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Side Chain Thiol-functionalized Poly(ethylene glycol) by Post-polymerization Modification of Hydroxyl Groups: Synthesis, Crosslinking and Inkjet Printing

Alexander Southan,[a] Eva Hoch,[a] Veronika Schönhaar,[b] Kirsten Borchers,[b] Christian Schuh,[b] Michaela Müller,[b] Monika Bach[a] and Günter E.M. Tovar*[a,b]

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Polymers with a poly(ethylene glycol) backbone and mercaptomethyl side chains were synthesized by post-polymerization modification of hydroxymethyl side chains in three steps. As the starting point of the synthetic route, linear copolymers of ethylene oxide and glycidol with molar contents of glycidol repeating units of approximately 20, 40, 60, 80 and 100% were used. The polymer-bound hydroxyl groups were converted to thiol groups in three steps, comprising tosylation, introduction of a triphenylmethyl protected thiol and thiol deprotection by acid treatment. The degree of thiol-functionalization was controlled by the degree of functionalization of the starting material. The degree of conversion of hydroxyl groups to thiol groups determined by ^{1}H NMR spectroscopy was quantitative for copolymers with approximately 20 and 40% glycidol repeating units and 92, 81 and 87% for copolymers with approximately 60, 80 and 100% glycidol repeating units, respectively. Exemplarily, poly(glycidylthiol) obtained by conversion of poly(glycidol) was crosslinked with poly(ethylene glycol) diacrylate (PEG-DA) to yield hydrogels which supported adhesion and proliferation of human fibroblasts 48 h after cell seeding. Spatially defined and surface attached gel structures were fabricated by subsequent inkjet printing of poly(glycidylthiol) and PEG-DA solutions onto acrylated glass slides.

Introduction

Polymers with a poly(ethylene glycol) (PEG) backbone are a highly interesting class of materials. The parent compound PEG itself is soluble in water and many organic solvents, is non-toxic and hydrolytically stable.[1–4] This chemical profile leads to bioinertness and makes it a much used material for various applications ranging from cosmetics,[5] drug delivery,[6] surface treatments[7] and tissue engineering.[8]

Characteristic for PEG itself is the lack of functional groups present per macromolecule. This is due to the synthesis of the polymer which involves the ring-opening polymerization (ROP) of ethylene oxide (EO).[1,9,10] The number of functional groups is generally limited by the number of end groups. In order to circumvent the limitation of only two functional groups per macromolecule for linear PEG derivatives, multi-arm or star-shaped PEGs have been developed.[11–13] Unfortunately, this approach does not allow tailoring the number of functional groups independently from the macromolecular architecture.

An approach which enables the introduction of a defined number of functional groups per macromolecule and the control of the polymer architecture at the same time is the localization of the functional groups in the polymer side chains. For polymers with a PEG backbone (PEG-based polymers), this can be achieved by ROP of functionalized epoxide monomers.[14] This way, different functional groups like hydroxyl,[15,16] amino,[17,18] furan,[19] chloromethyl[20] or vinyl ether groups[21] have been introduced. However, the number of known functionalized epoxide monomers which can be (co)polymerized is limited. Thus, post-polymerization modification of functional groups is often necessary, *e.g.* the thiol-ene addition of thiols to vinyl functionalities,[21] the conversion of chloromethyl into amino groups[22] or the introduction of ATRP initiators starting from poly(glycidol) with hydroxyl side chains.[23]

A functional group particularly relevant for polymer science is the thiol group due to its excessive reactivity, *e.g.* for crosslinking by disulfide formation[24] or post-polymerization reactions like the click thiol-ene or thiol-Michael reactions.[25,26] Also the binding of thiol-functionalized polymers to cell surfaces was shown by Bacalocostantis *et al.*[27] with possible implications for cell adhesion.

[a] *Institute of Interfacial Process Engineering and Plasma Technology IGVP, University of Stuttgart, Nobelstr. 12, 70569 Stuttgart, Germany. Fax: +49 711 9704200; Tel: +49 711 9704109; E-mail: Guenter.Tovar@igvp.uni-stuttgart.de*
[b] *Fraunhofer Institute for Interfacial Engineering and Biotechnology IGB, Nobelstr. 12, 70569 Stuttgart, Germany. Fax: +49 711 9704200; Tel: +49 711 9704109; E-mail: Guenter.Tovar@igb.fraunhofer.de*

The thiol group has not yet been successfully introduced into PEG-based polymers by the polymerization of functionalized epoxides. In fact, ROP experiments with sulfur containing epoxide compounds have not yet led to highly defined polymers since these compounds undergo manifold side reactions.[28–30]

Thus, the introduction of thiol groups could so far only be achieved by post-polymerization modification. As an attractive starting point, hydroxyl groups were used for a large variety of modifications.[31] Also thiol groups were introduced successfully by crosslinking esterfication of poly(glycidol) with 3,3'-dithiodipropionic acid and subsequent reduction of the disulfide crosslinks.[32] The drawback of this approach hydrolytic lability of the linker group.

In order to rule out the possibility of hydrolytic cleavage of the polymer side chains, the aim of this work was to synthesize PEG-based polymers with mercaptomethyl side chains. In order to show the general applicability of the post-polymerization approach, we aim to control the degree of thiol-functionalization, *i.e.* the fraction of repeating units carrying a thiol group, by the degree of hydroxyl-functionalization of the precursors. Finally, we will demonstrate the usefulness of the resulting polymers by inkjet printing to form hydrogels in a proof of principle experiment. We hypothesize that the thiol-functionalization has a significant effect on the interaction of the material with cells.

