of poly(γ-benzyl-L-glutamate-*co*-DL-propargylglycine) (**8**), the glycosylated poly(γ-benzyl-L-glutamate-*co*-DL-propargylglycine) obtained by "click" reaction with unprotected galactose (**9**), and the glycosylated poly(DL-propargylglycine-*co*-L-glutamaic acid) (**10**).

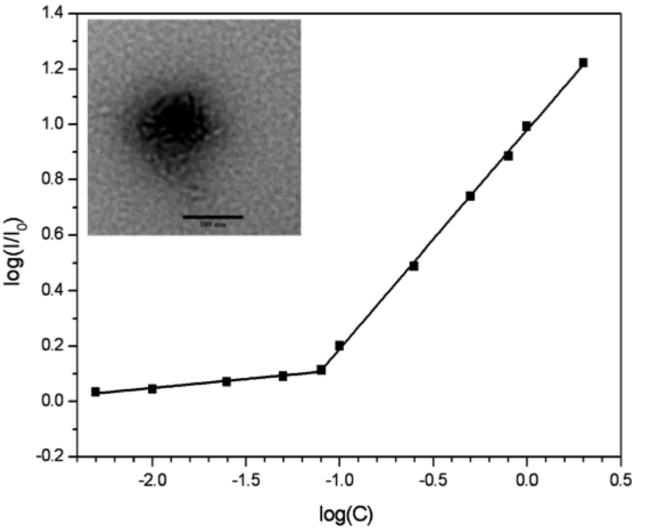


Figure 8. Relative fluorescence intensity of PNA as a function of the concentration of glycopeptides **7** in aqueous solution (*C*: concentration in mg/mL). The intersection of both lines marks the critical aggregation concentration. The inset shows a TEM micrograph of **7** in water (0.5 mg/mL); the scale bar represents 100 nm.

The glycosylation of both polymers was done with galactose, which can be readily converted to the corresponding azide. In order to investigate the influence of the free sugar hydroxy groups on the results of the "click" reaction both the acetyl-protected galactose **5** and the unprotected galactose **4** were employed for the glycosylation of **3**. Immediately noticeable was the improved solubility of the polypeptide in common organic solvents after conjugation with the acetylated galactose. A clear shift of the SEC trace to higher molecular weights was observed after attachment of both the protected and unprotected galactose (Figure 1). While in the latter case an M_n of 4150 g/mol was calculated from SEC, functionalization with the protected galactose produced a polymer with an M_n of 6450 g/mol in accordance with the higher molecular weight of the sugar units (Table 1). Spectroscopic evidence for the successful glycosylation was obtained from NMR spectra, which are in agreement with the proposed structure (not shown). An approximation of the click reaction yield was obtained from the MALDI−ToF spectrum of **6** (Figure 2). The spectrum reveals one dominating polymer species with repeating units corresponding to the propargylglycine with the protected galactose attached via a triazole unit and benzyl amide and amino end-groups (other small signals were identified as copper aggregates of **6**). Although any quantification of MALDI−ToF spectra has to be viewed with caution, this result suggests a very high glycosylation yield. Equally successful was the direct glycosylation of **3** with the unprotected galactose **4**. Direct evidence for the attachment of the sugar to the polymer backbone via the "click" reaction was obtained from NMR spectroscopy. Figure 4 shows the ^{13}C NMR spectrum of **7** in which peaks characteristic of the galactose (*i*−*o*) as well as the polypeptide carbons (*a*−*e*) can be identified. Most importantly, peaks *g* at 143 ppm and *h* at 123 ppm can be assigned to triazole carbons, which experience a significant downfield shift upon "click" reaction. Similarly, the ^{1}H NMR spectrum of **7** reveals proton peaks characteristic of the polymer backbone, the triazole and the sugar (Figure 3). Most significant are the downfield shifts of protons *d* and *e* to 3.0 and 7.97 ppm, respectively, upon formation of the triazole ring. Polymer **7** is soluble in water and polar organic solvents like DMF and DMSO, which suggests similarly high functionalization efficiency as for **6**. This is supported by the fact that all spectroscopic and solubility results are identical irrespective of whether **7** was synthesized directly from the unprotected galactose or from the protected galactose with subsequent deprotection.

