Superamphiphiles

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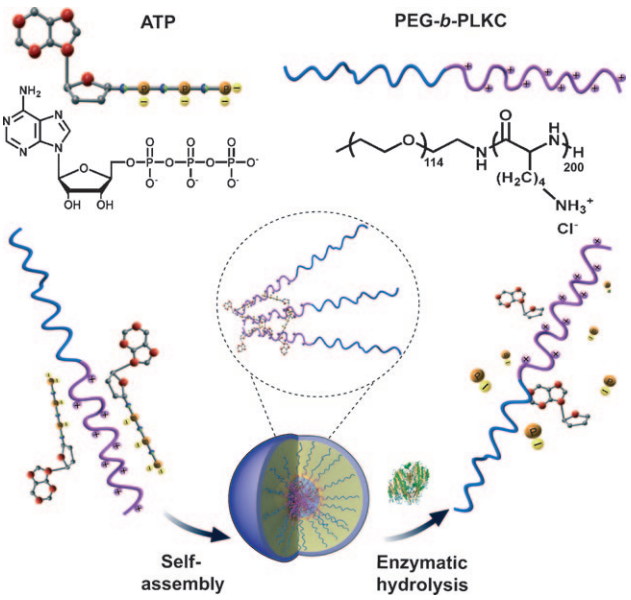
An Enzyme-Responsive Polymeric Superamphiphile**

*Chao Wang, Qishui Chen, Zhiqiang Wang, and Xi Zhang**

Stimuli-responsive polymers have developed greatly in recent years as a result of their prospective uses in biotechnology and drug-delivery systems.[1] Enzyme-responsive polymeric assemblies are particularly attractive because of their good biocompatibility and high degree of selectivity,[2] since overexpression of enzymes has frequently been implicated in the diseased state of cells. For example, some liposomes can be degraded by alkaline phosphatase, and a family of enzymes is always found in elevated concentrations in various types of tumor cells. Consequently, phosphatase-responsive systems are especially interesting for drug delivery and cancer therapy.[3] However, introducing enzyme-responsive sites into polymers generally requires tedious covalent synthesis, thus raising the cost of preparation. In addition, organic solvents and toxic reagents used in chemical synthesis may be incorporated into polymers and reduce their biocompatibility.

The new concept of "superamphiphile" has emerged as a powerful method of fabricating stimuli-responsive self-assemblies. Superamphiphiles are amphiphiles that are synthesized by noncovalent interactions.[4] Stimuli-responsive moieties can be linked to the amphiphiles on the basis of noncovalent interactions, greatly reducing the need for chemical synthesis.[5,6] The objective of the present study was to develop an inexpensive, highly efficient, and nontoxic procedure for producing enzyme-responsive polymeric self-assemblies based on the superamphiphile concept. An enzyme-responsive polymeric superamphiphile was successfully prepared by simply mixing a double-hydrophilic block polymer and a natural multicharged enzyme-responsive molecule in water. The superamphiphile self-assembles into spherical aggregates, which disassemble in response to enzymatic stimulus and subsequently release loaded molecules.

Adenosine 5′-triphosphate (ATP), which is generally acknowledged as an "energy currency" in most animate beings, plays an important role in most biological activities.[7] In this work, this natural molecule was used as a highly effective multinegatively charged and enzyme-responsive building block for fabricating polymeric superamphiphiles (Scheme 1). An important feature is that under physiological conditions ATP contains a hydrophobic adenine group and four negative charges. Another specific feature of ATP is that its phosphoanhydride bonds are enzyme-reactive and can be hydrolyzed by phosphatase, which results in a structural change from a multinegatively charged molecule into neutral adenine.[8] With these features in mind we chose the double-hydrophilic block copolymer methoxy-poly(ethylene glycol)$_{114}$-*block*-poly(L-lysine hydrochloride)$_{200}$ (PEG-*b*-PLKC), in which the PLKC segment is positively charged, for assembly with ATP. PEG-*b*-PLKC and ATP can form a polymeric superamphiphile in aqueous solution as a result of electrostatic interaction. ATP molecules noncovalently cross-link the positively charged polylysine segments, thus introducing hydrophobic adenine groups and resulting in the formation of self-assembled aggregates. Upon addition of phosphatase, the multiply negatively charged ATP is hydrolyzed to single-charged phosphate and a neutral adenine group. Hence, the PEG-*b*-PLKC–ATP complex dissociates, accompanied by disassembly of the self-assembled aggregates.



