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Cyclodextrin-Responsive Micelles Based on Poly(ethylene glycol)–Polypeptide Hybrid Copolymers as Drug Carriers

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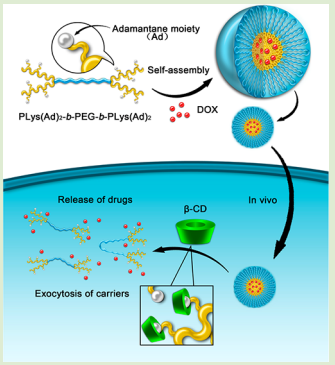
Supporting Information

ABSTRACT: Novel drug carriers based on poly(ethylene glycol) (PEG)–polypeptide copolymers, four-armed poly(ε-adamantane-L-lysine)$_2$-block-poly(ethylene glycol)-block-poly(ε-adamantane-L-lysine)$_2$ (PLys(Ad)$_2$-*b*-PEG-*b*-PLys(Ad)$_2$), have been prepared. The copolymers were synthesized via the ring-opening polymerization of amino acid *N*-carboxyanhydrides. The copolymers could spontaneously form core–shell micelles in aqueous solutions. It has been found that these micelles undergo triggered disassembly in response to an additional β-cyclodextrin (β-CD). The in vitro drug release in response to β-CD has been studied, and the result shows that the release of the entrapped drug doxorubicin (DOX) from the micelles could be accelerated by the addition of β-CD. Their cytotoxicity and cell internalization behavior were also investigated in detail. These micelles are expected to have great potential in controlled drug release applications.



In the past decades, "smart" materials have been extensively developed owing to their various applications, such as drug and gene delivery,[1−3] sensor,[4] and ionic channel.[5] Of various smart materials, amphiphilic copolymers with stimuli-responsive properties have attracted particular attention for their unique predominance.[6,7] These copolymers can self-aggregate into a variety of assemblies with highly organized structures, which can undergo a conformation switch in response to environmental stimuli. In the biological environment, the stimuli can be divided into two main families. The first one is the intrinsic stimuli from the organism itself, such as the temperature, pH value, and the GSH concentration.[8−11] In general, the differentiation between the pathological sites and the healthy tissue is subtle, which requires sophisticated design of polymer structure. The second one is the external stimuli, such as light, magnetic field, ultrasound, and voltage,[12−17] which is difficult to apply in patients. Thus, the developing of new modes of stimuli-responsive systems is very necessary.

Recently, chemical substances were exploited as a new stimulus for drug delivery system. Compared to conventional stimuli, the activity of chemical substances can be easily adjusted by external modification. The concentration of trigger molecules in vivo could be conveniently enhanced by oral administration or injection. Weber and his co-workers prepared stimuli-responsive hydrogels for the release of a bioactive agent in response to a clinically licensed drug, novobiocin.[18] Li and his co-workers reported supramolecular polymeric micelles constructed from ethylcellulose-graft-poly(e-caprolactone) (EC-g-PCL) and an α-cyclodextrin (α-CD) derivate, which respond to L-phenylalanine.[19] Furthermore, from the view of the clinical applications, the biocompatibility and biodegradability of the trigger molecules are essential.

The host–guest interaction between β-cyclodextrin (β-CD) and adamantane has been extensively exploited to fabricate drug delivery systems.[20−22] β-Cyclodextrin is a natural cyclic oligosaccharide composed of seven D(+)-glucose units.[23] It has a hydrophobic internal cavity, which has the ability of including "guest" molecules such as adamantane.[24] The affinity between the β-CD and its guest depends on its association constant. During various guest molecules, adamantane and its derivates have the strongest binging capacity with β-CD in aqueous solution,[25,26] which could exactly form a 1:1 inclusion complex with β-CD. The specific molecular recognition offers the possibility of developing a new trigger. Besides, β-CD is biocompatible, biodegradable, and extensively applied in food industries and drug delivery systems. However, to our best knowledge, there are few reports about the β-CD used as trigger stimuli.

