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Evaluating Chemical Ligation Techniques for the Synthesis of Block Copolypeptides, Polypeptoids and Block Copolypept(o)ides: A Comparative Study

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In this work, we describe the synthesis of block copolypeptides, polypeptoids and block copolypept(o)ides by chemical ligation techniques. Polysarcosine (PSar), poly(N-e-Trifluoroacetyl-L-Lysine) (PLys(TFA)) and poly(γ-Benzyl-L-Glutamate) (PGlu(OBzl)) homopolymers of different polarities and end group functionalities but with similar average degree of polymerization ($X_n=50$ and 100) could be obtained by ring opening polymerization (ROP) of α-amino acid N-carboxyanhydrides (NCA) and postpolymerization modification reactions. In the next step, these polymers were applied to copper(I)Acatalyzed azide-alkyne coupling (CuAAC), strain-promoted azide-alkyne coupling (SPAAC) and native chemical ligation (NCL). Our results suggest that all employed ligation techniques can be employed for the synthesis of block copolypeptides, polypeptoids and block copolypept(o)ides. SPAAC displayed for most conditions the highest ligation efficiencies (up to 86%) and from a practical point of view, was the most feasible method. NCL, however, performed very well for short hydrophilic polymers (up to 88%) and is favourable for the ligation of peptides from solid phase peptide synthesis (SPPS) to polysarcosine. As a proof of principle, we report a protocol for an efficient NCL coupling of polysarcosine to T-cell receptor core peptide (TCR CP), which is known to inhibit IL-2 production in antigen-stimulated T cells and, therefore, to suppress inflammation. In a comparative study, the influence of the ligation method, which directly relates to the chemical nature of the ligation site, did neither influence cytotoxicity nor complement activation. To conclude, chemical ligation techniques represent a complementary synthetic approach to the well-established sequential ring opening polymerization of NCAs.

Introduction

Synthetic polypeptides still see a steady increase of interest as synthetic material for an application in nanomedicine. The polypeptidic copolymer Copaxone® was among the 20 best selling drugs in 2013. Although the polymerization of α-amino acid N-carboxyanhydrides (NCA) has already been reported in the beginning of the last century by Hermann Leuchs, it took until the late 90’s for first reports on controlled polymerization to be published. While Deming employed metal complexes for the initiation of the polymerization, Cheng and coworkers used N-trimethylsilyl amines; Vayaboury et al. reported the use of primary amines as initiators at low temperatures, while Vicent and coworkers control the amine activity by ammonium salts with non-nucleophilic counterions. In addition, Peng et al. have employed rare earth borohydrides as initiators for the ROP of NCAs yielding α-hydroxy-ω-aminotelechelic polypeptides. All these aforementioned methods yield well-defined polypeptides.

These polymers - unlike most other biocompatible synthetic polymers - bear the potential to combine hydrolytic stability with in vivo degradability. In the case of enzymatic degradation, polypeptides can decompose into α-amino acids, which may be further metabolized in the organism, avoiding design limitation due to renal excretion or storage diseases. For these reasons, polypeptides have become intriguing candidates as materials in drug delivery applications. Although polymer-protein conjugates have already entered clinical practice, polymeric micelles, polyplexes and nanohydrogels are expected to shape the future of nanomedicines. These systems combine a functional core with a stealth-like corona and are formed by electro-static/entropic or polarity driven self-assembly of block copolymers. While in the first case, the cargo (siRNA, mRNA or pDNA) shapes the properties of the system, size and shape of the second are mainly determined by the hydrophobic–hydrophilic ratio and chemical nature of the individual polymer blocks within the amphiphilic block copolymer.
Recently, Birke et al. and Heller et al. have introduced polypept(o)ides as a class of functional block copolymers based on the polypeptoid polysarcosine (PSar) and functional polypeptides.\textsuperscript{12–15} PSar - poly(N-methyl glycine) - seems to be a particularly interesting alternative to polyethylene glycol (PEG) since it shows very similar physicochemical properties. It is soluble in aqueous solution and in organic solvents, e.g., DMSO or chloroform, and is exclusively a weak hydrogen bond acceptor which prevents the formation of other secondary structures than random coil.\textsuperscript{16,17} In addition, similar protein resistance compared to PEG has been reported.\textsuperscript{16,18} Kimura and coworkers as well as our research group have investigated PSar as hydrophilic block in amphiphilic block copolymers as alternative to PEG.\textsuperscript{19,20} Block copolypept(o)ides are readily synthesized by the sequential polymerization of NCAs, where the first block acts as macroinitiator for the second block. However, chemical ligation techniques are thought to present an important alternative pathway to block copolypept(o)ides whenever a functional block is required, which cannot be derived by NCA polymerization. This can be the case if the second block needs to possess a specific sequence and is, thus, synthesized by solid phase peptide synthesis (SPPS). Also, the second block might not tolerate deprotection conditions that are mandatory for the other. The class of chemical ligation reactions mostly relies on the selective reaction between two (bio)orthogonal groups, which are ideally absent from peptides and proteins (or biomolecules in general). Amongst these are azide, alkyne, tetrazine and many others that have been widely used for different in vitro and in vivo applications. They range from the labeling of biomolecules with fluorescent probes, affinity tags and isotope labels to the synthesis of glycopeptides and biohybrids such as polymer-protein conjugates, cell-surface labeling and the immobilization of proteins.\textsuperscript{21–24}

