Available online at www.sciencedirect.com
ScienceDirect
Journal of Controlled Release 121 (2007) 28–37
journal of controlled release
www.elsevier.com/locate/jconrel

Colloidally stable novel copolymeric system for gene delivery in complete growth media

Ankit Agarwal [a], Rita Vilensky [b], Anne Stockdale [a], Yeshayahu Talmon [b], Robert C. Unfer [c], Surya K. Mallapragada [a,*]

[a] Department of Chemical Engineering, Iowa State University, 3035 Sweeny Hall, Ames, IA 50011-2230, USA
[b] Department of Chemical Engineering, Technion-Israel Institute of Technology, Haifa-32000, Israel
[c] Iowa Cancer Research Foundation, Urbandale, IA-50322, USA

Received 8 February 2007; accepted 8 May 2007
Available online 17 May 2007

Abstract

Novel cationic pentablock copolymers based on poly(2-diethylaminoethylmethacrylate) (PDEAEM) and Pluronic F127 were evaluated as non-viral gene delivery vectors from a physiochemical point of view for stability and transfection efficiency in complete growth media. A novel strategy was introduced to sterically stabilize the polyplexes of such Pluronic-based cationic polymers against aggregation with serum proteins. As cationic pentablock copolymers condense plasmid DNA into nanoplexes of 100–150 nm diameter, unmodified Pluronic added to the formulation self-assemble with the pentablock copolymers on the surface of polyplexes and shield the cationic PDEAEM chains of pentablock copolymers sterically with its long poly(ethyleneoxide) chains. These coated polyplexes formed colloidally stable dispersions of 150–250 nm diameter in serum-supplemented buffers. Cryo-TEM micrographs also showed that coating polyplexes with unmodified Pluronic reduced aggregation in serum proteins. Pentablock copolymers preserved the integrity of plasmid DNA condensed inside the polyplexes and provided efficient resistance to its degradation by nucleases. Though the total amount of DNA retained by ExGen 500® polyplexes after nuclease digestion was more than that retained by pentablock copolymers, the amount of plasmid retained in supercoiled form was not significantly different. Polyplexes coated with unmodified Pluronic provided efficient transfection in SKOV3 cells in complete growth media, comparable to that provided by ExGen 500® in terms of number of cells transfected, and one order less in terms of total transgene protein expressed. These sterically shielded polyplexes also exhibited much lower cytotoxicities than uncoated polyplexes of pentablock copolymers, and significantly lower than the cytotoxicity of ExGen 500® at relevant concentrations. This colloidally stable, versatile, multi-component gene delivery system also forms thermo-reversible injectable hydrogels like Pluronics at physiological temperatures that can be used for sustained delivery of polyplexes, and is promising for systemic applications.

Keywords: Block-copolymers; Gene delivery; Colloidal stability; Transfection; Cytotoxicity

1. Introduction

The delivery of therapeutic genes to the targeted cells using non-viral vectors is widely being explored nowadays as a viable alternative to viral vectors, especially *via* cationic polymers [1,2]. More than a dozen new first generation and second-generation polymeric systems have been reported just in the past 5 years [3,4]. Researchers have increasingly recognized that dangers associated with domesticating viruses for gene delivery such as insertional mutagenesis, potential oncogenesis, immunogenicity and, long-term effect of the integrated transgene, along with production and packaging problems, can successfully be overcome by designing intelligent synthetic non-viral systems [5,6].

Cationic polymers electrostatically condense negatively charged DNA into nanoparticles, forming stable polymer/DNA complexes, "polyplexes" [7]. Though many of these polymeric vectors perform well *in vitro* in reduced serum conditions, they suffer from serious drawbacks when tested *in vivo* [8]. Binding of these polycations to DNA imparts excess positive surface charge to the complexes, which results in non-specific interactions with cellular blood components (erythrocytes), vessel endothelia and

* Corresponding author. Tel.: +1 515 294 7407; fax: +1 515 294 2689.
E-mail address: suryakm@iastate.edu (S.K. Mallapragada).

0168-3659/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2007.05.008

plasma proteins in an *in vivo* application [9]. In a systemic application, this leads to their aggregation and accumulation in the "first pass organs" such as lungs (consequently causing pulmonary embolism), liver and spleen, and finally opsonization and clearance by the reticuloendothelial system (RES), limiting their therapeutic applications [10,11]. Particle size, charge and stability of these polyplexes are key factors in determining their biodistribution, circulation time and transfection efficiency *in vivo* [12]. Different strategies have been developed to improve the *in vivo* stability and efficacy of first-generation polymers [13,14], resulting in a variety of second generation copolymers made by covalently linking polycations to a non-ionic water soluble polymer, such as poly(ethylene glycol) (PEG) [15–19], transferrin [20,21], or poly(*N*-(2-hydroxypropyl)methacrylamide (pHPMA) [22,23], forming a block or graft copolymer architecture. Adding such hydrophilic ligands or grafts to the polycations increases their aqueous solubility and shields their surface charge, creating a steric barrier against aggregation in blood streams or extra-cellular matrix [23,24].

Recently we reported the development of novel amphiphilic pentablock copolymers as gene delivery vectors [25]. These copolymers have triblock Pluronic F127 in the center, with cationic poly(2-diethylaminoethylmethacrylate) (PDEAEM) groups attached to their ends using an atom-transfer radical polymerization (ATRP) reaction scheme [26]. The copolymers form micelles in aqueous solutions because of the lower critical solution temperature (LCST) of hydrophobic poly(propyleneoxide) (PPO) chains and retain the thermoreversible gelation properties of the Pluronic. The cationic PDEAEM groups (pK_a ~7.3) electrostatically condense plasmid DNA into nanoparticles and provide a good buffering capacity at low pH that aids in the release of entrapped polyplexes from the acidic endosomal vesicles [27]. As in Pluronics, where the presence of hydrophobic PPO chains provide them with the unique ability to be incorporated into cell membranes [28,29], the PPO chains in the pentablock copolymers are expected to enhance cell interactions and increase translocation of polyplexes into the cells, with minimal damage to the cell membrane integrity, as compared to the cationic homopolymers PDEAEM or poly(2-dimethylaminoethylmethacrylate) (PDMAEM) [30]. These novel pentablock copolymers provided very good transfection efficiency, comparable to ExGen 500® (linear PEI), in reduced serum growth media, with minimal cytotoxicity [31] and thus are good candidates to be further investigated for gene therapy.

Here we report a novel method to sterically shield the surface charge of the polyplexes of such cationic pentablock copolymers and provide them colloidal stability in serum supplemented media. Since pentablock copolymers are derived from Pluronic F127, it was speculated that unmodified Pluronic added to the polyplex formulation would self-assemble with the pentablock copolymers on the surface of polyplexes and sterically shield the cationic chains of the pentablock copolymers with its long hydrophilic poly(ethyleneoxide) (PEO) chains. In the present work we have tested the stability and transfection efficiency of this multi-component gene-delivery system in serum supplemented media. The formulations were investigated from a physiochemical point of view by measuring their particle size, zeta-potential, and resistance of incorporated DNA towards nuclease digestion in serum containing buffers at various concentrations of constituent components. The goal was to investigate how efficiently, and at what weight ratios, adding free Pluronic stabilizes the polyplexes, and to assess the role of each component in the overall transfection process.

The knowledge obtained from the current work will be applied toward optimizing the design of this multi-component micellar system for ongoing *in-vivo* gene delivery studies in our labs. These pentablock copolymers are particularly promising toward clinical gene therapy because they are derived from Pluronics and are expected to retain their biological response modifying properties [32] such as sensitizing multi drug resistant (MDR) cancer cells [33], and overcoming blood brain barrier [28]. Complete understanding of this copolymer system is further important in its development as a controlled gene delivery system. The injectable aqueous pharmaceutical formulations of these copolymer/DNA complexes can form thermo-reversible gels in situ at physiological temperatures [26,31], a valuable characteristic which can be exploited for sustained delivery of polyplexes to localized tissues.

