International Journal of Pharmaceutics 427 (2012) 105–112

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Biodegradable hybrid recombinant block copolymers for non-viral gene transfection

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ARTICLE INFO

Article history:
Received 31 March 2011
Received in revised form
12 September 2011
Accepted 24 September 2011
Available online 1 October 2011

Keywords:
Elastin-like polypeptide (ELP)
Hybrid recombinant polymer
Gene delivery
Thermo-responsive
Diethylenetriamine (DET)

ABSTRACT

Thermal targeting of therapeutic genes can enhance local gene concentration to maximize their efficacy. However, lack of safe and efficient carriers has impeded the development of this delivery option. Herein, we report the preparation and evaluation of a hybrid recombinant material, p[Asp(DET)]$_{53}$ELP(1–90), that possess a thermo-responsive elastin-like polypeptide (ELP) segment and a diethylenetriamine (DET) modified poly-L-aspartic acid segment. The term, hybrid, indicates that the material was prepared by genetic engineering and synthetic chemistry. In summary, the thermal phase transition behavior and cytotoxicity of the biodegradable copolymer were studied. The polyplexes formed by the copolymer and pGL4 plasmid were characterized by dynamic light scattering and ζ-potential measurements. The polyplexes retained the thermal phase transition behavior conferred by the copolymer; however, they exhibited a two-step transition process not seen with the copolymer. The polyplexes also showed appreciable transfection efficiency with low cytotoxicity.

1. Introduction

Gene therapy is a promising therapeutic option that has the potential to cure diseases from the root. The majority of gene therapy clinical trials have utilized viruses for delivering therapeutic genes; however, due to several high profile incidents, there has been a growing concern over the immunogenicity and safety of viruses (Tan et al., 2008). As a result, non-viral gene delivery has attracted much attention during the past decade in an effort to avoid the use of viruses (Hart, 2000). Because of the drawback of poor gene transfer efficiency, non-viral gene delivery has yet to realize its potential. One approach to address the issue of poor gene delivery efficiency involves the use of stimuli-responsive polymers to specifically accumulate therapeutic gene at the disease site and/or facilitate intra-cellular DNA release from the delivery vehicles (Du et al., 2010; Ganta et al., 2008).

Stimuli-responsive polymers responding to various environmental cues such as heat, pH, light, and redox potential have been utilized in gene delivery (Berg et al., 1999; Cavallaro et al., 2006; Hinrichs et al., 1999; Murthy et al., 2003). Among these different polymers, the well-known thermo-responsive polymer, poly(*N*-isopropylacrylamide) (PNIPAAm), has garnered much attention due to the ease of synthesizing variants of PNIPAAm via free-radical polymerization. Clinical therapies have long used control of local temperature at the therapeutic target area with thermal ablation, hyperthermia, or cryoablation; thermo-responsive non-viral gene carriers present an attractive option exploiting thermal-based modalities. The majority of the literature in thermo-responsive polymeric gene delivery has emphasized the gene transfection enhancement accompanying hypothermia (Kurisawa et al., 2000; Lavigne et al., 2007; Sun et al., 2005; Turk et al., 2007). The main hypothesis regarding the transfection enhancement is that PNIPAAm releases DNA as it undergoes a globule to coil conformational change when the temperature is cooled below the polymer's lower critical solution temperature (LCST). However, two research groups reported a contradictory observation, where enhanced transfection was obtained by raising the temperature from below to above the polymer's LCST (Bisht et al., 2006; Zintchenko et al., 2006). Zintchenko et al. (2006) attributed this enhanced transfection above the LCST to increased cation numbers in the endosomal compartment resulting from polyplex aggregation, whereas Bisht et al. (2006) attributed this observation to greater cationic presentation on the surface of polyplexes. Though PNIPAAm has tenuous toxicity, it is not biodegradable, and its accumulation in the body can be a concern over long-term exposure. Therefore, it is imperative to develop the next generation of thermo-responsive polymers that boast biodegradability and biocompatibility and also exhibit no toxicity.

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doi:10.1016/j.ijpharm.2011.09.035

Elastin-like polypeptides (ELPs) are a class of genetically engineered, thermo-responsive polymers that consist of VPGXG pentapeptide monomers, where X is a guest residue that can be any amino acid except proline (Meyer and Chilkoti, 1999). Indeed, ELPs may be considered as a recombinant, protein-based polymer analogue of PNIPAAm. ELPs are water-soluble, biocompatible (Cappello et al., 1990; Rincon et al., 2006; Urry et al., 1991b), and thermally responsive with an inverse temperature phase transition (Urry, 1997). The inverse temperature phase transition of ELPs characterized by the transition temperature (T_t) can be modulated by changing the identity of the amino acid in the X position and the length of the repeating pentapeptide (Meyer and Chilkoti, 2002). ELPs are inert, biodegradable materials without unwanted toxic effects and host immune responses (Massodi and Raucher, 2007; Rincon et al., 2006). Numerous reports have also demonstrated the potential of ELPs as useful biomaterials for various biomedical applications (Betre et al., 2006; Frey et al., 2003; Meyer and Chilkoti, 1999; Meyer et al., 2001a; Nath and Chilkoti, 2003; Rao et al., 2002). Most noticeably, ELPs have been exploited for thermal targeting of drugs to solid tumors (Bae et al., 2007; Bidwell et al., 2007; Liu et al., 2006; Meyer et al., 2001b), and for gene therapy (Chen et al., 2008; Cresce et al., 2008; Haider et al., 2005).

