DOI: 10.1002/cmdc.200700093

pH-Responsive Multi-PEGylated Dual Cationic Nanoparticles Enable Charge Modulations for Safe Gene Delivery

May P. Xiong,[a] Younsoo Bae,[b] Shigeto Fukushima,[c] M. Laird Forrest,[a] Nobuhiro Nishiyama,[b] Kazunori Kataoka,*[b, c] and Glen S. Kwon*[a]

In gene therapy, the cytotoxicity of many polycations is undesirable and has been attributed to nonspecific membrane destabilizing effects and intracellular polyplex-mediated toxicity. To help prolong the pharmacokinetic profile of nonviral vehicles for gene delivery, the cationic surface charge of current systems is typically shielded through the conjugation of polyethylene glycol (PEG) chains to the particle surface. However, the design of an intelligent polycation with environment-sensing charge modulations is essential to minimize cytotoxicity and enhance gene expression. We have designed a novel di-cationic block copolymer, poly(aspartate-hydrazide)-block-*poly(L-lysine), capable of pH-mediated endosomal membrane disruption based on charge interactions, which has negligible toxicity elsewhere to the cell. The poly(L-lysine) segment, with a high pK_a value of ~9.4, preferentially forms a poly-ion complex with the negative phosphate groups of pDNA, whereas the pH-responsive poly(aspartate-hydrazide) segment, with the comparatively lower pK_a ~5.0, is characterized by a substantial fraction of unprotonated amino groups at physiological pH. As a consequence, complexation between such a polymer and pDNA leads to the formation of a two-layered nanoparticle. In particular, the nanoparticle possesses an unprotonated pH-responsive segment to serve as both a scaffold for acid-labile linkages of various moieties such as aldehyde-PEG and to transition from neutral to charged for disrupting endosomal membranes, and safely enhancing gene expression. Our system supports an endosomal escape mechanism based on charge interactions rather than the proton-sponge effect, and may be an important step towards engineering new classes of intelligent nonviral vectors.*

Introduction

Intelligent biomaterials that can emulate natural viruses by adapting to their environment with minimal toxicity to the cell are highly desired for gene therapy. These synthetic vehicles are often internalized in many different cell lines through receptor-mediated endocytosis into clathrin-coated vesicles that fuse to form early endosomes (pH 7.4–6), which become late endosomes (pH 6.0–5.5), and eventually lysosomes (pH 5.0).[1–3] Research to date has primarily emphasized gene delivery carriers equipped with endosomal buffering, also known as the proton sponge effect (PSE),[4] to escape acidic endolysosomes for mediating gene expression.[5,6] However, natural viruses are not equipped with endosomal buffering properties. Instead, they frequently exploit the decrease in pH to expose fusogenic domains that can disrupt the endosomal or lysosomal membrane, resulting in release of the virus into the cytoplasm. Similarly, endosomal membrane disruptions with charged surfaces may also play an important role in helping particles escape from endosomes.[7,8] Despite their positive attributes, many common polycations are cytotoxic due to nonspecific membrane destabilizing effects and intracellular polyplex-mediated toxicity.[9–12] From these observations, we have postulated that the appreciable effect of charge interaction on endosomal escape might be observed even in the absence of the PSE by designing a pH-responsive polycation with dual functional pK_a values (high and low) to emulate the escape mechanism used by natural viruses.

[a] *Dr. M. P. Xiong, Dr. M. L. Forrest, Prof. G. S. Kwon*
Division of Pharmaceutical Sciences
School of Pharmacy, University of Wisconsin
777 Highland Avenue, Madison, WI 53705-2222 (USA)
Fax: (+1) 608-262-5345
E-mail: gskwon@pharmacy.wisc.edu

[b] *Dr. Y. Bae, Dr. N. Nishiyama, Prof. K. Kataoka*
Center for Disease Biology and Integrative Medicine
Graduate School of Medicine, The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033 (Japan)
and
Center for NanoBio Integration, The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656 (Japan)
Fax: (+81) 3-5841-7139
E-mail: kataoka@bmw.t.u-tokyo.ac.jp

[c] *Dr. S. Fukushima, Prof. K. Kataoka*
Department of Materials Engineering
Graduate School of Engineering, The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656 (Japan)

Supporting information for this article is available on the WWW under http://www.chemmedchem.org or from the author.

Results and Discussion

Chemical synthesis

Figure 1 shows the chemical structure of the dual-cationic block copolymer, poly(aspartate-hydrazide)-*block*-poly(L-lysine) (abbreviated BC), based on functional pK_a differences between respective cationic blocks. The poly(L-Lys) (PLL) segment, with a high pK_a value of ~9.4, preferentially forms a poly-ion complex (PIC) with the negative phosphate groups of pDNA, whereas the pH-responsive poly(Asp-Hyd) segment, with a comparatively lower pK_a value of ~5.0, is characterized by a substantial fraction of unprotonated amino groups at physiological pH (Figure 1a). Some of these Asp-Hyd residues are coupled to aldehyde-PEG chains (ALD-PEG) through acid-labile hydrazone linkages to impart favorable stealth properties to the PIC (Figure 1b). As a consequence, the complexation between such a polymer and pDNA may lead to the formation of a two-layered particle possessing an unprotonated pH-responsive poly(Asp-Hyd) segment that functions as both a scaffold for acid-labile linkages of various moieties, and that has the ability to undergo a transition from neutral to charged for disrupting endosomal membranes, as illustrated in Figure 2. Therefore, the PIC described herein is formed from associations of such block copolymer chains: a) a pDNA condensing core made of PLL chains (known for not contributing to the PSE), and b) a shell composed of segments with repeating Asp-Hyd residues.

