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Research Paper

Intelligent Biosynthetic Nanobiomaterials (IBNs) for Hyperthermic Gene Delivery

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Purpose. Intelligent biosynthetic nanobiomaterials (IBNs) were constructed as recombinant diblock copolymers, notated as K_8-ELP(1–60), containing a cationic oligolysine (VGK_8G) and a thermosensitive elastin-like polypeptide (ELP) block with 60 repetitive pentapeptide units [$(VPGXG)_{60}$; X is Val, Ala and Gly in a 5:2:3 ratio].
Methods. K_8-ELP(1–60) was synthesized by recursive directional ligation for DNA oligomerization. Purity and molecular weight of K_8-ELP(1–60) were confirmed by SDS-PAGE and mass spectrometry. DNA polyplexes were prepared from K_8-ELP(1–60) and pGL3-Control (pGL3–C) plasmid DNA (pDNA) and stability was evaluated by gel retardation, DLS, and DNA displacement with heparin. Thermal transition profiles were studied by measuring the turbidity change at 350 nm and the polyplexes were used to transfect MCF-7 cells with a concomitant cytotoxicity assay.
Results. SDS-PAGE and MALDI-TOF studies showed highly pure copolymers at the desired molecular weight. K_8-ELP(1-60) condensed pDNA at a cation to anion (N/P) ratio above 0.25 with a tight distribution of particle size ranging from 115.5–32.4 nm with increasing N/P ratio. Thermal transition temperatures of K_8-ELP(1-60)/pDNA and K_8-ELP(1-60) alone were 44.9 and 71.5°C, respectively. K_8-ELP(1-60)/pDNA complexes successfully transduced MCF-7 cells with qualitative expression of enhanced green fluorescent protein (EGFP) and minimal cytotoxicity compared to branched poly(ethyleneimine) controls.
Conclusions. K_8-ELP(1-60) was successfully designed and purified through recombinant means with efficient and stable condensation of pDNA at N/P ratios>0.25 and polyplex particle size<115 nm. MCF-7 cells successfully expressed EGFP with minimal cytotoxicity compared to positive controls; moreover, polyplexes retained sharp, thermotransitive kinetics within a narrow T_t range at clinically relevant hyperthermic temperatures, where the decrease of T_t was due to the increased hydrophobicity upon charge neutralization.

KEY WORDS: nanobiomaterial; intelligent polymer; elastin-like polypeptide; gene delivery; gene vector; hyperthermia.

INTRODUCTION

As a model molecular medicine, gene therapy continues to show great promise for monomodal treatment of acute and chronic diseases (1,2), deleterious genetic predispositions (3), and additive or synergistic combination therapies with classical regimens, such as hyperthermia (4). The success of nonviral gene therapy depends largely upon the performance of delivery vectors to protect plasmid DNA (pDNA) in an active form by controlling its condensation and decondensation in sufficient numbers to elicit a therapeutic effect. Initially, viruses would appear to be the most efficient vectors; however, viral-based systems commonly compromise biocompatibility, patient safety, and induce high costs of scale-up. As such, nonviral approaches have emerged as an attractive alternative represented largely as artificial cationic formulations (5,6), liposomes (7,8), and polymers (9,10). Among these formulations, polymers are considered the most receptive for facile modification with various substituents or by block copolymerization. For example, recent studies have shown that poly(ethylene glycol) (PEG)-poly (L-lysine) (PLL) conjugated cationic block copolymers effectively condense pDNA into a core-shell polyion complex (PIC) micelle structure and reduce the toxicity of cationic polymers alone (11). Polymer PEGylation prevents hydrophobic aggregation by steric repulsion of polycation/pDNA complexes and clearance by the reticuloendothelial system (RES). Regarding the cationic block, it has been revealed

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ABBREVIATIONS: ELP, elastin-like polypeptide; ELP(1–60), ELP with 60 repetitive pentapeptide units $[(VPGXG)_{60}$, X is Val, Ala and Gly in a 5:2:3 ratio]; IBN, intelligent biosynthetic nanobiomaterial; ITC, inverse transition cycling; K_8, oligolysine (VGK_8G); RDL, recursive directional ligation; T_t (°C), phase transition temperature.

that the selection of optimal compositions such as molecular weight and pKa plays a pivotal role in facilitating the release and endosomal escape of pDNA (12,13) and eventually induces high transfection efficiency.

The synthesis of functional cationic block copolymers, however, is not simple and often requires specialized polymer chemistry. In particular, it is obvious that the gross physicochemical properties of synthesized polymers, such as molecular weight, polydispersity, reaction ratios, etc., are prone to changes between batches. Furthermore, more sophisticated chemistry becomes necessary for modification of block polymers with targeting ligands or implementation of new functionalities, consequently imposing a bottleneck for continued development by these complexities alone. In this study, we report a novel intelligent biosynthetic nanobiomaterial (IBN) platform for multi-targeted nonviral gene therapy. Thermosensitive cationic diblock copolymers were designed and synthesized for gene delivery through recombinant DNA cloning technology based upon recursive directional ligation (RDL), a facile biosynthetic route achieved by conventional bacterial culture expression (14). Block copolymers were composed of a cationic block from oligolysine (VGK_8G) and a thermosensitive elastin-like polypeptide (ELP) block with 60 repetitive pentapeptide units [$(VPGXG)_{60}$; X is Val, Ala and Gly in a 5:2:3 ratio], notated as K_8 and ELP(1–60), respectively. It must be clarified that K_8 and ELP(1–60) were selected herein as model blocks to exemplify our hypotheses that functional cationic diblock copolymers can be synthesized through biosynthesis with a high purity and yield at the precise molecular weight and the biosynthesized diblock copolymers composed of hydrophilic and cationic blocks would condense, protect, and transfer pDNA similar to PEGylated polycation formulations for gene delivery. It also should be noted that an oligolysine block was used instead of a cationic elastin block, where the guest residue X is replaced with lysine. Although a cationic elastin block $(VPGKG)_n$ has previously been developed (15), the cationic charge density is significantly lower compared to an oligolysine block; furthermore, polyplex assembly with such a system may be compromised as a result of steric interference. Hence, a (K_8) oligolysine block was selected to promote DNA condensation based on the concern over charge density and the polymer's DNA condensing ability (16,17).

