Investigation of *in vitro* biocompatibility of novel pentablock copolymers for gene delivery

Ankit Agarwal,[1] Robert Unfer,[2] Surya K. Mallapragada[1]
[1]*Department of Chemical and Biological Engineering, Iowa State University, Ames, Iowa 50011*
[2]*Iowa Cancer Research Foundation, Urbandale, Iowa 50322*

Received 23 October 2005; revised 27 April 2006; accepted 9 May 2006
Published online 15 November 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.30920

Abstract: Novel pentablock copolymers of poly(diethylaminoethylmethacrylate) (PDEAEM), poly(ethylene oxide) (PEO), and poly(propylene oxide) (PPO), (PDEAEM-*b*-PEO-*b*-PPO-*b*-PEO-*b*-PDEAEM), were synthesized as vectors for gene delivery, and were tested for their biocompatibility on SKOV3 (human ovarian carcinoma) and A431 (human epidermoid cancer) cell lines under different *in vitro* conditions using various assays to elucidate the mechanism of cell death. These copolymers form micelles in aqueous solutions and can be tuned for their cytotoxicity by tailoring the weight percentage of their cationic component, PDEAEM. Copolymers with higher PDEAEM content were found to be more cytotoxic, though their polyplexes were less toxic than the polycations alone. Pentablock copolymers displayed higher cell viability than commercially available ExGen 500® at similar N:P ratios. While cell death with ExGen was found to be accompanied by an early loss of cell membrane integrity, pentablock copolymers caused very little membrane leakage. Caspase-3/7 assay confirmed that none of these polymers induced apoptosis in the cells. These pentablock copolymers form thermo-reversible gels at physiological temperatures, thereby enabling controlled gene delivery. Toxicity of the polymer gels was tested using an agarose-matrix, simulating an *in vivo* tumor model where injected polyplex gels would dissolve to release polyplexes, diffusing through tumor mass to reach the target cells. Twenty five weight percent of copolymer gels were found to be nontoxic or mildly cytotoxic after 24 h incubation. Transfection efficiency of the copolymers was found to be critically correlated to cytotoxicity and depended on DNA dose, polymer concentration, and N:P ratios. Transgene expression obtained was comparable to that of ExGen, but ExGen exhibited greater cell death. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 81A: 24–39, 2007

Key words: block-copolymers; cytotoxicity; cationic; gene-delivery; temperature-sensitive

INTRODUCTION

In the recent past, biocompatible polymers have been widely explored for biomedical applications such as DNA and RNA delivery to targeted cells,[1–3] drug carrier systems for proteins and peptides,[4,5] and scaffolds for tissue regeneration.[6] The term biocompatibility implies that these polymers are non-cytotoxic, non-immunogenic, and demonstrate an appropriate host response in specific applications.[7,8]

Gene therapy treats a genetic deficiency by delivering genetic information in the form of nucleic acids to the targeted cells. Although great advances have been made in identifying target structures for gene therapy, and in the biotechnological production of nucleic acids, the progress has been mainly hampered by the lack of safe gene delivery systems that are efficient and nontoxic.[1,9] A variety of polycations (cationic polymers) have been proposed and investigated recently for gene delivery[10,11]; examples include poly-L-lysine (PLL),[12–14] polyethyleneimine (PEI),[15–17] polyamidoamine (PAMAM) dendrimers,[18] chitosan,[19,20] and methacrylate/methacrylamide polymers.[21,22] Cationic polymers condense negatively charged DNA through electrostatic interactions forming stable complexes called polyplexes. For an efficient DNA-delivery vector, these polyplexes should deliver their genetic payload with minimum damage to the cells such as cell membrane rupture, inflammation, or apoptosis. However, the existing polymeric gene-delivery systems are either toxic, aggregate *in vivo*, or they do not show good transfection efficiencies.[23,24]

Mechanisms and reasons for toxicity caused by polycationic macromolecules are not yet fully understood. It has been noted that the toxicity is dependent on the polymer molecular weight (MW), surface charge density, structure, flexibility, and three-dimensional

Correspondence to: S. K. Mallapragada; e-mail: suryakm@iastate.edu
Contract grant sponsor: US-DOE; contract grant number: W-7405-ENG-82
Contract grant sponsor: Bailey Career Development

arrangement of cationic charges.[7,25] Whether this toxicity is mediated by interaction of polycations with the cell membrane, or by activation of some intracellular signal transduction pathway after cellular uptake, is an issue of debate.[7,26–28] Contradictory studies have been reported and there is no general agreement on the causes of polymer toxicity. While Gebhart and Kabanov[29] showed that MW of chitosans did not affect cell viability, a very recently published report[30] shows that chitosan derivatives show dependence on size and MW for both toxicity and transfection efficiency. Florea et al.[26] showed that branched PEI produced similar toxicities across a range of MWs in COS-1 and Calu-3 cells, contradicting other studies on PEI done with different cell lines that showed that PEI toxicity increased with an increase in MW.[7,31] Researchers have also shown that transfection efficiency of polymer vectors is correlated to their toxicity.[29,32] Hill et al. studied poly(amidoamine)s (PAAs) and showed that only polymers that exhibited some toxicity were able to transfect A549 cells. Florea et al.[26] found that transfection efficiency of PEI was correlated with toxicity in Calu-3 cells, but not in COS-1 cell line. These studies suggest that toxicity of a particular polymer should be evaluated individually, and on more than one cell line.

Homopolymers of DMAEM (dimethylaminoethylmethacrylate) have previously been shown to complex DNA and transfect COS-7 and OVCAR cells.[22] However, they were toxic and exhibited only up to 3–6% transfection. Besides, good transfection was obtained only at high MWs ($M_w > 300$ kDa), which is not suitable for renal clearance in *in vivo* applications. Recently, we had reported novel pentablock copolymers PDEAEM-*b*-PEO-*b*-PPO-*b*-PEO-*b*-PDEAEM synthesized in our laboratory as promising nonviral vectors for gene delivery.[33] While their cationic component PDEAEM is responsible for condensation of DNA and endosomal escape of polyplexes, hydrophilic PEO chains in the copolymer shield the cationic surface charges of PDEAEM, thereby decreasing their toxicity. Besides, these copolymers form micelles,[34] which facilitate transport across the lipid bilayer of cell membranes. At higher concentrations and physiological temperatures, these micelles self-assemble to form thermoreversible gels,[35] a characteristic property that can potentially be used to form subcutaneously injectable systems for long-term gene delivery. Self assembly of these copolymers and the properties of their macroscopic gels have been discussed in detail earlier.[36,37] In the present study we have investigated the effect of various factors such as wt % of PDEAEM in the copolymers, their concentration in media, time of incubation, and N:P ratios (molar ratios of nitrogens (N) in pentablock copolymer to phosphates (P) in DNA) on the toxicity of the polymers, and have attempted to understand the mechanism by which these cationic copolymers cause cell death. Commercially available *in vitro* transfection reagent ExGen 500® has been used as a control for the study. The results obtained are intended to be used to tailor the formulations for *in vivo* studies, where suicide genes can be delivered to the localized tumors in a sustained fashion using injectable thermoreversible gels as depot of polyplexes, circumventing repeated administration to maintain the therapeutic levels of the protein.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle Medium (DMEM), OptiMEM I®, fetal bovine serum (FBS), 0.25% trypsin–EDTA solution, and Hank's Buffered Salt Solution (HBSS) were purchased from Invitrogen (Carlsbad, USA). HEPES salt was obtained from Sigma-Aldrich Co. (No. H4034) to make Hepes buffer saline. Lactate dehydrogenase (LDH) and MTT assay kits were also purchased from Sigma-Aldrich Co. (Tox-7 and Tox-1, respectively). *Renilla* luciferase assay system and Caspase-Glo® 3/7 assay kit were purchased from Promega Corporation (Madison, USA). The Qiagen Maxi Prep kit was purchased from Qiagen (Valencia, USA). ExGen 500 (written as ExGen henceforth), the *in vitro* transfection reagent, was purchased from Fermentas Life Sciences (Hanover, MD). DNase I was purchased from Ambion (Austin, USA). Low melting agarose of PCR grade from Fisher Scientific (cat no. BP2410) was used for making agarose matrices over the cells. Ultrapure water with at least 18 MΩ resistivity was used in all studies.

Polymers

The pentablock copolymers (Table I) were synthesized using oxyanionic or ATRP reaction schemes, which are discussed in detail elsewhere.[34,35] The chemical structure of pentablock copolymers is shown in Scheme I. Pluronic® F127 [$(PEO)_{100}$-*b*-$(PPO)_{65}$-*b*-$(PEO)_{100}$] was used as the macroinitiator in pentablocks A, B, and C and E, while pentablock D used Pluronic F68 [$(PEO)_{78}$-*b*-$(PPO)_{30}$-*b*-$(PEO)_{78}$]. Pentablock copolymers with different wt % of PDEAEM were investigated for gene delivery. MWs of the pentablock copolymers (Table I), as determined by NMR and gel permeation chromatography (described elsewhere[35]), varied from 15 to 22 kDa with polydispersities of up to 1.4. The pentablock A (containing 17% PDEAEM by wt) used in this study, however, had a higher polydispersity of 2.36.

Cells

The SKOV3 human ovarian carcinoma cell line, and A431 (ATCC CRL-1555), a human epidermoid carcinoma cell line obtained from ATCC™ (Virginia, USA), were used for cytotoxicity and transfection experiments. DU145, a human prostrate cancer cell line, obtained from Iowa Cancer Research Foundation, was also used for some transfection experiments.

TABLE I
Molecular Weights (MW) and Polydispersity Indices (PDI) of Different Pentablock Copolymers

Pentablock Copolymers	Wt % PDEAEM	M_w (GPC)	M_n (NMR)	PDI	nmol of Nitrogen/μg	Amount Containing 3 nmol of Nitrogen[a]
A	17	40,112	15,000	2.36	1.01	2.96 μg
B	26	23,516	17,300	1.23	1.45	2.08 μg
C	40	30,664	22,000	1.21	2.03	1.48 μg
D	60	20,365	19,973	1.34	2.73	1.10 μg
E	28	28,400	17,525	1.25	1.52	1.98 μg
ExGen	–	–	22,000	–	248.64	12.1 ng

[a]It is the amount of polymer required to condense 1 μg of plasmid DNA such that molar ratio of nitrogens of the polymer to the phosphates of DNA is 1, using the fact that 1 μg of DNA has 3 nmol of phosphate. Synthesis of pentablock D used Pluronic F68 as the macroinitiator while all others used Pluronic F127. M_w, weight–average MW, M_n, number–average MW.

Cell cultures were maintained in a humidified environment with 5% CO_2 at 37°C and passaged regularly to allow them to remain sub-confluent. Cells were fed with DMEM supplemented with 10% fetal bovine serum (FBS) and 1 μ*M* L-glutamine, unless otherwise stated. Neither antibiotics nor antimycotics were used to avoid the possibility of artificial membrane permeabilization effects from these agents.