The backbone of the block copolymer is derived from poly(β-benzyl-L-aspartate)-*block*-poly(L-lysine) (PBLA-*b*-PLL; see Supporting Information). PBLA was prepared by the ring-opening polymerization of β-benzyl-L-aspartate *N*-carboxyanhydride (BLA-NCA), initiated by the terminal $-NH_2$ group of butylamine to yield a polymer with narrow distribution and degree of polymerization (DP) of 36 (Figure 1S). The terminal amine on PBLA was used to initiate ring-opening polymerization of lysine(TFA) *N*-carboxyanhydride monomers (Lys-NCA) to yield PBLA-*b*-PLL(TFA) with DP = 53, in accordance with previous work in which a PLL length of ~50 was determined optimal for gene expression (Figure 2S).[5,13] Next, hydrazide groups were conjugated with a substitution efficiency near 100 mol% on PBLA, using a procedure reported by Bae et al.,[14,15] to give poly(Asp-Hyd)-*b*-PLL(TFA) (TFA = trifluoroacetyl). When deprotected, this polymer yields BC. Finally, ALD-PEG (M_W = 7000 g mol^{-1}) was synthesized following procedures reported by Nagasaki et al.[16] and characterized with ^{1}H NMR ($CDCl_3$; determined 91% acetal conversion into aldehyde groups) and gel permeation chromatography (GPC) (TSK-gel G3000PWXL and TSK-gel G4000PWXL; 10 mM LiCl in *N*,*N*-dimethylformamide (DMF); 0.8 mL min^{-1}; polydispersity index (PDI): 1.03). The final PEGylated block copolymers [poly(Asp-Hyd-PEG)-*b*-PLL] had either five ALD-PEG chains conjugated to each poly(Asp-Hyd) block to form the acid-labile hydrazone linkage (pH-PBC), or six COOH-PEG chains conjugated covalently by amide linkages to hydrazide groups to generate the non-hydrolysable control (cov-PBC), as shown in Figures 3S and 4S. There are substantial unconjugated hydrazide groups that remain on each block copolymer chain.

Physical characterization

The protonation profile of BC reveals that hydrazide groups do not significantly buffer in the critical range known to be essential for PSE-mediated escape of endosomes (pH 5.0–6.0), similar

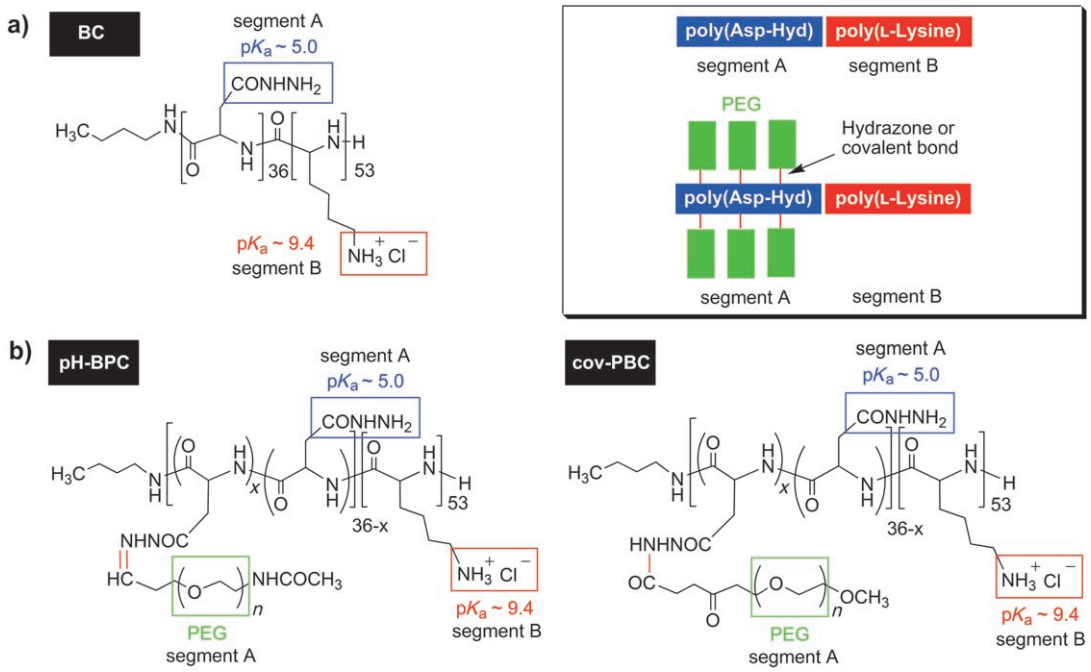

Figure 1. Structure of the block copolymers: a) poly(aspartate-hydrazide)-*block*-poly(L-lysine) (BC); b) pH-sensitive poly(aspartate-hydrazide-PEG)-*block*-poly(L-lysine) (pH-PBC), and covalent poly(aspartate-hydrazide-PEG)-*block*-poly(L-lysine) (cov-PBC). (See Supporting Information for synthesis.)

