Correlating Transfection Barriers and Biophysical Properties of Cationic Polymethacrylates

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Transfection efficiencies of several polymeric gene carriers were compared and correlated quantitatively to the amounts of cellular accumulation of plasmid DNA and to the expression of mRNA by quantitative real-time polymerase chain reaction (real-time PCR). Three polycations polymers with similar chemical structure were used in this study: poly(dimethylamino)ethyl methacrylate (PDMA) homopolymer, PEO-*b*-PDMA copolymer, and PEO-*b*-poly(diethylamino)ethyl methacrylate (PEO-*b*-PDEA) copolymer. Despite their similar chemical structures, the transfection efficiencies were significantly different. PEO-*b*-PDEA copolymer was significantly less efficient as gene carrier as compared to both PDMA and PEO-*b*-PDMA. Correlations between cytotoxicity, cellular uptake of plasmid DNA, expression levels of transgene and protein, and the physical properties of the polymers were observed. With the PEO-*b*-PDEA studies, cytotoxicity was due primarily to the excess of polymers that did not participate in the DNA binding. In addition, the inability of the polymer/DNA polyplexes to interact with cell effectively was identified as a critical barrier for high efficiency of transfection. This study demonstrated that the use of quantitative real-time PCR in combination with physical characterization techniques could provide useful insights into the transfection barrier at different cellular levels.

Introduction

In recent years, gene delivery has demonstrated great potential for treating genetic deficiencies.[1,2] Non-viral gene delivery systems such as cationic lipid and polymeric systems become an attractive route as viral delivery systems pose safety concerns unlikely to be resolved in the near future.[3−6] For instance, the LipofectAMINE lipid system[1] has been used for the delivery of plasmid DNA into human islets, and polyethylenimine (PEI) polymeric systems[7] have been used for in vivo delivery of antisense oligonucleotides. Cationic polymers are a leading class of gene delivery systems because of their molecular diversity that can be modified to fine-tune their physicochemical properties.[8,9] Polymeric systems commonly used in gene delivery include homopolymers, for example, poly(dimethylamino)ethyl methacrylate (PDMA),[10,11] block copolymers (e.g., PEI-*b*-Pluronic),[7] and polypeptide hydrogels.[12] However, despite the variety and versatility of these proposed non-viral transfection agents, there are several problems, such as low biocompatibility, toxicity, and, in particular, low transfection efficiency, that need to be addressed prior to their practical use. Successful transfection depends on the delivery of DNA transgene into the nucleus and the subsequent expression within the cell.[13−15] Understanding the interactions between the gene carriers with the components responsible for their cellular uptake and intracellular pathways is essential for the rational design of the high transfection efficiency carriers.

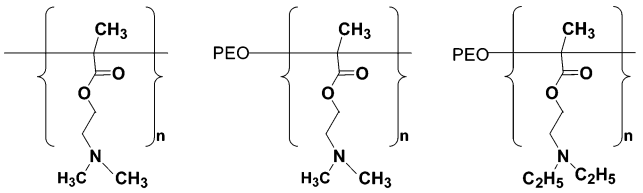

Figure 1. Chemical structures of polymers used in this study.

There are several methods to determine the amount of internalized plasmid DNA. The fluorescence labeling technique or fluorescence resonance energy transfer (FRET) approach is the most popular route.[16,17] However, fluorescence labeling could potentially affect the cell internalization behavior. On the other hand, using real-time polymerase chain reaction (PCR) for quantification of internalized plasmid DNA would not have such problem. Real-time PCR is the state-of-the-art technique to quantify nucleic acids for various analysis such as mutation detection and gene expression.[18,19] In general, good correlation between protein and mRNA expression levels is widely observed, but discordant correlation is not unusual.[20,21] Recently, an attempt to use real-time PCR to quantify the amount of plasmid DNA taken up by cells in transfection studies has been reported.[13] This approach could potentially lead to the identification of the cellular transfection barriers for different transfection vectors, which may be important for the design of an efficient gene delivery system.

In this study, three cationic polymethacrylate systems with similar chemical structures were chosen as model systems (Figure 1). PDMA is a cationic hydrophilic polymer, which has attracted considerable attention for gene therapy applications,[10,22,23] both as a homopolymer and as block copolymers

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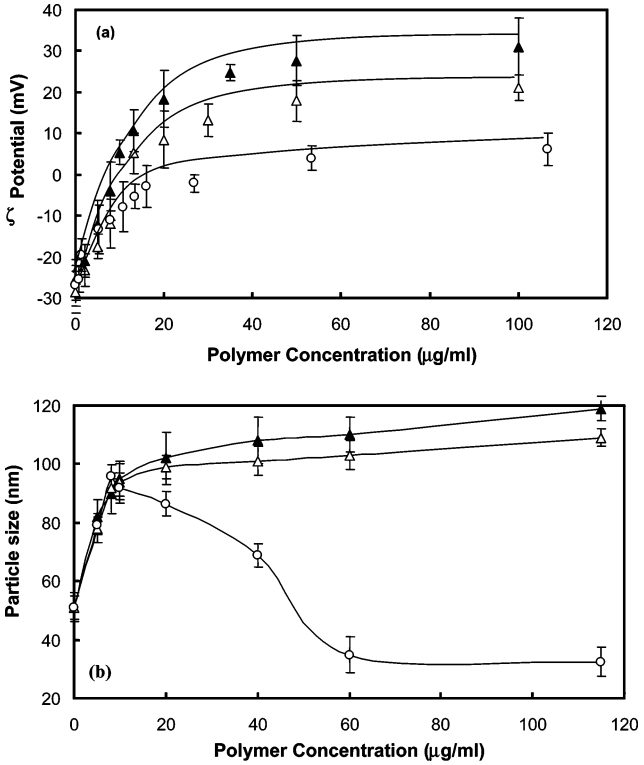

Figure 2. Physical properties of the polyplexes formed with plasmid EGFP DNA and varying polymer concentration. (a) Zeta potential. (b) Hydrodynamics radius of the polyplexes.