Plasmid DNA

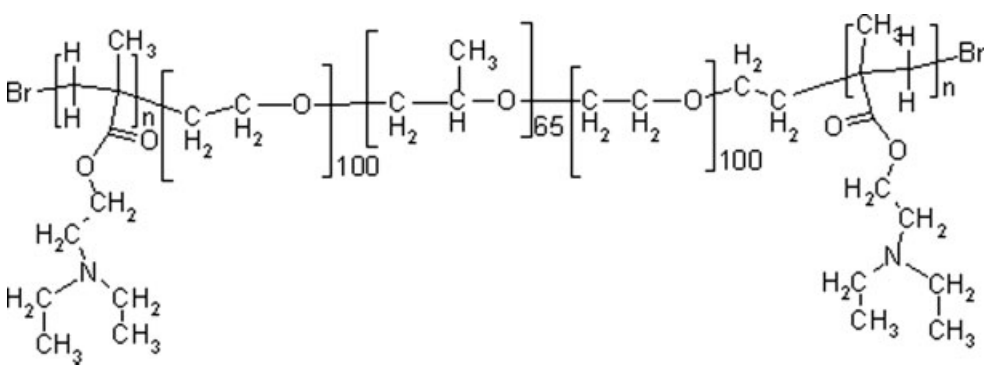
A 4.1 kb plasmid encoding *Renilla* luciferase (pRL-CMV) (Promega Corporation, Madison, USA) was used as the reporter gene. DH5α *E.coli* cells were transformed with the plasmid DNA and incubated in selective Luria-Bertani (LB) medium. Amplified plasmid DNA was purified using the Maxi-Prep DNA Purification Kit from Qiagen (Valencia, USA). The concentration and purity of the resulting DNA in a buffer (pH 7.5) of Tris-HCl and ethylenediaminetetraacetic acid (EDTA) was determined by measuring the absorbance at 260 and 280 nm. All DNA used had a 260/280 ratio of at least 1.80.

Polyplexes

Copolymer to DNA ratios are expressed as molar ratios of nitrogens (N) in pentablock copolymer to phosphates (P) in DNA, and written as N:P. The MW of the DEAEM monomer is 185 and the average MW of a nucleotide is ~308. Using the fact that 1 μg of DNA contains 3 nmol of phosphates, the amount of polymer required for corresponding N:P ratios was calculated (Table I). All polyplexes were formed by the same procedure. Copolymers were first dissolved in Hepes buffer saline (HBS – 20 m*M* of HEPES with 145 m*M* NaCl) pH 7.4, unless otherwise stated, to obtain a concentration of 1 mg/mL. This polymer solution was then diluted with the desired media or buffer in a polypropylene tube. After incubating for 5 min at room temperature, this diluted polymer solution was added to DNA (in TE buffer) contained in another tube. The tube was gently agitated and allowed to incubate for 30 min at room temperature.

Lactate dehydrogenase (LDH) assay

LDH is an integral cytosolic enzyme that is secreted out in the medium following the rupture of cell membrane.[25] Since the potential site of interaction of cationic macromolecules is the cell membrane, measuring the amount of LDH released in the medium has long been a preferred way to estimate membrane damage.[7] Cells were cultured in 96-well tissue culture plates at a density of $\sim 1.2 \times 10^4$ cells per well. After incubation overnight, growth media was removed and replaced with 200 μL polymer solutions in appropriate media. Cells were incubated with polymer solutions for 6–48 h, after which 100 μL of media was then collected in an optically clear 96-well microtiter plate, and LDH concentration was assayed using a commercial kit (Tox-7 from Sigma-Aldrich Co.) according to the supplier's protocol. The absorbance of each well was measured at 490 nm using a BioTek EL-340 plate reader (Winooski, USA). Background absorbance at 630 nm was subtracted from the main readings.



Scheme 1. Structure of pentablock copolymers.

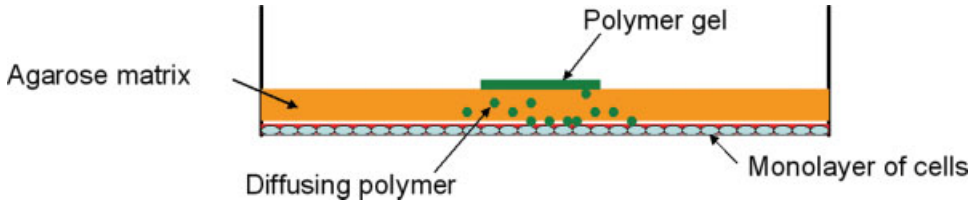


Figure 1. Diffusion of polymer through a 1% agarose matrix of DMEM with 10% FBS, simulating a tumor-model. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Media alone and media with only cells were used to obtain a background LDH level for normalization. Cells exposed to 0.1% Triton X-100 in DMEM were used as a positive control and set as 100% LDH release. The relative LDH release is defined by the ratio of LDH released over total LDH in the intact cells. Less than 10% LDH release was regarded as an acceptable level in our experiments. All samples were run in four replicates, and experiments were repeated twice.

MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used for the quantitative determination of cell viability. The assay is based on the cleavage of the cell membrane permeable yellow tetrazolium salt MTT into purple formazan by the ''succinate-tetrazolium reductase'' system (EC 1.3.99.1), which belongs to the respiratory chain of the mitochondria, and is active only in metabolically intact cells.

MTT assay was performed according to the method of Edmondson.[38] After incubating the monolayer of cells with polymer solutions for 6–48 h in a 96-well plate, as described before for the LDH assay, polymer solutions were aspirated and replaced with 200 μL of fresh DMEM without serum. Twenty microliters of MTT stock solution prepared in phosphate buffer saline (PBS) pH 7.4 was then added to each well giving a final MTT concentration of 0.5 mg/mL. After 4 h of incubation in a CO_2 incubator, the unreacted dye was removed by aspiration and the insoluble formazan crystals were dissolved by incubating with 200 μL dimethylsulfoxide (DMSO) for 2 h in a humidified atmosphere (37°C, 5% CO_2). Finally, the MTT absorbance was measured at 570 nm. Background absorbance measured at 630 nm was subtracted from the main readings. Viability was reported relative to control cells not exposed to the polymers.

Microscopic observations

After incubation with polymers, changes in morphology and detachment of cells from the dish were also observed using an Olympus IMT-2 (Melville, USA) inverted, phase-contrast light microscope equipped with objectives of 10× and 4× magnification.

Agarose diffusion assay

Our novel pentablock copolymers show sol–gel transitions. At w/w concentrations of 20% or more, aqueous solutions of the copolymers form a gel at physiological temperatures. The gels dissolve in presence of excess water, as the polymer concentration decreases. Polymer concentration in the gels is around 1000 times higher than the concentrations in aqueous solutions at which about 80% of the cells are metabolically viable. However, the polymer gels dissolve slowly, and in an *in vivo* situation where the polymer gel complexed with the therapeutic gene would be subcutaneously injected at the site of tumor, polyplexes would have to diffuse through a mass of tissues to reach the targeted cells. Therefore, to mimic this situation, the polymer gels (containing 25 wt % of polymer) were placed on the top of an agarose gel layer covering SKOV3 cells underneath. The agarose matrices were made in DMEM, containing 10% FBS and 1% agarose. The polymer was allowed to diffuse to the cells for 24 h. The experimental model is shown in Figure 1.

This method, derived from the work of Guess et al.,[39] was adapted from ISO procedures for cytotoxicity testing (ISO 109993–5. Biological evaluation of medical devices, Part 5: Tests for cytotoxicity: in vitro methods). Monolayers of SKOV3 cells were grown to confluence in 6 well plates, after which the culture medium was replaced with 3 mL serum supplemented (10%) DMEM, containing 1% agarose, to generate a protective agarose layer. To avoid heat denaturation of serum proteins, agar was melted and cooled to 45°C before adding it to DMEM containing 10% FBS. The plates were left at room temperature for 15 min to let the agarose solidify (melting point 32°C). After the agarose layer was formed, 3 mL of vital stain neutral red solution (0.01% in PBS) was added to each well, and the plate was left to incubate for 30 min at 37°C and 5% CO_2. Excess dye was then removed and polymer gels were placed at the center on top of these solidified agarose matrixes. The cells were then incubated for another 24 h. This assay is based on the migration or diffusion of toxic substances from the test article through the agarose to the cellular monolayer. The slow diffusion of leachable substances through the agarose results in a concentration gradient around the test article and a zone of dead cells if the leachable substances are toxic. Sample biocompatibility was estimated by observing cell lysis and zone of dead cells (marked by decolorized zones) under and around the specimen by light microscopy at 150× magnification.

The decolorized zones were scored as follows: 0, no decolorization detectable; 1, decolorization only under the specimen; 2, zone not greater than 5 mm from the specimen; 3, zone not greater than 10 mm from the specimen; 4, zone greater than 10 mm from the specimen; and 5, the total culture is decolorized. Cell lysis was defined as loss of cell membrane integrity, visible in light microscopy. Cell lysis was scored as follows: 0, no cell lysis detectable; 1, <20% cell lysis; 2, 20–40% cell lysis; 3, >40 to <60% cell lysis; 4, 60–80% cell lysis; and 5, >80% cell lysis.

For each specimen, one score was given, and the median score value for all parallels from each specimen was calculated for both the decolorization zone and the lysis zone. The cytotoxicity was classified as follows: 0–0.5, non-cytotoxic; 0.6–1.9, mildly cytotoxic; 2.0–3.9, moderately cytotoxic; and 4.0–5.0, markedly cytotoxic. The median (instead of the mean) was calculated to describe the central tendency of the scores because the results are expressed as an index in a ranking scale.

Characterization of polymer induced cell death

To elucidate whether the cell death induced by these polymers and polyplexes is apoptotic or necrotic in nature, staurosporine (Sigma, No. S-6942) was employed as a positive control for apoptosis. Staurosporine is an alkaloid that is a potent inhibitor of phospholipid/calcium-dependent protein kinase (protein kinase C), selectively inducing apoptosis.[40] SKOV3 cells were incubated in 96-well plates with polymers, polyplexes (as described earlier), and 200 n*M* staurosporine for 5 h. The effect of staurosporine on LDH release and metabolic activity of the cells was compared to that of the polymers and polyplexes. The Caspase-Glo^{TM} 3/7 Assay was employed to measure caspase-3 and -7 activities in the treated cell cultures. These caspases play key effector roles in apoptosis in mammalian cells, and their presence confirms the induction of apoptosis. The assay provides a proluminescent caspase-3/7 substrate that contains the tetrapeptide sequence DEVD (Asp-Glu-Val-Asp), a caspase-3/7 recognition site. The presence of activated caspases in the cells will result in the cleavage of the substrate, generating a ''glow-type'' luminescent signal produced by luciferase. Luminescence is proportional to the amount of caspase activity present. The assay was performed in a 96-well plate with SKOV3 cells, according to the supplier's protocol. Briefly, after incubating the cells with the test compounds for specified times in a white-walled 96-well plate, 100 μL of Caspase-Glo 3/7 Reagent was added to each well containing 100 μL of treated cells in culture medium. Prior to this, culture plates and reagent were allowed to equilibrate to room temperature. Luminescence was measured on a Veritas^{TM} Microplate Luminometer after incubating the treated cells with the reagent for 1–3 h.