2. Materials and methods

2.1. Materials

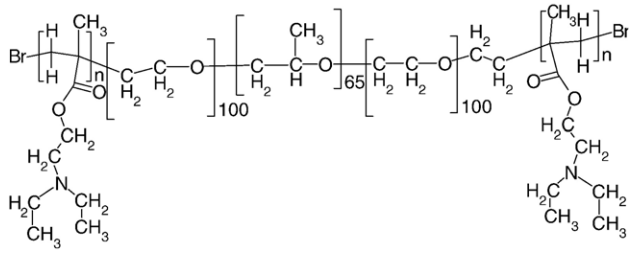
Dulbecco's Modified Eagle Medium (DMEM), OptiMEM I®, fetal bovine serum (FBS), 0.25% trypsin-EDTA solution, Hank's buffered salt solution (HBSS), Ultra-pure™ agarose, EDTA, TAE buffer and ethidium bromide were purchased from Invitrogen, Carlsbad, CA. HEPES salt, Heparin Sodium salt (cat # H-4784) and XTT (2,3-bis[2methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt) assay kit (Tox-2) were purchased from Sigma, St. Louis, MO. The Qiagen Maxi Prep kit was purchased from Qiagen, Valencia, CA. ExGen 500® (written as ExGen henceforth) and 6× TriTrack™ loading dye were purchased from Fermentas Inc, Hanover, MD. DNase I was purchased from Ambion Inc, Austin, TX.

2.2. Polymers

Pluronic F127 [$(PEO)_{100}$-b-$(PPO)_{65}$-b-$(PEO)_{100}$] micro pastille surfactant was donated by BASF (Florham Park, NJ) and used without further modification. The pentablock copolymers used for this reported study, $PDEAEM_8$-PEO_{100}-PPO_{65}-PEO_{100}-$PDEAEM_8$, had 20 wt.% of PDEAEM with M_n= 18,520 and M_w/M_n=1.14, as judged by ^{1}H NMR (in deuterated chloroform) and GPC (THF mobile phase, poly(methylmethacrylate) calibration standards) respectively, and were synthesized as previously reported [26], with their chemical structure shown in Scheme 1. It can be calculated that 1 μg of this copolymer has 1.03 nM of nitrogen residues.

2.3. Plasmid DNA and cell lines

A 4.1 kb plasmid pRL-CMV encoding Renilla luciferase (Promega, Madison, WI), and a 4.7 kb pEGFP-N1 (Clontech,



Scheme 1. Chemical structure of pentablock copolymers.

Mountain View, CA) plasmid encoding green fluorescence protein (GFP) were used as the reporter genes. Plasmids were amplified in DH5α *E. coli* cells and purified as reported earlier [25]. All DNA used had a A_{260}/A_{280} ratio of at least 1.80 in TE buffer, pH 7.4. The SKOV3 human ovarian carcinoma cell line was obtained from ATCC™ (Manassas, VA) and maintained as reported earlier [25].

2.4. Polyplex formulation

Copolymer to DNA ratios are expressed as N:P, molar ratio of nitrogen (N) in the cationic polymers to phosphate (P) in DNA, and were calculated using the fact that 1 μg DNA contains 3 nM of phosphates. Polyplexes were formed by following the precise order of mixing DNA, copolymers and Pluronic F127. Pentablock copolymers were first dissolved in 0.5× HBS (Hepes buffer saline-20 mM of HEPES with 145 mM NaCl), pH 7.0 at 4 °C. For luciferase transfection and cytotoxicity experiments in 96-well plates, polyplexes were prepared with 2.4 μg pRL-CMV in a final volume of 800 μl and were then divided into four equal parts for four wells in the plate, giving each well 0.6 μg of plasmid. First, an aliquot of DNA (1 μg/μL) in an Eppendorf tube was made up to 100 μL with 0.5× HBS buffer pH 7.0. Pentablock copolymer solution (2 mg/ml) was then added to DNA in the desired N:P ratio and incubated for 20 min at room temperature after gentle vortexing. If required, Pluronic F127 solution (25 mg/ml) in 0.5× HBS, pH 7.0 was added to the formulation to get the desired F127/pentablock copolymer wt. ratio with gentle vortexing and incubated for 10 mins. Final volume was made to 800 μL using desired growth media, OptiMEM I® or DMEM containing 10% FBS. For transfecting cells in 12-well plates with pEGFP, 3 μg of DNA per well was used and final volume was made to 1 ml for each well. For light scattering experiments, samples were prepared in 0.5× HBS buffer with 1 μg of DNA, and were made up to a final volume of 1 ml with either plain buffer or serum supplemented buffer, with final solute concentration between 0.3 to 0.8 mg/ml.

2.5. DNA condensation, nucleases resistance, serum stabilization

Electrophoretic mobility of plasmid DNA was evaluated using agarose gel electrophoresis. A total of 0.125 μg of DNA per lane was used. Polyplexes were prepared using 1 μg of pEGFP (0.1 μg/μL) in an Eppendorf tube, as outlined above, in 0.5× HBS, pH 7.0 buffer with a final sample volume of 100 μL and divided into 4 equal parts of 25 μL each for electrophoresis. To evaluate resistance to nuclease digestion, 25 μL polyplex solutions containing 0.25 μg plasmid DNA were incubated with 3 μL of 10× DNase I buffer and 1 μL of DNase I (2 IU/μL), giving 2 IU/μg DNA, for 15 mins at 37 °C. Immediately following incubation, 5 μL of 0.5 M EDTA was added and samples were placed on ice for 15 min to inactivate DNase I. To examine resistance to DNA degradation in serum, 25 μL polyplex solutions were incubated with 25 μL 0.5× HBS buffer containing 20% FBS for 30 min in a 37 °C incubator. Immediately following the incubation, nucleases were inactivated with EDTA and ice as mentioned earlier.

To asses the integrity of DNA inside the polyplexes, it was dissociated from the cationic polymers by adding heparin solution (100 mg/ml), an anionic glycosaminoglycan, in final concentration of 1% w/v and incuabating for 30 min at room temperature. After adding 5–7 μL of 6× loading buffer, samples were loaded on a 1% agarose gel stained with ethidium bromide (0.25 μg/mL). The gel was run in TAE buffer at 50 V for 2.5 h. Visualization and image capture was accomplished using a UV-transilluminator under a Kodak EDAS 290 digital imaging suite (Fisher Scientific, Pittsburg, PA). The band intensities were measured in arbitrary units using KODAK 1D image analysis software and all the experiments were repeated at least 4 times to obtain error bars.

2.6. Particle size and zeta(ζ) potential

Z-average diameter and polydispersity of the copolymers and polymer/DNA complexes were measured in Malvern disposable polystyrene cuvettes DTS 0012 at 37 °C by a Malvern Zetasizer Nano-ZS90 system, equipped with 4 mW 633 nm He-Ne laser (Malvern Instruments, Southborough, MA). Data analysis was carried out by the Dispersion Techonology Software (DTS) version 4.2. The instrument was calibrated with an aqueous polystyrene dispersion of 100 nm particles, using viscosity and refractive index of pure water at 37 °C. All water and buffers were double filtered using a 0.2 μm polyethersulfone (PES) sterile syringe filters (Fisher Scientific) to remove any dust particles. PES has both, very low protein binding and, very low extractables, and is recommended for filtering cell culture media. The test solutions were vortexed gently and incubated for 30 min at 37 °C before measurements. The ζ-potential of the polyplexes was measured at 37 °C in Malvern zeta potential cuvettes DTS 1060. The instrument was calibrated using a polystyrene dispersion with a known ζ-potential.

