Bioreducible Poly-L-Lysine–Poly[HPMA] Block Copolymers Obtained by RAFT-Polymerization as Efficient Polyplex-Transfection Reagents[a]

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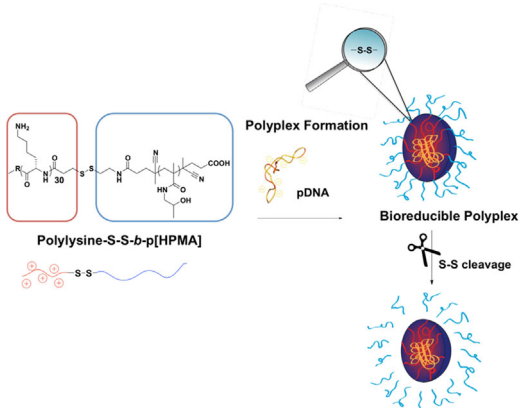
Polylysine-*b*-p[HPMA] block copolymers containing a redox-responsive disulfide bond between both blocks are synthesized by RAFT polymerization of pentafluorphenyl-methacrylate with a macro-CTA from N_{ε}-benzyloxycarbonyl (Cbz) protected polylysine (synthesized by NCA polymerization). This polylysine-*b*-p[PFMA] precursor block copolymer is converted to polylysine(Cbz)-*b*-p[HPMA] by postpolymerization modification with 2-hydroxypropylamine. After removal of the Cbz protecting group, cationic polylysine-*b*-p[HPMA] copolymers with a biosplittable disulfide moiety became available, which can be used as polymeric transfection vectors. These disulfide linked polylysine-S-S-*b*-p[HPMA] block copolymers show low cytotoxicity and increased transfection efficiencies (HEK-293T cells) compared to analogous blockcopolymers without disulfide group making them interesting for the transfection of sensitive immune cells.



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[a]**Supporting Information** is available from the Wiley Online Library or from the author.

1. Introduction

Cancer immunotherapy is considered a promising approach, aimed to recognize and eliminate malignant cells by exploiting the effectiveness and potency of the adaptive immune system.[1–3] Besides classical vaccination, the activation of dendritic cells (DCs) offers a promising strategy to induce cellular immune response.[4–6]

Especially, the transcriptional activation of DCs with antigen-encoding pDNA seems to be a very powerful method.[7,8] It facilitates the parallel activation of both T helper ($CD4^+$) and cytotoxic T ($CD8^+$) cells.[9,10] Moreover, pDNA vectors display a useful platform to incorporate additional moieties such as the DC-specific fascin promoter, which ensures that transcription of the encoding gene occurs selectively in DCs.[11,12]

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It gets only activated in DCs and ensures, thus, the selective read-out only in these cells. In addition, in case of DCs, only as few as about 1 000 DCs need to be transfected to induce a profound immune response in mice.[13] However, it is still challenging to find appropriate transfection vectors for sensitive immune cells and especially for in vivo applications.

Several drawbacks of using recombinant virus as transfection vectors, such as a risk in therapeutical applications, limitation in passenger DNA size, and difficulties in large scale production,[14–16] have led to intensive investigations on liposomal, lipidic, and polymeric transfection agents in the last decades. Especially polymer-based systems, more precisely polyplexes derived from pDNA and block copolymers consisting of a cationic block for pDNA complexation and a hydrophilic, biocompatible block for efficient shielding, seem most promising. This has nicely been shown by the innovating work of Wagner et al., and Kataoka and co-workers.[17–20] A biocompatible corona of the polyplexes, e.g., from PEG or p[HPMA] for stealth like properties, is thereby indispensable for systemic in vivo applications to (i) avoid unspecific interactions with serum proteins, to (ii) prevent extra- and intracellular degradation of the pDNA, and to (iii) prevent unspecific immune response due to stimulation of *toll like* receptors.[21–23] A DC specific uptake can be achieved by the incorporation of targeting ligands to the polyplex' surface to induce receptor mediated uptake. For DCs, a specific targeting of polymeric systems has been shown by the use of mannose or the monoclonal antibody aDEC-205.[24–26]

To improve the intracellular pDNA release, stimulus-responsive groups between the cationic core and the hydrophilic corona are needed. In this context, chemical moieties that respond to intracellular pH changes in the endosome or which are sensitive to the cytoplasmatic redox potential are highly suitable.[27,28] In the latter case, redox-stimuli responsive disulfide bonds seem applicable to achieve intracellular deshielding of polyplexes. They are quite stable under extracellular conditions. Intracellulary, however, they are rapidly cleaved due to a 50- to 1 000-fold increased concentration of glutathione.[29–31]

Especially in antigen presenting cells (APCs) like DCs the conditions for reductive splitting are already fulfilled in the endosome.[32] The removal of the shielding corona leads potentially to enhanced interactions of the cationic part of the polyplex with the endosomal membrane, which finally results in a facilitated endosomal escape. Therefore, disulfide groups are attractive to increase intracellular release of cargo, particularly of pDNA from polyplexes.[33,34]

Due to their biodegradability, peptidic polylysine as cationic core seems advantageous in comparison to poly[ethylenimine] (PEI), poly[amidoamidine] (PAMAM) and substituted (meth) acrylates[35–37] in terms of cytotoxicity. This is especially important when dealing with sensitive immune cells. Thus PEG, or poly[sarcosine] derivatives of polylysine seem most promising to transfect immune cells.[38,39] In this context, the use of clinically investigated poly[2-(hydroxypropyl methacrylamide)] (p[HPMA]) as a biocompatible, non-ionic hydrophilic corona appears quite reasonable too, since it features low immunogenicity[40,41] and has been used in clinical studies for anticancer vaccines.[42] Furthermore, p[HPMA] can be modified with targeting ligands like mannose or aDEC-205 antibodies to address DCs.[24,25]

The combination of reversible addition-fragmentation chain transfer polymerization (RAFT-polymerization) and reactive ester monomers allows the synthesis of multifunctional p[HPMA] based block copolymers of various topologies.[43,44] By this synthetic approach, we previously obtained polylysine-*b*-p[HPMA] block copolymers, which efficiently form polyplexes in the presence of pDNA and display reasonable transfection efficiencies at minimal cytotoxicity.[45] To increase transfection efficiencies, the incorporation of redox cleavable groups—like disulfide groups—seems suitable.

To achieve this, polylysine with carboxybenzyl (Cbz) blocked amino groups was selectively modified at the *N*-terminus with a chain transfer agent (CTA) including a disulfide bond. Then the synthesis of disulfide-linked polylysine-*b*-p[HPMA] block copolymers was realized via RAFT-polymerizatzion, that is—to some extent—compatible with disulfid bonds.[46,47] The disulfide containing polylsine-CTA could be successfully used for RAFT-polymerization of pentafluorophenyl methacrylate. The resulting block copolymers were finally converted to the desired polylysine-S-S-*b*-p[HPMA] with disulfide bonds. The formation and physicochemical characterization of the polyplexes as well as in vitro studies with HEK-293T cells to ascertain cellular uptake, transfection efficiencies, and toxicity are carefully investigated to analyze the influence of the bioreducible disulfide group.

2. Experimental Section

2.1. Materials

All chemicals (reagent grade) were obtained from Sigma–Aldrich (Deisenhofen, Germany). Chemicals were used without further purification unless otherwise indicated. Trifluoroethanol (TFE) was purchased from Acros Organics (Niederau, Germany). Protected L-lysine (NH_2-L-Lys(Cbz)-OH was obtained from Orpegen (Heidelberg, Germany). Oregon green (OG) cadaverine 488 was obtained from Invitrogen (Carlsbad, CA). Pentafluorophenol was purchased from Fluorochem (Derbyshire, UK). Dioxane and tetrahydrofuran used for synthesis of polymer were freshly distilled from sodium. 2,2-Azobis(isobutyronitrile) (AIBN) was recrystallized from diethyl ether, and was stored at −20 °C. Dialysis was performed using

Spectra/Por 3 membranes (MWCO 14 000 or 25 000 $g \cdot mol^{-1}$) obtained from Carl Roth (Karlsruhe, Germany). Sephadex Hi Trap desalting columns were purchased from GE Healthcare (Buc, France). The vector pGL3-Basic (agarose gel electrophoresis, FCS-measurements, CLSM) was obtained from Promega GmbH (Mannheim, Germany). pDNA encoding for Enhanced Green Fluorescent Protein (pEGFP-N1; transfection efficiency and toxicity by FACS) was purchased from Clonetech (Mountain View, CA). Gel Red was purchased from VWR (Darmstadt, Germany).

2.2. Characterization

^{1}H-, ^{19}F-NMR spectra were obtained at 300 or 400 MHz using a FT spectrometer from Bruker (Billerica, MA) and analyzed using MestReNova 6.0.2. IR spectra were recorded on a Bruker FT/IR-4100 using an ATR unit. SEC of hydrophilic HPMA-copolymers was performed in hexafluoroisopropanol (HFIP) containing 3 $g \cdot L^{-1}$ potassium trifluoroacetate as eluent and with the following components: column packed with modified silica (PFG columns particle size: 7 μm; porosity: 100 and 1 000 Å, respectively), refractive index detector G1362A RID and UV-detector UV-2075 plus from Jasco. Calibration was done using PMMA standards (PSS, Mainz, Germany). The flow rate was set to 0.8 mL $\cdot$ min^{-1} at a temperature of 40 °C. All hydrophilic HPMA polymers were purified by preparative SEC chromatography using Sephadex Hi Trap desalting columns as eluent with the following parts: pump (pU-2086 Plus series), UV/Vis detector (UV-2077 Plus), and a Jasco RI-detector (Jasco RI 2031 Plus series) from Jasco. The flow rate was set to 1.0 mL $\cdot$ min^{-1}. All elution diagrams were evaluated with PSS WinGPC.

2.3. Synthesis of 4-Cyano-4-((thiobenzoyl) sulfanyl) pentanoic Acid (Acid CTA)

4-Cyano-4-((thiobenzoyl) sulfanyl) pentanoic acid was synthesized according to the literature.[48] 300 MHz ^{1}H-NMR ($CDCl_3$): [δ/ppm] = 7.92–7.90 (d, 2H), 7.59–7.55 (t, 1H), 7.42–7.38 (t, 2H), 2.81–2.40 (m, 4H), 1.94 (s, 3H).

