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Pluronic-based cationic block copolymer for forming pDNA polyplexes with enhanced cellular uptake and improved transfection efficiency

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Abstract

PEGylated cationic polymers have been extensively studied for substituting virus as gene delivery vehicles. These polymers can produce water-soluble polyionic complexes (polyplexes) with plasmid DNA (pDNA) and show enhanced stability compared to non-PEGylated polyplexes. However, PEGylation always diminishes the transfection efficiency of polyplexes probably due to poor cellular internalization of the particles and difficulty in releasing the pDNA cargo from the complexes intracellularly for gene expression. As non-ionic surfactants, Pluronic block copolymers have been shown to interact with plasma membrane and promote cellular uptake of various small molecules and biomacromolecules. To evaluate whether Pluronic could improve the transfection efficiency of polyplexes, Pluronic P85- and PEG-based cationomers comprising poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl] aspartamide (P[Asp(DET)])} cationic blocks were synthesized and tested for their transfection ability. In this study, it was demonstrated that although the stability of the PEG-based polyplexes was better than that of the P85-based polyplexes based cationic polymers, the P85-based polyplex could achieve significantly higher transfection than the PEG counterparts. The improvement of gene delivering ability was shown to be correlated with the enhanced cellular internalization of the P85-based polyplexes.

Keywords

Pluronic; pDNA; Non-viral gene delivery; Polyplex

1. Introduction

Viral vectors are the most powerful carriers for gene delivery to treat various intractable diseases; however, there are still a lot of concerns for the safety issues related to the practical use of viruses for gene therapy in human [1,2]. In contrast, polyionic complexes (polyplexes) formed from plasmid DNA (pDNA) and cationic polymers are valid alternative vehicles for delivering nucleic acid materials for clinical use because of their low immunogenicity and high versatility in terms of chemical structure [3]; although generally the transfection ability of these non-viral vectors is still insufficient to elicit systemic therapeutic effects [4].

Introduction of hydrophilic poly(ethylene glycol) (PEG) molecules to the surface of nanoparticles is a very common method for increasing particle stability and thus circulation time [5–7], and extensive research has been done on PEGylated polyplexes for delivering nucleic acid materials [8,9]. Although PEGylation can improve the colloidal stability of the polyplexes, it also diminishes the cellular uptake of the polyplex particles by the target cells due to the steric hindrance of PEG [10,11]. To overcome this obstacle, reversible PEGylation using matrix metalloproteinase (MMP)-substrate peptide was shown to enable site-specific extracellular cleavage of PEG from the particles as abundant MMP enzyme was found to be localized at tumor tissues, and thus the particle uptake inhibition by PEG could be minimized [12]. Another strategy to increase cellular uptake is to install targeting ligands, for example, transferrin [13] and cyclic RGD peptide [14], which promotes the cell internalization of particles through receptor-mediated endocytosis.

PEG-based diblock cationomer, poly(ethylene glycol)-*block*-poly {*N*-[*N*-(2-aminoethyl)-2-aminoethyl] aspartamide (PEG-*b*-P[Asp (DET)]), has been shown that it is a promising delivery vehicle for pDNA, and studies have been done on the transfection ability, cytotoxicity [15], and biodegradability [16] of this material. Although polyplexes prepared from PEG-*b*-P[Asp(DET)] showed clear advantages in terms of transfection performance and toxicity over polyplexes prepared from other cationic polymers, for example, poly(L-lysine) [17], branched PEI, and linear PEI [18], PEG-*b*-P[Asp(DET)] polyplexes are always required to be prepared at high N/P ratios for transfection in order to surpass its competitors [10,15] and the polyplexes usually internalized into the cells at a much lower level compared to the polyplexes formed with non-PEGylated P[Asp(DET)] homopolymer [10]. This implies that further modifications on the P[Asp(DET)]-based polymer are required to further boost its performance especially at low N/P ratios. As an amphiphilic triblock copolymer with a hydrophobic poly (propylene oxide) block situated in between two hydrophilic poly (ethylene oxide) blocks, Pluronic block copolymer has been shown to interact with plasma membrane and lower the microviscosity of the membrane to promote cellular uptake of various small molecules [19] and biomacromolecules, including peptides [20,21] and nucleic acids [22]. Different Pluronics, including P85 [22], L92 [23], and P123 [24], have been used to conjugate with cationic polymers aiming at improving the delivery of nucleic acid-based materials. Enhanced delivery was observed with those Pluronic-based carriers; however, free Pluronic polymers usually are necessary to be included in the formulations to promote transfection efficiency and to allow the formation of colloiddally stable polyplexes.

In the present work, we employed the amphiphilic nature of Pluronic P85 to investigate whether the cellular internalization and transfection efficiency of polyplexes could be increased by replacing the hydrophilic PEG block with amphiphilic Pluronic P85 on (PEG-*b*-P[Asp(DET)]) diblock copolymer (Fig. 1). P85 was chosen as its HLB (hydrophilic-lipophilic balance) value which is 16 and which is a mid-range value within the Pluronic family. Also, certain degree of hydrophilicity was required for maintaining colloidal stability of particles as it was speculated that the Pluronic chains would be located at the surface of the particles after polyplex formation. P85-*b*-P[Asp(DET)] was synthesized using P85 amine as the initiator and the synthesized polymer was confirmed to be able to condense pDNA to produce nano-sized polyplex particles. The transfection efficiency and toxicity of the polyplexes formed with P85-*b*-P[Asp(DET)] were evaluated and compared to those of the PEG-*b*-P[Asp(DET)] polyplexes.

