Cell Transfection

DOI: 10.1002/anie.200800963

Charge-Conversion Ternary Polyplex with Endosome Disruption Moiety: A Technique for Efficient and Safe Gene Delivery**

*Yan Lee, Kanjiro Miyata, Makoto Oba, Takehiko Ishii, Shigeto Fukushima, Muri Han, Hiroyuki Koyama, Nobuhiro Nishiyama, and Kazunori Kataoka**

DNA or RNA delivery into target cells by synthetic nonviral vectors (lipoplexes and polyplexes) is widely recognized as a promising alternative to delivery with viral vectors, which encounter the safety issues inherent to their biological propensities.[1] Nevertheless, even in the case of nonviral vectors, the inconsistency between the delivery efficiency and the safety issue, particularly with regard to chemotoxicity, has been a major matter of concern. The vectors with high transfection efficiency often show high toxicity, whereas those with low toxicity frequently raise the issue of low transfection efficiency.

Various polycations with regulated basicity have been developed for the construction of polyplexes directed toward high transfection efficiency since Behr and co-workers introduced to the gene-delivery field the concept of endosomal escape through the "proton-sponge" effect hypothesized for polyethyleneimine (PEI), yet the toxicity of these polycations lends the polyplexes to only limited applications.[2] One of the main reasons for the limited success is probably that different, and even conflicting, functionalities of the polyplexes are required at each different stage of the delivery processes. For example, the moieties of high amine density in the polyplexes are important to overcome endosomal membrane barriers because their protonation potential contributes to endosome buffering as well as to membrane destabilization.[3] On the other hand, the positively charged nature of the polyplexes may induce nonspecific interactions with negatively charged serum components to form thrombi in the capillary and carries the risk of perturbing the structure of the plasma membrane to induce high cytotoxicity and excessive immune responses.[4] Shielding of the positive charges by covering the polyplex surface with polyanions[5] or poly(ethylene glycol) (PEG)[6] is a well-known practical solution to these problems, yet significant lowering of the transfection efficiency is inevitable, mainly due to the reduced cellular uptake and the impaired capacity for endosome escape. Therefore, much effort has been concentrated on the development of deshielding methods at a specific stage during the transfection process.[7]

Herein, we wish to communicate a novel approach to the design of polyplexes exerting both high transfection efficiency and lowered cytotoxicity by integrating a charge-conversion moiety into the polyplex structure. Maleic amide derivatives, *cis*-aconitic amide, and citraconic amide have negative charges at neutral pH values, but they degrade promptly at weakly acidic pH 5.5 to expose positively charged amines.[8] Therefore, if we cover the surface of the positively charged polyplexes with degradable amide-derivatized polymers to form ternary polyplexes (plasmid DNA/polycation/polyanion with the degradable side chain), the polyplexes maintain a neutral to negatively charged nature on the cell exterior, whereas the charge-conversion components are expected to turn positive in the acidic milieu of the endosome to facilitate the endosomal escape of the polyplexes through membrane disruption (Figure 1).

Initially, a polyplex between plasmid DNA (pDNA) and a polycation was prepared. As the polycation, we chose pAsp(DET) (Figure 2A), which had been proven by our group to be an endosome-disrupting and membrane-destabilization moiety with lower cytotoxicity than conventional polycations, including PEI.[9] The polyplexes showed positive surface charges with a zeta potential of approximately +40 mV because of the excess amount of polycations (N (amines in pAsp(DET))/P (phosphate in pDNA) ratio of 4–8). The polyplex was then added to 1–4 molar equivalents of the charge-conversion polymer pAsp(DET-Aco) (Figure 2A) to form the ternary polyplex. The pAsp(DET-Aco) should turn into pAsp(DET), which could also disrupt the endosome efficiently, at the endosomal pH value after degradation of the *cis*-aconitic amide moieties. Each ternary polyplex at various charge ratios showed unimodal size distribution with a mean diameter of about 130 nm, as measured by dynamic light scattering (DLS), even in the presence of excess pAsp(DET-Aco). Although there is a possibility of the formation of the binary polyplex between pAsp(DET-Aco)

