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Side-Chain Cysteine-Functionalized Poly(2-oxazoline)s for Multiple Peptide Conjugation by Native Chemical Ligation

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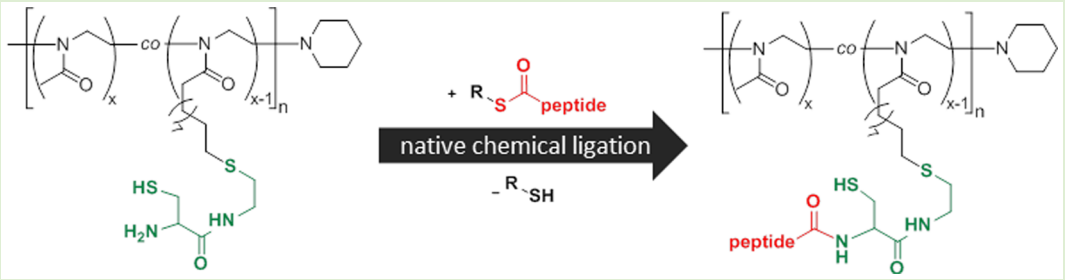
Supporting Information



ABSTRACT: We prepared statistical copolymers composed of 2-methyl-2-oxazoline (MeOx) in combination with 2-butenyl-2-oxazoline (BuOx) or 2-decenyl-2-oxazoline (DecOx) as a basis for polymer analogous introduction of 1,2-aminothiol moieties at the side chain. MeOx provides hydrophilicity as well as cyto- and hemocompatibility, whereas the alkene groups of BuOx and DecOx serve for functionalization with a thiofunctional thiazolidine by UV-mediated thiol−ene reaction. After deprotection the cysteine content in functionalized poly(2-oxazoline) (POx) is quantified by NMR and a modified trinitrobenzenesulfonic acid assay. The luminescent cell viability assay shows no negative influence of cysteine-functionalized POx (cys-POx) concerning cell viability and cell number. cys-POx was used for multiple chemically orthogonal couplings with thioester-terminated peptides through native chemical ligation (NCL), which was performed and confirmed by NMR and MALDI-ToF measurements.

INTRODUCTION

Poly(ethylene glycol) (PEG) is still the gold standard for stealth polymers and routinely used in the clinic, for example, in protein conjugates for prolonged circulation times.[1,2] In this, different PEGylation methods were developed keeping the protein active while a maximal shielding is ensured.[3] Nevertheless, there is growing evidence that PEGylated proteins can elicit antibody formation against PEG, which may limit therapeutic efficacy.[4,5] Additionally, in a more chemical view, the preparation of PEG using highly reactive oxirane monomers is not straightforward, and (bio)chemical functionalization of PEG is limited to the distal ends of the polymer chain.

Poly(2-oxazoline)s (POx) are an upcoming class of polymers as potential alternative to PEG.[6−8] POx can be used for protein conjugation[9] in most cases with the hydrophilic poly(2-methyl-2-oxazoline) (P(MeOx)) or poly(2-ethyl-2-oxazoline) (P(EtOx))[10−14] because they showed the same "stealth effect" as PEG[15,16] and are also known to be biocompatible.[17,18] Different reviews are published describing the enormous potential of poly(2-alkyl-2-oxazolines)s in context with biomedical applications and comparing POx with other water-soluble polymers like PEG.[19,20] The living cationic ring-opening polymerization is an easy and experimentally robust method to polymerize 2-substituted 2-oxazolines. It offers the advantage of controlled degree of polymerization and polymer architecture for polymers with low dispersity and broad variability in terms of the chemical functionalization of the polymer side chains through the use of functional monomers. Most of these monomers are liquid and easy manageable during polymerization. For example, oxazoline monomers with allyl side chains combine oxazoline-based advantages like biocompatibility with the opportunity of functionalization through thiol-ene reactions. Preparation of homopolymers based on 2-butenyl-2-oxazoline (BuOx) and copolymers in combination with 2-ethyl-2-oxazoline (EtOx) was shown to follow a living character and thiol−ene modification with six different thiols was performed to yield amphiphilic polymers.[21] BuOx homopolymers were recently used for the synthesis of DNA−polymers by using thiol−ene UV reaction or dicyclohexylcarbodiimide (DCC) coupling techniques.[22,23] Copolymers of EtOx with 2-decenyl-

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2-oxazoline (DecOx) moieties were also used for nanoparticle approaches.[24] The living character of the copolymerization of DecOx in combination with MeOx or EtOx has also been shown[25] and exploited for the preparation of cytocompatible hydrogels.[26] The same system was used for fabrication of POx-based polymer capsules using copper-catalyzed azide−alkyne cycloaddition (CuAAc) and thiol−ene chemistry for cross-linking.[27]

We have shown before with polyglycidols as model polymer that the introduction of thiazolidines (protected cysteines) at the side chain provide water-soluble polymers with cysteine capacity.[28] They especially permit chemoselective coupling using native chemical ligation (NCL), which describes the selective conjugation of cysteine moieties with thioesters via a stable peptide bond.[29] Thioesters rapidly exchange with 1,2-aminothiols yielding thioesters with an adjacent amine. These amine-functional thioesters rearrange fast to the final stable amide.[29,30] NCL reactions are widely used in the field of chemical biology to obtain challenging peptides and whole length proteins,[31,32] but they are also used in polymer chemistry for hydrogel cross-linking.[33]

Here we present an easy route to synthesize water-soluble side-chain cysteine-functionalized poly(2-oxazoline)s (cys-POx). The living cationic ring opening polymerization of 2-methyl-2-oxazoline (MeOx) and BuOx or DecOx followed by functionalization with 3-formyl-*N*-(2-mercaptoethyl)-2,2-dimethylthiazolidine-4-carboxamide (FTz4Cys, mercaptothiazolidine)[28] leads to side-chain cysteine-residues after deprotection. The degree of functionalization is predetermined by the statistical copolymerization of the monomers and is confirmed by NMR, GPC, and a modified trinitrobenzenesulfonic acid (TNBSA) assay. These cysteine-functionalized polymers are cytocompatible, which is proved by the "CellTiter-Glo luminescent cell viability assay" (LCV-assay). Cys-POx enables simple attachment of proteins by NCL and leads to polymer−protein conjugates in a chemically orthogonal and highly selective manner that is confirmed by NMR and MALDI-ToF measurements.

