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In Situ Polymer Analogue Generation of Azlactone Functions at Poly(oxazoline)s for Peptide Conjugation

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The physical and chemical stability of peptides for biomedical applications can be greatly enhanced through the conjugation of polymers. A well-known but rather underemployed selective coupling functionality is the azlactone group, which readily reacts with a number of different nucleophiles without the need for activation and the formation of any by-products. For example, azlactone functional polymers are used to react with peptides and proteins, rich in amino and thiol groups, to form polymeric beads for affinity-based column chromatography. So far, side chain functional azlactone polymers have been mainly synthesized by radical polymerization using 2-vinyl-4,4-dimethyl azlactone together with different acrylate monomers. Here, a new azlactone precursor equipped with a functional thiol is presented, which can be attached to any vinyl functional polymer by thiol–ene chemistry. Subsequently, the formation of the reactive azlactone ring can be performed in situ at high conversion rate without the need for illumination. This approach is tested on an azlactone side functional poly(2-oxazoline) by coupling amine containing molecules including a model peptide and is proven via ^{1}H NMR spectroscopy, IR spectroscopy, as well as HPLC measurements.

1. Introduction

Covalently linked peptide–polymer conjugates have been extensively researched for many biomedical applications as they can be used, for example, for drug delivery, for the stabilization of protein therapeutics or in tissue engineering and biosensing applications.[1–5] Besides selecting the appropriate peptide or protein, the polymer used for conjugation should be carefully chosen with regard to its physical and chemical properties as well as its biocompatibility and degradability.

The most prominent polymer for many biomedical applications is presently poly(ethylene glycol) (PEG), which can ameliorate the stability of the peptide structure regarding harsh environmental conditions.[3,5,6] However, other water-soluble polymers that offer multiple conjugation sites through multiple side chain functionalities can be interesting alternatives to PEG, which only offers α- or ω-end functionalization. One of those polymers are poly(2-alkyl-2-oxazoline)s (PO_X), which have already been explored for peptide–polymer conjugation by several researchers.[7,8] For example, Luxenhofer et al. attached a cyclic RGD peptide using copper mediated click-chemistry[9,10] and Schmitz et al.[11] attached thioester functional peptides to a multifunctional cysteine PO_X copolymer. PO_X is generally synthesized via cationic ring opening polymerization (CROP) and through a vast variety of monomers, different functional side chains can be easily incorporated into the polymer using appropriate copolymerization schemes.[12]

Another interesting functionality for such polymers is the electrophilic azlactone, which is basically a "masked" amino acid[13] and readily reacts with amines, thiols, and hydroxyls[14] without additional activation and the formation of any by-products. Based on these advantages, it could be a promising alternative to the commonly used *N*-hydroxysuccinimide active esters used in many conjugation reactions.[15–17] Azlactones have already been used in peptide ligation as they offer the opportunity to integrate chiral amino acids through stereoselective alkylation with subsequent ligation.[18] In polymer chemistry, 2-vinyl-4,4-dimethyl azlactone (VDMA) has been so far mainly polymerized by free radical polymerization using methacrylic acid as co-monomer, by atom transfer radical polymerization[19] to receive pure PVDMA or by reversible addition–fragmentation chain transfer (RAFT) polymerization with *N*-isopropyl acrylamide and *N,N*-dimethylacrylamide.[20] Surface anchored PVDMA brushes have also been used for protein immobilization, for example, RNase A, where the activity of the immobilized protein was close to the free enzyme.[21] Vinyl-functional azlactones were also applied for macromonomer functionalization, for example, amino group bearing gelatin was functionalized with 2-isopropenyl-4,4-dimethylazlactone at room temperature making it available for the formation of hydrogels via radical cross-linking, which was used to support in vitro cell growth.[22] Copolymers of poly(ethylene glycol methyl ether methacrylate-*ran*-vinyl azlactone-*ran*-glycidyl methacrylate)