Experimental

Intrumentation and Methods

^{1}H NMR spectra (500 MHz) were recorded on a "Avance 500" spectrometer (Bruker) with chloroform-d_1 as the solvent and tetramethylsilane as internal standard. Sample concentrations were about 20 mg per 0.5 mL deuterated solvent. Size exclusion chromatography (SEC) measurements in tetrahydrofuran (THF) with a polystyrene calibration were performed at 40 °C and with injection volumes of 50 μL on a "1260 Infinity GPC-SEC Analysis System" (Agilent Technologies) equipped with ultraviolet (UV) (255 nm) and refractive index (RI) detectors and a column combination of "PSS SDV Guard 10 μL" and "PSS SDV lin M 10 μL". Samples were prepared by dissolving the polymers at concentrations of approximately 2 mg mL^{-1} at 40 °C for 72 h and subsequent filtration through 0.2 μm PTFE syringe filters. Differential scanning calorimetry (DSC) measurements were performed on a "DSC 200 F3 Maia" (Netzsch) with sample masses of 15 mg in the temperature range from -100 °C to 100 °C with heating/cooling ramps of 10 K min^{-1} in Al crucibles under nitrogen. Glass transition temperatures were determined from the second heating curves. UV/VIS spectra were collected on a "UV-2450" photospectrometer (Shimadzu) in quartz glass cuvettes. For UV curing, a UV chamber with a UVA light intensity of 15 mW cm^{-2} was used. Thiol concentrations were determined by a photometric assay according to Egwim and Gruber in DMSO as the solvent.[33] Light and fluorescence microscopic images were collected using a "BZ-9000 Biorevo" microscope (Keyence). For inkjet printing, the piezoelectric inkjet printer Dimatix DMP-3000 (Fujifilm Dimatix Inc., AD Weesp/NL) was used. The printing head has 16 nozzles with a diameter of 21.5 μm, which are arranged in a line with a space of 254 μm between the nozzles. A drop spacing of 20 μm was selected.

Solvents, Reagents and Materials

Nitrogen "N 50" was purchased from Air Liquide (Düsseldorf, Germany) and dried by passing through a Varian "Gas Clean Moisture" filter prior to use. All the chemical syntheses were performed under an atmosphere of dry nitrogen. The following chemicals were purchased from Sigma-Aldrich: Glycidol (96%), ethyl vinyl ether (99%), *p*-toluenesulfonic acid monohydrate (98.5%), *p*-toluenesulfonyl chloride (TsCl, 99%), sodium hydroxide (NaOH, 97%), dimethyl sulfoxide (DMSO, anhydrous, >99.9%), tetrahydrofuran (THF, anhydrous, >99.9%), ethylene oxide (EO, 99.8%), calcium hydride (95%, +4 mesh), fluorescein diacetate (FDA), propidium iodide (PI), triphenylmethanethiol (TrtSH, 97%), triethylsilane (Et_3SiH, 99%), triethylamine (NEt_3, 99.5%), hydrochloric acid (HCl, 37%), potassium *tert*-butoxide (KO^tBu, ≥98%), KO^tBu solution in THF (1.0 M), poly(ethylene glycol) diacrylate (PEG-DA, average M_n 700) and chloroform-d_1 ($CDCl_3$, 99.8 atom-% D). They were used as received except for DMSO and THF (anhydrous, were further dried over molecular sieves 4 Å) and EO (was passed through a column of calcium hydride prior to use). Hydrogen peroxide (H_2O_2) solution (30%) and ammonium hydroxide solution (25%) were purchased from Fluka. Trifluoroacetic acid (TFA, ≥99.9%) was purchased from Carl Roth GmbH. 4,4'-dithiodipyridine (DTDP, 98%) was obtained from Alfa Aesar. 3-Acryloxypropyltrimethoxysilane (95%) was purchased from ABCR. Methanol (ACS reagent grade), ethanol (99.9%, HPLC grade), acetone (HPLC grade), dimethylformamide (DMF, ACS reagent grade), THF (HPLC grade) and dichloromethane (DCM, ACS reagent grade) were purchased from J.T. Baker. Water was withdrawn from a Barnstead GenPure xCAD water purification system (Thermo Scientific). 1-Ethoxyethyl glycidyl ether (EEGE) was prepared as described by Fitton *et al.*[34] Glass slides were obtained from Thermo Scientific. Hellmanex® II was received from Hellma Analytics. The radical photoinitiator 1-[4-(2-hydroxyethoxy)phenyl]-2-hydroxy-2-methyl-1-propan-1-one (Irgacure 2959) was a kind gift from Bodo Möller Chemie GmbH (Germany). Phosphate buffered saline (PBS) contained 137 mmol L^{-1} sodium

chloride, 2.7 mmol L^{-1} potassium chloride, 1.5 mmol L^{-1} potassium dihydrogen phosphate, 8.1 mmol L^{-1} sodium hydrogenphosphate, 1.1 mmol L^{-1} magnesium chloride and 0.9 mmol L^{-1} calcium chloride. Standard tissue culture dishes were obtained from Greiner Bio-One. Cell culture medium for the cultivation of human fibroblasts consisted of Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS; Invitrogen, Germany) and 1% (v/v) penicillin streptomycin. Human fibroblasts for cytocompatibility investigations were obtained from biopsies (after informed written consent was given) and isolated and expanded according to Kluger *et al.*[35] The fibroblasts were stored at -196 °C, defrosted before use and seeded at a density of 40,000 cells cm^{-2} in tissue culture flasks. Finally, the cells were harvested by trypsinization and a cell suspension with a cell density of $3x10^5$ cells mL^{-1} was prepared. The fibroblasts were used in passage 2.

Synthesis of Poly[EEGE-*co*-(ethylene oxide)]

Below, an experimental procedure for the copolymerization of EO and EEGE with a molar ratio of approximately 60:40 (sample number **2**) is given. The other polymerizations were carried out accordingly with identical total monomer concentrations (see Table 1 for details and product characteristics; for the homopolymerization of EEGE, the addition of EO was omitted.). In a Schlenk flask, 9.951 g EEGE (68.1 mmol, 43 eq.) were dissolved in 40 mL anhydrous DMSO. The solution was cooled to -20 °C in an ice-acetone bath and 4.161 g EO (94.5 mmol, 59 eq.) were distilled into the reaction flask. The frozen reaction mixture was warmed to room temperature, mixed well by stirring, frozen in liquid nitrogen and evacuated to a pressure of approx. 10^{-1} mbar. The reaction mixture was warmed to room temperature and the polymerization was initiated by addition of 1.6 mL of a 1 M KO^{t}Bu solution (1.6 mmol KO^{t}Bu, 1 eq.) in THF through a rubber septum. The polymerization was carried out *in vacuo* at 60 °C for 72 h. The solvent was removed under reduced pressure at 60 °C and a highly viscous, amber polymer was obtained (yield: 14.292 g, quantitative). ^{1}H NMR ($CDCl_3$, 500 MHz): δ/ppm = 1.19 (m, –CH_2–$C\underline{H}_3$), 1.29 (m, –CH–$C\underline{H}_3$), 3.3–3.8 (m, polyether backbone and $C\underline{H}_2$ next to backbone, –$C\underline{H}_2$–CH_3), 4.70 (m, –$C\underline{H}$–CH_3).