The significance of ELPs as useful thermosensitive biomaterials was originally suggested by Urry (18) and has been extensively studied by the pioneers of biopolymers in the last decade. Briefly, ELP libraries are composed of Val–Pro–Gly–Xaa–Gly (VPGXG) pentapeptide monomers, where "X" is any guest residue other than Pro (19). ELPs are characterized by high water-solubility, biocompatibility (20–22), and a reversible, rapid response to temperature with an inverse temperature phase transition (23). The rapid inverse temperature phase transition (T_t) is a function of the type and degree of guest residue (X) substitution, ionic state, and molecular weight (24) amongst other factors. Indeed, ELPs are highly soluble in aqueous solutions below the T_t but rapidly aggregate above the T_t. Furthermore, as this phase transition is completely reversible, ELPs may be used as a thermosensitive carrier for the delivery of therapeutic agents (25,26). This innate characteristic presents an opportunity for hyperthermic targeting of materials conjugated or associated with ELP following local or systemic administration and has been exploited to deliver drugs to solid tumors or *in situ* (27). *In vivo* studies also showed that cellular uptake and accumulation of ELPs in tumor tissues increased two fold with hyperthermia (28). Furthermore, it has been shown that by fusing cell penetration and therapeutic peptides, ELPs can deliver therapeutic cargos intracellularly with enhanced antiproliferative effects attributed to the ELP hyperthermic response (29,30).

DNA cloning technology has gained increasing attention as a novel tool for nonviral gene delivery vector preparation in the past few years, owing to its ability of creating structurally well-defined materials (31). The inherent characteristics of these biomaterials allow the study of desired properties for successful biosynthetic gene delivery vectors via a structure-function relationship difficult to achieve by synthetic polymers and hence the impetus for IBN platforms. Arís *et al.* (32) reported the first stable oligolysine encoding fusion protein, β-galactosidase, prepared by DNA cloning technology for gene delivery. The fusion protein consists of an *N*-terminal oligolysine domain for DNA condensation and an arginine–glycine–aspartic acid (RGD) sequence inserted on a solvent exposing loop of the protein for integrin targeting. In another report by Medina-Kauwe *et al.* (33), an oligolysine domain was cloned to the C-terminus of an adenoviral penton protein, showing 13% of transgene activity compared to lipofectin. Moreover, lysine-histidine (KH) repeats fused to basic fibroblast growth factor (bFGF) were prepared by Hatefi *et al.* (34) and showed enhanced transfection compared to KH repeats alone. It is of special note that to our knowledge, only the retated silk elastinlike polymers (SELP) pioneered by Ghandehari *et al.* have been investigated for matrix-mediated naked DNA and adenoviral gene therapy (35,36). However, there are no reports of systemic gene delivery utilizing thermosensitive ELP. Hyperthermic gene transfer by thermoresponsive polymers with enhanced transgene expression has been reported previously (37,38). Zintchenko *et al.* attributed the enhanced expression to an elevated "proton sponge" effect elicited by the large numbers of branched poly(ethyleneimine) (BPEI)-*b*-poly(*N*-isopropylacrylamide) (PNIPAM) copolymer molecules presenting in the endosome with hyperthermia. This phenomenon was hypothesized as the large, aggregated particles were internalized by cells at elevated temperature. This same mechanism could also enhance transfection efficiency with K_8-ELP(1–60) allowing endosomal buffering capability; however, this hypothesis is currently being rigorously studied.

On this basis, we designed IBNs as cationic protein-based diblock copolymers for systemic gene therapy containing electrostatically condensing oligolysine blocks and thermosensitive, particle stabilizing ELP blocks for systemic gene therapy. Herein we report the synthesis and characterization of K_8-ELP(1–60) block copolymers as a novel formulation for the development of thermosensitive nonviral gene vectors, an extension of the IBN platform. The evaluation was focused on the feasibility of K_8-ELP(1–60) and its DNA complexes for practical application in hyperthermic gene therapy.

MATERIALS AND METHODS

Materials

E. coli strain BLR(DE3) and Top10 cells were purchased from Novagen (Madison, WI) and Invitrogen (Carlsbad, CA), respectively. T4 DNA ligase, restriction enzymes, and pUC19 cloning vectors were obtained from New England Biolabs (Beverly, MA). Calf intestinal alkaline phosphatase (CIP), pGL3-C plasmid, and the CellTiter-Glo kit were obtained from Promega (Madison, WI). The pET-25b(+)SV2 and pUC19-ELP(1–30) plasmids were kindly gifted by Prof. Ashutosh Chilkoti (Duke University, Dept. of Biomedical Engineering). Large-scale DNA preps were conducted using PureLink™ Plasmid Filter Maxiprep Kits from Invitrogen (Carlsbad, CA). CircleGrow culture medium was purchased from Q-BIOgene (Carlsbad, CA). 5′-Phosphorylated oligonucleotides were synthesized at the UW-Madison Biotechnology Center. Precast Mini-Protein SDS-PAGE gels were purchased from BioRad, Inc. (Hercules, CA). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Mediatech, Inc. (Herndon, VA). BPEI and heparin sodium were purchased from Sigma (St. Louis, MO).