2.7. Cryo-TEM

Vitrified specimens of the polymer/DNA complexes were prepared for transmission electron microscopy (TEM) in a controlled environment vitrification system (CEVS) at 25 °C and 100% relative humidity, as previously described [34]. Briefly, a drop of the solution was applied onto a perforated holey-carbon film, supported on an electron microscopy 200-mesh copper grid, and held by tweezers in the vitrification

system chamber. The sample was blotted with a filter paper and immediately plunged into liquid ethane at its freezing point (−183 °C). The vitrification process captured the copolymers and their DNA condensates in the sample in a state as close as possible to the native state without the need for artifact-inducing staining-and-drying [35]. Samples were examined in a Philips CM120 or an FEI T12 G^2 cryo-dedicated transmission electron microscopes (Eindhoven, The Netherlands), operated at 120 kV, using either an Oxford CT-3500 (for the CM120; Oxford Istruments, Abingdon, England) or a Gatan 626 (for the T12; Gatan, Pleasanton, CA) cooling holders and transfer stations. Specimens were equilibrated in the microscopes below −178 °C, examined in the low-dose imaging mode to minimize electron beam radiation damage, and recorded at a nominal underfocus of about 2 μm to enhance phase-contrast. Images were acquired digitally by a MultiScan 791 (CM120) or a US1000 (T12) cooled charge-coupled-device cameras (Gatan, Pleasanton, CA), using the Digital Micrograph software.

2.8. Transfection and cytotoxicity

For transfection with luciferase plasmid, cells grown in 96-well plates up to 70% confluency were transfected with polyplex solutions in 200 μL media using 0.6 μg of DNA per well. Transfections with ExGen were performed according to manufacturer's protocol, with polyplexes prepared at N:P 6 and incubated with cells in complete growth media. Polymer solutions were aspirated after 3 h and cells were incubated for another 48 h in complete growth media until luciferase assay was performed. Cells were lysed with 40 μL Renilla Luciferase Assay Lysis Buffer and the luminescence was measured in arbitray Relative Luminescence Units (RLU) on an automated Veritas™ Microplate Luminometer using the Renilla Luciferase Assay System from Promega.

Luciferase activity (RLU) in each well was not normalized by the total amount of protein (mg) as that gives artificially high values (RLU/mg) in the samples where total protein level has been reduced by cell death. Instead, since all experiments were performed with same initial number of cells per well ($\sim 1.2\times10^4$) in a 96-well plate, luciferase expression is reported as RLU/well for each case, along with percentage cell viability found in each well using XTT assay. Briefly, XTT stock solution prepared in phosphate buffer saline (PBS) pH 7.4 was added to the cells grown in 200 μL media in 96-well plate to a final concentration of 0.2 mg/mL. After shaking the plates briefly on a gyratory shaker and incubating for 4 h at 37 °C, the concentration of formazan crystals formed in the viable cells [36] was found by measuring the absorbance at 450 nm on a BioTek EL-340 plate reader (Winooski, VT). Background absorbance measured at 630 nm was subtracted from the main readings.

SKOV3 cells grown in 12-well plates were transfected with the pEGFP plasmid following procedures similar to those used in 96-well plates. Cells were harvested 48 h after transfection and flow-cytometry was performed to count the number of GFP expressing cells using a Beckman-Coulter Epics ALTRA Fluorescence-activated cell sorter (Fullerton, CA) as described previously [25].

2.9. Statistics

Where appropriate, the data is presented as mean and standard deviation (SD) calculated over four data points at least. Significant differences between two groups were evaluated by Student's *t*-test and between more than two groups by one-way ANOVA analysis of variance, followed by Tukey's test. The level of significance was set at $p<0.05$, unless otherwise stated.

3. Results and discussion

3.1. Colloidal stability

The size distribution of the polymers and polyplexes in buffer solutions at pH 7.0 and 37 °C was measured in the absence and presence of serum, with different concentrations of unmodified Pluronic F127 added to the formluations. Polyplexes with N:P ratios from 5 to 30 were investigated. For most of the samples, single narrow peaks of the scattered light intensity were obtained, while for the samples with aggregates, intensity of the scattered light peaked at two different particle sizes, shown as unimodal or multimodal distribution of particle diameter in Fig. 1. Pentablock copolymers and Pluronic F127 had average cumulative micelle sizes of 33 nm and 23 nm diameter, respectively, while serum particles were around 9 nm, all with polydispersity-index (PDI) less than 0.1. A 2 mg/ml solution of pentablock copolymers and F127 (5:1 w/w) together had an average micelle size of 25±2 nm. All other results are presented in Fig. 1. All samples were investigated for up to five hours repeatedly to confirm dispersion stability over time. Fig. 1a shows that pentablock copolymers condensed plasmid DNA into stable polyplexes above N:P 5, with diameter less than 175 nm and low PDI. The size of the condensates decreased systematically on increasing the N:P ratios from 5 to 20, which reflects the process of DNA condensation induced by cationic polymers and has been explained in several studies by the coil-globule transition of plasmid DNA molecules upon complexing to take the compact conformation [19,37]. However, incubating these polyplexes in buffers supplemented with 10% FBS resulted in formation of large aggregates at high N:P ratios, represented by wider peaks (high polydispersity) and secondary peaks in Fig. 1b.

The effect of adding unmodified Pluronic to the formulations was evaluated by first measuring the resulting polyplex sizes in buffers without serum. Fig. 1c shows that at F127/pentablock copolymer wt. ratio 5:1, stable polyplexes of 145±11 nm diameter with low polydispersity were formed at all investigated N:P ratios. Small secondary peaks at 25–30 nm in the figure denote free particles (micelles) of excess pentablock and Pluronic self-assembled together. At higher N:P ratios, when polyplexes are actually smaller in size (notice N:P 20 Fig. 1a), but have higher surface charge, more F127 micelles attach to them to neutralize their surface charge. Thus particle size apparently remained constant at all N:P ratios in the presence of Pluronic. Fig. 1d shows the fate of these Pluronic stabilized polyplexes in buffers supplemented with 10% (v/v) FBS. Stable polyplexes of around 175 nm diameter with no aggregates in

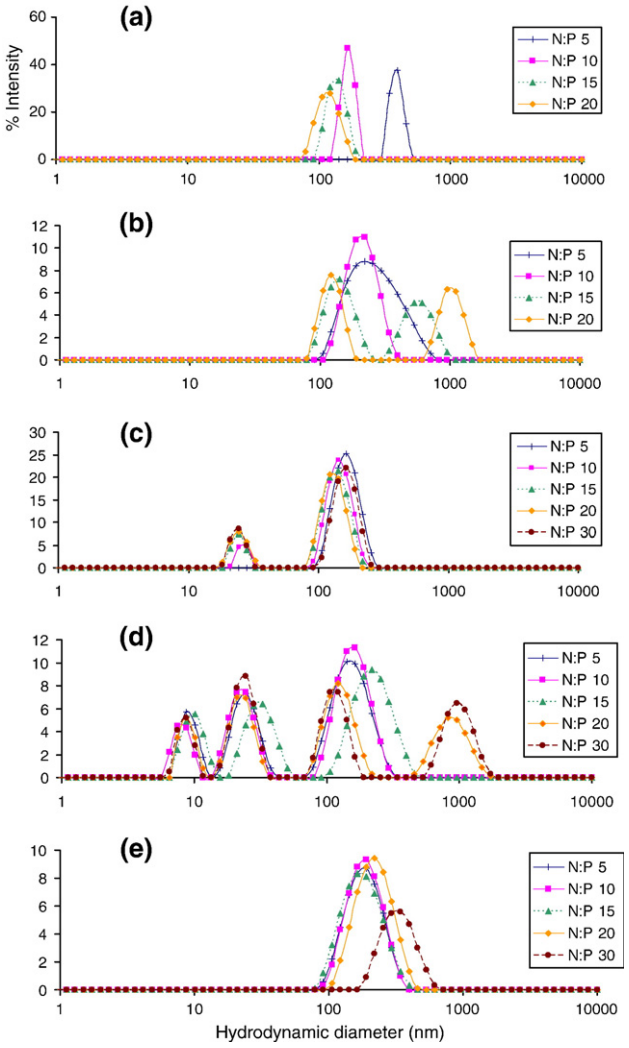


Fig. 1. Polyplex sizes at different N:P ratios in presence and absence of serum in 0.5× HBS buffer at 37 °C. Effect of Pluronic F127 on the stability of polyplexes in serum supplemented buffer is shown. (a) polyplexes in buffer, (b) polyplexes in buffer with 10% serum (c) polyplexes with 5:1 F127 in buffer, (d) polyplexes with 5:1 F127 in buffer with 10% serum, (e) polyplexes with 10:1 F127 in buffer with 10% serum.

solution were observed up to N:P 15. However, small distinct peaks of large aggregates of ~700±200 nm could be noticed at higher N:P ratios (Fig. 1d). Small peaks observed at ~8 nm and ~30 nm represent serum particles and free pentablock/Pluronic micelles, respectively, as had been noticed independently. These peaks however have been omitted in Fig. 1b and e to make the peaks of DNA condensates look more legible.