EXPERIMENTAL SECTION

Materials. Reagents and solvents were, unless stated otherwise, commercially available as reagent grade and did not require further purification. All Fmoc-protected amino acids were purchased from IRIS BioTech or Novabiochem. Acetic acid (VWR, p.a.), acetic acid anhydride (>98%, VWR), acetone (99.5%, Sigma-Aldrich), *N*,*N*′-carbonyldiimidazol (reagent grade, Sigma-Aldrich), 2-chloroethylamine hydrochloride (99%, Sigma-Aldrich), chloroform (>99%, VWR), cysteamine hydrochloride (AppliChem), Dawson Dbz NovaSyn TGR resin (Novabiochem), diethyl ether (Staub & Co, Nürnberg, Germany), 2,2-dimethoxy-2-phenyl acetophenone (DMPA, 99%, Sigma-Aldrich), *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide hydrochloride (purum, ≥98%, Sigma-Aldrich), *N*,*N*-diisopropylethylamine (Fisher Scientific), ethyl acetate (>99.5%, Merck, Darmstadt, Germany), formic acid (≥98%, Sigma-Aldrich), guanidine hydrochloride (Acros Organics), hydrochloride acid (HCl, 32%, Merck), hydroxybenzotriazole (IRIS Biotech GmbH), *N*-hydroxysuccinimide (98%, Sigma-Aldrich), magnesium sulfate (p.a., Merck), 4-mercaptophenylacetic acid (MPAA, Sigma-Aldrich), methanol (p.a., VWR), methyl *p*-toluenesulfonate (purum, ≥97.0%, Sigma-Aldrich), *p*-nitrophenylchloroformate (Sigma-Aldrich), 4-pentenoic acid (>98.0%, Sigma-Aldrich), piperidine (99.5%, Sigma-Aldrich), potassium hydroxide (Bernd Kraft), pyridine (≥99.8%, anhydrous, Sigma-Aldrich), sodium formate (>99.0%, Sigma-Aldrich), sodium chloride (≥99.8%, Sigma-Aldrich), sodium hydrogen phosphate (Sigma-Aldrich), sodium hydroxide (≥98%, Sigma-Aldrich), *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (IRIS Biotech GmbH), *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(6-chloro-1*H*-benzotriazol-1-yl)-uronium hexafluorophosphate (GL Biochem), triethylamine (≥99%, Sigma-Aldrich), trifluoroacetic acid (99%, ReagentPlus, Sigma-Aldrich), triisopropylsilane (Sigma-Aldrich), *tris*(2-carboxyethyl)-phosphine (TCI), and 10-undecenoxyl chloride (97%, Sigma-Aldrich) were used as received. Acetonitrile (99.9%, Sigma-Aldrich) and MeOx (Sigma-Aldrich) were dried over CaH_2 and distilled before use. Dichloromethane (≥99.5%, VWR) was dried over $CaCl_2$ and filtered before use.

Instrumentation. ^{1}H NMR spectra were recorded on a Bruker Fourier 300 at 300 MHz or a Bruker Fourier 600 at 600 MHz. Deuterated chloroform ($CDCl_3$), dimethyl sulfoxide (DMSO-d_6), and deuterium oxide (D_2O) spectra were recorded with nondeuterated solvent signals as internal reference.

UV-LEDs were obtained by Polymerschmiede (Aachen, Germany) having 4 UV-LED cubes with 11 W (each) and a wavelength of 365 nm.

GPC elugrams were recorded with a system from PSS, Agilent Technologies 1260 Infinity (pump and refractive index detector) using DMF as solvent with 1 g/L LiBr and a flow rate of 1.0 mL/min. We used one precolumn (PSS GRAM) and two columns with length = 300 mm, width = 8 mm, and particle size = 10 μm (PSS GRAM). For calibration, PEG standards (PSS) were used.

UV−vis absorption measurements were performed on a Genesys 10S Bio spectrophotometer at room temperature using a wavelength of 335 nm.

High-resolution mass spectra (HRMS) were measured on an Aquity UPLC system and an LCT Premier (Waters Micromass, Milford, MA) time-of-flight (TOF) mass spectrometer with electrospray ionization (ESI) using water and acetonitrile (10−90% gradient) with 0.1% formic acid as eluent.

HPLC purification of the peptides was performed on a JASCO LC-2000 Plus system using a reverse-phase C18 column consisting of a Smartline Manager 5000 with interface module, two Smartline Pump 1000 HPLC pumps, a 6-port-3-channel injection valve with 2.5 mL loop, a UV detector (UV-2077) and a high pressure gradient mixer running with water (0.1% TFA), and MeCN (0.1% TFA) gradient at 16 mL/min. Alternatively, preparative HPLC purification was performed on a Gilson PLC 2020 personal purification system using a Nucleodur VP 250/32 C18HTec 5 μm column from Macherey-Nagel at a flow rate of 32 mL/min.

Analytical HPLC was applied on a Shimadzu system with an SIL-20A autosampler, CBM-20A a controller, 2 LC-20AT pumps, a CTO-20A column oven, and a SPD-M20A UV/visible detector using an Agilent eclipse cdb-c18, 5 μm, 250 × 4.6 mm column with a flow rate of 1.0 mL/min or a Jasco system with an AS-2055 Plus autosampler, a Pu2 2089 Plus pump, and a UV-2077 Plus UV/visible detector using a Macherey -Nagel Nucleodur c18, 5 μm, 250 × 4 mm column with a flow rate of 1.0 mL/min.