Gene Construction for Diblock Copolymers

The forward and reverse DNA sequences of VGK_8G were 5′-GTGGGTAAAAAAAAGAAAAAAAAAA AGAAAGGC-3′ and 5′-TTTCTTTTTTTTTTTCTTTTTTT TACCCACGCC-3′, respectively. Oligonucleotides were annealed to form a double-stranded DNA cassette with *Pfl*M I compatible ends by heating an equimolar mixture of two oligonucleotides at 95°C for 5 min followed by slow cooling (1°C/min) to room temperature. pUC19-ELP(1–30) was digested with *Pfl*M I and enzymatically dephosphorylated using CIP. The linearized pUC19-ELP(1–30) vector was separated by low melting agarose gel electrophoresis and purified by a QIAGEN (Valencia, CA) QIAquick gel extraction kit. The annealed oligonucleotides and linearized vector were ligated at 16°C for 18 h. The ligation mixture was combined with 100 μl of chemically competent Top10 cells, transformed by heat shock, spread on CircleGrow medium agar plates supplemented with ampicillin (100 μg/ml), and incubated at 37°C overnight. Later, multiple colonies were chosen and grown further in 3 ml CircleGrow medium at 37°C for 12 h. Plasmids were isolated and purified using a Qiagen Miniprep kit. DNA sequencing and diagnostic digestion of the purified plasmids were performed using *Eco*R I and *Hind* III to confirm the putative insert. The K_8-ELP(1–30) encoded plasmid was doubly digested with *Pfl*M I and *Bgl* I and the resulting insert was gel purified. To obtain the K_8-ELP(1–60) gene, the K_8-ELP(1–30) insert was ligated into the linearized pUC19-ELP(1–30) vector, and the resulting clones were screened following the above protocol. The pET-25b(+)SV2 expression vector was modified by cassette mutagenesis further referenced as pET-25b(+)HC1. The forward and reverse DNA sequences of this cassette were 5′-TATGAGCGGGCCGGGCTGGCCGTGATA-3′ and 5′-AGCTTATCACGGCCAGCCCGGCCCGCTCA-3′, respectively. This cassette contains an *Sfi* I restriction site with flanking sequences compatible with *Pfl*M I and *Bgl* I digested inserts. Oligonucleotides were annealed by heating an equimolar mixture at 95°C for 5 min and then slowly cooled (1°C/min) to room temperature to form a double-stranded DNA cassette with *Nde* I and *Hind* III compatible ends. The cassette was inserted into the *Nde* I and *Hind* III restriction sites by restriction enzyme digestion and ligation. The resulting plasmid was confirmed by DNA sequencing. The pET-25b(+)HC1 was digested with *Sfi* I and enzymatically dephosphorylated using CIP followed by gel purification. *Pfl*M I and *Bgl* I digested K_8-ELP(1–60) was then ligated into the linearized pET-25b(+)HC1. Cloning was confirmed by diagnostic digestions using *Ava* I and *Xba* I. The plasmid containing the correct insert gene was transformed into the *E. coli* BLR (DE3) strain by heat shock.

Expression and Purification of the K_8-ELP(1–60) Diblock Copolymer

The K_8-ELP(1–60) diblock copolymers were expressed in *E. coli* BLR(DE3) at 37°C for 24 h on an orbital shaker at 225 rpm. The polymers were purified from the *E. coli* according to the inverse transition cycling (ITC) method described elsewhere (14) except that two rounds of PEI precipitation were used to remove any traces of chromosomal DNA. The concentration of the K_8-ELP(1–60) diblock copolymer solution was determined by UV spectrophotometry using a molar extinction coefficient of 5,690/M·cm at 280 nm. Purified polymers were characterized by SDS-PAGE and mass spectrometry to confirm purity and molecular weight. The polyacrylamide gel was visualized by Coomassie Blue staining. The molecular weight of the polymer was confirmed by electrophoresis comparing the corresponding band to a molecular weight standard and further verified by MALDI-TOF mass spectrometry.

Agarose Gel Electrophoresis of K_8-ELP(1–60)/pDNA Polyplexes

In order to confirm the ability of the polymers to condense pDNA, K_8-ELP(1–60)/pDNA polyplexes with various N/P ratios were electrophoresed on agarose gels. Variable concentrations of K_8-ELP(1–60) in phosphate buffered saline (PBS) were added to equal volume of 1 μg pGL3-C in PBS at room temperature to prepare the polyplexes while the N/P ratios were adjusted from 0.05 to 5. Electrophoresis was performed at 4°C on a 0.8% high melting agarose gel at 100 V for 90 min and visualized by ethidium bromide staining at a final concentration of 0.4 μg/ml.

Thermal Characterization of the Inverse Temperature Phase Transition

In order to measure the T_t of the complexes, K_8-ELP (1–60) was dissolved in PBS at 50 μM and 350 μl of the polymer solution was added to equal volumes of DNA solution at room temperature with various concentrations to adjust the N/P ratio. After incubation at 25°C for 30 min, 600 μl of the complex solution were subjected to the thermal transition study, which the optical density was monitored at 350 nm as a function of temperature using a Cary 100 UV-

visible spectrophotometer equipped with a multicell thermoelectric temperature controller (Varian, Inc., Palo Alto, CA). The rate for heating and cooling temperatures was 1°C/min and the T_t was determined as the temperature that exhibits half of the maximum optical density.

