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Protein Delivery

Charge-Conversional Polyionic Complex Micelles—Efficient Nanocarriers for Protein Delivery into Cytoplasm**

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In the postgenomic era, the elucidation of protein function is one of the most important challenges in biological fields as the development of protein-based therapeutics has great potential in medicinal science. Enhancement and knockout of a specific protein expression are among the various methods that have been used for fundamental research into protein function. The direct delivery of proteins into cells is probably one of the simplest and most decisive ways to examine protein function, as no interference or artifacts occur during the transcription–translation pathway. Moreover, an efficient in vivo protein delivery is essential for therapeutic applications. Although various protein-based biopharmaceuticals have been developed, the instability of proteins in serum and the lack of a delivery method into cytoplasm has limited further success.[1] Many research groups have therefore concentrated on the development of protein delivery methods[2] such as hydrogels, liposomes, nanotubes, or inorganic carriers, but a highly efficient delivery method that offers serum stability and generality has not yet been developed.

We report herein a novel approach for protein delivery based on polyionic complex (PIC) micelles, which are well-defined core–shell supramolecular structures formed through electrostatic interactions when diblock copolymers with both a neutral and an ionic block mix with their counterions.[3] Because the shell of the neutral block protects the core from external deactivation pathways such as enzymatic attack or aggregation, the PIC micelle can act as a molecular container. PIC micelles have also been used as delivery carriers for drugs or biomacromolecules because of their high stability, reduced immune response, and elongated circulation time, which arise from their biocompatible surfaces and high molecular weights.[4] We have successfully developed PIC micelles, which contain a block copolymer with poly(ethylene glycol) (PEG) as a neutral block and a poly(amino acid) as an ionic block,[5] for DNA and RNA delivery. However, the protein-containing PIC micelles dissociated immediately at a physiological salt concentrations, which has limited their biological application.[6] Stabilized PIC micelles could be obtained by cross-linking with glutaraldehyde; it was difficult to apply these micelles in the human body because of the toxicity of glutaraldehyde and the irreversibility of the cross-linking.[7] The salt stability of the PIC micelles is closely related to the charge density of its components. For example, PIC micelles of DNA with high charge density (−308 Da per charge) were stable, but those with lysozyme (+1980 Da per charge) dissociated rapidly at the physiological salt concentration.

Therefore, in order to obtain a higher micelle stability, we attempted to increase the charge density of the protein by employing a reversible conjugation. Citraconic amide and *cis*-aconitic amide, derivatives of the maleic acid amide, are stable at the normal physiological pH value of 7.4, but degrade at the endosomal pH value of 5.5 to expose primary amines, with a charge conversion from negative to positive.[8] If a protein has a sufficient amount of lysine groups that can be modified to citraconic amides or *cis*-aconitic amides, the pI (isoelectric point) of the protein decreases significantly. Moreover, because the *cis*-aconitic amide exposes two carboxylate groups per reacted amine group, the anionic charge density could be reversibly increased (Scheme 1). We expected that the PIC micelles that contain the modified protein would have an increased salt stability because of the high charge density, and that they could release the original protein after charge conversion in the endosome.

We selected equine heart cytochrome c (CytC; M_w = 12384 Da), an essential protein in the electron transfer of the mitochondria, as a model protein. The CytC is a cationic protein with a charge density of +1391 Da per charge, which arises from the presence of three aspartate, nine glutamate, two arginine, and 19 lysine units. However, CytC could not form the PIC micelles with poly(ethylene glycol)–poly[(*N*′-succinyl-2-aminoethyl(aspartamide)] (PEG–pAsp(EDA-Suc); **2**), an anionic block copolymer, in the presence of NaCl (150 mM). We modified CytC with citraconic anhydride and *cis*-aconitic anhydride to increase the charge density (the synthetic procedure for all block copolymers and the CytC modification method are described in detail in the Supporting Information). The resulting anionic proteins were Cyt–Cit (−501 Da per charge) and Cyt–Aco (−320 Da per charge).