Besides further evidence for the presence of galactose in the polymer, the FTIR spectra (Figure 5) provide valuable information about the secondary structure of the polypeptide after glycosylation. In the case of the protected galactose, the FTIR spectrum of **6** clearly shows the presence of a carbonyl band at 1744 cm^{-1} owing to the acetyl protecting groups. Interestingly, both amide bands are shifted to higher wave numbers when compared to the spectrum of **3**. The amide I band can now be found at 1664 cm^{-1} (1630 cm^{-1} in **3**). A similar shift of amide bands was observed for polymer **7**, which contains the unprotected galactose. Moreover, the characteristic OH bands between 1100 and 1050 cm^{-1} as well as a broad band centered at 3290 cm^{-1} confirm the presence of the unprotected galactose. The positions of the amide bands suggest a random coil conformation of the glycopeptide.[40] Apparently, the presence of the bulky galactose moieties prevents the formation of intermolecular hydrogen bonds and thus the arrangement of the polypeptides into β-sheets. However, in aqueous solution the CD spectrum of **7** clearly confirms β-sheet conformation by a characteristic minimum at 214 nm (Supporting Information). This apparent contradiction can be rationalized by the sample history; FTIR spectra were recorded from solid polymers obtained by evaporation of a DMSO solution. The interruption of hydrogen bonding between the amino acid units of the polypeptides by DMSO is apparently sufficient to prevent the formation of β-sheets and thus force the polymers into a random coil conformation in the solid state. In water, on the other hand, the glycopeptides can form weak hydrogen bonds sufficient enough to assemble as β-sheets.

While polymer **7** is readily soluble in water, we hypothesized that this behavior must cause the formation of aggregates at higher concentration. Indeed, dynamic light scattering (DLS) revealed the formation of aggregates in the range of 540 nm (0.5 mg/mL). In order to determine the critical aggregation concentration of **7** more accurately, we carried out fluorescent probe experiments in the presence of *N*-phenyl-1-naphthalamine (PNA). PNA strongly emits in a hydrophobic environment while it is quenched in polar media.[46] When the fluorescence of solutions of **7** in the presence of PNA was monitored at different concentrations, only low fluorescence was detected at concentrations below 0.1 mg/mL. At higher concentrations the fluorescence

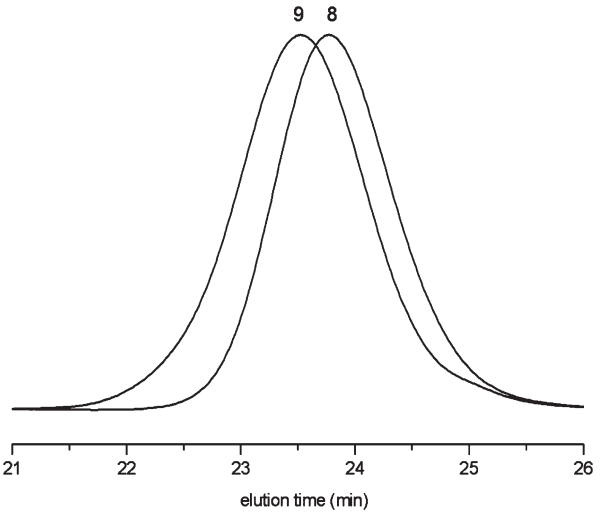


Figure 9. SEC traces (HFIP, PMMA standards) of poly(DL-propargylglycine-*co*-benzyl-L-glutamate) (**8**; M_n, 5800 g/mol; PDI, 1.15) and the glycosylated poly(DL-propargylglycine-*co*-benzyl-L-glutamate) obtained by "click" reaction with unprotected galactose (**9**; M_n, 7050 g/mol; PDI, 1.21).

drastically increased caused by the incorporation of PNA in the hydrophobic regions of the aggregates (Figure 8). The critical aggregation concentration of 0.079 mg/mL was determined by intersecting the two straight lines. A similar self-association was described by Li for glycosylated polyacrylates.[47] It appears that, although these highly glycosylated polymers are usually considered hydrophilic, hydrophobic interaction of the polypeptide backbone, and possibly hydrogen bonding via β-sheets as confirmed by CD spectroscopy, results in aggregation. TEM micrographs confirmed that these aggregates are nonuniform with large size variation in water (Supporting Information). From the enlarged micrograph shown in the inset of Figure 8 it seems that the aggregates consist of smaller tape-like assemblies. A detailed study into these phenomena is currently under way.

The glycosylation of copolymer **8** was carried out with the objective to investigate whether a selective deprotection of the benzyl ester groups of the γ-benzyl-L-glutamate can be achieved. The success of the click reaction and the presence of galactose in the copolymer were confirmed from both ^{1}H NMR (Supporting Information) as well as FTIR spectra (Figure 7). This coincides with an increase of the molecular weight of the copolymer from 5800 to 7050 g/mol (Figure 9). In contrast to the homopolymer, glycosylation does not result in a change of the polypeptide helical conformation as neither the amide I nor the amide II band experience a shift in the spectrum. The benzyl ester hydrolysis of the glycosylated copolymer **9** was carried under acid conditions with HBr. Of analytical relevance is the shift of the carbonyl band from 1731 (ester) to 1716 cm^{-1} (acid). Moreover, the ^{1}H NMR spectrum shows the complete disappearance of the signal at 7.5 ppm previously assigned to the aromatic benzyl ester protons (Supporting Information). Most importantly, signals of the galactose units are still present. These experiments emphasize a major advantage over recently reported systems in which the alkyne functionality was introduced via ester bonds (e.g., using glutamic acid) and are potentially prone to hydrolysis under these conditions.[29−31]