Scheme 1. Building blocks of the superamphiphile and the enzyme-responsive property of the self-assembled aggregates. The superamphiphile self-assembles into spherical aggregates, which disassemble upon addition of enzyme (calf intestinal alkaline phosphatase, CIAP) as a result of the enzymatic hydrolysis of ATP.

[*] C. Wang, Q. S. Chen, Prof. Z. Q. Wang, Prof. X. Zhang
Key Lab of Organic Optoelectronics & Molecular Engineering
Department of Chemistry, Tsinghua University
Beijing 100084 (P.R. China)
Fax: (86) 10-62771149
E-mail: xi@mail.tsinghua.edu.cn

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The superamphiphile was prepared by mixing different molar ratios of PEG-*b*-PLKC and ATP in a 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer solution (0.01M, pH 6.5), with the concentration of PEG-*b*-PLKC kept constant at 0.75 mg mL^{-1}. Although these solutions were clear to the naked eye, the dynamic light scattering (DLS) count rate depended strongly on the molar ratios. We define the compositional variation in terms of the charge ratio (r) between PEG-*b*-PLKC and ATP, where r = (200PEG-PLKC^{200+}):(4ATP^{4-}). The DLS count rates, shown in Figure 1a, were very low (even less than 10 kcps) until r reached

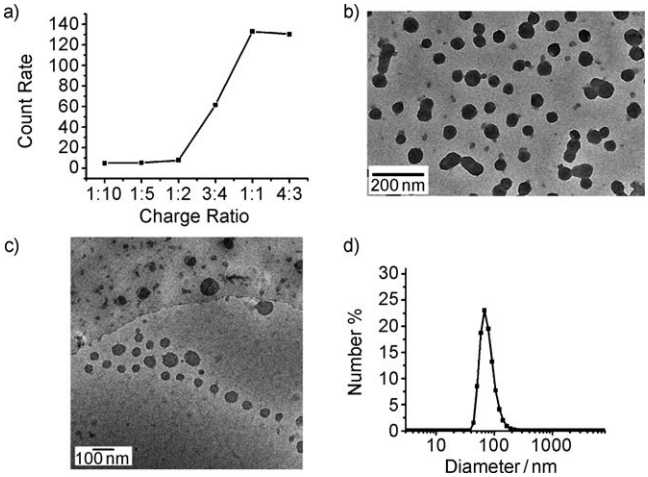

Figure 1. a) DLS count rates of the PEG-*b*-PLKC–ATP complex at different charge ratios. The concentration of the polymer was fixed at 0.75 mg mL^{-1}. b) TEM image, c) cryo-TEM image, and d) DLS data for the self-assembling aggregates formed by PEG-*b*-PLKC–ATP complex with polymer concentration 0.75 mg mL^{-1}. The DLS data are shown as the size probability distribution obtained by a CONTIN analysis.

0.75, which indicated that self-assembled structures began to form at $r > 0.75$. For $r > 1$, the count rate of the aggregates did not change, thus indicating complete complexation. This was confirmed by transmission electron microscopy (TEM): for $r = 0.5$, hardly any aggregates were observed on the copper grids (see Figure S1 in the Supporting Information). A few spherical aggregates began to appear at $r = 0.75$. When r reached 1, the entire field was covered with spherical aggregates, which confirmed complete complexation between PEG-*b*-PLKC and ATP (Figure 1b).