Previously, we have successfully cloned, expressed, and purified a novel ELP-based diblock copolymer bearing an oligo(lysine) block for pDNA condensation. The diblock copolymer successfully condensed pDNA and showed promising transfection efficiency. However, the biopolymer was limited at high concentrations by cytotoxicity (Chen et al., 2008). Furthermore, bacterial expression of diblock copolymers with a longer cationic block was restricted, thus prompting an alternative synthetic route. In contrast, cationic polymers possessing diethylenetriamine (DET) have been shown to exhibit minimal cytotoxicity and high gene transfection (Itaka et al., 2010; Kanayama et al., 2006; Lai et al., 2010). Herein, we report a new bioorganic ELP-based, cationic diblock copolymer, p[Asp(DET)]$_{53}$ELP(1–90) (where "1" represents a substitution of Val, Ala, and Gly in a 5:2:3 ratio at the X position and "90" indicates the polypeptide length of 90 repeating pentapeptides). This ELP-based cationic diblock copolymer is biodegradable, less toxic than previous biosynthetic biopolymers, and retains thermo-responsive behaviors for adjuvant therapy with clinically approved thermal ablation. This study assessed the thermal phase transition behavior, cytotoxicity, and transfection of p[Asp(DET)]$_{53}$ELP(1–90).

2. Materials and methods

2.1. Materials and cell culture

The plasmid encoding the ELP(1–30) polypeptide with repetitive pentapeptide units [(VPGXG)$_{90}$; X is Val, Ala, and Gly in a 5:2:3 ratio] was obtained from Prof. Ashutosh Chilkoti (Dept. of Biomedical Engineering, Duke University, USA). The ELP(1–30) gene was then used for oligomerization of the ELP(1–90) gene followed by expression of the ELP(1–90) polypeptide similarly to a previous report (Chen et al., 2008). β-Benzyl L-aspartate, anhydrous tetrahydrofuran (THF), anhydrous hexane, triphosgene, DET, and 25 kDa branched polyethyleneimine (BPEI) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and used as received. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin solution were purchased from Mediatech, Inc. (Herndon, VA, USA). The pGL4 vector, luciferase assay system, and Cell Titer-Blue® assay were obtained from Promega Corporation (Madison, WI). NaCl, Na_2HPO_4, KH_2PO_4, and KCl were purchased from Fisher Scientific (Waltham, MA).

COS-7 cells (African green monkey kidney cells; American type culture collection; Manassas, VA, USA) were maintained in DMEM supplemented with L-glutamine (4 mM), L-glucose (4.5 g/L), 10% (v/v) fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 μg/mL) in a 5% CO_2 humidified atmosphere at 37 °C and split twice a week.

2.2. Synthesis of thermo-responsive p[Asp(DET)]$_{53}$-ELP(1–90) diblock copolymer

β-Benzyl-L-aspartate *N*-carboxy-anhydride (BLA-NCA) was synthesized by the Fuchs–Farthing method using triphosgene (Daly and Poche, 1988). The poly(β-benzyl L-aspartate)-ELP(1–90) [p(BLA)ELP(1–90)] diblock copolymer was then synthesized by ring-opening polymerization of NCA using an ELP(1–90) polymer as the macro-initiator and BLA-NCA as the monomer. 138.5 mg (556.4 μmol) of BLA-NCA dissolved in 7 mL of anhydrous DMSO was transferred by a cannula to a flask containing 200 mg (5.56 μmol) lyophilized ELP(1–90) dissolved in 13 mL DMSO under an argon atmosphere. The solution was stirred at 40 °C for three days after which the polymer was precipitated in cold diethyl ether. The precipitation was repeated twice to remove residual DMSO and the polymer was collected by centrifugation at 1864 × *g* for 5 min after each precipitation. The product was then lyophilized (250 mg, 79.6% yield). ^{1}H NMR (DMSO – d_6): δ 0.73–0.90 (m, CH_3–valine), δ 1.16–2.06 (m; CH_3–alanine, β, γ CH_2–proline; β CH–valine), δ 3.50–4.34 (m; CH–alanine; CH_2–glycine; CH–proline; δ CH_2–proline; α CH–valine), δ 4.58 (b, CH–aspartate), δ 4.98 (b, CH_2–benzyl), δ 7.24 (b, C_6H_5–), δ 7.58–8.32 (m; NH). The degree of BLA polymerization was calculated from the integration ratio between methyl protons of valine (0.73–0.90 ppm) in the ELP block and the phenyl protons (7.24 ppm) of the benzyl protecting group of the poly(aspartate) p(Asp) side chain. DET (50 equivalent to the BLA repeating unit, 1.435 g, 14.14 mmol) was added to lyophilized p(BLA)ELP(1–90) (250 mg, 5.335 μmol) in 10 mL anhydrous DMF under argon. The reaction proceeded at 40 °C for 24 h; the reaction solution was transferred to a dialysis tube (6–8 kDa MWCO) and dialyzed against running deionized water until the solution pH became neutral. Anionic exchange was completed by twice dialyzing the solution against 0.01 N $HCl_{(aq)}$ for 1 h. Finally, the solution was dialyzed against running deionized H_2O until the solution pH became neutral. The p[Asp(DET)]$_{53}$ELP(1–90) copolymer solution was then freeze-dried producing a white fibrous solid (220 mg, 85.1% yield). Completion of the reaction was confirmed by the disappearance of the phenyl protons on ^{1}H NMR (D_2O) spectrum.

2.3. Thermal characterization of p[Asp(DET)]$_{53}$ELP(1–90) and polyplexes

The thermal phase transition behavior of p[Asp(DET)]$_{53}$ELP(1–90) and p[Asp(DET)]$_{53}$ELP(1–90)/pDNA polyplexes was studied by monitoring turbidity as a function of temperature. For all measurements, 600 μL of sample solution was loaded into a quartz cuvette, and the solution turbidity at 350 nm was monitored using a Cary 100 UV–vis spectrophotometer equipped with a temperature controller and a multi-cell holder (Agilent Technologies, Santa Clara, CA, USA) at a heating/cooling rate of 1 °C/min. Phase transition at slower (0.2 °C/min) or higher (5 °C/min) rates was initially tested, but no significant difference was found (data not shown). Experiments were modified as below to investigate the effects of polymer concentration, salt concentration, and pH on the thermal phase transition: (1) polymer concentration effects: a 100 μM stock solution of p[Asp(DET)]$_{53}$ELP(1–90) (concentration based upon ELP using the single tryptophan residue and an extinction coefficient of 5790 M^{-1} cm^{-1}) in phosphate buffered saline (PBS: 137 mM NaCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, and 2.7 mM KCl, pH 7.4) was prepared, and subsequently diluted to 1.0, 5.0, and 25 μM using

