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pH-Responsive Bioactive Glycopolypeptides with Enhanced Helicity and Solubility in Aqueous Solution

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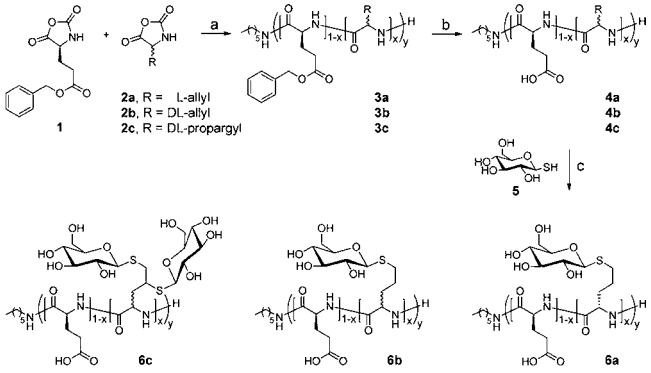
Supporting Information

ABSTRACT: Copolypeptides of L-glutamate and glucosylated L-/DL-allyl- or DL-propargylglycine were synthesized by ring-opening polymerization and thiol−ene/yne photochemistry in aqueous solution, allowing the mild introduction of sugar units (here, glucose) in the final step. The glucosylated and non-glucosylated samples adopt a random-coil conformation in neutral and basic media and an α-helical conformation in acidic media, the helical content depending on the number and configuration of allyl-/propargylglycine units. The glucocopolypeptides unveil enhanced helical stability and solubility down to pH 3.5. Turbidity assays proved the selective binding of the polymers to the plant lectin concanavalin A.

Stimuli-responsive polymers have attracted much interest because they are promising materials for advanced applications in biomedicine and life science (drug/gene delivery, diagnostics, tissue engineering, etc.) and bioinspired materials research, for instance, as mimics of adaptive biological systems.[1,2] The most frequently applied stimuli are pH, temperature, redox, light (also electrical or magnetic fields, etc.), which typically cause a drastic change in polymer solubility from hydrophilic to hydrophobic.[3] Polypeptide materials offer the additional feature of ordered conformations or secondary structure (e.g., α-helices), however, also accompanied by a decrease in hydrophilicity and solubility in water.[4−6] Stable polypeptide helices in water have been produced, such as poly[N^5-(ω-hydroxyalkyl)-L-glutamine],[7] poly[N^ε-(2-methoxyethoxy)acetyl-L-lysine],[8] poly(L-glutamate)s with elongated, charged side chains,[9,10] and glycosylated poly(L-cysteine)s.[11] This last example is quite exceptional because it shows a helix-to-coil transition that is triggered by oxidation yet irreversible, whereas the other systems are rather insensitive to any kind of external stimuli (pH, temperature, ionic strength, denaturing agents). Still, it remains a great challenge to produce stimuli-responsive polypeptide chains with good helix solubility in water.

Here we report the metal-free synthesis of glycosylated poly(L-glutamate)s by a combination of ring-opening copolymerization of amino acid *N*-carboxyanhydrides (NCAs) and photochemical thiol−ene/yne glycosylation in aqueous solution (Scheme 1). These glucocopolypeptides undergo pH-induced reversible coil-to-helix transitions, forming soluble α-helices in acidic media down to pH 3.5. Also, they can selectively bind to plant lectins via carbohydrate−protein interactions, making them potentially useful for applications in biomedicine and biomimetics.

Scheme 1. Synthesis of Glucocopolypeptides by NCA Copolymerization and Thiol−Ene/Yne Photochemistry[a]



[a]Reagents and conditions: (a) 1-hexylamine, DMF, 25 °C, 7 days; (b) MSA/anisole/TFA, 0−20 °C, 38 min; (c) Irgacure 2959, $h\nu$, 0.1 M aqueous acetate buffer, 25 °C, 12 h.

The NCAs of γ-benzyl L-glutamate (**1**), L- and DL-allylglycine (**2a** and **2b**, respectively), and DL-propargylglycine (**2c**) were synthesized using triphosgene in tetrahydrofuran (THF) and α-pinene as the HCl trap.[12] The monomers were isolated in high yields and purity [see the Supporting Information (SI)] after filtration through silica gel under an argon atmosphere (a modified version of the procedure of Kramer and Deming[13]).