Luciferase transfection

To determine the total protein expressed by a reporter gene per total cellular protein, a luciferase assay was employed, using pRL-CMV as the reporter gene. Cells were seeded in a 96-well plate up to 70% confluency prior to transfection, and were then transfected with various polyplex solutions in 200 μL OptiMEM I using 1 μg of DNA per well, unless otherwise stated. After 4–10 h incubation in OptiMEM I, the solution was replaced with fresh DMEM containing 10% FBS and incubated for 44 h. Cells were then lysed using a lysis buffer (*Renilla* Luciferase Assay Lysis Buffer, Promega) and the luminescence of the expressed reporter protein was measured on an automated Veritas Microplate Luminometer using the Promega *Renilla* Luciferase Assay System (Madison, USA). ExGen was used as a positive control and was expected to yield high efficiency of transfection. Cells exposed only to DNA (without polymer) were used as negative controls.

Total recovered cellular protein content of the cells was determined by a modified Bradford assay, using a CB-Protein Assay^{TM} Kit. Bovine serum albumin standards were prepared. Twenty microliters of the samples from luciferase detection protocol were placed in individual wells of a 96-well plate and diluted with nanopure water to 100 μL. CB-Protein Assay reagent (100 μL) was added into each well and mixed well. The plate was allowed to incubate for 15 min and absorbance was measured at 595 nm. The amount of protein was read from the standard curve.

Statistics

Where appropriate, the data is presented as mean and standard deviation (SD). Four samples were used for each case in all the experiments, and mean and SD were calculated over them. Significant differences between two groups were evaluated by Students' *t*-test and between more than two groups by one-way analysis of variance (ANOVA), followed by Tukey's test. The level of significance was set at $p < 0.05$, unless otherwise stated.

RESULTS

Effect of different wt % of PDEAEM on pentablock copolymer cytotoxicity

Pentablock copolymers containing different wt % of PDEAEM were incubated with SKOV3 and A431 cell lines in DMEM (10% FBS) for an extended period of 48 h to determine the effect of the cationic block on the polymers' cytotoxicity. After 48 h, the damage to cell membranes and their residual metabolic activity were evaluated using LDH and MTT assays, respectively. Figure 2(a,b) shows trends in the membrane leakage and cell viability of SKOV3 cells, respectively, and Figure 3(a,b) gives the same trends for A431 cell line, respectively. Two things are evident from these graphs. First, as the wt % of PDEAEM increases in the copolymers, there is a gradual increase in the damage to the cell membrane and a decrease in cell viability. Second, for each copolymer (except pentablock D containing 60% PDEAEM for A431 cells) a concentration can be noted from the graphs below which the polymer causes less than 10% membrane damage or allows >80% of the cells to still be metabolically viable. The copolymer containing 60% PDEAEM was found to be cytotoxic to A431 cells at almost all concentrations. The copolymers provide a unique way to tune the cytotoxicity for efficient use in gene therapy, as opposed to cationic polymers such as ExGen whose cytotoxicity can be controlled only by reducing the amount of polymer used. These results suggest that below a certain concentration, pentablock copolymers

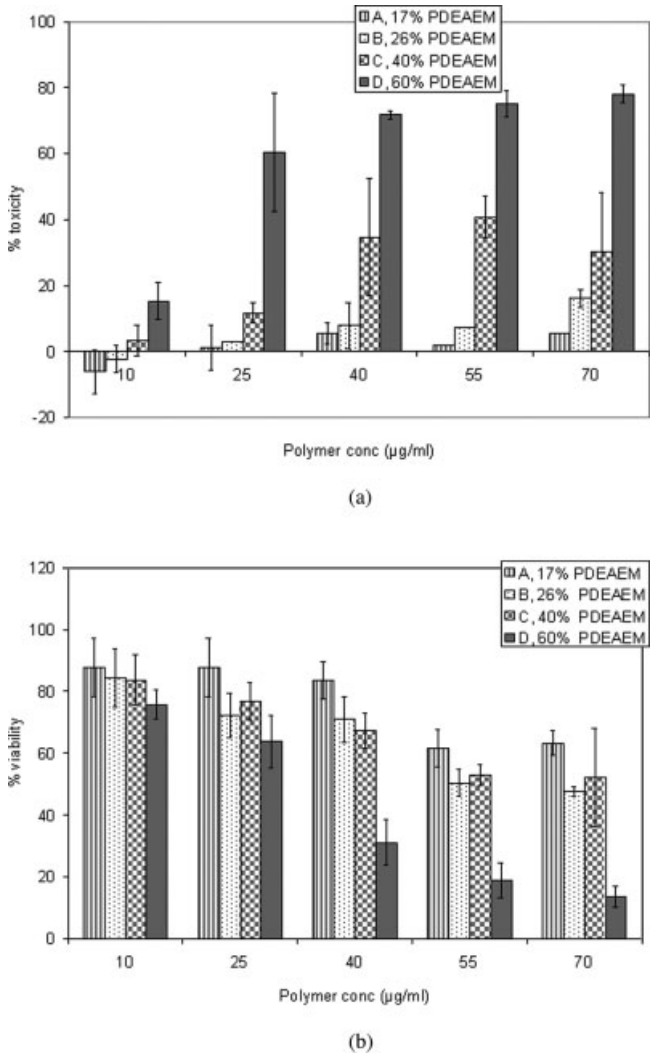


Figure 2. Cytotoxicity of pentablock copolymers with different wt % PDEAEM on SKOV3 cell line after 48 h incubation in FBS supplemented DMEM. (a) Percentage toxicity evaluated in terms of cell membrane damage using LDH assay and (b) residual metabolic activity evaluated by MTT assay, ($n = 4 \pm$ SD).

can have extended tissue-contact times of up to 48 h without causing significant damage to the cells, which is of significance for *in vivo* studies. Also, it can be observed that A431 cells are slightly more sensitive to the copolymers than SKOV3, exhibiting more toxicity at similar polymer concentrations.

Toxicity increases with exposure time

During *in vitro* transfection, cells were incubated with polyplexes in a low serum media OptiMEM I to avoid polymer loss because of binding with serum proteins. Since cells need to be grown in 10% serum supplemented media for good protein expression of the transfected gene, they were first incubated with polyplexes for a limited time, and then the polymer solutions were replaced with fresh media containing FBS to let the transfected gene be expressed.

Figure 4 shows the effect of incubation time of polyplexes with the cells on their toxicity. Polyplexes of pentablock copolymer B (26% PDEAEM), containing 1 µg plasmid pRL-CMV and different amounts of copolymer, were incubated with the SKOV3 cells for 6–14 h in OptiMEM I. For all polymer concentrations, it was observed that cell viability decreased on extending the incubation time of polyplexes with the cells. Typically at 50 µg/mL, it decreased from 100 to 60% when incubation time was extended from 6 to 14 h. After 14 h of exposure, cell viability was reduced to 60% even at lower copolymer concentrations of 30 µg/mL.

Cells were also incubated with just polymer solutions to examine how DNA complexation affects their cytotoxicity. Figure 5(a,b) shows that polyplexes were less toxic to the cells than the cationic polymers alone.

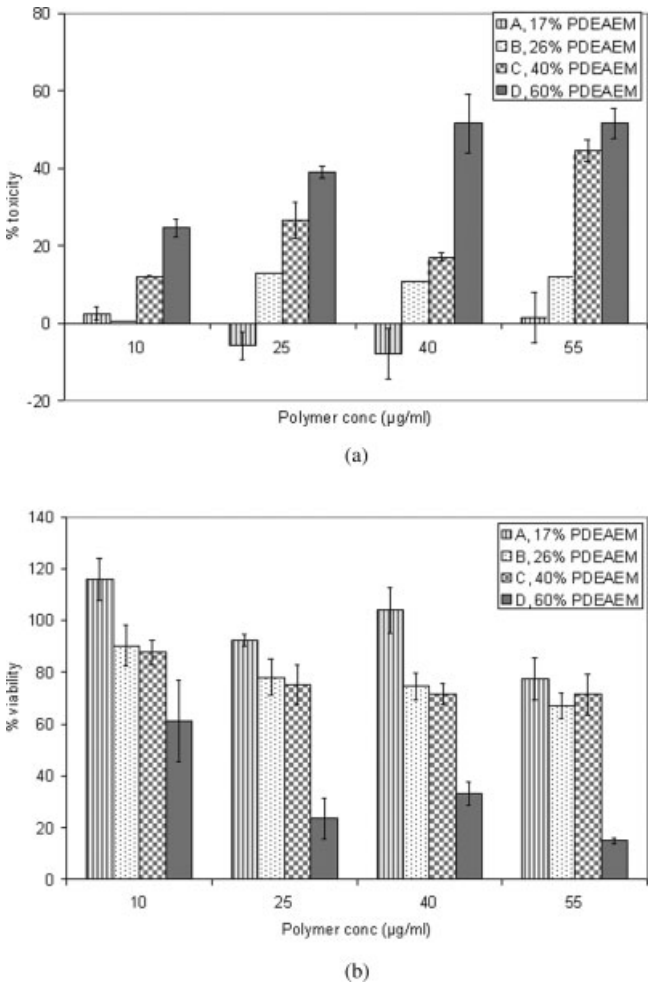


Figure 3. Cytotoxicity of pentablock copolymers with different wt % PDEAEM on A431 cell line after 48 h incubation in FBS supplemented DMEM. (a) Percentage toxicity evaluated in terms of cell membrane damage using LDH assay, (b) residual metabolic activity evaluated by MTT assay, ($n = 4 \pm$ SD).

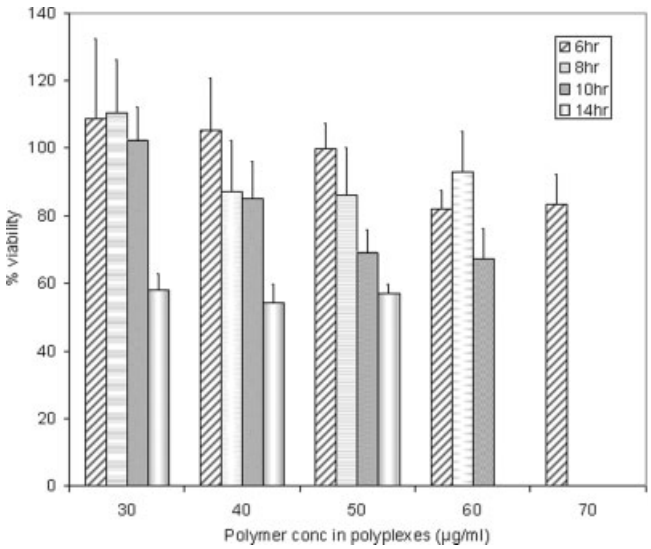


Figure 4. Dose- and time-dependent effect of the polyplexes of pentablock copolymer B on SKOV3 cells incubated in OptiMEM I. Residual metabolic activity was evaluated by MTT assay. Polyplexes contained 1 µg of pRL, ($n = 4 \pm$ SD).