Efficient ligation methods are characterized by a very high selectivity for product formation, easy purification and mild reaction conditions (room temperature and physiological pH) which is essential for many proteins in order to maintain their activity.\textsuperscript{22,25} Probably one of the best known chemical ligation techniques is the classical "click" reaction: Cu\textsuperscript{-}catalyzed cycloaddition of azides and terminal alkynes (CuAAC) developed by Sharpless and separately by Meldal and co-workers.\textsuperscript{26,27} In contrast to Huisgen's 1,3-dipolar cycloaddition,\textsuperscript{28,29} only the 1,4-substituted stereoisomer is obtained under much milder reaction conditions. CuAAC can be conducted in different solvents, including water and buffered saline at physiological pH. To avoid the use of copper(I) as catalyst, especially with regard to in vivo applications, the strain-promoted alkyne-azide cycloaddition (SPAAC) has been developed by Bertozzi et al.\textsuperscript{30} The initial idea dates back to discoveries of Wittig and Krebs who reported that cyclooctynes react with azides to form triazoles.\textsuperscript{30,31} For non-substituted cyclooctynes, the reaction rates are relatively slow (0.0012 M\textsuperscript{-1} s\textsuperscript{-1}), but the attachment of electron-withdrawing groups or additional ring-strain has been shown to significantly improve reaction rates.\textsuperscript{32} Azide-alkyne coupling reactions have been widely used in very different fields, including chemical biology, drug discovery, material science and polymer science.\textsuperscript{33–35} There are many examples where click chemistry has been successfully employed in the synthesis of nanocarriers for drug delivery.\textsuperscript{36,37} Native chemical ligation (NCL) is another interesting ligation technique since it results in an amide bond at the ligation site instead of an electron-poor aromatic system. The method was developed by Dawson and Kent in the early 90's.\textsuperscript{38,39} Today, it is one of the most effective practical methods for the total synthesis of proteins by using fully unprotected peptide fragments. This is an important aspect when considering the fact that coupling of protected peptide fragments is often
NCL can be performed in polar aprotic organic solvents like DMF with addition of base without racemization, which is potentially very useful for the ligation of hydrophobic peptide segments.\textsuperscript{41,42} While a lot of research has been devoted to all different kinds of ligation strategies, a systematic comparison of such methodologies for the synthesis of block copolymers is missing. Here, we report a comparative study of chemical ligation techniques, namely CuAAC, SPAAC and NCL, for the synthesis of block copolyketides, polypeptides and block copoly(ne)pentides. The individual polymers used for the individual ligations differ in chain length, polarity and chemical nature of end groups. We report optimized conditions for the individual reactions and provide coupling efficiencies. Furthermore, we describe the synthesis and purification of a polysarcosine-peptide conjugate derived by the described methodology, which is of amphiphilic nature. The employed T-cell receptor core peptide (TCR CP) is known to inhibit IL-2 production in antigen-stimulated T cells.\textsuperscript{43}

**Experimental Section**

**Materials and Methods**

Solvents were purchased from Sigma Aldrich. THF and n-hexane were dried over Na/K prior to use. DMF was purchased from Acros (99.8 %, Extra), dried over BaO and molecular sieves (3/4Å) and fractionally distilled in vacuo. Freshly distilled DMF was stored at -80 °C under exclusion of light. Before use, DMF was degassed under vacuum for at least 20 minutes to remove residual dimethylamine. Acetonitrile was dried over K₂CO₃ for 24h, over molecular sieve (3 Å) for another 24h and then distilled. Diphosgene was purchased from Alfa Aesar and used without further purification. Nevopentylamine (TCI Europe), propargylamine (Sigma Aldrich) and triethylamine (TEA) were dried over NaOH before use and distilled. 4-Mercaptopyrrolidinacetic acid (MPAA) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl) were purchased from Sigma Aldrich and used as received. 3-chloropropionylamino hydrochloride and sodium azide were purchased from Acros. Benzyl mercaptan was purchased from Alfa Aesar. Protected amino acids were bought from ORPEGEN. Dibenzylocyclooctyne-amine (DBCO-amine) was bought from Sigma Aldrich and used as purchased. TCR CP (CGLRILLKLV) was custom made by Peptide Specialty Laboratories GmbH. Millipore water was prepared using a MILLI-Q® Reference A+ System. Water was used at a resistivity of 18.2 MΩ·cm and total organic carbon of < 5 ppm. \textsuperscript{1}H and \textsuperscript{13}C NMR spectra were recorded on a Bruker AC 300 or AV 400 at room temperature. Spectra were calibrated using the solvent signals (\( \delta = 2.500 \) ppm for DMSO-d₆, \( \delta = 7.260 \) ppm for \( \text{CDCl}_3 \)) and total organic carbon of < 5 ppm. \textsuperscript{1}H and \textsuperscript{13}C NMR spectra were recorded on a Bruker AC 300 or AV 400 at room temperature. Spectra were calibrated using the solvent signals (\( \delta = 2.500 \) ppm for DMSO-d₆, \( \delta = 7.260 \) ppm for \( \text{CDCl}_3 \)). Degrees of polymerization were calculated by comparing the average of integrals of the benzylic methylene protons and benzylic protons of the protecting group (for PGLu(OBzl)), with the integrals of the ε-protons (for PLCys(TFA)), or with the average of integrals of methyl group and methylene protons (for PSar) in the 400MHz \textsuperscript{1}H NMR spectra.

Experimental procedures

3-AZIDO-L-AMINOPROPANE was synthesized according to literature.\textsuperscript{44} 3-chloropropionylamino hydrochloride (5.04 g, 0.039 mmol, 1 eq) and sodium azide (7.57 g, 0.116 mmol, 3 eq) were dissolved in 40 ml H₂O and heated to 80°C for 2h. The reaction mixture was concentrated in vacuo. 50 ml of diethyl ether were added following by addition of 3.5 g KOH in small portions with continuous stirring and cooling in an ice bath to keep the temperature below 10°C. The aqueous layer was extracted five times with 20 mL diethyl ether. The organic phases were combined and dried over sodium sulphate. The ether was evaporated in vacuo and the crude product (yellow oil) was purified by fractionation distillation and stored at -20°C (1.60 g, 0.016 mmol, 41%). \textsuperscript{1}H NMR (400 MHz, \( \text{CDCl}_3 \)) \( \delta \) [ppm] = 3.36 (t, \( J_{H,H} = 6.70 \) Hz, 2H, \( CH_2-N_3 \)), 2.79 (t, \( J_{H,H} = 6.80 \) Hz, 2H, \( CH_2-N_3 \)), 1.71 (p, \( J_{H,H} = 6.76 \) Hz, 2H, \( CH_2-CH_2-CH_3 \)), 2.21 (s, 2H, \( CH_2-N_3 \)). \textsuperscript{13}C NMR (400 MHz, \( \text{CDCl}_3 \)) \( \delta \) [ppm] = 49.13 (\( CH_2-N_3 \)), 39.31 (\( CH_2-N_3 \)), 32.43 (CH₂-CH₂-CH₂). IR azide ν [cm⁻¹] = 2088.5.