2. Materials and methods

2.1. Materials

Methoxy- ω -amino poly(ethylene glycol) (PEG-NH₂, M_n = 5000 g/mol, M_w/M_n = 1.04) was obtained from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan). Pluronic P85 was kindly

provided by BASF Corp. (Florham Park, NY). β -Benzyl-L-aspartate *N*-carboxyanhydride (BLA-NCA) was synthesized from L-Aspartic acid β -benzyl ester by the Fuchs–Farthing method using triphosgene [25]. L-Aspartic acid β -benzyl ester, triphosgene, 4-nitrophenyl chloroformate (*p*-NPC), 1,3-diaminopropane, diethylenetriamine (DET), benzene, *N,N*-dimethylformamide (DMF), dichloromethane (DCM), hexane, *N*-methyl-2-pyrrolidone (NMP), tetrahydrofuran (THF), and branched polyethylenimine (bPEI, $M_w = 25,000$ g/mol) were purchased from Sigma–Aldrich (St. Louis, MO). DET was distilled by conventional methods before use. DCM, DMF, hexane, NMP, and THF were purchased as anhydrous grade and used without further purification. Dialysis tubings (MWCO's 1000 and 6-8000) were purchased from Spectra/Por (Rancho Dominguez, CA). The pDNA coding for luciferase in pGL4 vector with a CMV promoter (Promega, Madison, WI) was amplified in DH5a *Escherichia coli* cells and purified using PureLink™ HiPure Plasmid Maxiprep Kit (Invitrogen, Carlsbad, CA). Luciferase Assay System Kit, CellTiter-Blue® Cell Viability Assay, and ethidium bromide (EtBr) were purchased from Promega (Madison, WI). RC DC Protein Assay Kit was purchased from Bio-Rad (Hercules, CA).

2.2. ^1H NMR and gel permeation chromatography (GPC) analyses

The ^1H NMR spectrum of each polymer was obtained with Varian Unity-Inova 400 MHz NMR spectrometer (Palo Alto, CA) with temperature regulated at 10 °C or 25 °C. Chemical shifts were reported in ppm relative to the residual protonated solvent resonance. Polymer molecular weight distributions were monitored using Agilent 1100 series equipped with TOSOH TSK-gel G3000PWXL and G4000PWXL columns and an internal refractive index (RI) detector. DMF with 10 mM LiCl was used as the eluent at a flow rate of 1 ml/min at 40 °C. PEG standards were used for calibration.

2.3. Synthesis of P85 amine (P85-NH₂)

P85 amine was prepared as previously described with modifications [26]. *p*-NPC (98 mg, 0.467 mmol) was dissolved in benzene and then added dropwise to P85 (2.15 g, 0.467 mmol) dissolved in benzene. The reaction mixture was stirred for 24 h at room temperature under an argon atmosphere. After reaction, benzene was evaporated under vacuum. The crude product was dissolved in methanol, precipitated in dry ice-chilled diethylether, and then subjected to centrifugation. The precipitation procedure was repeated at least 3 times to ensure the complete removal of unreacted *p*-NPC. The white solid obtained was dissolved in benzene and lyophilized. The degree of activation was determined to be 78% from the peak intensity ratio of the aryl protons of the nitrophenyl groups ($\text{NO}_2\text{-C}_6\text{H}_4\text{-}$, $\delta = 7.5$ and 8.25 ppm) to the methyl protons of P85 (-CH_3 , $\delta = 1.0$ ppm) in ^1H NMR spectrum taken in DMSO- d_6 at 25 °C. The activated P85 was subsequently reacted with 1,3-diaminopropane to obtain P85 amine. P85-*p*-NPC (1.08 g, 0.226 mmol) and excess amount of 1,3-diaminopropane (3.8 mL, 45.5 mmol) were dissolved in methanol. P85-*p*-NPC was added to 1,3-diaminopropane slowly and the reaction was allowed to proceed for 24 h at room temperature. The reaction mixture was transferred to a dialysis tubing (MWCO: 1000) and dialyzed extensively with methanol for 1 week. The product was then recovered by lyophilization. The degree of amine functionality was confirmed to be about 50% from the peak intensity ratio of the methylene protons of the propylamino moieties ($\text{-CH}_2\text{-}$, $\delta = 1.4\text{--}1.5$ ppm) to the methyl protons of P85 (-CH_3 , $\delta = 1.0$ ppm) in ^1H NMR spectrum. P85 molecules without amine functionality were not separated from P85-NH₂ at this stage. The purification would be done after the polymerization of PBLA described in the following section.

2.4. Synthesis of PEG-/P85-N-substituted polyaspartamide (PEG-/P85-b-P[Asp(DET)])

Block copolymer of PEG-*b*-poly(β -benzyl-L-aspartate) (PEG-*b*-PBLA) was synthesized as previously described [27] by ring-opening polymerization using β -benzyl-L-aspartate *N*-

carboxyanhydride (BLA-NCA) and α -methoxy- ω -amino poly (ethylene glycol) as the monomer and initiator, respectively. Briefly, BLA-NCA (835 mg, 3.35 mmol) dissolved in 1.5 mL DMF and 10 mL DCM was added to PEG-NH₂ (146 mg, 0.028 mmol) dissolved in 5 mL DCM. The polymerization was allowed to proceed for 96 h at 35 °C under an argon atmosphere. P85-*b*-poly(β -benzyl-L-aspartate) (P85-*b*-PBLA) was synthesized with the same procedure, while P85-NH₂ was used as the initiator of the polymerization. The product mixture was slowly added into diethylether. The precipitated polymer was then subjected to centrifugation. The precipitation procedure was repeated for at least 3 times to purify the polymer. The white crude solid obtained was dissolved in benzene and then freeze-dried. The degree of polymerization of the PBLA block of PEG-*b*-PBLA was determined to be 106 from the peak intensity ratio of the aryl protons of the benzyl groups (C₆H₅-, δ = 7.2–7.3 ppm) to the methylene protons of PEG (–OCH₂CH₂–, δ = 3.5 ppm) in ¹H NMR spectrum taken in DMSO-*d*₆ at 25 °C. The degree of polymerization of the PBLA block of P85-*b*-PBLA was estimated to be 105 using the methyl protons of P85 (–CH₃, δ = 1.0 ppm) for peak integration.