The cellular membrane damage was significantly reduced by the DNA complexation to the polymer, possibly due to the shielding of positive charges on the polycations. At N:P ratio of 8, with polymer concentration of ~80 µg/mL, the LDH release reduced from 86 to 7% after DNA complexation. Significant increase in the metabolic activity was also found at higher polymer concentrations corresponding to N:P ratios of 6 and above. This is in agreement with results seen by researchers for other polymers.[7,30,41]

Toxicity of pentablock copolymers was compared to ExGen at concentrations corresponding to same N:P ratios, thus actually comparing the concentration of their cationic components that are responsible for DNA condensation and endosomolysis. SKOV3 cells were incubated for 6 h with the polyplexes of two polymers in OptiMEM I. Figure 6(a) shows that polyplexes of ExGen caused extensive cell membrane damage, as compared to that caused by pentablock copolymers. At a high N:P ratio of 8, while pentablock copolymers showed only 10% LDH release, ExGen showed up to 85% LDH release. Further, as seen in Figure 6(b), MTT assay showed that at all N:P ratios, cell viability was significantly higher in the presence of the pentablock copolymers than with ExGen. These results clearly suggest that pentablock copolymers are less cytotoxic than ExGen, and perhaps the two polymers interact with the cells in a different fashion, inducing cell death by different mechanisms.

Apoptosis versus necrosis

Apoptosis is the carefully regulated process of cell death.[42] In contrast to the swelling and membrane rupture in necrosis, a cell undergoing apoptosis rapidly condenses into small enclosed fragments, which can then be phagocytosed by neighboring cells. Apoptosis can be characterized by the loss of mitochondrial membrane potential, activation of caspases, loss of plasma membrane asymmetry, and the condensing and eventual fragmentation of the cellular DNA.[43]

Damage to the cell membrane, which can be determined by the LDH assay, is known to occur either in necrotic cells or in the late stage of apoptosis. On the other hand, loss of mitochondrial inner transmembrane potential is often associated with the early stages of apoptosis and may be one of the central features of the process.[44] Collapse of this potential results in the decoupling of the respiratory chain, which reduces the ability of dying cells to reduce compounds such as tetrazolium salt MTT into colored formazan product, as can be determined by the MTT assay. Since staurospor-

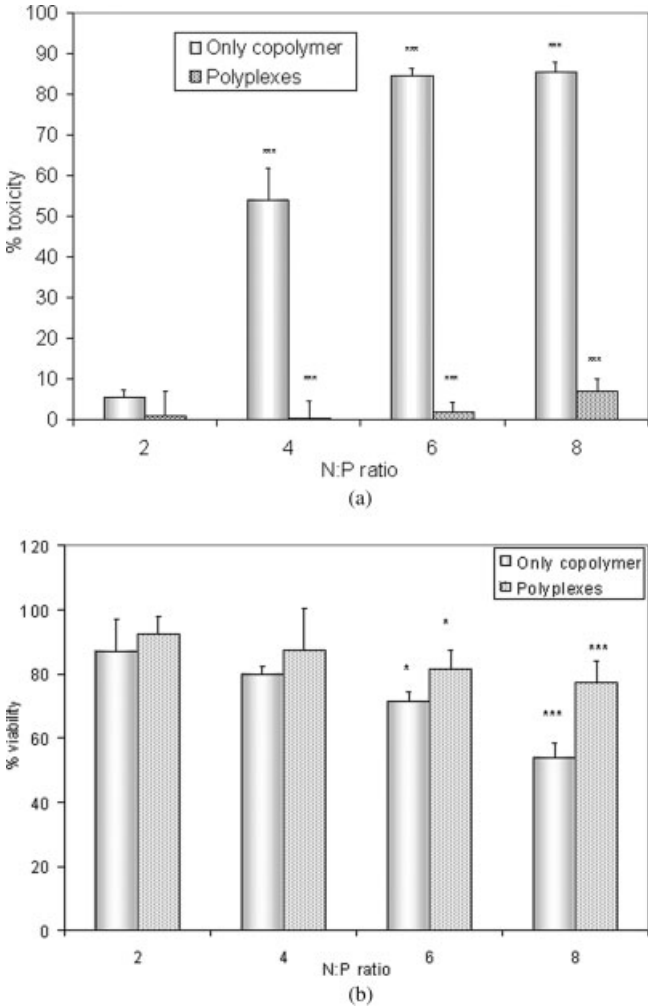


Figure 5. Comparing the cytotoxicity of polyplexes of pentablock copolymer B with that of the polycations alone on SKOV3 cells after 6 h incubation in OptiMEM I. (a) LDH assay and (b) MTT assay. Polyplexes contained 1 µg of pRL-CMV, ($n = 4 \pm$ SD). * indicates $p < 0.1$; ** indicates $p < 0.05$; *** indicates $p < 0.01$.

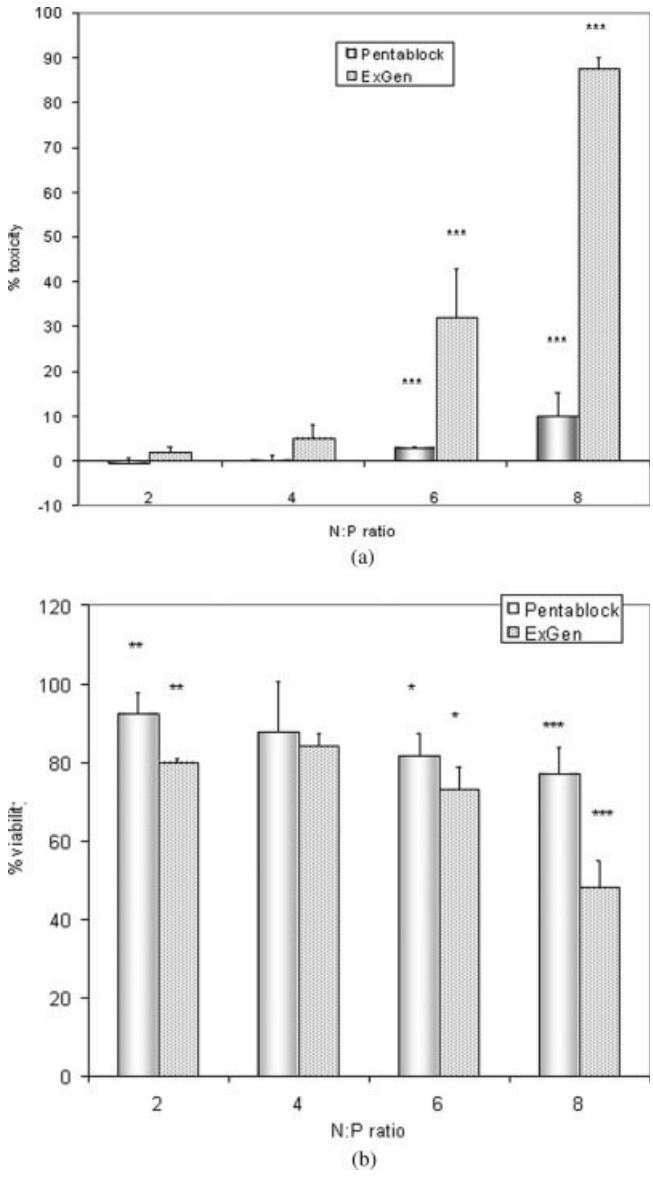


Figure 6. Cytotoxic effect of polyplexes of pentablock copolymer B and ExGen on SKOV3 cells after 6 h incubation in OptiMEM I. (a) LDH assay or (b) MTT assay. Polyplexes contained 1 μg of pRL-CMV, ($n = 4 \pm$ SD). * indicates $p < 0.1$; ** indicates $p < 0.05$; *** indicates $p < 0.01$.

ine selectively induces apoptosis into the cells, its effect on the LDH and MTT assays can be compared to those of ExGen and pentablock copolymers to try to understand the mechanism of cell death.

As expected in apoptosis, Figure 7 shows that cells incubated with 200 nm staurosporine for 5 h gave little LDH release, even though their metabolic activity was almost reduced to zero. Similar to this, as the concentration of pentablock copolymers increased, there was a significant decrease in metabolic activity of cells, but no significant change in the cell membrane damage. This hints that cell death in the presence of the pentablock copolymers might not be by necrosis, but might be through an apoptotic route. In contrast, as the concentration of ExGen increased, the decrease in metabolic activity of the cell population was accompanied with large LDH release. This indicates that cell death caused by the rapid loss of membrane integrity (necrosis) primarily accounted for the decrease in number of viable cells in the presence of ExGen.

The Caspase-Glo 3/7 Assay was performed to check the induction of apoptosis in the cells. SKOV3 cells were incubated with polymer–DNA complexes at different N:P ratios, and the activity of caspases was measured at three different times of incubation: 3, 4, and 6 h. Different polymer concentrations and times of incubation were used to empirically find the conditions that would activate caspases in the cells. Staurosporine was used as the positive control. Figure 8 shows the results of these caspase assays. Staurosporine induced

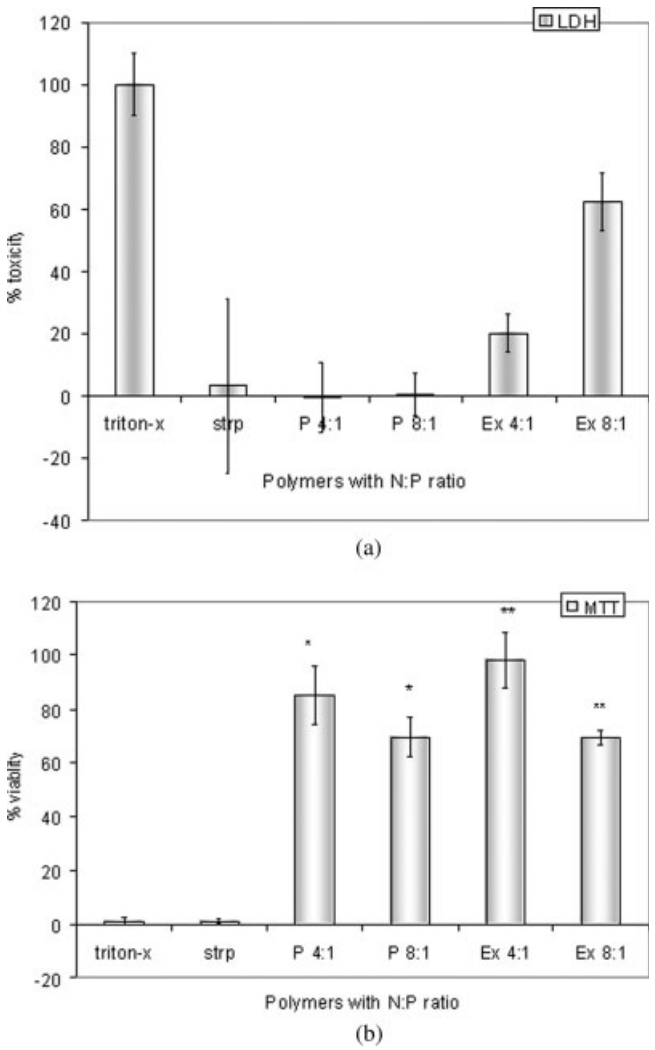


Figure 7. Dose-dependent effect of different polycations incubated with SKOV3 cells for 5 h. P, pentablock copolymer B; Ex, ExGen 500; strp, Staurosporine (200 n*M*). (a) Membrane damage by LDH assay, (b) percentage viability evaluated by MTT assay, ($n = 4 \pm$ SD). * indicates $p < 0.1$.

