Synthesis and Characterization of Poly(ethylene oxide)/polylactide/polylysine Tri-arm Star Copolymers for Gene Delivery

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ABSTRACT

Amphiphilic cationic poly(ethylene oxide)-S(polylysine)-poly(*D,L*-lactide) (mPEO-S(CK_n)-PLA) tri-arm star copolymers were synthesized by a combination of ring opening polymerization (ROP) and a thiol-disulfide exchange. The mPEO-S(CK_n)-PLA copolymers were found to be non-cytotoxic and could effectively condense GFP plasmid DNA into nanometer-sized complexes, as characterized by dynamic light scattering (DLS), suitable for endocytotic cellular uptake. *In vitro* DNA transfection studies showed that the amphiphilic structure is capable of DNA transfection and GFP expression. Addition of chloroquine into the medium further enhanced the DNA transfection efficiency.

KEYWORDS: star copolymers; gene transfection; ring-opening polymerization

INTRODUCTION

Amphiphilic block copolymers which are composed of a hydrophilic and a hydrophobic block can self-assemble into spherical micelles, worm-like micelles, vesicles and other ordered structures in block-selective solvents.[1-3] Micelles and vesicles formed by amphipihilic block copolymers, especially stimuli-responsive micelles and vesicles[4-5] have been widely investigated for biological applications such as gene therapy and drug delivery.[6-10]

Poly(ethylene oxide) (PEO) is often incorporated into amphiphilic copolymers as the hydrophilic block in order to both reduce interactions of polymer assemblies with serum proteins and to increase the stability of assemblies.[11-12] Poly(lactic acid) (PLA) is a biodegradable and biocompatible polyester which has been widely used as a hydrophobic block in amphiphilic block and triblock copolymers.[13-14] PEO-*block*-PLA diblock copolymers have been extensively studied as biological delivery systems because of their low cytotoxicity, biodegradability, and biocompatibility.[9-10, 13-15]

Although neutral block copolymer micelles and vesicles have been reported as gene delivery systems,[13] the use of cationic blocks or functional groups, which can pair with the anionic phosphate ester groups in nucleic acid backbones, has been widely adopted for the preparation of more efficient gene delivery systems. Amine-functionalized polymers including polyethylenimine (PEI) and its derivatives,[16-18] poly(*L*-lysine) (PLL),[19-21] and polyamidoamine (PAMAM) dendrimers[22-24] are

among the most widely applied cationic polymers in gene delivery systems. PLL is a biodegradable poly(amino acid) with pendent amine groups that can ionically interact with the negatively charged DNA backbone.[19-20] Although PLL shows great ability to condense DNA, it exhibits relatively high cytotoxicity despite the fact that the monomer *L*-lysine is not cytotoxic. Complexes formed by DNA and PLL show low gene transfection efficiency and need the addition of chloroquine to improve DNA transfection.[19-20] Since polyplexes of high molecular weight PLL with DNA aggregate or even precipitate in solution, addition of polyethylene oxide (PEO) blocks to PLL has been used to prevent the formation of insoluble precipitates.[25] The addition of hydrophobic components to PLL chains has been demonstrated to weaken DNA binding,[26] so the addition of PLL blocks to PEO-PLA copolymers provides a method through which to control the DNA release profile. PEG/PLL-based copolymer micelles with poly(leucine) hydrophobic blocks have been demonstrated to be effective delivery agents for plasmid DNA to 293T cells.[27]

Introduction of degradable linkages such as the hydrolytically degradable ester, phosphazene, or carbonate groups or bioreducible disulfide bonds into the polymer backbone or side chains, has been adopted as a strategy for reducing toxicity and improving biodegradability of cationic polymer gene vectors.[28-31] Among the degradable linkages that have been examined, disulfide bonds have been found to be stable during storage and systemic circulation. However, in extracellular environments, they are rapidly cleaved though a thiol-disulfide exchange reaction with glutathione within the cell.[28]

In this study, we have designed and synthesized an amphiphilic cationic tri-arm star triblock copolymer star-[poly(ethylene oxide); poly(*D,L*-lactide); poly(*L*-lysine)] (mPEO-S(CK_n)-PLA) which comprises a hydrophilic PEO block, a hydrophobic biodegradable PLA block, and a cysteine-terminated polylysine block (CK_n) with a cleavable disulfide linkage to the backbone of the star polymer, all coupled through a serine (S) core. The tri-arm star triblock copolymer mPEO-S(CK_n)-PLA is designed to condense DNA into structures that are stable in both aqueous and hydrophobic environments, through the effects of the PEO and PLA arms, respectively, and to release DNA inside cells through thiol-disulfide exchange. The DNA condensation ability and physicochemical properties of mPEO-S(CK_n)-PLA/DNA complexes were characterized at different N/P ratios (ratios of N atoms in CK_n blocks to P atoms in the plasmid DNA). Lastly, *in vitro* cytotoxicity and DNA transfection efficiency of the polymer/DNA complexes were also tested.

EXPERIMENTAL

Materials. Methoxy poly(ethylene glycol) amines, mPEO2k-amine (M_n = 2170 g/mol, 98.8%) and mPEO5k-amine (M_n = 5187 g/mol, 99.0%) were purchased from JenKem Technology (Beijing, China). 3,6-Dimethyl-1,4-dioxane-2,5-dione (*D,L*-lactide), 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) (98%), and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma-Aldrich. Triethylamine (TEA) (99%) was purchased from J. T. Baker. Trifluoroacetic acid (TFA) (99.5+ %), *N*-carbobenzoxyl-*L*-lysine (Z-*L*-lysine), 2-mercaptopyridine (98%), and dithiothreitol (DTT) (98%) were purchased from Alfa Aesar. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC•HCl) was purchased from Acros Organics. Hydroxybenzotriazole monohydrate (HOBt•H_2O) was purchased from Advanced ChemTech (Louisville, KY). *N*-(*tert*-Butoxycarbonyl)-*L*-serine (Boc-Ser-OH) (99%) and *S*-trityl-*L*-cysteinamide hydrochloride (NH_2-Cys(Trt)-H) (97%) were purchased from Aapptec (Louisville, KY). mPEO2k-amine and mPEO5k-amine were lyophilized from benzene before use. *D,L*-Lactide was recrystallized from THF, sublimed under vacuum. and stored in a N_2-filled dry box. DBU was distilled from CaH_2, dissolved in THF (20 mg/mL), and stored above 4 Å molecular sieves under N_2. TEA was passed through a short basic alumina column before

use. NH_2-Cys(Trt)-H was recrystallized from $CHCl_3$/MeOH (1:1 v/v). Other reagents were used as received. Z-Lysine-*N*-carboxyanhydride[32-33] and *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP)[34] were synthesized according to previous published procedures.

Instrumentation. Polymer average molecular weights (M_w and M_n) and molecular weight distributions ($Đ$) were estimated by gel permeation chromatography (GPC) with tetrahydrofuran (THF) as eluent at 40 °C at a flow rate of 1.0 mL/min on a set of two PLgel 5µm Mixed-D columns and a PL-ELS 1000 evaporative light scattering detector (Polymer Laboratories). Data were analyzed with Cirrus GPC software (Polymer Laboratories) based upon polystyrene standards (EasiCal PS-2, Polymer Laboratories). Block copolymer compositions were estimated by NMR spectroscopy (Varian 300 Hz, $CDCl_3$). Dynamic light scattering (DLS) experiments were carried out with a BIC 90 plus Particle Size Analyzer (Brookhaven Instruments Corp.) at a wavelength of 659 nm and a constant angle of 90°.