Sizes of K_8-ELP(1–60)/pDNA Polyplexes

The particle size of K_8-ELP(1–60)/pDNA polyplexes was determined by dynamic light scattering (DLS) measurements (NICOMP 380 ZLS instrument, Particle Sizing Systems, Santa Barbara, CA). Twenty-five micromolar polyplexes were prepared as described above, while the N/P ratio was adjusted from 1 to 10. Samples were prepared in triplicate to produce statistical parameters.

In Vitro pDNA Release Assay

The pDNA release profile of K_8-ELP(1–60)/pDNA polyplexes was evaluated by electrophoresis after mixing stable polyplexes with heparin for polyanion displacement. Polyplexes at N/P 1 were prepared at a final pDNA concentration of 50 μg/ml and 200 μl final volume. After incubation at 25°C for 30 min, 15 μl of the polyplex solution were transferred to 12 micro tubes and mixed with 15 μl of heparin solution at various concentrations. The mixed solutions were incubated at 25°C for another 30 min and then 20 μl of the solution were electrophoresed.

Cytotoxicity Assay

MCF-7 cells (human breast cancer) were seeded in 24-well plates at an initial density of 50,000 cells per well. The cells were preincubated at 37°C in a 5% CO_2 humidified atmosphere in 1 ml of DMEM containing 10% FBS and 1% penicillin/streptomycin for 24 h. Twenty microliters of the polyplex solution were pre-mixed with 179 μl of serum free DMEM containing 1% penicillin/streptomycin and 1 μl 20 mM chloroquine; 20 μl of the native copolymer solution were pre-mixed with 180 μl of serum free DMEM containing 1% penicillin/streptomycin, both solutions were then applied to the cells after the initial medium had been removed. After a 4 h incubation at 37°C, the medium was discarded and the cells were washed twice with PBS followed by adding fresh medium. The cells were incubated for another 20 h with serum containing DMEM and the viability of the cells was determined by a CellTiter-Glo kit.

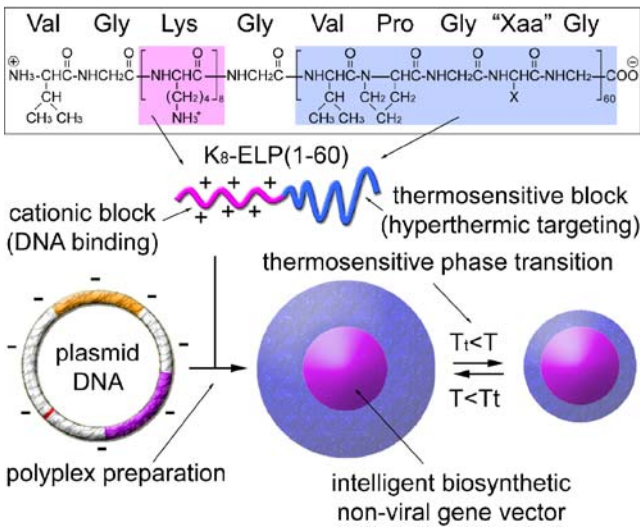

Fig. 1. The chemical structure of biosynthesized K_8-ELP(1–60) diblock copolymers and preparation of intelligent thermosensitive nonviral gene vectors.

In Vitro Transfection Study

MCF-7 cells were seeded in 24-well plates at an initial density of 50,000 cells per well. After cells were incubated until 70% confluent, 20 μl of polyplex solution were premixed with 179 μl of serum free DMEM containing 1% penicillin/streptomycin and 1 μl 20 mM chloroquine to facilitate ensonomal release. The initial medium was removed and the polyplex solutions were added to the cells. The cells were incubated for 4 h at 37°C after which the experimental medium was removed, cells washed twice with PBS, and fresh medium containing 10% serum was added. Cells were incubated for another 20 h before GFP gene expression was visualized.

Statistical Analysis

Groups were compared simultaneously by one-way ANOVA and Bonferroni post-test. Data were deemed statistically significant at $p<0.01$.

RESULTS

Gene Construction and Polymer Expression

The *Pfl*M I compatible ends were introduced to the K_8 encoding oligonucleotides to clone the pUC19 vector encoding the ELP(1–30) gene. RDL was then used for the extension of the ELP1 gene by DNA oligomerization (14). The K_8 gene was cloned into the vector prior to propagation of the ELP1 gene. This step facilitated the separation of the original ELP1 gene and the K_8-ELP(1–60) gene with a short K_8 block by diagnostic digestion and gel electrophoresis. The gene for the K_8-ELP(1–60) copolymer was then subcloned into the pET25b(+)HC1 vector, and the copolymers were expressed in the *E. coli* strain BLR(DE3). K_8-ELP(1–60) block copolymers (Fig. 1) were purified from the cell lysate by ITC with a yield of 17 mg/l. As shown in Fig. 2, sonicated cells were purified by centrifugation of the cell lysate to collect supernatant (Lane 2); PEI adding to the supernatant to remove chromosomal DNA contaminants (Lane 3); centrifugation at $T>T_t$ of ELP to separate supernatant (Lane 4) and ELP (Lane 5); repeating hot-spin to remove impurities in the supernatant (Lane 6); and purified ELP (Lane 7). Molecular weights of the obtained ELPs were compared with the standard (Lane 8). Surprisingly, SDS-PAGE showed the molecular weight of K_8-ELP(1–60) to be higher (ca. 35 kDa) than the expected 25 kDa, a phenomenon commonly observed in small, repetitive amino acids. Indeed, a MALDI-TOF mass spectrometry clearly showed the molec-