**Sarcosine-N-carboxyanhydride**

The synthetic of sarcosine NCA was adapted from literature and modified.\textsuperscript{45} Sarcosine (15.16 g, 170.2 mmol, 1 eq) was weighed into a pre-dried three-necked flask and dried under vacuum for 1 hour. 300 ml absolute (abs) THF was added under a steady flow of nitrogen. The apparatus was connected to two gas washing bottles filled with aqueous sodium hydroxide solution. Diphosgene (16.26 ml, 134 mmol, 0.8 eq) was added slowly via syringe. The colorless suspension was heated to 70°C yielding a clear solution after 3 hours of stirring. The solvent was evaporated under reduced pressure yielding a brown oil as crude reaction product. The oil was heated to 50°C and dried under reduced pressure to obtain an amorphous solid. The crude reaction product was redissolved in 60 ml THF and precipitated with 300 ml abs n-hexane. The precipitate was filtered off under N₂-atmosphere and dried with a stream of dry nitrogen for 60 - 90 minutes to remove residual traces of solvents. The next day, the product was dried in high vacuum for 2 hours in the sublimation apparatus and subsequently sublimated at 80 – 85 °C and < 1x10⁻² mbar. The product was collected from the sublimation apparatus in a glove box on the same day. Colorless crystals were obtained (50 – 67 %), mp = 6.80 Hz, 2H, \( CH_2-CH_2-CH_3 \)).
104.3 °C; 1H NMR (300 MHz, CDCl₃) δ [ppm] = 2.86 (s, 3H, NH-CH₂), 4.22 (s, 2H, NH-CH₂-CO).

N-γ-Trifluoroacetyl-L-lysine N-Carboxyanhydride
12.5 g (51.6 mmol) of N-γ-trifluoroacetyl-protected lysine were weighed into a pre-dried three-necked flask and dried under vacuum for 1 hour. The solid was suspended in 300 mL of absolute THF under a steady flow of dry nitrogen. The apparatus was connected to two gas washing bottles filled with aqueous sodium hydroxide solution. Diphosgene (5.50 mL, 45.6 mmol) was added slowly via syringe and the nitrogen stream was reduced. The colorless suspension was mildly refluxed for 2 hours, yielding a cloudy solution. Overnight, a steady flow of dry nitrogen was led through the solution removing excess phosgene and HCl. The solvent was evaporated under reduced pressure. The crude reaction product was dissolved in absolute THF and precipitated with an excess of dry n-hexane. The solid was collected by filtration under dry nitrogen atmosphere and washed with n-hexane. The crude product was recrystallized twice with absolute THF/n-hexane. 10.43 g of purified product (39.0 mmol; 75% yield; colorless needles, were obtained and stored in a Schlenk-tube at -80°C. mp = 101°C; 1H-NMR (300 MHz, DMSO-d₆) δ [ppm] = 1.42-1.24 (2H, m, -CH₂-CH₂-CH₂-CH₂-CH₂-CH₂-NH₂), 1.53-1.46 (2H, quin, 3JHH = 7.13 Hz, -CH₂-CH₂-CH₂-CH₂-CH₂-NH₂), 1.79-1.61 (2H, m, -CH₂-CH₂-CH₂-CH₂-CH₂-NH₂), 3.17 (2H, q, 3JHH = 6.3 Hz, -CH₂-NH₂), 4.43 (1H, t, 3JHH = 6.3 Hz, -CO-CH₂-), 9.09 (1H, s, -NH-CO-O-CO), 9.40 (1H, s, -NH-CO-CF₃).

γ-Benzyl-L-glutamate-N-carboxyanhydride
The synthesis of γ-benzyl-l-glutamate NCA was adapted from literature and modified. 9.75 g (41.1 mmol, 1 eq) H-Glu(OBzl)-OH were weighed into a three-necked round-bottomed flask equipped with a magnetic stir bar, a condenser, a rubber septum and a glass stopper and three-necked round-bottomed flask equipped with a magnetic stirrer, a condenser, a rubber septum and a glass stopper and a glass stopper. After 30 minutes, 100 mL abs. tetrahydrofuran (THF) was added to the solid, resulting in a colorless suspension. The suspension was heated to 70°C, diphosgene (4 mL, 32.9 mmol, 0.8 eq) was added to the mixture stirred for 1.5 h. The suspension turned into a clear, yellow solution. After the reaction mixture had cooled down, a steady flow of dry nitrogen was led through the solution removing excess of HCl and diphosgene. The next day, the resulting orange oil was evacuated to dryness. The product was weighed into a pre-dried three-necked flask and dried under vacuum. After 30 minutes, 100 mL abs. diethyl ether and centrifuged (4500 rpm at 4 °C for 15 min). After discarding the liquid fraction, new ether was added and the polymer was resuspended using sonication. The suspension was centrifuged again and the procedure was repeated. The polymer was then dissolved in H₂O and lyophilized to obtain a fluffy powder (277.7 mg, 96%). For different chain lengths, yields ranged from 87% to 96%. 1H-NMR: (400 MHz, DMSO-d₆) δ [ppm] = 0.88 – 0.79 (m, ini, -CH₂-CH₂-CH₂-NH₂), 2.58 – 3.11 (br, 3N₃, N-CH₂), 3.73 – 4.57 (br, 2N₃, -CO-CH₂-N₃).

Poly(γ-Benzyl-L-glutamate)
Polymerizations and work up were carried out as described above. For different chain lengths, yields ranged from 77% to 89%. 1H-NMR (400 MHz, DMSO-d₆) δ [ppm] = 0.82 (br, 9H (CH₃)), 1.56 – 2.44 (br, 4N₃, β,γ-CH₂), 3.71 – 4.44 (br, 1N₃, α-CH), 4.73 – 5.25 (br, 2N₃, -OCH₂Ph), 6.99 – 7.57 (br, 5N₃, -Ph), 7.65 – 8.71 (br, 1N₃, -NICO).

The polymerization using propargylamine, 3-azido-1-aminopropane and DBCO-amino as initiators was carried out in the same way. Yields were 88 and 77% (3-azido-1-aminopropane) and 93% (propargylamine) and 99 and 100% (DBCO-amine). Initiator signals used for integration: 3-azido-1-aminopropane 1H-NMR (400 MHz, DMSO-d₆) δ [ppm] = 7.86 – 7.10 (m, 8H, benzylic protons); initiator signals from propargylamine were not visible due to overlap with polymer backbone signals.