Synthesis of macroinitiator mPEO-S(Boc)-OH. mPEO5k-S(Boc)-OH and mPEO2k-S(Boc)-OH were prepared by an identical procedure adapted from the literature.[35] In a typical procedure, mPEO2k-amine (1.01 g, 0.465 mmol), Boc-Ser-OH (145 mg, 0.706 mmol), HOBt• H_2O (74 mg, 0.484 mmol) and EDC•HCl (93 mg, 0.485 mmol) were dissolved in CH_2Cl_2 (40 mL) and the resulting solution was cooled to 4 °C in an ice bath. Triethylamine (92 mg, 0.909 mmol) was dissolved in 5 ml CH_2Cl_2 and added to the above solution by syringe. The resulting solution was stirred at 4 °C for 2 h and then allowed to warm up to 25 °C slowly. After 20 h, the reaction mixture was washed with aqueous sodium bicarbonate (5%, 5 mL) once and then brine (5 mL) once. The organic fraction was dried over $MgSO_4$, filtered and concentrated by rotary evaporation and vacuum to afford mPEO2k-S(Boc)-OH as a white solid (965 mg, 88%) after lyophilization from benzene. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.0-3.4 (br, t, 4H per –OCH_2CH_2- repeat unit), 3.4(s, 3H, CH_3O-), 1.4 (s, 9H, Boc protecting group).

Synthesis of diblock copolymer mPEO-S(Boc)-PLA.[36-37] In a typical procedure to synthesize mPEO2k-S(Boc)-PLA2k, mPEO2k-S(Boc)-OH macroinitiator (128 mg, 0.054 mmol), and *D,L*-lactide (117 mg, 0.81 mmol) were dissolved in THF (5 mL) in a Schlenk tube inside a N_2-filled dry box. A solution of DBU in THF (0.06 mL, 0.13 M in THF, 7.8 µmol) was added to the polymerization solution by syringe. The sealed Schlenk tube was taken out of the dry box, and the solution was stirred at 25 °C for 2 h, at which point, benzoic acid (66.0 mg, 0.54 mmol) was added. The polymerization solution was concentrated and precipitated into diethyl ether. The white precipitate was re-dissolved in a minimal amount of THF and precipitated twice into hexanes/EtOAc (19:1 v/v) to afford a white solid (172 mg, ~70%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.2-5.0 (br, q, 1H per lactide repeat unit), 4.0-3.4 (br, t, 4H per –OCH_2CH_2- repeat unit), 3.4(s, 3H, CH_3O-), 1.8-1.6(br, d, 3H per lactide repeat unit), 1.4 (s, 9H, Boc protecting group).

Synthesis of mPEO-S(NH_2)-PLA. In a typical procedure, mPEO2k-S(Boc)-PLA2k (200 mg, 0.045 mmol) was dissolved in CH_2Cl_2 (2 mL). TFA (2 mL) was added, and the solution was stirred for 50 min. The solvents were removed by rotary evaporation. The residue was re-dissolved in THF and precipitated into isopropyl alcohol/Et_3N (19:1 v/v). The suspension was centrifuged at 6000 rpm for 15 min and the supernatant was discarded. The precipitation and centrifugation processes were repeated twice to afford a white solid (150 mg, 75%) ^{1}H NMR (300MHz, $CDCl_3$): δ 5.2-5.0 (br, q, 1H per lactide repeat unit), 4.0-3.4 (br, t, 4H per –OCH_2CH_2- repeat unit), 3.4(s, 3H, CH_3O-), 1.8-1.6(br, d, 3H per lactide repeat unit).

Synthesis of mPEO-S(PDP)-PLA. In a typical procedure, mPEO2k-S(NH_2)-PLA2k (120 mg, 0.028 mmol) was dissolved in $CHCl_3$ (4 mL).

SPDP (21.9 mg, 0.070 mmol) was added and the solution was stirred at 30 °C for 1.5 h under air. A second portion of SPDP (21.9 mg, 0.070 mmol) was added and the solution was stirred for another 1.5 h. The solvent was then removed by rotary evaporation. The crude product mixture was separated by silica gel chromatography using $CHCl_3$/MeOH (40:1 followed by 10:1) as eluent. mPEO-S(PDP)-PLA was eluted out in the 10:1 $CHCl_3$/MeOH eluent (R_f 0.7, yield: 60-90%). The degree of conjugation of PDP was quantified by measuring UV absorption (at 372 nm) of 2-mercaptopyridine cleaved from purified copolymers.[34] Briefly, a known amount of polymer (~2 mg) was dissolved in DMF (2 mL). DTT solution (20 µL, 20 mg/mL in DMF) was added, and the UV absorption of the solution was recorded every 2 minutes until it became constant (within 10 min). The conversions, back-calculated from the amount of 2-mercaptopyridine present in the sample solutions for UV measurements, were usually around 90% for mPEO2k and 60% for mPEO5k. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.5 (d, 1H, m-C_5NH_4-), 7.7-7.6 (d, 1H, o- C_5NH_4- ; dd, 1H, p-C_5NH_4-), 7.1 (d, 1H, m- C_5NH_4-), 5.3-5.0 (br, q, 1H per lactide repeat unit), 3.9-3.5 (br, 4H per –OCH_2CH_2- repeat unit), 3.4 (s, 3H, CH_3O-), 3.2 (t, 2H, -S-CH_2), 2.7 (br, 2H, CH_2-CO-), 1.7-1.4 (br, 3H per lactide repeat unit).

Preparation of trityl-cysteine-terminated poly(Z)lysine. NH_2-Cys(Trt)-H (10 mg, 0.028 mmol) was dissolved in dry DMF (10 mL) in a N_2-filled dry box. Z-Lysine-*N*-carboxyanhydride (345 mg, 1.13 mmol) was added to the solution. The solution was taken out of the dry box and stirred under active N_2 flow at 40 °C for 2-4 days. The DMF solution was concentrated and precipitated twice into diethyl ether to afford C(Trt)-K(Z)$_{40}$ as a white solid (320 mg, ~100%). ^{1}H NMR (300 MHz, DMSO-d^6,): δ 7.4-7.0 (br, Ar*H*, -N*H*CO-), 5.1-5.0 (br, -C*H*$_2$-Ar), 4.2-3.8 (br, α-C*H*), 3.0 (br, C*H*$_2$-NH), 1.9-1.2 (br, 6*H* per Lys side chain). SEC (H_2O/THF 1:9, PS standards): M_n = 3.9 kg/mol, $Đ$ = 1.6.

Preparation of Cys-Lys$_{40}$ (HS-CK$_{40}$).[38-39] C(Trt)-K(Z)$_{40}$ (200 mg, 18 µmol) was dissolved in TFA (3 mL) and stirred for 20 min. The solution was chilled to 4 °C and HBr/HOAc (5 mL) was added under N_2 flow, which resulted in the immediate evolution of visible fumes. The solution was allowed to warm up to room temperature slowly and stirred for another 45 min. The solution was added to isopropyl alcohol and the resulting suspension was centrifuged at 5500 rpm for 15 min. The supernatant was discarded and the solid was washed with isopropyl alcohol (20 mL×3) and dried under dynamic vacuum to afford polymer CK$_{40}$ (78 mg, 83%). ^{1}H NMR (300 MHz, DMSO-d$_6$): 8.1 (br, -CON*H*-), 4.2 (br, α-C*H*), 2.9 (br, C*H*$_2$SH), 2.8 (br, C*H*$_2$NH$_2$), 1.8-1.1 (br, 6*H* per Lys side chain).

Synthesis of mPEO-S(CK$_{40}$)-PLA. In a typical procedure, mPEO2k-S(PDP)-PLA2k (100 mg, 0.022 mmol) was dissolved in DMF (5 mL). HS-CK$_{40}$ oligomer (140 mg, 0.027 mmol) was added, and the solution immediately turned yellow. The solution was stirred for 20 min before it was dialyzed (MWCO = 3500 Da) against Nanopure water. The extent of conjugation of CK$_{40}$ was quantified prior to dialysis by measuring UV absorption (at 372 nm) of 2-mercaptopyridine present in crude product mixtures.[34] A known amount of crude product mixture (~3 mg) was dissolved in DMF (2 mL), and the UV absorption of the solution was recorded. The conversions, back-calculated from the amount of 2-mercaptopyridine present in the sample solutions from UV measurements, were usually greater than 90% for mPEO2k and 60% for mPEO5k. ^{1}H NMR (300 MHz, DMSO): δ 8.0 (br, -CON*H*-), 5.3-5.1 (br, 1H per lactide repeat unit), 4.8-4.6 (br, α-C*H*), 4.2 (br, α-C*H*), 4.0-3.3 (br, -N*H*$_2$), 3.5 (br, 4H per –OCH_2CH_2- repeat unit), 3.2 (s, 3H, C*H*$_3$O-), 2.75 (br, C*H*$_2$-NH$_2$), 1.7-1.1 (br, 3H per lactide repeat unit; 6H per Lys side chain).