MALDI measurements were performed on a Microflex LT system from Bruker (Am Studio 2D, 12489 Berlin, Germany) and processed and analyzed by FlexAnalysis 3 MALDI software. One μL of a 9:1 mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid (SDHB) matrix was added to 1 μL of water containing 0.1% TFA and 1 μL of sample. Subsequently, the solution was carefully mixed by pipetting and spotted. Analysis was performed in the linear positive-ion mode. For each spectrum 10 000 consecutive laser shots were accumulated (20 shots·sec^{-1}).

Synthesis. 2,2-Dimethylthiazolidine-4-carboxylic acid (Tz4CA) was synthesized according to Woodward et al.[34] 2,2-Dimethylthiazolidin-3-(*N*-formyl)-4-carboxylic acid (FTz4CA) was prepared as reported before.[28] FTz4Cys was prepared according to Kuhlmann et al.[28] using chloroform instead of DMF as solvent. Synthesis of BuOx and DecOx was prepared according to Gress et al.[21] or Kempe et al.[24] (Supporting Information).

Copolymerization of MeOx with BuOx or DecOx. Copolymerization of MeOx with BuOx was prepared according to Gress et al.[21] (Supporting Information). Copolymerization with DecOx was prepared slightly different from Dargaville et al.[25] using no microwave

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irradiation and acetonitrile as solvent. In brief, to a hot solution of methyl *p*-toluenesulfonate in dry acetonitrile (70 °C) was added a mixture of MeOx and DecOx under a dry argon atmosphere. Depending on the desired molecular composition of the copolymer, the ratio of MeOx and DecOx varied. The mixture was stirred for 48 h at 70 °C under an argon atmosphere. For end-capping of the polymer, piperidine (3 equiv) was added and additionally stirred overnight. The reaction mixture was precipitated into cold diethyl ether, was solved in water, and was submitted to dialysis against water (MWCO: 1 or 3.5 kDa) for 2 days. P[$(MeOx)_x$-*co*-$(BuOx)_{1-x}]_n$: ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.87 (1H, CH_2=C*H*CH_2), 5.01−4.91 (2H, CH_2=CHCH_2), 3.45 ((4 + 4)H, NC$H_2CH_{2(backbone)}$, $NCH_2CH_{2(backbone)}$), 3.04−2.95 (3H,C$H_3N_{(initiator)}$), 2.47 (2H, $COCH_2CH_2CH$=CH_2), 2.37 (2H, $COCH_2CH_2CH$=CH_2), 2.14 (3H,COCH_3). P[$(MeOx)_x$-*co*-$(DecOx)_{1-x}]_n$: ^{1}H NMR (300 MHz, $CDCl_3$): 5.81 (1H, CH_2=C*H*CH_2), 5.01−4.91 (2H, CH_2=$CHCH_2$), 3.45 ((4 + 4)H, NC$H_2CH_{2(backbone)}$, $NCH_2CH_{2(backbone)}$), 3.04−2.95 (C$H_3N_{(initiator)}$), 2.37−1.98 (3H, COCH_3; 2H, $COCH_2CH_2CH$=CH_2; 2H, $(CH_2)_7CH_2CH$=CH_2), 1.61 (2H, $CH_2CH_2(CH_2)_5$), 1.29 (10H, $CH_2(CH_2)_5CH_2$).

Polymer-Analogue Thiol−ene Reaction with FTz4Cys and P[(MeOx)$_x$-co-(BuOx)$_{1-x}$]$_n$ or P[(MeOx)$_x$-co-(DecOx)$_{1-x}$]$_n$. For the polymer functionalization the use of 3 equiv FTz4Cys related to every allyl-group is necessary. The polymer was dissolved in methanol, and argon was guided through the solution for 15 min. After the addition of FTz4Cys, DMPA (0.5 equiv) was added as UV-reactive radical starter. After 30 min of exposure with UV light (365 nm) the reaction was complete. Precipitation into cold diethyl ether two times and drying in vacuum revealed the product. P[$(MeOx)_x$-*co*-$(BuOxCA)_{1-x}]_n$: ^{1}H NMR of (300 MHz, D_2O): δ 8.35 (1H, NC*H*O), 4.88 (1H, NHCOC*H*N), 3.69−3.14 ((4 + 4)H, NC$H_2CH_{2(backbone)}$, $NCH_2CH_{2(backbone)}$; 2H, $COCH_2CH_2CH_2CH_2S$; 2H, SCH_2CH_2NH), 3.05−2.90 (3H, C$H_3N_{(initiator)}$), 2.67 (2H, $CH_2SCH_2CH_2NH$), 2.58 (2H, SCH_2CH_2NH), 2.05 (3H, COCH_3), 1.83−1.79 (6H, $(CH_3)_2C$), 1.59 (2H, $COCH_2CH_2CH_2CH_2S$). P[$(MeOx)_x$-*co*-$(DecOxCA)_{1-x}]_n$: ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.39 (1H, NC*H*O), 7.01−6.69 (1H, SCH_2CH_2NH), 4.98−4.70 (1H, NHCOC*H*N), 3.69−3.14 ((4 + 4)H, NC$H_2CH_{2(backbone)}$, $NCH_2CH_{2(backbone)}$; 2H, SCH_2CH_2NH; 2H, $COCHCH_2S$), 3.03−2.94 (3H, C$H_3N_{(initiator)}$), 2.62 (2H, $(CH_2)_5CH_2CH_2CH_2S$), 2.49 (2H, $(CH_2)_5CH_2CH_2CH_2SCH_2$), 2.26−1.97 (3H, COC$H_3$), 1.83−1.79 (6H, $(CH_3)_2C$), 1.64−1.47 (2H, $COCH_2CH_2(CH_2)_5$), 1.25 (2H, $CH_2(CH_2)_5CH_2$).