Finally, the ability of the synthesized glycopeptides **7** to interact with biological systems was assessed. Carbohydrates play a major role in biological recognition events mediated by specific carbohydrate-lectin interaction. While the exact mechanism of this interaction is still unknown, many studies show that it is highly specific and noncovalent. The *in vitro* evaluation of this

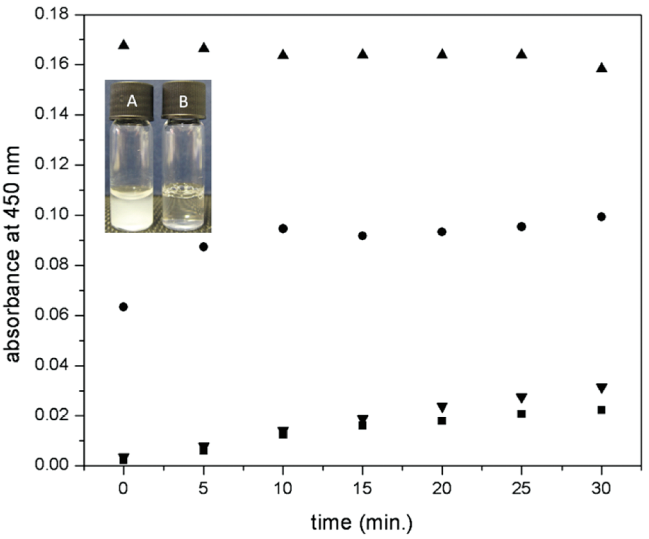


Figure 10. Absorbance (450 nm) of glycopeptides **7** solution upon reaction with two different lectins in PBS buffer: (▲) lectin RCA_{120}, glycopeptide concentration 2 mg/mL; (●) lectin RCA_{120}, glycopeptide concentration 0.1 mg/mL; (■)lectin RCA_{120}, PBS buffer without glycopeptide; (▼) lectin Con A, glycopeptide concentration 2 mg/mL. The inset shows the glycopeptide solution at 2 mg/mL with (A) RCA_{120} and (B) Con A present.

specific binding event is thus a first test for the ability of a synthetic glycopolymer to interact with biological systems, for example for the development of drug delivery, tissue engineering or other biomedical materials.[48] Typically, these tests are conducted by mixing the glycopolymer with a lectin that is selective for the sugar conjugated to the polymer.[4,8,49] A positive result is obtained by the appearance of a precipitation due to the aggregation of lectins measured as a reduced transparency of the solution. Since single sugar units only bind weakly to the lectin receptors, only multivalent binding will lead to lectin clustering and precipitation. *Ricinus communis* Agglutinin (RCA_{120}) is a known specific lectin for the selective binding of galactosyl residues. We therefore systematically investigated the change in absorbance of solutions of glycopeptides **7** with RCA_{120} at 450 nm. Upon addition of the glycopeptide in buffer solution to the lectin an immediate precipitation was visible (inset Figure 10). Further inspection of Figure 10 shows that the absorbance (i.e., turbidity) is higher for higher concentrations of glycopeptides. Moreover, at a glycopeptides concentration of 2 mg/mL the precipitation is so rapid that no change of absorbance was measured over time. At a concentration of 0.1 mg/mL the absorbance increases within the first 5 min of the experiment and reaches a plateau. As a control experiment, PBS buffer solution without glycopeptide was added to RCA_{120}. Only a slight increase in absorbance was detected, however, significantly lower than for the samples containing glycopeptides. When the same reaction was carried out with Concanavalin A lectin (Con A), which is selective for glucosyl and mannosyl but unable to bind galactosyl residues, no significant precipitation was monitored. The slight increase in absorbance monitored for Con A is in the range of the change detected for the control. These experiments confirm that the glycopeptides synthesized by "click" coupling of galactose to poly(DL-propargylglycine) are active in biorecognition. The lectin binding is selective and, depending on the concentration, instantaneous. In particular, no adverse effect of the triazole ring on the lectin binding was observed.

Conclusions

We have shown that synthetic glycopeptides can be readily obtained by Huisgen [3 + 2] cycloaddition of azide functional

galactose to alkyne-functional homo and copolypeptides. The fast and selective biorecognition was demonstrated by lectin clustering experiments. The fact that no labile bond was used to link the alkyne to the amino acid allows to apply common amino acid deprotection chemistry on the glycopeptides as was shown for the γ-benzyl-L-glutamate copolymer. The isosteric linkage thus improves the chemical and potentially metabolic stability while retaining biological activity. This presents a major advantage for the application of this approach for the synthesis of more complex polypeptides for a variety of structurally diverse biomimetic analogues.

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Supporting Information Available: Figures showing CD spectra, MALDI−ToF MS of the copolymer, ^{1}H NMR spectra of copolymers, and TEM of glycopeptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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