Preparation of triblock copolymer/GFP DNA complex. pEGFP-N1 plasmid DNA (4,700 bp) encoding Green Fluorescent Protein (GFP, Clontech) was diluted to 100 ng/µl in deionized

water. Complexes with different N/P ratios were prepared by adding solutions of mPEO2k-S(CK_{40})-PLA2k in deionized water solutions at various concentrations (42-1250 ng/µl) to equal volumes of plasmid solution (Table S1) and incubating the resulting solutions at 37 °C for 30 min before characterization by DLS and agarose gel electrophoresis. For the transfection and cytotoxicity tests, DMEM was used instead of deionized water.

Agarose gel electrophoresis. Agarose gel electrophoresis was performed to check the ability of the triblock copolymer to induce DNA condensation. In a typical procedure for mPEO2k-S(CK_{40})-PLA2k/GFP DNA complexes, 10 µl of each mPEO2k-S(CK_{40})-PLA2k/GFP DNA complex solution prepared as described above was mixed with 2 µl 6× loading buffer and loaded onto an 0.8 % agarose gel containing ethidium bromide (0.5 µg/mL).

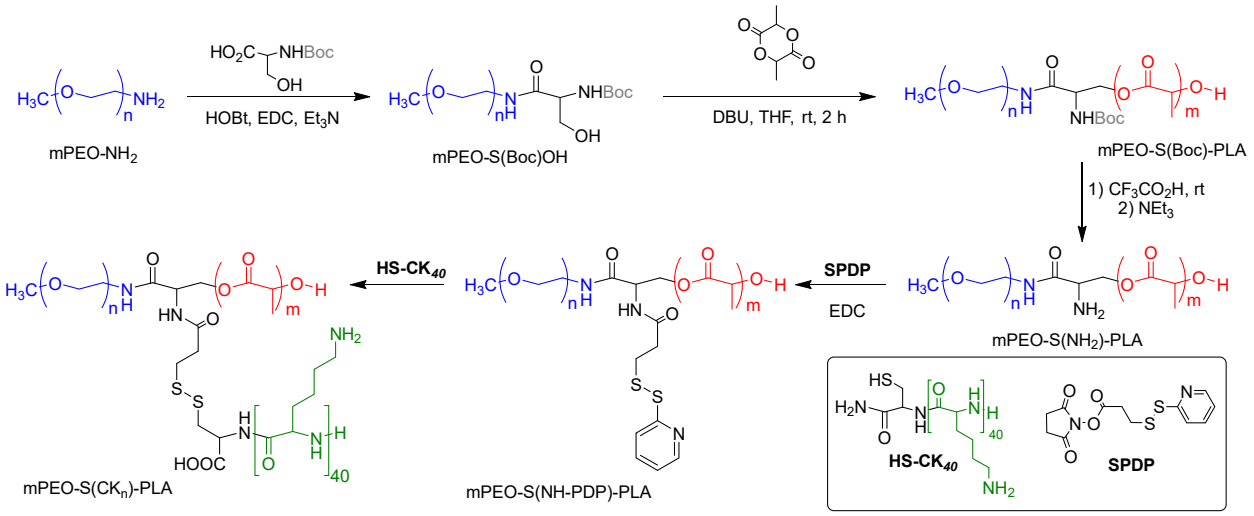
DNA Transfection. For DNA transfection using mPEO2k-S(CK_{40})-PLA2k/GFP DNA complexes, 1×10^5 HeLa cells were plated into each well of a 24-well plate one day prior to transfection. On the day of transfection, the medium was removed and 0.5 ml of a DMEM solution containing the mPEO2k-S(CK_{40})-PLA2k/GFP DNA complex (containing ~5µg of DNA) with or without 100 µM chloroquine was added to each well. The medium with mPEO2k-S(CK_{40})-PLA2k/GFP DNA was removed after a 4 h incubation at 37 °C in a humidified incubator (95% air, 5% CO_2) and 0.5 ml fresh complete culture medium (pH 7.4) containing 10% FBS was added. The cells were further incubated at 37 °C in the incubator for 48 h. Each transfection condition was carried out in triplicate. The transfected living cells were observed using fluorescence microscopy. DNA transfection of mPEO2k-S(CK_{30})/GFP DNA complexes in HeLa cells was carried out using the same protocol.

Flow Cytometry. Following transfection, HeLa cells were washed twice, trypsonized, resuspended, and then analyzed using a FACS 440 flow cytometer (BD Bioscience, Mountain View, CA, USA). An excitation wavelength of 488 nm was used with fluorescence emission measured at 530 ± 15 nm through fluorescence channel 1 (FL1). A minimum of 10,000 cells per sample were collected and the transfection efficiency was calculated from the ratio of the number of collected fluorescent (GFP expressing) cells to the number of total collected cells. Each transfection condition was carried out in triplicate.

Cytotoxicity Assay. The cytotoxicity of polymers was evaluated using the MTS colorimetric assay for determining viable cells (Promega). Before testing, HeLa cells in 100 µl of DMEM were seeded into 96-well plates at a density of $5\text{x}10^3$ cells/well. After incubation at 37 °C in a humidified incubator (95% air, 5% CO_2) for 24 h, 100 µL solutions of polymers in DMEM (pH 7.4) at different concentrations were added into the wells separately and the cells were further incubated for 24 h. Nearly 20 µL of MTS solution was added to each well and the cells were allowed to incubate for another 2 h. Cells without addition of MTS were used as blank to calibrate the microplate reader to zero absorbance. The absorbance of the solution in each well was measured at 490 nm with a microplate reader (BioTek ELx808). Untreated cells were used as a control (100% viable). The relative cell viability was calculated as a percentage relative to the untreated control cells. Each condition was performed in triplicate.

Statistical analysis. For the cytotoxicity data, each formulation was tested in quadruplicate and the results are presented as group mean +/- standard deviation. The results were then compared within each NP group using Independent Samples Median Tests. The significance of varying N/P ratio and pH on the transfection efficiency was determined using the Kruskal–Wallis non-parametric test for multiple comparisons. Cell cultures containing polymers at different N/P ratios and at various pH were compared to each other. Significance

SCHEME 1



for all tests was $p < 0.05$, and all data are expressed as mean S.D. The statistical software package SPSS 22 for Windows (SPSS, Chicago, IL, USA) was used for data analysis.

RESULTS AND DISCUSSION

Tri-arm star copolymers comprising a hydrophilic PEO arm, a hydrophobic biodegradable PLA arm, and a polylysine chain of well-defined length with a cleavable disulfide linkage to the remainder of the star polymer were synthesized as shown in Scheme 1**.** Briefly, Boc-serine was coupled to the amine terminus of methoxy poly(ethylene glycol) amine (mPEO-NH_2, M_n 2 kg/mol or 5 kg/mol) and the terminal hydroxyl group of the resulting polymer (mPEO-S(Boc)OH) was used as an initiation site for the polymerization of lactide with DBU as a catalyst for the preparation of diblock copolymers with a central Boc-protected amine group (mPEO-S(Boc)-PLA). Deprotection of the central amine group with trifluoroacetic acid, followed by coupling to (SPDP) resulted in the star polymer precursor mPEO-S(NH-PDP)-PLA, which was coupled with thiol-terminated polylysine (HS-CK_n) to afford the targeted tri-arm star copolymers mPEO-S(CK_n)-PLA.