[*] Dr. Y. Lee, S. Fukushima, Dr. N. Nishiyama, Prof. Dr. K. Kataoka
Division of Clinical Biotechnology, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033 (Japan)
Fax: (+81) 3-5841-7139
E-mail: kataoka@bmw.t.u-tokyo.ac.jp
Dr. K. Miyata, Dr. T. Ishii, Prof. Dr. K. Kataoka
Department of Bioengineering, University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033 (Japan)
Dr. M. Oba, Prof. Dr. H. Koyama
Department of Clinical Vascular Regeneration, University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033 (Japan)
Dr. K. Miyata, Dr. N. Nishiyama, Prof. Dr. K. Kataoka
Center for Nanobio Integration, University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656 (Japan)
Dr. M. Han, Prof. Dr. K. Kataoka
Department of Materials Engineering, University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656 (Japan)

[**] This work was supported by a Core Research for Evolutional Science and Technology (CREST) grant from the Japan Science and Technology Agency (JST).

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

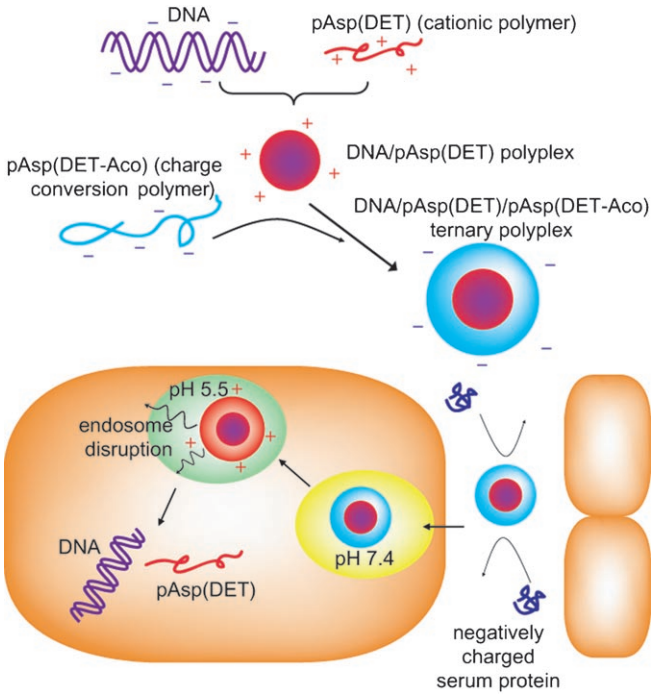

Figure 1. Diagram of the charge-conversion ternary polyplex with an endosome-disrupting function. pAsp(DET): poly{*N*-[*N*′-(2-aminoethyl)-2-aminoethyl]aspartamide}; pAsp(DET-Aco): poly(*N*-{*N*′-[(*N*′′-*cis*-aconityl)-2-aminoethyl]-2-aminoethyl}aspartamide).