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have been used as thin film coatings on silicon and polycarbonate substrates and tested with different peptides with thiol, amino, and cysteine N-terminus. It was found that the thioester bound peptides were not stable under cell culture conditions and would undergo hydrolysis. The coupling efficiency of peptides with primary amines at the N-terminus was rather low, but peptides with a cysteine at the N-terminus showed greater immobilization potential, which was explained by a rearrangement mechanism to the stable amide similar to the one observed during native chemical ligation (NCL).[23] It was furthermore shown by Ho et al. that it is possible to attach azlactone copolymers as well as heterobifunctional azlactone linkers to a more complex protein, lysozyme, with higher efficiency in DMSO than in aqueous buffer,[17,20] which can be explained by the fact that there is a competition between the amide bond formation and the undesired azlactone hydrolysis to carboxylic acids through the excess of water molecules.[16] A range of water-soluble copolymers of VDMA with anionic, polar uncharged, and zwitterionic monomers were synthesized by Gardner et al. and studied as possible macromolecular cross-linkers.[16] Their hydrolysis half-life depended on the one hand strongly on the used VDMA content of the polymer, in general solubility decreased with increasing VDMA content, and on the other hand also on the used co-monomer. Post-polymerization functionalization with VDMA was so far, to our knowledge, only performed by Ho et al.[20] at the ω-position of a poly(N-isopropylacrylamide) (PNIPAM), obtained by RAFT polymerization, using thiol–ene Michael addition with dimethylphenylphosphine as catalyst in tetrahydrofuran. However, it can be anticipated that post-polymerization functionalization with VDMA on a polymer with multiple thiol groups at the side chain might be difficult due to the reactivity of azlactone toward nucleophiles.

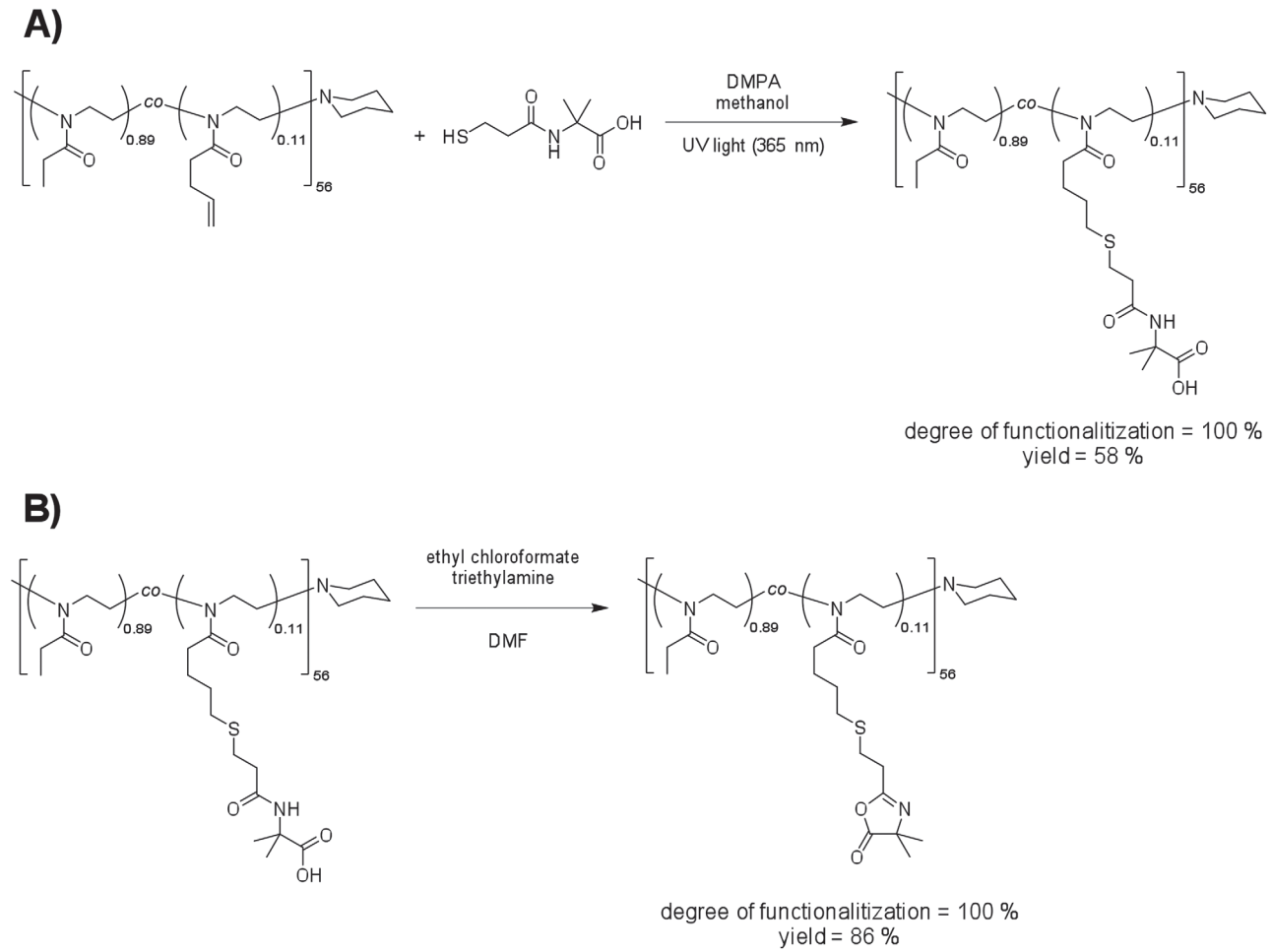
The aim of this work was to make the azlactone functionality accessible to water-soluble PO_X polymers through post-polymerization functionalization without the risk of any side reactions, which would allow selective and facile bioconjugation with this new class of biocompatible polymers.