Mixtures of NCAs **1** and **2a** (molar ratio 10:1), **1** and **2b** (10:1), and **1** and **2c** (5:1) at 4 wt % in *N*,*N*-dimethylformamide (DMF) were polymerized at 25 °C using 1-hexylamine as the initiator. After 1 week, the respective statistical co-polypeptides[14] **3a**, **3b**, and **3c** were precipitated in methanol, collected by centrifugation, and isolated in high yield (>85%). Results obtained by ^{1}H NMR analysis (chemical composition and molecular weight) and size-exclusion chromatography (SEC) (polydispersity index) (see the SI) are summarized in Table 1. Quantitative debenzylation of the co-polypeptides (>96% yield by ^{1}H NMR; see the SI) was achieved in less than 40 min by treatment with methanesulfonic acid (MSA) and anisole in trifluoroacetic acid (TFA) below room temperature, as described by Tsukada and co-workers,[15] thereby avoiding the risk of racemization of the polypeptide

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Table 1. Molecular Characteristics of the Copolypeptides Based on γ-Benzyl L-Glutamate and L-/DL-Allyl- or DL-Propargylglycine

sample	composition (monomers)	x^a	y^b	M_n (kg mol^{-1})^c	PDI^d
3a	**1**/**2a**	0.09	53	11.0	1.20
3b	**1**/**2b**	0.11	56	11.5	1.10
3c	**1**/**2c**	0.17	60	11.9	1.17

[a]Mole fraction of unsaturated amino acid units in the co-polypeptide, as determined by ^{1}H NMR analysis (400.1 MHz, TFA-d_1). [b]Number-average degree of polymerization, as determined by ^{1}H NMR end-group analysis. [c]Number-average molecular weight. [d]Apparent polydispersity index (as determined by SEC: eluent, *N*-methyl-2-pyrrolidone + 0.5 g/L LiBr; temperature, 70 °C; calibration, polystyrene).

backbone (as with alkaline hydrolysis) or reduction of unsaturated side chains (as with HBr/AcOH, TMSI/DCM, or H_2/Pd). The deprotected copolypeptides **4a**−**c** could be directly dispersed in water at neutral pH.

Glycosylation of the unsaturated peptide units was done with 1-thio-β-D-glucopyranose (**5**) (pK_a = 4.81, H_2O), which was obtained from its commercial sodium salt by treatment with DOWEX 50 acidic resin and lyophilization. To the 10−15 wt % solutions of copolypeptides **4a** and **4b** (1 wt % with respect to allylglycine units) in 0.1 M acetate buffer (pH 4.75) were added **5** (1.5 equiv with respect to allyl) and 4-(2-hydroxyethoxy)-phenyl 2-hydroxy-2-propyl ketone (Irgacure 2959) (0.1 equiv) as a photoinitiator. The mixtures were put under an argon atmosphere and irradiated for ∼12 h at room temperature with a medium-pressure mercury UV lamp (Heraeus TQ 150, 150 W), using the glass wall of the reaction vessel as a filter. Products **6a** and **6b** were isolated in high yield (>83%) after dialysis (RC 1000) against water and lyophilization. ^{1}H NMR analysis (see the exemplary spectrum of **6b** in Figure 1a)

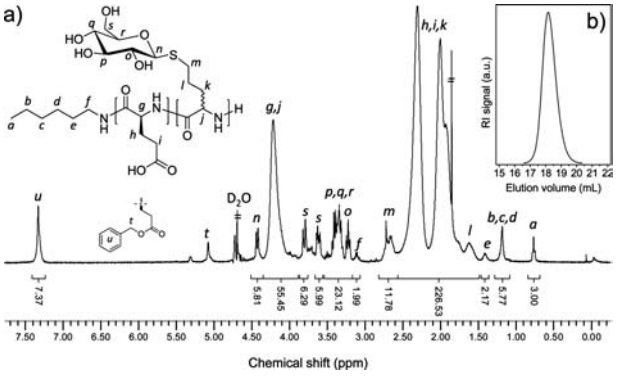

Figure 1. (a) ^{1}H NMR spectrum (400.1 MHz, D_2O) and (b) SEC refractive index (RI) trace (0.1 M aq $NaNO_3$) of glucocopolypeptide **6b**.

confirmed the complete disappearance of alkenes (5.0 and 5.7 ppm) and the presence of glucose units (3.0−4.0 and 4.5 ppm; −C*H*SCH_2− at 4.5 and 2.7 ppm).[16] SEC indicated a monomodal and apparently narrow molecular weight distribution (Figure 1b).