Synthesis of Poly[(ethylene oxide)-*co*-(glycidyl tosylate)]

Below, an experimental procedure for the tosylation of poly[EEGE-*co*-(ethylene oxide)] with a molar ratio of approximately 40:60 (sample number **7**) is given. The other tosylations were carried out accordingly with identical concentrations of the functional groups involved (see Table 2 for details and product characteristics). For the cleavage of the acetal protecting groups, 5.549 g of polymer sample **2** (26.8 mmol acetal groups, 1.0 eq.) were dissolved in 22 mL methanol and 2.7 mL 1 M hydrochloric acid (2.7 mmol H^+, 0.1 eq.) were added. The reaction mixture was stirred at room temperature for 5 h. The solution was neutralized with aqueous sodium hydroxide solution (1 M) and the solvent was removed at room temperature under reduced pressure. For tosylation of the liberated hydroxyl groups, the highly viscous, amber residue was dissolved in 10 mL water and a solution of 2.697 g sodium hydroxide (67.0 mmol, 2.5 eq.) in 10 mL water was added. The solution was cooled to 5 °C in an ice bath. A solution of 7.665 g *p*-toluenesulfonyl chloride (40.2 mmol, 1.5 eq.) in 30 mL THF was added dropwise, keeping the temperature below 25 °C. The reaction mixture was stirred at room temperature for 24 h. It was neutralized by addition of 1 M hydrochloric acid and the THF was removed by rotary evaporation at 40 °C. The liquid phase of the remaining aqueous mixture was decanted. The gummy residue was dissolved in 10 mL THF and precipitated in 100 mL methanol. The reprecipitation was repeated another two times and the precipitate dried in vacuum, yielding 6.271 g poly[(ethylene oxide)-*co*-(glycidyl tosylate)] (yield: 81%) as a tacky white residue. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ/ppm = 2.43 (s, Ar–$C\underline{H}_3$), 3.3–3.8 (m, PEG backbone), 3.8–4.2 (m, Ar–SO_3–$C\underline{H}_2$–), 7.34 (m, Ar–$\underline{H}$ ortho to methyl), 7.77 (m, Ar–$\underline{H}$ meta to methyl).

Synthesis of Poly[(ethylene oxide)-*co*-(glycidyl trityl thioether)]

Below, an experimental procedure for the synthesis of poly[(ethylene oxide)-*co*-(glycidyl trityl thioether)] with a molar ratio of approximately 60:40 (sample number **12**) is given. The other reactions were carried out accordingly with identical concentrations of the functional groups involved (see Table 3 for details and product characteristics). 1.785 g KO^{t}Bu (15.9 mmol, 1.5 eq.) were dissolved in 40 mL anhydrous THF and 5.130 g triphenylmethanethiol (18.6 mmol, 1.8 eq.) were added. A solution of 3.041 g of polymer sample **7** (10.3 mmol tosylate groups, 1.0 eq.) in 30 mL anhydrous THF was added and it was stirred at room temperature for 20 h. The reaction mixture was added dropwise to 450 mL methanol. The solvent was decanted, the resulting precipitate was dissolved in 40 mL THF and the reprecipitation was repeated in 400 mL methanol another two times. 3.470 g of a brittle white residue were obtained (yield: 84%). ^{1}H-NMR ($CDCl_3$, 500 MHz): δ/ppm = 2.1–2.5 (m, –$C\underline{H}_2$–S–CPh_3), 2.8–3.7 (m, PEG backbone), 7.20 (m, Ar-$\underline{H}$), 7.38 (m, Ar-$\underline{H}$).

Synthesis of Poly[(ethylene oxide)-*co*-glycidylthiol]

Below, an experimental procedure for the synthesis of poly[(ethylene oxide)-*co*-(glycidylthiol)] with a molar ratio

of approximately 60:40 (sample number **17**) is given. The other reactions were carried out accordingly with identical concentrations of the functional groups involved (see Table 4 for details and product characteristics). All liquids were degassed by five consecutive freeze-pump-thaw cycles directly before use. 1.305 g of polymer sample **12** (3.27 mmol triphenylmethyl groups, 1.0 eq.) were dissolved in 35 mL DCM. 3.5 mL of TFA and 3.5 mL of Et_3SiH were added. The solution was stirred at room temperature for 24 h. The solvents were removed *in vacuo* and the vicous residue was dissolved in 2.5 mL THF. The product was precipitated in 25 mL petroleum ether three times. 0.413 g of a highly viscous, colourless polymer were obtained (yield: 90%). ^{1}H-NMR ($CDCl_3$, 500 MHz): δ/ppm = 1.61 (m, –SH), 2.4–2.9 (m, –CH_2–SH), 3.4–4.1 (m, PEG backbone).

Gel Formation of Poly(glycidylthiol) and PEG-DA

As gel component 1, 0.706 g poly(glycidylthiol) **20** were dissolved in 3.8 mL degassed DMF. As gel component 2, 0.857 g PEG-DA were dissolved in 2.3 mL DMF and 0.35 mL NEt_3 were added. For gelation, 325 μL of gel component 1 (containing 51 mg polymer, 566 μmol thiol groups, 1 eq.) and 325 μL of gel component 2 (containing 79 mg PEG-DA, 226 μmol acrylate groups, 0.4 eq.) were mixed in a glass vial. The gelling time was monitored by the test tube inverting method which defines gelling to have occurred when the mixture does not flow under the influence of gravity for 1 minute.[36]

Investigation of Hydrogel Cytocompatibility

For cell experiments, the viscous gel precursor solution was pipetted into a cylindrical glass mold with a diameter of 16 mm and a height of 2.5 mm with a cylindrical indentation in the center with a diameter of 10 mm and a height of 1 mm, giving the gels a pan-like shape. The mold was covered with a glass slide and the gels were cured for 24 h at room temperature.

As a control for the cell experiments, hydrogels from PEG-DA without the addition of poly(glycidylthiol) **20** were prepared. For this purpose, 500 μL of a 30% (w/v) aqueous solution of PEG-DA (containing 150 mg PEG-DA) were mixed with 107 μL of a solution of Irgacure 2959 (28 mg Irgacure 2959 in 4 mL water) and 143 μL water, yielding a gel precursor solution with 20% (w/v) PEG-DA concentration. This solution was pipetted into an identical mold as the gels above, covered with a quartz glass pane and irradiated in the UV chamber for 10 minutes.