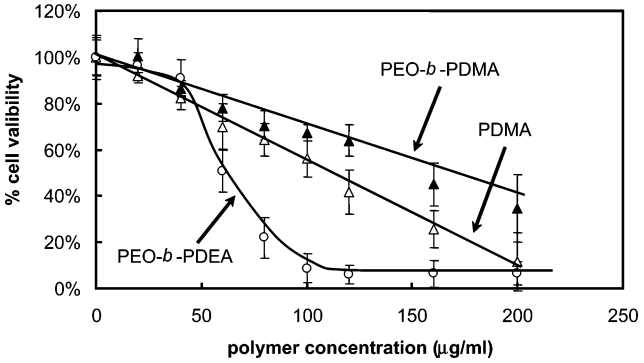

Figure 3. Cytotoxicity of various polymer/DNA complexes systems in Neuro2A cell. The cell viability was determined by MTS assay and was shown as the mean, and the error bars are the standard deviation of five determinations.

with polyethers, such as PEO-*b*-PDMA. PEO-*b*-PDEA is an amiphiphilic block copolymer capable of forming pH-dependent micelles. At physiological pH (pH 7.4), PDEA chains are somewhat hydrophobic and drive the formation of micelles, which are stabilized by the hydrophilic PEO segments above the critical micellization concentration (CMC). The cationic PDEA segments of the copolymer are able to bind to negatively charged DNA to form polymer/DNA complexes.[24,25]

Despite the similarities in the chemical structures of PDMA and PDEA, the number of cells transfected by their respective copolymers with PEO was found to be distinctively different. To identify the transfection barrier, we quantified and compared the amount of plasmid DNA accumulated in the cells and the expression level of the transgene using sequence-independent real-time PCR. Correlation between the real-time PCR results with the cytotoxicity data, biophysical properties of the polymer/DNA polyplexes, and the transfection efficiencies was observed. This study showed that quantifying the accumulation and

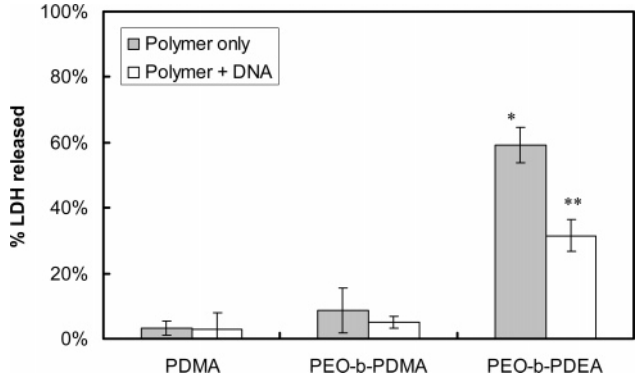

Figure 4. Membrane permeability measured in the presence of different polyplexes (50 μg/mL polymer and N/P ratio $\approx$10). The release of the cytoplasmic enzyme LDH is measured after 2 h incubation. Differences in the % LDH released between PEO-*b*-PDEA polymer (*) and polyplexes (**) were statistically significant based on the paired two-tailed *t*-test. A value of $P < 0.05$ was considered significant ($P = 0.0083$). Each value represents the mean $\pm$ standard deviation of five determinations.

expression of cellular transgenes using real-time PCR, in parallel with cytotoxicity measurements and biophysical studies, has enabled a better understanding of the factors influencing transfection efficiency.

Materials and Methods

Preparation of Plasmid DNA. Plasmid EGFP (pIRES-EGFP-EV71), an expression vector containing the enhanced green fluorescence protein (EGFP), was constructed by grafting picornaviral IRESes from Enterovirus 71 (strain 7423/MS/87) onto a pIRES-EGFP (Clontech Laboratories Inc., U.S.) backbone. The plasmid was amplified using *Escherichia coli* (DH5α) and purified using the Quantum Plasmid Miniprep kit (Biorad).

Polymer Synthesis. A full description of the synthesis and characterization of the polymers has recently been reported.[24] The degree of polymerization (DP) for the three polymer systems is about 65−75 repeat units of methacrylate monomer, which was determined using polystyrene GPC standard. The molecular weight of the PEO polymer (Dow Chemical) was $M_n \approx 5000$ Da.

Physical Properties Measurement. The zeta potential and the particle size measurements were carried out using the Brookhaven laser light scattering system, which consists of the Brookhaven Zeta PALS (phase analyzer light scattering) and the dynamics light scattering measurement. The plasmid DNA concentration used was about 5 μg/mL of 10 mM phosphate buffer. Polycation/DNA charge ratio (N/P ratio) was also calculated. The experimental details were described previously.[25]

Cell Culture and Cytotoxicity (MTS Assay) Study. Neuro2A cells (CCL-131, ATCC) were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37 °C, 5% CO_2, and 95% relative humidity. For cytotoxicity studies, cells (40 000 cells/well) were seeded into each of the 96 wells in a microtiter plate (Nunc, Wiesbaden, Germany) using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2*H*-tetrazolium) assay.[24]

Lactate Dehydrogenase (LDH) Assay. The amount of LDH was assayed with the Cytotoxicity Detection Kit (Roche Applied Science). Briefly, 40 000 Neuro2A cells were seeded into each 96-wells plate. After 24 h, cell culture medium was removed and replaced by 200 μL/well polymer solution. After 120 min of exposure, 100 μL of the supernatant was withdrawn from each well to a new plate, and 100 μL of substrate mix solution was then added. LDH released from cells catalyzes the conversion of INT-tetrazolium salt into a red formazan salt, which was quantified by measuring the absorbance at 490 nm

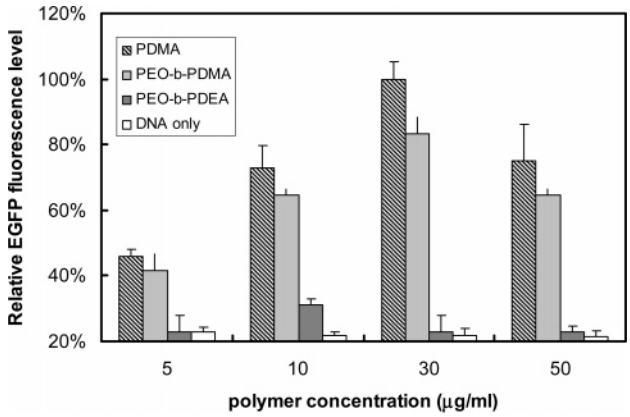

Figure 5. Relative EGFP protein fluorescence intensity level with various transfection systems normalized against the fluorescence intensity of 30 μg/mL of PDMA polyplexes. Each value represents the mean $\pm$ standard deviation of three determinations.

with the model 680 Microplate Reader (Bio-Rad, Hercules, CA). Controls for total LDH released were determined with cells treated with 0.1% (w/v) Triton X-100. The relative LDH release is defined by the ratio of LDH released over total LDH in the intact cells.