PBS; (2) salt concentration effects: 25 μM polymer solutions with salt concentrations of 0, 250, 500, and 1000 mM were prepared from phosphate buffers (containing 10 mM Na_2HPO_4 and 1.76 mM KH_2PO_4, pH 7.4) with various amounts of sodium chloride; and (3) pH effects: 25 μM polymer solutions were prepared in 10 mM Na_2HPO_4 buffer containing 140 mM NaCl, of which the pH were adjusted to 6, 7.4, and 9 by 0.01 N HCl or NaOH.

2.4. Titration of the p[Asp(DET)]$_{53}$ELP(1–90) diblock copolymer

The test polymers, BPEI and p[Asp(DET)]$_{53}$ELP(1–90), were prepared in 150 mM $NaCl_{(aq)}$ to a volume of 10 mL with a 2.8 mM amine concentration. Throughout the experiment, the temperature was maintained at 25 °C by a circulating water bath and the solutions were kept homogeneous by magnetic stirring. The degree of protonation versus pH was constructed from the change of H^+ levels in solution and amount of polymer used as described previously (Xiong et al., 2007).

2.5. Gel retardation assay of polyplexes

To confirm the ability of p[Asp(DET)]$_{53}$ELP(1–90) to electrostatically condense and retard agarose gel migration of pDNA, increasing N/P ratios (molar ratio of primary amine vs. DNA phosphate residues) were prepared. Briefly, polyplexes were formed by adding 10 μL of various concentrations of polymer in PBS to equal volume of 50 μg/mL pGL4 in PBS, the mixture was vortexed briefly and incubated at ambient temperature for 30 min before gel electrophoresis. All samples along with a free pGL4 control were loaded onto a 0.8% agarose gel containing 0.4 μg/mL ethidium bromide and electrophoresed for 45 min at 100 V.

2.6. Dynamic light scattering (DLS) and ζ-potential measurement

p[Asp(DET)]$_{53}$ELP(1–90)/pGL4 polyplexes were prepared similarly as previously described except that a low ionic strength phosphate buffer (4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, and 2.7 mM KCl; pH 7.4) was used instead of PBS to enable ζ-potential measurement. 500 μL and 750 μL polyplexes were subjected to DLS and ζ-potential measurements, respectively, at 25 °C on a Malvern Zetasizer Nano – ZS equipped with a He–Ne laser (Worcestershire, UK). The *Z*-average hydrodynamic diameter (D_h) was determined from the Stokes–Einstein relation, where the diffusion coefficients and polydispersity indices were calculated from cumulants analysis to the correlation function. The electrophoretic mobility of the polyplexes was measured by laser Doppler velocimetry. The mobility was used to calculate the ζ potential from the Henry equation, where the Smoluchowski approximation was used for the Henry's function in the equation. The thermal phase transition for the polyplexes was investigated as follow. 35 μL of 20, 100, and 500 μM polymers in PBS each were added to equal volume of 50 μg/mL pGL4, the mixed solution was vortexed briefly, and incubated at ambient temperature for 30 min. 630 μL PBS were then added to the mixed solution, resulting in the final polymer concentrations of 1.0, 5.0, and 25 μM with a 2.5 μg/mL pGL4 concentration.

2.7. Cytotoxicity

COS-7 cells were seeded in 96-well plates at an initial density of 5000 cells per well and incubated in 100 μL of DMEM with 10% FBS until 50–60% confluent. The medium was replaced with 90 μL DMEM and the cells were incubated for 1 h before dosing with polymers or the polyplexes. 10 μL of the sample solution was then added to each well and the plates incubated at 37 °C for 4 h. The medium was replaced with 100 μL fresh DMEM containing 10% FBS after dosing, and the cells were further incubated at 37 °C for an additional 44 h. Cell viability was probed by using the Cell Titer-Blue® assay; 20 μL assay solutions were added to each well followed by a 3 h incubation at 37 °C after which the results were read on a microplate reader (Molecular Devices, Sunnyvale, CA, USA) with an excitation at 560 nm and emission at 590 nm.

2.8. Transfection

COS-7 cells were seeded in 24-well plates at an initial density of 30,000 cells per well and incubated in 1 mL of DMEM with 10% FBS until 50–60% confluent. The medium was replaced with 360 μL DMEM and the cells were incubated for 1 h before transfection. 40 μL of sample solution was added to the medium in each well, and the cells were incubated at 37 °C for 4 h. The hyperthermic transfections were carried out at 43 °C for 30, 60, and 120 min, and then the temperature was lowered to 37 °C for the remainder of the 4 h transfection. The medium was replaced with 1.0 mL fresh DMEM containing 10% FBS and the cells were further incubated at 37 °C for an additional 44 h. The medium was removed and the cells washed once with 1.0 mL PBS pre-warmed in a water bath. Transgene expression was quantified by the luciferase assay. Luciferin reagent (100 μL) was added to a 20 μL sample and read immediately on a Berthold microplate luminometer (Oak Ridge, TN, USA) over a 10 s period with a 5 s delay. The total protein content was determined by a DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). 5 μL of sample solution were mixed with 20 μL Reagent A′ and 200 μL Reagent B. The mixture was incubated at room temperature for 15 min before reading the absorbance at 750 nm. The concentration of proteins was determined using a bovine gamma–globulin standard curve.