After curing, each gel was washed with 20 mL acetone for 8 h three times and once with 20 mL ethanol/water (70:30) for 2 h and were from now on handled under sterile conditions. Then, they were washed with 10 mL PBS buffer for 70 h, changing the washing buffer seven times at regular intervals. Finally, the gels were immersed in 10 mL cell culture medium and washed for 8 h three times. For cell cultivation, 50 μL of the fibroblast suspension described above (containing 15000 cells) were pipetted onto the hydrogel. The seeded hydrogels were kept under standard cultivation conditions (37 °C and 5% CO_2 in a humidified incubator) to allow cell adhesion and proliferation. After 3 h and 48 h, cell adhesion, morphology, and cell confluency were examined by fluorescence-based live/dead staining using FDA and PI.[37] For the FDA/PI staining the hydrogels were put into a petri dish and carefully washed with PBS. The rim of the gels was cut away in order to create a flat surface suitable for microscopy. 20 μL FDA/PI solution (10 μL of a 5 μg mL^{-1} FDA solution in acetone + 10 μL of a 0.5 μg mL^{-1} PI solution in PBS + 980 μL PBS) were pipetted onto each hydrogel and incubated for 1 min at 37 °C. The gels were investigated by fluorescence microscopy.

Inkjet Printing of Gel Formulation

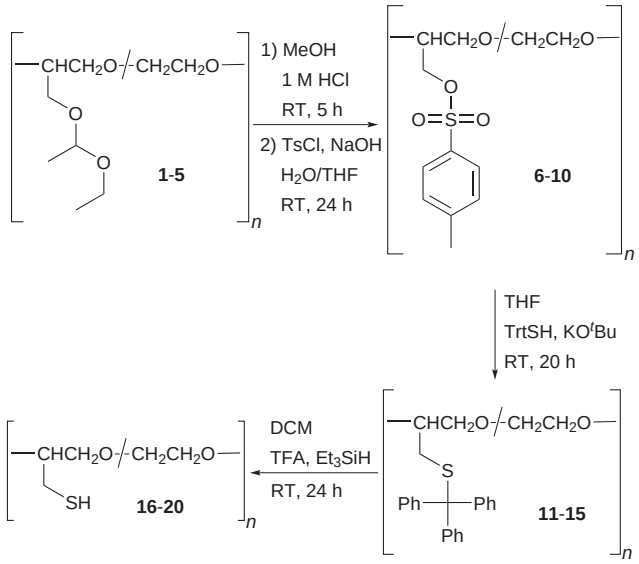
As a printing substrate, glass slides were functionalized with acrylate surface functionalities. For this purpose, glass slides were cleaned by heating in a 2% aqueous Hellmanex® II solution (v/v) to 40 °C for 45 minutes. Then, the glass surface was activated by heating to 70 °C in a 1:3 mixture (v/v) of H_2O_2 solution (30%) and ammonium hydroxide solution (25%) for 40 minutes. The glass slides were washed with water and ethanol and dried *in vacuo* for 60 minutes. Finally, they were immersed into a 5% solution (v/v) of 3-acryloxypropyltrimethoxysilane in ethanol/water (95:5, v/v) for 20 h under gentle agitation.

A spatially defined gel structure was formed by printing double layers of gel component 1 and gel component 2 formulated as described above onto the acrylate-functionalized glass slides. In total, four double layers were printed on top of each other, waiting 10 minutes after printing of each double layer before resuming the printing process. The printed gel structure was left to cure for 5 days at room temperature in an atmosphere saturated with DMF vapor. The printed gels were immersed carefully into DMF and shaken gently for 60 minutes. The glass slides were dried carefully in a stream of nitrogen, making sure not to dry the gels.

Results and Discussion

Synthetic Strategy

The introduction of hydroxymethyl groups into side chains of PEG-based polymer is well documented using the acetal protected monomer EEGE for homopolymers[15,38] and



Scheme 1 Synthetic route to the mercaptomethyl side chain functionalized PEG-based polymer poly[(ethylene oxide)-*co*-glycidylthiol] starting from poly[EEGE-*co*-(ethylene oxide)]. Free hydroxyl groups (not represented in this figure) in the side chains were generated by acidic treatment of the acetal protected starting polymers.

copolymers with EO or propylene oxide.[39,40] It was shown that poly[EEGE-*co*-(ethylene oxide)] can be synthesized as a nearly statistical copolymer by anionic ROP using alkoxide initiators.[40] Therefore, we used this system as the starting point of our synthetic strategy for the introduction of thiol functionalities into PEG side chains.

Starting from the acetal protected hydroxyl groups in the side chains, we envisioned that it should be possible to generate thiol groups in three steps (Scheme 1). The first step involves deprotection and activation of the hydroxyl groups by tosylation. The tosylate groups can then be displaced by a potent sulfur nucleophile, such as triphenylmethanethiol. The triphenylmethyl protective group can be removed in the final step by acidic treatment.

An acid labile protective group instead of a base labile one was chosen because of the greater stability of thiols in acidic environments and the less pronounced tendency to form disulfide bonds.[41] In the case of a multifunctional polymer, this would result in an insoluble polymer network which cannot be processed as simple as soluble polymers.

Prepolymer Synthesis and Deprotection

The synthesis of different copolymers of EO and EEGE as well as of a homopolymer of EEGE was carried out successfully. The different copolymer compositions and copolymer characteristics are summarized in Table 1. The gravimetric

Table 1 Poly[EEGE-*co*-(ethylene oxide)] copolymer characteristics of different compositions ([a])number average molecular weight (M_n) and weight average molecular weight (M_w) determined by SEC. [b])Glass transition temperature (T_g) determined by DSC. [c])Theoretical molar fraction of EEGE ($p\%_{Gr}$) in the monomer mixture before polymerization determined gravimetrically. [d])Molar fraction of EEGE repeating units ($p\%_{NMR}$) in the copolymer determined by ^{1}H NMR spectroscopy.).