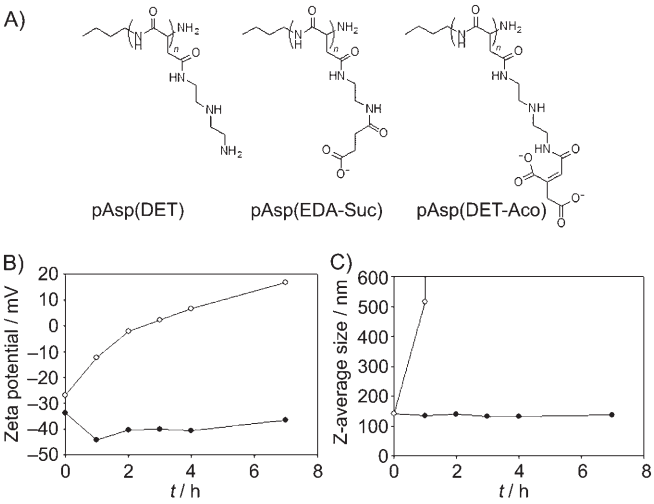

Figure 2. A) The structures of the polycation pAsp(DET), the non-charge-conversion polyanion poly[(*N*′-succinyl-2-aminoethyl)aspartamide] (pAsp(EDA-Suc)), and the charge-conversion polyanion pAsp(DET-Aco). B) The charge conversion of the ternary polyplex of DNA/pAsp(DET)/pAsp(DET-Aco). C) The change of hydrodynamic diameter of the ternary polyplex. ○: results at pH 5.5; ●: results at pH 7.4.

and pAsp(DET) without DNA, the formation of the DNA-containing ternary polyplex was confirmed by gel electrophoresis assays (see the Supporting Information).

The charge-conversion behavior of the ternary polyplex was monitored from the change in the zeta potential, as illustrated in Figure 2B. The ternary polyplex maintained a zeta potential of around −40 mV at pH 7.4. However, the zeta potential at pH 5.5 increased gradually from negative to positive; this result indicates the charge conversion due to the degradation of the *cis*-aconitic amide moieties. After incubation for 2 h at pH 5.5, the zeta potential reached 0 mV. As a negative control, we used a non-charge-conversion polyanion with a similar structure, pAsp(EDA-Suc) (Figure 2A). The ternary polyplex with pAsp(DET) and pAsp(EDA-Suc) maintained a zeta potential of around −40 mV at pH 5.5 and pH 7.4, and it showed no sign of charge conversion (see the Supporting Information).

The charge conversion also induced a dramatic size change in the ternary polyplex. As shown in Figure 2C, the ternary polyplex maintained a diameter of around 130 nm at pH 7.4, but there was an immediate increase in its size at pH 5.5, even after 1 h. After 2 h, large aggregates with a diameter of over 1 μm had formed. The reason for the aggregation is probably the reduction in the repulsive forces due to the partial charge neutralization after 1 h and the complete neutralization after 2 h at pH 5.5, as indicated from the data of the zeta potential measurements.

For the potential in vivo applications, the polyplex stability in a solution of serum proteins should be addressed. In a solution of bovine serum albumin (BSA), the ternary polyplexes maintained their original diameter, whereas the positive polyplex of pAsp(DET) showed a prompt increase in diameter, even after 1 h of incubation (Figure 3A). The improved stability of the ternary polyplex was probably due to the repulsive forces between the anionic ternary polyplex and the BSA; this could be a merit for future systemic applications.

The transfection was performed by using human umbilical vein endothelial cells (HUVEC). Only limited transfection reagents have been available for these cells in the past because they are very difficult to transfect and sensitive to toxicity.[10]