Herein we designed novel β-CD-sensitive micelles based on adamantane-modified PEG–polypeptide hybrid copolymers. The polypeptide segments have good biocompatibility, biodegradability, and biofunctionality due to their structure and function similar to those in the natural proteins.[27−30] Considering that high molecular weight hydrophobic polypeptide segments commonly have poor solubility even in organic solvent, due to their α-helical conformations,[31] the copolymers were designed as a star shape which has a higher hydrophobic proportion with shorter polypeptide chains. Compared with the linear copolymers, star-shaped copolymers may exhibit higher drug loading and different assembly behavior according to

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previous reports.[32−34] In this study, we prepared novel amphiphilic four-armed poly(ε-adamantane-L-lysine)$_2$-block-poly(ethylene glycol)-block-poly(ε-adamantane-L-lysine)$_2$ (PLys(Ad)$_2$-*b*-PEG-*b*-PLys(Ad)$_2$). The copolymers could self-assemble into core−shell micelles in aqueous solution. To study the self-assembly behavior of this coplymer, the morphology and size distribution were characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS), respectively, and we also explored the cytotoxicity and cell internalization behavior of the self-assembled micelles to evaluate the potential for in vivo drug delivery. Meanwhile, their size changes as well as the in vitro drug release behavior of the self-assembled micelles in response to β-CD were also investigated in detail.

For the synthesis of the four-armed block copolymer PLys(Ad)$_2$-*b*-PEG-*b*-PLys(Ad)$_2$, the macroinitiator tetraamino-modified PEG (TAPEG) was first prepared according to our previous work.[35] The four primary amine end groups of the TAPEG were used to initiate the polymerization of Lys(Ad)-NCA monomers. The synthetic route is shown in Scheme S1 (Supporting Information). Ad-COOH was first obtained from the reaction of succinic anhydride and adamantanamine. Then it was converted to NHS esters and coupled to N_α-Z-L-lysine to give the conjugate N_α-Z-L-lysine(Ad). The successful synthesis of N_α-Z-L-lysine(Ad) was testified by ^{1}H NMR spectroscopy, shown in Figure 1a. The resonance signals of the adamantane