In Vitro Transfection. Various cationic polymers at different polycation/DNA ratios were mixed with 4 μg of purified plasmid per well of transfected cells. The polycation/DNA mixtures were diluted to a final volume of 1 mL with serum-free medium (SFM) and incubated for 20 min at 25 °C. Two identical sets of six-well plates (one set was used for plasmid DNA accumulation and the other for RNA level measurements) were seeded with 0.5×10^6 cells per well and incubated at 37 °C, 5% CO_2, and 95% relative humidity for 24 h prior to transfection. The polycation/DNA complexes were then added into the wells and incubated for a further 6 h. For RNA and protein analyses, the culture medium was replaced with DMEM supplemented with 10% FCS, and the cells were cultured for 48 h. Thereafter, the cells in each well were counted and lysed with 0.1% Triton X-100, and the EGFP protein fluorescence intensity levels were measured fluorometrically (BMG Labtechnologies FLUOstar optima).

Total DNA, RNA Extraction, and cDNA Synthesis. For the plasmid DNA accumulation study, after 6 h incubation with various plasmid DNA/polyplexes or naked plasmid DNA, the cells were washed with 3× PBS solution to remove unbound plasmid DNA. The cells were then trypsinized (0.1%) and pelleted, following which they were lysed in 50 mM NaOH/1% SDS and heated at 95 °C for 7 min to ensure the inactivation of DNAses and the release of total DNA. Total DNA was precipitated in the presence of 0.5 M NaCl, 75% ethanol and centrifuged at 14 000*g* for 15 min. The pellet was washed with 70% ethanol, air-dried, and then resuspended in 50 μL of TE (10 mM Tris, 1 mM EDTA, pH 8) buffer.

Total RNA was extracted after 48 h of transfection using Trizol according to manufacturer's instructions (Invitrogen). The final pellet containing total RNA was air-dried and dissolved in diethylpyrocarbonate-treated (DEPC) water. The amount of total RNA was quantified using UV spectroscopy, and the integrity of the total RNA was validated by denaturing agarose gel electrophoresis.

Real-Time PCR Using SYBR Green I. Real-time PCR was carried out on the iCycler iQ (Bio-Rad, Hercules, CA), following an initial denaturation for 3 min at 95 °C, by 40 cycles of 60 s denaturation at 95 °C, 30 s annealing at 60 °C, and 60 s extension at 72 °C. Fluorescence detection was carried out during the annealing phase. The reaction was carried out in a total volume of 40 μL in 1× XtensaMix-SG (BioWORKS), containing 2.5 mM MgCl, 10 pmol of primers, and 0.5 U DNA polymerase (DyNAzyme II, Finnzymes, Finland). All real-time PCR reactions were carried out simultaneously with linearized plasmid standards and a negative control (water). Forward primer (ATCCGCAGCGACATCAACCT) and reverse primer (ACGCCCT-TGCTCTTCATCAG) were designed to amplify a fragment of 238 base pairs within the EGFP open reading frame based on the vector sequence. The concentrations of EGFP in all of the samples were interpolated from standard curves. The threshold cycle (C_t) was calculated by multiplying the average standard deviation of the relative fluorescent units (RFU) gathered from the first 8−10 cycles of amplifications by a factor of 10, using the Optical interface v3.0B.

Results

Physical Properties of Polyplexes. The surface charge and the particles size of the polyplexes were initially investigated using the laser light scattering technique (Figure 2). PEO-*b*-PDEA polymer when polyplexed with plasmid DNA showed significantly lower surface charge potential than that of the other two systems. Notably, the zeta potential of the PEO-*b*-PDEA polyplexes was found to be slightly above zero as compared to PDMA polyplexes (+30 mV). The particle size of the positively charged PDMA polyplexes remained fairly constant with increasing polymer concentrations, while the near neutral charged PEO-*b*-PDEA polyplexes showed structural rearrangement into a smaller particle at higher polymer concentrations (Figure 2b). The morphology of the PEO-*b*-PDEA polyplexes at different concentrations has previously been reported.[25]

In Vitro Cytotoxicity Testing. The cytotoxic effects of various polymer/DNA complexes on Neuro2A cells were measured using MTS assay after 24 h (Figure 3). Similar results were obtained when MTS assay was carried out in the absence of serum 6 h after exposure to the polymer/DNA. Despite the similarities in the chemical structures of the polymers, the effects on cell viability were different. Cytotoxicity varied linearly with concentration for both PDMA and PEO-*b*-PDMA where greater than 80% of the cells were viable at polymer concentrations of 50 μg/mL or less. For the PEO-*b*-PDEA system, however, a sigmoidal variation in cell viability with polymer concentration was observed with a sharp increase in the cytotoxicity (<50% cell viability) for polymer concentrations above 50 μg/mL. Interestingly, as shown in Figure 2b, PEO-*b*-PDEA polyplexes showed significant changes in particle size at polymer concentration of about 50 μg/mL. Above this critical concentration, PEO-*b*-PDEA/DNA complexes undergo significant structural rearrangement to form compact micelle-like structures, driven by the amiphiphilic nature of this copolymer.[25] This observation suggests that the amiphiphilic micelle-like structures may be related to higher cytotoxicity as compared to homopolymer PDMA/DNA complexes that do not undergo structural transformation.

Based on these cytotoxicity results, all subsequent transfection studies were carried out at polymer concentrations 50 μg/mL and below, where cytotoxicity was minimal for all three polymer systems.