Poly(N-γ-Trifluoroacetyl-L-lysine)
Polymerizations were carried out as described above. After completion of the reaction had been confirmed by IR spectroscopy (disappearance of the NCA peaks (1853 and 1786 cm⁻¹)). Directly after completion of the reaction, the polymer was precipitated in cold diethyl ether and centrifuged (4500 rpm at 4 °C for 15 min). After discarding the liquid fraction, new ether was added and the polymer was resuspended using sonication. The suspension was centrifuged again and the procedure was repeated. The polymer was then dissolved in H₂O and lyophilized to obtain a fluffy powder (277.7 mg, 96%). For different chain lengths, yields ranged from 87% to 96%. 1H-NMR: (400 MHz, DMSO-d₆) δ [ppm] = 0.88 – 0.79 (m, ini, -CH₂-CH₂-CH₂-NH₂), 2.58 – 3.11 (br, 3N₃, N-CH₂), 3.73 – 4.57 (br, 2N₃, -CO-CH₂-N₃).

The polymerization using 3-azido-1-aminopropane, propargylamine and DBCO-amino were carried out in the same way. Yields were 91 and 96% (3-azido-1-aminopropane), 88 and 91% (propargylamine) and 99 and 100% (DBCO-amine). Initiator signals used for integration: 3-azido-1-aminopropane 1H-NMR (400 MHz, DMSO-d₆) δ [ppm] = 1.71 – 1.61 (m, 2H, -CH₂-CH₂-N₃). DBCO-amine 1H-NMR (400 MHz, DMSO-d₆) δ [ppm] = 7.86 – 7.10 (m, 8H, benzylic protons); initiator signals from propargylamine were not visible due to overlap with polymer backbone signals.

Polysarcosine
In a typical experiment, 461.8 mg of SarNCA (4.012 mmol) were transferred under nitrogen counter flow into a pre-dried Schlenk-tube, equipped with a stir bar and again dried in vacuum for 30 minutes. The NCA was then dissolved in 3.5 mL of dry DMF. A stock solution of 9.38 µl (7.00 mg, 0.080 mmol, 1/100 eq, M/I = 100) of neopentylamine in 2 mL DMF was prepared and 1 mL of this stock solution were added to the monomer solution via syringe. The solution was stirred at 40°C and kept at a constant pressure of 1.25 bar of dry nitrogen via the Schlenk-line to prevent impurities from entering the reaction vessel while allowing CO₂ to escape. Completion of the reaction was confirmed by IR spectroscopy (disappearance of the NCA peaks (1853 and 1786 cm⁻¹)). After precipitation the reaction mixture had cooled down, a steady flow of dry nitrogen was led through the solution removing excess of HCl and diphosgene. The next day, the resulting orange oil was evacuated to dryness. The product was weighed into a pre-dried three-necked flask and dried under vacuum. After 30 minutes, 100 mL abs. diethyl ether and centrifuged (4500 rpm at 4 °C for 15 min). After discarding the liquid fraction, new ether was added and the polymer was resuspended using sonication. The suspension was centrifuged again and the procedure was repeated. The polymer was then dissolved in H₂O and lyophilized to obtain a fluffy powder (277.7 mg, 96%). For different chain lengths, yields ranged from 87% to 96%. 1H-NMR: (400 MHz, DMSO-d₆) δ [ppm] = 0.88 – 0.79 (m, ini, -CH₂-CH₂-CH₂-NH₂), 2.58 – 3.11 (br, 3N₃, N-CH₂), 3.73 – 4.57 (br, 2N₃, -CO-CH₂-N₃).
S-BENZYL THIOSUCCINIC ACID

The synthesis was adapted from the literature.47 In a round bottomed flask, equipped with a rubber septum and a magnetic stir bar, succinic anhydride (2.26 g, 22.6 mmol, 1 eq) and 4-(dimethylamino)-pyridine (138 mg, 1.15 mmol, 0.05 eq) were dissolved in 25 mL dry acetonitrile/pyridine 9:1 (v/v) and stirred at room temperature for 10 min under a nitrogen atmosphere. Benzyl mercaptan (2.95 mL, 3.11 g, 25.1 mmol) was added and the reaction mixture was stirred for 4 days at room temperature. For purification, the solvent was evaporated in vacuo and the residue was dissolved in 30 mL of aqueous sodium hydrogen carbonate solution (pH 8.2). The aqueous phase was extracted three times with 20 mL of diethyl ether. The aqueous phase was then acidified with 5 M HCl and colorless crystals precipitated from the solution, which were filtered off and subsequently washed with cold 0.1 M HCl and with ice-cold water. The product was dried in a vacuum desiccator. The reaction flask was rinsed with aqueous NaHCO₃ solution and the above steps were repeated. Overall yield of colorless crystals: 2.63 g (11.7 mmol, 52 %). FD-MS = 224.2 g/mol. ¹H NMR (400 MHz, CDCl₃): δ [ppm] = 7.24 (t, J=7.8 Hz, 2H, -CH₃), 8.34 (s, 1H, AOCO), 11.1 (s, 1H, ACOO) ppm. The aqueous solution was evaporated in vacuo and the residue was dissolved in 30 mL of dry DMF while cooling the flask with an ice bath. 2 73.1 (paraAC), 128.8 (orthoAC), 128.9 (metaAC), 137.4 (A benzyl), 1.36 (s, 9nH, AC(2)), 2.74 (t, J=6.8 Hz, 2H, -CH₂CH₂CO₂), 7.57 – 6.95 (m, 5H, -benzyl), 11.1 (s, 1H, -COOH). ¹³C NMR (100 MHz, CDCl₃): δ [ppm] = 29.1 (-CH₂CO₂), 33.4 (-SCH₂), 37.9 (-CH₂CH₂CO₂), 127.5 (para-C), 128.8 (ortho-C), 128.9 (meta-C), 137.4 (-CH₂CO₂), 178.32 (ypsoAC), 197.1 (-COOH).