Thiol−ene photoadditions were also performed using a 26 W energy-saving bulb (Exo Terra PT2187 ReptiGlo 5.0, λ = 290−690 nm; see the UV−vis spectrum in the SI) as a "green" (more energy/cost-effective and easier/safer to handle) light source instead of the 150 W medium-pressure mercury lamp. With this energy-saving lamp, the glycosylation of **4b** with **5** went to completion within ∼6 h or <12 h in the presence or absence of a photoinitiator, respectively.

Copolypeptide **4c** carries pendant propargyl units (10 per chain on average), to each of which ideally 2 molar equiv of **5** can be added, as demonstrated by Dondoni and co-workers for a tripeptide.[17,18] Glycosylation of **4c** was performed, applying the same conditions as described before, using 3 equiv of **5** with respect to propargyl. ^{1}H NMR analysis of the product **6c** (see the spectrum in the SI) confirmed the attachment of glucose to the polymer chain but also showed new signals at 5.7 and 6.3 ppm, which are attributable to vinyl sulfide (−C*H*=C*H*S−) as the intermediate monoaddition product.[18,19] Quantitative NMR analysis suggested that an average of 1.4 glucose units were added per propargyl unit (14 glucose units per chain). While the first addition step occurred nearly quantitatively (∼90% yield by ^{1}H NMR), the second addition of thiosugar was more difficult, possibly because of steric constraints.

The copolypeptides **4a**−**c** and **6a**−**c** are quite soluble in water (>90 mg mL^{-1}) at neutral pH, at which the glutamate carboxyl groups are ionized (pK_a ≈ 4.3).[4] Like poly(L-glutamic acid), samples **4a**−**c** started to precipitate from water at pH ∼4.7, as (partial) protonation of the carboxylates rendered the chains less hydrophilic. Samples **6a**−**c**, on the other hand, remained soluble down to pH 3.5. The enhanced solubility of the glucocopolypeptides in acidic media is attributed to the presence of the highly water-soluble glucose units. Even though the weight fraction of glucose was rather low ($w_{glucose}$ < 0.2), the chains were dissolved on a molecular level and did not tend to form aggregates, as exemplarily verified for **6b** by dynamic light scattering.

The pH-dependent conformations of the peptide chains in ∼0.02 wt % aqueous solution were analyzed by circular dichroism (CD) spectroscopy; the spectra are collected in Figure 2a. Generally, at pH 6.5 or higher, the CD spectra are characteristic of a random-coil conformation with a maximum at λ = 218 nm. Below pH 6, the chains adopt an α-helical conformation, as indicated by two minima at λ = 208 and 222 nm. Notably, the CD spectra recorded at different pH show an isodichroistic point at λ = 204 nm, suggesting that the transition is exclusively between the random-coil and α-helix conformations, thereby excluding the presence of, for instance, β-sheets.[20]

The value of the mean residual ellipticity at λ = 222 nm, $[\theta]_{222}$ (Figure 2b), can be used to estimate the helicity of peptide chains: % α-helix = $[(-[\theta]_{222} + 3000)/39000] \times 100\%$.[21] Accordingly, the helicities of **4a**−**c** were found to be 54%, 42%, and 24%, respectively, at pH 4.78. For comparison, poly(L-glutamic acid) with an average of ∼50 repeat units reaches a maximum helicity of 71% ($[\theta]_{222} = -24.641 \times 10^3$ deg cm^2 dmol^{-1}) at pH 4.75 (see ref 4). The presence of just a few L-allylglycine units (five per 53 amino acid units in **4a**), has a noticeable effect on the α-helical conformation of the poly(L-glutamate) chain, decreasing the helical content by 17%. In fact, L-allylglycine could be identified as a non-helix-forming amino acid, though the secondary structure of poly(L-allylglycine) could not be revealed yet (CD data not shown). The helicity further decreased upon incorporating racemic defects (**4b**) and increasing their number (**4c**), accompanied with a shortening of L-glutamate segments, as expected. The helicities of the glucocopolypeptides **6a**−**c** were found to be 76%, 65%, and 41%, respectively, at pH 4.5, which are considerably higher than those of the non-glucosylated chains. Notably, **6a** has a higher