LDH is a cytoplasmic enzyme that is normally not secreted outside the cells and is used as an indicator of cell membrane disruption.[26,27] The LDH assay was used to compare the extent of cell membrane disruption in the presence of various polymer systems after 2 h. Neither PDMA nor PEO-*b*-PDMA polymer systems induce significant damage to the cell membrane (<10% of LDH was released). However, PEO-*b*-PDEA caused a significant disruption of the cell membrane (Figure 4). The amount of LDH released in the presence of free PEO-*b*-PDEA polymer (59% LDH released) was significantly higher than the PEO-*b*-PDEA + DNA polyplexes (32% LDH released), where both polyplexes and excess polymer chains were present.

These results indicate that the surface active PEO-*b*-PDEA polymers were capable of forming self-assembly aggregates,

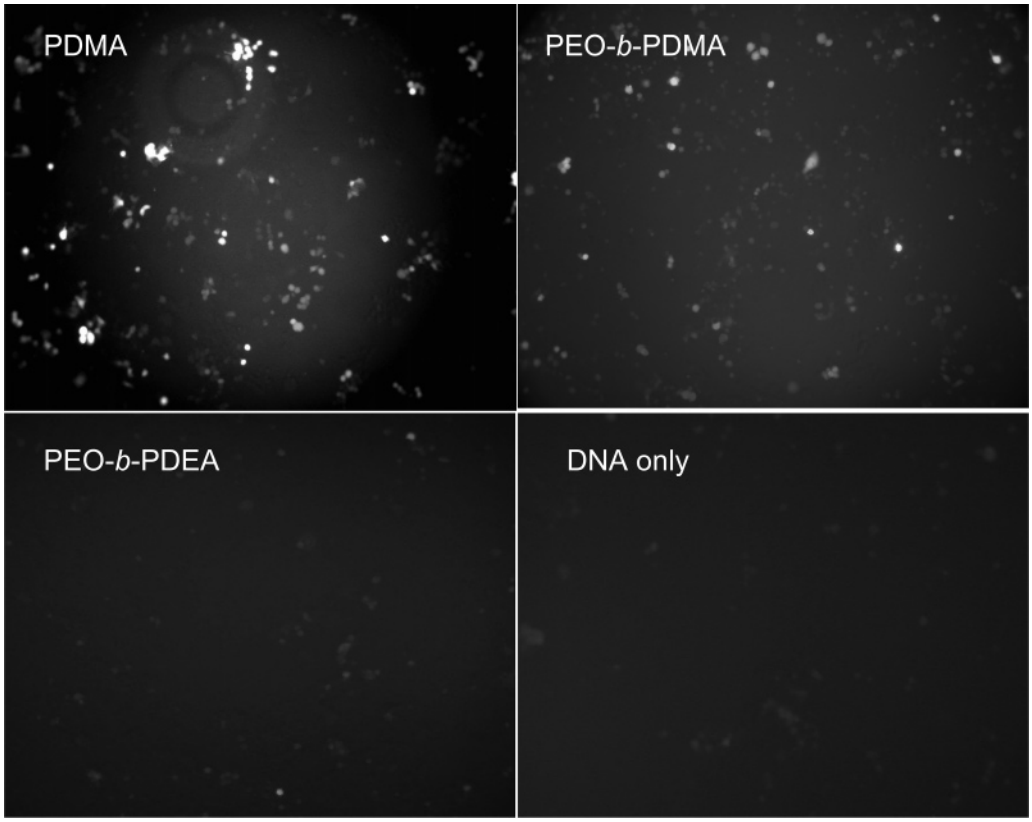

Figure 6. Fluorescence microscopy images of EGFP-transfected Neuro2A cells with various transfection systems at optimum polymer concentration (N/P ratio ≈5−10).

which may result in significantly higher cytotoxicity as compared to the hydrophilic PDMA polymers.

In Vitro Transfection of Neuro2A Cells. Fluorescence spectrometry was used to evaluate the transfection efficiencies at the protein level for the various polymer systems. The data are normalized against the highest intensity in the experiment that is the fluorescence level of 30 μg/mL of PDMA polyplexes. The fluorescence levels were significantly higher when using either PDMA or PEO-*b*-PDMA as compared to PEO-PDEA polyplexes (Figure 5). The number of cells expressing EGP was found to correlate with the fluorescence measurements (Figure 6). Two other cell-lines (Hela and PC12) showed the same trend of transfection efficiencies.

The results above show that, although PDMA and PDEA polymers have similar chemical structures, they exhibited significant differences in the physical, cytotoxicity, and transfection efficiencies. To identify the bottleneck in the transfection pathway, we next investigated the accumulation of the plasmid DNA and the expression of the transgene.

Real-Time PCR Results. The amounts of mRNA expressed and the amounts of plasmid DNA accumulated in the cells were quantified by real-time PCR (Figure 7b and c). As a control in quantifying mRNA, no significant amplification of the transgene was observed when reverse transcription was omitted (data not shown). The amplification of the standards in Figure 7a yielded a slope of 3.47, corresponding to a high amplification efficiency of about 90%. The dynamic range of amplification was greater than 5 log dilutions (10^{-16}−10^{-21} mol). Amplification signals detected in the no-template control (W) case were due to the formation of primer-dimer, as verified by gel electrophoresis (data not shown).

High expression levels of EGFP transcripts were observed in cells transfected with PDMA or PEO-*b*-PDMA polyplexes (C_t value ≈ 12) as compared to cells transfected with naked EGFP (C_t value ≈ 22). The difference in the C_t values of studies using naked DNA and PDMA polyplexes was about 10 cycles, which is about a 1000-fold difference in the amounts of transgene uptaken (Figure 7b and c). The amount of EGFP plasmid uptaken by the cells using PEO-*b*-PDEA polyplexes (C_t value ≈ 18) was significantly lower than that using PDMA polyplexes. Because a similar rank order was observed between the protein and mRNA expression levels with the use of various polymers, the translation of mRNA to protein is unlikely to be a major transfection barrier. The real-time PCR results for plasmid DNA accumulation at the cellular level are shown in Figure 7c. Six hours after the transfection, DNA (both genomic and plasmid DNA) was extracted, and the plasmid DNA was then quantified by real-time PCR. The overall trends for the various systems at the DNA cellular level were similar to those observed at the mRNA level.