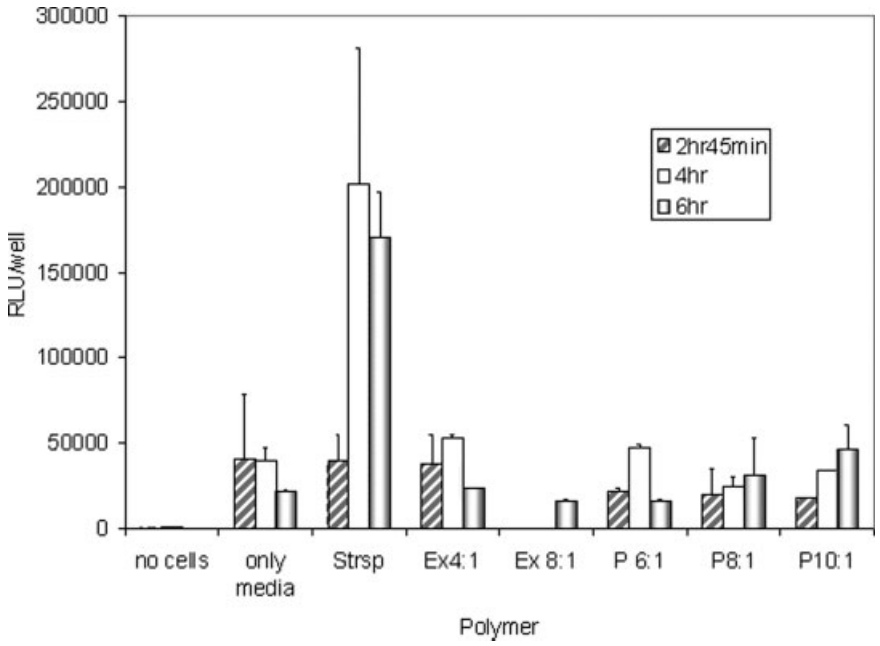


Figure 8. Activity of Caspases 3/7 in SKOV3 cells after incubation with different compounds for specified times, as found using Caspase-Glo 3/7 assay. Ex, ExGen 500; P, Pentablock copolymer B; Strsp, 200 n*M* Staurosporine. All solutions were made in OptiMEM I media. ($n = 4 \pm$ SD).

apoptosis by activating caspases after 4 and 6 h of incubation, but no caspase activity was detected after 165 min incubation. However, no significant caspase activity was detected at any incubation time for any tested N:P ratio of pentablock copolymers' or ExGen polyplexes. At higher incubation times or higher polymer concentrations, increased cell death was clearly visible using light microscopy, as it has been shown in LDH and MTT assays earlier.

These results indicate that neither ExGen nor pentablock copolymers induced apoptosis in the treated cells. However, they did affect the cells differently. Unlike ExGen, that extensively ruptured the cellular membrane leading to cell death, pentablock copolymers appear to cause cell death by some alternative mechanism.[45–47] This has implications in minimizing the inflammatory processes accompanying cell death in the presence of the pentablock copolymers as opposed to the presence of ExGen.

Luciferase transfection

The transfection efficiency of polymers depends on the amount of polymer used (N:P ratio) to condense the DNA, the amount of the DNA dose, and time for which cells are incubated with the polyplexes. The upper limit of all these factors in turn depends on the cytotoxicity of the polymer. As N:P ratio increases, amount of free polymer in the media increases, increasing the cell death, thereby effectively decreasing reporter protein expression. A higher DNA dose would generate more reporter protein, but will require higher amount of polymer for condensation, thus again being limited by the polymer toxicity profile. Also, increased exposure of cells to the polyplexes would allow polyplexes an extended period of time to enter into the cells, but that would again increase the toxicity, as shown earlier in Figure 4.

Keeping these above stated factors in mind, several different carcinoma cell lines were transfected with luciferase plasmid using pentablock copolymers, while ExGen was used as a positive control. Ideal N:P ratios and ideal incubation times of polyplexes with the cell lines were obtained empirically for both the polymers, following manufacturer's protocol for ExGen. Figure 9 shows the transfection obtained with pentablock B using 1 μg pRL in SKOV3, A431, and DU145 cells. Polyplexes were incubated for 10 h with the cells. As can be observed, pentablock copolymers were able to transfect all three cell lines. However, amount of gene expression obtained at similar N:P ratios was different in different cell lines. While luciferase expression increased significantly in all three cell lines by increasing N:P ratio from 4 to 6, the increase was much higher in DU145 cells than in SKOV3 or A431 cell lines. Also, while in SKOV3 cells luciferase expression peaked off at N:P ratio 8 and then decreased, it was almost the same (not significantly different) for different N:P ratios in A431 cells.

As observed in Figure 9, the amount of luciferase protein expressed in the SKOV3 cells increased with the amount of copolymer used to condense the DNA (N:P ratio). However, after a certain polymer concentration, the luciferase expression decreased on further increasing the N:P ratio. Similar trends were observed

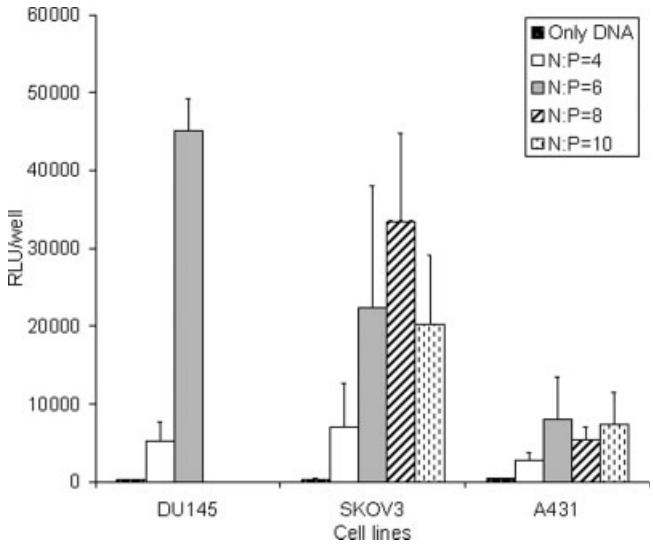


Figure 9. Luciferase expression obtained in DU145, SKOV3, and A431 cell lines by transfecting them with polyplexes of pentablock copolymer B and 1 μg DNA at different N:P ratios, ($n = 4 \pm$ SD).

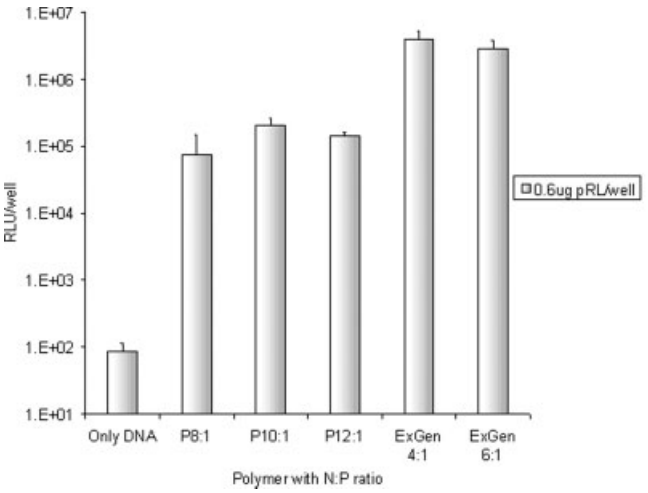


Figure 11. Luciferase expression in SKOV3 cells after transfecting 0.6 μg of DNA with pentablock copolymer E and ExGen at different N:P ratios. Polyplexes were incubated with cells for 3.5 h in OptiMEM I, ($n = 4 \pm$ SD).

in Figure 10 for ExGen. Toxicity of the polymers at these N:P ratios can be correlated from Figures 5 and 6.

In Figure 11, comparing the transfection efficiency of pentablock copolymers and ExGen, it can be observed that maximum amount of luciferase expressed using pentablock copolymer E was approximately only 19 times less than that given by ExGen. Polyplexes of both the polymers were incubated in OptiMEM I with the cells for 3.5 h using 0.6 μg of pRL/well in a 96-well plate. It should be noted here that ExGen has been optimized over the years to give this good transfection, while optimum formulation of pentablock copolymers is still being investigated. Again, this figure also shows that there is an optimum concentration for both the polymers at which they give maximum transfection. Above that concentration, increased toxicity camouflages expression of the transfected gene.

Luciferase activity(RLU) in each well is 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 the cell death. Instead, since all experiments were performed with same initial number of cells per well ($\sim 1.2 \times 10^4$) in a 96-well plate, luciferase expression is reported as RLU/well for each case.

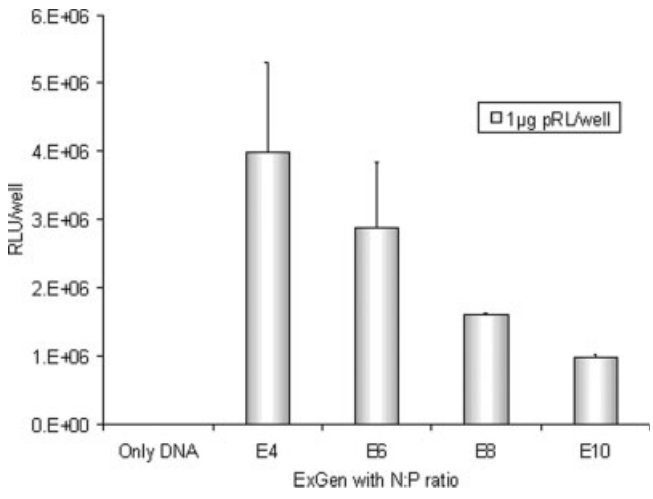


Figure 10. Luciferase expression in SKOV3 cells after transfecting 1 μg of DNA per well in a 96-well plate with ExGen at different N:P ratios. Polyplexes were incubated with cells for 11 h in OptiMEM I, ($n = 4 \pm$ SD).