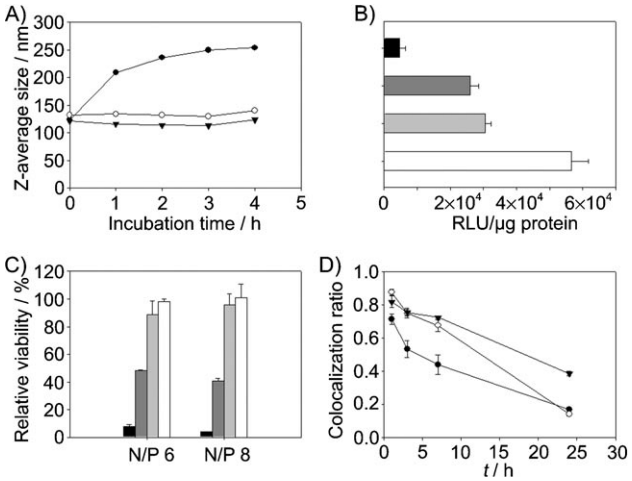

Figure 3. A) The stability of the polyplex in BSA solution. B) The transfection activity of the various vectors. C) The relative viability of HUVEC transfected with the various vectors. D) The colocalization ratio of the red fluorescence of cyanine-5-labeled DNA with the green fluorescence of LysoTracker Green (see Figure 4). Error bars indicate the standard error. Black bars: ExGen 500; ● and dark gray bars: pAsp(DET) polyplex; ▼ and light gray bars: pAsp(EDA-Suc) ternary polyplex; ○ and white bars: pAsp(DET-Aco) ternary polyplex.

The resulting transfection data with luciferase pDNA are summarized in Figure 3B. The N/P ratios between DNA and pAsp(DET) in both the simple polyplex and the ternary polyplex were 6, the value at which they showed the highest transfection efficiency. The ternary polyplexes were formed by addition of two molar equivalents of pAsp(DET-Aco) or pAsp(EDA-Suc) to the simple polyplex. The control ternary polyplex with pAsp(EDA-Suc) showed similar transfection efficiency to the simple polyplex of pAsp(DET), whereas the charge-conversion ternary polyplex of pAsp(DET-Aco) showed a tranfection efficiency that was more than ten times higher than that of ExGen 500, a commercially available transfection reagent of linear PEI, and two times higher than that of the pAsp(DET) polyplex. Even though the negative surface charge of the ternary complex was not helpful for the cellular uptake and endosomal escape, it increased the stability of the complex in the presence of the serum proteins, as shown in Figure 3A, and reduced the toxicity, so that the non-charge-conversion ternary polyplex (DNA/pAsp(DET)/pAsp(DET-Suc)) showed similar transfection efficiency to the simple polyplex. With the introduction of the charge-conversion endosome-disrupting moiety into the ternary polyplex on the basis of that stability and low toxicity (DNA/pAsp(DET)/pAsp(DET-Aco)), the transfection efficiency was still more increased. The transfection results with yellow-fluorescence-protein (YFP) pDNA, which also showed the appreciable transfection efficiency of the ternary polyplex system, is summarized in the Supporting Information.

The cytotoxicity, as measured by an MTT viability assay, is shown in Figure 3C. At N/P ratios of 6 and 8, which were the optimal ratios for the transfection, ExGen 500 showed very high toxicity with a viability below 10%, and the pAsp(DET) polyplex also showed the viability to be decreased to 50%. One of the main reasons for the decreased viability was probably the positive surface charge of the polyplexes inducing membrane toxicity.[11] However, the ternary polyplexes, which had negatively charged surfaces at the cell exterior, showed almost no cytotoxicity at both N/P ratios.

For the confirmation of the enhanced endosomal escape of the charge-conversion ternary polyplex, the intracellular distribution of the polyplex was investigated by confocal laser scanning microscopy (CLSM) by using cyanine-5-labeled pDNA (Figure 4). The yellow fluorescence changes to red when the polyplex is released from the acidic vasicular organelles. The positively charged pAsp(DET) polyplex showed significant endosomal escape, even only after 3 h, and over 80% of the DNA had escaped after 24 h. Both ternary polyplexes showed low endosomal escape after 3 h. However, the charge-conversion ternary polyplex from pAsp(DET-Aco) showed similar levels of endosomal escape to the positive pAsp(DET) polyplex after 24 h, whereas large portions (over 40%) of the non-charge-conversion ternary polyplex with pAsp(EDA-Suc) still remained in the endosomes.