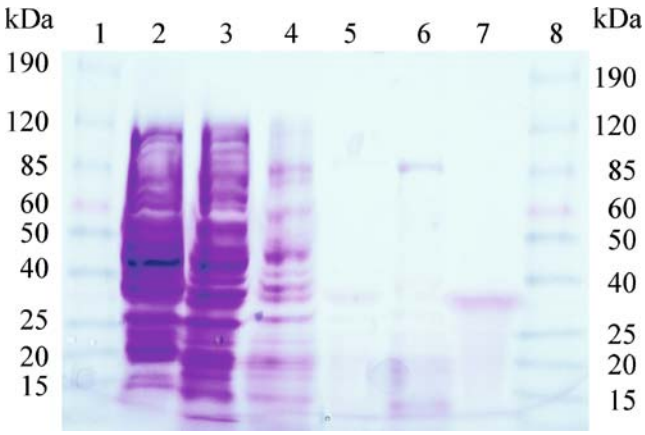

Fig. 2. SDS-PAGE at different purification stages. (Lanes 1 and 8) with a molecular weight marker from 10 - 190 kDa; (Lane 2) sonicated cell lysate; (Lane 3) supernatant collected after PEI precipitation; (Lane 4) supernatant collected after the first hot spin; (Lane 5) pellet collected after the first hot spin; (Lane 6) supernatant collected after the second hot spin; and (Lane 7) purified K_8-ELP(1-60) diblock copolymers collected after the second hot spin. SDS-PAGE with a 4-20% gradient polyacrylamide gel at 100V., for 150 min. The gel was then stained by Coomasive Blue.

ular weight of K_8-ELP(1–60) was 25,317 Da (Fig. 3) with an expected result of 25,445 Da. A previous report showed the difference could be attributed to the removal of the N-terminal methionine by methionyl-aminopeptidase (39). The relatively low yield compared to the general yields for ELP1 homopolymer (>200 mg/l) is probably due to copolymer loss during ITC as a result of the cationic K_8 block's effect on increasing the thermotransition of the block copolymers.

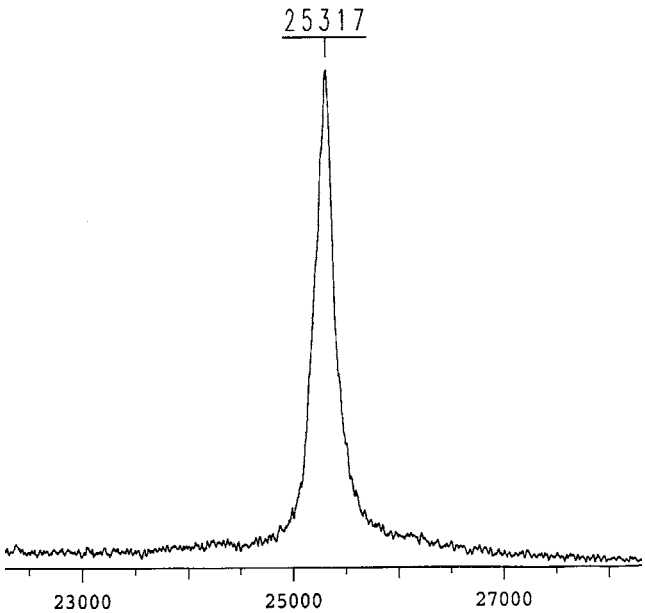

Fig. 3. Molecular weight distribution of K_8-ELP(1-60) diblock copolymers measured by MALDI-TOF mass spectrometry. The expected M.W. was 25445 kDa, which was different from the 25317 kDa observed M.W. possibly due to the *N*-terminal methionine deletion (39).

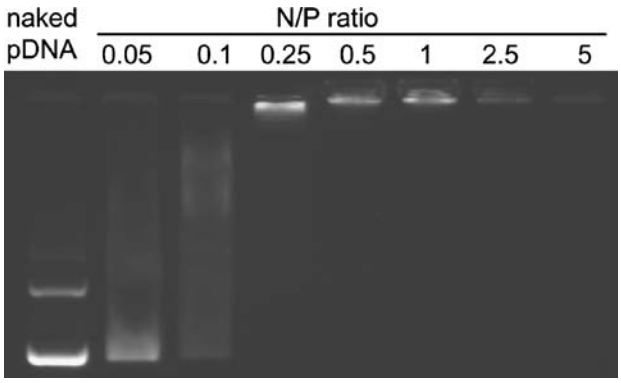

Fig. 4. Gel retardation assay of cationic elastin diblock copolymers and pDNA. Gel conditions were 0.8% high melting agarose, 100V, 90 min, and 0.4 **μg/ml** ethidium bromide.

Preparation and Characterization of K_8-ELP(1–60)/pDNA Polyplexes

Gel retardation assays show that K_8-ELP(1–60) condenses pDNA effectively (Fig. 4) at low N/P ratios. It is of special interest that pDNA condensation was shown at N/P 0.25 with EtBr exclusion at N/P 2.5 determined by EtBr staining. These results suggest that K_8-ELP(1–60)/pDNA electrostatic interactions may not be the only driving force and that other cooperative effects, possibly hydrophobic, could be involved as the thermotransition profiles changed as the N/P ratios increased drastically (Fig. 5). The T_t of K_8-ELP(1–60) was 71.5°C at 25 μM and considering that the T_t of ELP(1–60) is 62.1°C (data not shown), it is suggested that the addition of the oligolysine moiety induced the increase in T_t as postulated by Urry (23) wherein polar amino acid side chains increase the T_t of ELP polymers. As expected the T_t of polyplexes at N/P 1 dropped to 44.9°C and the temperature range of the phase transition was narrow, suggesting a monodisperse system of electroneutral K_8-ELP(1–60)/pDNA complexes, a cooperative hydrophobic effect might explain the lowering of the T_t upon complex formation. The positive charges on the copolymers should neutralize the negative charges on pDNA, thus leaving the copolymers more hydrophobic relative to the native, protonated state; consequently, the resulting hydrophobicity may depress the T_t (40). It should be emphasized that this temperature (44.9°C) is within clinically relevant temperatures for hyperthermic, adjuvant therapy (41). When the N/P ratios increased, however, the T_t increased as expected and approached that of free K_8-ELP(1–60) (T_t 71.5°C) with a corresponding depression of transition kinetics as shown by the broad transition curve. These results suggest a mixed population consisting of free K_8-ELP(1–60) block copolymers combined with K_8-ELP(1–60)/pDNA polyplexes at N/P>1. The T_t of polyplexes at N/P 10 was as high as that of free K_8-ELP(1–60) block copolymers. Parallel DLS measurements (Table I) showed that the particle size of the polyplexes decreased from 115.5–32.4 nm as N/P ratios increased with a small rise at N/P 10. The sample groups were deemed statistically significant following one-way ANOVA and a Bonferroni post-test ($p<0.001$). The size and distribution are promising in terms of *in vivo* systemic gene delivery applications. There-