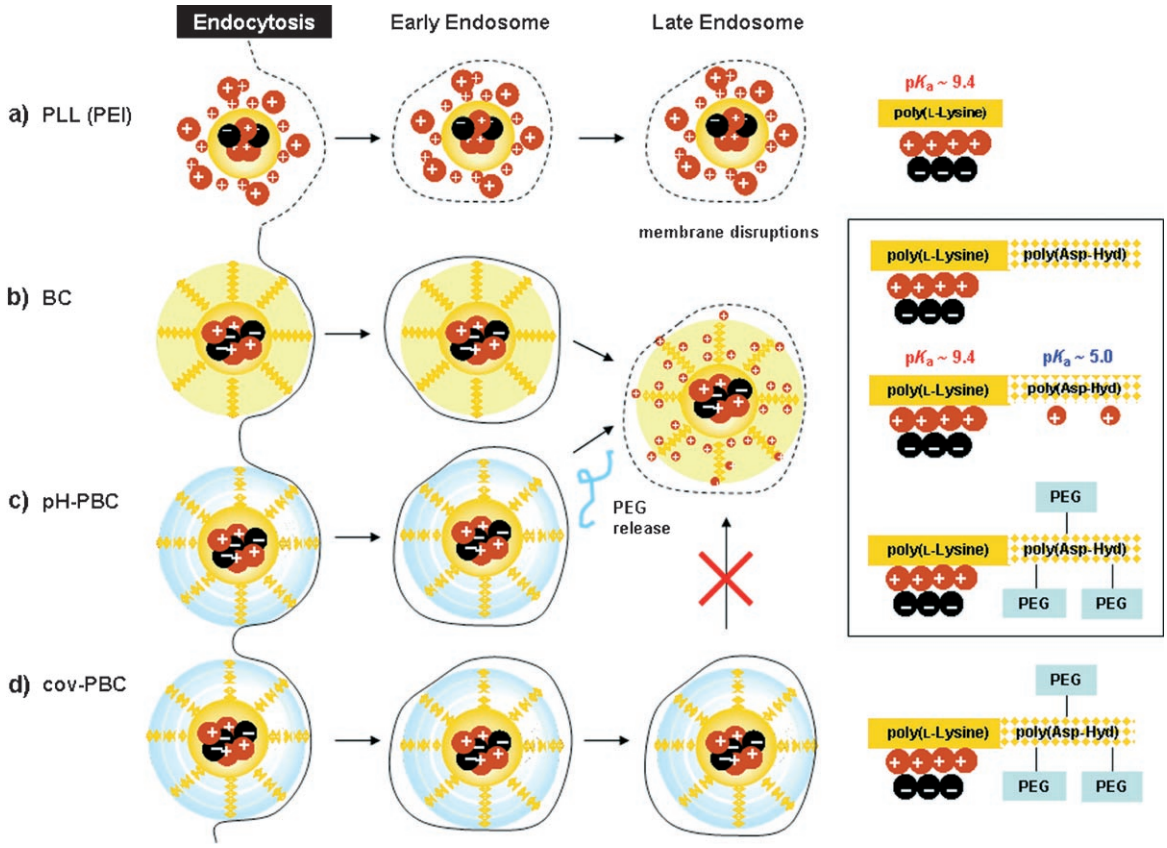

Figure 2. a) Cationic polyplexes formed with PLL (or PEI) are cytotoxic to cells. In contrast, neutral shielded particles [parts b), c), and d)] cause minimal membrane damage during endocytosis: Following a decrease in pH, hydrazide amino groups protonate (b) and/or acid-labile PEG chains are released (c). This increases endosomal membrane disturbances with the resulting charged particle and enhances escape into the cytosol (pH 7.4), whereupon sensing the new pH, hydrazide amino groups deprotonate again, once more imparting neutral properties to the PIC and minimizing intracellular toxicity. d) cov-PBC particles cannot release PEG chains following a decrease in pH, and this may minimize particle interactions with the endosomal membrane.

to PLL (Figure 3), and furthermore do not contribute significantly to pGL3 condensation at physiological pH, because mostly PLL groups undergo electrostatic interactions with pDNA (Figure 4). This suggests that the PIC formed is composed of a PLL–DNA core with poly(Asp-Hyd) segments on the surface. The effect of pH on colloidal properties of the PIC was also investigated (Figure 5). At pH 7.4, PICs formed with BC have mostly unprotonated hydrazide groups exposed on the surface (~5.0 mV), and counterion screening causes particles to exhibit aggregation behavior (diameter ~440 nm). However, as hydrazide groups on PICs become protonated with decreasing pH, charged cationic surfaces are generated (~35 mV), overcoming van der Waals interactions and resulting in electrostatic repulsions between individual particles (~100 nm). For pH-PBC, the size and ζ profiles are more informative than the averages reported (Figure 5, insert). Although average $\zeta < 8$ mV and average diameter is < 100 nm over the range of pH, the system is represented by distinct multimodal populations, indi-

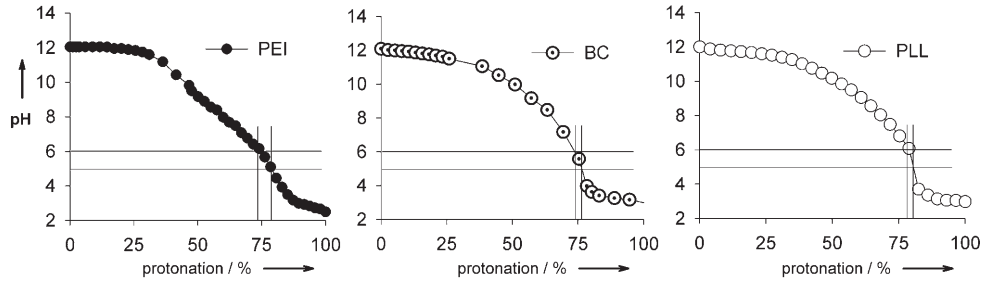

Figure 3. Protonation profiles comparing branched PEI, BC, and PLL. As expected, PEI can buffer over a relatively wide pH range. BC carries little buffering capacity, similar to PLL (note: error bars omitted to simplify profile). Table 1S summarizes the mol% of various amines on each polymer; only side chain terminal amines are considered. The data indicate that the presence of hydrazide groups on BC does not contribute significantly to endosomal buffering in the critical range of pH 5.0–6.0 essential to the PSE.