The quantitative analyses of the CLSM images are summarized in Figure 3D. The charge-conversion polyplex showed similar behavior to the non-charge-conversion polyplex until 3 h, but it showed less colocalization ratio after 7 h,

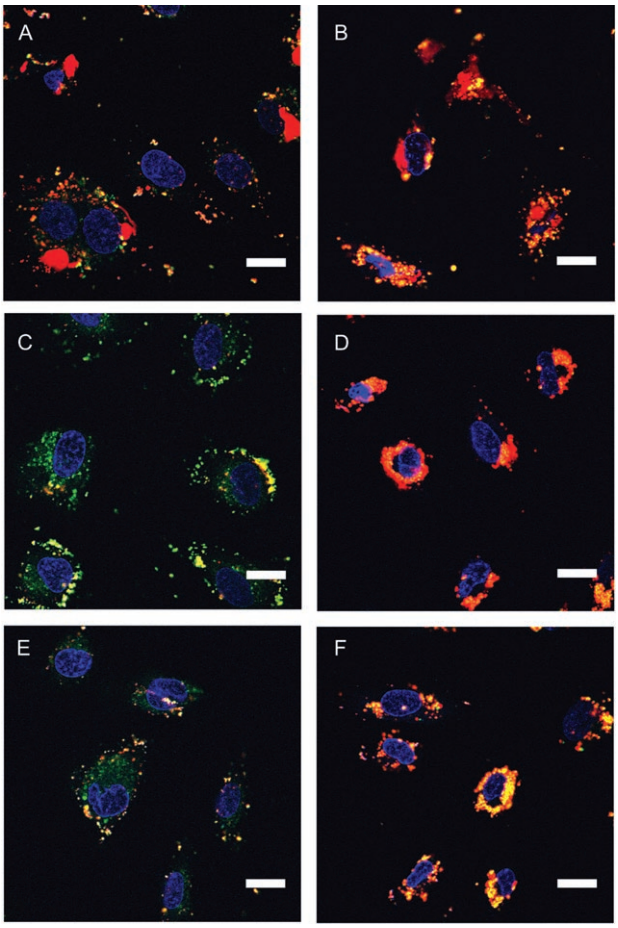

Figure 4. CLSM images of HUVEC transfected with pAsp(DET) polyplex (A and B), pAsp(DET-Aco) ternary polyplex (C and D), and pAsp(EDA-Suc) ternary polyplex (E and F). (A, C, and E) are images after 3 h of transfection; (B, D, and F) are images after 24 h of transfection. Plasmid DNA labeled with cyanine 5 (red) was used. The cell nuclei were stained with Hoechst 33342 (blue), and the late endosome and lysosome were stained with LysoTracker Green (green). Each scale bar represents 20 μm.

and finally had a similar ratio to the positive pAsp(DET) polyplex after 24 h. By considering that the endosomal acidification and the charge conversion required some time, the CLSM data were reasonable and agreed with the luciferase transfection data.

In summary, we have developed ternary polyplexes that express negative charges at the pH value of the cell exterior and that turn positive to disrupt the endosome at endosomal pH values. Eventually, these polyplexes achieved appreciably high transfection activity and low toxicity against sensitive primary cells (HUVEC). The transfection efficiency of this ternary polyplex system could be enhanced more by the conjugation of appropriate ligands, such as an RGD peptide for active internalization through binding of the integrin receptor.[12] The concept of our charge-conversion ternary polyplex with an endosome-disrupting moiety could easily be applied to various sensitive primary cells, the efficient and non-chemotoxic transfection of which is one of the most important and urgent issues in the biomedical field. Also, the stability of the ternary polyplex in the presence of negatively

charged serum proteins could be helpful for the development of in vivo gene vectors.

Received: February 28, 2008
Revised: April 10, 2008
Published online: June 4, 2008

Keywords: charge conversion · DNA · gene delivery · polymers · transfection

[1] a) D. W. Pack, A. S. Hoffman, S. Pun, P. S. Stayton, *Nat. Rev. Drug Discovery* **2005**, *4*, 581 – 593; b) E. Mastrobattista, M. A. E. M. Aa, W. E. Hennink, D. J. A. Crommelin, *Nat. Rev. Drug Discovery* **2006**, *5*, 115 – 121.