Synthesis of diblock copolymer mPEO-S(Boc)-PLA

The macroinitiator mPEO-S(Boc)-OH was synthesized by the coupling of Boc–protected serine to amine-terminated PEO (mPEO-NH_2).[35] The Boc group was chosen for compatibility with lactide polymerization conditions and for its ability to be removed without degradation of the polylactide block. Based on a comparison of the ^{1}H NMR spectra for mPEO-NH_2 and mPEO-S(Boc)-OH (Figure S1), the disappearance of the triplet peak (δ = 3.2 ppm) corresponding to the protons of the methylene group adjacent to the terminal amine group of mPEO-NH_2 confirmed complete conversion to mPEO-S(Boc)-OH. The presence of the singlet (δ = 1.4 ppm) in the mPEO-S(Boc)-OH spectrum arising from the Boc methyl protons confirms the success of the coupling reaction.

The serine hydroxyl group of mPEO-S(Boc)-OH was used to initiate the ring opening polymerization (ROP) of lactide with DBU[36-37] at room temperature to afford the diblock copolymer mPEO-S(Boc)-PLA. The molecular weights of the PLA blocks prepared could be controlled by the molar ratio of lactide to mPEO-S(Boc)-OH macroinitiator and ranged from 3-20 kg/mol in the samples prepared and dispersities ($Đ$) were narrow (< 1.2) for all polymerizations (Table 1). When the low molecular weight macroinitiator mPEO2k-S(Boc)-OH was used to initiate the ring-opening polymerization of

TABLE 1 mPEO-S(Boc)-PLA prepared by DBU-catalyzed solution polymerization.[a]

Entry	Polymer	LA/MI	$M_{n,theory}$[b] (kg mol^{-1})	$M_{n,NMR}$[c] (kg mol^{-1})	$M_{n,SEC}$[d] (kg mol^{-1})	$Đ$[d]
1	PEO2k-PLA1k	5	3.1	3.1	3.2	1.06
2	PEO2k-PLA2k	10	3.8	3.7	3.9	1.08
3	PEO2k-PLA2.5k	15	4.4	4.5	4.5	1.06
4	PEO5k-PLA6k	45	11.3	11.1	5.6	1.18
5	PEO5k-PLA10k	75	15.1	14.6	7.8	1.17

[a] PEO2k-S(Boc)-OH or PEO5k-S(Boc)-OH macroinitiator (MI), DBU, PEO2k-S(Boc)-OH loading 1 mol%-relative to lactide with PEO2k MI or 5 mol%-relative to lactide with PEO5k MI. [MI] = 10.8 mM with PEO2k MI; [MI] = 4.8 mM with PEO5k MI. Polymerizations were carried in THF at room temperature for 2h. [b] $M_{n,\ theory}$ is reported based upon the conversion determined by comparison of integrated peak areas of lactide protons against PLA protons in the ^{1}H NMR of crude polymer sample. [c] $M_{n,NMR}$ was estimated by comparison of integrated peak areas for protons in the PLA block against protons in the PEO block in the ^{1}H NMR of purified polymer sample. [d] $M_{n,SEC}$ and M_w/M_n were estimated by SEC against polystyrene standards

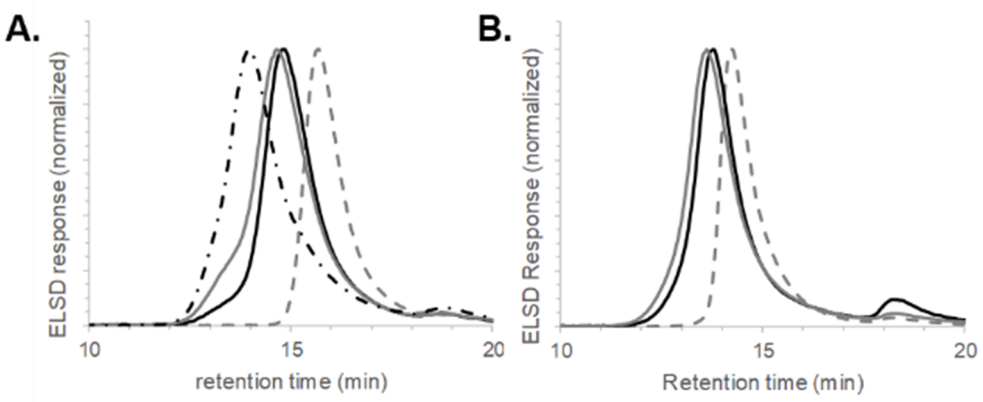


FIGURE 1 Comparative size exclusion chromatograms for PEO-S(Boc)-PLA diblock copolymers. (A) PEO2k series (from left to right): PEO2k-S-PLA2.5k (black ·-·), PEO2k-S-PLA2k (grey solid line), PEO2k-S-PLA1k (black solid line), PEO2k macroinitiator (grey ---); (B) PEO 5k series (from left to right): PEO5k-S-PLA10k (grey solid line), PEO5k-S-PLA6k (black solid line), PEO5k macroinitiator (grey - - -). All chromatograms were acquired on an evaporative light scattering detector (ELSD) with tetrahydrofuran eluent at a flow rate of 1 mL/min and normalized.

D,L-lactide, high conversions and narrow molecular weight distributions were obtained at the monomer feed ratios investigated ([lactide]/[mPEO5k-S(Boc)-OH] from 5 to 15) (Table 1: entries 1-3; Figure 1A). When the higher molecular weight macroinitiator mPEO5k-S(Boc)-OH was used to initiate the ring-opening polymerization of *D,L*-lactide (Table 1: entries 4-5), high conversions were obtained with monomer feed ratios investigated ([lactide]/[mPEO5k-S(Boc)-OH] from 45 to 105). The SEC chromatograms for macroinitiator mPEO5k-S(Boc)-OH and mPEO5k-S(Boc)-PLA samples showed narrow molecular weight distributions and clear shifts (Figure 1B).The presence of the peak corresponding to Boc methyl protons (1.4 ppm) in the ^{1}H NMR spectrum of all diblock copolymers confirmed retention of the Boc group after polymerization (Figure S2c).

Synthesis of triblock copolymer mPEO-S(CK_n)-PLA

A critical step in the synthesis of the star copolymer is the selective deprotection of the amine group at the block junction of mPEO-S(Boc)-PLA without degradation of the PLA block. Initially, a Z-protected serine was used at the block junction. After lactide polymerization, attempted cleavage of the Z group with HBr/HOAc, led to an undesirable amount of hydrolysis of the PLA block evident by NMR. The Boc-protected serine residue was chosen for further experiments as it has been reported to be selectively removable by treatment with TFA/CH_2Cl_2 (1:1) at room temperature [40]. After exposing mPEO-S(Boc)-PLA sample to 1:1 TFA/CH_2Cl_2, selective removal of the Boc group without degradation of the PLA block was observed. ^{1}H NMR spectra of the deprotected polymers show disappearance of the peak corresponding to the Boc methyl groups with the little change to the integration ratio between PLA peaks and PEO peaks.