END GROUP FUNCTIONALIZATION

All end group modifications with S-benzyl thiosuccinic acid and Boc-L-Cys(Trt) were carried out in the same way. End group modification of Plys(TFA) was conducted in situ without prior isolation of the homopolymer. For lyophilization, it was preceded as for homopolymer synthesis. 183.0 mg (0.073 mmol, 1 eq) PSar were weighed into a flame-dried Schlenk-flask and dried under vacuum for 30 minutes. The polymer was then dissolved in 2 mL of dry DMF. In another Schlenk-flask, 163.9 mg (0.731 mmol, 10 eq) S-benzyl-thiosuccinic acid, together with 305.4 mg (0.805 mmol, 11 eq) N,N,N’-tetramethyl-1-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) and 108.4 mg (0.802 mmol, 11 eq) hydroxybenzotriazole (HOBt) were dissolved in 2 mL of dry DMF while cooling the flask with an ice bath. 273.1 µL (207.6 mg, 1.606 mmol, 22 eq) DPEA were directly added and the mixture was stirred for 30 minutes with cooling. The resulting activated ester was subsequently added to the polymer solution and stirred at room temperature for 65 h.

For purification, the reaction mixture was precipitated in diethyl ether and centrifuged (4500 rpm at 4 °C for 15 min). After discarding the liquid fraction, the new diethyl ether was added and the polymer was resuspended using sonication. The suspension was centrifuged and the procedure was repeated. Products were freeze-dried according to homopolymer synthesis. Deprotection of trityl and boc protecting groups was judged to be complete when the corresponding chemical shifts had disappeared in ¹H NMR.

CUAAC, HYDROPHILIC CONDITIONS

Polymers were weighed into a Schlenk tube (1 eq of each), equipped with a magnetic stir bar and dissolved in 2 mL H₂O. A stock solution of ascorbic acid in water was prepared and 50 mol% were added to the mixture (0.5 mL). The solvent was degassed by freeze-pump-thaw cycles. 15 µL of a CuSO₄·5H₂O solution was added (5 mol%). After a third freeze-pump-thaw cycle, the reaction mixture was heated to 40 °C for 3 days, followed by dialysis against H₂O to remove residual copper and lyophilized, yielding a fluffy colourless solid.

CUAAC, HYDROPHOBIC AND AMPHIPHILIC CONDITIONS

Polymers were dissolved in 2 mL of dry DMF in a Schlenk tube (1 eq of each), equipped with a magnetic stir bar. PMDETA was added via a pipette with N₂ counterflow. The mixture was degassed by three freeze-pump-thaw cycles and transferred to a second Schlenk tube, containing Cu(II)Br (2 eq) with a syringe that had been previously purged with nitrogen. The reaction mixture was heated to 40 °C for 70 h, precipitated in H₂O/methanol 1:9. Polymers were freeze-dried from dioxane. Samples were analyzed by HIFIP GPC.

SPAAC

Ligations between hydrophilic polypeptides were conducted in H₂O. Ligations between hydrophobic and hydrophilic/ampiphilic polypeptides were conducted in DMF. In a typical experiment, 1 eq of each polymer was weighed into a round-bottomed flask and dissolved in the appropriate solvent (c = 100 mg/mL). The reaction mixture was stirred for 70 h at 40 °C. Aqueous samples were dialyzed against water (MWCO = 1000 and 3500 g/mol respectively) and lyophilized. Samples in DMF were precipitated in diethyl ether, washed twice and freeze-dried from dioxane. Samples were analyzed by HIFIP GPC.

NCL, HYDROPHILIC CONDITIONS

2 mL of 10 mM PBS-buffer (pH = 7.4) were degassed for 45 minutes and MPAA and TCEP·HCl with final concentrations of 50 mM and 30 mM respectively. Polymers were transferred into a Schlenk tube (1 eq each, c = 1 mM), equipped with a stir...
bar, the degassed buffer was added, the pH was adjusted if necessary, and the reaction mixture was heated to 40 °C for 3 days. The reaction was quenched by addition of 1 M HCl until the reaction mixture reacted slightly acidic. The precipitated solid was separated from the reaction mixture by centrifugation (13,300 rpm, 15 minutes) and the liquid fraction was freeze-dried and analyzed by HFIP GPC. For cell experiments, samples were dialyzed against phosphate buffer (pH = 7.0) to remove residual MPAA.

NCL, Amphiphilic Conditions
1.4 mM of polypeptide and 50 mM of MPAA were weighed into a Schlenk tube and dried under vacuum for 30 minutes. DMF was degassed by three freeze-pump-thaw cycles and 0.5 mL were added to the solids by using an N₂-purg ed syringe. 40 mM of TEA were added also via a purged syringe and the reaction mixture was heated to 40°C for 70 h in an argon atmosphere. The reaction mixture was precipitated in diethyl ether and freeze-dried from H₂O. Samples were analyzed by HFIP GPC after treatment with TCEP overnight in MeOH and subsequent freeze-drying by addition of an excess of water.

NCL with TCR CP (CGLRILLLKV)
1 mL 4 M urea buffer (pH = 7.09) were degassed by leading N₂ through the solution for 30 minutes. 50 mM of MPAA and 20 mM TCEP-HCl were added to the buffered solution, the pH was readjusted to 7.0 with 1 N NaOH and degassed again. 9.10 mg (9.99 x 10⁻⁴ mmol, 1 eq) polysarcosine-S-benzylthiosuccinate (X₅₅ = 124, determined by ¹H NMR) and 2.30 mg (2.04 x 10⁻³ mmol, 2 eq) of TCR CP were weighed into a colorless solid and freeze-dried from H₂O. Samples were analyzed by TCEP/HCl over night in MeOH and subsequent freeze-drying by addition of an excess of water.

Determination of Human Complement Fragment 5a Concentration in Human Serum
200 µL of freshly obtained human serum were mixed with 200 µL of aqueous polymer solution to yield a final polymer concentration of 1 mg/mL, respectively. Serum-polymer solutions were incubated for 1 hour at 37 °C. Polymer-induced activation of complement system was assessed by applying the “Complement Component 5a (C5a) ELISA Kit” (antibodies-online GmbH, Aachen, Germany) according to manufacturer’s protocol. For this, samples were diluted 1:2 and 1:4 using “Reference Standard and Sample Diluent” provided by the kit and subjected to further analysis.

Results and Discussion
Polymer Synthesis and Functionalization
The aim of this work is to investigate the performance and the feasibility of the three most prominent ligation techniques – CuAAC, SPAAC and NCL – in a head-to-head comparison for the synthesis of block copolypeptides, polypeptide(ides) and block polypeptide(ides). Therefore, we have synthesized various polypeptide(ides) bearing C-terminal and N-terminal functionalities (see scheme 1).