Polymer gel cytotoxicity studies

Since the agarose matrix was transparent, images of the cells around the polymer gel and directly below the gel were taken using a light microscope after 24 h of incubation. Figure 12 shows these images of cells at 60× and 150× magnification. Since the cells were treated with the vital stain Neutral Red before placing the polymer gel on them, a decolorized zone on the plate can be observed if there were any cell deaths. The images show that there was a small decolorized spot (diameter < 1 mm) directly below the polymer gel. However, the cells around it were stained red and seemed to be as healthy as those far away from the polymer gel. There was also no visible significant cell lysis around the polymer gel. The polymer gels of pentablock copolymers A, B, and C were tested, and all of them appeared to be either non-cytotoxic or mildly cytotoxic. The results with their cytotoxicity scores are summarized in Table II. The modulus and dissolution rate of these polymer gels depend on their MW.[35] Copolymers with higher MW chains form stronger gels that dissolve over a longer period. Therefore, pen-

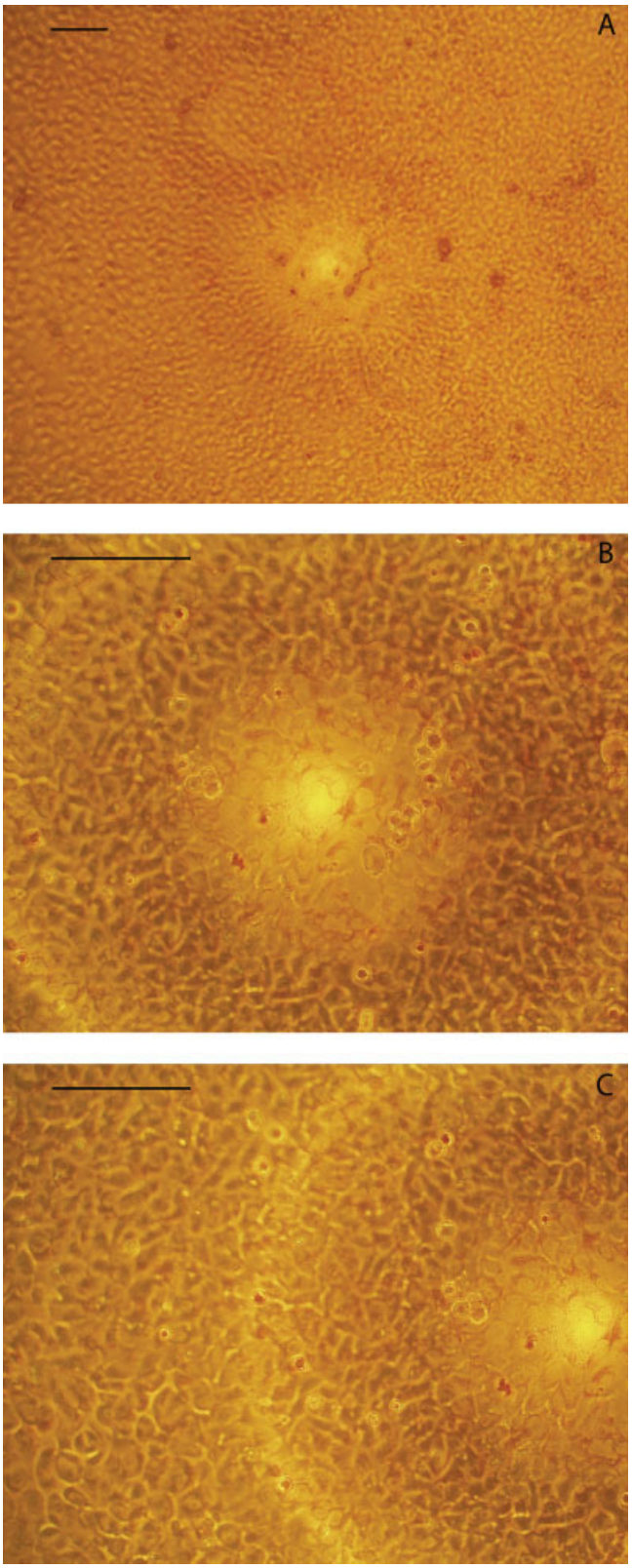


Figure 12. SKOV3 cells under agarose–gel matrix after 24 h incubation with a 25 wt % gel of pentablock copolymer A placed on top of agarose matrix at (a) ×60 magnification, (b,c) ×150 magnification—regions right below and around the polymer gel. Scale bar = 1 mm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tablock B, which had lowest M_w (weight average MW), did not have a high modulus at 25 wt %, and dissolved and spread faster on the agarose matrix thus, causing cell death over larger radii.

DISCUSSION

In this study we have evaluated cytotoxicity of new pentablock copolymers under different *in vitro* conditions. Different assays and cell types were used to determine various aspects of toxicity. MW of the synthesized copolymers was maintained below 20 kDa, as that is the cut-off mass for renal excretion by the kidney, thus assuring final removal of copolymer from the body in an *in vivo* study. Copolymers with different wt % of PDEAEM blocks were studied to assess the effect of this cationic group on copolymers' toxicity. PDEAEM blocks of the copolymers, containing tertiary nitrogens, condense DNA and, are responsible for their pH buffering capacity that helps in the release of polyplexes from endosomes. Therefore, increasing PDEAEM content in the copolymers would increase both the amount of DNA they can condense and their transfection efficiency. However, it was observed that as the wt % of PDEAEM increased from 17 to 60%, cell viability decreased significantly in SKOV3 and A431 cancer cell lines. Copolymers were incubated with cells in FBS supplemented DMEM for 48 h at different concentrations. Copolymers with higher percentages of PDEAM (pentablock C and D) caused more leakage of the cell membrane, followed by decrease in metabolic activity, while those with up to 26% PDEAEM did not cause much cell membrane damage even at high concentrations. This increase in toxicity of copolymers can be explained by the fact that copolymers with higher wt % of PDEAEM block have higher cationic surface charge, and thus higher charge density. This higher cationic charge resulted in increased damage to the anionic cell membranes, as has been suggested by researchers for other polycations.[7] However, for all the copolymers (except for pentablock D on A431 cells), a concentration can be determined from the graphs below which they exhibited less than 10% LDH release or >80% cell viability even after 48 h of incubation. This is of great sig-

TABLE II
Summary of Test Results from Agarose Overlay Analysis[a]

Specimen	Zone Index	Lysis Index	Cytotoxicity Score	Comment
Pentablock A	0.5	0	0.5	Non-cytotoxic
Pentablock B	2	1	1	Mildly cytotoxic
Pentablock C	1	1	1	Mildly cytotoxic

[a]Approximately 4 mg of polymer gels containing 25 wt % of the polymer were placed on the top of agarose layer.

nificance for gene therapy applications involving longer tissue-contact time *in vivo*.

A431 cells were found to be more sensitive to the copolymers, exhibiting comparatively less cell viability than SKOV3 cells at same polymer concentrations, especially in terms of cell membrane damage. This might be due to the different compositions of the membranes and glycocalyx of different cell lines.[7]

Since pentablock copolymers C and D, containing 40 and 60 wt % PDEAEM, respectively, showed good transfection only at concentrations where high cell death was also observed (transfection data not shown), further detailed screening of other *in vitro* conditions was reported only with the pentablock copolymer B (26% PDEAEM).

Polyplexes were found to be less toxic than the polycations alone. Complexing DNA with the polymer reduced the LDH release by up to 80% at N:P ratio of 8. A significant increase in cell metabolic activity was also observed. This indicates that free cationic copolymers perhaps damage cells due to their positive surface charge interacting with cellular lipid membranes and other internal cell organelles. On binding with DNA, some of this surface charge is shielded, thus reducing their toxicity. Changes in the conformation of polycationic macromolecules on binding with DNA might also be a reason for this reduced toxicity. This is very much in agreement with other researchers,[7,41] but in contrast with Gebhart et al.,[29] who reported using an MTT assay with Cos-7 cells that polyplexes of PEI(50K) and ExGen reduced the percent survival of cells by 40% compared to the polycations alone. At higher N:P ratios, though some of the polycation is used to condense DNA, the rest of it is available in the free charged form to interact with the cells, thus explaining decrease in cell viability.

Toxicity of the polyplexes of pentablock copolymers was found to increase as their time of incubation with the cells in OptiMEM I increased from 6 to 14 h. Though at lower concentrations, polyplexes were not toxic even up to 10 h of incubation time, at higher concentrations, cell metabolic activity decreased significantly. This suggests that to transfect cells *in vitro*, there is an upper time-limit for which polymer–DNA solutions can be incubated with the cells in OptiMEM I. After that, the polymer–DNA solution should be replaced with fresh media containing FBS for good growth of the cells.

The toxicity of pentablock copolymers was compared to ExGen (22 kDa linear PEI). Both polymers seemed to affect the cells in different ways. While ExGen caused extensive damage to the cell membrane integrity, followed by a decrease in metabolic activity of the cells, pentablock copolymers showed no significant cell membrane damage even when the metabolic activity decreased below 80%. At the same N:P ratios, however, pentablock copolymers exhibited significantly higher cell viability than ExGen.

These results led to investigation of the different mechanisms by which the two polymers interact with the cells. The cytotoxicity of polycationic macromolecules with different structures is influenced by various properties such as MW, charge density, three-dimensional arrangements of the cationic residues, structure, and conformational flexibility.[25,48] The types of amines in the polymer have also been reported to play a role in the toxicity. Ferruti et al.[13] had reported, based on his study with modified PLL, that polymers with tertiary amines exhibit lower toxicity than those with primary and secondary residues. Dekie et al.[49] had also noted that presence of primary amines on the poly L-glutamic acid derivatives had a significant toxic effect on red blood cells. The observations presented here agree with other studies and show that the pentablock copolymers, that have tertiary nitrogens, are significantly less toxic than ExGen, which has series of primary nitrogens, even though their molecular masses are almost the same (close to 20 kDa).