At F127/pentablock copolymer wt. ratio 10:1, colloidally stable polyplexes were formed in serum supplemented buffers even at high N:P ratios. As shown in Fig. 1e, polyplexes of ~200 nm diameter were formed up to N:P 20. At N:P 30, average particle size increased to 337±90 nm. These results again indicate that more F127 is needed to stabilize the particles with larger surface charge density at higher cationic copolymer concentrations. Since the size of polyplexes coated with unmodified Pluronic is larger than the uncoated ones at high N:P ratios (compare Fig. 1c and a), it would take fewer such polyplexes to make same size aggregates (700 nm and larger), suggesting that smaller amount of plasmid is lost to the aggregates in these free Pluronic stabilized formulations.

Zeta potential of the particles in different formulations was measured in plain buffers containing no serum proteins. The measurements confirmed that pentablock copolymers and their polyplexes have excessive cationic surface charge. Pentablock copolymers by themselves gave zeta potential of +6.0±1.3 mV (with peak width of 10 mV), and serum particles showed −6 mV (with peak width of 12 mV). After DNA condensation, the zeta potential of copolymer/DNA complexes (at N:P 20) was still around +2.2±0.23 (with peak width of 12 mV). However, when free F127 was added to the polyplex formulations in wt. ratio 5:1 (F127/pentablock copolymer), the zeta-potential was reduced to almost zero (0.037±0.5 mV, with peak width of 11 mV).

Cryo-TEM images in Fig. 2a show that polyplexes not coated with free Pluronic F127 formed large masses of aggregates when incubated with buffers supplemented with 10% FBS. Short thread like structures of polyplexes entangled with a large number of platelets and globules of serum proteins were observed. However, polyplex formulations containing 5:1 w/w Pluronic/pentablock copolymer had fine thread like

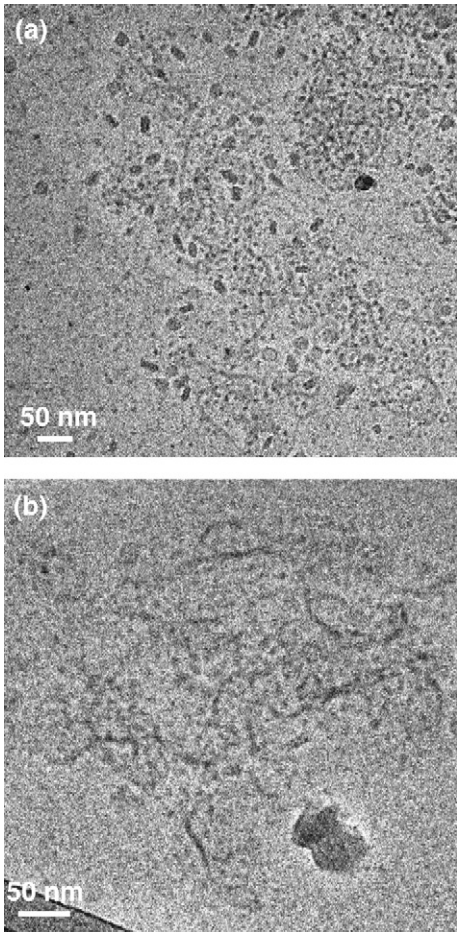


Fig. 2. Micrographs of pentablock copolymer/DNA complexes (N:P 10) in a serum supplemented (10% v/v) buffer with, (a) no free Pluronic, or (b) free Pluronic (5:1 w/w Pluronic: pentablock copolymer) added to the formulation.

structures of polyplexes with significantly fewer numbers of globules of serum proteins around them (Fig. 2b). The representative micrographs shown here suggest that charged polyplexes attract more serum proteins to them, forming large aggregates, and when cationic surface charge of polyplexes is sterically shielded by unmodified Pluronic, fewer globules of serum proteins accumulate on the polyplexes leaving their extended fine thread-like structure intact.

3.2. DNA integrity and protection

A plasmid DNA can exist in one of the three conformations: linear (after getting nicked), open circular (with only one strand nicked), or supercoiled. A varied degree of supercoiling might also exist depending upon the writhes in the plasmid. Fig. 3a shows that pEGFP plasmid released using heparin from the polyplexes of ExGen (lane 2), or of pentablock copolymers formed at different N:P ratios and Pluronic F127 concentrations (lanes 3–8), has the same band intensity in each conformation as the native plasmid in lane 1 (band intensity data not shown here). Absence of a band representing linear DNA confirms that the plasmid was not nicked by condensation with polymers and its integrity was maintained. No effect of adding unmodified Pluronic to the formulations was observed.

DNA released from the polymer/DNA complexes post incubation with DNase I was also run on the agarose gels. Fig. 3b shows that pentablock copolymers and ExGen provide partial protection to the plasmid DNA against degradation by nucleases. Lane1 contains native plasmid DNA that exist in two conformations — open circular and supercoiled, which is completely digested by DNase I in lane 9, confirming the activity of nucleases. Lanes 2–8 contain various polyplexes of ExGen and pentablock copolymer after incubation with DNase1. DNA bands on the top of these lanes confirm that there is some DNA left condensed with the polymers after nucleases digestion. To examine the amount and integrity of DNA left inside the polyplexes in lanes 2–8, they were incubated with heparin and run in lanes 10–16, respectively. Plasmid DNA released from the polyplexes of ExGen (lane 10) had little fraction in the supercoiled form, with most of it converted to open circular state. The DNA band between supercoiled and open circular plasmid bands can either be a linear plasmid, or a relaxed supercoiled form of the plasmid with relatively less number of writhes and twists, reduced while dissociating from the cationic polymer. Comparing this to the plasmid released from polyplexes of pentablock copolymers in lanes 11–16, two points can be inferred. First, the total amount of DNA retained in polyplexes after nuclease digestion was greater with ExGen than with pentablock copolymers. Second, the total amount of DNA retained in supercoiled form, which many would agree is the fraction with greatest integrity, was almost the same with both cationic polymers. Nuclease resistance provided by pentablock copolymers did not improve on increasing the N:P ratio from 10 to 20, or by adding free Pluronic to the formulations. Densitometry data obtained over four similar experiments is plotted in Fig. 3c and clearly reiterates above stated inferences.