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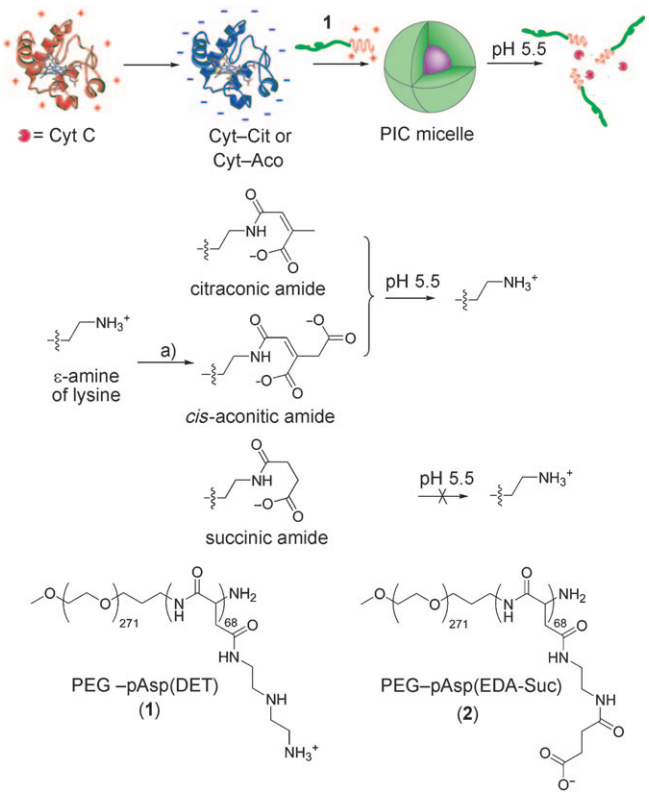

Scheme 1. Schematic representation showing the preparation of the charge-conversional PIC micelles containing CytC derivatives and PEG–pAsp(DET). a) Citraconic anhydride (or *cis*-aconitic anhydride/ succinic anhydride).

The formation of the PIC micelle containing the modified CytC and a block copolymer, PEG–poly[*N*-{*N*′-(2-aminoethyl)-2-aminoethyl}aspartamide] (PEG–pAsp(DET); **1**), was examined because PEG–pAsp(DET) has been reported to efficiently deliver DNA into cytoplasm and to have minimal toxicity.[9] The pH-sensitive endosome-destabilization activity of the pAsp(DET) block was shown to be the main reason for the high delivery efficiency.[10] Dynamic light scattering (DLS) measurements showed the PIC micelles to have a unimodal size distribution with diameters of about 50 nm and PDI values of about 0.05, even at physiological salt concentration (150 mM NaCl; Table 1). The spherical shape of the micelles was confirmed by using AFM (Figure 1). The spherical PIC micelles were formed at the N/C (amine/carboxylate) ratio of 2. Considering that one *N*′-(2-aminoethyl)-2-aminoethyl group has one positive charge at pH 7.4[11] because of the pKa difference between two amines, the PIC micelles could be formed at the charge ratio (+/−) of 1. Consequently, we succeeded in forming stable and stoichiometric PIC micelles under physiological salt conditions, by increasing the charge density of the protein without cross-linking.

Table 1: The formation of the PIC micelles between the block copolymer and CytC derivatives.

Protein	Charge density [Da per charge][a]	pI[a]	Diameter [nm][b]	PDI[b]
CytC[c]	+1391	9.57	n.d.	n.d.
Cyt–Cit[d]	−501	3.71	43.3	0.046
Cyt–Aco[d]	−320	3.47	50.1	0.055

[a] The calculation is described in the Supporting Information. [b] Determined by using DLS. [c] Compound **2** was used as the anionic block copolymer. [d] Compound **1** was used as the cationic block copolymer.