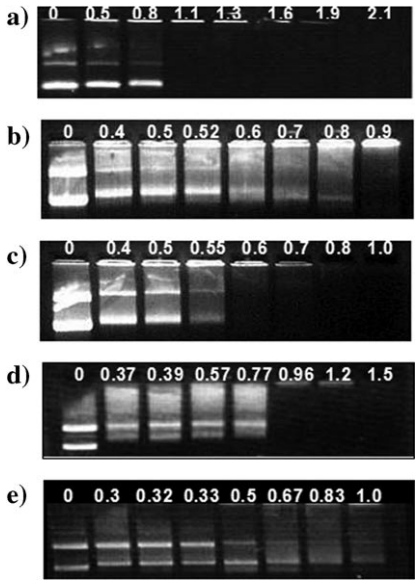

Figure 4. Isoelectric points (pI) of the polymer in complex with 1 μg pGL3; values indicate equivalent N/P ratios: a) PEI (25 000 g mol^{-1}), pI (*w*/*w*) = 0.20 μg, N/P = 1.1; b) BC (poly(Asp-Hyd)$_{36}$-PLL$_{49}$) (11 000 g mol^{-1}), pI (*w*/*w*) = 0.65 μg, N/P = 0.9; c) PLL$_{44}$ (9200 g mol^{-1}), pI = 0.40 μg, N/P = 0.7; d) pH-PBC (five PEG chains per BC, 46 000 g mol^{-1}), pI = 2.5 μg, N/P = 0.96; e) cov-PBC (six PEG chains per BC, 53 000 g mol^{-1}), pI = 4 μg, N/P = 1.0. Electrostatic interactions occur mostly between lysine groups and pGL3, as BC and PBC polymers have similar N/P ratios of ~1.

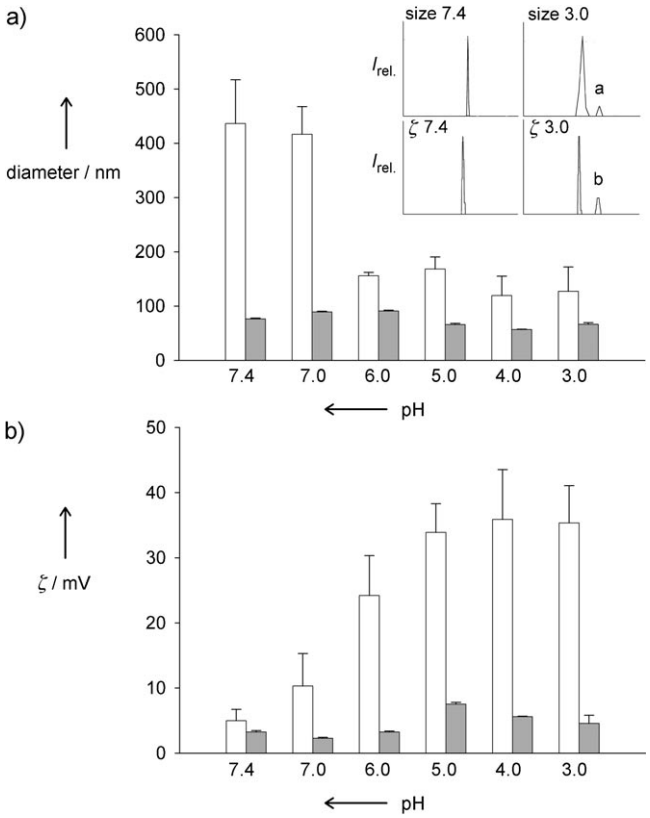

Figure 5. a) Size and b) ζ profiles for BC (white) and pH-PBC (gray) at the indicated pH values. Particles were formed at N/P = 20 (to concur with the transfection experiments) with 2 μg pGL3 in water, incubated for at least 30 min, and diluted with low-ionic-strength buffers of appropriate pH before size measurement. The same sample was then injected into the instrument to obtain ζ potentials. At pH 7.4, BC particles are ~440 nm with ζ potentials of ~5 mV. At pH 3.0, BC particles are ~100 nm with ζ potentials of ~35 mV. For pH-PBC, the particles are ~80 nm in diameter with a ζ value of ~3 mV at pH 7.4. At pH 3.0, the average size is ~70 nm and ζ ~5 mV. However, pH-PBC profiles under acidic conditions (insert) point to particle instability as PEG is released. Large aggregates (peak a) with higher ζ values (peak b) appear (multimodal populations were observed for all acidic pH values). In contrast, cov-PBC had single population profiles (data not shown). (Note: the values reported were calculated by averaging the sums of peak intensities multiplied by magnitudes.)

cative of reversible PEG release. This is in sharp contrast to the single population observed with cov-PBC under acidic conditions (data not shown). pH-Release studies confirm that, in a closed system, the hydrazone linkage is reversible and exhibits equilibrium behavior at various pH values over prolonged incubation (Figure 6).

Biological characterization

In addition, subsequent lactate dehydrogenase (LDH) release studies show that our dual-cationic polymer does not cause membrane toxicity when complexed with pDNA. At physiological pH, free PEI, PLL, and BC polymer chains have cationic properties that cause high levels of membrane toxicity to cells, relative to PEG-shielded polymers (Figure 7 a,c). However, if BC (20 μg mL^{-1}) is complexed with pGL3, the presence of many unprotonated hydrazides on the surface of the PIC causes negligible membrane toxicity (Figure 7 b). As BC concentration is increased in the presence of pGL3 (40 μg mL^{-1}), LDH release also increases, but that is most likely due to the presence of excess free polymers in solution with respect to pDNA concentration (Figure 7 d). Our results imply that freely accessible charged polymers cause membrane-associated toxicity, but that membrane damage is minimized when the cationic PLL block is concealed within the PIC core.