The effects of polymer concentrations used in transfection and the levels of transgene plasmid DNA and mRNA in cells are shown in Figure 8. The levels of mRNA were normalized against GAPDH expression. The level of cellular DNA is normalized to total number of cells for each experiment. Both mRNA and plasmid DNA accumulation at cellular levels were similar when transfected with either PDMA or PEO-*b*-PDMA polyplexes, suggesting that the presence of PEO does not have a significant effect on the transfection pathway. For the PEO-*b*-PDEA polyplexes, the cellular plasmid DNA content was significantly lower than that of the PDMA and PEO-*b*-PDMA systems. This observation suggests that the possible barrier to the use of the PEO-*b*-PDEA system may reside in the inability of the polyplexes to be associated and/or transported across the cellular membrane and not due to the inability of the polyplex to release the plasmid or enhanced degradation of the plasmid intracellularly.

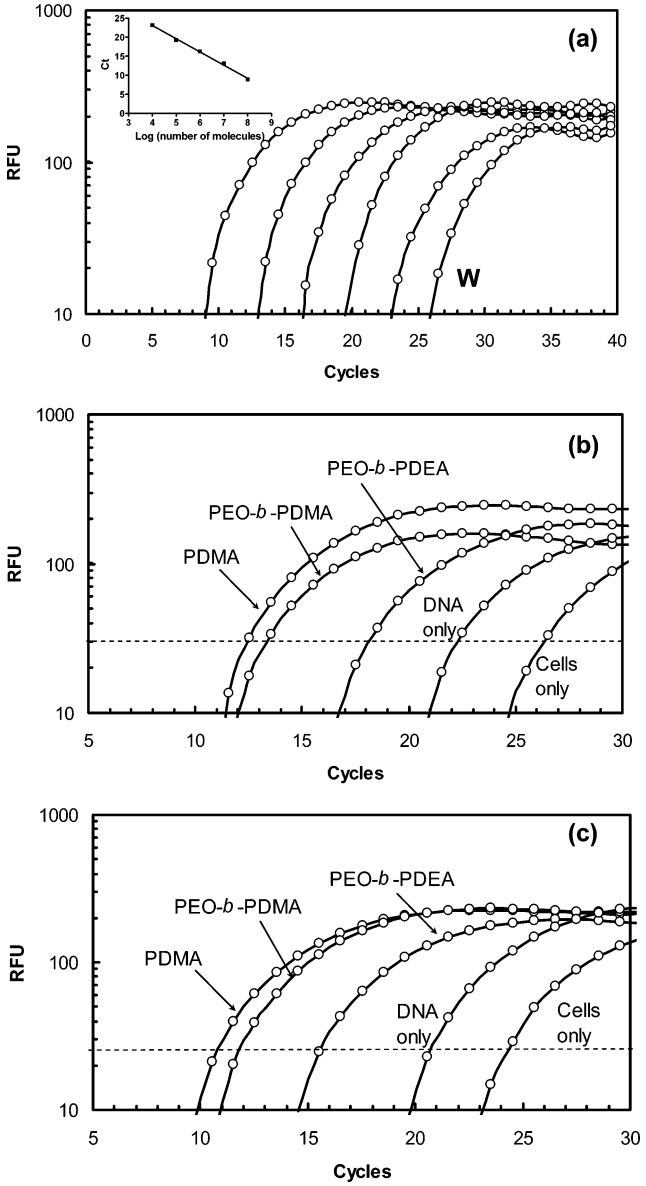

Figure 7. Real-time PCR results. (a) Amplification of five log dilution of EGFP standards. Negative controls with no templates (W) were carried out simultaneously. Inset: the slope of the plot of *Ct* versus −log mol of the EGFP standards in triplicates was 3.47 ± 0.07 (r^2 = 0.99). (b) Amplification of various optimized transfection agents at mRNA level. The expressions of GAPDH were similar in all of the samples. (c) Amplification of various optimized transfection agents at DNA cellular uptake level. The amplification signals from non-transfected cells (cells only) were due to the formation of primer-dimer. The experiments were repeated twice.

Discussion

We have evaluated three polycation vectors with similar chemical structures for their DNA transfection efficiency. Researchers have reported that, for particle sizes formed after polyplexing, smaller than 200 nm facilitate endocytosis[28] and increase diffusion in the cytoplasm.[29] However, this study shows that, despite the similarities in chemical structures, the PEO-*b*-PDEA polyplex showed notable differences in transfection efficiencies. There are a number of possibilities for this observation, which include the failure of this polyplex to interact with the membrane, low cellular internalization, problems in trafficking to the correct compartments, low unpacking efficiency, unfavorable nuclear mechanisms in transcription, and translation of the transgene.[6]

To elucidate the potential barriers in transfection when using PEO-*b*-PDEA polyplex, it was essential to quantify the cellular accumulation of transgene plasmid DNA and the expressed mRNA. Quantitative real-time PCR results showed that high amounts of plasmid DNA accumulated in cells soon after transfection with PDMA and PEO-PDMA but not PEO-*b*-PDEA polyplexes, suggesting that the transfection barrier is at the extracellular membrane level. This raised an apparent paradox as PEO-*b*-PDEA polyplexes exhibited high toxicity at concentrations comparable to those of the other polymers used. To address this issue, LDH released on exposure to the polyplexes showed that the membrane disruption effect of PEO-*b*-PDEA polyplexes was significantly less than the free PEO-*b*-PDEA polymers, indicative that the membrane disruption is primarily due to the uncomplexed PEO-*b*-PDEA polymers. Similar observations showing that free polymers caused higher cytotoxicity than their polyplexes have been reported previously.[30,31]

A hypothetical model illustrating the possible mechanism of membrane−polyplexes interaction of the different polymethacrylate systems in in vitro cell transfection is shown in Figure 9.