Hydrolysis of Thiazolidine Side Chains. The deprotection of the thiazolidine ring took place in 0.1 M HCl at 70 °C for 5 days. After dialysis against diluted acetic acid (0.01 M) and lyophilization the functionalized polymer was complete. P[$(MeOx)_x$-*co*-$(BuOxCys)_{1-x}]_n$: ^{1}H NMR (300 MHz, D_2O): δ 3.92−3.14 ((4 + 4)H, NC$H_2CH_{2(backbone)}$, $NCH_2CH_{2(backbone)}$; 2H, $COCH_2CH_2CH_2CH_2S$; 2H, SCH_2CH_2NH), 3.03−2.89 (3H, C$H_3N_{(initiator)}$), 2.67 (2H, $CH_2SCH_2CH_2NH$), 2.55 (2H, SCH_2CH_2NH), 2.07 (3H, COCH_3), 1.89 (3H, $(CH_3)_2C$) 1.58 (2H, $COCH_2CH_2CH_2CH_2S$). P[$(MeOx)_x$-*co*-$(DecOxCys)_{1-x}]_n$: ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.92−3.14 ((4 + 4)H, NC$H_2CH_{2(backbone)}$, $NCH_2CH_{2(backbone)}$; 2H, SCH_2CH_2NH; 2H, $COCHCH_2S$), 3.00 (C$H_3N_{(initiator)}$), 2.65 (2H, $CH_2)_5CH_2CH_2CH_2S$), 2.53 (2H, $(CH_2)_5CH_2CH_2CH_2SCH_2$), 2.32−1.79 (3H, COC$H_3$), 1.52 (2H, $COCH_2CH_2(CH_2)_5$), 1.24 (2H, $CH_2(CH_2)_5CH_2$).

Quantification of Cysteine Side Chains. TNBSA Assay. A solution mixture of 50 μL of sample solution (10 mg·mL^{-1} of calibration compound and polymer) with 50 μL of 10 mg·mL^{-1} H_2O_2/0.1 M $NaHCO_3$ (500 μg, 14.7 μmol, 3 equiv with respect to ethanolamine) solution and 4.950 mL of $NaHCO_3$ (0.1M) was prepared. The 99 μg·mL^{-1} stock solution was stored at 37 °C for 1 h before a concentration series was prepared ranging from 99.0 to 0.59 μg·mL^{-1} with sample volumes of 500 μL (Table S1 in the Supporting Information (SI)). 500 μL of 0.1 M $NaHCO_3$ without H_2O_2 was used as blank solution. After the addition of 250 μL of TNBSA solution (0.085 μmol TNBSA, 0.01% in 0.1 M $NaHCO_3$) all samples were stored at 37 °C for 2 h; meanwhile, they turned yellow depending on the concentration. The samples were diluted with 250 μL of 10% SDS solution and 125 μL of 1 M HCl. The samples were closed, shaked, and opened to remove the produced CO_2. Finally, the absorbance of each sample (1 mL) was measured at 335 nm using UV−vis cuvettes, and the concentrations of the polymers and amines were calculated. The amount of cysteines per polymer chain could be calculated by the relation between amine concentration and polymer concentration.

Cell Culture Materials. Human fibroblasts (isolated from foreskin) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) containing 10% fetal bovine serum (FBS) in the presence of 100 units·mL^{-1} penicillin and 0.1 mg·mL^{-1} streptomycin.

Cytocompatibility Test. Cytocompatibility tests (SI, Figure S16) were measured utilizing the CellTiter-Glo LCV-assay (Technical Bulletin, Promega Corporation, Madison, WI). When added to cells, the assay reagent produces luminescence in the presence of ATP from viable cells. Human fibroblasts in cell medium were plated in 24-well plates at a density of 50 000 cells per well and incubated for 24 h under standard cell culture conditions (37 °C, 5% CO_2). Four polymers were tested at concentrations of 10, 1, and 0.1 mg·mL^{-1} with different compositions: P[$(MeOx)_{0.95}$-*co*-$(BuOxCys)_{0.05}]_{44}$, P[$(MeOx)_{0.95}$-*co*-$(DecOxCys)_{0.05}]_{54}$, P[$(MeOx)_{0.90}$-*co*-$(BuOxCys)_{0.10}]_{55}$, and P-[$(MeOx)_{0.90}$-*co*-$(DecOxCys)_{0.10}]_{57}$. Test samples were solubilized in medium to the desired treatment concentration and cells were treated with 100 μL of each solution.

For the luminescence tests the overlap was remoted, 110 μL of medium and 110 μL of the assay reagent were added to each well, and cell lysis was induced on an orbital shaker for 2 min. Plates were incubated at room temperature for 10 min to stabilize the luminescence signal, and results were read on an Elisa Reader Spectrafluor Plus (Tecan, Deutschland, Crailsheim). All plates had control wells containing medium without cells to obtain a value for background luminescence.