We next compared the metabolic activity of our polymers both in free form and in complex with pGL3 to investigate the potential intracellular toxicity often associated with cationic polyplex-mediated uptake.[12] The free polymers (PEI and BC) with accessible cationic components caused greater metabolic toxicity to the cell following internalization (Figure 8 a). PEG-shielded polymers exhibited low metabolic toxicity. On the other hand, PICs with concealed cationic PLL cores (BC, pH-PBC, and cov-PBC) gave little evidence of metabolic toxicity to the cell, in contrast to PEI-formulated particles (Figure 8 b). These results correspond well with the LDH assay data.

Finally, we looked at the transfection efficiency of these polymers in five very different cell lines and found that pH-PBC and BC (both nontoxic) consistently resulted in higher gene expression relative to cov-PBC (Figure 9) and PLL (data not shown). Note that the trend in transfection between the polymers and across different cell lines is not consistent. This might be a result of the fact that in the absence of targeting ligands, various cell lines use different modes of particle internalization (such as clathrin and/or caveolae-mediated processes) that can ultimately affect uptake pathways and particle fate within the cell.[17–19] Importantly, however, there is a consistent

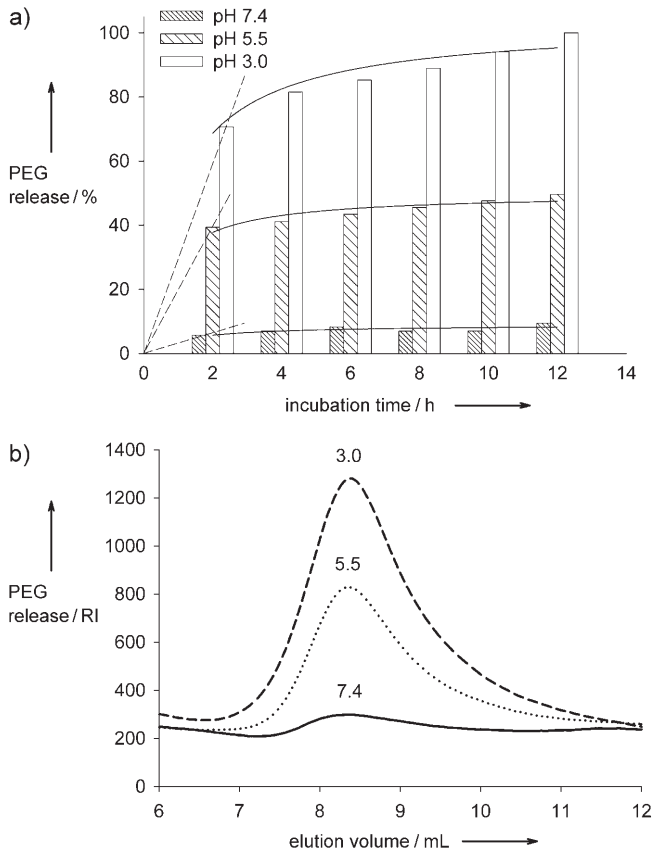

Figure 6. PEG release monitored by GPC: pH-PBC was injected into an OHpak SB-806M GPC column (injection volume: 20 μL; polymer: 2 mg mL^{-1}; of HEPES eluent: 10 mM, pH 7.4, 0.75 mL min^{-1}, 30 °C; Shodex, Kawasaki, Japan), and PEG release was detected by refractive index (RI) for 12 h. a) PEG falls off upon incubation at pH < 7.0, but the hydrazone bond is relatively stable at pH 7.4, with less than 10% PEG released over 12 h. The reversible nature of the hydrazone bond leads to an equilibrium state in the release profiles at different pH's. b) Sample curves at $t = 2$ h are shown with pH values indicated.

enhancement in gene expression for BC and pH-PBC compared with cov-PBC in the cell lines investigated. This demonstrates that a dynamic approach for release of PEG from BC is beneficial to increase endosomal membrane disruptions with charged particles, as the presence of PEG can minimize membrane interactions in the absence of charge.[20]

It is of significant interest that our dual-cationic PICs suggest an endosomal escape mechanism for polyplexes which might be attributed to disruptions based on cationic-mediated cellular membrane interactions rather than through the PSE. In other words, selectively controlling interactions between polyplexes and the cell membrane is a new and facile approach for the design of effective and high-performance nonviral gene vectors. This results in nonviral vectors that are both safe to the cell while retaining high transfection efficiencies. Indeed, unlike PEI, which induces effective transfection activity but also causes high levels of nonspecific membrane-associated toxicity (Figure 2a), neutral shielded particles (BC, pH-PBC, and cov-PBC) result in cellular uptake with minimal toxicity to the membrane (Figure 2b–d). Upon sensing a decrease in pH, PEG chains are released from pH-PBC PICs, and the remaining BC particle acquires favorable cationic properties for disrupting endosomal membranes. After the particles escape into the cytosol (pH 7.4), hydrazide groups start to deprotonate upon sensing the new pH, imparting neutral properties to the PIC again. This is very similar to how natural viruses respond to their environment by exposing a fusogenic domain at low pH and concealing it at higher pH, and may explain the low metabolic toxicity of PICs in the cell.