While C-terminal azide or alkyn functionalities were introduced via primary amine initiators in the ring opening polymerization of α-amino acid N-carboxyanhydrides, N-terminal functionalities, namely cysteine and a thioester moiety, have been introduced by postpolymerization modification using peptide-coupling chemistry.

Determination of Cellular Toxicity of Polymers with MTT Assay
Cytotoxicity of polymers was investigated with MTT assay using the “CellTiter 96® Non-Radioactive Cell Proliferation Assay Kit” (Promega, Madison, USA) according to manufacturer’s protocol. HEK 293T cells were seeded onto 96-well culture plates at a concentration of 1.5 x 10⁴ cells per well. Cells were incubated for 24 hours with 100 µL of medium containing 0.1 mg/mL and 0.5 mg/mL and 1.0 mg/mL of polymers, respectively (hexaplicates). The next day, 30 µL of MTT substrate solution were added to each well and cells were cultured for another 2 hours. Subsequently, 100 µL of “Solubilization Solution/Stop Mix” were added to each well. After 1 hour, the absorbance at 570 nm was measured.

The polymers have been synthesized from the corresponding NCAs as previously reported (see scheme 2). The employed NCAs, namely SarNCA, Lys(TFA)NCA and Glu(OBzl)NCA, were synthesized according to the Fuchs-Farthing method.
Melting points (Glu(OBzl)NCA: mp = 93.7 °C, Lys(TFA): mp = 101 °C, SarNCA: mp = 104.3 °C) were in good agreement with the literature or even higher than reported which accounts for high purity. All synthesized NCAs were stored at -80°C under a nitrogen atmosphere over several months without any detectable degradation or oligomerization.

While heterotelechelic homopolymers with alkyn or azide endgroups have been synthesized directly using 3-azido-1-aminopropane (APA), propargylamine (PA) and DBCO-amine as initiators for the ROP, polypept(o)ides for NCL were further characterized by HFIP-GPC and 1H NMR. Analytical data of homopolymers is summarized in Table 1.

Table 1: Characteristics of the synthesized homopolymers applying neopentylamine (NPA), 3-azido-1-aminopropane (APA), DBCO-amine (DBC0) and propargylamine (PA) as initiators.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Initiator</th>
<th>X&lt;sub&gt;n&lt;/sub&gt;(NMR)</th>
<th>M&lt;sub&gt;GPC&lt;/sub&gt; (kg/mol)</th>
<th>Đ&lt;sub&gt;GPC&lt;/sub&gt;</th>
<th>Đ&lt;sub&gt;n&lt;/sub&gt;(NMR)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Mod.&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>PSar</td>
<td>NPA</td>
<td>58</td>
<td>16.1</td>
<td>0.06</td>
<td></td>
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<tr>
<td>P2</td>
<td>PSar</td>
<td>NPA</td>
<td>117</td>
<td>24.3</td>
<td>0.09</td>
<td></td>
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<tr>
<td>P3</td>
<td>PSar</td>
<td>NPA</td>
<td>46</td>
<td>13.3</td>
<td>1.05</td>
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<tr>
<td>P4</td>
<td>PSar</td>
<td>APA</td>
<td>44</td>
<td>11.8</td>
<td>0.05</td>
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<tr>
<td>P5</td>
<td>PSar</td>
<td>APA</td>
<td>96</td>
<td>20.6</td>
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<tr>
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<tr>
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<td>n.d.</td>
<td>25.0</td>
<td>1.10</td>
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<tr>
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<td>DBCO</td>
<td>55</td>
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<tr>
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<td>PSar</td>
<td>DBCO</td>
<td>119</td>
<td>24.6</td>
<td>1.06</td>
<td></td>
</tr>
<tr>
<td>P10</td>
<td>PGlu(OBzl)</td>
<td>APA</td>
<td>64</td>
<td>14.8</td>
<td>1.05</td>
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<td>P11</td>
<td>PGlu(OBzl)</td>
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<td>P12</td>
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<td>1.47</td>
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<tr>
<td>P13</td>
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<td>PA</td>
<td>117</td>
<td>19.9</td>
<td>1.06</td>
<td></td>
</tr>
<tr>
<td>P14</td>
<td>PGlu(OBzl)</td>
<td>DBCO</td>
<td>n.d.</td>
<td>14.6</td>
<td>1.07</td>
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</tr>
<tr>
<td>P15</td>
<td>PGlu(OBzl)</td>
<td>DBCO</td>
<td>n.d.</td>
<td>24.9</td>
<td>1.07</td>
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<tr>
<td>P16</td>
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<td>9.0</td>
<td>1.14</td>
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<tr>
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<td>111</td>
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<td>n.d.</td>
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<tr>
<td>P19</td>
<td>PLys(TFA)</td>
<td>APA</td>
<td>n.d.</td>
<td>17.5</td>
<td>1.11</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> X<sub>n</sub> was determined by 1H-NMR in DMSO-d<sub>6</sub> by relating end group signals to the ones of the repeating unit.

<sup>b</sup> M<sub>n</sub> was determined relative to PMMA standards.

The degree of polymerization was calculated by 1H NMR by comparing the signal of the end group with the ones of the repeating units. Monomodal Poisson-like molecular weight distributions were ensured by HFIP GPC. Unfortunately, for some polymer-end group combinations, the degree of polymerization (X<sub>n</sub>) could not be obtained by 1H NMR due to an overlap of end group proton signals with polymer backbone signals (see experimental section). A comparison of M<sub>n(GPC)</sub> and Đ<sub>GPC</sub> values of these polymers to their homologues with different end groups ensured that, even in the absence of NMR studies, these polymers are of comparable chain length.

In general, homopolymers possess low dispersities (Đ ≤ 1.1) and high end-group integrities (see table 1). This is remarkable since especially in the case of PLys(TFA), the dispersities determined by GPC are even overestimated due to the coexistence of secondary structures differing in hydrodynamic volume as previously reported by Huesmann and coworkers. For PSar and PGlu polymers, dispersities are below 1.1 (see Table 1) and GPC elograms indicate Poisson-like molecular weight distributions (see supporting information).