*Native Chemical Ligation of Peptide **1** with Cysteine-Functionalized POx.* The NCL between peptide 1 (2.24 mg, 4.1 μmol, 5 equiv) and cysteine-functionalized POx (poly[$(MeOx)_{0.90}$-*co*-$(DecOxCys)_{0.10}]_{55}$, 5 mg, 0.8 μmol, 1 equiv) was carried out in ligation buffer (6 M Gn·HCl, 100 mM NaH_2PO_4, 50 mM TCEP, 200 mM MPAA, pH 7.0) for 4 h. The reaction volume was 170 μL, which corresponds to a concentration of peptide 1 of 5 mM, while the concentration of the polymer was 1 mM. Then, an additional portion of peptide 1 (1.1 mg, 2.0 μmol, 2.5 equiv) was added to the ligation mixture and the reaction was allowed to proceed overnight. The final mixed ratio between peptide (M_n as nonthioester = 393.16 g·mol^{-1}) and polymer (M_n = 6150 g·mol^{-1}) was 7.5:1. The reaction was quenched by the addition of H_2O containing 0.1% TFA to a total volume of 2 mL and subsequently submitted to HPLC (JASCO) on a C18 column. P[$(MeOx)_x$-*co*-$(DecOxPep)_{1-x}]_n$: ^{1}H NMR (600 MHz, D_2O): δ 7.34−7.24 (5H, $Ar_{(phenylalanine)}$), 4.25 (1H, $ArCH_2CH_{(phenylalanine)}$), 3.92 (2H, $NHCH_2COOH_{(glycine)}$), 3.75−3.14 ((4 + 4)H, NC$H_2CH_{2(backbone)}$, $NCH_2CH_{2(backbone)}$; 2H, SCH_2CH_2NH; 2H, $COCHCH_2S$), 2.59 (2H, $(CH_2)_5CH_2CH_2CH_2S$), 2.46 (2H, $(CH_2)_5CH_2CH_2CH_2SCH_2$), 2.32−1.79 (3H, COC$H_3$), 1.46 (2H, $COCH_2CH_2(CH_2)_5$), 1.20 (2H, $CH_2(CH_2)_5CH_2$).

*Native Chemical Ligation of Peptide **2** with Cysteine-Functionalized POx.* Cysteine-functionalized POx (P[$(MeOx)_{0.90}$-*co*-$(DecOxCys)_{0.10}]_{60}$) was dissolved in filtered and degassed ligation buffer (6 M guanidine hydrochloride (GnHCl), 200 mM NaH_2PO_4, 200 mM MPAA, 10 mM *tris*(2-carboxyethyl)phosphine (TCEP)). The pH was readjusted to 7.2 by the addition of NaOH or HCl, and the mixture was added to peptide **2** to give a final concentration of 4 mM with respect to the peptide. The reactions were left overnight at RT before they were quenched by the addition of H_2O containing 0.1% TFA to a total volume of 2 mL and subsequently submitted to HPLC (JASCO) on a C18 column. The mixed ratios between peptide (M_w = 2443 g·mol^{-1}) and polymer (M_w = 5800 g·mol^{-1}) were 1:1, 3:1, and 6:1.

RESULTS AND DISCUSSION

Thiazolidine Synthesis (Protected Cysteine). Synthesis and characterization of the thiofunctional cysteine derivative have been done as reported in detail elsewhere.[28] In brief,

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Table 1. Composition, Molecular Weights, and Dispersities of Different Polymers with Molar Fractions (x) and Repeating Units (n)[a]

polymer	$x_{theoretical}$	$x_{determined}$	$n_{theoretical}$	$n_{determined}$	M_n (NMR)[b]	M_n (GPC)[c]	M_w (GPC)[c]	PDI
				$P[(MeOx)_x\text{-}co\text{-}(BuOx)_{1-x}]_n$				
I	0.95	0.95	47	44	3800	3550	3800	1.06
II	0.90	0.90	50	55	4900	4500	5300	1.17
				$P[(MeOx)_x\text{-}co\text{-}(DecOx)_{1-x}]_n$				
III	0.95	0.95	47	44	4000	3550	3750	1.12
IV	0.90	0.90	50	55	5300	4800	5800	1.,20

[a]Composition in the copolymer is identical with the composition in the feed. [b]Determined by ^{1}H NMR spectroscopy (300 MHz, $CDCl_3$ or D_2O). [c]Determined by GPC (DMF with LiCl) using PEG calibration.

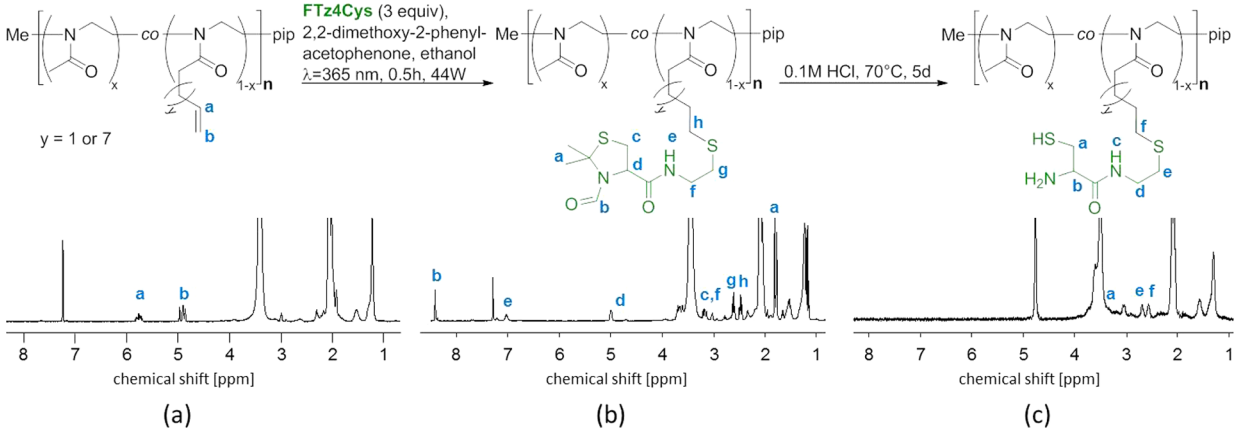

Figure 1. ^{1}H NMR of (a) $P[(MeOx)_{0.90}\text{-}co\text{-}(BuOx)_{0.10}]_{50}$ (in $CDCl_3$), (b) $P[(MeOx)_{0.90}\text{-}co\text{-}(BuOxCA)_{0.10}]_{50}$ (in $CDCl_3$), and (c) $P[(MeOx)_{0.90}\text{-}co\text{-}(BuOxCys)_{0.10}]_{50}$ (in D_2O).

literature-known formation of the HCl-salt of Tz4CA by treatment of L-cysteine hydrochloric salt with acetone[34] was performed first, followed by formylation of the amine according to literature.[35] Subsequently, the carboxylic functionality was mildly activated using *N*,*N*-carbonyldiimidazole in chloroform for 2 h at room temperature under an inert atmosphere. The addition of cysteamine hydrochloric salt in pyridine under an inert atmosphere and workup by recrystallization led to the desired product. ^{1}H NMR spectrum (SI, Figure S2) in DMSO-d_6 showed all intended signals with characteristic chemical shifts.