3. Results and discussion

3.1. Synthesis of thermo-responsive p[Asp(DET)]$_{53}$-ELP(1–90) diblock copolymer

The p(BLA)ELP(1–90) diblock copolymer was prepared (Fig. 1) as previously described (Bae et al., 2003). The polymerization took place under an inert atmosphere to prevent premature termination of the reaction. The product was collected via precipitation in cold ether and characterized by ^{1}H NMR in DMSO-d_6 (results shown in the materials and methods section). The degree of polymerization was calculated from the ratio between the methyl protons of valine in the ELP block and the phenyl protons of the benzyl protecting group on the p(Asp) side chain, and the copolymer appeared to have 53 repeating BLA units. Quantitative aminolysis of the p(BLA) side chain was then performed with DET under anhydrous conditions (Fig. 1), and completion of the aminolysis reaction was confirmed by the complete disappearance of the phenyl protons on the ^{1}H NMR spectrum in D_2O (data not shown).

3.2. Thermal characterization of p[Asp(DET)]$_{53}$ELP(1–90)

The thermal transition behavior of ELPs has been well studied. It was reported that the ELPs phase transition temperature is an inverse logarithmic function of ELP concentration (Meyer et al., 2001a; Urry et al., 1985). In the case of p[Asp(DET)]$_{53}$ELP(1–90) diblock copolymer, the same concentration dependent phase transition behavior of pure ELPs was evidenced by the phase transition profile shift towards lower temperatures with increasing concentration (Fig. 2a). Furthermore, at higher polymer concentrations, not only did the phase transition occur at a lower temperature, but the maximum turbidity of the solution also increased. High solution turbidity was likely due to the increased number of particles at higher copolymer concentrations because turbidity is a function

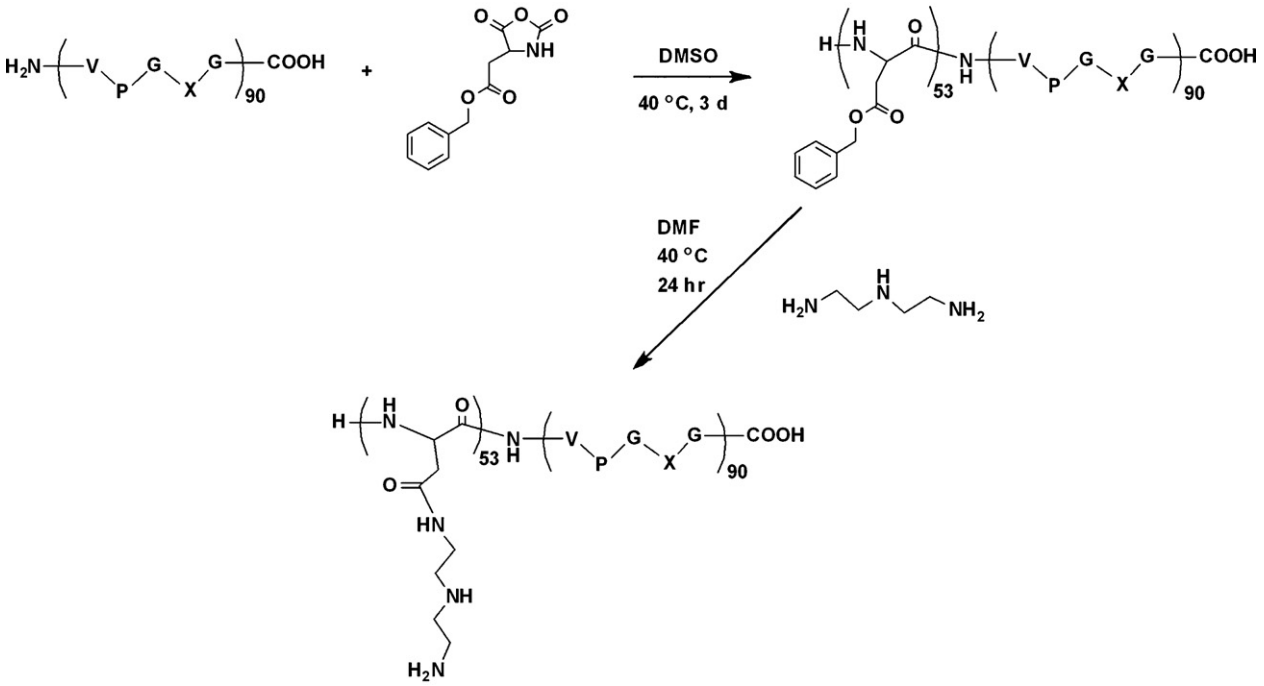

Fig. 1. Reaction scheme of p[Asp(DET)]$_{53}$ELP(1–90).

of both the particle size and the number of particles that act as light scattering centers.

The T_t at each concentration was defined as the temperature with the maximum first derivative of OD_{350} and calculated from the raw turbidity data. When the numerical values of T_t at each concentration were plotted against the logarithmic concentration (Fig. 2b), a linear relationship was obtained that mimics previous reports of ELPs concentration dependent phase transition (Meyer et al., 2001a). It should also be noted that the T_t of 25 μM p[Asp(DET)]$_{53}$ELP(1–90) was 3 °C higher that of ELP(1–90) alone. The increased T_t could be attributed to the presence of the hydrophilic DET moieties that could disrupt intra- and intermolecular ELP hydrophobic interactions, thus raising the T_t (Urry et al., 1991a). However, this observation is in sharp contrast with a previous report that the T_t of cationic ELPs drastically increased with a 2–5% cationic content (Lim et al., 2008), despite that the cationic content in p[Asp(DET)]$_{53}$ELP(1–90) was 10%. Nevertheless, in that report, lysine residues were incorporated into the ELP pentapeptide repeating sequence, whereas the cationic DET in this report existed as a separate block outside of the ELP sequence. It is plausible that the cationic residues being physically distant from the ELP sequence have less interaction with the ELP backbone; therefore, a lesser T_t shift was observed.