The free amine group was then treated with *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP)[34] to afford the active disulfide intermediate mPEO-S(NH-PDP)-PLA. In the ^{1}H NMR spectrum of mPEO-S(NH-PDP)-PLA (Figure S2d), peaks at δ = 8.6, 7.6,and 7.0 ppm were attributed to the aromatic pyridyl protons and peaks at δ = 2.8 and 3.2 ppm were attributed to the protons in the two methylene groups of the PDP segment. The extent to which 2-pyridyl disulfide units were incorporated in the mPEO-S(NH-PDP)-PLA was estimated to be around 90% for mPEO2k-based copolymers and 60% for mPEO5k-based copolymers by analysis of the amount of 2-pyridinethiol released by UV spectroscopy after the addition of excess dithiothreitol.[34]

A poly(*L*-lysine) block with one cysteine residue at the C-terminus (HS-CK_{40}) was synthesized in two steps. First, the amine group of *S*-trityl-protected cysteinamide (NH_2-Cys(Trt)-H) was used to initiate the ring-opening polymerization of Z-lysine-*N*-carboxyanhydride to afford the Trt-cysteine-terminated poly(Z-lysine). Based on the integration of aromatic protons (δ = 7.2 ppm) and benzyl protons (δ = 5.0 ppm) in ^{1}H NMR spectra of the resulting polymers (Figure S3), the conversion of Z-lysine-*N*-carboxyanhydride to poly(Z-lysine) was typically 85-90%. In a subsequent step, the Trt and Z protecting groups were removed by treating the polymer sequentially with TFA and HBr/HOAc under N_2 flow.[38-39] The disappearance of the aromatic peaks and benzylic methylene protons in the ^{1}H NMR spectra of the unprotected polymer indicated the successful removal of both Trt and Z protecting groups.

The target tri-arm star copolymer mPEO-S(CK_{40})-PLA was obtained by the direct reaction of mPEO-S(NH-PDP)-PLA diblock copolymer with HS-CK_n in DMF. Coupling efficiencies were estimated by spectrophotometric analysis of the amount of 2-mercaptopyridine released during the coupling reaction. ^{1}H NMR spectra of the resulting mPEO-S(CK_{40})-PLA star copolymers acquired in DMSO-d_6 (lysine peaks are not readily visible in $CDCl_3$—see Figure S2e) after dialysis to remove any unreacted HS-CK_n (Figure S4) show the appearance of peaks that can be attributed to backbone methine protons (δ = 4.2 ppm) and α-amino methylene protons (δ = 2.75 ppm) in the polylysine block. The conversions estimated by UV spectroscopy were usually above 90% for mPEO2k-based copolymers and 60% for mPEO5k-based copolymers. Because of the relatively low SPDP and PLL coupling efficiencies with the mPEO5k-based copolymers, only the mPEO2k polymers were subsequently used in transfection studies.

Cytotoxicity of mPEO-S(CK_n)-PLA

Evaluation of the cytotoxicity of cationic polymers is essential prior to their application as gene delivery systems. The cytotoxicity of mPEO2k-S(CK_{40})-PLA2k tri-arm star copolymers was evaluated by an MTS assay in HeLa cells at various concentrations, and compared with the corresponding polylysine HS-CK_{40} and branched polyethyleneimine (PEI, average M_w = 25 kg/mol), which is a commonly used as a

transfection reagent.[16-18] A much wider range of polymer concentrations (N/P from 3 to 120) than was used in transfection studies was investigated in order to better understand the cytotoxicity of these new polymers. mPEO2k-S(CK_{40})-PLA2k triblock copolymers showed no toxicity at all conditions examined while the corresponding polylysine HS-CK_{40} and branched PEI showed relatively low toxicity at low concentrations (N/P ratios from 3-10) but increased toxicity at higher concentrations (N/P ratios from 30-120) (Figure 2). At these higher concentrations the toxicity of HS-CK_{40} and PEI reached ~80% in comparison to the triblock copolymer and this difference was statistically significant (Figure 2).

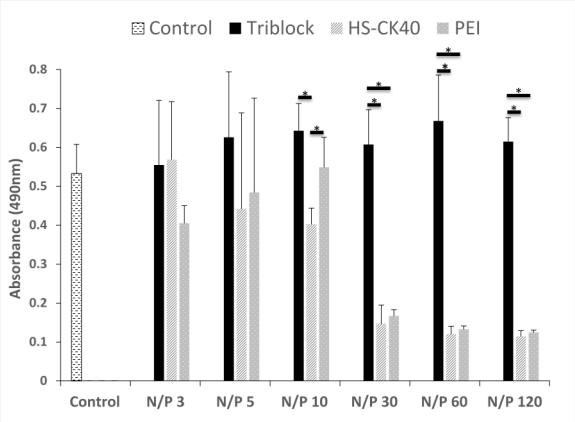


FIGURE 2 Quantitative cytotoxicity analysis of the triblock copolymer mPEO2k-S(CK_{40})-PLA2k in HeLa cells using the corresponding poly(lysine)$_{40}$ (HS-CK_{40}) and branched polyethyleneimine (PEI, average M_w = 25 kg/mol) as reference. Error bars represent the std.dev over four independent experiments. *p < 0.015.

Further, to visualize these data, we also show microscopy images of the cells in the presence of the triblock copolymer mPEO2k-S(CK_{40})-PLA2k at both N/P ratios of 3 and 120 as representative examples (Figure 3). While cells exposed to mPEO2k-S(CK_{40})-PLA2k at both N/P ratios as well as with HS-CK_{40} or PEI at N/P 3 (Figure 3B-E) resembled healthy untreated control cells (Figure 3A), those in the presence of PLL_{40} or PEI at an N/P ratio of 120 were dying as evidenced by cellular fragmentation (Figure 3F and G).

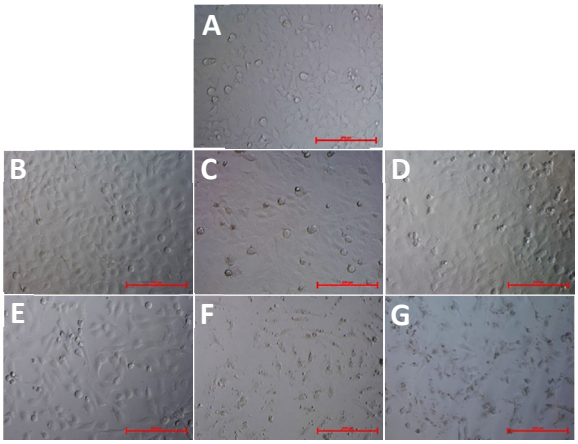


FIGURE 3 Qualitative cytotoxicity analysis of HeLa cells with triblock copolymers PLL and PEI at N/P 3 and N/P 120. (A) Control untreated cells; (B) mPEO2k-S(CK_{40})-PLA2k at N/P ratio of 3; (C) HS-CK_{40} at N/P ratio of 3; (D) PEI at N/P ratio of 3; (E) mPEO2k-S(CK_{40})-PLA2k at N/P ratio of 120; (F) HS-CK_{40} at N/P ratio of 120; (G) PEI at N/P ratio of 120. Scale bar = 200 µm.

Characterization of mPEO-S(CK_{40})-PLA/DNA complexes

mPEO2k-S(CK_{40})-PLA2k deionized water solutions at various concentrations (42 ng/mL to 1250 ng/mL) to equal volumes of GFP plasmid DNA solution (100 ng/mL) were mixed to form complexes with different N/P ratios (from 0.5 to 15) (Table S1). The hydrodynamic diameters of mPEO2k-S(CK_{40})-PLA2k triblock copolymer/DNA complexes were measured by DLS and revealed that the triblock copolymer mPEO2k-S(CK_{40})-PLA2k could condense GFP plasmid DNA into complexes with diameters ranging from 130–220 nm (Figure 4) which were appropriate for endocytotic cellular uptake.[41]

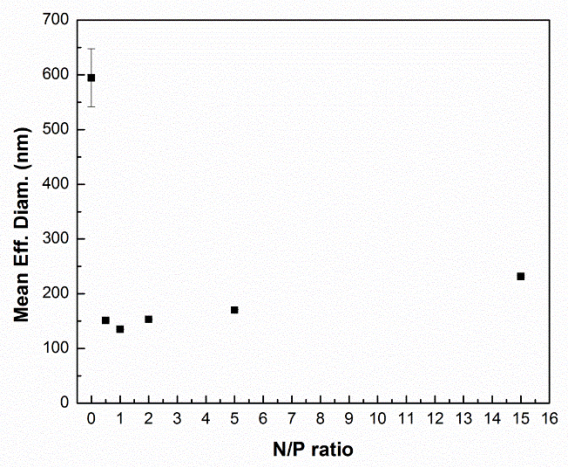


FIGURE 4 DLS particle size measurements of triblock copolymer mPEO2k-S(CK_{40})-PLA2k/GFP plasmid DNA complexes in deionized water at various N/P ratios. N/P = 0 corresponds to the plasmid DNA without any additives. Error bar for N/P = 0 represents the mean st.dev. (n = 5). Mean st.dev. values for diameters measured for samples with N/P > 0 are smaller than the data markers (< 2.5 nm).