In this model, the positively charged PDMA polyplexes (Figure 9a) are thought to adsorb to the cellular membrane walls due to electrostatic interactions, after which they are internalized into the cells. Unbound PDMA or PEO-*b*-PDMA polymer chains could similarly adsorb electrostatically onto the cell membrane but do not cause significant membrane disruption, as they could not penetrate the hydrophobic segment of the cell membranes. This suggestion is consistent with the evidence from studies on the LDH release, which indicated minimal membrane disruption. Previous studies have shown that PDMA polymer has a minimum effect on the membrane and that, by increasing the interaction between PDMA and cell membrane, improvements in transfection efficiencies have been observed.[32,33]

Unlike the other two polymer systems, PEO-*b*-PDEA polyplexes by themselves do not interact favorably with the membrane. As electrostatic interactions are known to be the main driving force for the interaction of polyplexes with the cell membrane, especially in the absence of a target ligand,[34,35] it is likely that the near-to-neutrally charged surfaces of PEO-*b*-PDEA polyplexes may be unfavorable to allow significant interaction with the membrane. This lowered electrostatic interaction with cell membranes results in lower cellular uptake and hence poorer transfection when compared to the PDMA systems. The excessive interaction of the membrane with the free amiphiphilic PEO-*b*-PDEA polymer chains may contribute to the disruption of the membrane, thus reducing the cellular accumulation of plasmid DNA. Thus, the complexation of PEO-*b*-PDEA polymer chains with plasmid DNA reduced the amount of unbound polymer, resulting in lower cytotoxicity. The mechanism of membrane destabilization due to amiphiphilic PEO-*b*-PDEA may be similar to other types of surfactants and polymers that are known to disrupt phospholipids containing membrane.[36−38]

In recent years, a large number of novel cationic polymer and surfactant systems have been reported and characterized for gene transfection. Yet, in some instances, the transfection systems may have good biophysical characteristics with enhanced endosomal escape[39] or resistance to enzymatic degradation[29] or even with targeted moiety[40] properties but showed poor expressions of the transgene at the protein levels. The reasons for the poor correlation between their biophysical properties and transfection efficiencies have yet to be defined, and the strategies described in this study will be useful in elucidating potential barriers to transfection.

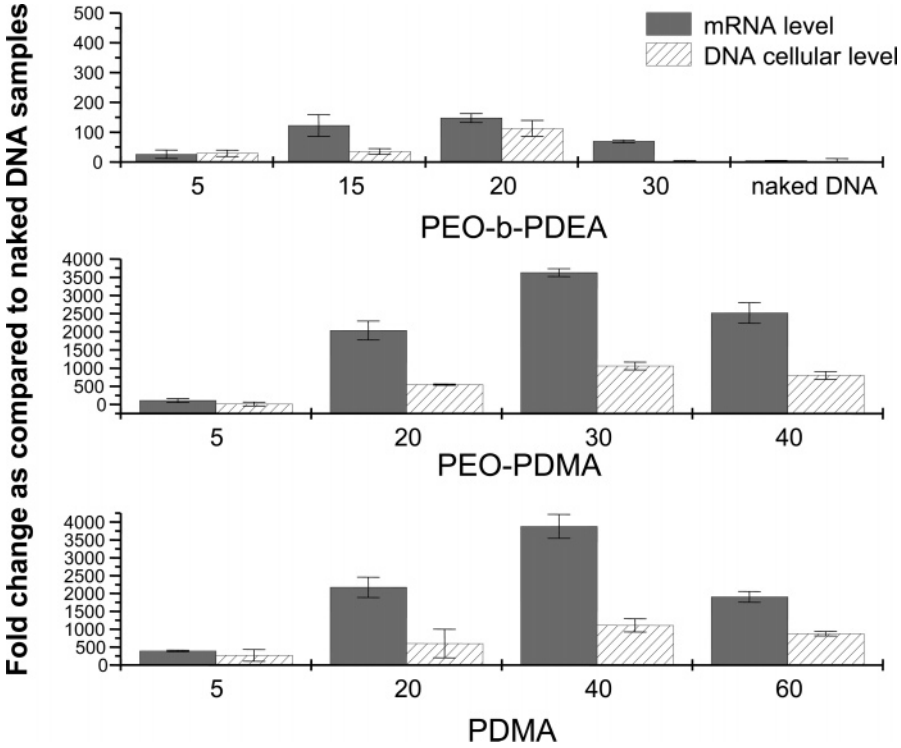

Figure 8. Transfection efficiencies of polyplexes as compared to naked DNA. The results from EFGP mRNA measurements were normalized against the expression of GAPDH. The amounts of plasmids in the cells were normalized against the number of cells plated. The ratios of the amount of EGFP mRNA and plasmids were then compared to the amounts found in cells transfected with naked DNA alone. The results are expressed as mean ± standard deviation of four determinations. The experiment was repeated twice.

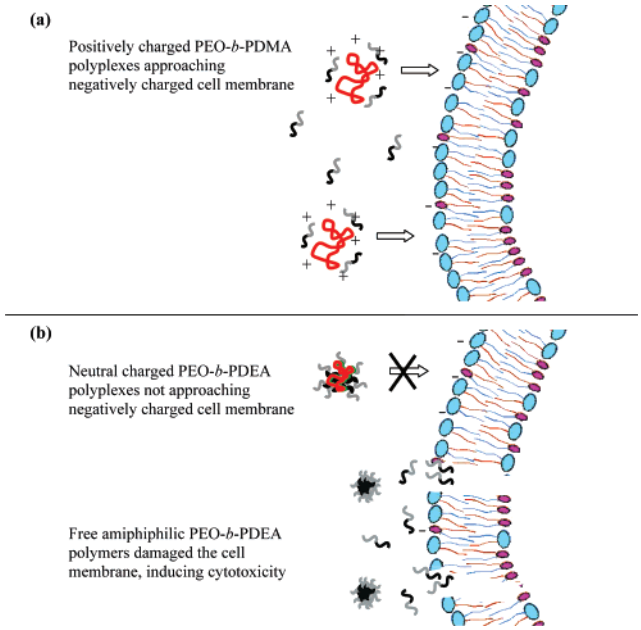

Figure 9. Hypothetical model of cellular membrane−polyplexes interactions for (a) PDMA and PEO-*b*-PDMA polyplexes, and (b) PEO-*b*-PDEA polyplexes during the in vitro cell transfection.