2. Results and Discussion

Preliminary experiments, data not shown, revealed that the immediate attachment of vinyl dimethylazlactone at multi thiol-functional PO_X copolymers through radical mediated as well as Michael addition-based thiol–ene reaction led to cross-linking of the individual dissolved polymer chains, which was to be expected since attached azlactone rings can be easily opened by remaining thiols of the polymer side chains. Hence, a new mercaptoacyl amino acid, *N*-(3-mercapto-1-oxopropyl)-2-methylalanine (MOMA), based on the non-proteinogenic amino acid 2-aminoisobutyric acid was synthesized adapted from Condon et al.[24] to overcome this problem. The synthesis is a two-step approach, in the first step, the amine group of the amino acid is transformed into an amide by addition of 3-bromopropionyl chloride and a thioester is introduced through the addition of thiobenzoic acid in a slightly alkaline environment. In the second step, the thioester is deprotected using ammonia, resulting in the mercaptoacyl amino acid MOMA. The thiol group of MOMA can subsequently be used for thiol–ene reaction with vinyl functionalities at PO_X side chains. For this purpose, poly(2-ethyl-2-oxazoline-*co*-2-butenyl-2-oxazoline) (P(EtOx-*co*-ButEnOx)) was synthesized by cationic ring opening polymerization in a microwave reactor according to literature.[25] The theoretical chain length, which was set by the initiator to monomer ratio, matched with the experimental chain length as determined by ^{1}H NMR end group analysis. Size exclusion chromatography (SEC) revealed a monomodal polymer with a dispersity of 1.14 (Figure S7, Supporting Information). The functionalization of the copolymer P(EtOx-*co*-ButEnOx) with MOMA (**Scheme 1**A) was carried out in methanol with a photo-initiator at a wavelength of 365 nm. ^{1}H NMR analysis revealed that all vinyl groups of the copolymer had reacted and only a small excess of the thiol compound MOMA had to be used (Figure S6, Supporting Information). The IR spectrum of the functionalized copolymer revealed the stretching vibration of the carboxylic acid (C=O) at 1730 cm^{-1} and the bending vibration of the secondary amide (N—H) at 1540 cm^{-1} of the attached MOMA, in addition to the carbonyl vibration of the numerous amides of the polymer backbone at 1633 cm^{-1} (**Figure 1**). The ring closure was efficiently performed in situ at the side chain using ethyl chloroformate and triethylamine (Scheme 1B). The successful formation of the azlactone ring could be observed by ^{1}H NMR analysis (absence of secondary amide proton at 8.0 ppm) (Figure S6, Supporting Information) and by IR spectroscopy where the vibration of the carboxylic acid and the secondary amide of MOMA had disappeared and a new peak at 1815 cm^{-1} for the carbonyl group of the azlactone ring appeared (Figure 1). The SEC revealed that the modified polymer was still monomodal after the functionalization and no observable side reaction had occurred, which fulfilled our aim to functionalize polymers at the side chain without any undesirable cross-linking (Figure S8, Supporting Information).

It is known from literature that the azlactone ring can react with amine, thiol, and hydroxyl groups but can also undergo a reaction with cysteine that is similar to that of the native chemical ligation[23,26] (**Scheme 2**A). The advantage of using a cysteine terminated peptide for the peptide–polymer conjugation is on the one hand that is was shown that these are more efficiently bound than primary amines[23] and on the other hand, after rearrangement, a free thiol is still available for further reactions at the linkage site, which could, for example, be used for subsequent thiol–ene reactions to form chemically cross-linked hydrogels.

To test the azlactone functionality at the side chain, we chose two small molecules, cysteamine and cysteine methyl ester (CME) and a model peptide with the sequence CGGGF bearing an N-terminal cysteine and an aromatic amino acid, which would be visible in the ^{1}H NMR spectroscopy and HPLC analysis (Scheme 2B). The reaction was carried out in dry DMSO as the azlactone ring is easily opened by water molecules and as catalyst triethylamine was added following the protocol of Ho et al.[20] After the reaction, the modified polymer was dialyzed for three days against water to make sure that any non-bound reaction partners were removed. The ^{1}H NMR spectrum of the freeze-dried sample of P(EtOx-*co*-ButOxAL)-Cysteamine showed a new singlet in the region for amine groups at 7.59 ppm with a relative integral of 5, which would account for a functionalization of 91% (**Figure 2**A). As it is also possible that the thiol initially reacted with the azlactone group, the missing 9% might have reacted with the thiol end



Scheme 1. Reaction scheme of the polymer analogue functionalization of side functional PO_x with azlactone. A) Thiol–ene reaction with azlactone precursor MOMA. B) Ring closure of azlactone at the side chain of PO_x.