For many electrostatic complexes, as the molar charge ratio increases precipitation will occur, therefore limiting the range of their applicability. Turbidity measurements of different charge ratios of PEG-*b*-PLKC–ATP were obtained by UV/Vis absorbance at 600 nm (turbidity = 1–10^{-A}). The results showed hardly any difference in turbidity for molar ratios from 1:10 to 10:1, thus indicating that the system has good water solubility (see Figure S2 in the Supporting Information).

More information on the size and structure of these self-assembled species was obtained from cryogenic transmission electron microscopy (cryo-TEM) and DLS studies. The pH 6.5 MES solution of PEG-*b*-PLKC–ATP at $r = 1$ with PEG-*b*-PLKC concentration 0.75 mg mL^{-1} was used as the standard solution. Cryo-TEM images (Figure 1c) show that spherical aggregates are formed in aqueous solution, in accordance with the conventional TEM observations. The average diameter of the spherical objects was 70–80 nm from the cryo-TEM results. The average size of the spherical aggregates was confirmed by DLS, which revealed a hydrodynamic diameter of 68 nm (Figure 1d). It should be noted that when the concentration of the PEG-*b*-PLKC–ATP complex changed from 20 times to 1/20 times that of the standard solution, spherical aggregates were still formed, which suggests that the complex is very stable to dilution, as indicated in the DLS count rate measurements (see Figure S3 in the Supporting Information). Interestingly, the size of the spherical aggregates decreased slightly with an increase of the concentration. Considering their good stability and electrostatic cross-linked nature, the spherical aggregates are in many ways reminiscent of the "polyplexes" of DNA and polycations that are used for gene delivery.[9]

^{31}P NMR and HPLC measurements were performed to demonstrate enzymatic cleavage of the phosphate groups of the superamphiphiles. A 150 U L^{-1} concentration of alkaline phosphatase (CIAP), which is comparable with the average amount of alkaline phosphatase present in a healthy adult, was added to PEG-*b*-PLKC–ATP solution. Figure 2a shows

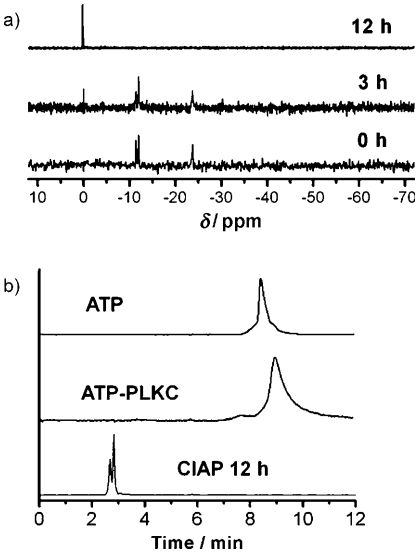

Figure 2. a) ^{31}P NMR spectra of PEG-*b*-PLKC–ATP complex solution at various times after addition of 150 U L^{-1} CIAP. b) HPLC data for pure ATP, PEG-*b*-PLKC–ATP complex, and the complex 12 h after the addition of 150 U L^{-1} CIAP.

^{31}P NMR spectra of the superamphiphile at different times after addition of CIAP. After 3 hours the ATP peaks were weakened and a new peak appeared at about 1 ppm, which corresponded to the released phosphoric acid. After 12 hours only peaks for phosphoric acid were observed, thus indicating that all of the phosphoanhydride bonds had been cleaved. The HPLC results in Figure 2b confirm the enzymatic hydrolysis of ATP within the PEG-*b*-PLKC–ATP complex. The peak with retention time about 9 minutes corresponds to ATP

disappearing 12 hours after treatment with CIAP, which confirmed that all the ATP molecules had been hydrolyzed by CIAP. Since free ATP can be hydrolyzed by CIAP in about 10 minutes, the different hydrolysis rate is probably the result of a dynamic equilibrium between the self-assembled and unassembled states of the superamphiphiles, and the enzyme attacks only the freely soluble species.