Synthesis of Pentafluorophenyl-(4-phenylthiocarbonylthio-4-cyanovalerate) (PFP-CTA). Pentafluorophenyl-(4-phenylthiocarbonylthio-4-cyanovalerate) was synthesized according to the literature.[49] 300 MHz ^{1}H-NMR ($CDCl_3$): [δ/ppm] = 7.95–7.92 (d, 2H), 7.61–7.57 (t, 1H), 7.44–7.30 (t, 2H), 3.10–2.51 (m, 4H), 1.99 (s, 3H). 376 MHz ^{19}F-NMR ($CDCl_3$): [δ/ppm] = −152.85 (d, 2F), −157.60 (t, 1F), −162.21 (t, 2F).

2.4. Synthesis of 2-((2-Nitrophenyl) disulfanyl) ethanamine

4 g (355 mmol; 1 eq) of 2-mercaptoethylamine hydrochloride and 10 g (53 mmol, 1.5 eq.) of 2-nitrophenylsulfanyl chloride were suspended in 100 mL of glacial acetic acid and stirred for 3.5 h at 70 °C. The solvent was evaporated under reduced pressure and the crude product was dissolved in 12 mL of dimethylformamide (DMF). After diluting with 100 mL of chloroform the desired product crystallized as a yellow solid. 50 mL of chloroform were added to the filtered product, and the mixture was stirred for 30 min at room temperature. This procedure was repeated twice, yielding 6.5 g (28.4 mmol, 80%) of 2-((2-nitrophenyl) disulfanyl) ethanamine as a yellow solid.

300 MHz ^{1}H-NMR (dmso-d_6): [δ/ppm] = 8.33–8.27 (m, 2H), 8.24 (br. s, 2H), 7.95–7.89 (t, 1H), 7.59–7.54 (t, 1H), 3.05 (s, 4H).

FD-MS: [m/z] = 230.0 ([M + H], calc. 230.0[M + H]).

2.5. Synthesis of 3-((2-Aminoethyl) disulfanyl) propanoic Acid

The synthesis of 3-((2-aminoethyl) disulfanyl) propanoic acid was adapted from the literature.[50] In brief, 4 g (17.4 mmol, 1 eq.) of 2-((2-nitrophenyl)-disulfanyl) ethanamine and 1.84 g (17.4 mmol, 1 eq.) of 3-mercaptopropanoic acid were dissolved in 50 mL of water in a round bottom flask. 4.8 mL (34.8 mmol, 2 eq.) triethylamine were added and the red colored reaction mixture was stirred for 30 min at room temperature. Afterward, the solution was acidified (pH 5) using 1M hydrochloric acid (change in color from red to yellow) and extracted five times with ethyl acetate. The combined aqueous phases were evaporated under reduced pressure to obtain the colorless crude product, which was purified by flush column chromatography (EtOAc/AcOH/H_2O 8:2:1; R_f = 0.28). After additional recrystallization from a solution of 90% *n*-pentanol in water, 580 mg (2.5 mmol, 14.5%) of 3-((2-aminoethyl) disulfanyl) propanoic acid were obtained as a colorless solid.

300 MHz ^{1}H-NMR (D_2O): [δ/ppm] = 3.37–3.31 (t, 2H), 2.99–2.92 (q, 4H), 2.70–2.66 (t, 2H).

ESI-MS: [m/z] = 182.04 ([M + H], calc. 182.02 [M + H]).

IR υ [cm^{-1}]: 2 950 (br), 2 619 (w), 2 350 (w), 2 031 (w), 1 698 (s), 1 620 (s), 1 510 (s), 1 407 (vs), 1 247 (s), 1 092 (s), 947 (w), 903 (w), 772 (w), 656 (w).

mp: 152.8 °C.

2.6. Synthesis of 3-((2-(4-Cyano-4-((phenylcarbonothioyl) thio) pentanamido)-ethyl)-disulfanyl) propanoic Acid (Disulfide CTA)

The disulfide CTA was obtained by reacting 370 mg (0.83 mmol, 1.3 eq.) PFP-CTA, 0.22 mL (1.6 mmol, 2.5 eq.) of triethylamine and 116 mg (0.64 mmol, 1 eq.) of 3-((2-aminoethyl)-disulfanyl) propanoic acid in 10 mL of THF. The reaction was stirred at room temperature for 4 h under argon atmosphere and light exclusion. The solvent was removed by evaporation under reduced pressure and the crude was dissolved in 50 mL of dichloromethane. The solution was extracted 3 times with pure water and once with brine. The organic phase was dried over magnesium sulfate and the solvent was removed under reduced pressure. Further purification was carried out via flash chromatography (chloroform/ethanol/acetic acid 40:1:0.1; R_f = 0.30) yielding 70 mg (0.16 mmol; 25%) of the disulfide CTA as a red solid.

300 MHz ^{1}H-NMR ($CDCl_3$): [δ/ppm] = 7.92–7.89 (d, 2H,), 7.59–7.55 (t, 1H), 7.42–7.37 (t, 2H), 3.64–3.58 (q, 2H), 2.98–2.94 (t, 2H), 2.85–2.77 (m, 4H), 2.67–2.35 (m, 4H), 1.93 (s, 3H).

ESI-MS: [m/z] = 465.06 ([M + Na], calc. 465.05 [M + Na]); 907.14 ([M + M + Na], calc. 907.10 [M + M + Na]).

2.7. Synthesis of 3-((2-(4-Cyano-4-((phenylcarbonothioyl) thio) pentanamido) ethyl)-disulfanyl) propanoic Acid-pentafluorophenyl-ester (Disulfide-PFP-CTA)

A round bottom flask was loaded with 60 mg (0.13 mmol, 1 eq.) of 3-((2-(4-cyano-4-((phenylcarbonothioyl) thio) pentanamido) ethyl) disulfanyl) propanoic acid in 5 ml of abs. THF and 37 μL (0.27 mmol, 2 eq.) of triethylamine. Under argon atmosphere 46 μL (0.27 mmol, 2 eq.) of pentaflourophenyl triflouroacetate were added dropwise and the reaction was stirred for 4 h at room temperature under light exclusion. The solvent was removed by evaporation under reduced pressure, the crude was dissolved in 25 mL of dichloromethane and extracted with water. The organic phase was dried over magnesium sulfate and the solvent was removed by using the rotary evaporator. Further purification was conducted via flash chromatography (chloroform/ethanol/acetic acid 40:1:0.1%; $R_f = 0.43$) yielding 74 mg (0.12 mmol, 94%) of the disulfide-PFP-CTA as a red solid.

300 MHz ^{1}H-NMR ($CDCl_3$): [δ/ppm] = 7.92–7.89 (d, 2H), 7.59–7.54 (t, 1H), 7.42–7.37 (t, 2H), 3.66–3.60 (q, 2H), 3.15–3.01 (m, 4H), 2.86–2.81 (t, 2H), 2.67–2.38 (m, 4H), 1.94(s, 3H).

376 MHz ^{19}F-NMR ($CDCl_3$): [δ/ppm] = −162.30 (t, 2F), −157.76 (t, 1F), −152.77 (d, 2F).

2.8. Synthesis of Pentafluorophenyl Methacrylate (PFMA)

Pentafluorophenyl methacrylate (PFPMA) was prepared according to literature.[51] 300 MHz ^{1}H-NMR ($CDCl_3$): [δ/ppm] = 6.45 (s, 1H), 5.91 (s, 1H), 2.09 (s, 3H). 376 MHz ^{19}F-NMR ($CDCl_3$): [δ/ppm] = −152.71 (d, 2F), −158.12 (t, 1F), −162.41 (t, 2F)

2.9. Synthesis of Neopentyl-Ammonium Tetrafluoroborate

Neopentyl-ammonium tetrafluoroborate was synthesized as previously described.[52]

2.10. Synthesis of N_ε-Benzyloxycarbonyl-L-Lysine-*N*-Carboxyanhydride (Lys(Cbz) NCA)

N_ε-Benzyloxycarbonyl-L-lysine-*N*-carboxyanhydride was synthesized as previously described.[52]

2.11. Synthesis of Polylysine with N_ε-Benzyloxycarbonyl (N_ε-Cbz) Protecting Group with NCA-Polymerization (HOOC-poly[L-Lys(N_ε-Cbz)]$_{30}$-NH_2)

HOOC-poly[L-Lys(N_ε-Cbz)]$_{30}$-NH_2 was synthesized by polymerization of α-aminoacid-*N*-carboxy anhydrides (NCA-polymerization) with N_ε-benzyloxycarbonyl-L-lysine-*N*-carboxyanhydride (Lys (Cbz) NCA) as monomer and neopentyl-ammonuim tetrafluoroborate as initiator as previously described.[52] In brief, 333 mg (1.09 mmol) of the monomer (Lys(N_ε-Cbz) NCA) were transferred into a *Schlenk tube* in a nitrogen counterflow and dissolved in 3.3 mL of abs. DMF. Afterward, 5.73 mg (0.036 mol) of the initiator neopentylammonuim tetrafluoroborate were added through a stock solution in DMF. The resulting solution was stirred 3 d at 40 °C under a constant nitrogen flow. The polymer was precipitated in cold ethyl ether, dissolved in dioxane/water mixture andlyophilized yielding 241 mg of the polymer as a colorless powder.

400 MHz ^{1}H-NMR (dmso-d_6): [δ/ppm] = 8.49–7.89 (m, 30H), 7.26–7.16 (m, 180H), 4.97–4.94 (m, 61 H), 4.33–3.66 (m, 30H), 2.93 (br. s, 61H), 2.04–1.15 (m, 180H), 0.82–0.79 (d, 9H).