It was reported that the phase transition of ELPs is a two-step process (Yamaoka et al., 2003); the phase transition initially begins with a structural transformation from a random coil to a β-spiral confirmation, with the hydrophobic side chain exposed to the aqueous environment. At the same time, intra-molecular aggregation is prohibited due to the rigid backbone of the β-spiral confirmation formed in the initial step. As a result, further aggregation of ELPs after the initial conformational change proceeds through intermolecular interactions. This phenomenon distinguishes ELPs from other synthetic thermo-responsive polymers such as PNIPAAm, which do not exhibit a concentration-dependent phase transition.

It is known that the ELP thermal phase transition is sensitive to the addition of salt, where T_t decreases with increasing NaCl concentration (Cho et al., 2008; Luan et al., 1991; Meyer and Chilkoti, 1999). The presence of salts can significantly depress T_t by competing against the polypeptide for hydration or weaken the hydrophobic hydration of the polypeptide, depending on the type of salt (Cho et al., 2008). It was further reported by Lim et al. that a cationic ELP deviated from this relationship at 5% cationic content (Lim et al., 2008), but in Fig. 2c, it can be seen that the T_t of p[Asp(DET)]$_{53}$ELP(1–90) decreased linearly with increasing NaCl concentration resembling unmodified ELPs. Again, the difference between Fig. 2c and what was reported in the literature could be attributed to the cationic residues being physically distant from the ELP sequence.

3.3. Titration of the p[Asp(DET)]$_{53}$ELP(1–90) diblock copolymer

The DET functionality has two amino groups with distinct pK_a values of 6.0 and 9.5, among which only the primary amine is charged under physiological pH, thus giving DET containing polymers a unique pH responsiveness that could be exploited for endosomal release of bioactive compounds (Itaka et al., 2010; Kanayama et al., 2006). However, we speculated that the protonation state would shift the thermal transition profile at different pH as previously reported for ELPs with glutamic acid or histidine at the X position (MacKay et al., 2010). Therefore, it was necessary to investigate the pH-dependent phase transition behavior. First, we demonstrated the protonation state of p[Asp(DET)]$_{53}$ELP(1–90) through potentiometric titration (Fig. 3a), the degree of protonation of the diblock copolymer at each pH was then calculated from the titration data (Fig. 3b). It is obvious from the data that p[Asp(DET)]$_{53}$ELP(1–90) exhibited a two-step protonation profile corresponding to the different pK_a of DET and is consistent with the literature report (Kanayama et al., 2006). Subsequently, the thermal phase transition of p[Asp(DET)]$_{53}$ELP(1–90) at different pH was studied by monitoring the sample turbidity at 350 nm. In Fig. 3c, the transition curves shifted slightly as pH changed from 9 to 6. The overall trend of the T_t shift can be explained by increased hydrophobicity of the copolymer as more DET becomes deprotonated at pH 9 than 6. However, the change

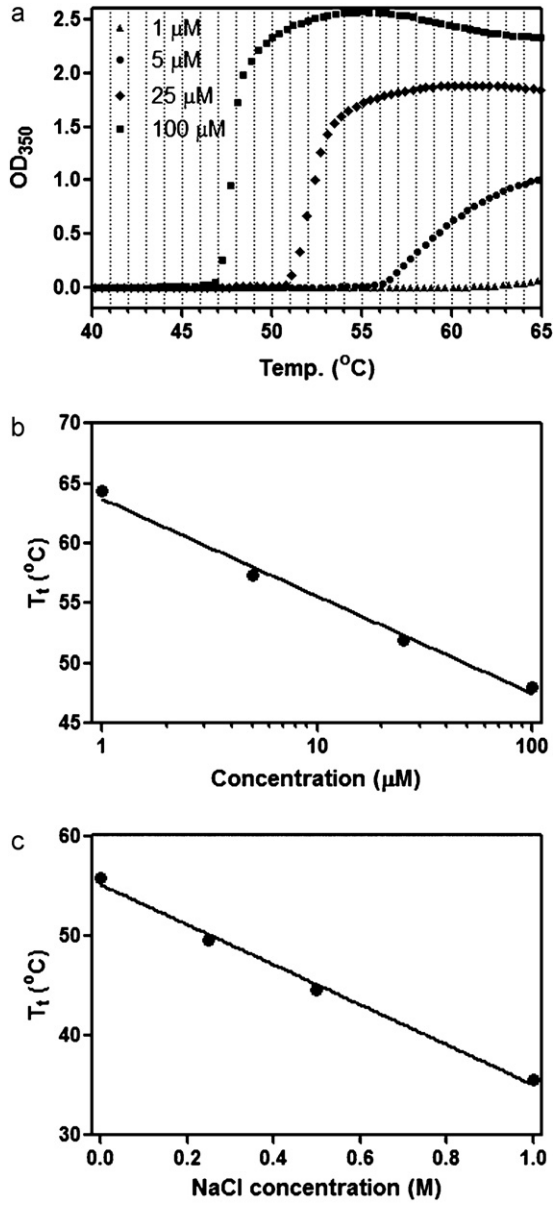

Fig. 2. Turbidity was monitored at 350 nm with a heating/cooling rate of 1 °C/min. (a) Concentration dependent thermal transition profile of p[Asp(DET)]$_{53}$ELP(1–90). (b) T_t of p[Asp(DET)]$_{53}$ELP(1–90) as a function of polymer concentration. (c) T_t of 25 μM p[Asp(DET)]$_{53}$ELP(1–90) as a function of NaCl concentration.