Polymer Analogue Functionalization. We used BuOx or DecOx with allyl groups for further functionalization via thiol−ene chemistry. Table 1 shows polymers with different composition, molecular weights, dispersities, molar fractions, and repeating units. We used cationic ring-opening polymerization without microwave irradiation and resulted in narrow dispersities. Nevertheless, some GPC elugrams were fronted or broader than expected, referring to possible literature-known side reactions.[36,37]

Mercaptothiazolidine was introduced by thiol−ene reaction with 2,2-dimethoxyphenylacetophenone (DMPA) as radical source, UV irradiation (λ = 365 nm), and 3 equiv of mercaptothiazolidine with respect to the allyl groups in degassed methanol. Figure 1 shows representative for all copolymer compositions the ^{1}H NMR spectra of poly-$[(MeOx)_{0.90}\text{-}co\text{-}(BuOx)_{0.10}]_{50}$. (See the Supporting Information for all spectra.) The signals at δ 4.94 and 5.81 (Figure 1a) are assigned to the allyl group of BuOx.

After UV reaction with marcaptothiazolidine and precipitation into cold diethyl ether (Figure 1b), the signals of the allyl function have vanished, indicating a complete consumption of the UV-reactive allyl groups. Additionally, the newly appearing signals at δ 1.83 (doublet, two methyl groups of the acetale protected cysteine), at δ 4.70 and 4.98 (methine proton next to the protected amine of the cysteine function), at δ 7.01 (amide proton of the side chain), and at δ 8.39 (formyl group of the thiazolidine ring) can be assigned to the introduced thiazolidine.

GPC measurements in DMF showed a small increase from M_n = 2.9 to 3.7 kg·mol^{-1} (PEG standards, Figures S5 and S7 in the SI), along with an increase in dispersities, further supporting successful coupling of the thiazolidine component. In all cases the molecular weights determined by GPC fall below the calculated ones. We assign this deviation to the PEG standards used for calibration of GPC and their different dependence of the hydrodynamic radius on the molecular weight compared with POx derivatives. However, also molecular interactions due to the introduced side chains may contribute.

Under acidic conditions the N-formyl group can be cleaved and the resulting thiazolidine-ring subsequently equilibrates to cysteine and acetone in aqueous solution. Refluxing the polymer at 70 °C for 5 days in 0.1 M HCl solution leads to a hydrolysis of the thiazolidine ring. Dialysis against H_2O produced oxidized polymers with gel structures that were only soluble in reductive media. We suppose that the H-exchange of amines and nearby thiols is very fast and leads to the formation of ammonium thiolates. They may rapidly build up disulfide bridges and generate gel-structures. It is, however, unclear to what extent these gels are physically or covalently cross-linked. In any case, dialysis against acetic acid (0.01 M) instead of water ensured permanently protonated thiols during work up

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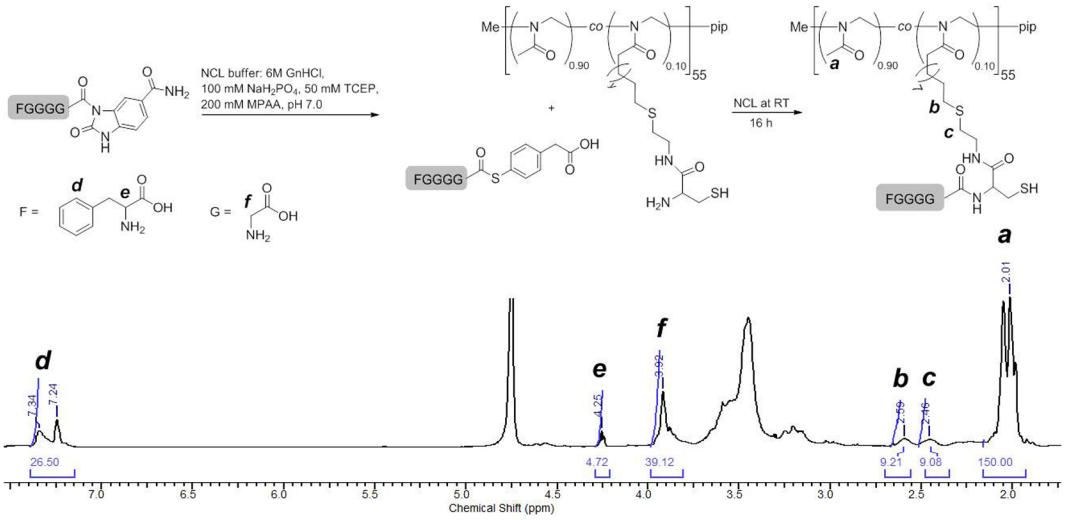

Figure 2. ^{1}H NMR of polymer−peptide conjugates bearing five peptides per polymer chain (in D_2O).

and yielded stable products of cysteine-functional POx that could be lyophilized.

Quantification of the Cysteine Functionality. We used the TNBSA assay under oxidative conditions to quantify cysteine functionality in POx as described in detail in a related publication with functionalized polyglycidols.[28] TNBSA is able to react with thiols and amines, resulting in a trinitrophenyl complex that can be quantified at a wavelength of 335 nm by UV−vis spectrophotometry. For unambiguous quantification of the amines we oxidized all thiol functions prior to the quantification assay by treatment with hydrogen peroxide for 1 h at 37 °C. A concentration series of the polymer between 7.92 and 99.00 μmol·mL^{-1} yielded an average content of 5.0 ± 0.6 amines (cysteines) per polymer chain with a polymer bearing five cysteine functions calculated by NMR spectroscopy.