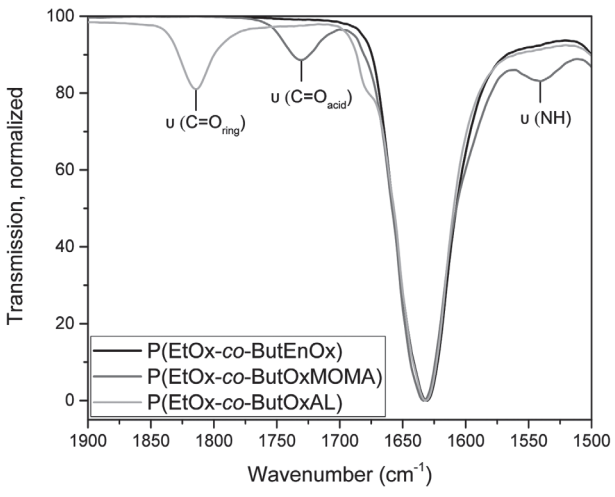
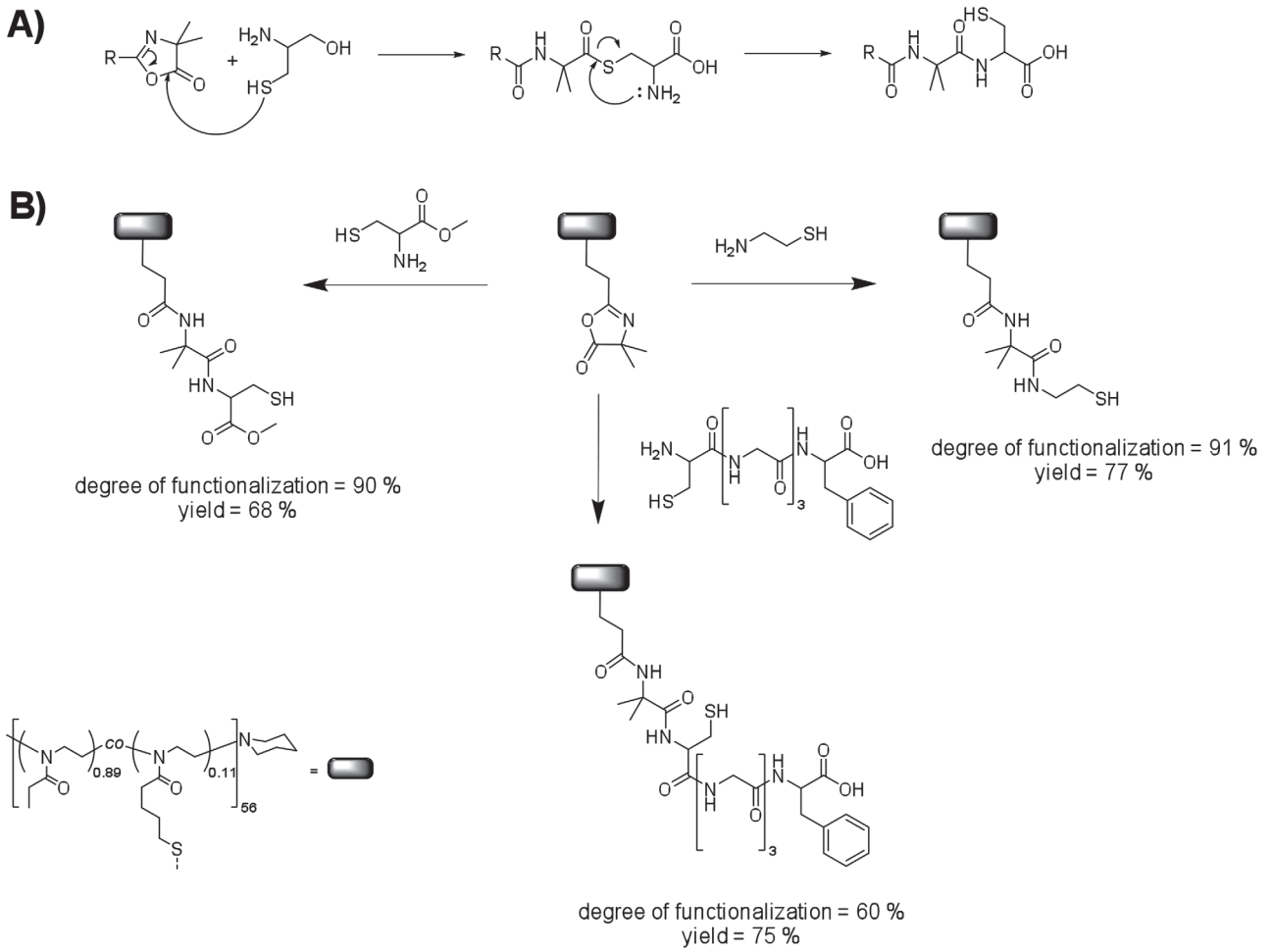