Conclusions

We have evaluated the transfection efficiency of three cationic poly(methacrylate) systems at various levels: protein, mRNA, and plasmid DNA accumulation. Although the three polymers are similar structurally, their transfection efficiencies were significantly different. The results of real-time PCR together with cytotoxicity and physical properties measurements suggested that, in the case of PEO-*b*-PDEA, cellular uptake is probably the major barrier to transfection. Furthermore, the amiphiphilic nature of the free polymer may be responsible for the disruption of the membrane, leading to significant cytotoxicity at high concentrations. For PDMA and PEO-*b*-PDMA polyplexes, the transfection efficiencies were significantly better than PEO-*b*-PDEA, and this may be due to the electrostatic adsorption of the positively charged polyplexes to the cell surface. This study demonstrated that transfection barriers could be investigated in greater detail by correlating the expression levels at the protein, mRNA, and cellular plasmid DNA levels using real-time PCR with the physical properties of the gene carrier. These insights into the structure−transfection relationship could prove to be useful for the optimization of other polymeric gene delivery system.

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References and Notes

(1) Mahato, R. I.; Henry, J.; Narang, A. S.; Sabek, O.; Fraga, D.; Kotb, M.; Gaber, A. O. Cationic lipid and polymer-based gene delivery to human pancreatic islets. *Mol. Ther.* **2003**, *7*, 89−100.
(2) Pinthus, J. H.; Waks, T.; Kaufman-Francis, K.; Schindler, D. G.; Harmelin, A.; Kanety, H.; Ramon, J.; Eshhar, Z. Immuno-gene therapy of established prostate tumors using chimeric receptor-redirected human lymphocytes. *Cancer Res.* **2003**, *63*, 2470−2476.
(3) Roth, C. M.; Sundaram, S. Engineering synthetic vectors for improved DNA delivery: Insights from intracellular pathways. *Annu. Rev. Biomed. Eng.* **2004**, *6*, 397−426.
(4) Pannier, A. K.; Shea, L. D. Controlled release systems for DNA delivery. *Mol. Ther.* **2004**, *10*, 19−26.
(5) Niidome, T.; Huang, L. Gene therapy progress and prospects: Nonviral vectors. *Gene Ther.* **2002**, *9*, 1647−1652.