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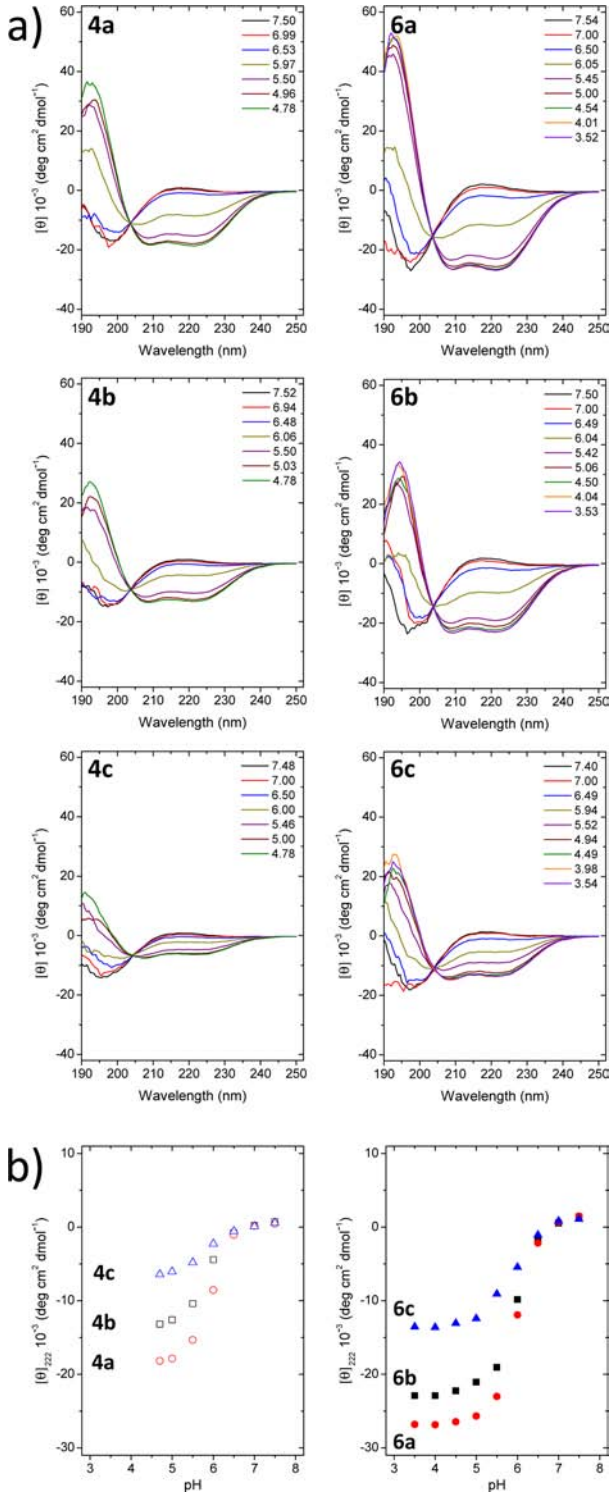

Figure 2. pH dependence of (a) the CD spectra and (b) the mean residual ellipticities at 222 nm of ~0.02 wt % aqueous solutions of copoly(L-glutamate)s **4a–c** and glucosylated copoly(L-glutamate)s **6a–c**.

helical content than the poly(L-glutamate). **6a–c** are soluble down to pH 3.5 (see above) without further increase in the helicity (see Figure 2b). Hence, the formation of the α-helix appears to be facilitated by the presence of additional hydrophilic glucose side chains. Steric effects can be excluded because the glucose units are far-removed from the backbone and also present in just low numbers. The increase in helicity may be due to attractive side-chain interactions (as found by Lotan et al.[7] for the stabilization of poly[N^5-(ω-hydroxyalkyl)-L-glutamine] α-helices by hydrophobic side-chain interactions; also see refs 8 and 9), here arising from hydrogen bonding between the COOH groups. The helicity reached a maximum value at about pH 4, where most of the carboxylic acid groups ($pK_a \approx 4.3$) are protonated and the number of hydrogen bonds is highest.