Conclusions

In summary, we have designed an intelligent polycation with dual functional pK_a values capable of safely emulating the escape mechanism used by natural viruses. This novel dual-cationic block copolymer, poly(Asp-Hyd)-*b*-PLL, is pH-mediated to disrupt endosomal membranes based largely on charge interactions, and is therefore minimally toxic to the cell elsewhere. This is a mechanism separate from the traditional PSE followed by osmotic rupture of the vesicle. Furthermore, the significant presence of unconjugated hydrazides on the block copolymer can serve as a scaffold for easily incorporating many additional pH-sensitive functionalities, in addition to ALD-PEG, to the PIC to boost gene expression further. Examples of this include PEG-shielded fusogenic peptides for endosomal escape, nuclear localization signals (NLS), dynein-binding moieties, and additional masked targeting peptides for nuclear localization.[21,22] Therefore, these findings offer a new platform for generating more effective and complex multifunctional biomaterials for increasing gene expression.

Experimental Section

General materials. Branched polyethylenimine (M_W = 25 000 g mol^{-1}) and poly(L-lysine) hydrobromide (M_W = 9200 g mol^{-1}) were purchased from Sigma–Aldrich (Milwaukee, WI, USA). All materials were used without further purification. The pDNA encoding firefly luciferase (pGL3, 5.3 kb) was obtained from Promega (Madison, WI, USA), transformed into electrocompetent DH5α cells, propagated in LB broth (1 L) supplemented with ampicillin (100 μg mL^{-1}), and purified with a plasmid Maxiprep kit (BioRad, Hercules, CA, USA). All pDNA had purity levels of 1.8 or greater by UV/Vis (A_{260}/A_{280}). For cell culture work, Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, phosphate buffered saline (PBS), fetal bovine serum (FBS), trypsin–EDTA (0.25% trypsin, 2.21 mM EDTA in Hank's balanced salt solution (HBSS)) and penicillin/streptomycin were purchased from Cellgro (Mediatech, Herndon, VA, USA).

Physical characterization. pH titration measurements for polymers were obtained with an Orion micro-combination pH/sodium electrode (Thermo Electron Corporation, Waltham, MA, USA; see Supporting Information for general procedures). Dynamic light scattering and ζ potential data were obtained with a nanoZS 90 or a Malvern 3000HS series zetasizer (Malvern Instruments, UK). Polymers were mixed to form PICs at N/P = 20 by addition of polymer to an equal volume of pGL3 in Milli-Q water. The sample was allowed to incubate at room temperature for at least 15 min before dilution with the appropriate buffer ranging from pH 7.4 to pH 3.0. Particle diameters and ζ potentials were measured following dilution at each pH value.

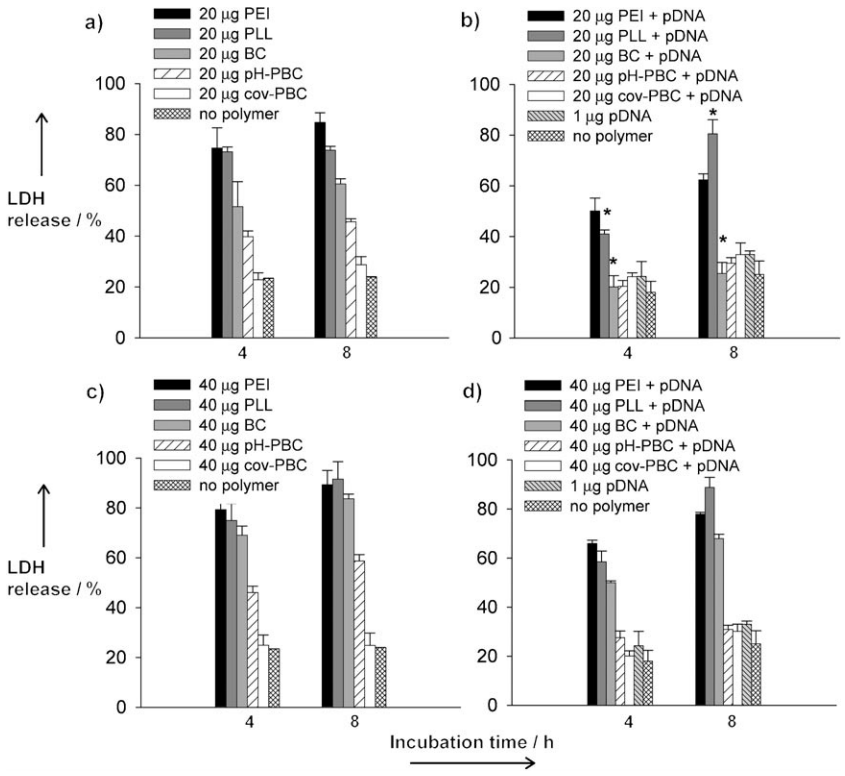

Figure 7. Membrane toxicity of polymers at concentrations of 20 and 40 μg mL^{-1} toward MDA-MB-231 (human breast cancer) cells in DMEM serum-free media. Polymers were incubated both in free form [parts a) and c)] and also pre-complexed to 1 μg pGL3 [parts b) and d)]. Free polymers with cationic components (PEI, PLL, BC) are toxic to cells after just 4 h incubation. However, complexed polymers with concealed PLL (BC, pH-PBC, cov-PBC) effect significantly less LDH release at 20 μg than the static polycations (*$p < 0.05$ using one-way ANOVA). At 40 μg, the presence of excess free polymers in solution with respect to pGL3 (see BC) re-establishes membrane toxicity.