2.12. Synthesis of Polylysine-Chain Transfer Agents (Polylysine-CTAs)

In a typical reaction procedure 500 mg (0.063 mol, 1 eq.) of HOOC-poly[Lys(N_ε-Cbz)]$_{30}$-NH_2were dissolved in dry NMP. In a separate flask 55.6 mg (0.13 mmol, 2 eq.) of pentafluorophenyl-(4-phenylthiocarbonylthio-4-cyanovalerate) (PFP-CTA) and 55.8 mg (0.25 mmol, 4 eq.) of N^1, N^1, N^6, N^6-tetramethylnaphthalene-1,8-diamine("proton sponge") were dissolved in 1.5 mL of dry NMP and added to the polylysine with free amino terminus. The resulting mixture was stirred overnight under argon atmosphere in the dark. The NMP was removed under reduced pressure, 1 mL of TFE was added and the polymer was precipitated in cold ethyl ether. The product was dissolved with 5 mL TFE and purified by silica gel flash chromatography (eluent: ethyl acetate/methanol 10:1 → 1:1 → 1:2). The product was finally dissolved in a TFE/water mixture and lyophilized yielding 414 mg (0.05 mmol) of the product as a pink powder.

400 MHz ^{1}H-NMR (dmso-d_6): [δ/ppm] = 7.89–7.87 (d, 1.63H), 7.68–7.65 (t, 0.82H), 7.49–7.45 (t, 1.62H), 7.31–7.14 (m, 170H), 4.98–4.95 (m, 68H), 4.21–3.74 (m, 34H), 2.94 (s, br, 68H), 1.92–1.18 (m, 216H), 0.83–0.80 (m, 9H).

GPC (HFIP): $\overline{M}_n = 11\,300\ g \cdot mol^{-1}$. $\eth = 1.11$.

2.13. Synthesis of Polylysine-Disulfide-Chain Transfer Agent (Polylysine-Disulfide-CTA)

500 mg (0.063 mmol, 1 eq.) of HOOC-poly[Lys(N_ε-Cbz)]$_{30}$-NH_2were dissolved in 5 mL of abs. *N*-methyl-2-pyrrolidone (NMP). A separate flask was loaded with 76 mg (0.13 mmol. 2 eq.) of 3-((2-(4-cyano-4-((phenylcarbonothioyl) thio) pentanamido) ethyl)-disulfanyl) propanoic acid-pentafluoro-phenyl ester (disulfide-PFP-CTA) and 55.8 mg (0.25 mmol, 4 eq.) of N^1,N^1,N^6,N^6-tetramethylnaphthalene-1,8-diamine in 1.5 mL of abs. NMP. This solution was added to the polylysine solution under argon atmosphere. The reaction was stirred over night at room temperature and light exclusion. Then the solvent was removed by evaporation under reduced pressure, the crude was dissolved in 1 mL of trifluoroethanol, and finally the polymer was precipitated in cold ethyl ether. Afterward, the product was purified by flash chromatography (eluent: ethyl acetate/methanol 10:1 → 1:1 → 1:2), dissolved in trifluoroethanol again and slowly added into water dropwise. The solution was lyophilized and 428 mg (0.054 mmol, 80%) of the product were obtained as a pink solid.

400 MHz ^{1}H-NMR (dmso-d_6): [δ/ppm] = 7.90–7.88 (d, 1.72H), 7.69–7.65 (t, 0.86H), 7.51–7.47 (t, 1.72H), 7.31–7.14 (m, 150H), 4.97–4.94 (m, 60H), 4.19–3.81 (m, 30H), 2.93(s, br, 60H), 1.99–1.23 (m, 180H), 0.82–0.79 (m, 9H).

GPC (HFIP): $\overline{M}_n = 11\,000\ g \cdot mol^{-1}$, $\eth = 1.13$.

2.14. Synthesis of Polymers

Polymerizations were carried out in *Schlenk tubes* with a TFE/dioxane mixture (1:8) as solvent after four freeze–thaw cycles at 60 °C for 18 h in an oil bath. AIBN was used as initiator with a ratio of (disulfide) polylysine-CTA/initiator of 6:1. Block copolymers were precipitated from TFE/dioxane in hexane. The end group was removed with an access of 4,4-azobis(4-cyanovaleric acid) (ACVA). Conversion of poly [PFMA] to poly[HPMA] was carried out by postpolymerization modification with 2-hydroxypropylamine and complete conversion was monitored by ^{19}F-NMR spectroscopy. Each polymer was synthesized with and without the fluorescence dye Oregon Green 488. Labeled polymers were used for cell uptake experiments and FCS-measurements. Unlabeled polymers for agarose gel electrophoresis and determination of transfection and toxicity.

2.15. Synthesis of Block Copolymers Polylysine-*b*-Poly[HPMA] (P1–P3) and Polylysine-S-S-*b*-p[HPMA] (P4–P6) by RAFT-Polymerization

In a typical reaction procedure a *Schlenk tube* was loaded with 125 mg (107 mg, 0.012 mmol with CTA end-group) of disulfide-polylysine-CTA were diluted in a mixture of 600 μL TFE and 5 mL abs. dioxane, 500 mg (2 mmol) of the monomer PFMA and 0.34 mg (0.002 mmol) of the initiator AIBN were added via stock solution (molar ratio: AIBN/CTA/monomer 1/6/1 000). After four freeze–thaw cycles the resulting solution was stirred for 2 d at 60 °C in an oil bath. The polymer was precipitated from TFE/dioxane (1:8) in hexane three times yielding 394 mg of a pink powder. The polymer was dissolved in TFE/dioxane (1:8) and stirred for 4 h with 120 mg (0.44 mmol, 25 eq.) of 4,4-azobis(4-cyanovaleric acid) (ACVA) at 85 °C for removal of end groups. The polymer with removed end groups was precipitated three times from dioxane/TFE (1:8) in hexane/ethyl ether (2:1) obtaining 366 mg of a colorless powder. Subsequently, 50 mg of the block copolymer were dissolved in 1 mL abs. DMSO with 3 mL abs. dioxane, 1 mg (0.002 mmol) of Oregon Green 488 cadaverine, 71 μL (0.52 mmol) NEt_3 and 30 mg (0.4 mmol) 2-hydroxypropylamine were added and stirred for 3 d at 35 °C. After complete conversion the HPMA block copolymer was precipitated in ethyl ether and dialyzed against pure water (MWCO 14 000 g · mol^{-1}) obtaining 27 mg of the disulfide polylysine-*b*-p [HPMA] block copolymer with N_ε-Cbz-protected polylysine.

GPC (HFIP): $\overline{M}_n = 32\,100$, $Đ = 1.48$

In case of the disulfide linked block copolymer (polylsysine-S-S-*b*-p[HPMA] A–C), deprotection was carried out by reaction with 200 μL methane sulfonic acid ($MeSO_3H$) in 1.5 mL TFA for 3 h at room temperature. 1.5 mL of water was added and the reaction was stirred for additional 3 h at room temperature. After codestillation with toluene and dichloromethane the cationic block copolymer was first dialyzed against 10 mM NaCl solution and then against pure water (MWCO 14 000 or 25 000 g · mol^{-1}) and finally lyophilized obtaining 15 mg of an orange powder. The same polymer was synthesized without Oregon Green 488.

400 MHz 1H-NMR (dmso-d6): [d/ppm] = 7.40 (br. s 1H), 4.71 (br. s, 1H), 4.23 (br. s,0.12H), 3.67 (br. s, 1H), 2.91 (br. s, 2H), 1.69–0.84 (m, 12H).

GPC (HFIP): $\overline{M}_w / \overline{M}_n = 1.43$

Deprotection of the N_ε-benzyloxycarbonyl protecting group (N_ε-Cbz) of the polymer without disulfide bond (polylsysine-*b*-p[HPMA] A–C) was carried out by reaction with 100 μL 33 wt% HBr in 1.5 mL acetic acid for 90 min at room temperature. 1.5 mL of water was added and stirred for 90 min at room temperature. After codestillation with toluene and dichloromethane the cationic block copolymer was dialyzed against pure water (MWCO 14 000 or 25 000 g · mol^{-1}) and lyophilized.

2.16. Formation of Polymer–pDNA Complexes (Polyplex Micelles)

Desired amounts of pDNA were dissolved in water bio reagent grade in an *Eppendorf tube*. The cationic block copolymers P2 and P4 were dissolved in 10 mM Tris–HCl buffer ($c = 1$ mg · mL^{-1}) and slowly added to the pDNA in solution in different amounts as indicated. The resulting polyplexes were mixed by frequently pipetting and vortexting. The polyplexes were formed one day before the experiments. Complexes of pDNA and Jet-PEI were prepared according to manufacture instructions. pGL3-Basic vector (Promega GmbH Mannheim, Germany) was used for formation of polyplexes for agarose gel electrophoresis, FCS-measurements and CLSM. pEGFP-N1pDNA (Clonetech, Mountain View, CA) was used for determination of transfection efficiency and toxicity by FACS analysis.

2.17. Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed to determine optimal pDNA polymer mixing ratios. In brief, 160 ng pGL3-Basic vectorpDNA was mixed with the different polymersin different ratiosas described earlier and filled into the slots of a 0.5 wt% agarose gel with 6× loading buffer. The experiments were conducted at 120 V for 20 min and the polyplexes were visualized by staining the pDNA with GelRed and detected with UV light at 365 nm.

2.18. Fluorescence Correlation Spectroscopy

Fluorescence correlation spectroscopy (FCS) was performed using a commercial setup (Zeiss, Oberkochen, Germany) that consisted of the module ConfoCor 2 and an inverted microscope (Axiovert 200), equipped with a Zeiss C-Apochromat 40×/1.2 W water immersion objective. For excitation the 488 nm line of an argon laser was used, and collected fluorescence was filtered through a LP505 long pass emission filter before reaching an avalanche photodiode detector that enables single-photon counting. Eight-well polystyrene-chambered coverglass (Laboratory-Tek, Nalge Nunc International, Penfield, NY) was used as a sample cell. For each solution, a series of 10 measurements with a total duration of 5 min was performed. The confocal observation volume was calibrated using a reference dye with a known diffusion coefficient (i.e., Alexa Fluor 488).

2.19. Cell Culture

Adherent HEK 293-T cells were grown in culture medium (DMEM, supplemented with 10% FBS, 4 mM l-glutamine, 0.1 M 2-mercaptoethanol, 100 U · mL^{-1} penicilin and 100 μg · mL^{-1}streptomycin) and maintained at 37 °C at a humidified atmosphere with 10% CO_2 under sterile conditions. Prior to experiments, cells were harvested,

counted and the required number of cells (cells $\cdot$ mL^{-1}) was adjusted. All experiments with polyplexes formed by pDNA and block copolymer with bioreducible disulfide group (P4) were carried out without 0.1 M 2-mercaptoethanol.