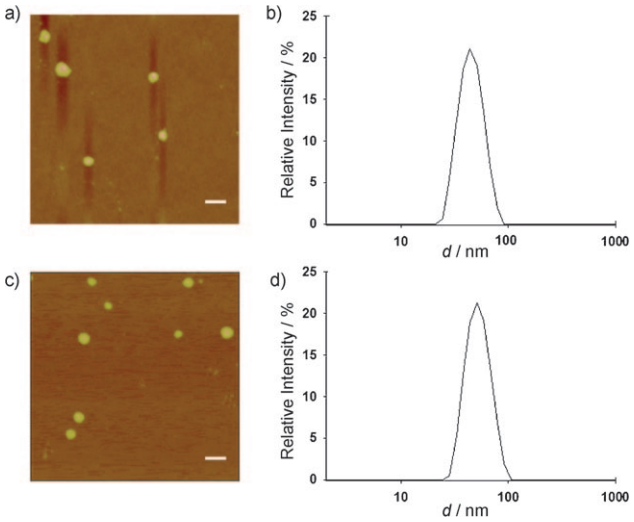

Figure 1. AFM images of the PIC micelles containing a) Cyt–Cit and c) Cyt–Aco. DLS distributions of the PIC micelles containing b) Cyt–Cit and d) Cyt–Aco (N/C ratio = 2). Scale bars: 200 nm.

The resulting citraconic amide and *cis*-aconitic amide in Cyt–Cit and Cyt–Aco showed rapid degradability at pH 5.5 (see Figure S1 in the Supporting Information). At pH 5.5, about 80% of the modified lysine reverted to the original lysine within 2 hours, whereas at pH 7.4, only 20–30% reverted, even after 24 hours. As the degradation took place concurrently with the charge conversion from negative to positive, the corresponding dissociation of the PIC micelles was expected to occur. The dissociation was analyzed by using the fluorescence quenching–dequenching method.[12] The fluorescence intensity of the Alexa Fluor 488 labeled CytC derivatives in the core of the PIC micelles was reduced significantly because of the probe–probe quenching effect (<20%). However, the protein release from the PIC micelles induced the recovery of the fluorescence intensity (Figure 2). Over 50% of Cyt–Cit was released from the PIC micelles within 4 hours at pH 5.5, whereas only 10% was released even after 8 hours at pH 7.4. Experiments with Cyt–Aco showed similar release profiles but with a slower rate, which is probably because Cyt–Aco has a higher charge density than Cyt–Cit. The bioactivity of the released CytC from the PIC micelles was also analyzed with a 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay (see Figure S4 in the Supporting Information).[13] No difference was observed between the released CytC and the native CytC, which means that the modification–reversion cycle does not affect the activity of CytC. Because the only modification was the change of the amino acids from hydrophilic (+) to hydrophilic (−), extreme conformational denaturation that affected the protein activity was probably limited.

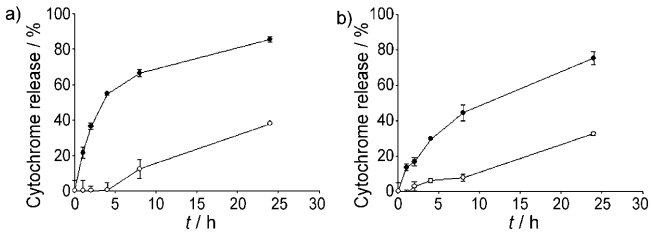

Figure 2. Release of the CytC derivatives from the PIC micelles containing a) Cyt–Cit and b) Cyt–Aco at 37 °C at pH 5.5 (●) and pH 7.4 (○). Each error bar represents the standard deviation of three experiments.

Finally, the delivery efficiency of the charge-conversional PIC micelles on a human hepatoma cell line (HuH-7) was examined. The intracellular distribution of the CytC derivatives labeled with Alexa Fluor 488 (green) was investigated by using confocal laser scanning microscopy (CLSM). The cell images after incubation for 24 h are shown in Figure 3. Because the late endosome and lysosome were stained by LysoTracker Red (red), the CytC in the endosome was detected as yellow. The yellow fluorescence turned to green after protein release from the endosome (see Figure S5 in the Supporting Information for the quantification of the green and red fluorescence colocalization). The native CytC and succinyl CytC (Cyt–Suc), the non-charge-conversional anionic derivative, were used as the controls. As shown in Figure 3a, almost no green fluorescence was detected when the cells were incubated with the native CytC. The lack of green fluorescence was expected, because it is difficult for hydrophilic proteins to penetrate through the plasma membrane. The cells incubated by the PIC micelles containing Cyt–Suc and the polymer **1** showed approximately yellow fluorescence (colocalization ratio (CR) = 0.803); Figure 3b), which means that significant cellular uptake but no endosomal escape occurred. Because the PIC micelles containing Cyt–Suc and **1** did not show any dissociation, even after 24 h at pH 5.5 (see Figure S3 in the Supporting Information), the low efficiency of the endosomal escape is quite reasonable when it is considered that direct contact between the cationic (pAsp(DET)) block and endosomal membrane is important for endosomal escape to occur.[14]