Figure 1. Stacked IR spectra of the original polymer P(EtOx-*co*-ButEnOx), the copolymer functionalized with the mercaptoacyl amino acid MOMA and the copolymer after ring closure showing specific carbonyl vibrations.

of the cysteamine molecule. However, since the thioester is not stable and can be easily hydrolyzed as shown by Schmitt et al.,[23] it is possible that the thioesters were cleaved during dialysis and only the stable amide remained conjugated to the polymer chain. This experiment also shows that the amine group seems to be more reactive under the conditions tested. In addition, the carbonyl ring vibration at 1815 cm^{-1} in the IR spectrum of P(EtOx-*co*-ButOxAL)-Cysteamine completely disappeared again and a slightly larger peak of the vibration of the two secondary amide groups, which were introduced into the side chain by the attached cysteamine molecule, became visible (Figure S9A, Supporting Information). The successful reaction of P(EtOx-*co*-ButOxAL) with CME could also be detected with ^1H NMR spectroscopy with the appearance of an additional signal for the secondary amide group at 7.84–7.75 ppm and the additional alkyl signals of the CME molecule (Figure 2B). The integrals of the CME molecules that were attached to the polymer account for a functionalization degree of the available azlactone groups of approximately 90%, which is similar to the value obtained for cysteamine. The IR spectrum also shows the disappearance of the carbonyl ring vibration at 1815 cm^{-1} and a



Scheme 2. A) Reaction of azlactone with cysteine function. Adapted with permission.[23] Copyright 2016, American Chemical Society. B) Reaction scheme of azlactone functionality with cysteine methyl ester, cysteamine, or model peptide CGGGF.

new peak of the carboxylic ester of CME showing at 1740 cm^{-1} in addition to the vibration of the secondary amide group at 1540 cm^{-1} (Figure S9B, Supporting Information). The attachment of the peptide CGGGF to the polymer side chain was also visible in the ^{1}H NMR spectrum where the aromatic signals of the phenyl alanine amino acid showed at 7.22 ppm with an relative integral of 15.4, which would account for approximately three functionalized azlactone side chains (Figure 2C). The IR spectrum of the peptide functionalized polymer (Figure S9C, Supporting Information) shows the disappearance of the carbonyl ring vibration but also the appearance of the vibration of the carboxylic acid of the MOMA molecule, which can be explained by the fact that not all azlactone groups reacted with the peptide and were later opened by water molecules during dialysis. Nevertheless, there are also peaks visible originating from the attached peptide, for example, the valency vibration at 3280 cm^{-1} and the deformation vibration at 1525 cm^{-1} of the secondary amide groups. To further prove the attachment of the peptide, the compound was measured on a HPLC system. SEC did not prove applicable for analysis of the polymer peptide conjugate as the peptide would strongly interact with the column material. The HPLC trace of the peptide, the original polymer P(EtOx-*co*-ButEnOx), as well as the azlactone functionalized polymer and of the product of P(EtOx-*co*-ButOxAL) with CGGGF at the wavelength of 254 nm is shown in Figure S10, Supporting Information. Due to the aromatic ring of phenylalanine, the peptide appears as a strong signal at a retention time of 12.4 min. The unmodified polymer does not absorb light at this wavelength; hence it is barely visible. The azlactone functionalized polymer shows a slight absorption. The trace of the polymer with the attached peptide does not show any signal at 12.4 min, which confirms that the peptide signals that were visible in the NMR spectrum do not originate from the remaining free non-bound peptide. Instead, the signal intensity of the polymer–peptide conjugate has increased, confirming the successful attachment of the peptide to the polymer.