The side chains of PBLA block were then substituted with DET through aminolysis reaction to obtain PEG-*b*-P[Asp(DET)] [15]. Dried PEG-*b*-PBLA (202 mg, 7.49 μ mol) was dissolved in 4 mL NMP and added dropwise to 4.3 mL (39.6 mmol) distilled DET (50 equiv to benzyl group of PBLA segment) dissolved in 4 mL NMP. The reaction mixture was stirred for 1 h at 15 °C under argon atmosphere. The polymer was then precipitated in diethylether and subsequently dried and dissolved in 10 mM PBS containing 150 mM NaCl (pH 7.4) and dialyzed against PBS three times in a dialysis tubing (MWCO: 6-8000) at 4 °C. The polymer solution was then dialyzed against distilled water overnight at 4 °C, lyophilized, and the polymer was collected as the hydrochloride salt form. The substitution was confirmed by the disappearance of the aryl protons of the benzyl groups (C₆H₅-, δ = 7.2–7.3 ppm) and the emergence of methylene protons of the DET moieties (–CH₂CH₂NHCH₂CH₂–, δ = 2.6–3.6 ppm). Quantitative introduction of DET was also confirmed by the peak intensity ratio of the methylene protons of PEG (–OCH₂CH₂–, δ = 3.5 ppm) or the methyl protons of P85 (–CH₃, δ = 1.0 ppm) to the methylene protons of the DET moieties (–CH₂CH₂NHCH₂CH₂–, δ = 2.6–3.6 ppm) in the ¹H NMR spectrum taken in D₂O at 10 °C.

2.5. Preparation of polyionic complex (polyplex)

The stock solutions were prepared by dissolving pDNA and synthesized polymers in 10 mM Tris-HCl buffer (pH 7.5) at concentrations of 50 μ g/mL and 10 mg/mL, respectively. Polyplexes were formed by adding different amounts of polymer solutions (1/3 total volume) to pDNA solutions (2/3 total volume) at various N/P ratios and incubated at room temperature for at least 30 min before experiments. The final concentration of pDNA in all the samples was kept at 33 μ g/mL. The polyplexes were formed in the same way in all experiments.

2.6. Gel retardation analysis

Polyplexes with different N/P ratios were loaded on a 0.8 wt% agarose gel containing 0.004% ethidium bromide and electrophoresed at 100 V for 45 min with a running buffer of 40 mM Tris-acetic buffer with 2 mM EDTA. The migrated pDNA was visualized by UV.

2.7. Dynamic light scattering (DLS) and ζ -potential measurements

The DLS and ζ -potential measurements were done at 25 °C using a Zetasizer Nano-ZS (Malvern Instruments, UK) equipped with a He-Ne ion laser (λ = 633 nm) for the incident beam at a detection angle of 173°. Polyplex samples formed at 33 μ g pDNA/mL with different N/P ratios were adjusted to 10 μ g pDNA/mL using 10 mM Tris-HCl buffer (pH 7.5). The polyplex samples (50 μ L) were transferred into micro cuvettes and then followed

by the measurement. The data obtained were analyzed by the cumulant method, and the hydrodynamic diameters of samples were then calculated by the Stokes–Einstein equation and the sizes were reported as Z-average diameters. The ζ -potential of polyplexes was estimated using a Dip cell (Malvern Instruments, UK) and the electrophoretic mobility was measured. The ζ -potential was then calculated by the Smoluchowski equation.

2.8. In vitro transfection

MDA-MB-231 human breast cancer cell line and A549 human lung cancer cell line were obtained from ATCC and cultured in DMEM and RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin, respectively. MDA-MB-231 was seeded at 50,000 cells/well and A549 was seeded at 30,000 cells/well on 24-well plates and incubated for 24 h. After the cells reached to about 60% confluence, polyplex solutions (30 μ L) were prepared with different polymers synthesized at varying N/P ratios, diluted with 270 μ L of fresh culture medium containing 10% FBS, and then added to the cells. The pDNA amount for each well was kept at 1 μ g. The polyplexes were allowed to incubate with the cells for 24 h. The polyplex-containing medium was then removed and 1 mL of fresh DMEM with 10% FBS was added to each well. The cells were then maintained at 37 °C for another 24 h before luciferase assay. For luciferase assay, the cells were washed with 1 $\times$ PBS (1 mL) after medium removal. 140 μ L of lysis reagent was then added to each well and the cells were incubated at room temperature for 15 min. 100 μ L of luciferase substrate was added to 20 μ L of the lysate and the luminescence intensity was measured by an Orion microplate luminometer (Berthold Detection Systems, Oak Ridge, TN). The obtained luciferase activity was then normalized with the amount of total proteins present in the lysates determined by the RC DC Protein Assay Kit.

2.9. In vitro cytotoxicity

MDA-MB-231 cells were seeded at 5000 cells/well on 96-well plates in DMEM containing 10% FBS and incubated for 24 h. Polyplex solutions (10 μ L) prepared at different N/P ratios and free polymer solutions with different amine concentrations were diluted with fresh medium (90 μ L) and then added to the cells. The cells were incubated with polyplexes or free polymers for 24 h before refreshing medium. The cells were incubated for another 24 h and the assay was carried out according to the manufacturer's protocol (CellTiter-Blue® Cell Viability Assay). The fluorescence signals were measured 4 h after adding the dye. The results were represented as percentages of cell viability determined using untreated cells.

2.10. Flow cytometry measurements

pDNA was labeled with Label IT® Tracker™ Intracellular Nucleic Acid Localization Kit, Cy™5 (Mirus, Madison, WI) according to the manufacturer's protocol. MDA-MB-231 cells were seeded at 100,000 cells/well on 6-well plates in DMEM containing 10% FBS and incubated for 24 h. Polyplex solution prepared with Cy5-labeled pDNA for each polymer at N/P ratio of 10 was added to the cells after dilution with fresh DMEM medium containing 10% FBS and incubated for 4 h. The concentration of pDNA was kept at 2 μ g/well. After removing the polyplex-containing medium, the cells were rinsed with PBS 3 times. The cells were trypsinized with 500 μ L of trypsin-EDTA and collected by centrifugation at 300 g for 2 min. The cell pellets were resuspended in 500 μ L PBS. The Cy5 fluorescence intensity of each sample was then measured using BD LSR II cytometer (BDBiosciences, Franklin Lakes, NJ) equipped with a 633 nm laser for excitation.

2.11. Confocal laser scanning microscopy

MDA-MB-231 cells were seeded at 100,000 cells/well on sterile coverslips on 6-well plates in DMEM containing 10% FBS and incubated for 24 h. Polyplex solution prepared with 2

µg Cy5-labeled pDNA for each sample at N/P ratio of 10. The polyplexes were added to the cells after dilution with fresh DMEM medium containing 10% FBS and incubated for designated time periods. Polyplex containing medium were then removed and the cells were rinsed with PBS. After staining the acidic late endosomes and lysosomes with 75 nM LysoTracker Green (Invitrogen) in DMEM for 30 min at 37 °C, the cells were rinsed again with PBS and then fixed with 4% formaldehyde for 10 min at room temperature. The cells were washed with PBS and the cell nuclei were stained with 3 mM DAPI (Invitrogen) in PBS for 5 min at room temperature. The cells were rinsed with PBS and the coverslips were mounted on glass slides with SlowFade mounting medium (Invitrogen) and then the microscopy was done using FluoView 1000 confocal microscope (Olympus) with a 100 × objective. The excitation wavelengths used were 405 nm for DAPI (blue), 488 nm for LysoTracker Green (green), and 635 nm for Cy5 (red).