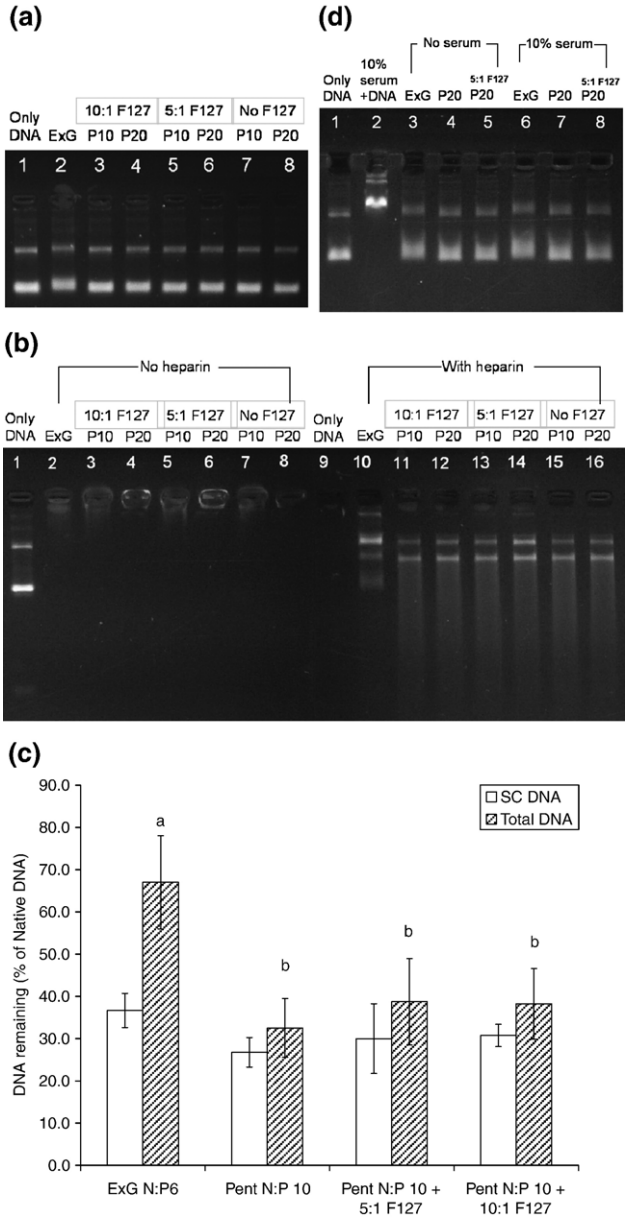


Fig. 3. (a) Integrity of pEGFP plasmid released from the polyplexes after incubating them with heparin. Lane 1: native plasmid with heparin; lane 2: ExGen at N:P 6; lane 3,4: pentablock copolymer at N:P 10 and 20 respectively, with 10:1 F127; lane 5,6: pentablock copolymer at N:P 10 and 20 respectively, with 5:1 F127; lane 7,8: pentablock copolymer at N:P 10 and 20 respectively, with no F127. (b) effect of DNase1 on the stability of condensed plasmid. Lane 1: native pEGFP plasmid. All other lanes have polyplexes incubated with DNase1. Lane 2: ExGen N:P 6; lane 3, 4: pentablock N:P 10 and 20 respectively with 10:1 F127, lane 5, 6: pentablock N:P 10 and 20 respectively with 5:1 F127, lane 7, 8: pentablock N:P 10 and 20 respectively with no F127; lane 9: naked plasmid. Lanes 10–16 contain plasmid released from polyplexes in lanes 2 to 8 respectively after incubation with heparin. (c) densitometry analysis on the plasmid DNA retained in polyplexes after DNase I digestion, presented in Fig. 3b ($n=4\pm$SD). SC: fraction of the supercoiled DNA retained, ExG: ExGen, pent: pentablock copolymer, a and b indicates significant difference with $p<0.05$. (d) integrity of pEGFP plasmid released from the polyplexes before and after incubating with 10% serum. Lane 1: naked plasmid, lane 2: naked plasmid after incubation with seurm; lane 3: ExGen at N:P 6; lane 4, 5: pentablock copolymer at N:P 20, with no Pluronic, and with 5:1 Pluronic, respectively; lane 6,7,8: polyplexes in lane 3, 4, and 5 after incubation with serum.

Fig. 3d shows the stability of polyplexes in serum proteins. Complete conversion of supercoiled plasmid DNA in lane 1 to open circular form in lane 2 after incubation with serum suggests that molecules of plasmid DNA got nicked by nucleases in serum proteins. However, the integrity and topology of plasmid DNA released from the polyplexes of ExGen and pentablock copolymers before (lanes 3–5) and after incubation with serum proteins (lanes 6–8) is intact as in native plasmid DNA in lane 1. This confirms that pentablock copolymers protect condensed DNA against degradation in serum. No effect of adding unmodified Pluronic to the formulations was observed. The results again suggest that low transfection efficiency of pentablock copolymers without unmodified Pluronic in serum-supplemented media is not because of the degradation of condensed DNA inside its polyplexes. Instead, it is due to the loss of plasmid DNA to the large aggregates of polyplexes and serum proteins. Also, the sole function of unmodified Pluronic added to the system is to provide colloidal stability to the polyplexes and prevent aggregation, and not to protect DNA against degradation by serum proteins.

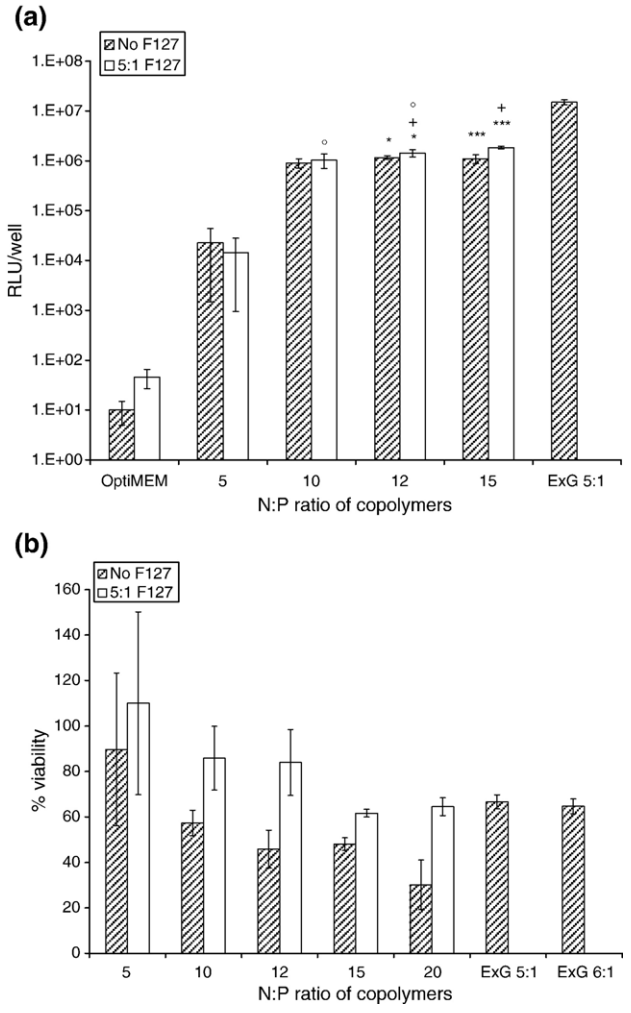


Fig. 4. (a): Effect of free Pluronic F127 on the transfection efficiency of pentablock copolymers at different N:P ratios in OptiMEM I® media. $+p<0.05$, $*p<0.1$, $°p<0.1$, $***p<0.005$ ($n=4\pm$SD). (b): effect of free Pluronic F127 on the cytotoxicity of pentablock copolymers' polyplexes at different N:P ratios in OptiMEM I® media. Polyplexes containing 0.6 μg pRL-CMV were incubated with SKOV3 cells for 3 h in Opti-MEM I® and cell viability was measured after another 48 h of incubation in complete growth media ($n=4\pm$SD).

3.3. Transfection and cytotoxicity

DNA condensates of pentablock copolymers showed remarkably high transfection efficiency in the reduced serum media OptiMEM I®. As shown in Fig. 4a, transgene expression of luciferase obtained with pentablock copolymers at N:P 10 and above was only one order less than that obtained with ExGen (which could be due to the better resistance to nuclease digestion provided by ExGen, as evident in Fig. 3b). The luciferase expression increased on increasing the N:P ratios, but it was concomitant with a reduction in the viability of the cells (Fig. 4b). Thus, a increase in transfection efficiency of copolymers at higher N:P ratios was offset by an increase in toxicity, displaying an overall reduced protein expression per well. However, when F127 was added to the polyplex solutions, there was a significant decrease in the cytoxicity of the copolymers. This in turn increased the total luciferase expression per well at higher N:P ratios, and an overall higher expression was obtained with such a formulation at N:P 15. Though cells transfected with ExGen gave one order of magnitude higher luciferase expression than those with pentablock copolymers at N:P 12, the cell viability in the presence of ExGen was reduced to 65% as compared to 90% with the pentablock copolymers.