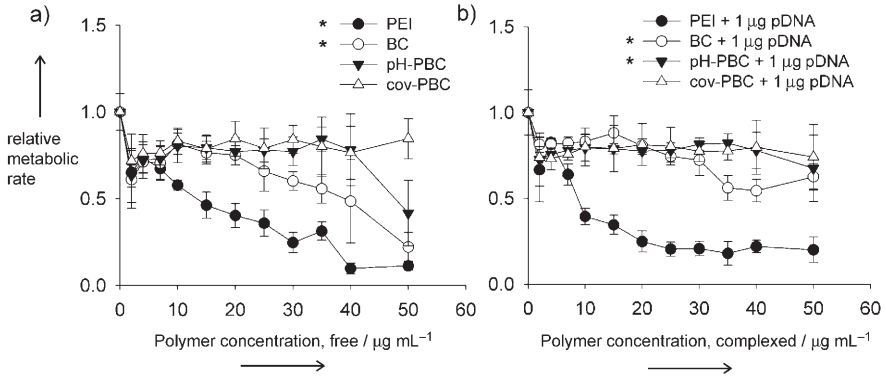

Figure 8. Metabolic toxicity of polymers incubated for 4 h at various concentrations toward MDA-MB-231 (human breast cancer) cells in DMEM serum-containing media. Polymers were incubated in both free form and pre-complexed to 1 μg pGL3. a) Free polymers with cationic components are toxic to cells at higher concentrations, with PEI being more toxic than BC (*$p < 0.05$ using two-way ANOVA). b) When complexed, BC results in negligible toxicity to the cell, similar to pH-PBC particles (*$p > 0.05$ using two-way ANOVA).

Cellular LDH release studies. Polymer interactions with cell membranes were evaluated by monitoring LDH release from MDA-MB-231 human breast cancer cells by using the CytoTox 96 non-radioactive assay from Promega (Madison, WI, USA). One-way ANOVA was used to statistically compare LDH data. MDA-MB-231 cells were seeded in 96-well plates at 25 000 cells well^{-1}. After 18 h, the plates were washed with PBS (2×) and serum-free DMEM (90 μL) was added. Each 96-well plate was divided into two sections: maximum LDH and experimental LDH release. Polymer (free or complexed to 1 μg of pGL3) was added to both sections appropriately and incubated with the cells for 4 or 8 h. At the end of each incubation period, PBS was added to the experimental LDH wells (10 μL for volume correction) and 50 μL of the final supernatant was removed and put into a separate 96-well plate. Next, 10 μL of lysis solution (Triton-X, 9% *v/v*) was added to the remaining maximum release wells and incubated for 1 h at 37 °C. Then, 50 μL of the supernatants from these lysed cells were used as controls for determining maximum LDH release. LDH substrate (50 μL) was then added to the separate 96-well plates containing supernatants, covered with aluminum foil, and shaken at room temperature for 30 min before reading the absorbance ($\lambda = 492$ nm).

Cellular metabolic toxicity studies. Metabolic toxicity was assessed through the resazurin dye (Sigma–Aldrich, Milwaukee, WI, USA) by incubating the respective polymers (free or complexed with 1 μg pGL3) in serum-containing media and monitoring the toxic effects of the polymers on the metabolic rates of cells through reduction of the dye. Briefly, 96-well plates were seeded at 20 000 cells well^{-1} and incubated for 24 h. Free polymer at increasing concentrations of 0–50 μg mL^{-1} was added to wells and incubated in serum-containing media for 4 h before refreshing the media. After 18 h incubation, 10 μL of resazurin dye (60 μM in PBS) was added to each well and incubated for 4 h before measuring fluorescence ($\lambda_{ex} = 560$ nm, $\lambda_{em} = 590$ nm). Cell viability is reported as relative metabolic rate with respect to controls without polymers. Two-way ANOVA was used to compare the data.

Cell transfection studies. MDA-MB-231 (human breast cancer), C2C12 (murine myoblast) and MCF-7 (human breast cancer) cell lines were obtained and cultured according to ATCC protocols. COS-7 cells (Green African monkey kidney) were obtained from David M. Lynn (University of Wisconsin). 4T-1 cells (murine colon cancer) were obtained from the Small Molecule Screening Facility

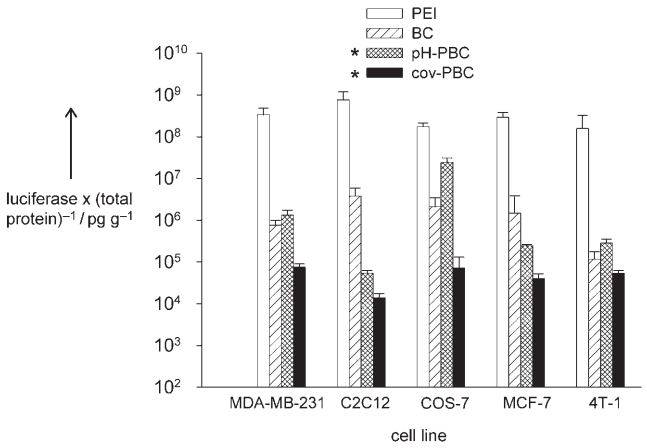

Figure 9. Transfection efficiency of the polymers in MDA-MB-231 (human breast cancer), C2C12 (murine myoblast), COS-7 (Green African monkey kidney), MCF-7 (human breast cancer) and 4T-1 (murine colon cancer) cells when incubated in the respective serum-containing media for 4 h. All polymers were formed at N/P = 20 (optimal ratio determined in tests with N/P = 7–50) with 2 μg pGL3. pH-PBC and BC PICs consistently effect significantly higher gene expression relative to cov-PBC across all cell lines (*$p < 0.05$ using one-way ANOVA in each case).