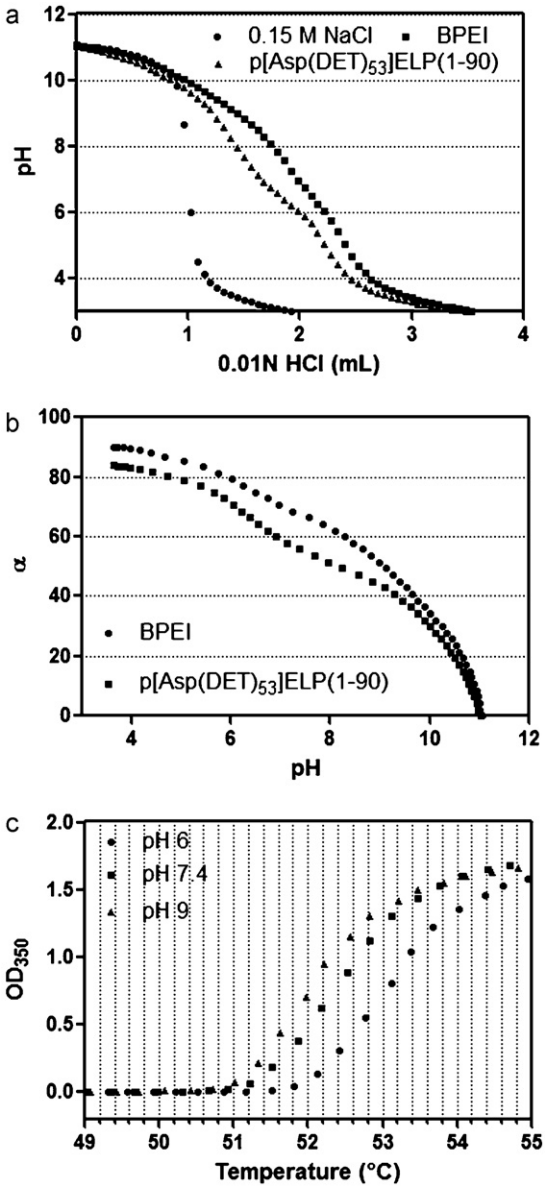

Fig. 3. Potentiometric titration of p[Asp(DET)]$_{53}$ELP(1–90) at 25 °C. (a) pH of p[Asp(DET)]$_{53}$ELP(1–90) and BPEI solutions and the volume of 0.01 N HCl added. (b) Degree of ionization of the polymers as a function of pH. (c) pH-dependent thermal transition profile of 25 μM p[Asp(DET)]$_{53}$ELP(1–90).

in T_t was expected to be much higher, further suggesting that the DET block does not impair aggregation of the ELP block.

3.4. Cytotoxicity of p[Asp(DET)]$_{53}$ELP(1–90)

The p[Asp(DET)]$_{53}$ELP(1–90) polymer was well tolerated by COS-7 cells even into the mM range of amine concentration (Fig. 4). By comparison, cells were completely eradicated at a much lower amine concentration in the case of BPEI. Safe excipients are essential to successful drug product development and this same principle should hold true for developing polymeric gene delivery system. ELPs have been shown to be non-toxic (Urry et al., 1991b). Considering the toxic effect exerted on a living organism by many cationic polymers, these preliminary results suggest that the DET-based cationic diblock copolymer could potentially be a safe gene delivery vehicle as previously reported (Itaka et al., 2010; Kanayama et al., 2006; Lai et al., 2010).

3.5. Gel retardation assay of polyplexes

Polyplexes were formed by mixing the p[Asp(DET)]$_{53}$ELP(1–90) polymer with pGL4 plasmid DNA. The negative charges on pGL4 were neutralized by complexation with the positively charged copolymer via electrostatic interaction. Successful formation of polyplexes was confirmed by gel electrophoresis (Fig. 5), as the mobility of pGL4 was gradually retarded between N/P 1 and 2 with increasing amount of cationic copolymer. Complete retardation of pGL4 mobility at an N/P ratio between 2 and 5 was due to the loss of negative charges. It has been suggested that the neutralization of negative charges on plasmid DNA (pDNA) leads to a structural change of pDNA due to hydrophobic collapse of the random coiled pDNA (Tecle et al., 2003), and subsequently, the formation of nano-sized particles that have dimensions smaller than native pDNA (Bloomfield, 1996).

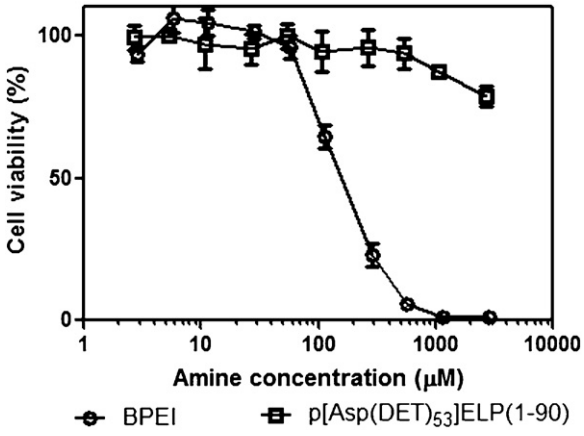

Fig. 4. COS-7 cell cytotoxicity as a function of amine concentration of p[Asp(DET)]$_{53}$ELP(1–90) and BPEI polymers measured by Cell Titer-Blue® assay (data reported as mean ± SD, $n = 4$).

3.6. Dynamic light scattering and ζ-potential measurement

As the colloidal properties often dictate the cellular entry mechanism and physical stability of polyplexes (Smith et al., 1997), they were further characterized by DLS and ζ-potential measurement. The hydrodynamic diameters of polyplexes were found to be between 90 and 100 nm (PdI < 0.25) at N/P > 2 (Fig. 6). The polyplexes sizes obtained were slightly larger than those in previous reports of DET containing polymers (Kanayama et al., 2006; Lai et al., 2010), which might be due to the larger copolymer used in this investigation. Furthermore, the polyplexes were found to be physically stable in PBS when the particle size was tracked over a 24 h period (data not shown). This characteristic has critical implications to the successful development of gene delivery systems, as greater shelf stability provides a wider application window that simplifies the logistics over manufacturing, distribution, and storage of therapeutics.