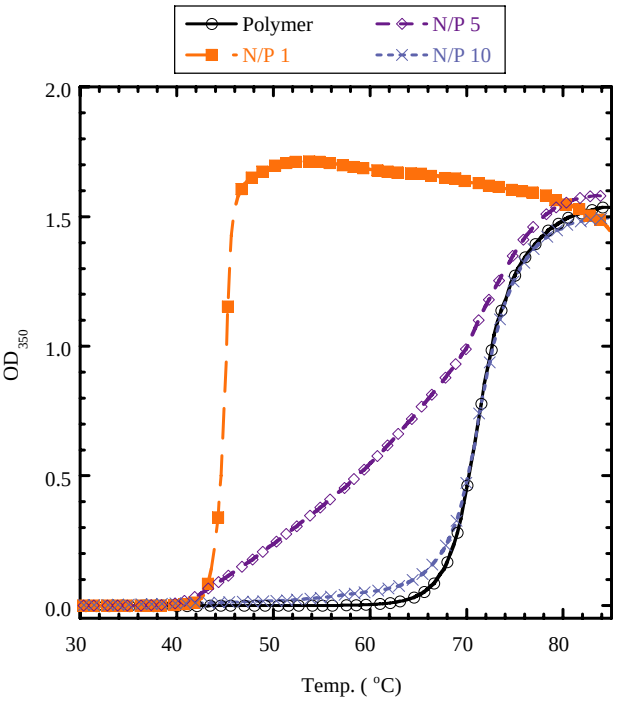

Fig. 5. Thermal transition profiles of the native cationic elastin diblock copolymer and polyplexes at N/P ratios 1 to 10 at 25 μM copolymer concentration. The heating rate was 1°C/min with turbidity monitored as OD_{350}.

fore, it was concluded that K_8-ELP(1–60) block copolymers successfully condensed pDNA to form a nanoparticulated polyplex which shows clear thermosensitivity at proximal hyperthermic temperatures. Furthermore, neither turbidity nor physical aggregation was observed with K_8-ELP(1–60)/pDNA polyplexes 30 minutes post formulation, which suggests a particle stabilization effect from the ELP similar to PEG.

DNA Release

DNA release is essential for polyplexes to transfect cells, while stability of the polyplexes is a prerequisite to protect DNA. In order to evaluate the stability as well as the DNA releasing property of the polyplexes, heparin was mixed with K_8-ELP(1–60)/pDNA polyplexes in various concentrations (Fig. 6). There are a large number of anionic proteins in the body that may disrupt polyplexes, as such heparin is commonly used as a model anionic species to monitor DNA dissociation from polyplexes (42). Under assumed equilibrium conditions, stable polyplexes at N/P 1 were initially mixed with heparin at 0.019 USP units/μg DNA concentration. However, DNA release did not begin at heparin concentrations <0.19 USP units/μg DNA, and polyplexes were completely disrupted with pDNA release at 0.47 USP units heparin/μg DNA. Addition of 4–6 USP units of heparin/ml of whole blood is usually employed to prevent coagulation; therefore, these results indicate that the polyplexes were stable in the presence of anionic proteins (6 USP units of heparin/ml) corresponding to physiological conditions. The results suggest that K_8-ELP(1–60)/pDNA polyplexes may safely deliver and release its DNA cargo intracellularly.

Observation of EGFP Transfection

In order to assess the performance of K_8-ELP(1–60)/pDNA polyplexes as a nonviral gene carrier, MCF-7 cells were transfected by polyplexes containing pEGFP-N3. As shown in Fig. 7, intense fluorescence of the enhanced green fluorescence protein (EGFP) was observed in the cells 24 h post transfection using polyplexes at N/P 1. Negative controls and naked pDNA did not show EGFP transgene expression. It must be mentioned that EGFP transfection was observed in limited cells while positive controls transfected large numbers of cells. While the positive transfection results are promising, there is substantial work to be done as these results show that transgene expression may be determined by cellular interactions such as exposure time, cell cycle, polyplex uptake, and thermal conditions.

In Vitro Cytotoxicity

In vivo transfection is generally accompanied by longer exposure times at the targeted site than *in vitro* experimental conditions, hence it is of vital importance to assess cytotoxicity. Fig. 8 shows the cell viability against N/P ratios of the polyplexes where toxicities of the polyplexes were relatively low across all the N/P ratios tested. K_8-ELP(1–60) block copolymers showed a concentration dependent cytotoxicity (Fig. 8), however, the cytotoxicity of the K_8-ELP(1–60) block copolymers is extremely low compared to PEI or PLL. At a 47.7 mg/ml copolymer concentration, nearly 72% of the cells were viable, which is in sharp contrast with less than 30% viable cells with the same concentration of PEI and PLL (43). Moreover, the relatively higher toxicity was induced presumably by the free cationic charge as evidenced by the results of increasing the N/P ratio. These results indicate that although free K_8-ELP(1–60) block copolymers do induce cytotoxicity, this cytotoxicity is effectively neutralized with the formation of polyplexes.