3. Results

3.1. Synthesis of PEG-*b*-P85-*b*-P[Asp(DET)]

Block copolymers of PEG-*block*-poly(aspartamide) and P85-*block*-poly(aspartamide) were synthesized according to Scheme 1. Initially, P85 was modified to introduce a primary amine for subsequent polymerization. P85 was activated with 1 M equivalent amount of 4-nitrophenyl chloroformate in order to minimize the production of P85 with both hydroxyl ends activated. The activated P85 was then reacted with excess amount of 1,3-diaminopropane to prevent P85-dimer formation. After purifications, ¹H NMR showed that the activation percentage of P85 was about 70% at the first step, while the amine functionality was approximately 50% after the second reaction. GPC analysis confirmed that the obtained P85 with terminal amino group contained no dimers and the molecular weight polydispersity was determined to be 1.11. The ring-opening polymerization of β-benzyl L-aspartate *N*-carboxyanhydride (BLA-NCA) was then carried out using α-methoxy-ω-amino poly(ethylene glycol), and the modified P85 amine, as the initiators to prepare PEG-*b*-PBLA, and P85-*b*-PBLA, respectively. The degrees of polymerization of the PBLA blocks were confirmed to be 106 for PEG-*b*-PBLA and 105 for P85-*b*-PBLA. GPC measurements revealed that the molecular weight polydispersity was 1.09 and 1.18 for PEG-*b*-PBLA and P85-*b*-PBLA, respectively. The introduction of diethylenetriamine (DET) to the PBLA blocks was done by aminolysis reaction with excess amount of DET relative to the benzyl ester groups of PBLA at low temperature. After protonation of the DET side chains and purification using dialysis at 4 °C to prevent degradation of the polymers, ¹H NMR obtained with the obtained polymers showed that the removal of benzyl ester groups was complete and the substitution rates for both PEG-*b*-P[Asp(DET)] and P85-*b*-P[Asp(DET)] were close to 100%. Table 1 summarized the compositions and physical characteristics of the synthesized polymers.

3.2. Formation and stability of polyplexes

Polyplexes were formed by adding stock polymer solution to pGL4 pDNA solution directly at different N/P ratios. To confirm the polyplex formation between the pDNA and polymers, PEG-*b*-P[Asp(DET)] or P85-*b*-P[Asp(DET)], agarose gel electrophoresis was employed. As shown in Fig. 2, the amount of free pDNA decreased with N/P ratio. Complete pDNA retardation was observed at N/P ratio > 2 for each sample, which indicated that no free pDNA was present and all pDNA was trapped inside the polyplex particles at N/P ratio > 2. As the N/P ratio was calculated based on the total number of primary and secondary amino groups on the polymers and it has been reported previously that there is around 50% protonation of the DET side chains at pH 7.4 [18], N/P 2 would be the stoichiometric mixing ratio between the pDNA and polymers and the minimal ratio of complete pDNA retardation. The results obtained are consistent with published data [10].

After confirming the polyplex forming ability of the polymers, the diameters of the polyplexes formed at various N/P ratios were measured by dynamic light scattering technique and the results are shown in Fig. 3A. Within the N/P ratio range tested, all the polyplexes maintained sizes below 90 nm. The sizes of polyplexes with N/P > 5 stayed the same at about 70 nm for both PEG-*b*-P[Asp (DET)] and P85-*b*-P[Asp(DET)] polyplexes. Moreover, there was no aggregation observed for the polyplexes formed at stoichiometric mixing ratio (N/P 2) which implied that the PEG and P85 chains on the polyplex surface could minimize the interactions among polyplex particles even the particles possessed neutral charges.

The ζ -potential of the polyplexes was determined by laser-Doppler electrophoresis. As seen in Fig. 3B, the ζ -potential increased with N/P ratio of the polyplexes. The ζ -potential was almost neutral (0 mV) at N/P ratio of 2 and this is consistent with the stoichiometry at this N/P ratio as all negative charges on the pDNA molecules are neutralized by the positive charges on the polymer molecules with no free polymer theoretically present. The ζ -potential values of both PEG-*b*-P[Asp(DET)] and P85-*b*-P[Asp (DET)] polyplexes leveled off at N/P 5 and the values stayed the same up to N/P 20. The maximum ζ -potential values obtained in this study are + 10 mV and +15 mV for PEG-*b*-P[Asp(DET)] and P85-*b*-P[Asp(DET)] polyplexes, respectively. The difference in maximum ζ -potential values revealed that the charge shielding efficiency of the PEG layer may be higher than that of the P85 layer surrounding the polyplex particle.

Colloidal stability is one of the important factors governing polyplexes for *in vivo* applications; therefore, the stability of the polyplexes was examined under physiological salt concentration of 150 mM NaCl based on the particle size measurements. If the polyplexes are not stable, salt-induced aggregation will occur due to the loss of electrostatic repulsion after the charges on the polyplexes are screened by salt. The sizes of the polyplexes in solvent containing 150 mM NaCl were monitored for 12 h at 25 °C by DLS. The size measurement was done every 30 min after salt addition. Both polyplexes formed with PEG-*b*-P[Asp(DET)] and P85-*b*-P[Asp(DET)] showed growth in size within the 12 h time period. The size of the PEG-*b*-P[Asp(DET)] polyplexes increased from 100 nm to about 140 nm, while the P85-*b*-P[Asp(DET)] polyplexes showed a more significant size increase from 120 nm to 270 nm (Fig. 4A). Although the size increased substantially for the P85-*b*-P[Asp(DET)] polyplexes, the polydispersity (PDI) remained below 0.18 after 12 h incubation (Fig. 4B). This implies that the uniform size distribution of the polyplexes prepared from P85-*b*-P[Asp(DET)] could still be maintained with the growth in particle size. The particle size polydispersity did not change significantly for PEG-*b*-P[Asp(DET)] polyplexes.