Charge density of the polycations, resulting from the number and three-dimensional arrangement of the cationic residues, together with the flexibility of the polycations, is another important factor influencing cytotoxicity. These factors determine the accessibility of the cationic charges to the cell surface.[50,51] Rigid molecules have more difficulty in attaching to the cell membrane than flexible ones. Interaction of cationic macromolecules with membrane proteins and phospholipids disturbs membrane function and structure.[28,52] ExGen is a linear and flexible polycation with a very high charge density (248 nmol of nitrogen residues per microgram), thus causing more damage to the anionic cell membranes. On the other hand, pentablock copolymers, which exist as spherical micelles in an aqueous environment, have more of a globular structure with comparably less charge density (1–3 nmol of nitrogen residues per microgram), thus causing less damage to the cell membrane. Other such examples of polymers that show good biocompatibility because of their globular structures are PAMAM and cHSA (cationized human serum albumin), as reported by Fischer et al.[7] Another advantage of pentablock copolymers is that they have hydrophilic chains of PEO that shield the surface charge of the cationic PDEAEM, further decreasing their toxicity. In addition to this, the hydrophobic chains of PPO, which are known to interact with the cellular lipid membranes inducing structural changes,[53,54] enable easy access of pentablock copolymers into the cells and help in their translocation within the cells.[55,56] Furthermore, Pluronic micelles had been shown to enhance sealing of permeablized membranes damaged by ionizing radiations or electroporation, thus preventing cell necrosis[57,58] and increasing the rate of wound and burn healing.[59,60] Since pentablock copolymers form similar micelles, they might also be exhibiting these biological-response

modifying activities of Pluronic, thus explaining less cell membrane damage caused by the copolymers. These characteristics of pentablock copolymers are advantageous for *in vivo* studies, since less leakage of cell membranes of the treated cells would cause less inflammation to the surrounding cells.

The mechanism of cytotoxicity caused by polycations is not fully understood. In this study, two different possible mechanisms were observed. While the toxic effects of ExGen seemed to principally result from its interaction with the cell membrane, causing rapid rupture of cell membrane, followed by decrease in metabolic activity, the cytotoxicity of pentablock copolymers appeared to involve some mechanism other than just a membrane lytic effect.

To obtain further insight into the mechanism of cell death and differentiate between apoptotic and necrotic routes, a Caspase-Glo 3/7 assay was employed. Demonstration of biochemical changes in the cells such as activation of caspases is commonly used to characterize apoptosis. Cells treated with positive control Staurosporine showed high activity of caspases, thus confirming induction of apoptosis. Staurosporine induced cell death showed typical features of apoptosis in MTT and LDH assays, such as complete loss of metabolic activity while cell membrane was still intact. For ExGen and pentablock copolymers, different polymer concentrations were tested for different incubation times with the cells. However, no significant caspase activity was detected for any of them, indicating that apoptosis was not occurring with either of the polymer. MTT assays, however, indicated reduced cell viability at these concentrations. This suggests that cells were dying, but not by apoptosis. An early and rapid loss of plasma membrane integrity by ExGen suggests a necrotic type of cell death, as noted by other researchers.[7] However, since pentablock copolymers neither cause damage to the cell membrane nor induce apoptosis, but still reduce metabolic activity at high concentrations, additional mechanism of cytotoxity involving an intracellular route seems to be involved. There is a possibility that these polycations have specific interactions with a membrane component after cellular uptake,[45,46] that activate some signal transduction pathways inside the cell,[47] leading to cell death.

Pentablock copolymers were tested for their transfection efficiency on different cell lines under various *in vitro* conditions using a luciferase plasmid. Pentablock copolymer B, containing 26% (w/w) PDEAEM, gave appreciable transfection in all the three cell lines tested. Since different cell lines have different composition of their membranes and glycocalyx, and different cell division rates, the rates of entry of polyplexes across the cell membrane and nuclear membrane differ in each of them. This affects both, the toxicity and the transfection efficiency of the polymers, and explains why the level of luciferase expression was different in different cell lines at similar N:P ratios. While transfection in SKOV3 cells peaked at N:P ratio 8 and then decreased at N:P 10, it was not significantly different in A431 cells at N:P ratios of 6, 8, and 10. These results suggest that different cell lines have different optimum conditions under which they give best transfection, and should therefore be evaluated individually.

Figure 10 shows that the transfection efficiency of polymers peaks at certain N:P ratios and decreases after that. This means that above these N:P ratios, the toxicity of polymers increases to the extent that the cells die before they can express the transfected gene. Thus, at those high N:P ratios, even though transfection is good, not too many cells are left viable to express the transfected gene, thereby displaying less luciferase expression per well. The only way to work at higher N:P ratios and obtain high transfection while not increasing the polymer toxicity is to reduce the amount of DNA dose, as demonstrated in experiments with pentablock copolymer E (containing 28% (w/w) PDEAEM) (Fig. 11). The maximum amount of total luciferase expression obtained in SKOV3 cells with pentablock E was only 19 times less than the maximum given by ExGen. Two reasons can well explain this high gene expression by pentablock E in Figure 11 compared to pentablock B in Figure 10. First, it had slightly higher content of PDEAEM than pentablock B, thus being able to condense more DNA per polymer micelle. Second, in experiments with pentablock E, only 0.6 μg of pRL/well was used (instead of 1 μg used otherwise). Since lesser amount of polymer was required to condense 0.6 μg DNA than 1 μg DNA, it was possible to work at high N:P ratios (up to 10:1) with 0.6 μg DNA and get higher transfection without compromising toxicity. It should be noted that with pentablock B complexed with 1 μg plasmid [Fig. 10], luciferase expression peaked at N:P 8:1, and it decreased beyond that value. These results clearly indicate that transfection efficiency of the polymers is critically correlated with their cytotoxicity, and that it can be optimized by adjusting the DNA dose and corresponding N:P ratios. Another thing to be noted is that though ExGen gives higher transfection than pentablock copolymers, it is also accompanied with high toxicity, as shown in Figure 6 for corresponding N:P ratios. So some of the transfection observed might be from the cells that had subsequently died.

The agarose–matrix experiments simulating a tumor-model suggested that polymer gels, containing up to 1000 times higher polymer concentration than that used for experiments in liquid growth medium, did not kill the cells after diffusing through the agarose–matrix. Pentablock copolymers A, B, and C, containing different wt % of PDEAEM block were tested on these agarose–gel matrices. Polymer gels of all these polymers were found to be nontoxic or mildly toxic. This

indicates that in an *in vivo* experiment, a polymer gel can be implanted at the site of tumor, releasing polymer micelles over a period of time, without causing significant cell death in the vicinity. Thus, such a gel could even deliver genes complexed with the polymer in a sustained fashion to the targeted cells, providing extended gene expression, and maintaining desired level of the expressed therapeutic proteins without repeated injections. Complexes of DNA and pentablock copolymers have also been found to form gels, as reported earlier by our group,[33] and these gels were found to dissolve in excess buffer to release complexed plasmids, and not free plasmid.

It should be clearly noted here that in our polymer–DNA gel system, gels dissolve to release polyplexes, which are subsequently up taken by the targeted cells. Recently, several polymer systems, such as hydrogels of gelain,[61] implantable polymer matrices (EVAc, poly (ethylene-co-vinyl acetate)), and injectable microspheres (PLGA and PLA),[62] have been reported in the literature that encapsulate naked DNA and release it in a controlled fashion to the cells. In these cases, however, the encapsulation materials are inert and do not aid in the transfection. No system has been reported till date to our knowledge that delivers complexed DNA (polyplexes) to the cells in a sustained fashion. Since polymer–DNA complexes give much higher transfection than naked DNA, it is evident that our novel polymer–DNA gels can be instrumental in improving the gene therapy.

CONCLUSIONS

Pentablock copolymers were tested for their cytotoxicity under various *in vitro* conditions. Toxicity of these copolymers was found to increase on increasing the wt % of their cationic block PDEAEM, and can thus be tuned by tailoring the cationic content in the copolymers while still preserving their DNA complexation properties. Below certain concentrations, different pentablock copolymers could be incubated with cells in complete media for up to 48 h without exhibiting significant cell death. Polyplexes were found to be less toxic than polycations alone, as DNA condensation shields their surface charges. However, polyplexes caused more cell death at longer incubation time with the cells in OptiMEM I. Polyplexes of pentablock copolymers were found to be much less toxic than ExGen. While ExGen caused rapid loss of cell membrane integrity, followed by decrease in cell viability, pentablock copolymers caused less than 10% membrane leakage even at high concentrations where metabolic activity was reduced to $<80\%$. None of the polymers, however, were found to induce apoptosis in the cells. Pentablock copolymers were hypothesized to cause cell death by activating some signal transduction pathway once they get into the cells. Optimum conditions that showed maximum transgene expression with minimal cell death were obtained by varying the DNA dose, the polymer concentration, and N:P ratios. The transfection was found to be correlated to the toxicity of the polyplexes. Transfection obtained with pentablock copolymers was comparable to that shown by ExGen. Pentablock copolymers form thermoreversible gels at higher concentrations and physiological temperatures. The agarose–matrix experiments with the 25 wt % polymer gels proved that they were nontoxic or mildly toxic. This suggests that if formed subcutaneously at the site of tumors, pentablock copolymer gels can release polyplexes over a period of time, which can then diffuse through tumor tissues to the targeted cancer cells without damaging neighboring healthy tissues. The thermoreversible gelation features along with the good transfection efficiencies and tunable cytotoxicities make these new copolymers promising vectors for gene delivery.

The authors thank Michael Determan for the synthesis and characterization (gel permeation chromatography, NMR) of pentablock copolymers.

References

1. Schmidt-Wolf GD, Schmidt-Wolf IGH. Non-viral and hybrid vectors in human gene therapy: An update. Trends Mol Med 2003;9:67–72.
2. Merdan T, Kopecek J, Kissel T. Prospects of cationic polymers in gene and oligonucleotide therapy against cancer. Adv Drug Deliv Rev 2002;54:715–758.
3. Brigger I, Dubernet C, Couvreur P. Nanoparticles in cancer therapy and diagnosis. Adv Drug Deliv Rev 2002;54:631–651.
4. Jeong B, Kim SW, Bae YH. Thermosensitive sol-gel reversible hydrogels. Adv Drug Deliv Rev 2002;54:37–51.
5. Kost J, Langer R. Responsive polymeric delivery systems. Adv Drug Deliv Rev 2001;46(1–3):125–148.
6. Lauffenburger DA, Schaffer DV. The matrix delivers. Nat Med 1999;5:733, 734.
7. Fischer D, Li Y, Ahlemeyer B, Krieglstein J, Kissel T. In vitro cytotoxicity testing of polycations: Influence of polymer structure on cell viability and hemolysis. Biomaterials 2003;24:1121–1131.
8. De Groot CJ, Van Luyn MJA, Van Dijk-Wolthuis WNE, Cadee JA, Plantinga JA, Otter WD, Hennink WE. In vitro biocompatibility of biodegradable dextran-based hydrogels tested with human fibroblasts. Biomaterials 2001;22:1197–1203.
9. Verma IM. A tumultuous year for gene therapy. Mol Ther 2000;2:415, 416.
10. Liu F, Huang L. Development of non-viral vectors for systemic gene delivery. J Controlled Release 2002;78(1–3):259–266.
11. Schatzlein AG. Non-viral vectors in cancer gene therapy: Principles and progress. Anti-Cancer Drugs 2001;12:275–304.
12. Gondsho A, Irie K, Susaki H, Iwasawa H, Okuno S, Sugawara T. Tissue-targetting ability of saccharide-poly(L-lysine) conjugates. Biol Pharma 1994;17:275–282.
13. Ferruti P, Knobloch S, Ranucci E, Gialnasi E, Duncan R. A novel chemical modification of poly-L-lysine reducing toxicity while preserving cationic properties. Proc Int Symp Control Rel Bioact Matter 1997;24:45, 46.