Center (University of Wisconsin) and cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin. The luciferase assay system was purchased from Promega. Luminescence was measured with a microplate Tropix luminometer (Applied Biosystems). Protein content was obtained using the DC protein assay kit from BioRad (Hercules, CA, USA), absorbance was measured with the Spectramax 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA), and results were fit to a known protein calibration curve. Cells were seeded at either 300000 cells well^{-1} in 6-well plates or 150000 cells well^{-1} in 12-well plates and cultured for 24 h. The next day, wells were aspirated, washed with PBS (2×1 mL), and appropriate medium for each cell line was added (supplemented with 10% FBS and 1% penicillin/streptomycin). Complexes (2 μg pGL3 well^{-1}) formed at the indicated N/P ratio (N/P = 1 is defined as the minimum amount of polymer required to retard 1 μg of pGL3 on a 0.75% agarose electrophoretic gel) were added to wells 4 h before refreshing the media. After 12 h, plates were assayed with the Promega luciferase assay system. Luciferase relative light units (RLUs) were converted into units of concentration by using known luciferase concentration standards. One-way ANOVA was used to compare transfection data. A value of $p < 0.05$ was considered statistically significant.

Acknowledgements

This work was partially supported by NIH grant R01 AI-43346-08. M.P.X. acknowledges the NSF for an EAPSI fellowship to the University of Tokyo and the PhRMA foundation for a predoctoral fellowship in pharmaceutics.

Keywords: block copolymers · gene delivery · intracellular trafficking · nanoparticles · polymers

[1] M. L. Forrest, D. W. Pack, *Mol. Ther.* **2002**, *6*, 57–66.
[2] J. Rejman, V. Oberle, I. S. Zuhorn, D. Hoekstra, *Biochem. J.* **2004**, *377*, 159–169.
[3] F. M. Brodsky, C. Y. Chen, C. Knuehl, M. C. Towler, D. E. Wakeham, *Annu. Rev. Cell Dev. Biol.* **2001**, *17*, 517–568.
[4] O. Boussif, F. Lezoualc'h, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix, J. P. Behr, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 7297–7301.
[5] S. Fukushima, K. Miyata, N. Nishiyama, N. Kanayama, Y. Yamasaki, K. Kataoka, *J. Am. Chem. Soc.* **2005**, *127*, 2810–2811.
[6] M. Oishi, K. Kataoka, Y. Nagasaki, *Bioconjugate Chem.* **2006**, *17*, 677–688.
[7] Z. Y. Zhang, B. D. Smith, *Bioconjugate Chem.* **2000**, *11*, 805–814.
[8] G. F. Walker, C. Fella, J. Pelisek, J. Fahrmeir, S. Boeckle, M. Ogris, E. Wagner, *Mol. Ther.* **2005**, *11*, 418–425.
[9] J. P. Clamme, J. Azoulay, Y. Mely, *Biophys. J.* **2003**, *84*, 1960–1968.
[10] W. T. Godbey, K. K. Wu, G. J. Hirasaki, A. G. Mikos, *Gene Ther.* **1999**, *6*, 1380–1388.
[11] W. T. Godbey, K. K. Wu, A. G. Mikos, *Biomaterials* **2001**, *22*, 471–480.
[12] S. M. Moghimi, P. Symonds, J. C. Murray, A. C. Hunter, G. Debska, A. Szewczyk, *Mol. Ther.* **2005**, *11*, 990–995.
[13] K. Itaka, K. Yamauchi, A. Harada, K. Nakamura, H. Kawaguchi, K. Kataoka, *Biomaterials* **2003**, *24*, 4495–4506.
[14] Y. Bae, S. Fukushima, A. Harada, K. Kataoka, *Angew. Chem.* **2003**, *115*, 4788–4791; *Angew. Chem. Int. Ed.* **2003**, *42*, 4640–4643.
[15] Y. Bae, W. D. Jang, N. Nishiyama, S. Fukushima, K. Kataoka, *Mol. Biosyst.* **2005**, *1*, 242–250.
[16] Y. Nagasaki, T. Kutsuna, M. Iijima, M. Kato, K. Kataoka, S. Kitano, Y. Kadoma, *Bioconjugate Chem.* **1995**, *6*, 231–233.
[17] J. Rejman, A. Bragonzi, M. Conese, *Mol. Ther.* **2005**, *12*, 468–474.
[18] K. von Gersdorff, N. N. Sanders, R. Vandenbroucke, S. C. De Smedt, E. Wagner, M. Ogris, *Mol. Ther.* **2006**, *14*, 745–753.
[19] C. Goncalves, E. Mennesson, R. Fuchs, J. P. Gorvel, P. Midoux, C. Pichon, *Mol. Ther.* **2004**, *10*, 373–385.
[20] M. P. Xiong, M. L. Forrest, A. L. Karls, G. S. Kwon, *Bioconjugate Chem.* **2007**, *18*, 746–753.
[21] E. Mastrobattista, M. A. van der Aa, W. E. Hennink, D. J. Crommelin, *Nat. Rev. Drug Discovery* **2006**, *5*, 115–121.
[22] R. M. Sawant, J. P. Hurley, S. Salmaso, A. Kale, E. Tolcheva, T. S. Levchenko, V. P. Torchilin, *Bioconjugate Chem.* **2006**, *17*, 943–949.
[23] A. von Harpe, H. Petersen, Y. Li, T. Kissel, *J. Controlled Release* **2000**, *69*, 309–322.

Received: April 25, 2007
Revised: May 28, 2007
Published online on June 20, 2007