3.3. In vitro transfection efficiency

The effect of substituting PEG with P85 on the block copolymers on gene delivery ability of the polyplexes was evaluated from the luciferase gene expression in the cells after transfection. The transfection experiments were carried out in MDA-MB-231 human breast cancer cell line and A549 human lung cancer cell line using polyplexes prepared from PEG-*b*-P[Asp(DET)] and P85-*b*-P[Asp (DET)] at various N/P ratios. Polyplexes formed with bPEI at N/P 10 was used as a control as N/P 10 was determined to be optimal N/P ratio for transfection without causing massive cell death. Fig. 5A shows that P85-*b*-P[Asp(DET)] was the best transfecting agent in the study with MDA-MB-231 cells. It is an efficient vehicle for pDNA delivery at both low and high N/P ratios. The transfection efficiency of P85-*b*-P[Asp(DET)] polyplexes did not increase a lot with N/P ratios. Within the N/P ratio range tested, the luciferase expressions are within the 1 order of magnitude. Furthermore, the P85-*b*-P[Asp (DET)] polyplexes achieve higher luciferase expression than bPEI polyplexes at every single N/P ratio. On the other hand, PEG-*b*-P [Asp(DET)] polyplexes showed lower

transfection ability than P85-*b*-P[Asp(DET)] polyplexes, and the gene expression level obtained with PEG-*b*-P[Asp(DET)] polyplexes was highly dependent on the N/P ratio at which the polyplexes formed. Quantitatively, the luciferase expression level was about 3 orders of magnitude higher for P85-*b*-P[Asp(DET)] polyplexes at N/P 5, while the difference diminished to 1 order of magnitude at N/P 40 in MDA-MB-231 cells. A549 cells were also used for comparing the transfection efficiency of different polyplexes (Fig. 5B) and a similar tendency was observed. The difference in luciferase gene expression was clearly observed between PEG-*b*-P[Asp(DET)] and P85-*b*-P[Asp(DET)] polyplexes formed at low N/P ratios, and the expression level became more comparable at high N/P ratios. Free P85 block copolymers at various concentrations were also added to the polyplex solution for transfection study; however, improvement in transfection efficiency could not be observed and significant cell death was detected at high concentrations of free P85 under the tested conditions (data not shown).

3.4. In vitro cytotoxicity

The toxicity caused by the PEG-*b*-P[Asp(DET)], P85-*b*-P[Asp(DET)], and bPEI polymers was evaluated using MDA-MB-231 cells based on the change of cellular metabolism. The study was done with different polyplexes at the N/P ratio range (N/P 5–40) used in transfection study. As shown in Fig. 6A, P85-*b*-P[Asp(DET)] polyplexes showed slightly higher toxicity than PEG-*b*-P[Asp(DET)] polyplexes did; however, they could still maintained about 90% cell viability at N/P ratio of 40. On the contrary, polyplexes formed with bPEI caused significant toxicity to the cells. The cell viability decreased to 15% at N/P 20 and almost 0% at N/P 40. The intrinsic toxicity of free polymers was also investigated. No substantial toxicity caused by free PEG-*b*-P[Asp(DET)] or P85-*b*-P[Asp(DET)] polymers was observed even up to amine concentration of 1000 μ M, while bPEI polymers elicited appreciable toxicity towards the cells (Fig. 6B). 50% growth inhibition was observed at amine concentration of approximately 100 μ M and complete growth inhibition was obtained at 250 μ M.

3.5. Cellular uptake study

Uptake of polyplexes prepared with different cationic polymers at N/P 10 by MDA-MB-231 cells was quantified by flow cytometry. After 4 h incubation with the cells, polyplexes formed with P85-*b*-P [Asp(DET)] showed highest level of cellular internalization based on the mean fluorescence intensity detected from the Cy5-labeled pDNA, which was 3 times higher than that of bPEI polyplexes and about 10 times higher than that of the polyplexes formed with PEG-*b*-P[Asp(DET)] (Fig. 7A). The overall trend of cellular uptake of polyplexes is the same as the one obtained with transfection experiments. Comparing to PEG-*b*-P[Asp(DET)], P85-*b*-P[Asp(DET)] promoted a more narrow distribution and a higher level of the amount of polyplex uptake within the cell population (Fig. 7A and B).

3.6. Intracellular trafficking

To gain more insight into the intracellular localization of polyplexes after cellular uptake, confocal laser scanning microscopy was employed to collect images of cells incubated with polyplexes for different time durations. Cell nuclei were stained with DAPI (blue). pDNA was labeled with Cy5 (red), while late endosomes and lysosomes were stained with LysoTracker Green as shown in green in the confocal images. Perfect colocalization of pDNA and late endosomes/lysosomes produced yellow pixels due to the overlapping of the red and green fluorescence. As seen in Fig. 8, larger amount of red fluorescence could be observed with the P85-*b*-P [Asp(DET)] polyplexes at all the time points tested in this study, and this is consistent with the results obtained from the flow cytometry analysis. The polyplexes prepared from P85-*b*-P[Asp(DET)] interacted with the cellular membrane as fast as 30 min after polyplex addition as majority of the polyplexes was found to be localized at

the cell surface (Fig. 8A). Polyplexes progressively arrived at the late endosomes and lysosomes between 2 and 4 h as more yellow pixels were observed at 4 h after incubation started (Fig. 8B and C). After 24 h, fraction of the polyplexes successfully escaped from the acidic vesicles as red fluorescence was detected in the cytoplasm and around those vesicles. Yellow pixels could still be seen at this time point which represents partial escape of polyplexes from late endosomes and lysosomes (Fig. 8D). There was no substantial difference observed in term of intracellular trafficking between polyplexes formed with PEG-*b*-P[Asp(DET)] and P85-*b*-P[Asp(DET)] except the total amount of Cy5 fluorescence detected.

4. Discussion

PEGylated polyplexes have been extensively studied for delivering nucleic acid materials ultimately for therapeutic purposes. On the one hand, PEGylation enhances the colloidal stability of polyplexes and minimizes the recognition of the polyplexes by the reticuloendothelial system, but on the other hand, PEGylation reduces the interactions between the polyplexes and the target cells and which leads to hampered cellular uptake of the polyplexes and subsequently diminished transfection efficacy [28]. In this regard, the PEG block of PEG-*b*-P[Asp(DET)] polymer was substituted with amphiphilic Pluronic P85 to investigate whether the cellular uptake and transfection efficiency could be improved through promoting polyplex particle and cell membrane interaction.