It is well known that charged colloids are more stable than neutral colloids, because the electrostatic repulsion between two charged surfaces prevents aggregation. However, charged surfaces are often associated with higher cytotoxicity (Stefanelli et al., 1999, 2000). As such, a dilemma arises regarding stability and toxicity. It was reported that the introduction of polymers with large hydrodynamic volume such as poly(ethylene glycol) (PEG) at the colloid surface imparts a neutral surface and could physically separate the colloids and maintain colloidal stability. The p[Asp(DET)]$_{53}$ELP(1–90)/pGL4 polyplexes were negatively charged at N/P 1, but the ζ-potential became neutral at N/P > 2 (Fig. 6). Even at N/P 20, the highest N/P ratio tested, the ζ-potential was only slightly positive. When compared to the high ζ-potential (>30 mV) of p[Asp(DET)] (Miyata et al., 2008), this result suggested that the neutral ELP(1–90) block of the current diblock copolymer was able to effectively shield the positive charges.

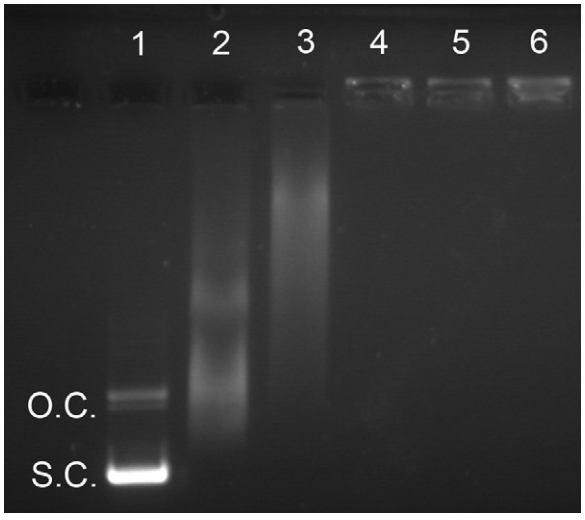

Fig. 5. Gel retardation of p[Asp(DET)]$_{53}$ELP(1–90)/pGL4 polyplexes. O.C. indicates the open circular and S.C. the super coil form of pGL4. Lane 1: naked pGL4, 2: N/P 1, 3: N/P 2, 4: N/P 5, 5: N/P 10, 6: N/P 20.

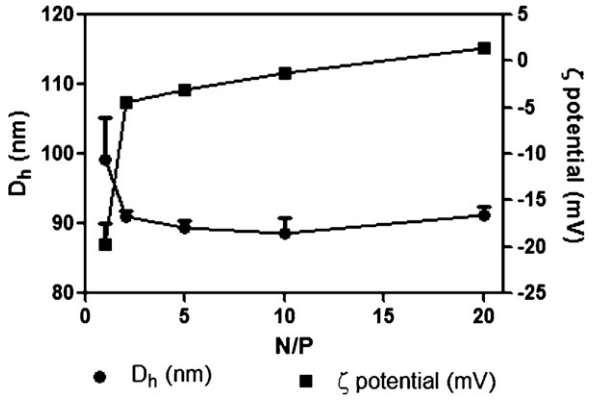

Fig. 6. DLS and ζ-potential measurements of p[Asp(DET)]$_{53}$ELP(1–90)/pGL4 polyplexes with 25 μg/mL pGL4 concentration and various copolymer concentrations at different N/P ratios (data reported as mean ± SD, $n = 3$).

3.7. Thermal characterization of polyplexes

The underlying goal of this research is to develop a thermal targeting polymer for gene delivery. The p[Asp(DET)]$_{53}$ELP(1–90) diblock copolymer shows a phase transition about its T_t in response to a change in temperature and demonstrates that the copolymer and pGL4 self-assembled into nanosize polyplexes. In order to verify that the polyplexes could undergo a similar phase transition, the OD_{350} of polyplexes as a function of temperature was monitored. As expected, the polyplexes showed a sharp inverse temperature phase transition. At an ELP 25 μM copolymer concentration, the polyplexes largely retained the turbidity profile of the naked copolymer, (Fig. 7) aside from a subtle increase in the optical density from 40 to 52 °C, which was the temperature where the bulk transition started. At the same time, we monitored the size of the polyplexes by DLS as a function of temperature (Fig. 8a). It was found that the size of the polyplexes slowly increased with temperature up to 40 °C, when an abrupt increase in size proceeded. The polyplexes' size continued to increase to more than 1 μm, and at 52 °C the bulk transition was apparent when the size grew to the measurement limit at 6 μm. In addition, the phase transition of the polyplexes was partially irreversible; during cooling, the bulk disaggregated at a slower rate than aggregation, a common characteristic of ELP, but the OD_{350} value flattened out at a value slightly higher than zero even after the bulk transition had disappeared. This subtle increase in OD_{350} has been observed by other researchers and was identified as the formation of micelle-like structures (Dreher et al., 2008; Meyer and Chilkoti, 2002; Simnick et al., 2010). However, the formation of those micelle-like structures was reported to be reversible. Again the cooling was monitored by DLS. Below 52 °C, the bulk transition disappeared as the size returned to around 1 μm and no further size reduction was observed even with the temperature returning to 25 °C (Fig. 8b). These larger particles were therefore responsible for the minute increase of turbidity observed in the thermal scan. As a result, we

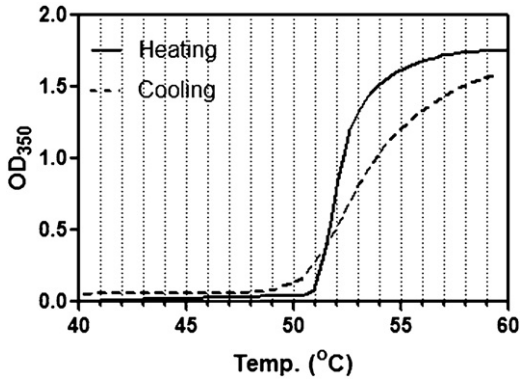

Fig. 7. Turbidity of p[Asp(DET)]$_{53}$ELP(1–90)/pGL4 polyplexes in PBS at N/P 357 (25 μM copolymer and 2.5 μg/mL pGL4) monitored at 350 nm with a heating/cooling rate of 1 °C/min.

concluded that the polyplexes underwent an irreversible primary aggregation below the copolymer's T_t, followed by a reversible secondary aggregation at temperatures about the T_t. The aggregation is critical for thermal targeting, as particles aggregate at the disease site in response to external heating, which facilitates the particles deposition. Both the copolymer and the polyplexes aggregated at elevated temperatures $T_t \sim 52$ °C. Although the T_t of the polyplexes is not clinically relevant, the T_t may be modulated by changing the ELP block composition or molecular weight. Therefore, the thermal responsiveness of this copolymer can be used for thermal targeting of therapeutic pDNA to hyperthermic disease sites.