[2] a) 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; b) M. Neu, D. Fischer, T. Kissel, *J. Gene Med.* **2005**, *7*, 992 – 1009.

[3] a) M. X. Tang, C. T. Redemann, F. C. Szoka, *Bioconjugate Chem.* **1996**, *7*, 703 – 714; b) R. Wattiaux, N. Laurent, S. W.-D. Coninck, M. Jadot, *Adv Drug Delivery Rev.* **2000**, *41*, 201 – 208.

[4] a) A. C. Hunter, *Adv. Drug Delivery Rev.* **2006**, *58*, 1523 – 1531; b) M. Ogris, S. Brunner, S. Schüler, R. Kircheis, E. Wagner, *Gene Ther.* **1999**, *6*, 595 – 605.

[5] V. S. Trubetskoy, S. C. Wong, V. Subbotin, V. G. Budker, A. Loomis, J. E. Hagstrom, J. A. Wolff, *Gene Ther.* **2003**, *10*, 261 – 271.

[6] M. Han, Y. Bae, N. Nishiyama, K. Miyata, M. Oba, K. Kataoka, *J. Controlled Release* **2007**, *121*, 38 – 48.

[7] a) E. R. Gillies, A. P. Goodwin, J. M. Fréchet, *Bioconjugate Chem.* **2004**, *15*, 1254 – 1263; b) M. Krämer, J. F. Stumbé, H. Türk, S. Krause, A. Komp, L. Delineau, S. Prokhorova, H. Krautz, R. Haag, *Angew. Chem.* **2002**, *114*, 4426 – 4431; *Angew. Chem. Int. Ed.* **2002**, *41*, 4252 – 4256.

[8] a) Y. Lee, S. Fukushima, Y. Bae, S. Hiki, T. Ishii, K. Kataoka, *J. Am. Chem. Soc.* **2007**, *129*, 5362 – 5363; b) D. B. Rozema, D. L. Lewis, D. H. Wakefield, S. C. Wong, J. J. Klein, P. L. Roesch, S. L. Bertin, T. W. Reppen, Q. Chu, A. V. Blokhin, J. E. Hagstrom, J. A. Wolff, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 12982 – 12987.

[9] a) N. Kanayama, S. Fukushima, N. Nishiyama, K. Itaka, W.-D. Jang, K. Miyata, Y. Yamasaki, U.-I. Chung, K. Kataoka, *ChemMedChem* **2006**, *1*, 439 – 444; b) K. Masago, K. Itaka, N. Nishiyama, U. Chung, K. Kataoka, *Biomaterials* **2007**, *28*, 5169 – 5175.

[10] a) V. Zaric, D. Weltin, P. Erbacher, J. S. Remy, J. P. Behr, D. Stephan, *J. Gene Med.* **2004**, *6*, 176 – 184; b) J. J. Green, G. T. Zugates, N. C. Tedford, Y. H. Huang, L. G. Griffith, D. A. Lauffenburger, J. A. Sawicki, R. Langer, D. G. Anderson, *Adv. Mater.* **2007**, *19*, 2836 – 2842.

[11] a) D. Fischer, Y. Li, B. Ahlemeyer, J. Krieglstein, T. Kissel, *Biomaterials* **2003**, *24*, 1121 – 1131; b) S. M. Moghimi, P. Symonds, J. C. Murray, A. C. Hunter, G. Debska, A. Szewczyk, *Mol. Ther.* **2005**, *11*, 990 – 995.

[12] a) K. Temming, R. M. Schiffelers, G. Molema, R. J. Kok, *Drug Resist. Updates* **2005**, *8*, 381 – 402; b) M. Oba, S. Fukushima, N. Kanayama, K. Aoyagi, N. Nishiyama, H. Koyama, K. Kataoka, *Bioconjugate Chem.* **2007**, *18*, 1415 – 1423.