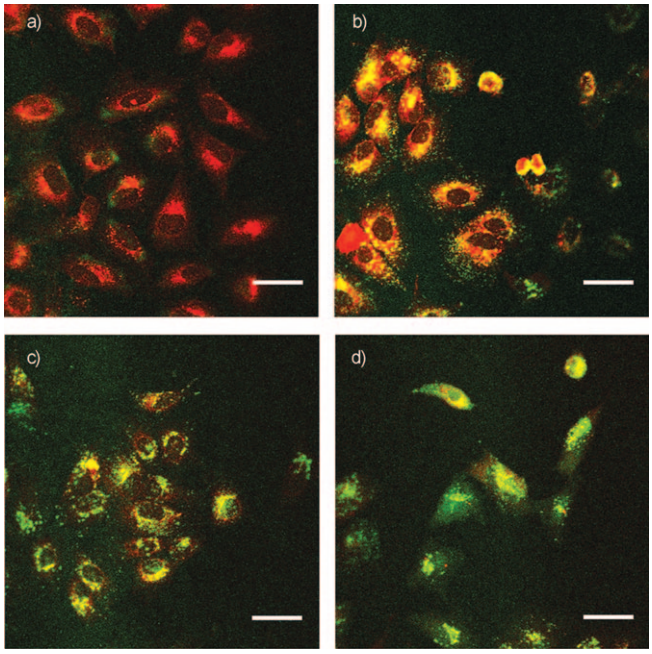

Figure 3. CLSM images of HuH-7 delivered by a) free native CytC, b) Cyt–Suc PIC, c) Cyt–Aco PIC, and d) Cyt–Cit PIC micelles after 24 h transfection. Each CytC derivative was labeled with Alexa Fluor 488 (green). The late endosome and lysosome were stained with LysoTracker Red (red). Scale bars: 50 μm.

In contrast, the charge-conversional PIC micelles containing Cyt–Aco or Cyt–Cit showed strong green fluorescence as well as yellow fluorescence (Figure 3c,d). It was assumed that the polymer **1** released from the PIC micelle could come into direct contact with the endosomal membrane to induce the efficient escape of the CytC. When the two charge-conversional PIC micelles are compared, micelles containing Cyt–Cit (CR = 0.498) showed more efficient endosomal release and resulting cytosolic distribution than Cyt–Aco (CR = 0.682). This result is probably due to the higher sensitivity of Cyt–Cit to the pH reduction over Cyt–Aco. The faster dissociation of the Cyt–Cit micelles in the endosome could lead to faster endosomal escape and diffusion into the cytoplasm.

In summary, we have developed an efficient method, which is based on charge-conversional PIC micelles, of protein delivery into cytoplasm. The stability of the PIC micelle under physiological salt conditions was significantly improved by increasing the charge density of the protein without any cross-linking. The charge conversion of the protein induced the efficient endosomal release, especially in the case of the PIC micelles containing Cyt–Cit. The long circulation time of the PIC micelles and controlled release activity of the charge-conversional moiety were combined in our charge-conversional PIC micelles, which could make them highly valuable for in vivo protein delivery. Moreover, when considering that the molecular weight of the PIC micelles is well over several megadaltons, these charge-conversional PIC micelles could potentially be optimal for the intracellular delivery of high-molecular-weight membrane-impermeable proteins.