14. Zauner W, Brunner S, Buschle M, Ogris M, Wagner E. Differential behaviour of lipid based and polycation based gene transfer systems in transfecting primary human fibroblasts: A potential role of polylysine in nuclear transport. Biochim Biophys Acta Gen Subj 1999;1428:57–67.
15. Boussif O, Lezoualch F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethyleneimine. Proc Natl Acad Sci USA 1995;92:7297–7301.
16. Iwai M, Harada Y, Tanaka S, Muramatsu A, Mori T, Kashima K, Imanishi J, Mazda O. Polyethylenimine-mediated suicide gene transfer induces a therapeutic effect for hepatocellular carcinoma in vivo by using an Epstein-Barr virus-based plasmid vector. Biochem Biophys Res Commun 2002;291:48–54.
17. Godbey WT, Barry MA, Saggau P, Wu KK, Mikos AG. Poly (ethylenimine)-mediated transfection: A new paradigm for gene delivery. J Biomed Mater Res 2000;51:321–328.
18. Tang MX, Fedemann CT, Szoka FC Jr. In vitro gene delivery by degraded polyamidoamine dendrimers. Bioconjugate Chem 1996;7:703–714.
19. Richardson SCW, Kolbe HVJ, Duncan R. Potential of low molecular mass chitosan as a DNA delivery system: Biocompatibility, body distribution and ability to complex and protect DNA. Int J Pharmaceut 1999;178:231–243.
20. Erbacher P, Zou S, Bettinger T, Steffan AM, Remy JS. Chitosan-based vector/DNA complexes for gene delivery: Biophysical characteristics and transfection ability. Pharmaceut Res 1998;15:1332–1339.
21. Van De Wetering P, Schuurmans-Nieuwenbroek NME, van Streenbergen MJ, Crommelin DJA, Hennink WE. Co-polymer of 2-(dimethylamino)ethyl methacrylate with ethoxytriethylen glycol methacrylate or N-vinyl-pyrroliodone as gene transfer agents. J Controlled Release 2000;64:193–203.
22. Van De Wetering P, Cherng JY, Talsma H, Crommelin DJA, Hennik WE. (2-Dimethylamino)ethyl-methacrylate based copolymers as gene transfer agents. J Controlled Release 1998;53:145–153.
23. Brazeau GA, Attia S, Poxon S, Hughes JA. In vivo myotoxicity of selected cationic macromolecules used in non-viral gene delivery. Pharmaceut Res 1998;15:680–684.
24. Dash PR, Read ML, Barret LB, Wolfert MA, Seymour LW. Factors affecting blood clearance and in vivo distribution of polyelectrolyte complexes for gene delivery. Gene Ther 1999; 6:643–650.
25. Choksakulnimitr S, Masusa S, Tokda H, Takakura Y, Hashida M. In vitro cytotoxicity of macromolecules in different cell culture systems. J Controlled Release 1995;34:223–241.
26. Florea BI, Meaney C, Junginger HE, Borchard G. Transfection efficiency and toxicity of polyethylenimine in differentiated Calu-3 and nondifferentiated COS-1 cell cultures. AAPS PharmSci 2002;4:E12.
27. Hill IRC, Garnett MC, Bignotti F, Davis SS. In vitro cytotoxicity of poly(amidoamine)s: Relevance to DNA delivery. Biochim Biophys Acta Gen Subj 1999;1427:161–174.
28. Morgan DML, Larvin VL, Pearson JD. Biochemical characterisation of polycation-induced cytotoxicity to human vascular endothelial cells. J Cell Sci 1989;94:553–559.
29. Gebhart CL, Kabanov AV. Evaluation of polyplexes as gene transfer agents. J Controlled Release 2001;73(2/3):401–416.
30. Kean T, Roth S, Thanou M. Trimethylated chitosans as non-viral gene delivery vectors: Cytotoxicity and transfection efficiency. J Controlled Release 2005;103:643–653.
31. Fischer D, Bieber T, Li Y, Elsässer H-P, Kissel T. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: Effect of molecular weight on transfection efficiency and cytotoxicity. Pharmaceut Res 1999; 16:1273–1279.
32. Van De Wetering P, Cherng J-Y, Talsma H, Hennink WE. Relation between transfection efficiency and cytotoxicity of poly(2-(dimethylamino)ethyl methacrylate)/plasmid complexes. J Controlled Release 1997;49:59–69.
33. Agarwal A, Unfer R, Mallapragada SK. Novel cationic pentablock copolymers as non-viral vectors for gene therapy. J Controlled Release 2005;103:245–258.
34. Anderson BC, Cox SM, Bloom PD, Sheares VV, Mallapragada SK. Synthesis and characterization of diblock and gel forming pentablock copolymers of tertiary amine methacrylates, poly (ethylene glycol) and poly(propylene glycol). Macromolecules 2003;36:1670–1676.
35. Determan MD, Cox JP, Seifert S, Thiyagarajan P, Mallapragada SK. Synthesis and characterization of temperature and pH responsive pentablock copolymers. Polymer 2005;46:6933–6946.
36. Determan MD, Guo L, Thiyagarajan P, Mallapragada SK. Supramolecular self-assembly of multiblock copolymers in aqueous solution. Langmuir 2006;22:1469–1473.
37. Determan MD, Chieh-Tsung L, Thiyagarajan P, Mallapragada SK. Self-assembly of temperature and pH-responsive pentablock copolymers. Polym Mater: Sci Eng 2005;23:808.
38. Edmondson J. A rapid and simple MTT-based spectrophotometric assay for determining drug sensitivity in monolayer cultures. J Tissue Culture Methods 1988;11:15–17.
39. Guess WL, Rosenbluth SA, Schimdt B. Agar diffusion method for toxicity screening of plastics on cultured cell monolayers. J Pharm Sci 1965;54:1545–1547.
40. Koh J-Y, Wie MB, Gwag BJ, Sensi SL, Canzoniero LMT, Demaro J, Csernansky C, Choi DW. Staurosporine-induced neuronal apoptosis. Exp Neurol 1995;135:153–159.
41. Godbey WT, Wu KK, Mikos AG. Poly(ethylenimine) and its role in gene delivery. J Controlled Release 1999;60(2/3):149–160.
42. Miller LJ, Marx J. Apoptosis. Science 1998;281:1301.
43. Lincz LF. Deciphering the apoptotic pathway: All roads lead to death. Immunol Cell Biol 1998;76:1–19.
44. Green DR, Reed JC. Mitochondria and apoptosis. Science 1998; 281:1309–1312.
45. Leroy D, Schmid N, Behr J-P, Filhol O, Pares S, Garin J, Bourgarit JJ, Chambaz EM, Cochet C. Direct identification of a polyamine binding domain on the regulatory subunit of the protein kinase casein kinase 2 by photoaffinity labeling. J Biol Chem 1995;270: 17400–17406.
46. Arnold LJJ, Dagan A, Gutheil J, Kaplan NO. Antineoplastic activity of poly(L-lysine) with some ascites tumor cells. Proc Natl Acad Sci USA 1979;76:3246–3250.
47. Vepa S, Scribner WM, Natarajan V. Activation of endothelial cell phospholipase D by polycations. Am J Physiol Lung Cell Mol Physiol 1997;272 (4 Part 1):L608–L613.
48. Rihova B. Biocompatibility of biomaterials: Hemocompatibility, immunocompatiblity and biocompatibility of solid polymeric materials and soluble targetable polymeric carriers. Adv Drug Deliv Rev 1996;21:157–176.
49. Dekie L, Toncheva V, Dubruel P, Schacht EH, Barrett L, Seymour LW. Poly-glutamic acid derivatives as vectors for gene therapy. J Controlled Release 2000;65(1/2):187–202.
50. Ryser HJ. A membrane effect of basic polymers dependent on molecular size. Nature 1967;215:934–936.
51. Singh AK, Kasinath BS, Lewis EJ. Interaction of polycations with cell-surface negative charges of epithelial cells. Biochim Biophys Acta Prot Struct Mol Enzymol 1992;1120:337–342.
52. Morgan DML, Clover J, Pearson JD. Effects of synthetic polycations on leucine incorporation, lactate dehydrogenase release, and morphology of human umbilical vein endothelial cells. J Cell Sci 1988;91:231–238.
53. Batrakova EV, Miller DW, Li S, Alakhov VY, Kabanov AV, Elmquist WF. Pluronic P85 enhances the delivery of digoxin to the brain: In vitro and in vivo studies. J Pharmacol Exp Therapeut 2001;296:551–557.

54. Kostarelos K, Tadros TF, Luckham PF. Physical conjugation of (tri-) block copolymers to liposomes toward the construction of sterically stabilized vesicle systems. Langmuir 1999;15:369–376.
55. Melik-Nubarov NS, Pomaz OO, Dorodnych TY, Badun GA, Ksenofontov AL, Schemchukova OB, Arzhakov SA. Interaction of tumor and normal blood cells with ethylene oxide and propylene oxide block copolymers. FEBS Lett 1999;446:194–198.
56. Batrakova EV, Li S, Vinogradov SV, Alakhov VY, Miller DW, Kabanov AV. Mechanism of pluronic effect on P-glycoprotein efflux system in blood-brain barrier: Contributions of energy depletion and membrane fluidization. J Pharmacol Exp Therapeut 2001;299:483–493.
57. Hannig J, Zhang D, Canaday DJ, Beckett MA, Astumian RD, Weichselbaum RR, Lee RC. Surfactant sealing of membranes permeabilized by ionizing radiation. Radiat Res 2000;154:171–177.
58. Lee RC, Hannig J, Matthews KL, Myerov A, Chen C-T. Pharmaceutical therapies for sealing of permeabilized cell membranes in electrical injuries. Ann N Y Acad Sci 1999;888:266–273.
59. Agren MS. An amorphous hydrogel enhances epithelialisation of wounds. Acta Dermato-Venereol 1998;78:119–122.
60. Cao YL, Lach E, Kim TH, Rodriguez A, Arevalo CA, Vacanti CA. Tissue-engineered nipple reconstruction. Plast Reconstructive Surg 1998;102:2293–2298.
61. Kushibiki T, Tomoshige R, Fukunaka Y, Kakemi M, Tabata Y. In vivo release and gene expression of plasmid DNA by hydrogels of gelatin with different cationization extents. J Controlled Release 2003;90:207–216.
62. Luo D, Woodrow-Mumford K, Belcheva N, Saltzman WM. Controlled DNA delivery systems. Pharmaceut Res 1999;16: 1300–1308.