Table I. Size Distribution of K_8-ELP(1–60)/pDNA Polyplexes at N/P 1, 2.5, 5, and 10

	N/P ratios of the polyplexes			
	1	2.5	5	10
Hydrodynamic radius±SD (nm)	115.5±9.0	47.0±6.1	32.4±1.0	93.3±6.61

Hydrodynamic radius reported as mean±SD (n=3)

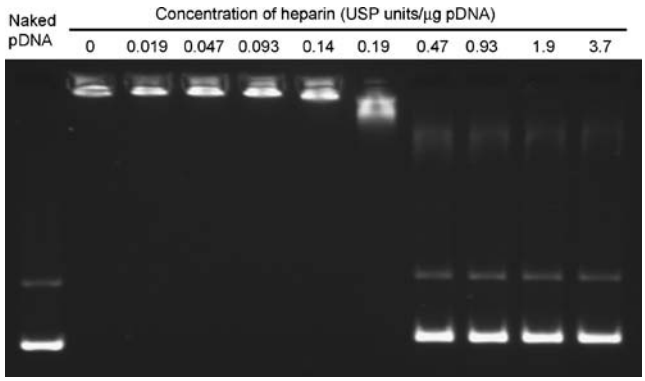

Fig. 6. DNA displacement by heparin sodium with K_8-ELP(1–60)/pDNA N/P 1 polyplexes.

DISCUSSION

Recombinant DNA cloning technology presages continued biosynthesis of polymers providing precise, genetic control over polymer structure and function for medicinal applications (31). Protein-based polymers, such as ELPs, are a class of such polymers with favorable properties for a wide range of biomedical applications; however, facile genetic engineering remains constrained by the available amino acids and host-strain expression. For example, expression of oligo-lysine ELP block copolymers could be difficult due to the repetitive amino acid block often flagged as deleterious by host organisms; moreover, oligo-lysine ELP may also pose a cytotoxic effect due to the high cationic charge, thereby destabilizing anionic membranes of organelles and/or cells. Yet despite these hurdles, success with lysine repeats is reported in the literature where fusion proteins containing oligo-lysines were successfully expressed in prokaryotic expression systems for gene transfer applications (32,33). Another interesting study was reported by Kiick *et al.* (44), which reported the incorporation of non-natural amino acids into biosynthetic polypeptides, further diversifying the application of polypeptides as functional materials. Herein we report for the first time IBNs consisting of cationic elastin diblock copolymers for multimodal gene therapy (Fig. 1). These gene carriers were designed for electrostatic pDNA condensation by the oligo-lysine block and contributing particle stability and thermal targeting by the ELP block. Results from SDS-PAGE and MALDI-TOF mass spectrometry showed that thermosensitive cationic block copolymers were successfully prepared with a high purity and precise molecular weight (Figs. 2 and 3), albeit at yields significantly lower than ELP homopolymers. Expression levels were most likely compromised by the high T_t of the native cationic elastin diblock copolymers thereby limiting the effectiveness of ITC purification or perhaps compartmentalization by inclusion bodies, nevertheless, studies are ongoing to increase the yields. Surmising coulombic interactions between the anionic phosphate backbone of the pDNA and cationic oligo-lysine, pDNA condensation was observed at N/P 0.5 and near EtBr exclusion at N/P 2.5 (Fig. 4). As expected, particle size measurements of the K_8-ELP(1–60)/pDNA polyplexes decreased steadily from N/P 1 (~115 nm) to N/P 5 (~30 nm; Table I). K_8-ELP(1–60)/pDNA polyplexes at N/P 10 increased in particle size presumably due to the presence of free or non-condensing K_8-ELP(1–60) copolymers. Future study will include characterization by atomic

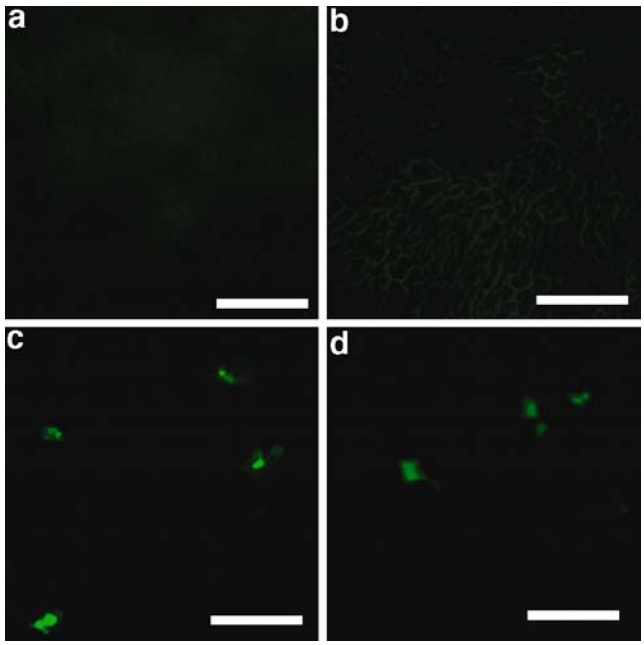

Fig. 7. Fluorescent micrograph of MCF-7 cells transfected with pEGFP: **a** non-treated cells; **b** naked pDNA; **c** BPEI/pDNA N/P 10; and **d** K_8-ELP(1–60)/pDNA N/P 1. *Scale bar* represents 500 μm.