	M_n [a])/ g mol^{-1}	M_w/M_n [a])	T_g [b])/ °C	$p\%_{Gr}$/ % [c])	$p\%_{NMR}$/ % [d])
1	2760	1.49	-64.9	21.7	21.0
2	3860	1.42	-63.8	41.9	40.4
3	3580	1.70	-63.9	60.2	58.1
4	4700	1.67	-62.1	81.5	79.7
5	3980	1.88	-61.7	100	100

polymer yields of samples **1**–**5** were quantitative. Also, the copolymer compositions determined by ^{1}H NMR were near the theoretical values defined by the monomer mixture before copolymerization (Figure 1). The M_w/M_n values were between 1.4 and 1.9, increased with the EEGE content of the monomer mixture and were higher than reported so far in the literature. Also the molecular weights determined by SEC are below the theoretical values which should correspond to an average degree of polymerization of approximately 100. This indicates to more chain transfer reactions taking place when DMSO is used as the polymerization solvent than *e.g.* in THF[40] or diglyme[23] and is comparable to the values obtained by Taton *et al.*.[15] However, monomodal molecular weight distributions (MWDs) were obtained (Figure 2). The main objective of this study is to show the feasibility of thiol-functionalization by post-polymerization reactions of polymers **1**–**5**. The shapes of the MWDs should therefore be preserved during the reaction sequence.

For post-polymerization modification of the polymer side chains, the acetal protective groups were cleaved by treatment with hydrochloric acid, yielding poly[(ethylene oxide)-*co*-glycidol]. Since acidic degradation of a poly(glycidol) backbone with hydrochloric acid was reported,[15] the reaction mixture was neutralized before removal of the solvent *in vacuo*. The sodium chloride formed during neutralization should not interfere with the following reaction and the poly[(ethylene oxide)-*co*-glycidol] obtained was used directly without further purification for the next step.

Tosylation of Hydroxyl Groups in the Side Chains

The general feasibility of the tosylation of a side chain hydroxyl-functionalized PEG-based polymer was demonstrated by us using poly(glycidol) as the starting material.[42] Here we show that this approach is also suitable for the tosylation of copolymers of ethylene oxide and glycidol. The char-

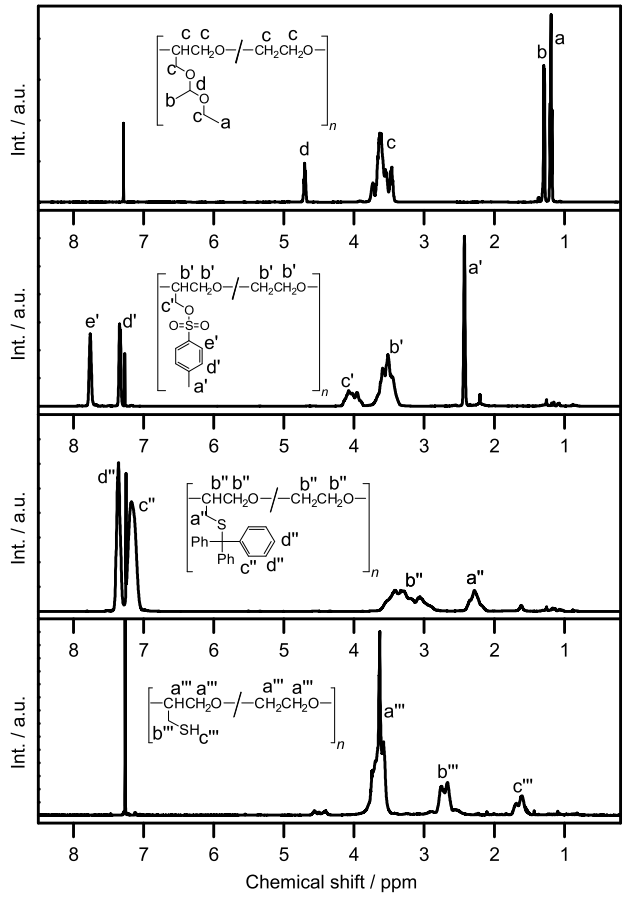


Fig. 1 ^{1}H NMR spectra of polymer samples **3**, **8**, **13** and **18** (from top to bottom).

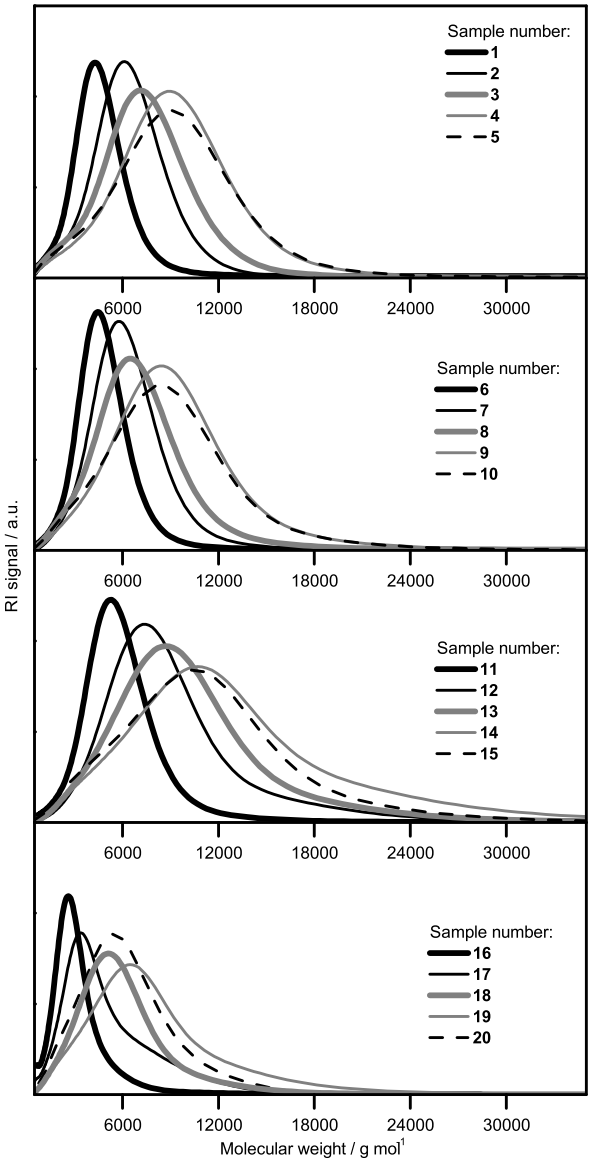


Fig. 2 Molecular weight distributions (MWDs) of all polymer samples synthesized in this study determined by SEC relative to polystyrene standards. The monomodal shape of the MWD of the starting material (samples **1**–**5**) was maintained throughout the reaction sequence as shown in Scheme 1, indicating that the polymer backbone remained unaltered and that no crosslinking took place.

acteristics of the resulting poly[(ethylene oxide)-*co*-(glycidyl tosylate)] copolymers are summarized in Table 2. Polymer samples **6**–**10** were directly derived from samples **1**–**5**. Polymer samples **6**–**10** were generally highly viscous liquids to tacky solids that were insoluble in water and methanol, but soluble in THF, DCM and chloroform.