P85-*b*-P[Asp(DET)] was prepared by the polymerization of BLA-NCA using P85 modified with amine functionality as the initiator and the subsequent aminolysis reaction to substitute the benzyl ester protecting groups with diethylenetriamine (DET) for introducing cationic moieties (Scheme 1). The complexation of pDNA by the polymers synthesized, PEG-*b*-P[Asp(DET)] and P85-*b*-P[Asp(DET)], was confirmed by gel retardation analysis as shown in Fig. 2. Complete retardation was observed at $N/P > 2$, which corresponds to $N^+/P > 1$ at physiological pH 7.4 due to the mono-protonated state of the DET cationic side chains. Physical properties such as particle size and ζ -potential were characterized as they are important factors governing whether the delivery system can be employed for future *in vivo* studies. The polyplexes formed with both block copolymers have sizes at about 70 nm when N/P ratio is 5 or greater (Fig. 3A). Although close-to-neutral ζ -potential was observed at the stoichiometric N/P ratio of 2 (Fig. 3B), no aggregation could be observed based on the size measurements and this reveals that both PEG and P85 chains can provide certain level of protection for the polyplexes when the particles are experiencing minimal repulsive force at this critical N/P ratio. The values of ζ -potential of the polyplexes also leveled off at N/P 5 and stayed at around +10 mV and +15 mV for PEG-*b*-P[Asp(DET)] polyplexes and P85-*b*-P[Asp(DET)] polyplexes, respectively. As previously reported that the ζ -potential can go up to +30 mV for particles prepared from non-PEGylated P[Asp(DET)] homopolymers [29], the ζ -potential result suggests that the PEG and P85 chains on the surface of the particles can effectively shield the charges on the particles, while the PEG chains provided better charge shielding effect as lower ζ -potential was observed. The lower charge masking ability of P85 can be owing to its lower molecular weight (PEG: 5000 g/mol, and P85: 4600 g/mol) and its amphiphilic nature, which may result in a thinner P85 palisade surrounding the polyplex compared to PEG.

Polyplex stability is important for *in vitro* and especially crucial for *in vivo* systemic gene delivery. The effect of introducing P85 to polyplex surface on stability of polyplexes in solution with physiological salt concentration was monitored for 12 h based on the change of particle size (Fig. 4A) and polydispersity (Fig. 4B) as salt can screen the charges on the polyplexes and minimize the charge repulsion force among polyplex particles. PEG-based polyplexes demonstrated superior stability compared to P85-based polyplexes. The increase

in particle size and change in polydispersity were small for PEG-based polyplexes, while the size increased about 2 times for the polyplexes prepared with P85-*b*-P[Asp(DET)] within the 12 h period. The lower stability of the P85-based polyplex may be related to the more hydrophobic P85 layer surrounding the polyplex which provides less protection to the polyplex particle against salt-induced aggregation compared to the more hydrophilic PEG layer. When the particles lose charges in physiological salt environment, they will have higher chance to interact with one another, and at the same time the hydrophobicity of the P85 chains may provide the driving force for the polyplex particles to come together based on hydrophobic interaction. This result allows us to know that modifications on polyplex to improve the stability are necessary for future *in vivo*, especially systemic delivery applications.

The substitution of PEG block with Pluronic P85 on the block cationomer led to enhanced transfection efficiency of the polymers in particular when the polyplexes were formed at low N/P ratios. High transfection efficiency could be achieved in both MDA-MB-231 and A549 cell lines with low N/P ratio polyplexes prepared with P85-*b*-P[Asp(DET)], and increased N/P ratio did not contribute to better transfection result (Fig. 5). On the contrary, the transfection ability of PEG-based polyplexes was highly dependent on N/P ratio. PEG-based polyplexes prepared at high N/P ratios, for example N/P 40, are required to achieve results comparable to P85-based polyplexes. Study has shown that free cationic polymers exist when N/P ratio is higher than 4 for this specific PEG block copolymer [30]. This implies that large excess amount of PEG-*b*-P[Asp(DET)] polymers is required to coexist with the polyplexes in order to achieve high transfection and this will impose an obstacle for *in vivo* applications because injected materials are diluted rapidly in physiological environments and it may be impossible for large amount of free polymers to concentrate at the target sites. As P85-*b*-P[Asp(DET)] polyplexes were shown to possess high transfection ability even the N/P ratio was low as 5 and without the presence of large excess amount of polymers, this could be an advantage of using this gene delivery vehicle for *in vivo* applications. Previous studies have shown that including free Pluronic block copolymers in different polyplex formulations (branched PEI, Superfect [31], and poly(2-*N*-(dimethylaminoethyl) methacrylate [23]) could enhance the transfection; nonetheless, no increase in transfection efficiency was observed in this study using the combination of free P85 and P85-*b*-P[Asp(DET)] as the free Pluronic and polyplex-forming agent, respectively. Although no definite conclusion can be drawn whether free Pluronic can promote better transfection of P85-*b*-P[Asp(DET)] polyplexes at this stage, it is possible that the testing conditions may play a crucial role for utilizing free Pluronic as transfection enhancer.

The mechanism for the improved transfection performance by P85-*b*-P[Asp(DET)] polyplexes was further elucidated by the analyses of cellular uptake and intracellular trafficking of the polyplexes. It was confirmed by both flow cytometry (Fig. 7) and confocal microscopy (Fig. 8) that the cellular internalization of the P85-*b*-P[Asp(DET)] polyplexes was much more efficient than that of the PEG-based polyplexes. The P85-*b*-P[Asp(DET)] polyplexes started interacting with the cell membrane extensively within 30 min and the polyplexes accumulated inside the cells at a much higher level during a 24 h time course than the PEG-*b*-P[Asp(DET)] polyplexes did. All these data are consistent with the hypothesis that the amphiphilic characteristic of P85 substantially facilitates the cellular uptake of the polyplex particles by increasing the interaction between the particles and the cellular membrane. The amphiphilic nature of P85 helps improve the internalization of particles, but on the other hand it is also possible that P85 may cause excess disruption of the cell membrane and lead to undesired toxicity or cell death. However, significant toxicity caused by P85-*b*-P[Asp(DET)] polyplexes or free polymers could not be observed in this study based on the cellular metabolic level (Fig. 6); therefore, P85-*b*-P[Asp(DET)] is an efficient synthetic delivery vehicle for pDNA with very low toxicity.