2.20. Cellular Toxicity Determined by Flow Cytometry

Assessment of cytotoxicity on the level of single cells, HEK 293-T cells were seeded onto 12-well cell culture plates (1.5×10^5 cells in 1 mL) one day before experiments. The culture media was changed and cells were incubated with naked pDNA (pEGFP-N1, 5 μg per well, negative control), polyplexes derived from polymer P2 or P4 (5 μg pDNApEGFP-N1at N/P 7) or complexes with Jet-PEI (2.5 μg pEGFP-N1). The culture media was changed after 24 h andcells were harvested (PBS)/2 mM EDTA). After resuspension in HBSS buffer, the cells were coincubated with Alexa Fluor 647-conjugated Annexin V (BioLegend, San Diego, CA) for identification of early apoptotic cells and 7-AAD (BioLegend, San Diego, CA) to detect necrotic cells. Samples where analyzed by flow cytometry using a FACS Canto II flow cytometer quipped with BD FACS Diva software (BD Biosciences, San Diego, CA). Data were analyzed using FlowJo software (FLOWJO, Ashland, OR).

2.21. Transfection Efficiency Determined by Flow Cytometry

Assessment of transfection efficiency on the level of single cells was performed using pDNA encoding for Enhanced Green Fluorescent Protein (EGFP) (pEGFP-N1). In brief, HEK 293-T cells were seeded onto 12-well cell culture plates (1.5×10^5 cells in 1 mL) 1 d before experiments. The culture media was changed and cells were incubated with naked pDNA (5 μg pEGFP-N1 per well, negative control), polyplexes derived from polymers P2 or P4 (5 μg pEGFP-N1 at N/P 7) or complexes with Jet-PEI (2.5 μg pEGFP-N1). The culture media was changed after 24 h, cells were harvested (PBS)/2 mM EDTA) and analyzed by flow cytometry (see above) for determination of EGFP positive cells.

2.22. Confocal Microscopy

HEK-293T cells were seeded on a μ-slide (cover glass with chambers) in a 8-well (Ibidi) format with 2×10^5 cells $\cdot$ mL^{-1}. 24 h after incubation at 37 °C the medium was changed to OptiMEM and the cells were incubated with different polyplexes composed of pGL3-Basic vector and polymers P2 or P4 (N/P 7) at 37 °C. Afterward, the cells were analyzed in a 8-well format by confocal microscopy. Pictures were recorded with a Leica Microscope (with "live cell Unit") and analyzed with Leica Application suite software.

3. Results and Discussion

The combination of controlled radical polymerization (RAFT-polymerization) of reactive ester monomers, like pentafluorophenyl methacrylate and sequential postpolymerization with 2-hydroxypropylamine can afford multifunctional p[HPMA] based block copolymers of various topologies.[44,53] These p[HPMA] based block copolymers are usually obtained by sequential RAFT polymerization of two different methacrylate monomers, including pentafluorophenyl methacrylate, with low molecular weight chain transfer agents (CTAs).

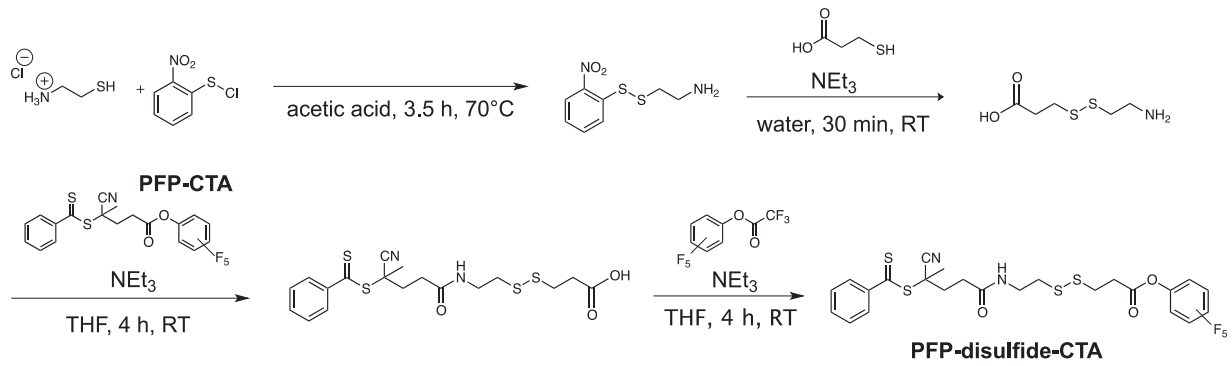
We modified this synthetic strategy to obtain semi-peptidic polypeptide-*b*-p[HPMA] block copolymers, more precisely cationic polylysine-*b*-p[HPMA] block copolymers, which can be used for pDNA delivery (transfection). Therefore, we use macromolecular-polylysine chain transfer agents (polylysine-CTAs) for RAFT-polymerization of PFMA.[45] Postpolymerization modification with 2-hydroxypropylamine and deprotection of the polylysine block under acidic conditions lead to cationic polylysine-*b*-p[HPMA] block copolymers. We now further extended this synthetic approach to obtain cationic polylysine-*b*-p[HPMA] block polymers with a bioreducible disulfide group between the blocks to increase transfection properties.

3.1. Polymer Synthesis

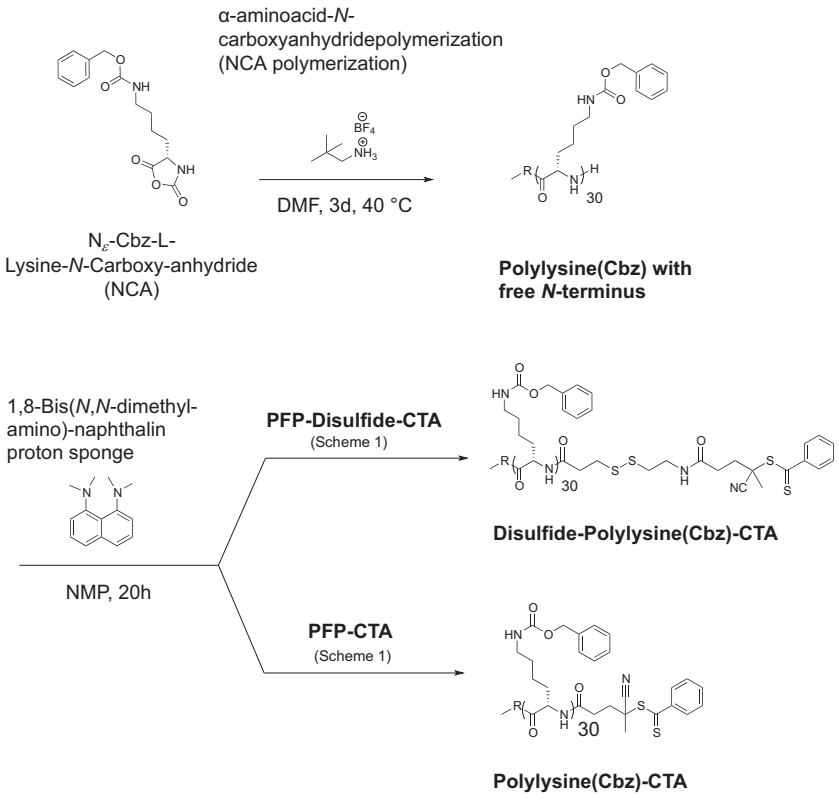
Cationic polylysine-*b*-p[HPMA] block copolymers with a bioreducible disulfide bond were synthesized by RAFT-polymerization of the reactive ester monomer pentafluorophenyl methacrylate with macromolecular, peptidic disulfide-polylysine chain transfer agents with a blocked N_ε-side group (Disulfide-Polylysine(Cbz)-CTA). The p[PFMA] block of the resulting hydrophobic block copolymers polylysine(Cbz)-S-S-*b*-poly[PFMA] A–C was reacted with 2-hydroxypropylamine (HPA) to prepare poly[*N*-(2-hydroxypropyl methacrylate (p[HPMA]). After final removal of the lysine's N_ε-protecting group under acidic conditions, cationic polylysine-S-S-*b*-p[HPMA] block copolymers A–C (P4–P6) are obtained. Simultaneously, the same block copolymers were synthesized without a disulfide bond (polylysine-*b*-p[HPMA] block copolymers A–C (P1–P3) (Scheme 1–3).

For the synthesis of bioreducible structures, an asymmetric disulfide-CTA (PFP-disulfide-CTA) was synthesized in multiple steps by reaction with the conventional PFP-CTA (Scheme 1). The resulting PFP-disulfide CTA contains both, a dithioester group for RAFT-polymerization and an activated pentafluorophenyl ester moiety for furher modification.

In parallel, poly-L-lysine with N_ε-benzyloxycarbonyl protecting groups (i.e., (polylysine(Cbz) was obtained by polymerization of corresponding α-amino acid-*N*-carboxy anhydrides (Scheme 2). NCA-polymerization was used since well-defined polylsine(Cbz) can be synthesized with a high degree of polymerization and in good yields (g scale)[52] compared to solid phase peptide synthesis (SPPS). In



Scheme 1. Multistep synthesis of low molecular weight chain transfer agent with disulfide bond between the dithioester and the activated ester group (PFP-disulfide-CTA).