In order to assess the success of the reaction, the degree of conversion of the side chain hydroxyl groups was calculated from the ^{1}H NMR signals (Figure 1). The ^{1}H NMR spectrum showed the characteristic tosyl group related signals at a chemical shift of 2.43 (methyl group), 7.34 and 7.77 ppm (aromatic protons). The percentage $p\%_{Ts}$ of repeating units carrying a tosyl group was calculated as:

$$p\%_{Ts} = \frac{4 \cdot Int(Ts)}{9 \cdot Int(BB) + Int(Ts)} \quad (1)$$

Here, Int(Ts) is the sum of the tosylate related ^{1}H NMR signal intensities (signals a', c', d' and e' in Figure 1) and Int(BB) is the signal intensity of the backbone protons (signal b' in Figure 1). For copolymer samples **6** and **7** with copolymer samples **1** and **2** as precursors carrying 21.0 and 40.4% functionalized repeating units, practically quantitative conversion of the hydroxyl side chains to the corresponding tosylate side chains was achieved. For polymer samples **8**–**10**, the degree of conversion decreased with increasing degree of functionalization of the precursor due to steric hindrance of the remaining

Table 2 Poly[(ethylene oxide)-*co*-(glycidyl tosylate)] copolymer characteristics of different compositions ([a]Degree of tosylation ($p\%_{Ts}$) of polymer repeating units determined by ^{1}H NMR and equation 1. For other symbol explanations, see Table 1).

	M_n/ g mol^{-1}	M_w/ M_n	T_g/ °C	$p\%_{Ts}$ [a]/ %	yield/ %
6	3320	1.34	-24.5	23.4	77
7	4410	1.25	-4.7	39.6	81
8	3920	1.55	7.6	54.6	86
9	5060	1.60	17.7	69.9	76
10	4370	1.67	28.1	87.1	78

hydroxyl groups. In case of the homopolymer **10**, 87.1% of the hydroxyl side chains were converted. The remaining hydroxyl groups were not expected to participate in or interfere with the following reactions and thus were not considered to be problematic.

Together with the increase of the degree of tosylation, the glass transition temperature (T_g) increased. It closely followed the prediction made by the Fox equation[43] with a T_g for PEG of -67 °C,[44] showing that the degree of functionalization is well controlled.

In all cases, the tosylation proceeded without altering the shape of the MWD (Figure 2). This also proves that during the preceding deprotection step, no polymer backbone cleavage occurred. Looking at the corresponding M_w/M_n value pairs, the M_w/M_n values generally decreased a little. We speculate that this was due to the loss of low molecular weight oligomers during the reprecipitation in methanol.

Nucleophilic Displacement of the Tosylate Groups in the Side Chains

In the reaction step following the tosylation, a thiol nucleophile is needed. We chose triphenylmethanethiol which carries an acid labile protecting group and is commercially available in sufficient purity, unlike similar examples described in the literature like 4-methoxybenzylthiol,[45] 2,4,6-trimethoxybenzylthiol[46,47] or xanthenylthiol.[48,49] Additionally, the triphenylmethyl protecting group (trityl group) can be cleaved quantitatively by treatment with TFA with Et_3SiH as a cation scavenger at room temperature.[50,51] These deprotection conditions should leave the PEG backbone unaltered, making the triphenylmethyl protecting group suitable for our purpose.

The utilization of a protected thiol instead of the direct method using the sulfide anion offers two main advantages: a) The formation of crosslinks during tosylate replacement is not possible. With sulfide anions this could only be avoided using a large excess of sulfide. b) The triphenylmethyl protected polymers are expected to be shelf-stable at room temperature.

Table 3 Poly[(ethylene oxide)-*co*-(glycidyl trityl thioether)] copolymer characteristics of different compositions ([a]Degree of triphenylmethyl thioether functionalization ($p\%_{STrt}$) of polymer repeating units determined by ^{1}H NMR and equation (2). For other symbol explanations, see Table 1).

	M_n/ g mol^{-1}	M_w/ M_n	T_g/ °C	$p\%_{STrt}$ [a]/ %	yield/ %
11	3930	1.30	2.2	25.8	72
12	5560	1.34	26.2	41.0	84
13	5650	1.47	35.6	55.7	91
14	5910	1.70	73.2	67.8	95
15	5050	1.69	79.9	87.3	74

This is not the case for the tosylated polymers described above of which the MWDs changed upon storage. The storage of polymers with free thiol groups is also expected to be difficult due to the formation of disulfide bridges under the influence of oxygen.

The characteristics of the poly[(ethylene oxide)-*co*-(glycidyl trityl thioether)] copolymers (sample numbers **11**–**15**) resulting from the reaction of poly[(ethylene oxide)-*co*-(glycidyl tosylate)] with different degrees of functionalization (sample numbers **6**–**10**) are shown in Table 3. The polymers were brittle solids (polymer samples **12**–**15**) or highly viscous liquids (polymer sample **11**). They were insoluble in water and methanol but soluble in THF, DCM and chloroform.

Again the success of the reaction was assessed with ^{1}H NMR spectroscopy (Figure 1). The tosylate group derived signals disappeared completely and a new set of signals of the triphenylmethyl protecting group at 7.20 and 7.38 ppm was observed. The degree of functionalization $p\%_{STrt}$ of polymer repeating units with triphenylmethyl thioether groups was calculated in analogy to equation (1):

$$p\%_{STrt} = \frac{4 \cdot Int(STrt)}{17 \cdot Int(BB') + Int(STrt)} \qquad (2)$$

Here, Int(STrt) is the sum of the triphenylmethyl thioether related signals intensities (signals a", c" and d" in Figure 1) and Int(BB)' is the backbone related signal intensity (signal b" in Figure 1). The resulting values for $p\%_{STrt}$ were practically identical with the corresponding values for $p\%_{Ts}$, showing a complete conversion of the polymer bound tosylate groups in spite of the large steric demand of the triphenylmethyl group.