5. Conclusions

In this study, we demonstrated that substituting the hydrophilic PEG shell with amphiphilic Pluronic P85 layer on the surface of polyplex could lead to higher transfection efficiency of the P[Asp (DET)]-based pDNA delivery system. The amphiphilic nature of the P85 shell promoted cellular uptake of the polyplex particles and subsequently increased the transfection ability of the particles. Another advantage of using this P85-based polyplex for gene delivery is no excess polymers are necessary for high transfection efficiency and this is especially beneficial for *in vivo* applications. However, it has been shown in this study that the stability with respect to salt-induced aggregation of the P85-based polyplexes is lower than that of the PEG-based polyplexes; therefore, further improvements on the P85-based polyplex stability may be required before it can be used for *in vivo*, particularly systemic pDNA delivery.

References

1. Lehrman S. Virus treatment questioned after gene therapy death. *Nature*. 1999; 401:517–8. [PubMed: 10524611]
2. Cavazzana-Calvo M, Lagresle C, Hacein-Bey-Abina S, Fischer A. Gene therapy for severe combined immunodeficiency. *Annu Rev Med*. 2005; 56:585–602. [PubMed: 15660528]
3. Merdan T, Kopecek J, Kissel T. Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. *Adv Drug Deliv Rev*. 2002; 54:715–58. [PubMed: 12204600]
4. Pack DW, Hoffman AS, Pun S, Stayton PS. Design and development of polymers for gene delivery. *Nat Rev Drug Discov*. 2005; 4:581–93. [PubMed: 16052241]
5. Klibanov AL, Maruyama K, Torchilin VP, Huang L. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. *FEBS Lett*. 1990; 268:235–7. [PubMed: 2384160]
6. Senior J, Delgado C, Fisher D, Tilcock C, Gregoriadis G. Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: studies with poly(ethylene glycol)-coated vesicles. *Biochim Biophys Acta*. 1991; 1062:77–82. [PubMed: 1998713]
7. Yokoyama M, Okano T, Sakurai Y, Ekimoto H, Shibasaki C, Kataoka K. Toxicity and antitumor activity against solid tumors of micelle-forming polymeric anticancer drug and its extremely long circulation in blood. *Cancer Res*. 1991; 51:3229–36. [PubMed: 2039998]
8. Merdan T, Kunath K, Petersen H, Bakowsky U, Voigt KH, Kopecek J, et al. PEGylation of poly(ethylene imine) affects stability of complexes with plasmid DNA under *in vivo* conditions in a dose-dependent manner after intravenous injection into mice. *Bioconjug Chem*. 2005; 16:785–92. [PubMed: 16029019]
9. Ogris M, Brunner S, Schuller S, Kircheis R, Wagner E. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther*. 1999; 6:595–605. [PubMed: 10476219]
10. Takae S, Miyata K, Oba M, Ishii T, Nishiyama N, Itaka K, et al. PEG-detachable polyplex micelles based on disulfide-linked block cationomers as bioresponsive nonviral gene vectors. *J Am Chem Soc*. 2008; 130:6001–9. [PubMed: 18396871]
11. Mishra S, Webster P, Davis ME. PEGylation significantly affects cellular uptake and intracellular trafficking of non-viral gene delivery particles. *Eur J Cell Biol*. 2004; 83:97–111. [PubMed: 15202568]
12. Hatakeyama H, Akita H, Kogure K, Oishi M, Nagasaki Y, Kihira Y, et al. Development of a novel systemic gene delivery system for cancer therapy with a tumor-specific cleavable PEG-lipid. *Gene Ther*. 2007; 14:68–77. [PubMed: 16915290]
13. Ogris M, Steinlein P, Carotta S, Brunner S, Wagner E. DNA/polyethylenimine transfection particles: influence of ligands, polymer size, and PEGylation on internalization and gene expression. *AAPS PharmSci*. 2001; 3:E21. [PubMed: 11741272]
14. Oba M, Fukushima S, Kanayama N, Aoyagi K, Nishiyama N, Koyama H, et al. Cyclic RGD peptide-conjugated polyplex micelles as a targetable gene delivery system directed to cells