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[1] I. M. Tomlinson, *Nat. Biotechnol.* **2004**, *22*, 521 – 522.
[2] a) K. Y. Lee, S. H. Yuk, *Prog. Polym. Sci.* **2007**, *32*, 669 – 697; b) N. W. S. Kam, Z. Liu, H. Dai, *Angew. Chem.* **2006**, *118*, 591 – 595; *Angew. Chem. Int. Ed.* **2006**, *45*, 577 – 581; c) I. I. Slowing, B. G. Trewyn, V. S. Y. Lin, *J. Am. Chem. Soc.* **2007**, *129*, 8845 – 8849.
[3] a) A. Harada, K. Kataoka, *Macromolecules* **1995**, *28*, 5294 – 5299; b) A. V. Kabanov, T. K. Bronich, V. A. Kabanov, K. Yu, A. Eisenberg, *Macromolecules* **1996**, *29*, 6797 – 6802; c) M. A.

Cohen Stuart, N. A. M. Besseling, R. G. Fokkink, *Langmuir* **1998**, *14*, 6846 – 6849.
[4] a) G. S. Kwon, K. Kataoka, *Adv. Drug Delivery Rev.* **1995**, *16*, 295 – 309; b) K. Kataoka, A. Harada, Y. Nagasaki, *Adv. Drug Delivery Rev.* **2001**, *47*, 113 – 131; c) M. Harada-Shiba, K. Yamauchi, A. Harada, I. Takamisawa, K. Shimokado, K. Kataoka, *Gene Ther.* **2002**, *9*, 407 – 414.
[5] a) K. Kataoka, H. Togawa, A. Harada, K. Yasugi, T. Matsumoto, S. Katayose, *Macromolecules* **1996**, *29*, 8556 – 8557; b) S. Katayose, K. Kataoka, *Bioconjugate Chem.* **1997**, *8*, 702 – 707; c) K. Itaka, N. Kanayama, N. Nishiyama, W. D. Jang, Y. Yamasaki, K. Nakamura, H. Kawaguchi, K. Kataoka, *J. Am. Chem. Soc.* **2004**, *126*, 13612 – 13613.
[6] a) A. Harada, K. Kataoka, *Macromolecules* **1998**, *31*, 288 – 294; b) A. Harada, K. Kataoka, *J. Am. Chem. Soc.* **1999**, *121*, 9241 – 9242; c) M. Jaturanpinyo, A. Harada, X. Yuan, K. Kataoka, *Bioconjugate Chem.* **2004**, *15*, 344 – 348.
[7] X. Yuan, Y. Yamasaki, A. Harada, K. Kataoka, *Polymer* **2005**, *46*, 7749 – 7758.
[8] a) J. K. Shetty, J. E. Kinsella, *Biochem. J.* **1980**, *191*, 269 – 272; b) Y. Lee, S. Fukushima, Y. Bae, S. Hiki, T. Ishii, K. Kataoka, *J. Am. Chem. Soc.* **2007**, *129*, 5362 – 5363; c) Y. Lee, K. Miyata, M. Oba, T. Ishii, S. Fukushima, M. Han, H. Koyama, N. Nishiyama, K. Kataoka, *Angew. Chem.* **2008**, *120*, 5241 – 5244; *Angew. Chem. Int. Ed.* **2008**, *47*, 5163 – 5166.
[9] 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.
[10] K. Miyata, M. Oba, M. Nakanishi, S. Fukushima, Y. Yamasaki, H. Koyama, N. Nishiyama, K. Kataoka, *J. Am. Chem. Soc.* **2008**, *130*, 16287 – 16294.
[11] K. Masago, K. Itaka, N. Nishiyama, U. Chung, K. Kataoka, *Biomaterials* **2007**, *28*, 5169 – 5175.
[12] a) S. Kwon, J. H. Carson, *Anal. Biochem.* **1998**, *264*, 133 – 140; b) Y. Lee, H. Mo, H. Koo, J. Y. Park, M. Y. Cho, G. Jin, J. S. Park, *Bioconjugate Chem.* **2007**, *18*, 13 – 18; c) B. Z. Packard, A. Komoriya, D. D. Toptygin, L. Brand, *J. Phys. Chem. B* **1997**, *101*, 5070 – 5074.
[13] R. E. Childs, W. G. Bardsley, *Biochem. J.* **1975**, *145*, 93 – 103.
[14] S. Takae, K. Miyata, M. Oba, T. Ishii, N. Nishiyama, K. Itaka, Y. Yamasaki, H. Koyama, K. Kataoka, *J. Am. Chem. Soc.* **2008**, *130*, 6001 – 6009.