Like for the previous step, the introduction of the protected thiol group was possible without altering the shape of the MWD (Figure 2). Also the M_w/M_n values were comparable proving the stability of the polymer backbone under the reaction conditions. Furthermore the T_g values increased with increasing content of the trityl protected thiol-functionalized repeating unit in accordance with the theoretical prediction of the Fox equation. All analytical data show that for all degrees

Table 4 Poly[(ethylene oxide)-*co*-glycidylthiol] copolymer characteristics of different compositions ([a])Degree of thiol functionalization ($p\%_{SH,NMR}$) of polymer repeating units determined by ^{1}H NMR. [b)] Degree of thiol functionalization ($p\%_{SH,DTDP}$) of polymer repeating units determined by photometric assay using DTDP. For other symbol explanations, see Table 1).

	M_n/ g mol^{-1}	M_w/M_n	$p\%_{SH,NMR}$/ % [a)]	$p\%_{SH,DTDP}$/ % [b)]	yield/ %
16	2140	1.31	20.5	20.9	72
17	2420	1.63	42.1	41.6	90
18	3340	1.52	55.1	56.4	86
19	3920	1.55	66.3	64.2	88
20	3450	1.43	87.0	87.3	82

of functionalization, the substitution of the tosylate groups by triphenylmethanethiol was successful. The characterization data of polymer samples **11**–**15** remained unaltered during storage at room temperature for at least six months. Thus, the polymers with protected thiol groups can be prepared in larger quantities and can be deprotected directly before free thiols are needed.

Deprotection of Thiol Groups in the Side Chains

For the cleavage of the triphenylmethyl protecting group, a modified literature protocol was applied.[50] The characterization data for the resulting copolymer samples **16**–**20** are given in Table 4. The quantitative deprotection of the thiol groups was confirmed by ^{1}H NMR analysis by the disappearance of the triphenylmethyl related signals. The signal intensitiy of the newly formed thiol proton at a chemical shift of 1.61 ppm was used to calculate the degree of functionalization of the copolymers. The resulting values are practically identical with the data obtained for the precursors (polymer samples **11**–**15**). Additionally, a photometric assay for thiol quantification using 4,4'-dithiodipyridine (DTDP) confirmed the ^{1}H NMR data.[33] Polymer samples **16**–**20** were viscous, colorless polymers which were soluble in THF, DMF, DCM, chloroform and acetone. The T_g values of the thiol-functionalized polymers were not measured because the polymers aged rapidly in contact with air which could not be completely excluded during sample preparation for DSC measurements.

Since acidic treatment of PEG-based polymers may lead to backbone degradation[15] and disulfide formation of thiols may lead to crosslinking of the polymers, the shape of the MWDs have to be closely watched after the deprotection. If the polymers are handled under strictly oxygen free conditions, the shape of the MWD is similar to the precursors whereas the M_w/M_n value slightly decreases, probably due to loss of the low molecular weight fraction during reprecipitation. The polymers can also be stored for at least two months without alteration of the MWDs. If oxygen traces are present during the reaction or storage, insoluble polymer networks are formed within a few hours. Therefore, the strict exclusion of oxygen is crucial for the successful preparation of polymer samples **16**–**20** for the applications described below.

Cytocompatibility of Hydrogels of Polymer Sample 20

The characterization data collected for polymer samples **16**–**20** indicate that well-defined products were obtained. The reactivity of the polymer-bound thiol groups was further exploited by gel formation using PEG-DA as a crosslinker, DMF as solvent and NEt_3 as a catalyst. At a total polymer concentration in the mixture of the two gel precursor solutions of 20% (w/v), polymer samples **16**–**20** successfully formed gels at room temperature between 250 (sample **20**) and 290 s (sample **16**). In order to test the response of human fibroblasts to surfaces containing the thiol functionalized polymers, gels prepared from polymer sample **20** were seeded with human fibroblasts. Sample **20** was selected because it has the highest thiol content and the effect of the thiol-functionalization should be well pronounced.

For the cell experiments, the gels were washed excessively with different solvents in order to remove the DMF entirely. The hydrogel surfaces were then seeded with human fibroblasts and they were left to adhere and proliferate. After 3 and 48 h culture time, the cells were stained with FDA and PI and assessed qualitatively by fluorescence microscopy compared to cells seeded onto a photopolymerized PEG-DA gel (control without poly(glycidylthiol) **20**) and a standard tissue culture dish. The results are shown in Figure 3.

On the PEG-DA gel surfaces, only very few cells were observed at both observation times. This observation is in accordance with earlier investigations which showed that PEG surfaces exhibit protein repellent behavior leading to the failure of cell attachment.[8] In contrast, the hydrogels containing polymer sample **20** did not show this behavior. After 3 h culture time, well-adhered fibroblasts were observed on the hydrogel surfaces. After 48 h, the living cells covered the entire hydrogel surfaces. Additionally, the hydrogel surfaces enabled the cells to proliferate and form a confluent cell layer. The cell proliferation and morphology were comparable to the results obtained with the standard cell culture dishes. This indicates that the gels were not cytotoxic in contact with the fibroblasts.

The reason for the different behavior of the cells on hydrogels with or without polymer sample **20** cannot be determined by these experiments because the polymer properties of sample **20** like hydrophilicity and functionality were significantly different from pure PEG. Therefore, it cannot be clearly stated if the thiol-functionalization actively supported the cell attachment or if the hydrogel surfaces supported the adhesion due to a change of their hydration status[52,53] or the loss of their pro-

