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A Protein Nanocarrier from Charge-Conversion Polymer in Response to Endosomal pH

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Smart polymers, whose characteristics change in response to an external signal, such as electric potential, magnetic field, temperature, light, and pH, etc., are spotlighted in various research fields including analytical chemistry, tissue engineering, and drug delivery.[1] Especially, the smart polymers, which are sensitive to biosignals, that is, reductive potential[2] or pH, are very attractive in the drug delivery field requiring selective controlled-release. Some pH-sensitive polymers are even facilitating the endosomal escape of drugs by a membrane interaction and/or an increase in the local osmotic pressure.[3]

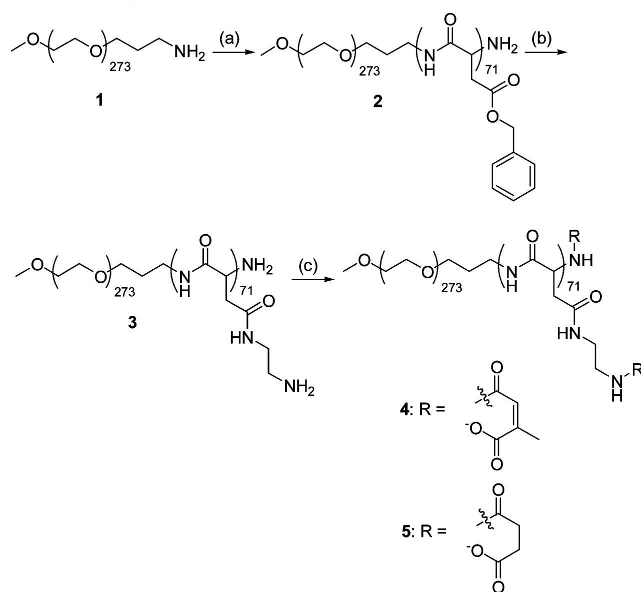
pH-Sensitive polymers developed so far that release drugs in the endosomal component use acetal, hydrazone, and orthoester bonds, etc.[4] Although they showed a selective degradation in the endosome, their drug release in response to pH was relatively passive and slow. A more active and prompt response to a small pH drop is needed for more effective drug delivery, for example, the specific release in the early endosome.

Citraconic anhydride is an α-methyl derivative of maleic anhydride, which can be used to mask the charge of proteins. As shown in Scheme 1, amide bonds are formed from the reaction between primary amines and citraconic anhydride. The resulting amides have negative charges owing to the carboxylate groups at the end. The citraconic amide is stable at both neutral and basic pH, but it becomes unstable at acidic pH and promptly degrades back into the cationic primary amine. It has been reported that the citraconic amide degrades around pH 5.[5] Therefore, we considered that the citraconic amide could provide a pH-dependent degradability to the polymers that can be selectively functionalized during the early endosome in a cell. Because the degradation was directly related to the charge-conversion, it could also provide an abrupt change in the interaction with counter-ions.

In this study, we synthesized a block copolymer with combtype side groups of the citraconic amide and characterized their physicochemical properties such as the degree of degradability and the charge conversion. With this polymer, we also developed a novel nanocontainer that can promptly release its protein cargo by generating a repulsive electrostatic force owing to the charge-conversion at the endosomal pH.

The synthesis of the diblock copolymer, poly(ethylene glycol)-poly[(*N'*-citraconyl-2-aminoethyl)aspartamide] (PEG-pAsp(EDA-Cit)) (**4**) is illustrated in Scheme 1. The diblock copolymer **2** was synthesized as previously reported.[6] Briefly, the ring-opening polymerization of *β*-benzyl-L-aspartate *N*-carboxy-anhydride (BLA-NCA) was initiated by the terminal primary amino group of α-methoxy-*ω*-amino poly(ethylene glycol) (M_n = 12 000) (**1**), and the reaction produced **2**. The prepared **2** was further modified into PEG-poly[(2-aminoethyl)aspartamide] (**3**) by aminolysis with excess ethylenediamine. Finally, **4** was synthesized from **3** and the citraconic anhydride in the pyridine solvent. The detailed synthetic procedures are described in the Supporting Information.

Scheme 1. Synthesis of PEG-pAsp(EDA-Cit): (a) BLA-NCA/DMF; (b) Ethylenediamine/DMF; (c) Citraconic Anhydride (or Succinic Anhydride)/pyridine



The pH-dependent degradation rates of the citraconic amide of **4** are shown in Figure 1. The degradation rate was calculated by measurement of the primary amine concentration in the polymer at 37 °C. The fluorescamine method was used for the quantification of the amine concentration.[7] In the meantime, PEG-pAsp(EDA-Suc) (**5**) was synthesized as the negative control by mixing **4** with succinic anhydride instead of citraconic anhydride. Although **5** has a structure similar to **4**, **5** does not degrade under acidic pH conditions. The experiments showed that approximately 80% of the citraconic amides was degraded in the acetate buffer (pH 5.5) within 1 h, while 60% of the citraconic amides remained intact in the phosphate buffer (pH 7.4) even after 5 h. No degradation was observed in the case of the succinic amides under both pH conditions.