Cell Toxicity Tests. A number of tetrazolium-dye-based assays such as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide), or WST (water-soluble tetrazolium) are usually applied as standard methods to assess cell viability. In all of these assays, reductive enzymes (reductases) generate a dye that can subsequently be measured colorimetrically and the intensity can be correlated with cell viability. Because of the reductive properties of our cysteine-containing polymers, we measured luminescence, corresponding to ATP production, as a marker of cell viability with the LCV-assay to verify the cytocompatibility of the polymers. Therefore, we incubated human fibroblasts with four functionalized polymers that varied in composition and cysteine moiety: P[$(MeOx)_{0.95}$-*co*-$(BuOxCys)_{0.05}$]$_{44}$, P[$(MeOx)_{0.95}$-*co*-$(DecOxCys)_{0.05}$]$_{54}$, P[$(MeOx)_{0.90}$-*co*-$(BuOxCys)_{0.10}$]$_{55}$, and P-[$(MeOx)_{0.90}$-*co*-$(DecOxCys)_{0.10}$]$_{57}$.

All samples with concentrations between 10 and 0.1 mg·mL^{-1} were analyzed concerning cell number and cell viability after 48 h of incubation (SI, Figure S16). In general, all polymers were proved to be cytocompatible up to concentrations of 1 mg·mL^{-1}. However, P[$(MeOx)_{0.95}$-*co*-$(DecOxCys)_{0.05}$]$_{54}$ resulted in cell activity and cell numbers under 40 or 20% at 10 mg·mL^{-1}. Also, P[$(MeOx)_{0.90}$-*co*-$(BuOxCys)_{0.10}$]$_{55}$ caused reduction of cell numbers down to 50% after 48 h of treatment. There is no clear dependency on the length of the hydrophobic side chain linker or degree of functionalization of the polymer, so the negative influence on cell vitality or cell number of these polymers at high concentrations of 10 mg·mL^{-1} remains unclear or might be due to minor impurities in the polymer itself, yet we can summarize that no cytotoxicity concerning human fibroblasts was determined up to concentrations of 1 mg·mL^{-1}.

Native Chemical Ligation. To prove the accessibility and chemical reactivity of the cysteine-functionalities we used NCL for multiple polymer−peptide conjugate formation. Therefore, we employed a polymer bearing five cysteine side chains (calculated by NMR) and coupled C-terminal thioester peptide with polymer/peptide ratio 1:5 (Figure 2). As a model peptide we applied a sequence of phenylalanine and glycine. NCL was performed overnight at RT and at a pH of 7.2 under reductive conditions with MPAA as an aromatic NCL catalyst. HPLC was applied to remove salts and excess MPAA. Polymer-containing fractions were collected and showed no unreacted peptide by UV/MS.

Figure 2 shows the NMR of polymer−peptide conjugates. The degree of polymerization and functionalization was predetermined by the copolymerization of MeOx and DecOx. We used the peak at δ 2.0 (which can be assigned to the methyl group of MeOx) as reference for the calculation of the attached peptide sequences.

After NCL, all predicted signals are visible in the NMR spectrum. Besides the signals of the polymer, new signals of the attached peptide are evident with accurate integrals at δ 3.9 (methylene group of glycine), δ 4.2 (methine group of phenylalanine), and δ 7.2 to 7.3 (aromatic unit of phenylalanine). MALDI ToF measurements of the polymer before and after NCL show a broader signal after NCL, as expected (Supporting Information). The attachment of small peptides to cysteine-functionalized POx was possible without limitation.

Because the chosen peptide is biologically irrelevant and has no further function, we additionally proved the reactivity of NCL with tau[390−410] sequence (Thz-EIVYKSPVVSGD-TSPRHLSN, Thz: thiazolidine protected cysteine, 2443 g·mol^{-1}), which is sterically demanding and known in the research area of Alzheimer's disease. We employed a 5800 g·mol^{-1} polymer bearing six cysteine side chains (calculated by NMR) and coupled the peptide to polymer/

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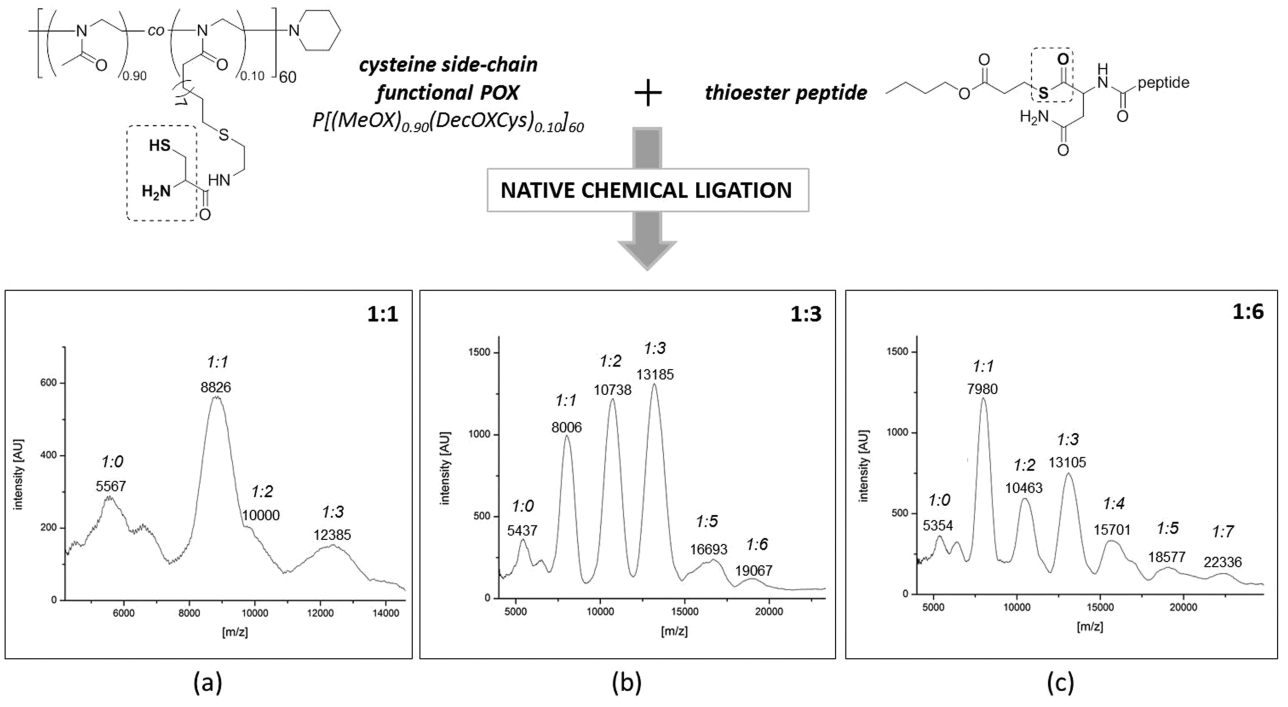