Fig. 5 shows the transfection efficiency and cytotoxicity of the copolymers in complete growth media containing 10% FBS. Little transfection was obtained by incubating the cells with uncoated polyplexes of pentablock copolymers, as most of these charged complexes aggregated with serum proteins and couldn't get across the cell membrane. This is also evident from the increased cell viability (Fig. 5b) in complete growth media with polyplexes at N:P ratios 10, 15, and 20, as compared to that in OptiMEM I® (Fig. 4b). Since most of the charged complexes formed neutralized aggregates with serum proteins, few cationic complexes were left in the complete media to damage the cells. However, when F127 was added to the formulation in F127/pentablock wt. ratio 5:1, significantly higher transfection was obtained at all N:P ratios, with total luciferase expression at N:P 15 and higher as good as that obtained with pentablock copolymers in the serum-free media. This confirms that most of the polyplexes were prevented from aggregating with serum proteins and could get across the cell membrane to deliver their DNA payload to the nucleus. This charge shielding effect of F127 was also evident from the reduced toxicity of the polyplexes coated with F127 (Fig. 5b), compared to unshielded polyplexes, as the reduced cationic surface charge caused less inhibition of the cell's metabolic viability.

Different concentrations of free Pluronic were added to the polyplex solutions to shield their surface charge and to find the optimum formulation. Results with F127/pentablock wt. ratio 10:1 are also presented here for comparison. As shown in Fig. 5, though there is no significant effect of higher F127 concentration at lower N:P ratios, a significant increase in transfection efficiency and cell viability is observed at N:P 25 and 30. This can be explained by the fact that at higher N:P ratios a larger amount of

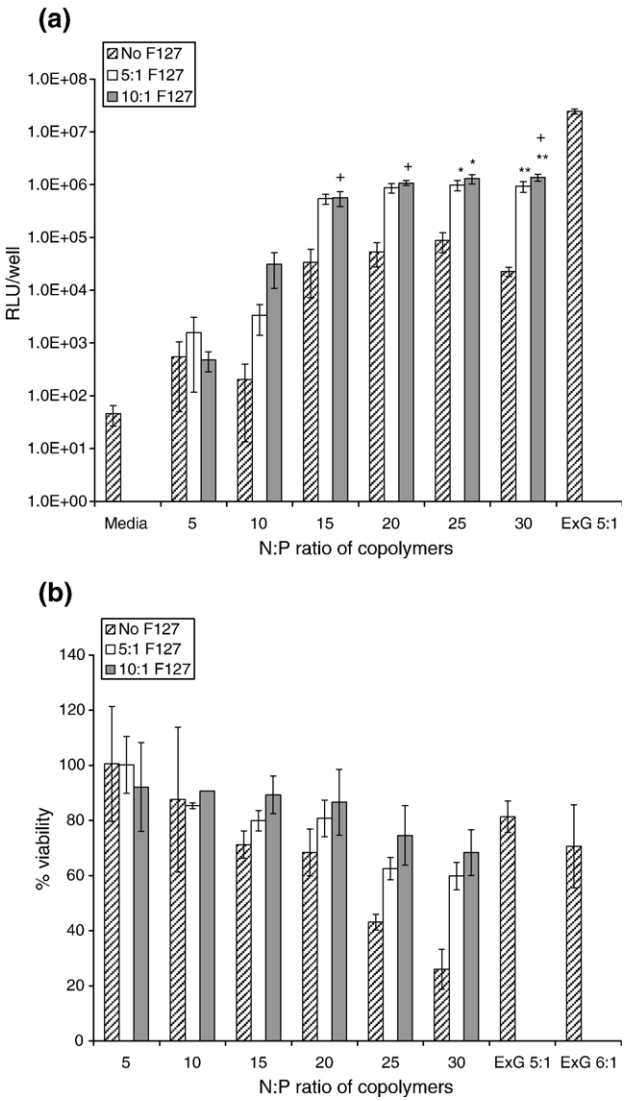


Fig. 5. (a): Effect of free Pluronic F127 on transfection efficiency of pentablock copolymers at different N:P ratios in complete growth media containing 10% FBS. $+p<0.05$, $*p<0.1$, $**p<0.05$ ($n=4\pm$SD). (b): effect of free Pluronic F127 on cytotoxicity of pentablock copolymers' polyplexes at different N:P ratios in complete growth media containing 10% FBS. Polyplexes containing 0.6 μg pRL-CMV were incubated with SKOV3 cells for 3 h in complete growth media and then replaced with fresh media. Cell viability was measured after another 48 h ($n=4\pm$SD).

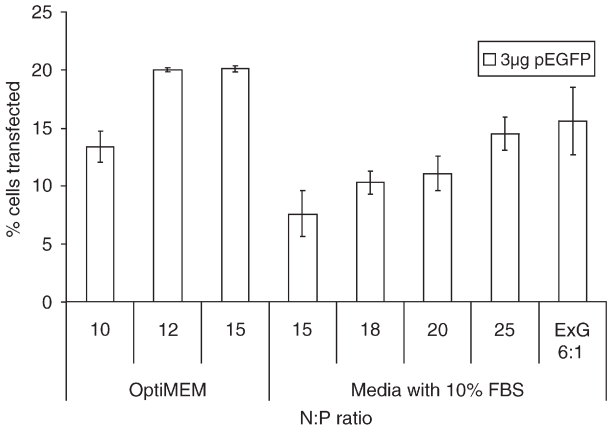


Fig. 6. Percentage of cells expressing GFP after transfection with pEGFP using pentablock copolymers at different N:P ratios, stabilized with free Pluronic (5:1 w/w Pluronic/pentablock copolymer), in the reduced serum growth media OptiMEM I® or complete growth media supplemented with 10% FBS.

unbound cationic copolymer is present in the media, and the polyplexes have higher surface charge density. Thus, there is an increase in both, the total charged surface area to be shielded (because of increased free copolymer micelles), and total surface charge to be shielded. Hence, an increased concentration of free F127 is required at these high N:P ratios to form stabilized dispersions in serum-supplemented media. This also suggests that for an *in vivo* formulation, where large doses of DNA will be injected in a single dose, higher amount of free F127 should be used to shield higher concentration of copolymers in the solution.

SKOV3 cells were also transfected with a pEGFP plasmid in the presence and absence of serum to evaluate transfection efficiency of polymers in terms of percentage of cells expressing the transgene. Pentablock copolymer/DNA complexes with F127/pentablock wt. ratio 5:1 were used. As reported in Fig. 6, similar to results obtained with luciferase transfection, the transfection efficiency of copolymers increased with the N:P ratio. The pentablock copolymers transfected up to 21% cells in reduced serum media and up to 17% cells in complete growth media, which is comparable to the transfection efficiency obtained with ExGen. This suggests that pentablock copolymers can successfully deliver the plasmid DNA to the nucleus of as many cells as ExGen and their transfection efficiency (in terms of total gene expression) is limited by the amount of gene delivered.