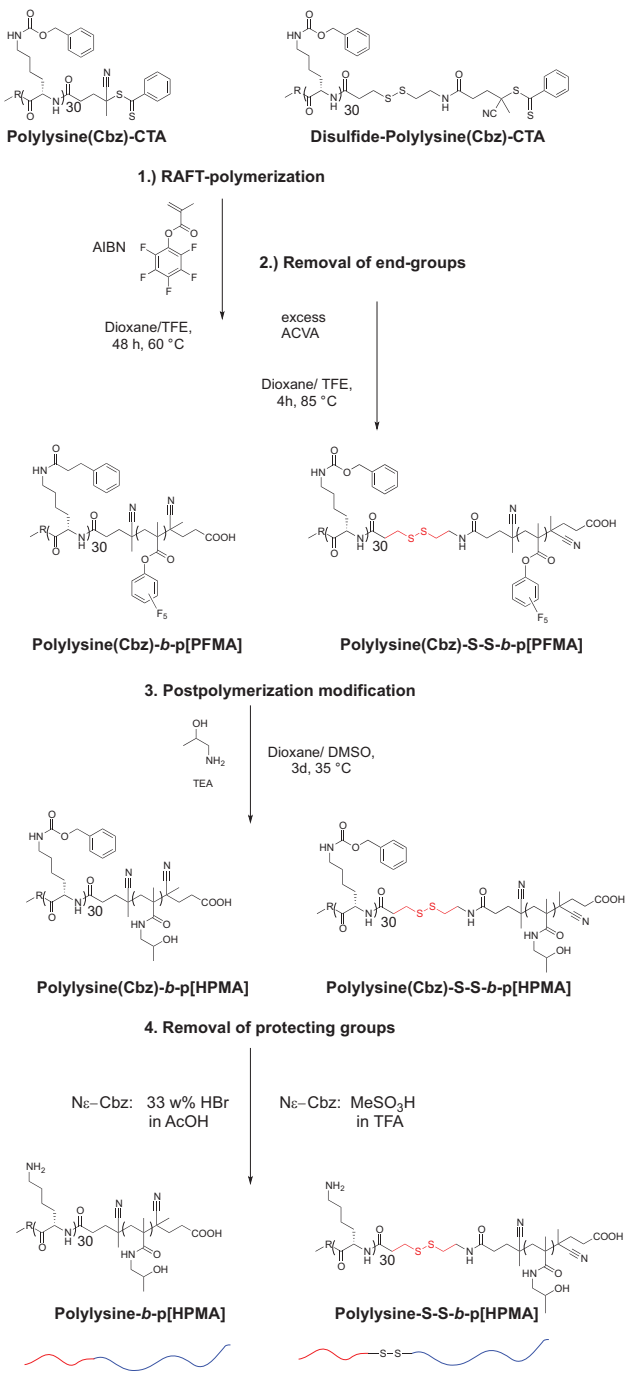
Scheme 2. Synthesis of poly-L-lysine with N_ε-benzyloxycarbonyl (N_ε-Cbz) protecting group by polymerization of α-amino acid-*N*-carboxy anhydrides (NCA-polymerization) and subsequent modification at the *N*-terminus with PFP-disulfide-CTA or PFP-CTA obtaining disulfide-polylsine(Cbz)-CTA and polylysine(Cbz)-CTA.

previous work of Kataoka et al., it could be shown that the optimal length of the polylysine block for pDNA complexation varies between a degree of polymerization of $30 \leq X_n \leq 50$.[39] Moreover, we achieved the highest transfection efficiency for polylysine-*b*-p[HPMA] polymers with polylysine obtained by NCA-polymerization with $X_n = 30$.[45] That is why we chose polylysine with a degree of polymerization of 30 to prepare disulfide-polylysine(Cbz)-CTA. For the synthesis of macromolecular polylysine CTA with and without redox cleavable disulfide bond polylysine$(Cbz)_{30}$ was modified with PFP-CTA or rather PFP-disulfide-CTA to obtain polylysine(Cbz) CTA and disulfide-polylysine(Cbz)-CTA (Scheme 2). Both CTAs were characterized by GPC in HFIP (Table 1).

The macromolecular, peptidic chain transfer agents polylysine(Cbz)-CTA and disulfide-polylysine(Cbz)-CTA have then been applied for RAFT-polymerization of pentafluorophenyl methacrylate (PFMA) leading initially to hydrophobic block copolymers polylysine(Cbz)-*b*-p[PFMA] A–C and polylysine(Cbz)-S-S-*b*-p[PFMA] A–C (Scheme 3). A mixture of dioxane/

Table 1. Characterization of disulfide-polylysine-CTA and polylysine(Cbz)-CTA with GPC in HFIP.

CTA	X_N (NMR)	$\overline{M}_n$ (HFIP-GPC) [g · mol^{-1}]	$Đ$ (HFIP-GPC)
Polylysine(Cbz)-CTA	30	11 300	1.11
Disulfide-polylysine(Cbz)-CTA	30	11 000	1.13



Scheme 3. Synthesis of Polylysine-*b*-poly[HPMA] A–C **P1–P3** and polylysine-S-S-*b*-p[HPMA] A–C (**P4–P6**) by RAFT polymerization of PFMA with disulfide-polylysine-CTA or polylysine CTA, subsequent end-group removal with ACVA, postpolymerization modification with 2-hydroxypropylamine, and protecting group cleavage under acidic conditions.

trifluoroethanol (TFE) (8:1) was thereby used to ensure solubility of both the polylysine-CTAs and PFMA monomer. Afterward, the dithioester end-group was removed with an excess of 4,4-azobis(4-cyanovaleric acid) (ACVA) to prevent undesired side reactions.[54] Noteworthily, the disulfide bond showed stability both toward the polymerization conditions and during end-group removal (this observation follows in partresults reported in ref.[55]). Postpolymerization modification with 2-hydroxypropylamine (HPA) yielded block copolymers without (polylysine(Cbz)-*b*-p[HPMA] A–C) and with disulfide bond between the two blocks (polylysine(Cbz)-S-S-*b*-p[HPMA] A–C) (Scheme 3, Table 2).

For each CTA, three different polymers have been synthesized. The AIBN/polylysine CTA/monomer (PFMA) ratios have thereby been chosen to obtain block copolymers with large p[PFMA] or rather p[HPMA] blocks after polymeranalogous modification, since the size of the hydrophilic p[HPMA] block should be much bigger than the cationic part for efficient shielding of the charged polyplexes.[39,56] Supporting Information S1 and S2 display the elution diagrams of both polylysine(Cbz)-CTA and disulfide-polylysine(Cbz)-CTA and the corresponding block copolymers polylysine(Cbz)-*b*-poly[HPMA] A–C without disulfide group and polylysine(Cbz)-S-S-*b*-poly[HPMA] A–C with disulfide, respectively. It shows the strong increase in molecular weight during successful RAFT polymerization. However, measurements of N_ε-benzyloxycarbonyl-protected polylysine block copolymers by GPC in HFIP (Table 2) may give incorrect results due to secondary structures of protected polylysine.[52] A more precise characterization of the molecular weight of polylysine-*b*-poly[HPMA] block copolymers was achieved by ^{1}H-NMR-measurements after deprotection of the polylysine(Cbz) block (P1–P6) (Table 3).

To verify that the redox cleavable disulfide bond is still existing (non reacted) after all reaction steps, the protected block copolymers polylysine(Cbz)-S-S-*b*-poly[HPMA] have been treated with tris(2-carboxy ethyl-phosphin) (TCEP) in aqueous solution overnight and directly submit to GPC measurements with HFIP as solvent. Figure 1 shows the elution profile for every block copolymer before and after treatment with TCEP. In all cases it can nicely been seen that reduction leads to a decrease in molecular weight due to cleavage of the disulfide bond. The shift to lower molecular weights is relatively small, since the shown p[HPMA] block is quite larger than the polylysine block. In addition, a signal corresponding to the size of the cleaved polylysine block increases in size with an elution volume of about 18 ml. This clearly proves the presence of an intact disulfide bond between the polylysine and the p[HPMA] block (Figure 1).

In a final step the N_ε-benzyloxycarbonyl-protecting group of the polylysine block was removed under acidic conditions (Scheme 3). In case of the **polylysine(Cbz)-*b*-poly[HPMA] A–C** 33 wt% HBr in acetic acid can be used for an efficient deprotection without side reactions.[45,52] In

Table 2. Characterization of **polylysine(Cbz)-*b*-p[HPMA]** block copolymers **A–C** without disulfide and **polylysine(Cbz)-S-S-*b*-p[HPMA] A–C** with disulfide by GPC in HFIP.

Polymer	Polylysine-CTA used	$\overline{M}_n$ Polylysine CTA (HFIP-GPC) [g · mol^{-1}]	Ratio AIBN/ Polylysine-CTA/monomer (PFMA)	$\overline{M}_n$ Polylysine(Cbz)-*b*-poly[HPMA] (HFIP-GPC) [g · mol^{-1}]	*Đ* (HFIP-GPC)
Polylysine(Cbz)-*b*-p[HPMA] A	Polylysine(Cbz) 30-CTA	11 300	1/6/708	18 700	1.40
Polylysine(Cbz)-*b*-poly[HPMA] B	Polylysine(Cbz) 30-CTA	11 300	1/6/2067	38 500	1.47
Polylysine(Cbz)-*b*-poly[HPMA] C	Polylysine(Cbz) 30-CTA	11 300	1/6/2638	55 100	1.35
Polylysine(Cbz)-S-S-*b*-p[HPMA] A	Disulfide-polylysine(Cbz) 30-CTA	11 000	1/6/1000	32 100	1.48
Polylysine(Cbz)-S-S-*b*-p[HPMA] B	Disulfide-polylysine(Cbz) 30-CTA	11 000	1/6/1714	41 500	1.50
Polylysine(Cbz)-S-S-*b*-p[HPMA] C	Disulfide-polylysine(Cbz) 30-CTA	11 000	1/6/2224	47 100	1.54

Table 3. Characteristics of polylysine-*b*-poly[HPMA] A–C (P 1–3) and polylysine-S-S-*b*-p[HPMA] A–C (P4–6).

Polymer	$\overline{M}_n$ (NMR) polylysine-*b*-poly[HPMA]	*Đ (HFIP-GPC)* polylysine(X)-*b*-poly[HPMA]	Ratios X_n (poly[HPMA]) to X_n (polylysine) (NMR)
Polylysine-*b*-poly[HPMA] A P1	9 400	1.57	61:30 (2:1)
Polylysine-*b*-poly[HPMA] B P2	30 500	1.48	200:30 (6:1)
Polylysine-*b*-poly[HPMA] C P3	57 100	1.32	390:30 (13:1)
Polylysine-S-S-*b*-p[HPMA] A P4	36 800	1.43	230:30 (7:1)
Polylysine-S-S-*b*-p[HPMA] B P5	46 900	1.49	300:30 (10:1)
Polylysine-S-S-*b*-p[HPMA] C P6	65 000	1.52	430:30 (14:1)

case of the **polylysine(Cbz)-S-S-*b*-poly[HPMA] A–C** block copolymers, deprotection with 33 wt% HBr leads to cleavage of disulfide bond, probably due to readily oxidation of bromine (data not shown). That is why deprotection was carried out with methane sulfonic acid in trifluoroacetic acid (TFA).[57] (Supporting information S3 shows the ^{1}H-NMR spectra of successful removal of the N_ε-benzyloxycarbonyl-protecting group of polylysine-S-S-*b*-poly[HPMA]). Figure 2 additionally displays the GPC elution profile of the block copolymer **polylysine(Cbz)-S-S-*b*-poly[HPMA] A**, before and after deprotection (**polylysine-S-S-*b*-poly[HPMA] A, P4)** as well as after reduction (=cleavage) with TCEP. Since, the elution profile shows no significant difference between the blue and the red line, deprotection with methane sulfonic acid does not lead to disulfide bond cleavage compared to treatment with TCEP (green line) (Figure 2).