In conclusion, the synthesis of the azlactone precursor MOMA, its facile attachment to vinyl functionalities and the ring closure in situ at very high yields, opens a new route to decorate a large variety of vinyl functional polymers with azlactones. The azlactone functional polymers can further be used to subsequently attach different peptides, or even proteins, using orthogonal synthesis approach. This conjugation route is not solely limited to thiol-containing peptides, which can be attached to

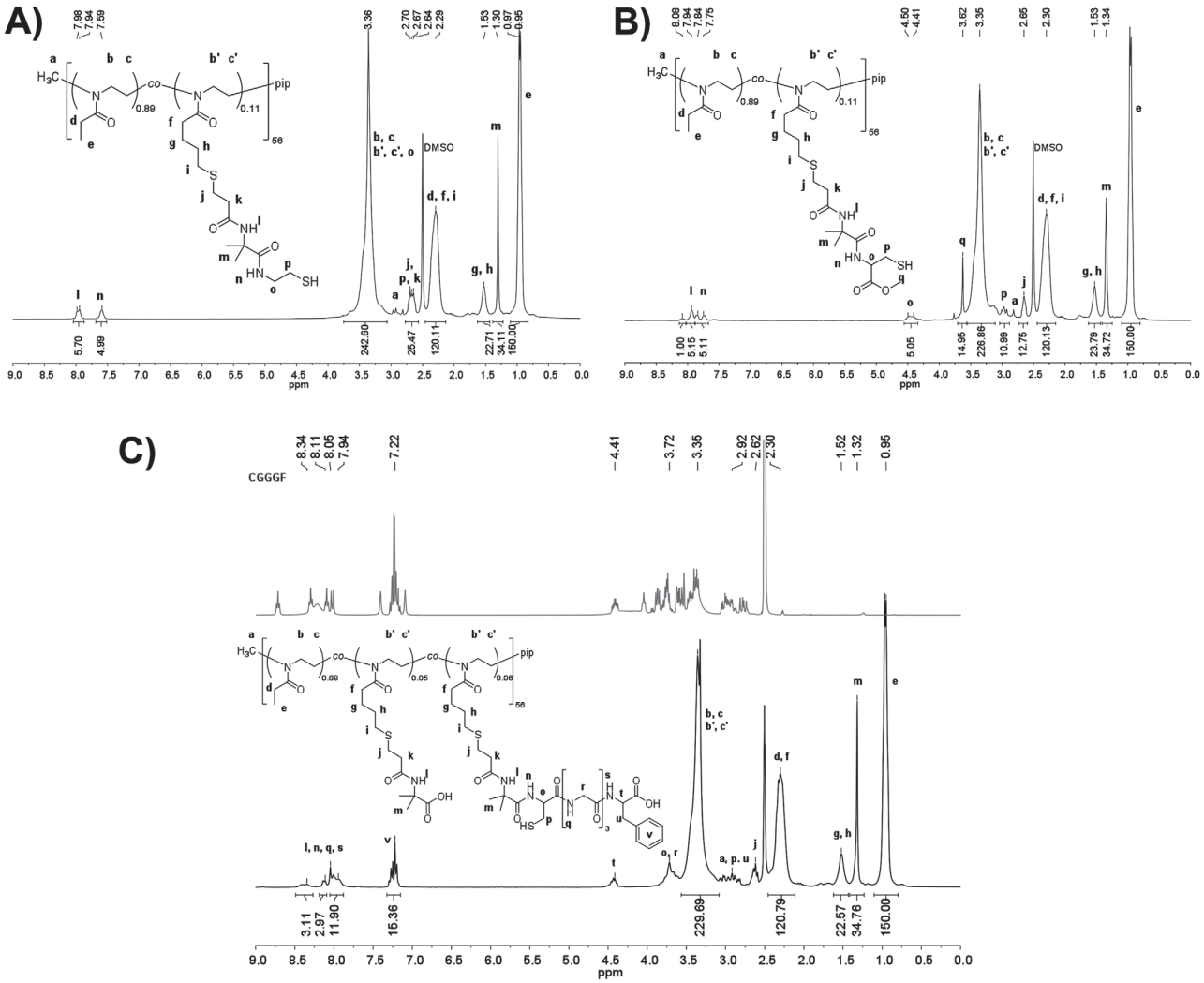


Figure 2. A) ^{1}H NMR in DMSO-d_6 of P(EtOx-*co*-ButOxAL) which has reacted with cysteamine with the secondary amide of the opened azlactone ring at 7.96 ppm and the secondary amide of the reacted cysteamine at 7.59 ppm. B) ^{1}H NMR in DMSO-d_6 of P(EtOx-*co*-ButOxAL) which has reacted with cysteine methyl ester with the secondary amide of the opened azlactone ring at 7.94 ppm and the secondary amide of the reacted cysteine methyl ester at 7.84 and 7.75 ppm (doublet). C) ^{1}H NMR in DMSO-d_6 of P(EtOx-*co*-ButOxAL) which has reacted with the peptide CGGGF (in gray) with the secondary amide of the opened azlactone ring at 8.13 ppm and the aromatic signals of the phenyl alanine amino acid at 7.22 ppm. The integral value of the aromatic signal indicates that 3 of the 5.5 possible side chains were functionalized with the peptide.

polymers using thiol–ene chemistry,[27] but offers the flexibility to readily attach a variety of peptides via their amine functions.