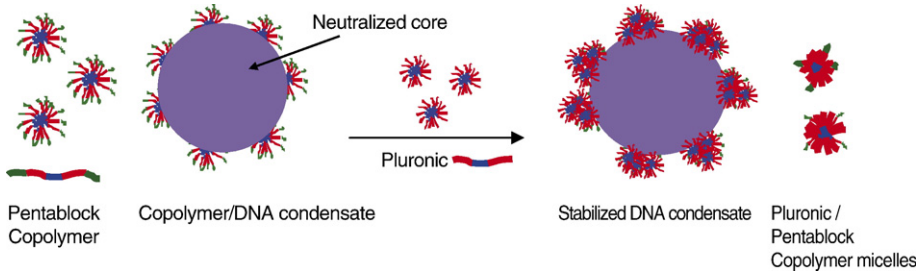


Fig. 7. Schematic (drawn not to scale) showing how adding free Pluronic to a solution of pentablock copolymer/DNA complexes would sterically shield the charged PDEAEM groups (labeled green) on their surface. PEO chains are indicated in red, and PPO chains in blue. Purple core consist of electrostatically neutralized polymer/DNA condensates.

4. General discussion

This work reports a novel multi-component polymeric gene delivery system and critically evaluates its colloidal stability and transfection efficiency. Plasmid DNA was condensed into nanoplexes with novel cationic pentablock copolymers composed of PDEAEM-b-PEO-b-PPO-b-PEO-b-PDEAEM and the nanoplexes were coated with unmodified Pluronic to sterically shield their surface. Pluronics have previously been shown to enhance the uptake of plasmid DNA and cell transfection when added along with cationic polymers [38]. When poly(*N*-ethyl-4-vinylpyridinium bromide) (pEVP-br) and DNA complexes were mixed with 1% Pluronic P85, it intensified their uptake by non-specific endocytosis in the eukaryotic cells and significantly increased their transfection efficiency [39]. Gebhart et al showed that when free Pluronic was added to the unstable complexes of P123-g-PEI(2 K) and DNA, it sterically shielded the hydrophobic PPO chains of P123 grafted on the copolymer and formed more hydrophilic stable dispersions that provided enhanced transfection efficiency [40]. However, the copolymers still exhibited low level of DNA protection against DNase I and gave much lower transfection efficiency as compared to ExGen. A similar self-assembly of unmodified Pluronic with the novel pentablock copolymers is exploited in the presented polymeric gene delivery system.

Dynamic light scattering and cryo-TEM micrographs confirmed that adding unmodified Pluronic F127 to the polyplexes of pentablock copolymers reduced their aggregation with serum proteins. The plausible mechanism of the process is sketched in Fig. 7 and can be explained as follows. Pluronic F127 has same hydrophobic PPO core as the pentablock copolymers. Thus, when F127 is added to the polyplex solutions, hydrophobic PPO blocks of F127 tend to self-assemble with the hydrophobic PPO blocks of the extra pentablock copolymers on the surface of polyplexes. While the two hydrophobic cores self-assemble, the long PEO chains of F127 sterically shield the cationic PDEAEM chains of the pentablock copolymers. By adding F127 at a w/w ratio (F127/pentablock copolymer) of 5:1 or more to the formulation, several F127 molecules are available per extra pentablock molecules on the polyplex surface. Thus, stable nanoscale polyplexes (nanoplexes) with condensed DNA in the core and hydrophilic PEO chains of F127 and pentablock copolymers in the corona are formed. The hydrophilic surface of nanoplexes with PEO chains prevents the aggregation of particles with each other too. Furthermore, extra molecules of cationic pentablock copolymer self assemble with free Pluronic molecules to arrange them in a dynamic equilibrium to form sterically stabilized micelles with reduced zeta-potential. Since both the copolymers have the same amphiphilic architecture, the resulting polyplex solutions still retain the thermo-gelling properties, and form thermo-reversible hydrogels at 37 °C.

The order of addition of F127 to the formulation was also investigated by observing its impact on the size of the particles and their transfection efficiency. Among several investigated formulations two in particular were important — one, reported above, where Pluronic was added to the formulations after DNA condensation with the pentablock copolymers, and second, where Pluronic was added to the DNA aliquots before adding pentablock copolymer to the formulation. No significant difference was observed (data not shown), and the first formulation was adopted for the rest of the study. It should be noted though that the polymer/DNA complexes were formed in serum-free buffers. The formation of large aggregates of polyplexes with serum proteins is a very rapid, irreversible process, and therefore their surface charge needs to be shielded before serum is added to the system.

Finally, this is a dynamic gene delivery system that can be easily tailored to specific therapeutic applications. Pluronic F127 was used is this study because first, it has long hydrophilic chains, and second, it is already approved for use in pharmaceutical preparations, which means the toxicological data exists, and can therefore speed the preclinical development of pentablock copolymer formulations. However, there are many other Pluronics that may be used in these formulations as unmodified Pluronic, or can be substituted in pentablock copolymers for F127. A detailed review on Pluronics with their structure, characteristics, and drug delivery applications is discussed elsewhere [32]. The wt.% of cationic PDEAEM chains can also be tailored in the pentablock copolymers conferring varying degrees of cytotoxicity, transfection efficiency, and pH-sensitivity to the polyplexes [25,31].

5. Conclusions

In summary, we have reported a novel colloidally stable multi-component copolymeric gene delivery system. Cationic pentablock copolymers condensed the plasmid DNA in nanoplexes and unmodified Pluronic self-assembled on the nanoplexes to sterically shield their surface charge. The nanoplexes formed stable dispersions and gave excellent transfection efficiency in complete growth media with enhanced biocompatibility. Nanoplexes provided an efficient protection to condensed plasmid DNA against nucleases and serum proteins and, did not aggregate with serum proteins. These qualities of this novel copolymeric system, together with its ability to form injectable thermo-reversible self-assembled hydrogels, makes it a versatile gene delivery system, and warrants good performance in *in vivo* applications.

Acknowledgements

We acknowledge Michael D Determan for the synthesis of pentablock copolymers, and thank undergraduate student Shytyug Loh for assistance with the laboratory work. This work was supported by a Bailey Career Development grant and US-DOE through contract number W-7405-ENG-82. The cryo-TEM work was performed at the Hannah and George Krumholz Laboratory for Advanced Microscopy at the Technion, part of the Technion Project on Complex Fluids.

References

[1] D. Putnam, Polymers for gene delivery across length scales, Nat. Mater. 5 (2006) 439–451.