The ratio of the p[HPMA] block to the polylysine segments and the resulting molecular weight of the block copolymers were calculated by ^{1}H-NMR-spectroscopy, since GPC measurement might give false results due the cationic character of polylysine (rod shape rather than random coil). Table 3 displays the characteristics of the final cationic block copolymers without (P 1–3) and with disulfide bond (P 4–6). For in vitro studies P2 and P4 have been chosen, since they exhibit similarand most optimal block ratios of cationicpolylysine to the shielding p [HPMA] block (1:6 for P2 and 1:7 for P4; Table 3). Figure 3 displays the elution profiles for the (Cbz)-polylysine-CTA and disulfide (Cbz)-polylysine-CTA and the corresponding

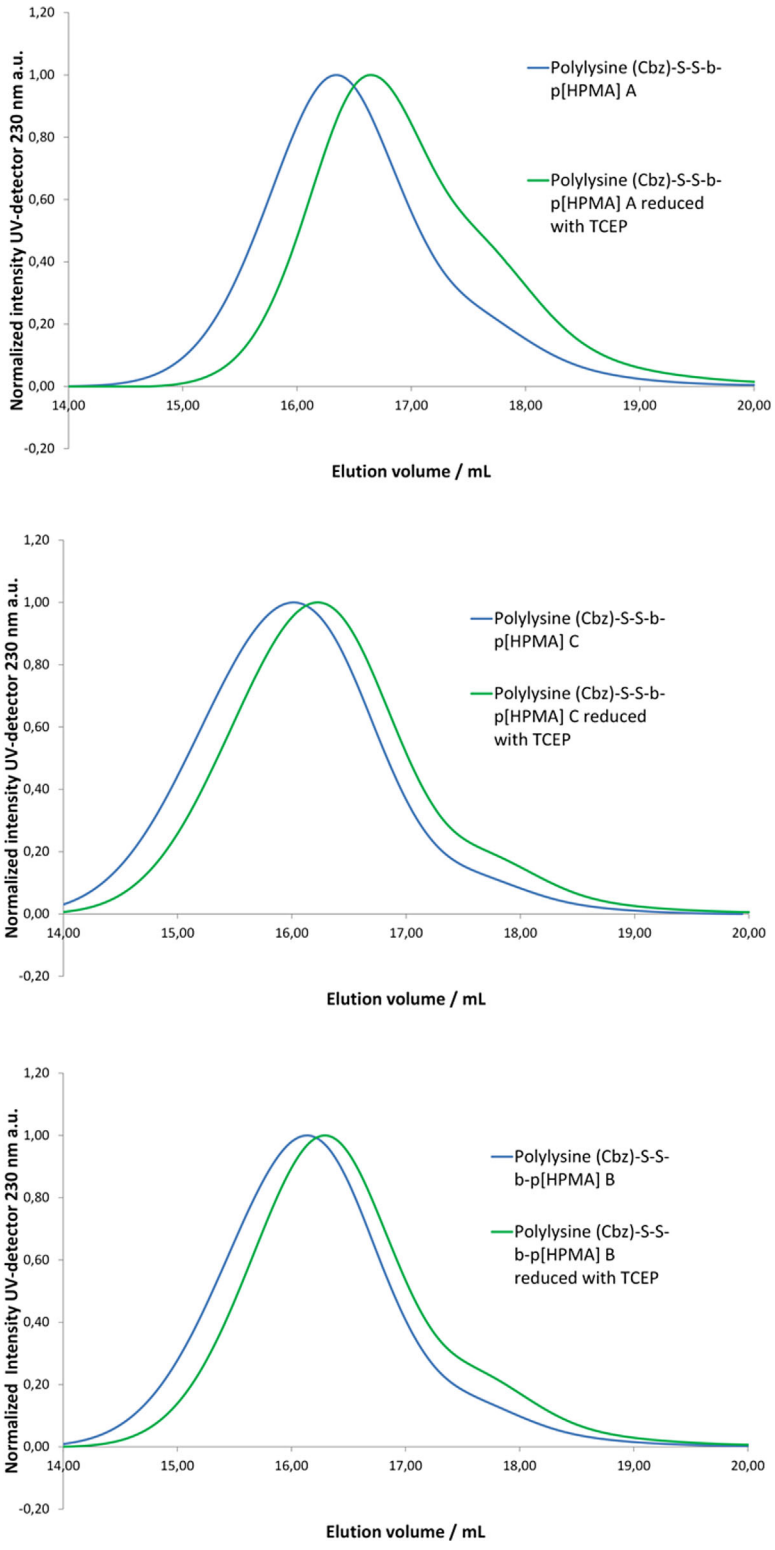


Figure 1. Proof of presence of a disulfide bond by directed reduction of block copolymers **polylysine(Cbz)-S-S-*b*-poly[HPMA] A–C** through overnight treatment with TCEP.

cationic block copolymers P2 and P4 derived from RAFT polymerization and several polymeranalogues reaction steps.

These results nicely show that an asymmetric disulfide could be synthesized in a multistep reaction, which contains both a dithioester group for RAFT-polymerization and an activated PFP group (PFP-disulfide-CTA) (Scheme 1). Additionally, a well defined polylysine (X_n:30) with N_ε-benzyloxycarbonyl protecting group (polylsine (Cbz)) obtained by NCA-polymerization could be modified with this PFP-disulfide (Cbz)-CTA selectively at the *N*-terminus obtaining a macromolecular, peptidic chain transfer agents with a biocleavable disulfide group (disulfide-polylysine(Cbz)-CTA) (Scheme 2). Controlled RAFT polymerization utilizing both the CTA with disulfide (disulfide-polylysine(Cbz)-CTA) and without disulfide bond (polylysine(Cbz)-CTA) and PFMA monomer (rather hydrophobic), and subsequent postpolymerization modification with 2-hydroxypropylamine leads to polylysine(Cbz)-S-S-*b*-p[HPMA] A–C and polylysine(Cbz)-*b*-p[HPMA] A–C. Different molecular weights of the block copolymers could be obtained by varying polylysine (Cbz)-CTA to monomer ratios (Table 2). Most importantly, the presence of the disulfide bond for the polylysine(Cbz)-S-S-*b*-p[HPMA] A–C block copolymers was proved by direct reduction (=cleavage) with TCEP, thus showing that the disulfide is stable during synthesis. After removal of the N_ε-benzyloxycarbonyl protecting group of the polylysine block under acidic conditions cationic block copolymers P 1–3 and P 4–6 were obtained. Noteworthily, for the deprotection of disulfide containing polymers, methane sulfonic acid in TFA had to be used instead of HBr in acetic acid to prevent undesired cleavage of the disulfide bond. Cationic polymers with disulfide (P4) and without disulfide (P2) have been chosen for detailed in vitro transfection experiments with HEK-293T cells due to their similar and most optimal block lengths ratio of cationic polylysine to the (p[HPMA]) shielding block (1:6 for P2 and 1:7 for P4) in order to test the significance of the disulfide bond.

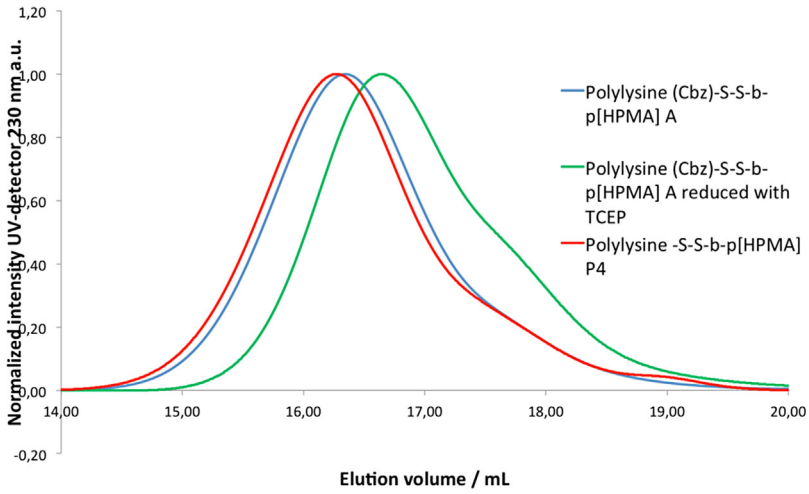


Figure 2. GPC elution profile of **polylysine(Cbz)-S-S-*b*-poly[HPMA] A** (blue), deprotected **polylysine-S-S-*b*-poly[HPMA] A** (red) and cleaved block copolymer after reduction with TCEP (green).

3.2. Polyplex Formation and Physicochemical Characterization

Polyplexes were formed by simple mixing of pDNA with the different cationic block copolymers P2 and P4. Thereafter, the corresponding polymers were dissolved in TRIS-buffer and added to the pDNA (pGL3-Basic vector or pEGFP-N1) in water at the desired concentrations, thoroughly mixed and incubatedfor 18 h (Scheme 4).

In order to test the capability of pDNA condensation and to determine suitable polymer to pDNA ratios we performed agarose gel electrophoresis (Figure 4). As to be expected increased retardation of pDNA was observed with increasing amounts of polymer added. For both block copolymers (P2 and P4) complete encapsulation of the pDNA was observed. A more precise expression of polymer to pDNA ratio is the N/P value. The N/P value of 1 implies equal amounts of negative charges on pDNA and positive charges on the polymer. 1 μg of pDNA represents 3.07×10^{-9} mol of negative charges. With the known molecular weights of block copolymers and the number of positive charges on each chain (equal to the degree of polymerization of polylysine), the amount of polymer needed for N/P 1, etc. can be calculated. It can nicely be seen that both polymers P2 and P4 mediate pDNA encapsulation at N/P ratios of higher or equal to one. For transfection, N/P ratios of 7 have been used to ensure absence of free, non complexed pDNA (Figure 4, white arrows).