Since the PEG-*b*-PLKC–ATP complex is enzyme-responsive, we considered the possibility that this characteristic could be introduced to the self-assembled spherical aggregates described above. Cryo-TEM images showed that hardly any aggregates were present in the solution after CIAP treatment, thus indicating disassembly of the spherical aggregates (Figure 3a). The disassembly process was monitored using the count rate data from DLS (Figure 3b), which reveal that the aggregates disappeared in about 4 hours. It was already shown that for $r < 0.75$ there were hardly any aggregates present, hence most of the aggregates disappeared even when about half the ATP remained.

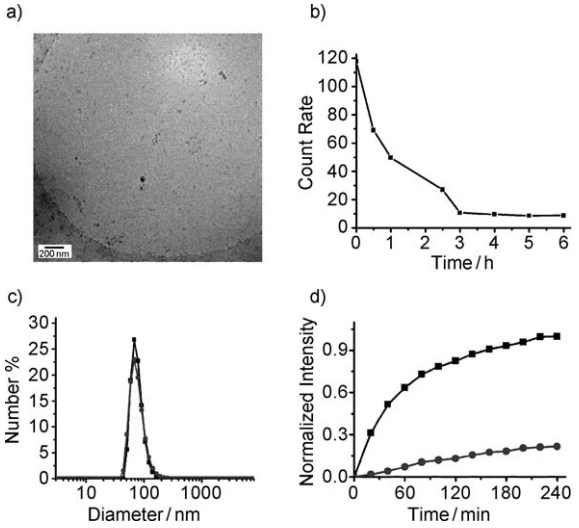

Figure 3. a) Cryo-TEM image of the PEG-*b*-PLKC complex after treatment with 150 U L^{-1} CIAP. b) Count rate of the complex at different times after the addition of CIAP. c) Hydrodynamic diameter of the PEG-*b*-PLKC–ATP complex before (●) and after addition of denatured CIAP (■). d) Release of HPTS encapsulated in the spherical aggregate formed by PEG-*b*-PLKC–ATP complex with (■) and without CIAP (●).

To prove that the protein CIAP itself is not a factor that contributes to the change in PEG-*b*-PLKC–ATP, a control experiment was carried out in which the same amount of denatured CIAP (treated in boiling water for 2 h) was added to the solution. DLS results showed that there was no significant change in either the average diameter or the count rate of the aggregates (Figure 3c), thus eliminating the possibility of the enzyme protein being a factor.

The PEG-*b*-PLKC–ATP aggregate was further studied as a possible medium for encapsulation and release of guest molecules upon enzyme stimulus. The trisodium salt of 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) as a model guest molecule was loaded into the spherical aggregates; excess HPTS was removed by dialysis. The dialysis process not only ensured removal of HPTS that was not encapsulated, but also showed that the PEG-PLKC–ATP–HPTS complex is sufficiently stable in an aqueous medium. The release kinetics of HPTS with and without addition of CIAP was studied through fluorescence emission spectroscopy, which showed the process of controlled release of HPTS from the PEG-*b*-PLKC aggregates. Figure 3d shows that the release rate of the solution treated with CIAP was significantly greater than that in the absence of phosphatase, which confirmed the enzyme-controlled releasing property of the aggregates.

In conclusion, based on the concept of a superamphiphile, we have developed a new way of preparing enzyme-responsive polymeric systems utilizing the electrostatic interactions between a double-hydrophilic block copolymer and a natural enzyme-responsive molecule. Compared with conventional enzyme-responsive polymers, this new method, which does not require covalent synthesis, is very simple, highly efficient, and nontoxic. A noteworthy aspect of this model is that no organic solvent but only water is used in the entire process. The self-assembled spherical aggregates exhibit good responsiveness and releasing properties to phosphatase. In addition, the amount of enzyme used for the controlled release experiment was about the average concentration of alkaline phosphatase present in a healthy adult, which emphasizes the highly efficient responsiveness to enzymes. Thus, the present study provides a route to the fabrication of enzyme-responsive polymeric superamphiphiles for controlled self-assembly and disassembly. It is anticipated that this novel system will have great potential in drug-delivery applications.

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