3.8. Cytotoxicity and transfection of polyplexes

The cytotoxicity of polyplexes largely remained unchanged compared to the copolymer with 80% cell viability at N/P 357 corresponding to a 2.68 mM amine concentration, which in turns translated into a 25 μM copolymer concentration (Fig. 9).

Transfection efficiency was tested using luciferase reporter assay. The luciferase activity started low at low N/P ratios; however, the activity gradually increased reaching a maximum at N/P

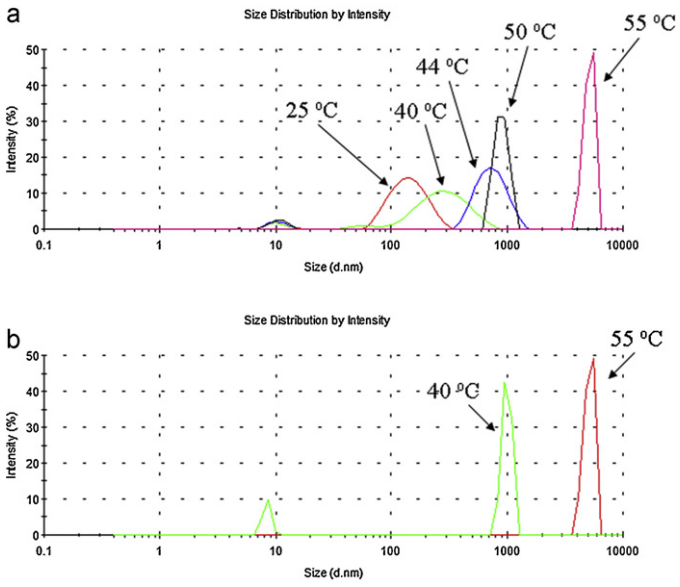

Fig. 8. Temperature effect on the stability of 25 μM p[Asp(DET)]$_{53}$ELP(1–90)/pGL4 polyplexes (N/P 357) in PBS assessed by DLS. (a) Particle size as a function of temperature. (b) Particle size remained large even after the polyplex solution temperature was lowered to 40 °C.

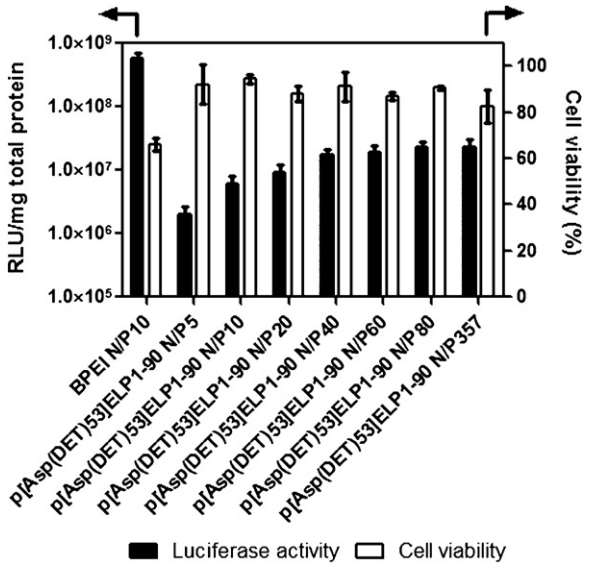

Fig. 9. Transfection efficiency and cytotoxicity of p[Asp(DET)]$_{53}$ELP(1–90)/pGL4 polyplexes (data reported as mean ± SD, $n = 3$ for transfection data and $n = 4$ for cytotoxicity data).

40 (Fig. 9). It has been documented that high N/P ratios of DET-based diblock copolymers are necessary for higher transfection efficiency (Kanayama et al., 2006), and this same trend was also observed.

4. Conclusions

Poor transfection efficiency of polymeric gene delivery has hampered its development as a viable delivery option and tremendous efforts have been spent on addressing this issue. While often overlooked, the safety profile of such systems is critical for successful clinical applications. The US Food and Drug Administration demands a thorough understanding and full characterization of the property of excipients used in pharmaceuticals. Although polymeric gene delivery is still at an early developmental stage, it would greatly benefit the development later if the FDA demands are addressed earlier. Following this principle, it would be natural to use a biodegradable polymer for gene delivery. In this study, biodegradable p[Asp(DET)]$_{53}$ELP(1–90) was synthesized as a hybrid recombinant block copolymer for thermo-sensitive gene transfection. The polyplexes formed by the copolymer and pGL4 plasmid were characterized by DLS, ζ-potential measurements. Neutral polyplexes were less than 150 nm. The polyplexes retained thermal phase transitions conferred by the ELP backbone; however, they exhibited a two-step transition process not seen with the copolymer. The polyplexes also showed an appreciable transfection efficiency with low cytotoxicity. These results demonstrate that the copolymer was well tolerated by cells, which is in sharp contrast to BPEI's cytotoxicity.

Acknowledgements

The authors would like to acknowledge the School of Pharmacy, University of Wisconsin–Madison for funding this research, and would also like to thank Mr. Tsz Chung Lai and Ms. Tracy Williamson for their valuable suggestions on the manuscript.

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