Next, we performed agarose gel electrophoresis of complexes at various N/P ratios (ranging from 0 to 15) to visualize the condensation capability of mPEO2k-S(CK_{40})-PLA2k triblock copolymers with DNA (Figure 5). The uncomplexed naked plasmid DNA migrated (Figure 5: lane 2) through the gel in the expected two bands (supercoiled [large bright] and circular single stranded [lower] structures). In addition, a couple of faint bands appear above the supercoiled plasmid DNA indicating the presence of nicked/relaxed circle and linear DNA. Since triblock alone (control) did not contain any DNA, the lane appears empty (Figure 5: lane 3). Neutralization of DNA backbone phosphate groups by PLL ammonium groups resulted in a significant reduction in DNA migration distance (Figure 5: lanes 4-8). Specifically, N/P ratios of 15, 5, 2, and 1 resulted in minimal migration of the complexed DNA into the gel (Figure 5: Lanes 4-7, respectively). At an N/P ratio of 0.5 (Figure 5: Lane 8), uncomplexed DNA was detected at the same migration distances observed in the control (Figure 5: Lane 2): a low quantity of supercoiled plasmid DNA (arrowhead) and a larger amount of circular single-stranded plasmid DNA (arrow), which indicates that complexation was incomplete. The fact that there is a smear present in the gel above the DNA bands (Figure 5: Lane 8), may also indicate that there is a distribution of complexes with different N/P ratios, as well as some of the nicked/relaxed circular and linear DNA. Collectively, these DNA retardation results, especially in the presence of various N/P ratios from 1-15 are consistent with previous studies showing minimal migration of polycation/DNA complexes through an agarose gel.[42] Further, although uncomplexed or partially complexed DNA will migrate in the gel, the DNA retardation observed with increasing N/P ratio (1-15) is probably due to the larger polyplexes that contain lower net negative charges.

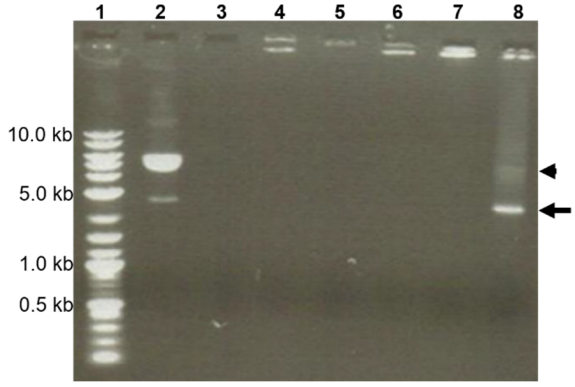


FIGURE 5 Agarose gel electrophoresis analysis of triblock copolymer mPEO2k-S(CK_{40})-PLA2k/GFP plasmid DNA complexes at various N/P ratios. Lane 1: 10kb DNA ladder; Lane 2: Naked plasmid DNA; Lane 3: triblock copolymer mPEO2k-S(CK_{40})-PLA2k; Lanes 4-8: The N/P ratios of mPEO2k-S(CK_{40})-PLA2k/DNA complexes were 15, 5, 2, 1 and 0.5, respectively. For lanes 2 and 8, the arrowhead indicates uncomplexed supercoiled DNA and the arrow indicates uncomplexed circular single-stranded plasmid DNA, respectively.

In vitro transfection

To study the DNA transfection efficiency of mPEO2k-S(CK_{40})-PLA2k triblock copolymers *in vitro*, HeLa cells were transfected with mPEO2k-

S(CK_{40})-PLA2k/GFP plasmid DNA complexes. For comparison, HeLa cells were also transfected by HS-CK_{40}/plasmid DNA complexes. The qualitative data for N/P ratio of 5 is shown as an example (Figure 6) but the quantitative data includes that with N/P ratios of 3, 5, and 10 as well as different pH values (Table 2). Specifically, mPEO2k-S(CK_{40})-PLA2k/GFP plasmid DNA complexes qualitatively showed higher gene transfection activity than HS-CK_{40}/GFP plasmid DNA complexes (Figure 6, A and C). Chloroquine diphosphate, which is known to interfere with the endocytosis process (it can raise the pH of endocytotic vesicles and reduce delivery to lysosomes and degradation by lysosomal enzymes[19]), was added to enhance cell transfection which it did as shown for both conditions (Figure 6, B and D). We used flow cytometry to measure the percent of transfected cells and the data are summarized in Table 2. Although a higher transfection efficiency was observed at N/P 5 as compared to N/P 3 and N/P 10, this increase was not statistically significant. Besides the N/P ratio, the pH of the medium can also affect transfection and thus we tested this as well. By varying the buffer pH from 7.0 to 7.6, the highest transfection efficiency was obtained at pH 7.2, but again it was not statistically significant. Enhancement of transfection efficiency by chloroquine was statistically significant only and in agreement with the fluorescence microscopy analysis (Figure 6, B and D).

TABLE 2 HeLa cell transfection by mPEO2k-S(CK_{40})-PLA2k/GFP plasmid DNA complexes.

Conditions	Avg % cells transfected
N/P=3, pH=7.4	0.36 ± 0.05
N/P=5, pH=7.4	0.43 ± 0.07
N/P=10, pH=7.4	0.29 ± 0.06
N/P=5, pH=7.0	0.48 ± 0.11
N/P=5, pH=7.2	0.78 ± 0.04
N/P=5, pH=7.6	0.44 ± 0.19
N/P=5, pH=7.4, with Chloroquine	1.75 ± 0.55[a]

[a] p = 0.033 only as compared to N/P=10, pH=7.4

At this point, we do not know why the DNA transfection efficiency of our tri-arm star copolymers is low. We speculate that it may be a function of the amount of DNA used (~5 μg within each complex). Perhaps a higher starting amount of DNA could also proportionally increase the transfection efficiency. Further, we do not know the structure of the DNA within the complex. While it is known that supercoiled DNA results in the highest levels of transfection efficiency, we have not determined the structural state of the plasmid DNA in our complexes (especially given our electrophoresis data, Figure 5). It is possible that other DNA release strategies, such as the self-catalyzed polycations developed by Monteiro and co-workers, would also be advantageous.[43-44] Clearly, additional work is needed to further improve the transfection efficiency of our tri-arm star copolymer/DNA system.

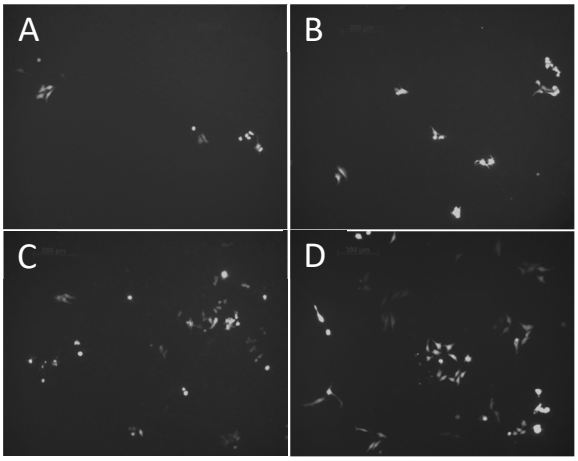


FIGURE 6 Fluorescence images of GFP expression in HeLa cells transfected by PLL_{40}/GFP plasmid DNA complexes and mPEO2k-S(CK_{40})-PLA2k/GFP plasmid DNA complexes at N/P ratio 5 and pH 7.4. (A) PLL_{40}, N/P=5; (B) HS-CK_{40}, N/P=5, with chloroquine; (C) mPEO2k-S(CK_{40})-PLA2k, N/P=5 and (D) mPEO2k-S(CK_{40})-PLA2k, N/P=5, chloroquine. Scale bar = 200 μm.