Neopentyl-terminated polymers were further modified at the N-term by standard peptide coupling chemistry using HOBt/HBTU/DIPEA (see figure 1). PSar was functionalized with S-benzyl thiosuccinic acid (BTS) and Boc-L-Cys(Trt) (Cys) respectively. PLys(TFA) was functionalized with Cys. Modification of polypeptides with BTS did not give reliable degrees of end group modification. Although it has been shown that N-terminal thioester synthesis can be achieved for low molecular weight peptides, the required long reaction times (2 days) for quantitative N-terminal modification led to aminolysis of the thioester, resulting in an inactivation of the end group by succinimide formation. Cyclisation cannot occur for PSar because of its methyl-substituted N-terminus. The degree of end group modification was determined by comparing the integral of the neopentylamine initiator’s t-butyl group at 0.83 – 0.85 ppm with the integral of the benzylic protons’ signal of S-benzyl thiosuccinic acid (SH, 7.2 – 7.3 ppm) and of the trityl protons’ signal of Boc-L-Cys(Trt), respectively, (15N H, 7.2 – 7.3 ppm and 9NH, 1.35 ppm) in DMSO-d<sub>6</sub>. For modification of PLys(TFA), only the trityl protons’ signals were clearly detectable and used for determination of the degree of end group modification (see figure 1A). Determined degrees of modification and analytical data of end group modified polymers can be found in table 2. Within the limits of accuracy, NMR data suggests nearly quantitative modification for the synthesized polymers (see table 2). In addition, MALDI-TOF MS analysis of PSar polymers (X<sub>n</sub> = 50) containing five different functionalities (PA, DBCO, APA, NPA+BTS, NPA+Boc-L-Cys(Trt)) needed for ligation reactions further confirms the NMR data (see supporting information).
The covalent attachment of respective end groups to the polymer was verified by diffusion ordered spectroscopy (DOSY) (see figure 1B).

Finally, polymers modified with Boc-L-Cys(Trt) were subsequently deprotected in TFA for 30 minutes at room temperature using TIPS as scavenger. Complete deprotection was monitored by $^1$H NMR (see Fig. 1A). Deprotection in TFA was chosen since PSar and PLys(TFA) are both soluble in TFA and for purification, the reaction mixture was precipitated in diethyl ether to easily remove residual scavenger and resulting hydrophobic products of removed protecting groups (Trt).

**Ligation experiments**

Copper(I)-catalyzed alkyne-azide coupling (CuAAC), strain-promoted alkyne-azide 1,3-cycloaddition (SPAAC) and native chemical ligation (NCL) were performed as ligation techniques and reaction conditions (see scheme 3) have been optimized. Ligation experiments were performed for hydrophilic, hydrophilic/hydrophobic (amphiphilic) and hydrophobic conditions to provide coupling protocols for the synthesis of block copolymers consisting of blocks with different polarities. The employed polymer combinations are listed in table 3. Two different degrees of polymerization (M/I ratio = 50, 100) were designed to study the influence of chain length on ligation efficacy. PSar was employed as hydrophilic and non-ionic block, while PLys(TFA) and PGlu(OBzl) served as hydrophobic blocks, with PLys(TFA) being slightly more polar than PGlu(OBzl) due to the amide bond in the side chain. Ligations were carried out between polypept(o)ides with similar chain lengths (50/50 and 100/100) to facilitate analysis of coupling product formation by HFIP GPC.

![Scheme 3: Chemical ligation techniques used for block copolymer synthesis; a) PBS (pH 7.4), 50 mM MPAA/20 mM TCEP/HCl; b) DMF (abs)/50 mM MPAA/40 mM TEA; c) H$_2$O/50 mol% sodium ascorbate/5 mol% CuSO$_4$; d) DMF (abs)/2 eq CuBr/4 eq PMDETA; e) H$_2$O; f) DMF.](image)

![Table 3: Polymer combinations for ligation experiments.](table)

<table>
<thead>
<tr>
<th>Ligation type</th>
<th>Hydrophilic</th>
<th>Amphiphilic</th>
<th>Hydrophobic</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuAAC</td>
<td>PSar/PSar</td>
<td>PSar/PGLu(OBzl)</td>
<td>PLys(TFA)/PGLu(OBzl)</td>
</tr>
<tr>
<td>SPAAC</td>
<td>PSar/PSar</td>
<td>PSar/PGLu(OBzl)</td>
<td>PLys(TFA)/PGLu(OBzl)</td>
</tr>
<tr>
<td>NCL</td>
<td>PSar/PSar</td>
<td>PSar/PLys(TFA)</td>
<td>Not accessible</td>
</tr>
</tbody>
</table>

NCL was only performed for hydrophilic and amphiphilic conditions for the reasons explained above and GPC samples were pretreated with TCEP-HCl to avoid misinterpretation by oxidation (dimer formation). All ligations were performed at a 1:1 molar ratio of polymers and 40 °C for 70h to ensure comparability between individual ligation reactions. To quantify relative coupling efficiencies, GPC traces were fitted by Gaussian distribution (see figure 2), from which the peak areas were used to calculate respective contents. This methodology is enabled since the GPC plots of homopolymers themselves can be fitted with negligible deviation by Gaussian distribution as expected for polymers having a Poisson-like molecular weight distribution. Ranges of determined efficiencies are shown in figure 2 as well as elugrams of...
ligations between short polypeptide(s) (50/50) under hydrophilic conditions for CuAAC, SPAAC and NCL. Corresponding fit curves used for efficiency determination are also displayed.

Determined efficiencies for individual ligation experiments ranged from 53 to 88% (see supporting information). It has to be noted that residual unreacted homopolymer can also be attributed to a non-equimolar ratio of the two employed homopolymers, since due to the intrinsic uncertainty of determined chain lengths, an exact 1:1 ratio of functional end groups is hardly achievable. Coupling efficiencies are likely to be slightly overestimated for amphiphilic CuAAC ligation products, since copper was removed by precipitation in water/MeOH to enable NMR analysis. This was done in order to prove triazole formation (see supporting information). In general, precipitation of the coupling reaction is not expected to significantly affect the molecular weight distribution of ligation products, since both individual homopolymers already precipitate under the reported conditions. We want to emphasize here that coupling efficiencies were primarily determined for better comparability of the different techniques. Nevertheless, these results indicate that block copolymer synthesis is feasible with all three presented ligation techniques with good efficiencies.