The helix-to-coil transition of **6a–c** was monitored by CD spectroscopy throughout 10 cycles between pH 7.5 and 3.5 (see the SI), and the results suggested that the process is fully reversible. Also, the addition of NaCl in physiological amount (0.9 wt %) had no noticeable effect on the conformation, especially the helicity at pH 3.5 (see the SI).

The ability of the glucocopolypeptides to interact with biological systems, here the legume lectin concanavalin A (ConA), was also examined by turbidity assays.[22,23] At neutral or physiological pH, the multivalent ligands **6a–c** (adopting a random-coil conformation) are supposed to bind to ConA (a tetrameric protein structure with four sugar binding sites)[24] leading to cross-linking, clustering, and ultimately precipitation of the protein.[25−27] Samples **6a–c** and **4a–c** as controls (1.0 mg mL^{-1} = 77–190 mM glucose, 80 μL) were incubated with ConA (2.0 mg mL^{-1} = 19 μM ConA, 800 μL) in HEPES buffer solution at pH 7.0 at room temperature. The turbidity was monitored by measurement of the absorbance at 450 nm over a period of 40 min. The curves in Figure 3 indicate that ConA

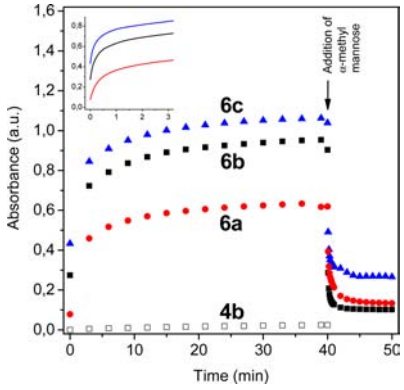

Figure 3. Turbidity curves for the precipitation of ConA with **4b** and **6a–c** in HEPES buffer at pH 7.0 at room temperature.

precipitated from the solution containing **6a–c** within just a few minutes, which was not the case with the non-glucosylated **4b** (or with **4a** and **4c**; data not shown), thereby confirming selective binding of the lectin to the glucose units of the polypeptide chains. The selectivity of the binding was further supported by the fact that the addition of α-methylmannose, which is a monovalent ligand with a stronger affinity to ConA than glucose, led to disruption of the protein–polymer clusters and instantaneous clearing of the solution (Figure 3). From the initial slope of the turbidity curve (Figure 3 inset), which is a measure of the ConA clustering kinetics, it was found that the binding rate is correlated with the number of glucose units per polypeptide chain (i.e., the epitope density).[27] It also appears that the clustering kinetics is hardly affected by the presence of doubly glucosylated units (local high epitope density), as in **6c**.

In summary, we have described the controlled synthesis of statistical copolypeptides of L-glutamate and L-/DL-allyl- or DL-propargylglycine and subsequent (eventually "green") glucosy-

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lation by thiol−ene/yne photochemistry in aqueous solution. This method allows the introduction of the sugar moieties as the final step under mild conditions, paving the way for more complex and less stable sugars. All of the glucosylated and non-glucosylated copoly(L-glutamate)s adopted a random-coil conformation in neutral and basic media and an α-helical conformation in acidic media, with the helical content depending on the number and configuration of allyl-/propargylglycine units. The glucosylated copolypeptides exceeded the helical stability of unfunctionalized poly(L-glutamate)s and were soluble down to pH 3.5. A maximum helicity of ∼76% was reached prior to precipitation. Turbidity assays proved the selective binding of the glucocopolypeptides to the plant lectin ConA.

The pH responsiveness and enhanced solubility of the α-helical structure together with the biological activity make these copolymers excellent candidates for the study of more complex carbohydrate−protein binding systems. Current work is devoted to the synthesis of amphiphilic glycocopolypeptide block copolymers for potential use as smart carrier systems for targeted drug release and the production of coated surfaces as mimics of extracellular matrices for tissue engineering.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures; ^{1}H and ^{13}C NMR data for all NCA monomers (**1** and **2a−c**); and ^{1}H NMR, CD, SEC, and analytical ultracentrifugation data for copolypeptides **4a−c** and **6a−c**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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