3. Experimental Section

Materials and instruments used can be found in the Supporting Information.

Synthesis of MOMA: The synthesis of MOMA is a two-step synthesis adopted from Condon et al.[24] to produce mercaptoacyl amino acids. The first step is the synthesis of *N*-[3-(benzoylthio)-1-oxopropyl]-2-methylalanine, which is deprotected in the second step to reveal the free thiol. 2-aminoisobutyric acid (6.0 g, 58 mmol, 1 equiv.) was dissolved in 90 mL of 1.5 M NaOH in a round bottom flask with stirrer and cooled down in an ice bath. 3-bromopropionyl chloride (9.966 g, 58 mmol, 1 equiv.) was added via syringe. The solution was left stirring for 0.5 h at room temperature and the pH was checked for neutrality. After slight adjustment with concentrated hydrochloric acid, the solution was further stirred for 3 h at room temperature. An aqueous solution of potassium carbonate (0.7 M) and thiobenzoic acid (8.837 g, 64 mmol, 1.1 equiv.) was added and the reaction mixture was stirred overnight. Concentrated HCl was added until the pH was acidic and a white solid was precipitated. Ethyl acetate (100 mL) was added in which the precipitate dissolved. The organic phase was then separated, and the solvent was removed under reduced pressure. *N*-[3-(benzoylthio)-1-oxopropyl]-2-methylalanine was received by crystallization from cold diethyl ether.

3 g (10.157 mmol) of *N*-[3-(benzoylthio)-1-oxopropyl]-2-methylalanine was diluted in 11 mL of water and 7.5 mL of concentrated ammonia was added. The suspension cleared momentarily with a white precipitate forming after a few minutes and the suspension was stirred for 2 h. 10 mL of water was added and the white precipitate was filtered. The aqueous solution was washed three times with 15 mL of ethyl acetate. Concentrated HCl was added until the pH was acidic and the product as a white precipitate formed. The product was filtered out and freeze-dried to dryness.

Yield: 0.858 g, 4.486 mmol, 44.17%.

^{1}H NMR (300 MHz, DMSO-d_6, ppm): δ = 12.11 (s, 1H, COOH), 8.09 (s, 1H, NH), 2.62 (t, 2H, CH_2), 2.37 (q, 2H, CH_2), 2.22 (s, 1H, SH), 1.33 (s, 6H, 2 × CH_3).

^{13}C NMR (300 MHz, DMSO-d_6, ppm): δ = 174.37 (carboxylic acid C=O), 168.57 (amide C=O), 53.61 (CO—C$(CH_3)_2$—NH), 38.66 (CO—CH_2—CH_2—S, hidden by solvent peaks), 23.82 (C$(CH_3)_2$), 18.86 (CO—CH_2—CH_2—S).

EI-MS m/z: $[M + H]^+$ calculated for $C_7H_{13}NO_3S$, 191.6; found, 191.0

IR spectrum (cm^{-1}): ν = 3308 (amide, NH valency), 3247–2688 (CH_2, CH_3 valency), 1720 (C=O valency), 1612 (NH deformation), 1545 (NH deformation), 1465–672 (CH_2, CH_3 deformation).

Raman spectrum (cm^{-1}): ν = 3000–2870 (C—H), 2572 (S-H), 773 (C-$S_{aliphatic}$).

Synthesis of P(EtOx-co-ButEnOx): The main monomer 2-ethyl-2-oxazoline (EtOx) was distilled to dryness before use and the co-monomer 2-butenyl-2-oxazoline (ButEnOx) was prepared according to Gress et al.[28]

The random copolymer was prepared in a microwave reactor at 100 °C for 2 h with the initiator methyl tosylate (MeTos) and the solvent acetonitrile following the protocol of Wiesbrock et al.[29] The initiator to monomer ratio determined the chain length and was set at 1:50:5 (MeTos:EtOx:ButEnOx). The polymer was terminated using 3 equiv. of piperidine and received as a white powder after precipitation in cold diethyl ether from chloroform.

^{1}H NMR of P(EtOx$_{0.89}$-*co*-ButEnOx$_{0.11}$)$_{56}$ (300 MHz, CD_3CN, ppm): δ = 7.61 (d, aromat., tosylate anion), 7.17 (d, aromat., tosylate anion), 5.85 (m, 5.5H, CH=CH_2), 4.99 (m, 11H, CH=CH_2), 3.42 (m, 222H, NCH_2CH_2 backbone), 2.96 (d, 2H, CH_3N), 2.86 (s, 1H, CH_3N), 2.33 (m, 133H, NCH_2CH_3, $NCH_2CH_2CH_2CH$), 1.76–1.37 (m, 10H, N$(CH_2)_5$), 1.03 (m, 150H, NCH_2CH_3).