As expected, in general, ligations between shorter polypeptides are easier to realize than ligations between longer polypeptides/polysarcosine. This is likely due to increased steric demand and decreased probability of meeting between reactive chain ends. Generally, azide alkyne coupling reactions gave better results than NCL, even if NCL showed very high (up to 88%) coupling efficiencies for hydrophilic conditions. When the two different types of azide alkyne coupling reactions are directly compared, SPAAC seems to be slightly superior to CuAAC. Not only are coupling efficiencies slightly better compared to CuAAC, especially for amphiphilic and hydrophobic conditions, but also, for SPAAC, no additional catalyst is necessary. Therefore, the reaction is very simple to conduct and it is not required to work in dry solvents or under inert conditions. When functionalities for click chemistry can be easily introduced into both blocks as shown for homopolymers in this study, SPAAC appears to be the method of choice. On the other hand, chain length determination by \(^1\)H NMR is a lot more reliable when neopentylamine (9 equivalent protons) or other initiators with clearly detectable signals are used.

In case of a peptide already bearing an N-terminal Cys residue (synthesized either by SPPS or by biotechnological means), NCL can become the preferred method. However, NCL is optimized for biological applications and is only very easy to conduct in aqueous buffered solutions. In organic solvents, however, the reaction demands working under absolutely dry conditions to avoid hydrolysis of the thioester. If no reducing agent is added, it is also necessary to work under oxygen free conditions to avoid cystine formation, which inhibits the cysteine to further take part in chemical ligation. For the synthesis of amphiphilic conjugates, also mixtures of organic solvents and aqueous buffered solutions are an option. Nevertheless, it needs to be kept in mind that end group modification might not always be quantitative and is also dependent on chain length, which further limits this method for block copolymer synthesis.

In this work, the main focus was set on a direct comparison between different ligation techniques and for the purpose of feasible monitoring. Purification of block copolymers, e.g., block copolymer(s), however, can be easily conducted whenever two polymers or peptides, which differ significantly in chemical properties like polarity (solubility) or in size are used as for example shown by Lecommandoux and coworkers. Thus, the ligation approach is also complementary pathway to the sequential ring opening polymerization for the synthesis of amphiphilic block copolymer(s).

In Fig. 2, HFIP GPC elugrams of ligation products from CuAAC, SPAAC and NCL (X = 50, hydrophilic) and Gauss-fits used to determine efficiencies displayed in the table below.

**Evaluation of Cytotoxicity and Complement Activation of Ligation Site**

Since all of the aforementioned methods showed promising results as ligation techniques for block copolymer synthesis, we performed studies on cytotoxicity and complement activation for resulting short, hydrophilic ligation products (PSar\(_{50}\) + PSar\(_{50}\)) of all three ligations (L1, L2, L3). Therefore, an MTT assay was performed for block copolymers derived by either CuAAC, SPAAC or NCL, which did not indicate significant differences between individual ligation types and also compared to a reference PSar homopolymer (see supporting
information). CuAAC and SPAAC ligations result in a triazole moiety as ligation site opposed to NCL, where a native amide bond is formed. It could be suspected that these electron deficient aromatic rings, which are not common to the body, would cause activation of the immune system. As an initial test, polymers L1, L2 and L3 were incubated with human serum for 1h at 37°C and the concentration of human complement factor 5a (C5a) was determined by ELISA (see figure 5). Compared to human serum itself, no increased concentration of C5a could be detected.

Fig. 3: Concentration of C5a determined by ELISA after incubation of L1(CuAAC), L2(SPAAC) and L3(NCL) with human serum for 1h at 37°C.

Hence, these preliminary experiments suggest that the nature of the ligation site does neither affect toxicity nor complement activation of these polymers, which is a first crucial requirement for applications in nanomedicine. Nevertheless, future research needs to address different types of immune responses apart from complement activation.

NCL with SPPS peptide TCR CP
For peptides synthesized by SPPS, NCL is a very effective method since end group functionalities can be easily introduced during the cleavage from the resin. Therefore, we used a BTS-modified PSar for conjugation with a considerably hydrophobic peptide sequence with 9 amino acids bearing a cysteine residue at the N-terminus (CGLRILLKV). This peptide sequence is called the T-cell receptor core peptide (TCR CP) and has shown to inhibit IL-2 production in antigen-stimulated T cells and thus to suppress inflammation in some animal models T cell-mediated disease.53,54

PSar$_{124}$-BTS was conjugated to TCR CP in a mixture of 4 M urea buffer/THF 1:1 in the presence of MPAA and TCEP-HCl, while using an excess of TCR CP (2 eq). After purification by precipitation and dialysis, the conjugate was analysed by $^1$H NMR, DOSY and HFIP GPC. DOSY data (see figure 4) clearly demonstrates covalent attachment to the polymer since corresponding signals appear as one diffusing species. After successful synthesis of the conjugate, the HFIP GPC elugram displayed bimodal molecular weight distribution with a second peak at double molecular weight (see figure 4). This second peak disappears after treatment with TCEP-HCl overnight in H$_2$O. The higher molecular weight peak can, thus, be attributed to dimer formation, which only becomes possible once the free thiol moiety is incorporated into the polymer, thus, further proves effective coupling of the decapeptide to polysarcosine.

In conclusion, a well-defined conjugate can be obtained under reductive conditions, which can be easily purified by precipitation and dialysis. The conjugate is currently under investigation with respect to inhibition IL-2 production in different inflammation models.

Conclusions
We have demonstrated that semitelechelic polypeptides and polypeptoids with low dispersities can be synthesized by using varying functionalized primary amines as initiators or by subsequent peptide coupling at the N-terminus as postpolymerization modification. End group modifications using HOBT/HBTU/DIPEA coupling at N-termini allowed for the quantitative introduction of cysteine and thioester functionalities to PSar and of cysteine to PLys(TFA). In the latter case, the introduction of the thioester moiety was not possible in a reliable way, due to the occurrence of thioester aminolysis. Furthermore, three different ligation techniques were investigated for block copolymer synthesis. Successful ligation of polymers was analyzed by HFIP GPC and elugrams were fitted by Gaussian distributions to compare individual ligation techniques under different polarity conditions and for two different average chain lengths ($X_n = 50,100$). SPAAC
proved to be most efficient under the given circumstances and straightforward in synthesis. Furthermore, no differences in cytotoxicity or complement activation for ligation products have been detected in preliminary in vitro experiments. Moreover, native chemical ligation has been successfully applied to the synthesis of polysarcosine-TCR CP conjugate and stresses the usefulness of the this method for coupling with peptides synthesized by SPPS or even natural protein sequences bearing an N-terminal cysteine residue.

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