For a more detailed characterization of the polyplexes we performed fluorescence correlation spectroscopy (FCS) studies of Oregon Green 488-labeled polymers P2 and P4 before and after mixing with pDNA (pGL3-Basic vector) (supporting information, Figure S4). FCS is a very sensitive technique that allows precise measurement of the hydrodynamic radii of fluorescent species in solution[58] and thus it is especially suitable to study the formation of complexes between the relatively small fluorescent polymers and the significantly larger non-fluorescent pDNA. Thereby, we found a hydrodynamic radius of 58 nm for the polyplex formed by pDNA and P2 and of 40 nm for the polyplexes formed by pDNA and P4 (bothat N/P 7). For comparison the hydrodynamic radii of P2 and P4, were found to be 4.1 and 3.0 nm respectively (Figure S4). Thus, the polymers P2 and P4 show similar pDNA encapsulation properties and hydrodynamic diameters as expected.

3.3. Biological Evaluation of Polyplexes Derived from pDNA and Polymers P2 and P4 with HEK 293-T Cells

3.3.1. Cellular Binding and Uptake

In order to determine cellular binding and successful internalization, polyplexes composed of Oregon Green 488-labeled polymers P2 and P4 and pDNA (pGL3-Basic vector) (N/P 7) were subjected to in vitro experiments using HEK 293-T cells. Confocal laser scanning microscopy (CLSM) was performed after incubating the cells with the polyplexes for 24 h (Figure 5). The images reveal that both polyplexes P2 and P4 are successfully internalized by HEK-293T cells. Thus, the precondition for a transfection is given.

3.3.2. Transfection Efficiencies

For a successful transfection, the pDNA needs to escape from endosomal compartments and it needs to be released from the polyplexes after entering the cell in order to be expressed. To determine transfection efficiencies, we employed fluorescence activated cell sorting (FACS) to detect expression of a pDNA-encoded fluorescence reporter. This method is advantageous compared to the conventional *Luciferase assay*, since transfection efficiencies can be determined on single cell level.[38,45] Thus, a deeper insight in transfection efficiencies is obtained. Thereto, polyplexes were formed with pDNA, which encodes for *enhanced green fluorescent protein* (pEGFP-N1). Hence, transfected cells are detectable by FACS measurements due to EGFP expression (this cannot be done with Luciferase assays due to fast disappearance of bioluminescence). HEK-293T cells were incubated with polyplexes formed by pEGFP-N1 and polymers P2 and P4, eachat N/P values of 7. Additionally, cells were treated with naked pDNA (neg. control) and polyplexes with commercial, polymeric transfection reagent Jet-PEI (pos. control). Frequencies of transfected

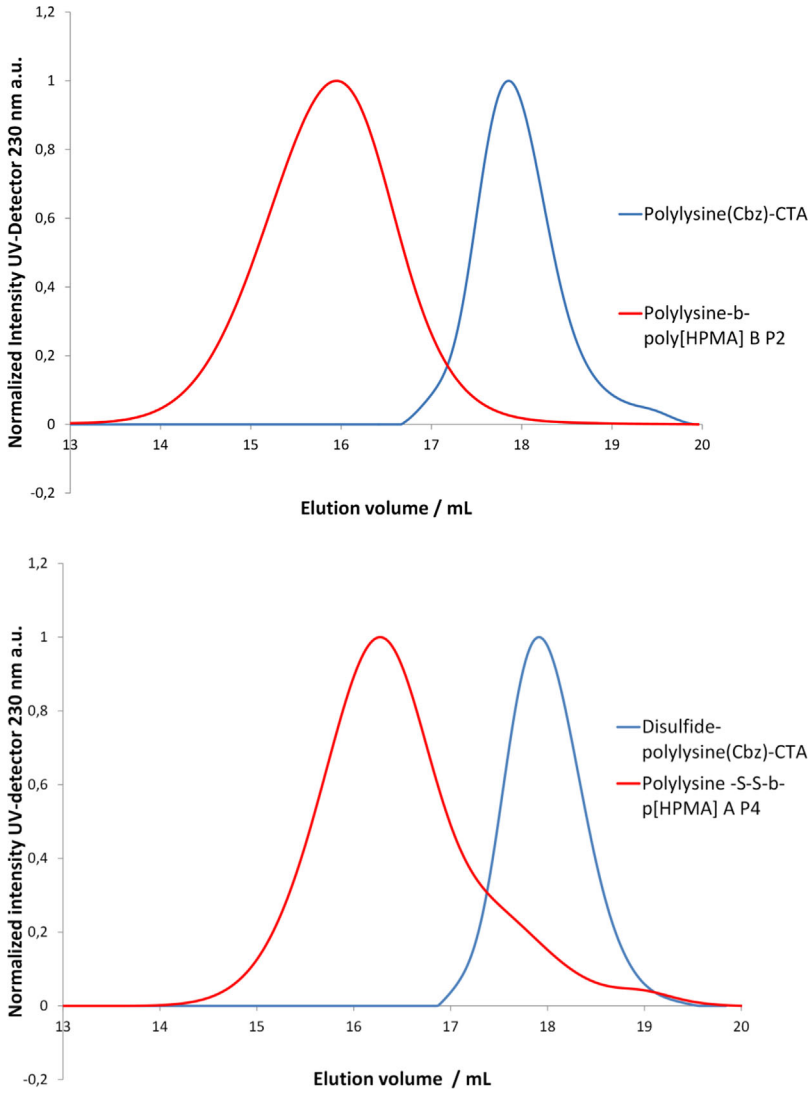


Figure 3. GPC elution profiles for the polylysine(Cbz)-CTA and disulfide(Cbz)-polylysine-CTA and the corresponding cationic block copolymers P2 and P4, respectively, in HFIP.

cells were determined by FACS measurements 24 h after incubation (Figure 6). The mean fluorescence intensities of EGFP of one representative experiment are given in Figure S5.

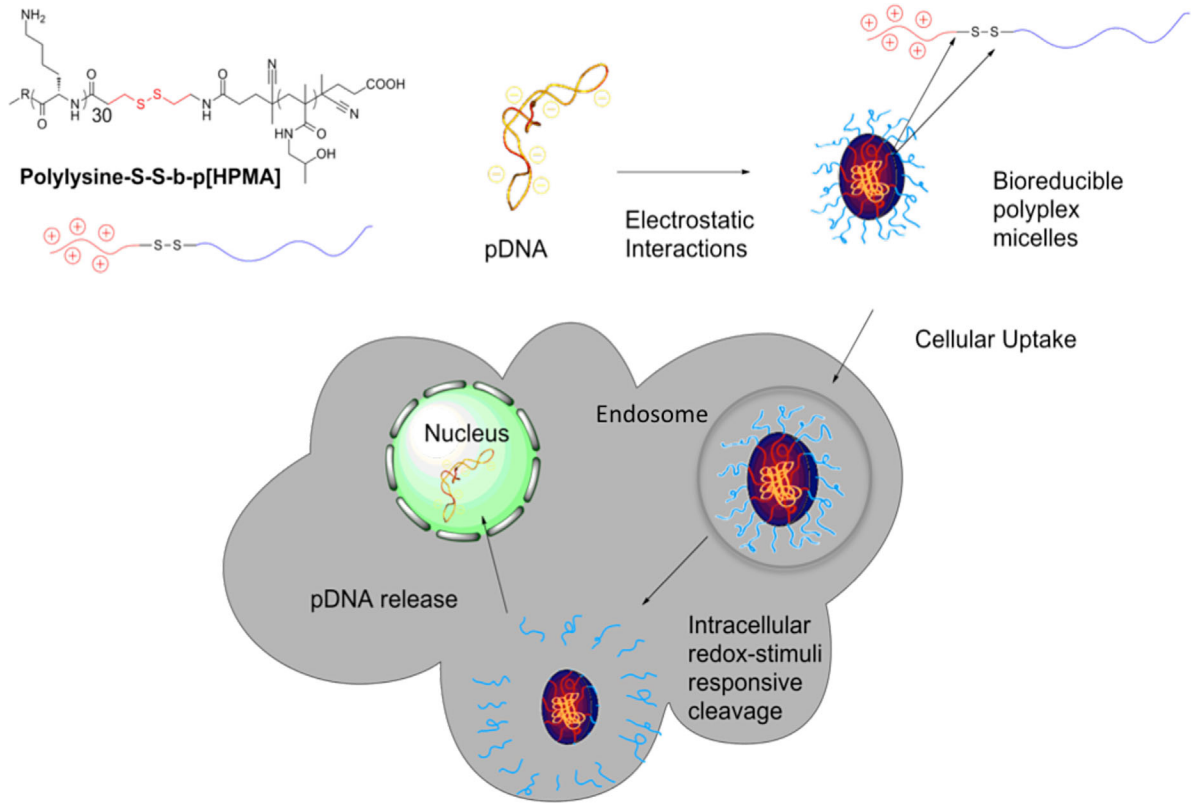
We aimed to increase the transfection efficiency by introducing a bioreducible disulfide bond between the complexing polylysine and the shielding p[HPMA] block. While treatment with naked pDNA resulted in no detectable transfection (as to be expected), low transfection efficiencies were obtained with polyplexes composed of pDNA and polymer P2. In contrast, polyplexes composed of pDNA and polymer P4 yielded strong transfection, in a pDNA dose-dependent manner. At higher dose of pDNA (5 μg), P4-derived polyplexses mediated a higher frequency of $EGFP^+$ cells than obtained for the positive control (JetPEI-derived polyplexes). Interestingly, in case of JetPEI either dose of pDNA resulted in comparable transfection of HEK-293 T cells. This shows—first of all—that the disulfide bond mediates a very strong increase in efficiency (since the polymers are almost identical, except for the disulfide bond in P4), due to an intracellular reductive cleavage of the p[HPMA] corona and improved release of cargo pDNA. Importantly, bioreducible polyplexes with P4 are as effective as the commercial transfection reagent Jet-PEI. This very nicely shows, that the redox-stimuli responsive, intracellular cleavage of the p[HPMA] corona leads to a strong increase in transfection efficiency.