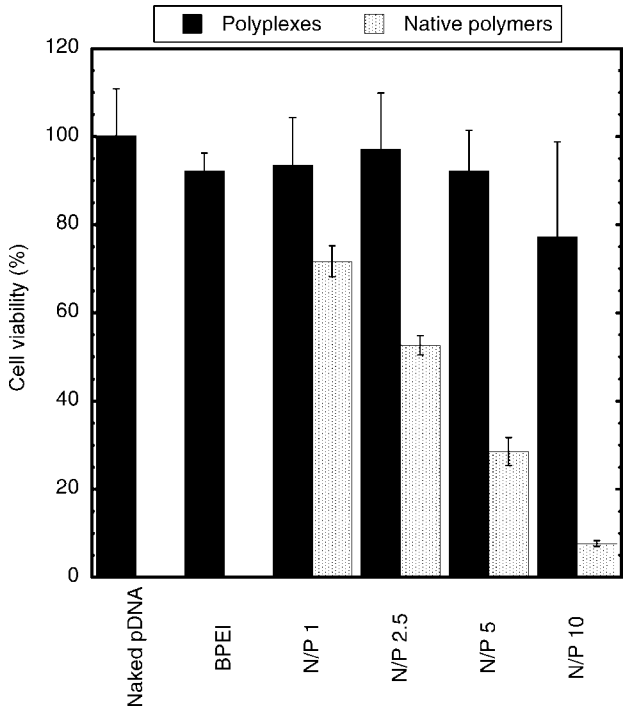

Fig. 8. Cytotoxicity of the polyplexes and native copolymers. The concentrations of native copolymers used in the study were the same as used in the polyplexes, which were 47.700, 119.25, 238.50, and 477.00 μg/ml for N/P 1, 2.5, 5, and 10 respectively. Data reported as mean±SD (n=4).

force microscopy, transmission electron microscopy, and determination of the critical association number to better elucidate the assertion that these polyplexes are micellar in structure.

For hyperthermic targeting of K_8-ELP(1–60)/pDNA polyplexes, an appropriate thermotransitive profile is paramount. While native cationic block copolymers exhibited thermotransitive kinetics outside the range of clinically relevant hyperthermic temperatures, subsequent mixing with pDNA formed polyplexes with T_t~45°C (Fig. 5), which is proximal to clinically hyperthermic temperatures (37). Previous reports have shown that ELPs aggregate due to hydrophobic collapse when heated above their transition temperatures (T_t) and return soluble upon cooling below T_t (41). As such, thermal transition behaviors of both the copolymer and the polyplexes, as a function of temperature, were monitored by the optical density at 350 nm (OD350). We hypothesized that the addition of a small cationic block, K_8, to the ELP block, would not greatly alter the transition profile following charge neutralization and polyplex equilibrium. Indeed, as shown in Fig. 5, the block copolymer K_8-ELP(1–60) exhibits a transition temperature of 71.5°C at 25 μM block copolymer concentrations, which was 9.4°C higher than the T_t of ELP(1–60). Interestingly, when K_8-ELP(1–60) block copolymers form polyplexes with pDNA, the T_t decreased to ~45°C with a 1°C T_t range between 25 and 75% of the maximum absorbance. This behavior was encouraging considering that the copolymer consists of a thermo-insensitive cationic block predicted to alter the phase transition of the putative thermosensitivity of the ELP block. The results indicate that the K_8 block promotes a stable and efficient complexation with pDNA. For a thermally responsive gene delivery system to be successful, the copolymer/pDNA complex should transition within therapeutically relevant temperatures at the therapeutic site and release the pDNA cargo. The thermal transition profile of the complexes depends upon the N/P ratio and exhibits a single transition curve at N/P 1 but evolves into a bimodal curve at N/P>1 with higher N/P ratios approaching the unimodal thermotransitive kinetics of the native cationic elastin diblock copolymer. The bimodal curve suggest two cationic block copolymer populations, copolymers establishing the polyplexes and unbound free copolymer. The thermotransitive uniformity of the complex is critical for successful targeted transfection in terms of higher transfection and lower cytotoxicity. Therefore, the K_8-ELP(1–60)/pDNA polyplexes at N/P 1 with a single thermal transition profile are encouraging.

The hallmark of gene therapy is to achieve targeted transgene expression with minimal toxic repercussions; hence, transgene expression and concomitant cytoxocity studies were completed. Concerns of pDNA release were allayed by the heparin displacement assay (Fig. 6) in which the anionic heparin effectively dissociated pDNA from the cationic elastin diblock copolymers. While not a conclusive study, experiments are in-progress to further evaluate the stability of K_8-ELP(1–60)/pDNA polyplexes by DN*ase* protection. Regarding the transfection efficiency, the K_8-ELP(1–60)/pDNA polyplexes showed visual, transfection levels relatively less effective than BPEI (Fig. 7), but cytotoxicity remained low (Fig. 8). Most notably, the polyplexes transfected cells at N/P <1 suggesting that ELP-based cationic block copolymers may undergo cooperative interactions between electrostatic and hydrophobic means during polyplex formation. Based upon these preliminary results we conclude that recombinant cationic diblock copolymers, such as K_8-ELP(1–60), show tremendous potential as intelligent biosynthetic nanobiomaterials for multiplatform therapeutic applications.

CONCLUSION

The overall objective is to create an IBN platform for multimodal gene delivery, drug delivery, and therapeutic fusion proteins. As an extension of IBNs, and based upon the preliminary data, we conclude that recombinant cationic elastic diblock copolymer, K_8-ELP(1–60), shows tremendous potential for continued development of the IBN platform.

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