SEC (DMF): M_n = 6577 g mol^{-1}, M_w = 7471 g mol^{-1}, $Đ$ = 1.14, elugram can be found in the Supporting Information.

Functionalization of P(EtOx-co-ButEnOx): The copolymer P(EtOx$_{0.89}$-*co*-ButEnOx$_{0.11}$)$_{56}$ was dissolved in methanol and the solution was degassed with argon for 15 min. MOMA (1.5 equiv. per double bond function of the copolymer) and 0.5 equiv. of the photo-initiator 2,2-dimethoxy-2-phenylacetophenone were added and the solution was stirred under UV light (λ = 365 nm) for 30 min. The solvent was removed under reduced pressure and the polymer was dialyzed against water for 1 d to remove excess MOMA. The polymer was received as a white powder after freeze-drying.

^{1}H NMR of P(EtOx$_{0.89}$-*co*-ButOxMOMA$_{0.11}$)$_{56}$ (300 MHz, DMSO-d_6, ppm): δ = 8.03 (s, 5.7H, NH), 3.35 (m, 248H, NCH_2CH_2 backbone), 2.96 (d, 2H, CH_3N), 2.86 (s, 1H, CH_3N), 2.61 (m, 14H, SCH_2CH_2CO), 2.30 (m, 124H, CCH_2CH_3, $CCH_2CH_2CH_2CH$), 1.52 (m, 27H, $CCH_2(CH_2)_2CH_2S$), 1.32 (s, 37H, C$(CH_3)_2$), 1.03 (m, 150H, CCH_2CH_3).

IR spectrum (cm^{-1}): ν = 3040–2755 (CH_2, CH_3 valency), 1730 (C=O valency, carboxylic acid), 1633 (C=O valency, amide), 1542 (NH deformation), 1507–990 (CH_2, CH_3 deformation).

The ring-closure at the side chain of P(EtOx$_{0.89}$-*co*-ButOxMOMA$_{0.11}$)$_{56}$ was performed similar to the synthesis of the monomer 2-vinyl-4,4-dimethylazlactone[15] by dissolving the dry polymer in DMF and adding triethylamine (3 equiv. per MOMA function at the side chain). After cooling the reaction mixture to 0 °C, ethyl chloroformate (2 equiv. per MOMA functionality) was added dropwise and the reaction mixture was stirred for 3 h at 0 °C. The white crystalline was removed by centrifugation and the solvent was removed under reduced pressure. The polymer was precipitated three times from dry dichloromethane in cold diethyl ether. The residual solvent was removed under reduced pressure and the polymer was received as a slightly yellowish powder and stored under inert atmosphere.

^{1}H NMR of P(EtOx$_{0.89}$-*co*-ButOxAL$_{0.11}$)$_{56}$ (300 MHz, DMSO-d_6, ppm): δ = 3.35 (m, 247H, NCH_2CH_2 backbone), 2.96 (d, 2H, CH_3N), 2.86 (s, 1H, CH_3N), 2.75 (m, 23H, SCH_2CH_2C), 2.55 (m, 11H, $CCH_2(CH_2)_2CH_2S$), 2.29 (m, 110H, CCH_2CH_3, $CCH_2(CH_2)_2CH_2S$), 1.52 (m, 22H, $CCH_2(CH_2)_2CH_2S$), 1.31 (s, 37H, C$(CH_3)_2$), 1.03 (m, 150H, CCH_2CH_3).

SEC (DMF): M_n = 6987 g mol^{-1}, M_w = 7776 g mol^{-1}, $Đ$ = 1.11, elugram can be found in the Supporting Information.

IR spectrum (cm^{-1}): ν = 3040–2755 (CH_2, CH_3 valency), 1815 (C=O valency, azlactone carbonyl), 1633 (C=O valency, amide), 1507–990 (CH_2, CH_3 deformation).

Conjugation Reactions to Azlactone Functional POx Copolymer: 0.100 g of P(EtOx$_{0.89}$-*co*-ButOxAL$_{0.11}$)$_{56}$ was dissolved in 4 mL of dry DMSO and degassed with argon for 15 min. 3 equiv. (for every azlactone functionality) of cysteamine, cysteine methyl ester or the peptide CGGGF was added. As base, 10 equiv. of triethylamine was added, and the solution was stirred for 12 h under inert atmosphere. The product was dialyzed against acidified water (pH 3) for 3 days and received as a white powder after lyophilization.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

azlactone, peptide conjugation, polymer-analogue functionalization, polyoxazoline

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