Figure 3. MALDI-ToF measurements of polymer–peptide conjugates with polymer/peptide feed ratios of 1:1, 1:3, and 1:6 with corresponding estimated maxima of the signals and the amount of peptides at each polymer. The molecular weight of the pure polymer was M_n = 5800 g·mol^{-1}, as calculated by NMR measurements.

peptide ratios 1:1, 1:3, and 1:6 (Figure 3). NCL was performed under the same conditions as in the first experiment. Even here we used HPLC to remove salts and excess MPAA. Polymer-containing fractions were collected and showed no unreacted peptide by UV/MS.

The results of MALDI-ToF measurements are shown in Figure 3 and demonstrate the successful formation of polymer–peptide conjugates. For interpretation of the MALDI spectra, it is important to note that dispersity of the polymer and the multitude of polymer species formed especially in the case of copolymers are known to impede the resolution of MALDI spectra. Polymer analogous functionalization with the thiazolidine and the subsequent functionalization with peptides further intensify these effects in our study, so the MALDI-ToF spectra are not ideally resolved, yet they allow us to extract the most important information.

The signal at 5567 g·mol^{-1} in Figure 3a reveals uncoupled cysteine-functionalized POx. For a 1:1 molar ratio between polymer and peptide in the reaction mixture, the signal at 8826 g·mol^{-1} indicates the main species in this sample showing the conjugation of one cys-POx with one peptide. The signal for double-conjugated peptide merely occurs as shoulder of the main signal at molecular masses around 10 000 g·mol^{-1}, and the weak signal at 12385 g·mol^{-1} may be attributed to triple conjugation of peptides to the polymer. In the case of a polymer/peptide ratio 1:3 (Figure 3b), signals at 8006, 10 738, and 13 185 g·mol^{-1} can be assigned to conjugates of one polymer chain with one, two, and three peptides. However, the signals at 16 693 and 19067 g·mol^{-1} reveal that also conjugation of one polymer with five or six peptides occurred.

A possible signal around 15 000 g·mol^{-1} that could be assigned to conjugates with four peptides cannot be assigned unambiguously. To provoke the formation of high conjugate numbers we also applied a 6-fold excess of peptide with respect to the polymer (Figure 3c). Nearly all expected signals were obtained, with most intensive signals in the range of one to three peptides per polymer chain (7980, 10 463, and 13 105 g·mol^{-1}). Despite the higher excess of peptide, higher signals are very broad and weak, which could be a hint of sterically hindrance caused by the relatively big peptide. Nevertheless, maxima with shoulders are visible at 15 701, 18 577, and 22 336 g·mol^{-1}, showing attendance of higher conjugates. We assume that the overlay of different species led to a broad signal distribution. Table 2 summarizes all observed masses identified with MALDI-ToF measurements in the different feed ratios of polymer/peptide 1:1, 1:3, and 1:6.

CONCLUSIONS

We successfully demonstrate a concept allowing the synthesis of water-soluble cysteine side-chain-functionalized POx based on MeOx copolymerized with BuOx or DecOx. The multifunctional prepolymers with reactive allyl groups enable thiol–ene reactions with mercaptothiazolidines. Deprotection led to

Table 2. Theoretical and Observed Masses of 1:1, 1:3, and 1:6 Polymer Functionalized with the Model Peptide

polymer/peptide molar ratio	$M_{n,theo}$/g·mol^{-1} $[M + H]^+_{cal}$	$M_{n,obs}$/g·mol^{-1}		
		$M_{1:1}$	$M_{1:3}$	$M_{1:6}$
1:0	5800	5567	5437	5354
1:1	8082	8826	8006	7980
1:2	10364	10000	10738	10463
1:3	12646	12385	13185	13105
1:4	14928			15701
1:5	17210		16693	
1:6	19492		19067	18577
1:7	21774			22336

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side-chain cysteine-functionalized POx, which was confirmed by NMR and GPC. The cysteine amount was quantified by a modified TNBSA-assay in which thiol groups were oxidized before quantification and cytocompatibility was confirmed by LCV-assay. We show that multiple NCLs per polymer with C-terminal thioester peptides is possible efficiently with low-molecular-weight peptides and that steric effects occur for larger sequences such as the tau[390−410] sequence with a molecular weight of 2443 $g \cdot mol^{-1}$. Future studies will focus on the exploitation of the multiple selective coupling of peptides to these polymers to assess multivalency effects in cell interaction studies.

■ ASSOCIATED CONTENT

Supporting Information

Additional information about synthesis and characterization of all substances, NMR and GPC analysis of all polymers, quantification of cysteine side chains by TNBSA, and data of LCV assay as well as MALDI TOF measurements of polymer−peptide conjugates. 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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