CONCLUSIONS

A new type of amphiphilic cationic tri-arm star triblock copolymer mPEO-S(CK_n)-PLA was synthesized and characterized. The polymer can

form complexes with plasmid DNA and its amphiphilic character can potentially stabilize the polymer/DNA complexes in both aqueous and hydrophobic environments. Compared to the corresponding polylysine, we observed successful gene transfection and a reduction in cytotoxicity for the tri-arm star copolymer complexes. These results suggest that the tri-arm star triblock structure could be considered as a safe gene delivery system.

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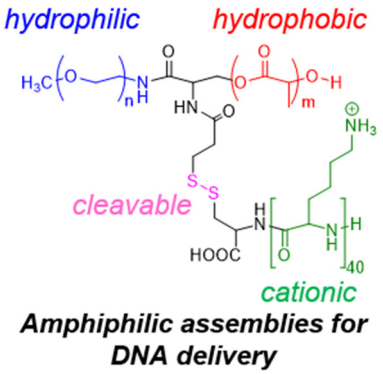
GRAPHICAL ABSTRACT

Tianyuan Wu, Yu Cai, Xia Zhao, Chai K. Ngai, Benjamin Chu, Benjamin Hsiao, Michael Hadjiargyrou, Robert B. Grubbs

Synthesis and Characterization of Poly(ethylene oxide)/polylactide/polylysine Tri-arm Star Copolymers for Gene Delivery

Star copolymers with hydrophilic, hydrophobic, and cationic arms can be synthesized by a combination of ring-opening polymerization and coupling steps. The star copolymers have low toxicity to cells and model studies with GFP and HeLa cells show they can be used to deliver DNA to cell nuclei.



Synthesis and Characterization of Poly(ethylene oxide)/polylactide/polylysine Tri-arm Star Copolymers for Gene Delivery

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Table S1. Calculated polymer/DNA mass ratios for PEO2k-S(CK40)-PLA2k/GFP DNA complexes with different N/P ratios.

N/P ratio	Mass ratio of polymer to DNA	PEO2k-S(CK_{40})-PLA2k Concentration (ng/µL)	GFP DNA plasmid Concentration (ng/µL)
0.5	0.42	42	100
1	0.83	83	100
2	1.67	167	100
5	4.18	418	100
15	12.5	1250	100

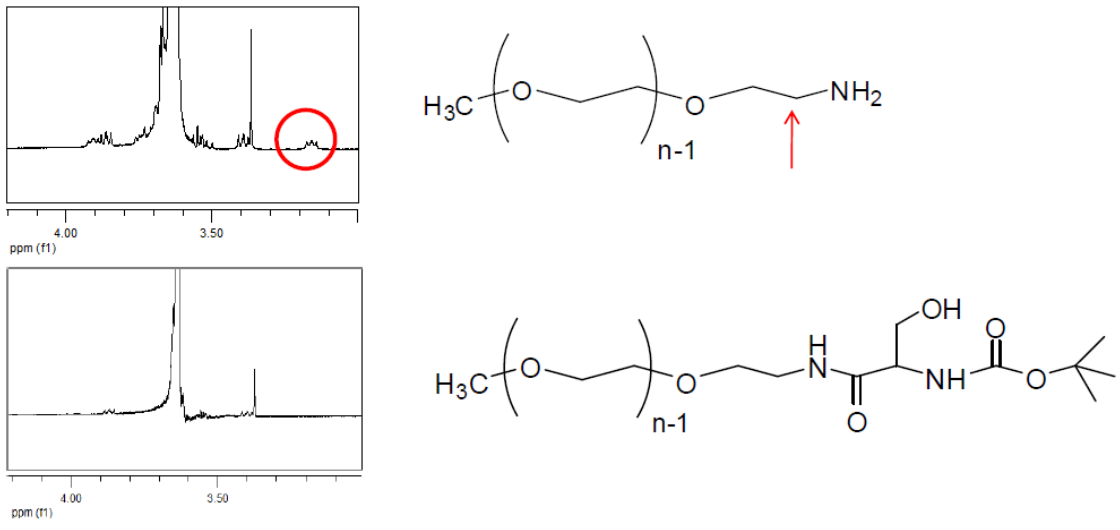

Figure S1. Comparison between ^{1}H NMR spectra ($CDCl_3$) of mPEO2k-NH_2 (top) and mPEO2k-S(Boc)-OH (bottom) from δ 3.0-4.0 ppm showing the disappearance of the peak corresponding to the methylene protons adjacent to the amine group after coupling to Boc-serine.

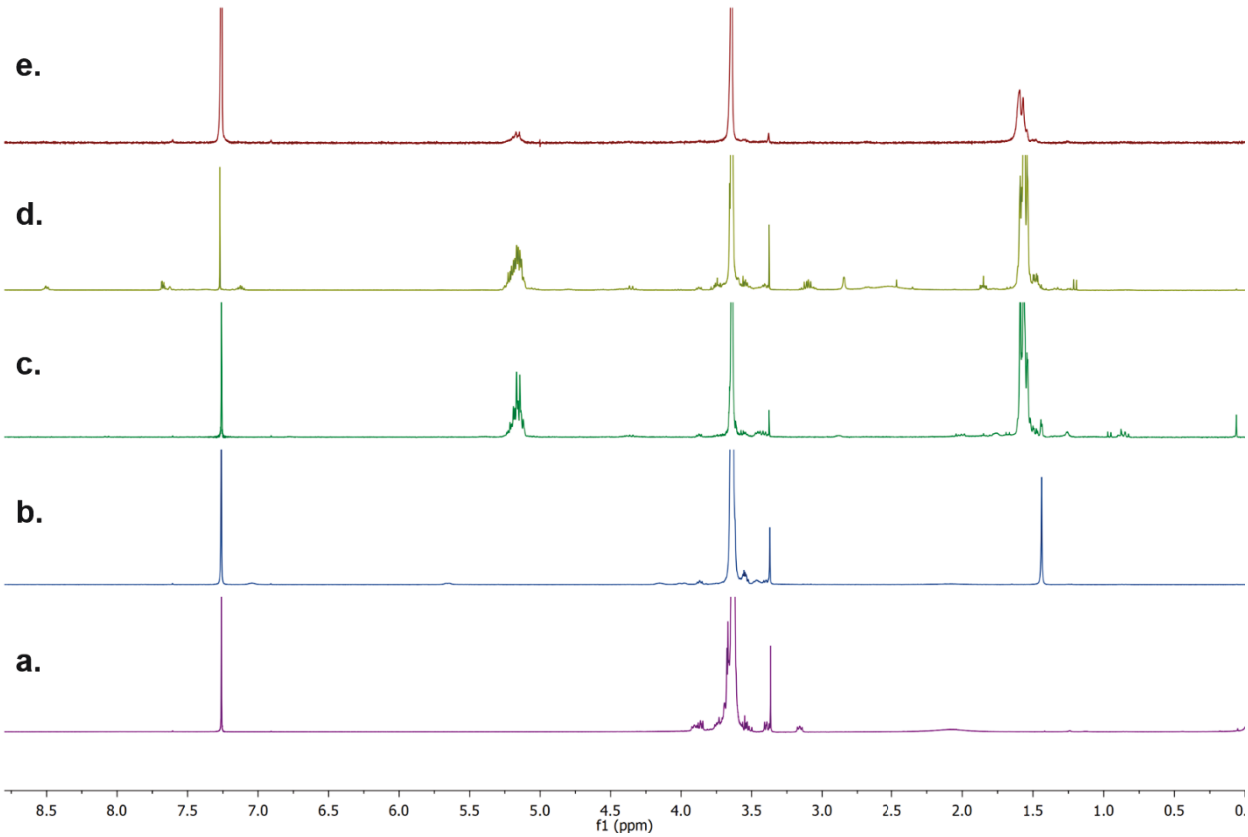

Figure S2. ^{1}H NMR spectra ($CDCl_3$, 300 MHz) for a) PEO2k-NH_2, b) PEO2k-S(Boc)-OH, c) PEO2k-S(Boc)-PLA2k d) PEO2k-S(NH-PDP)-PLA2k and e) PEO2k-S(CK_{40})–PLA2k.