- possessing $\alpha(v)\beta(3)$ and $\alpha(v)\beta(5)$ Integrins. *Bioconjug Chem.* 2007; 18:1415–23. [PubMed: 17595054]
15. Kanayama N, Fukushima S, Nishiyama N, Itaka K, Jang WD, Miyata K, et al. A PEG-based biocompatible block cationomer with high buffering capacity for the construction of polyplex micelles showing efficient gene transfer toward primary cells. *Chemmedchem.* 2006; 1:439–44. [PubMed: 16892379]
 16. Itaka K, Ishii T, Hasegawa Y, Kataoka K. Biodegradable polyamino acid-based polycations as safe and effective gene carrier minimizing cumulative toxicity. *Biomaterials.* 2010; 31:3707–14. [PubMed: 20153891]
 17. Miyata K, Fukushima S, Nishiyama N, Yamasaki Y, Kataoka K. PEG-based block cationomers possessing DNA anchoring and endosomal escaping functions to form polyplex micelles with improved stability and high transfection efficacy. *J Control Release.* 2007; 122:252–60. [PubMed: 17643543]
 18. Han M, Bae Y, Nishiyama N, Miyata K, Oba M, Kataoka K. Transfection study using multicellular tumor spheroids for screening non-viral polymeric gene vectors with low cytotoxicity and high transfection efficiencies. *J Control Release.* 2007; 121:38–48. [PubMed: 17582637]
 19. Batrakova EV, Li S, Alakhov VY, Miller DW, Kabanov AV. Optimal structure requirements for pluronic block copolymers in modifying P-glycoprotein drug efflux transporter activity in bovine brain microvessel endothelial cells. *J Pharmacol Exp Ther.* 2003; 304:845–54. [PubMed: 12538842]
 20. Batrakova EV, Vinogradov SV, Robinson SM, Niehoff ML, Banks WA, Kabanov AV. Polypeptide point modifications with fatty acid and amphiphilic block copolymers for enhanced brain delivery. *Bioconjug Chem.* 2005; 16:793–802. [PubMed: 16029020]
 21. Yi X, Batrakova E, Banks WA, Vinogradov S, Kabanov AV. Protein conjugation with amphiphilic block copolymers for enhanced cellular delivery. *Bioconjug Chem.* 2008; 19:1071–7. [PubMed: 18447367]
 22. Yang Z, Sahay G, Sriadibhatla S, Kabanov AV. Amphiphilic block copolymers enhance cellular uptake and nuclear entry of polyplex-delivered DNA. *Bioconjug Chem.* 2008; 19:1987–94. [PubMed: 18729495]
 23. Bromberg L, Deshmukh S, Temchenko M, Iourtchenko L, Alakhov V, Alvarez-Lorenzo C, et al. Polycationic block copolymers of poly(ethylene oxide) and poly(propylene oxide) for cell transfection. *Bioconjug Chem.* 2005; 16:626–33. [PubMed: 15898731]
 24. Vinogradov SV, Batrakova EV, Li S, Kabanov AV. Mixed polymer micelles of amphiphilic and cationic copolymers for delivery of antisense oligonucleotides. *J Drug Target.* 2004; 12:517–26. [PubMed: 15621677]
 25. Darly WH, Poche D. The preparation of N-carboxyanhydrides of α -amino-acids using bis(trichloromethyl)carbonate. *Tetrahedron Lett.* 1988; 29:5859–62.
 26. Li JT, Carlsson J, Lin JN, Caldwell KD. Chemical modification of surface active poly(ethylene oxide)-poly (propylene oxide) triblock copolymers. *Bioconjug Chem.* 1996; 7:592–9. [PubMed: 8889022]
 27. Harada A, Kataoka K. Formation of polyion complex micelles in an aqueous milieu from a pair of oppositely-charged block-copolymers with poly (ethylene glycol) segments. *Macromolecules.* 1995; 28:5294–9.
 28. Hatakeyama H, Akita H, Harashima H. A multifunctional envelope type nano device (MEND) for gene delivery to tumours based on the EPR effect: a strategy for overcoming the PEG dilemma. *Drug Deliv Rev.* 2010
 29. Miyata K, Oba M, Nakanishi M, Fukushima S, Yamasaki Y, Koyama H, et al. Polyplexes from poly(aspartamide) bearing 1,2-diaminoethane side chains induce pH-selective, endosomal membrane destabilization with amplified transfection and negligible cytotoxicity. *J Am Chem Soc.* 2008; 130:16287–94. [PubMed: 19006313]
 30. Oba M, Miyata K, Osada K, Christie RJ, Sanjoh M, Li W, et al. Polyplex micelles prepared from omega-cholesteryl PEG-polycation block copolymers for systemic gene delivery. *Biomaterials.* 2011; 32:652–63. [PubMed: 20932567]

31. Sriadibhatla S, Yang Z, Gebhart C, Alakhov VY, Kabanov A. Transcriptional activation of gene expression by pluronic block copolymers in stably and transiently transfected cells. *Mol Ther*. 2006; 13:804–13. [PubMed: 16199206]

Fig. 1.
Schematic illustration of enhanced cellular uptake by Pluronic-based polyplexes.

Fig. 2.
Gel retardation analysis of the polyplexes.

Fig. 3. Cumulant diameters (A) and ζ -potential values (B) of polyplexes formed at different N/P ratios in Tris-HCl buffer (pH 7.5) at 25 °C. Results are expressed as mean $\pm$ SD ($n = 3$).

Fig. 4. Time-dependent change of cumulant diameter (A) and polydispersity (B) of the polyplexes prepared at N/P 10 and incubated in Tris-HCl buffer (pH 7.5) containing 150 mM NaCl at 25 °C. Error bars in (A) represent SD ($n = 3$).

Fig. 5.

In vitro transfection efficiency of polyplexes formed at different N/P ratios in MDA-MB-231 (A) and A549 (B) cell lines. Polyplexes formed with bPEI at N/P 10 were used as control. Results are expressed as mean $\pm$ SD ($n = 3$). * $P < 0.005$.

Fig. 6.

In vitro cytotoxicity of polyplexes prepared at different N/P ratios (A) and free polymers at various amine concentrations (B) in MDA-MB-231 cells. 100% cell viability was determined from untreated cells. bPEI was used as control. Results are expressed as mean $\pm$ SD ($n = 3$).

Fig. 7. Cellular uptake analysis of polyplexes (N/P 10) in MDA-MB-231 cells. (A) Flow cytometric data reported as mean of the relative fluorescence intensity $\pm$ SD ($n = 3$). (B) Flow cytometric histogram profiles of different polyplexes showing the distribution of Cy5 fluorescence intensity. * $P < 0.005$. ** $P < 0.001$.

Fig. 8.

Intracellular trafficking analysis by confocal laser scanning microscopy. Polyplexes containing Cy5-labeled pDNA (red) were formed with P85-*b*-P[Asp(DET)] (A–D) and PEG-*b*-P[Asp(DET)] (E–H) at N/P 10 and incubated for different time periods (A, E: 0.5 h, B, F: 2 h, C, G: 4 h, D, H: 4 h and subsequent 20 h polyplex free incubation) with MDA-MB-231 cells before images were taken. Cell nuclei and late endosomes/lysosomes were stained with DAPI (blue) and LysoTracker Green (green), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Scheme 1.

Syntheses of Pluronic (P85) amine (A) and PEG-/P85-b-P[Asp(DET)] block copolymers (B).

Table 1

Characteristics of the polymers used in this study.

Polymer	Initiator				Total MW (g/mol) ^d	M_w/M_n ^b
		MW (g/mol) ^a	M_w/M_n	–NH ₂ %		
PEG- <i>b</i> -P[Asp(DET)]	5200	1.04 ^a	98.4 ^a	106	30300	1.09
P85- <i>b</i> -P[Asp(DET)]	4600	1.11 ^b	50 ^c	105	29600	1.18

^a Information provided by manufacturers.

^b Determined by GPC.

^c Determined by ¹H NMR.

^d Calculated based on the HCl salt form of the polymers.