3.3.3. Cytoxicity of Polyplexes

For efficient systems it remains most challenging to increase the transfection efficiency, but to keep a low cytoxicity at the same time. In fact, toxicity is a natural result of the polycationic nature of the vector, which is—on the other side—needed for DNA condensation and endosomal escape. The need for low toxicity becomes especially important when dealing with sensitive immune cells such as DCs and it is an essential precondition for in vivo applications of transfection agents.

A precise determination of cytotoxicity can be achieved via FACS measurements. By staining the cells with Annexin V, which binds to phosphatidylserine at the extracellular membrane, early apoptotic cells are detected. The dye 7-AAD, which enters disintegrated cell membranes only, intercalates with chromosomal DNA, and serves to stain necrotic cells. Cells that are double-positive for Annexin V and 7-AAD represent a late apoptotic state. This approach is advantageous to conventional MTT assays, since more detailed information on cytotoxicity is obtained on single cell-level.

To assess potential cytotoxicity of polyplexes composed of pDNA and P2 and P4, we performed according FACS measurements. 24 h after transfection of HEK-293 T cells with naked pDNA (pEGFP-N1) as a negative control, and according polyplexes using Jet-PEI (pos. control).



Scheme 4. Formation of bioreducible polyplexes with negatively charged pDNA and cationic, redox-stimuli responsive polymer polylysine-S-S-*b*-p[HPMA] (**P4**) for improved transfection properties, due to intracellular polyplex deshielding (cleavage of the p[HPMA] corona).

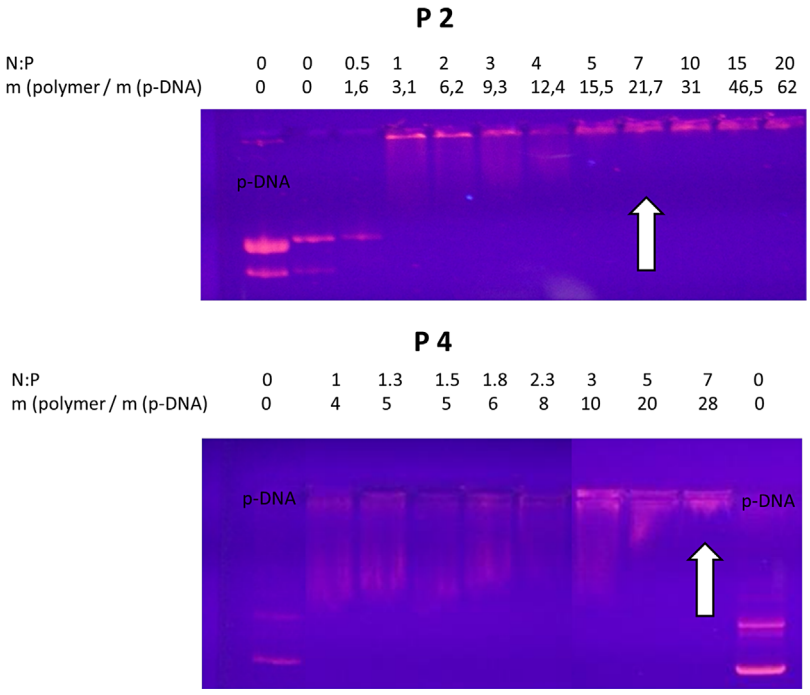


Figure 4. Agarose gel electrophoresis with pDNA and polyplexes composed of pDNA and polymers **P2** and **P4** at different N/P ratios as indicated.

As shown in Figure 7, transfection of HEK-293 T cells with polyplexes yielded no enhanced apoptosis or necrosis as compared with the negative control. These results clearly indicate that introduction of a disulfide group mediates a strong increase in transfection efficiency without increasing cytotoxicity and, thus, makes polyplexes composed of pDNA and P4 highly suitable transfection reagents for sensitive cells.

4. Conclusion

The multistep synthesis of a disulfide CTA allows the preparation of N_{ε}-benzyloxycarbonyl (Cbz) protected polylysine chain transfer agents (CTAs) with an internal disulfide group. Subsequent RAFT polymerization experiments with pentafluorphenyl-methacrylate demonstrate that this disulfide group is inert under the conditions of

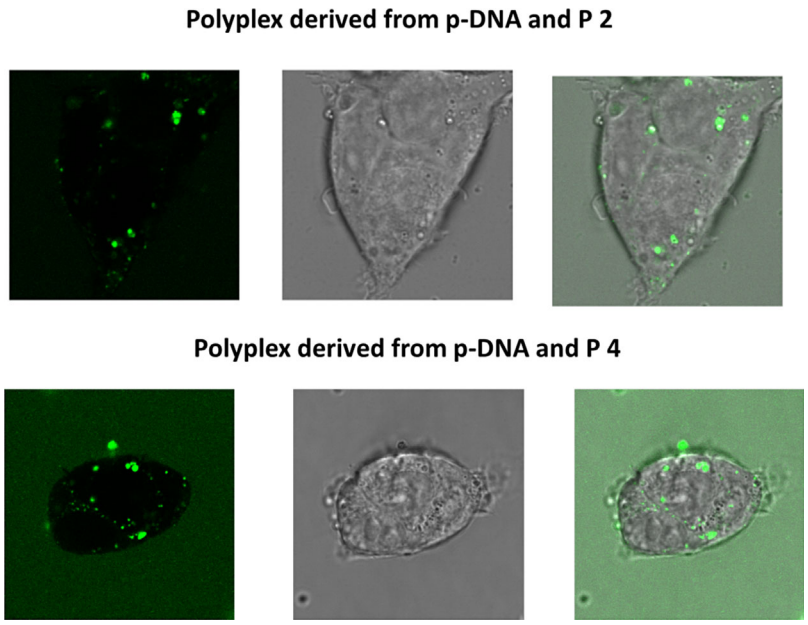


Figure 5. CLSM of HEK-293 T cells 24 h after incubation with polyplexes composed of pDNA and polymers P2 and P4 (N/P 7) (green: polymer dye Oregon Green 488).

radical polymerization. It gets, thus, possible to obtain block copolymers from Cbz protected polylysine and poly(PFMA). Conversion of the reactive ester block with 2-hydroxypropylamine and deprotection of the polylysine block makes block copolymers from the cationic poly(lysine) and the biocompatible poly(HPMA) available, which are linked by a disulfide group. They can thus be split under reductive conditions.

The resulting cationic block copolymer forms polyplexes with pDNA in a size range of 40 nm in radius at optimized N/P values of 7. Transfection experiments with HEK-cells demonstrate that polyplexes containing the bioreducible disulfide groups possess a better transfection efficiency than polyplexes from analogues blockcopolymer, but without disulfide group. Finally transfection efficiencies of polylysine-S-S-*b*-p[HPMA] block copolymers are comparable to these of Jet-PEI and in addition they show lower cytotoxicity. This makes the new polylysine-S-S-*b*-p[HPMA biosplittable block copolymers interesting for the transfection of sensitive immune cells.

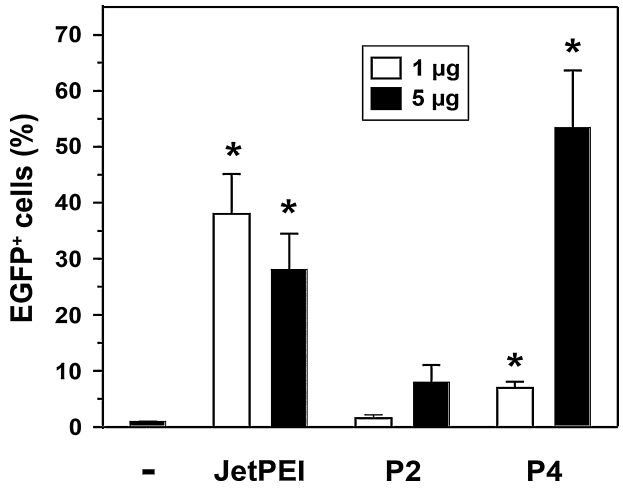


Figure 6. Frequencies of transfected HEK-293T cells as determined with FACS using pDNA encoding for EGFP (for polyplexes derived from or P2 and P4 (N/P 7)). Two different pDNA concentrations (1 µg: white bars, 5 µg: black bars) were used in parallel. FACS analysis was performed 24 h after the onset of incubation. Naked pDNA served as a negative control, and polyplexes with Jet-PEI as a positive control (*y*-axis: Frequencies of GFP-positive cells; data represent the mean ± SEM of 3 independent experiments). Statistical significant difference: versus negative control (−). $^{*}P < 0.05$.

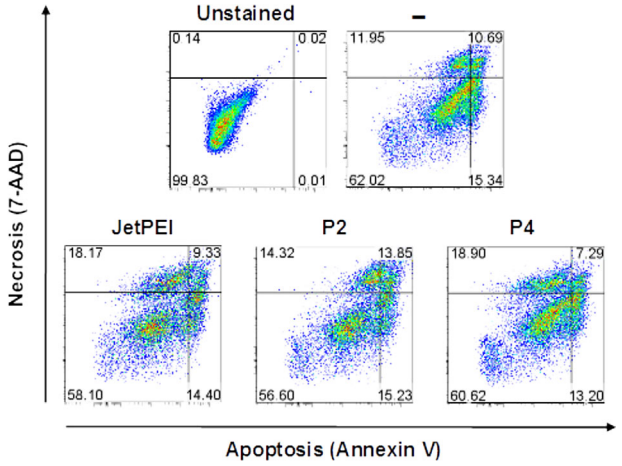


Figure 7. Frequencies of apoptotic/necrotic HEK-293 T cells as assessed 24 h after transfection with naked-pDNA as negative control (−), polyplexes derived from Jet-PEI, and polyplexes composed of **P2** and **P4** (each N/P 7). Frequencies of early apoptotic (Annexin V^+), late apoptotic (Annexin V^+7-AAD^+) and necrotic (7-AAD^+) cells are indicated. Untreated HEK-293 T cells left untreated and unstained were used for gating. Graphs are representative for one of two independent experiments.

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