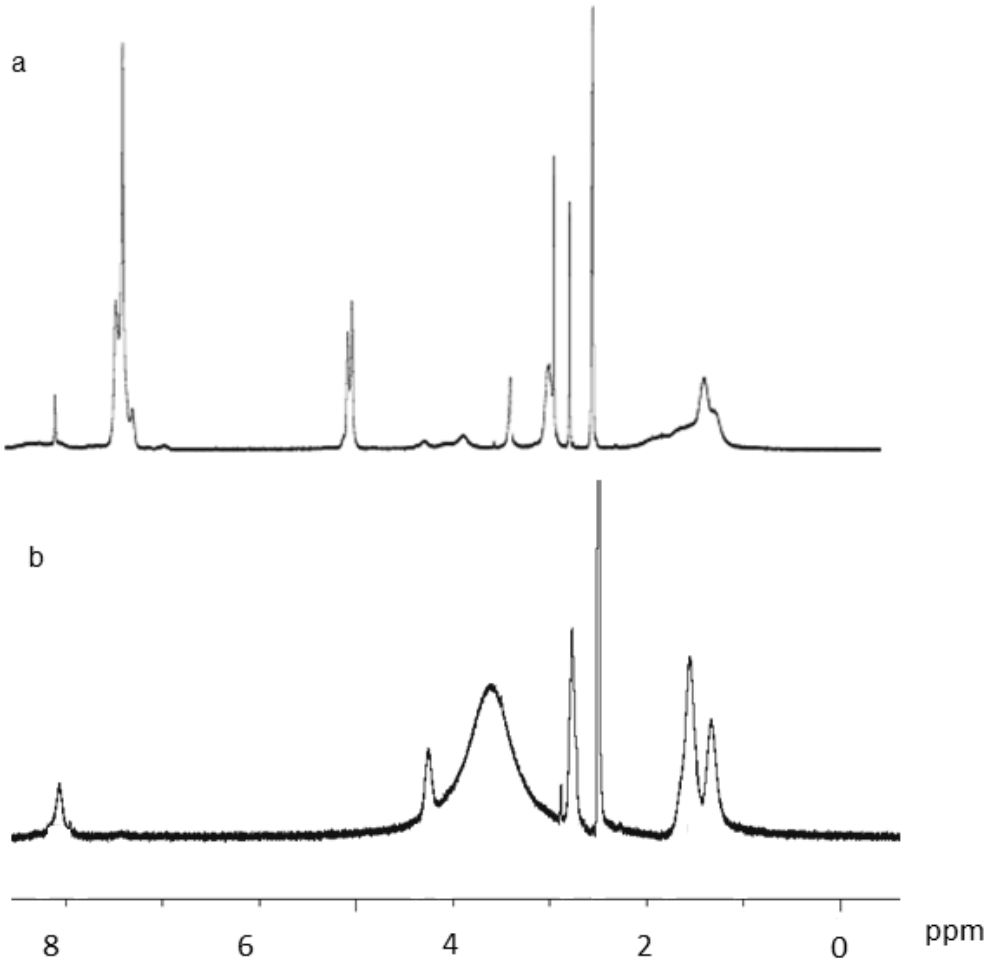

Figure S3. ^{1}H NMR spectrum (300 MHz) of a) Cys(Trt)-poly(Z)lysine and b) Cys-Lys$_n$ (HS-CK$_n$) in DMSO-d^6 (δ = 2.50 ppm). Solvent peaks in a): DMF (δ = 8.0 ppm and δ= 2.9, 2.7 ppm) and water (δ = 3.4 ppm). Solvent peaks in b): water (br, δ= 3.4 ppm).

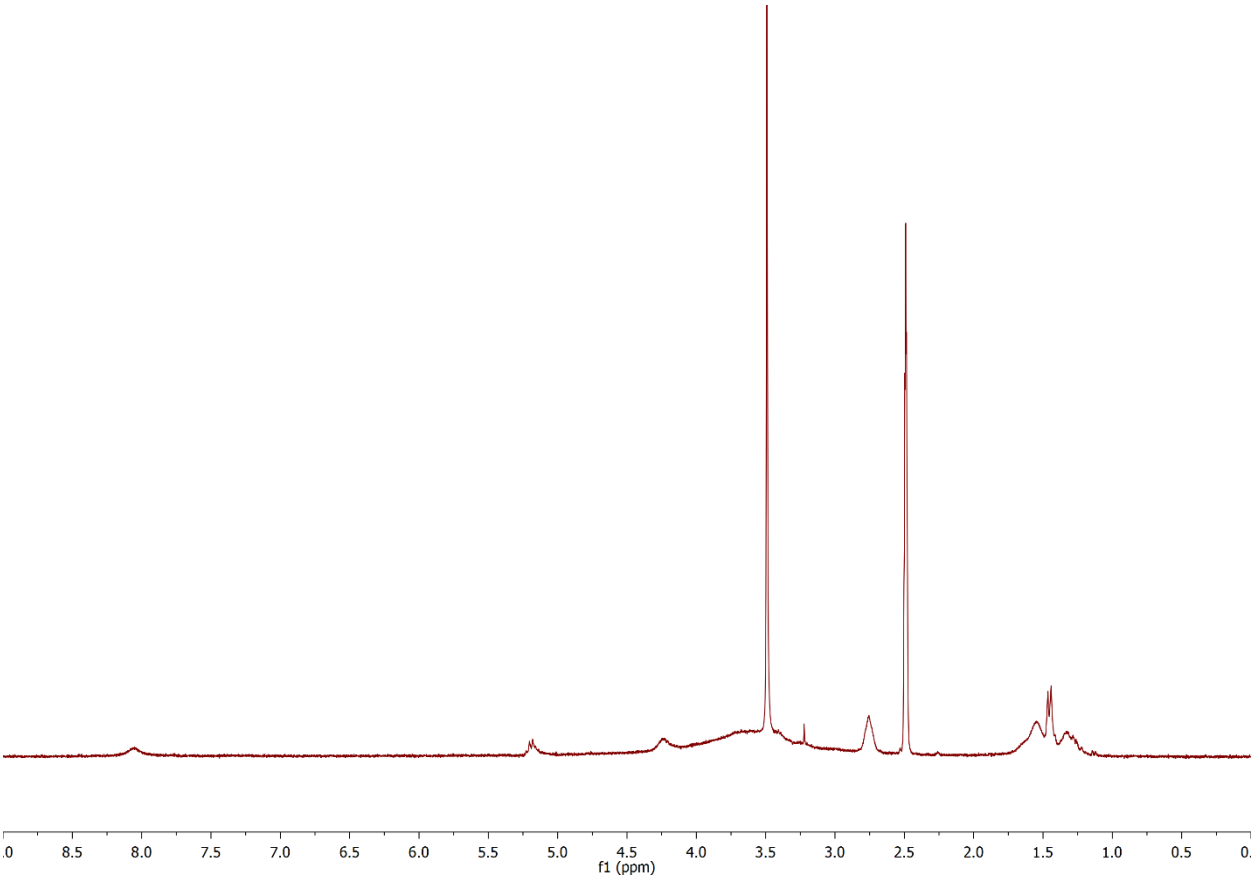

Figure S4. ^{1}H NMR spectrum (300 MHz) of PEO2k-S(CK_{40})–PLA2k in DMSO-d^{6} (δ = 2.50 ppm).