[2] M. Thomas, A.M. Klibanov, Non-viral gene therapy: polycation-mediated DNA delivery, Appl. Microbiol. Biotechnol. 62 (1) (2003) 27–34.
[3] J.K. Vasir, V. Labhasetwar, Polymeric nanoparticles for gene delivery, Expert Opin. Drug Deliv. 3 (3) (2006) 325–344.
[4] T.G. Park, J.H. Jeong, S.W. Kim, Current status of polymeric gene delivery systems, Adv. Drug Deliv. Rev. 58 (4) (2006) 467–486.
[5] C.M. Henry, Gene delivery-without viruses, Chem. Eng. News 79 (48) (2001) 35–41.
[6] A. El-Aneed, An overview of current delivery systems in cancer gene therapy, J. Control. Release 94 (1) (2004) 1–14.
[7] P.L. Felgner, Y. Barenholz, J.P. Behr, S.H. Cheng, P. Cullis, L. Huang, J.A. Jessee, L. Seymour, F. Szoka, A.R. Thierry, E. Wagner, G. Wu, Nomenclature for synthetic gene delivery systems, Hum. Gene Ther. 8 (5) (1997) 511–512.
[8] F. Verbaan, I. van Dam, Y. Takakura, M. Hashida, W. Hennink, G. Storm, C. Oussoren, Intravenous fate of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes, Eur. J. Pharm. Sci. 20 (4-5) (2003) 419–427.
[9] R.I. Mahato, Y. Takakura, M. Hashida, Nonviral vectors for in vivo gene delivery: physicochemical and pharmacokinetic considerations, Crit. Rev. Ther. Drug Carr. Syst. 14 (2) (1997) 133–172.
[10] M. Ogris, E. Wagner, Targeting tumors with non-viral gene delivery systems, Drug Discov Today 7 (8) (2002) 479–485.
[11] D.B. Fenske, I. MacLachlan, P.R. Cullis, Long-circulating vectors for the systemic delivery of genes, Curr. Opin. Mol. Ther. 3 (2) (2001) 153–158.
[12] P.R. Dash, M.L. Read, L.B. Barrett, M.A. Wolfert, L.W. Seymour, Factors affecting blood clearance and in vivo distribution of polyelectrolyte complexes for gene delivery, Gene Ther. 6 (4) (1999) 643–650.
[13] U. Lungwitz, M. Breunig, T. Blunk, A. Gopferich, Polyethylenimine-based non-viral gene delivery systems, Eur. J. Pharm. Biopharm. 60 (2) (2005) 247–266.
[14] S.J. Hwang, M.E. Davis, Cationic polymers for gene delivery: designs for overcoming barriers to systemic administration, Curr. Opin. Mol. Ther. 3 (2) (2001) 183–191.
[15] M. Lee, S.W. Kim, Polyethylene glycol-conjugated copolymers for plasmid DNA delivery, Pharm. Res. 22 (1) (2005) 1–10.
[16] T. Kawano, M. Yamagata, H. Takahashi, Y. Niidome, S. Yamada, Y. Katayama, T. Niidome, Stabilizing of plasmid DNA in vivo by peg-modified cationic gold nanoparticles and the gene expression assisted with electrical pulses, J. Control. Release 111 (3) (2006) 382–389.
[17] Jeong Soon Jang, So Yeon Kim, Sang Bong Lee, Kyung Ok Kim, Joong Soo Han, Young Moo Lee, Poly(ethylene glycol)/poly([epsilon]-caprolactone) diblock copolymeric nanoparticles for non-viral gene delivery: the role of charge group and molecular weight in particle formation, cytotoxicity and transfection, J. Control. Release 113 (2) (2006) 173–182.
[18] A.M. Funhoff, S. Monge, R. Teeuwen, G.A. Koning, N.M.E. Schuurmans-Nieuwenbroek, D.J.A. Crommelin, D.M. Haddleton, W.E. Hennink, C.F. van Nostrum, Peg shielded polymeric double-layered micelles for gene delivery, J. Control. Release 102 (3) (2005) 711–724.
[19] K. Itaka, K. Yamauchi, A. Harada, K. Nakamura, H. Kawaguchi, K. Kataoka, Polyion complex micelles from plasmid DNA and poly(ethylene glycol)-poly(-lysine) block copolymer as serum-tolerable polyplex system: physicochemical properties of micelles relevant to gene transfection efficiency, Biomaterials 24 (24) (2003) 4495–4506.
[20] M.L. Kyung, S.K. In, B.L. Yong, C.S. Sang, C.L. Kang, J.O. In, Evaluation of transferrin-polyethylenimine conjugate for targeted gene delivery, Arch. Pharm. Res. 28 (6) (2005) 722–729.
[21] R. Kircheis, L. Wightman, A. Schreiber, B. Robitza, V. Rossler, M. Kursa, E. Wagner, Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application, Gene Ther. 8 (1) (2001) 28–40.
[22] T.E. Robert, C. Carlisle, Simon S. Briggs, Jon A. Preece, Karel Ulbrich, Leonard W. Seymour, Polymer-coated polyethylenimine/DNA complexes designed for triggered activation by intracellular reduction, J. Gene Med. 6 (3) (2004) 337–344.
[23] D. Oupicky, M. Ogris, K.A. Howard, P.R. Dash, K. Ulbrich, L.W. Seymour, Importance of lateral and steric stabilization of polyelectrolyte gene delivery vectors for extended systemic circulation, Mol. Ther. 5 (4) (2002) 463–472.
[24] F.J. Verbaan, C. Oussoren, C.J. Snel, D.J.A. Crommelin, W.E. Hennink, G. Storm, Steric stabilization of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes mediates prolonged circulation and tumor targeting in mice, J. Gene Med. 6 (1) (2004) 64–75.
[25] A. Agarwal, R. Unfer, S.K. Mallapragada, Novel cationic pentablock copolymers as non-viral vectors for gene therapy, J Control Release 103 (1) (2005) 245–258.
[26] M.D. Determan, J.P. Cox, S. Seifert, P. Thiyagarajan, S.K. Mallapragada, Synthesis and characterization of temperature and ph responsive penta-block copolymers, Polymer 46 (18) (2005) 6933–6946.
[27] N.J. Zuidam, G. Posthuma, E.T.J. De Vries, D.J.A. Crommelin, W.E. Hennink, G. Storm, Effects of physicochemical characteristics of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes on cellular association and internalization. J. Drug Target. 8 (1) (2000) 51–66.
[28] E.V. Batrakova, S. Li, S.V. Vinogradov, V.Y. Alakhov, D.W. Miller, A.V. Kabanov, Mechanism of pluronic effect on *p*-glycoprotein efflux system in blood-brain barrier: contributions of energy depletion and membrane fluidization, J. Pharmacol. Exp. Ther. 299 (2) (2001) 483–493.
[29] N.S. Melik-Nubarov, O.O. Pomaz, T.Y. Dorodnych, G.A. Badun, A.L. Ksenofontov, O.B. Schemchukova, S.A. Arzhakov, Interaction of tumor and normal blood cells with ethylene oxide and propylene oxide block copolymers, FEBS Lett. 446 (1) (1999) 194–198.
[30] P. van de Wetering, J.Y. Cherng, H. Talsma, D.J.A. Crommelin, W.E. Hennik, (2-dimethylamino)ethyl-methacrylate based copolymers as gene transfer agents, J. Control. Release 53 (1998) 145–153.
[31] A. Agarwal, R. Unfer, S.K. Mallapragada, Investigation of in vitro biocompatibility of novel pentablock copolymers for gene delivery, J. Biomed. Mater. Res. Part A 81A (1) (2007) 24–39.
[32] A.V. Kabanov, V.Y. Alakhov, Pluronic block copolymers in drug delivery: From micellar nanocontainers to biological response modifiers. Crit. Rev. Ther. Drug. Carr. Syst. 19 (1) (2002) 1–72.
[33] E.V. Batrakova, S. Li, W.F. Elmquist, D.W. Miller, V.Y. Alakhov, A.V. Kabanov, Mechanism of sensitization of mdr cancer cells by pluronic block copolymers: selective energy depletion, Br. J. Cancer 85 (12) (2001) 1987–1997.
[34] J.R. Bellare, H.T. Davis, L.E. Scriven, Y. Talmon, Controlled environment vitrification system: an improved sample preparation technique, J. Electron Microsc. Tech. 10 (1) (1988) 87–111.
[35] Y. Talmon, Staining and drying-induced artifacts in electron microscopy of surfactant dispersions, J. Colloid Interface Sci. 93 (2) (1983) 366–382.
[36] N.W. Roehm, G.H. Rodgers, S.M. Hatfield, A.L. Glasebrook, An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt xtt, J. Immunol. Methods 142 (2) (1991) 257–265.
[37] S.M. Mel'nikov, V.G. Sergeyev, K. Yoshikawa, Discrete coil-globule transition of large DNA induced by cationic surfactant, J. Am. Chem. Soc. 117 (9) (1995) 2401–2408.
[38] A. Kabanov, J. Zhu, V. Alakhov, Pluronic block copolymers for gene delivery- advances in genetics, in: M.C.H. Leaf Huang, Ernst Wagner (Eds.), Non-viral vectors for gene therapy, Part 1, Second ed., Academic Press, New York, 2005, pp. 231–261.
[39] I. Astafieva, I. Maksimova, E. Lukanidin, V. Alakhov, A. Kabanov, Enhancement of the polycation-mediated DNA uptake and cell transfection with pluronic p85 block copolymer, FEBS Lett. 389 (3) (1996) 278–280.
[40] C.L. Gebhart, S. Sriadibhatla, S. Vinogradov, P. Lemieux, V. Alakhov, A.V. Kabanov, Design and formulation of polyplexes based on pluronic-polyethyleneimine conjugates for gene transfer, Bioconjug. Chem. 13 (5) (2002) 937–944.