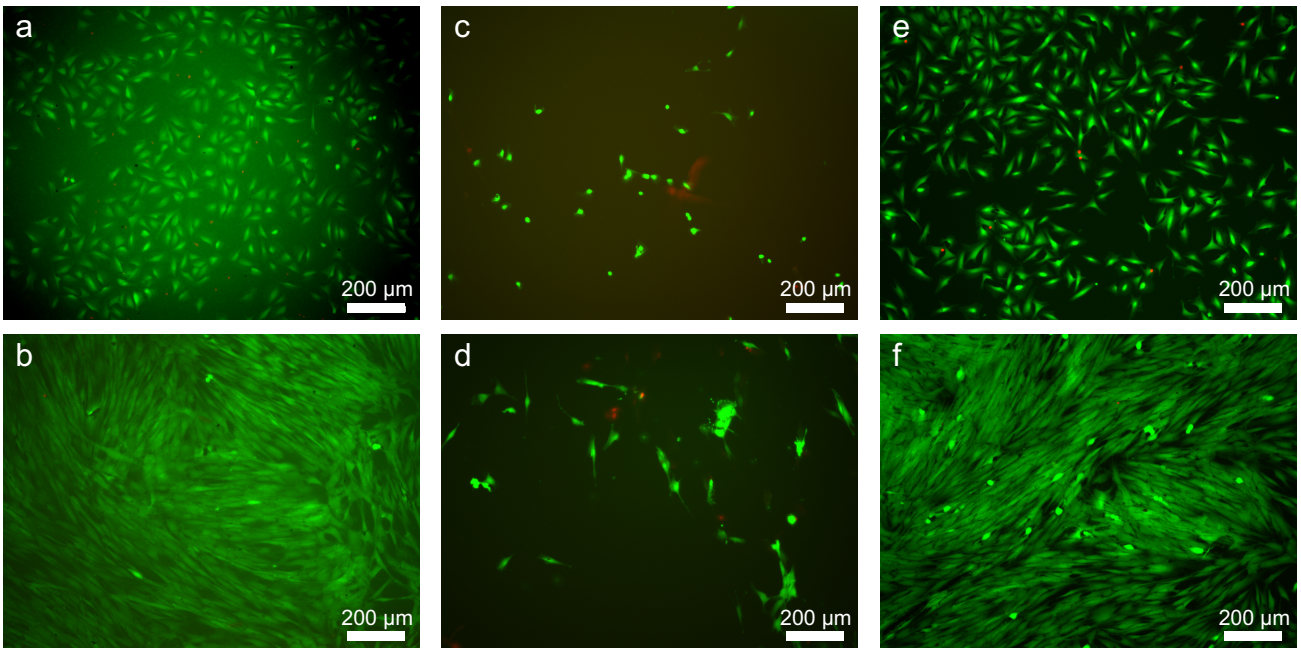


Fig. 3 Fluorescence microscopic images of human fibroblasts seeded onto different hydrogels after FDA/PI staining for assessment of the cell viability. Images a,c and e were taken 3 h after cell seeding, images b, d and f 48 h after cell seeding. (a,b) On the gels formed by thiol-functionalized polymer sample **20** and PEG-DA the cells adhered and showed their cell-type specific morphology after proliferation. (c,d) In contrast, on pure, photo-crosslinked PEG-DA gels, very little cell attachment could be observed. (e,f) In the standard tissue culture dish, similar cell morphology and attachment to the surface were observed like in images a and b.

tein repellent behavior. Thus, a protein layer from the cell culture medium may have been deposited onto the gel surface in a first step which then enabled the cells to adhere.[54]

Both the quantification of the fibroblast response as well as the characterization of protein adsorption on hydrogel surfaces depending on the degree of thiol-functionalization of the gels are important for a full understanding of the material properties. These questions are a matter of ongoing experiments in our laboratory. However, the results presented here allow the hypothesis that the synthetic route yielding polymer samples **16**–**20** led to polymeric materials which are ready for potential applications in cell culture and tissue engineering.

Inkjet Printing of Polymer sample 20 with PEG-DA

The thiol-Michael reaction has been widely used for hydrogel formation by mixing the solutions of a thiol component and an acrylate component.[55] It was also described for the modification of thiol[56,57] or acrylate[58,59] modified surfaces. However, no report is given so far which combines these two approaches with inkjet printing which additionally offers spatial control for the generation of three-dimensional structures. So far, inkjet printing for the formation of chemically crosslinked hydrogels was mainly used for the printing of hydrogel precursor solutions which could be cured photochemically.[60,61]

For the proof of principle inkjet printing experiments, we used the hydrogel formulation described above for the cell experiments. In order to prevent the gel precursor solution to solidify inside the printing head, the two components (solutions of polymer sample **20** and PEG-DA) were printed one after another, resulting in printed patterns composed of double layers of the two components. As a pattern which demonstrates the spatial control of the inkjet printing technique for hydrogel formation, an image of the chemical structure of polymer sample **20** was printed onto an acrylate-functionalized glass slide (Figure 4 a). In this example, four double layers were printed on top of each other.

For successful crosslinking the two components have to mix with each other. The formation of a crosslinked polymer network was assessed by washing the printed pattern with DMF for 1 h. The gel structure on the glass surface did not detach or dissolve, showing that both the crosslinking and the surface binding were successful (Figure 4 b). For optimization and closer characterization of the printing process, the ink formulation or the drop spacing may be changed and the mixing of the two components should be evaluated together with the mechanical properties of the gels. Here, we showed the general feasibility of the formation of chemically crosslinked polymer gels by conjugate addition by inkjet printing.

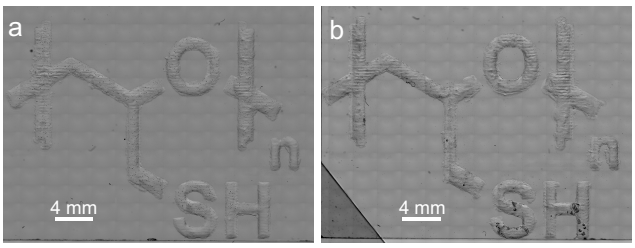


Fig. 4 Microscopic images of printed gels onto acrylate-functionalized glass slides. The printed pattern was generated by alternate deposition of solutions of the thiol-functionalized polymer **20** and of PEG-DA in eight consecutive layers. (a) Image directly after printing, (b) image after curing and washing in DMF.

Conclusions

We successfully developed a synthetic route yielding mercaptomethyl side chain functionalized PEG-based polymers starting from hydroxymethyl side chain functionalized PEG-based polymers in three reaction steps. The degree of functionalization was controlled successfully by the starting material. Well defined products were obtained and their potential applications for tissue engineering and inkjet printing were demonstrated. As a next step, the technique of inkjet printing may be used to create spatially defined patterns useful for cell culture. The platform defined by the poly[(ethylene oxide)-*co*-(glycidyl tosylate)] copolymers can be used as a starting point for versatile developments in side chain functionalized PEG-based polymers in the future.

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Table of Contents Entry

Novel thiol side chain functionalized PEG-based polymers were synthesized by post polymerization reactions of hydroxyl functionalized polymers. Applications of the novel polymers in cell culture and inkjet printing were demonstrated.