We previously reported that the formation and characterization of the PIC micelles between the PEG-polyaspartate (PEG-pAsp) and lysozyme, of which the isoelectric point occurs at pH 10.[8]

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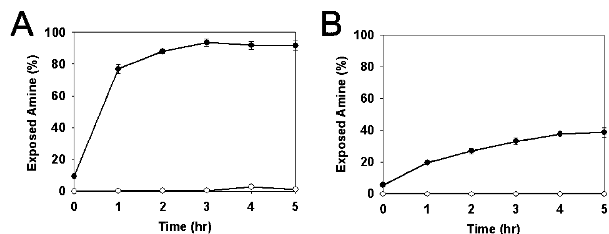

Figure 1. The degradation of citraconic amide (●) and succinic amide (○) in 4 and 5 at pH 5.5 (A) and pH 7.4 (B). The data are expressed as mean values (± standard deviation) of three experiments.

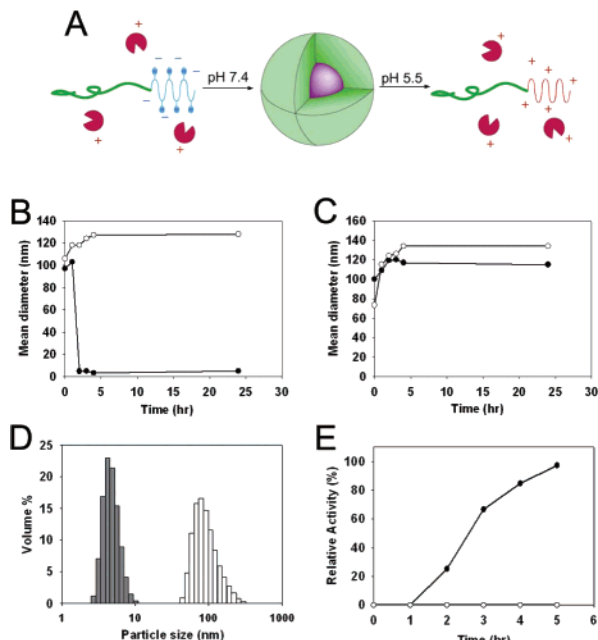

Figure 2. Formation and dissociation of the PIC micelles: (A) schematic diagram for the formation and dissociation of the PIC micelles; the time course of the mean diameter of the PIC micelles of (B) PEG-pAsp(EDA-Cit) and (C) PEG-pAsp(EDA-Suc); (D) the DLS histogram of the PIC micelles at 0 h (white) and at 2 h (gray) at pH 5.5; and (E) the relative lysozyme activity of the PIC micelles. The black dots (●) represent the data at pH 5.5 and the white dots (○) are at pH 7.4.

Following the experimental protocols, the PIC micelles were prepared by mixing **4** and the lysozyme. The pH-dependent stability of the micelles was analyzed by dynamic light scattering (DLS) measurements. The measurements demonstrated that the PIC micelles were successfully formed. More notably, it also suggested that these micelles would be destabilized at the endosomal pH by the degradation of the citraconic amides in **4** accompanied by the charge conversion from negative to positive (Figure 2A).

Figure 2B demonstrates that the size of the micelles prepared from **4** and the lysozymes was stabilized with a diameter of about 130 nm at pH 7.4 even after incubation at 37 °C for 24 h. However, the PIC micelles were promptly dissociated at pH 5.5 within 2 h. The pH dependent profiles of the PIC micelles from **5** and the lysozymes are shown in Figure 2C. As expected, the succinic amide-based micelles were stable at both pH values after 24 h. Figure 2D shows the size distribution of the citraconic amide-based micelles before and after the dissociation. The micelles showed a unimodal size distribution before the dissociation. The distribution around 4.8 nm after a 2-hr incubation resulted from the single lysozyme molecule.[8]

Figure 2E shows the activity of the lysozyme released from the PIC micelles. The lysozyme activity was measured by the well-known method using the *Micrococcus luteus* cell suspension.[9] The relative activity is expressed as a percentage of the free-lysozyme activity. Because the activity depends on the pH, the free-lysozyme activity at each pH was used for the calculation of relative activity.

At pH 7.4, no lysozyme activity was observed for over 5 h. However, at pH 5.5, the PIC micelles showed a lysozyme activity after 2 h, and it increased and reached 97% of the free-lysozyme activity after 5 h. Interestingly, it took 3 h more to observe the full lysozyme activity after the dissociation of the micelles. Considering the fact that the micelles dissociated within 2 h, it is suggested that there is a weak interaction between the lysozyme and block copolymer chains at least 3 h after the dissociation of the micelles. Because the direct contact between lysozyme and the bacterial cell wall is required for the full activity, PEG chain bound to lysozyme, even though the binding is very weak, can reduce the lysozyme activity. After 80% degradation, **4** cannot form the PIC micelles with lysozyme, but it can still repress the lysozyme activity below 5% (data not shown). Nevertheless, it was confirmed that the citraconic amide-based micelles can selectively dissociate and release the lysozyme while maintaining its enzymatic activity by responding to the change in pH.

In summary, we synthesized the charge-converting block copolymer using the citraconic amide as a pH-sensitive charge masking group. The citraconic amide-based block copolymer was selectively degraded in response to the endosomal pH and formed PIC micelles with the cationic model protein, that is, the lysozyme. Most notably, the micelles selectively released the active lysozyme promptly by sensing the change in pH corresponding to the acidic conditions in the intracellular endosomal compartments. Therefore, it was concluded that our stimuli-sensitive block copolymers are promising designs for future drug and gene delivery systems.

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Supporting Information Available: Materials and methods, the temperature-depending degradation rate of the polymer and dissociation rate of the PIC micelles. This material is available free of charge via the Internet at http://pubs.acs.org.

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