(6) Pack, D. W.; Hoffman, A. S.; Pun, S.; Stayton, P. S. Design, and Development of polymers for gene delivery. *Nat. Rev.* **2005**, *4*, 581−593.
(7) Belenkov, A. I.; Alakhov, V. Y.; Kabanov, A. V.; Vinogradov, S. V.; Panasci, L. C.; Monia, B. P.; Chow, T. Y. K. Polyethyleneimine grafted with pluronic P85 enhances Ku86 antisense delivery and the ionizing radiation treatment efficacy in vivo. *Gene Ther.* **2004**, *11*, 1665−1672.
(8) Putnam, D.; Gentry, C. A.; Pack, D. W.; Langer, R. Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 1200−1205.
(9) Azzam, T.; Eliyahu, H.; Makovitzki, A.; Linial, M.; Domb, A. J. Hydrophobized dextran-spermine conjugate as potential vector for in vitro gene transfection. *J. Controlled Release* **2004**, *96*, 309−323.
(10) Mastrobattista, E.; Kapel, R. H. G.; Eggenhuisen, M. H.; Roholl, P. J. M.; Crommelin, D. J. A.; Hennink, W. E.; Storm, G. Lipid-coated polyplexes for targeted gene delivery to ovarian carcinoma cells. *Cancer Gene Ther.* **2001**, *8*, 405−413.
(11) van Dijk-Wolthuis; van de Wetering; Hinrichs, W.; Hofmeyer, L.; Liskamp, R.; Crommelin, D.; Hennink, W. A versatile method for the conjugation of proteins and peptides to poly[2-(dimethylamino)-ethyl methacrylate]. *Bioconjugate Chem.* **1999**, *10*, 687−692.
(12) Megeed, Z.; Haider, M.; Li, D. Q.; O'Malley, B. W.; Cappello, J.; Ghandehari, H. In vitro and in vivo evaluation of recombinant silk-elastinlike hydrogels for cancer gene therapy. *J. Controlled Release* **2004**, *94*, 433−445.
(13) Varga, C.; Tedford, N.; Thomas, M.; Klibanov, A.; Griffith, L.; Lauffenburger, D. Quantitative comparison of polyethylenimine formulations and adenoviral vectors in terms of intracellular gene delivery processes. *Gene Ther.* **2005**, 1−10.
(14) Chesnoy, S.; Huang, L. Structure and function of lipid-DNA complexes for gene delivery. *Annu. Rev. Biophys. Biomol. Struct.* **2000**, *29*, 27−47.
(15) Wang, K. C.; Wu, J. C.; Chung, Y. C.; Ho, Y. C.; Chang, D. T.; Hu, Y. C. Baculovirus as a highly efficient gene delivery vector for the expression of hepatitis delta virus antigens in mammalian cells. *Biotechnol. Bioeng.* **2004**, *89*, 464−473.
(16) Ouahabia, A.; Thiryb, M.; Schiffmannc, S.; Fuksd, R.; Nguyen, H.; Ruysschaerta, J. M.; Vandenbrandena, M. Intracellular visualization of BrdU-labeled plasmid DNA/cationic liposome complexes. *J. Histochem. Cytochem.* **1999**, *47*, 1159−1166.
(17) Kinoshita, A.; Fukumoto, H.; Shah, T.; Whelan, C. M.; Irizarry, M.; Hyman, B. Demonstration by FRET of BACE interaction with the amyloid precursor protein at the cell surface and in early endosomes. *J. Cell Sci.* **2003**, *116*, 3339−3346.
(18) Wong, M. L.; Medrano, J. F. Real-time PCR for mRNA quantitation. *BioTechniques* **2005**, *39*, 75−85.
(19) Too, H. P. Real time PCR quantification of GFR alpha-2 alternatively spliced isoforms in murine brain and peripheral tissues. *Mol. Brain Res.* **2003**, *114*, 146−153.
(20) Gygi, S. P.; Rochon, Y.; Franza, B. R.; Aebersold, R. Correlation between protein and mRNA abundance in yeast. *Mol. Cell. Proteomics* **1999**, *19*, 1720−1730.
(21) Chen, G. A.; Gharib, T. G.; Huang, C. C.; Taylor, J. M. G.; Misek, D. E.; Kardia, S. L. R.; Giordano, T. J.; Iannettoni, M. D.; Orringer, M. B.; Hanash, S. M.; Beer, D. G. Discordant protein and mRNA expression in lung adenocarcinomas. *Mol. Cell. Proteomics* **2002**, *1*, 304−313.
(22) Zabner, J.; Fasbender, A.; Moninger, T.; Poellinger, K.; Welsh, M. Cellular and molecular barriers to gene transfer by a cationic lipid. *J. Biol. Chem.* **1995**, *270*, 18997−19004.
(23) Bromberg, L.; Deshmukh, S.; Temchenko, M.; Iourtchenko, L.; Alakhov, V.; Alvarez-Lorenzo, C.; Barreiro-Iglesias, R.; Concheiro, A.; Hatton, T. A. Polycationic block copolymers of poly(ethylene oxide) and poly(propylene oxide) for cell transfection. *Bioconjugate Chem.* **2005**, *16*, 626−633.
(24) Tan, J. F.; Ravi, P.; Too, H. P.; Hatton, T. A.; Tam, K. C. Association behavior of biotinylated and non-biotinylated PEO-*b*-PDEAEMA. *Biomacromolecules* **2005**, *6*, 498−506.
(25) Tan, J. F.; Too, H. P.; Hatton, T. A.; Tam, K. C. Aggregation behavior and thermodynamics of binding between PEO-PDEAEMA and plasmid DNA. *Langmuir* **2006**, *22*, 3744−3750.
(26) Moghimi, S. M.; Symonds, P.; Murray, J. C.; Hunter, A. C.; Debska, G.; Szewczyk, A. A two-Stage poly(ethylenimine)-mediated cytotoxicity: Implications for gene transfer/therapy. *Mol. Ther.* **2005**, *11*, 990−995.
(27) Vihola, H.; Laukkanen, A.; Valtola, L.; Tenhu, H.; Hirvonen, J. Cytotoxicity of thermosensitive polymers poly(*N*-isopropylacrylamide), poly(*N*-vinylcaprolactam) and amphiphilically modified poly(*N*-vinylcaprolactam). *Biomaterials* **2005**, *26*, 3055−3064.
(28) Noria, A.; Kopecek, J. Intracellular targeting of polymer-bound drugs for cancer chemotherapy. *Adv. Drug Delivery Rev.* **2005**, *57*, 609−636.
(29) Fischer, D.; Dautzenberg, H.; Kunath, K.; Kissel, T. Poly(diallyldimethylammonium chlorides) and their *N*-methyl-*N*-vinylacetamide, copolymer-based DNA-polyplexes: role of molecular weight and charge density in complex formation, stability, and in vitro activity. *Int. J. Pharm.* **2004**, *280*, 253−269.
(30) Kunath, K.; von Harpe, A.; Fischer, D.; Kissel, T. Galactose-PEI−DNA complexes for targeted gene delivery: degree of substitution affects complex size and transfection efficiency. *J. Controlled Release* **2003**, *88*, 159−172.
(31) Fischer, D.; Li, Y. X.; 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.
(32) Dailey, L. A.; Kleemann, E.; Merdan, T.; Petersen, H.; Schmehl, T.; Gessler, T.; Hanze, J.; Seeger, W.; Kissel, T. Modified polyethylenimines as non viral gene delivery systems for aerosol therapy: effects of nebulization on cellular uptake and transfection efficiency. *J. Controlled Release* **2004**, *100*, 425−436.
(33) Christiaens, B.; Dubruel, P.; Grooten, J.; Goethals, M.; Vandekerckhove, J.; Schacht, E.; Rosseneu, M. Enhancement of polymethacrylate-mediated gene delivery by Penetratin. *Eur. J. Pharm. Sci.* **2005**, *24*, 525−537.
(34) Mounkes, L. C.; Zhong, W.; Cipres-Palacin, G.; Heath, T. D.; Debs, R. J. Proteoglycans mediate cationic liposome-DNA complex based gene delivery in vitro and in vivo. *J. Biol. Chem.* **1998**, *40*, 26164−70.
(35) Ryhanen, S. J.; Saily, M. J.; Paukku, T.; Borocci, S.; Mancini, G.; Holopainen, J. M.; Kinnunen, P. K. J. Surface charge density determines the efficiency of cationic gemini surfactant based lipofection. *Biophys. J.* **2003**, *84*, 578−587.
(36) de la Maza, A.; Coderch, L.; Gonzalez, P.; Parra, J. L. Subsolubilizing alterations caused by alkyl glucosides in phosphatidylcholine liposomes. *J. Controlled Release* **1998**, *52*, 159−168.
(37) Vial, F.; Rabhi, S.; Tribet, C. Association of octyl-modified, poly(acrylic acid) onto unilamellar vesicles of lipids and kinetics of vesicle disruption. *Langmuir* **2005**, *21*, 853−862.
(38) Warisnoicharoen, W.; Lansley, A. B.; Lawrence, M. J. Toxicological evaluation of mixtures of nonionic surfactants, alone and in combination with oil. *J. Pharm. Sci.* **2003**, *92*, 859−868.
(39) Funhoff, A. M.; van Nostrum, C. F.; Koning, G. A.; Schuurmans, N. M. E.; Crommelin, D. J. A.; Hennink, W. E. Endosomal escape of polymeric gene delivery complexes is not always enhanced by polymers buffering at low pH. *Biomacromolecules* **2004**, *5*, 32−39.
(40) Dauty, E.; Remy, J. S.; Zuber, G.; Behr, J. P. Intracellular delivery of nanometric DNA particles via the folate receptor. *Bioconjugate Chem.* **2002**, *13*, 831−839.

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