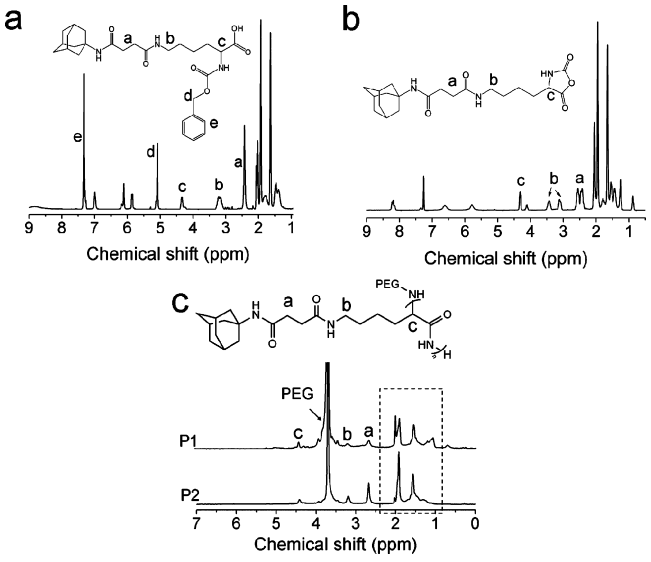


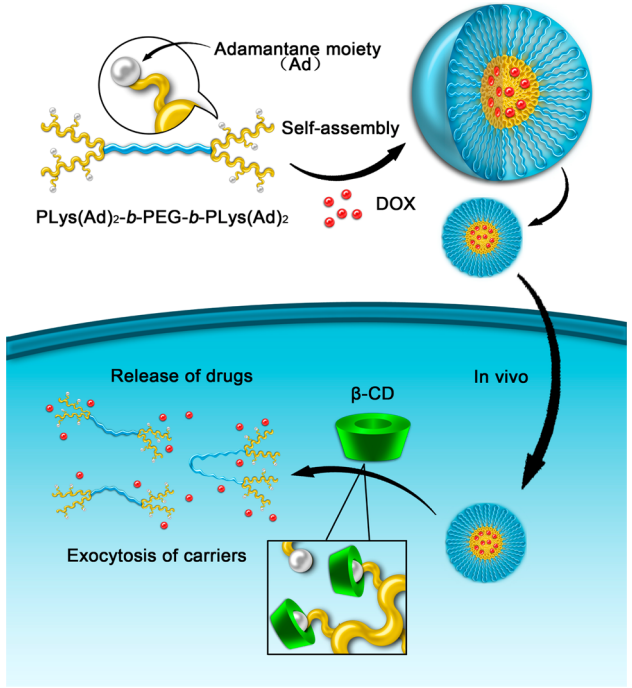
Figure 1. ^{1}H NMR spectra of (a) N_α-Z-L-lysine(Ad); (b) Lys(Ad)-NCA; (c) the copolymers P1 and P2.

moiety appeared at 2.04, 1.96, and 1.64 ppm. The peak at 3.19 ppm was assigned to the protons of the −NH−CH_2− in the lysine moiety. The preparation of Lys(Ad)-NCA was accomplished using Cl_2CHOCH_3. The ^{1}H NMR spectrum of Lys(Ad)-NCA was shown in Figure 1b. The peak appearing at 8.20 ppm was assigned to the protons of −CO−NH− in the NCA ring. Peaks at 5.20 and 7.30 ppm disappeared, which was attributed to the protons of the benzyloxycarbonyl groups, which demonstrated the successful synthesis of Lys(Ad)-NCA.

PLys(Ad)$_2$-*b*-PEG-*b*-PLys(Ad)$_2$ was prepared via the ring-opening polymerization (ROP) of amino acid *N*-carboxyanhydrides initiated by the primary amine groups of TAPEG. The copolymers with TAPEG/Lys(Ad)-NCA feed weight ratios of 1/1 and 1/2 were coded as P1 and P2, respectively. As shown in Figure 1c, the peak at 8.20 ppm disappeared, and characteristic signals of PEG protons at 3.60 ppm appeared after the ROP. The FT-IR spectra of the copolymers were illustrated in Figure S1 (Supporting Information). The peak at 1652 and 1542 cm^{-1}, the typical amide I and II bands, increased with the weight ratio of PLys(Ad) segments, which revealed the ring-opening polymerization of Lys(Ad)-NCA. The number molecular weight of P1 and P2 measured by SEC-MALLS was 9100 with M_w/M_n = 1.022 and 27 700 with 1.016.

As shown in Scheme 1, the amphiphilic copolymer could self-assemble into micelles in water after a dialysis procedure.

Scheme 1. Schematic Illustration of the Formation of Micelles and Triggered Drug Release Mechanism



Pyrene was used as the hydrophobic probe to indicate the formation of micelles. The critical micelle concentration (CMC) of copolymer P1 and P2 was 91 and 33 mg L^{-1}. Micellization of PLys(Ad)$_2$-*b*-PEG-*b*-PLys(Ad)$_2$ was further predicted by the ^{1}H NMR spectrum of the lyophilized micelles in D_2O. The signals of the PLys(Ad) segment almost disappeared, which suggested that the hydrophobic PLys(Ad) core was embedded in the hydrophilic PEG outer shell.

The morphology and size distribution of micelles in water were observed by TEM and DLS. The mean hydrodynamic diameter of P1 micelles was 250 nm with a polydispersity of 0.24. To P2, it was 180 nm with a PDI of 0.13. The TEM images (Figure 2a and 2c) showed that the micelles exhibit a spherical shape and well dispersed without aggregation during drying. The mean diameter measured by TEM was around 50 nm, which was smaller than the hydrodynamic radius (R_h) measured by the DLS, which may be attributed to the shrinkage caused by the water evaporation in vacuum.

As we know, polypeptides display stable secondary structures, such as α-helices, β-sheets, and random coils, due to their intramolecular cooperative hydrogen bonding.[36−38] These secondary structures could strongly influence the self-assembly behavior of the polypeptide chains.[39] To investigate

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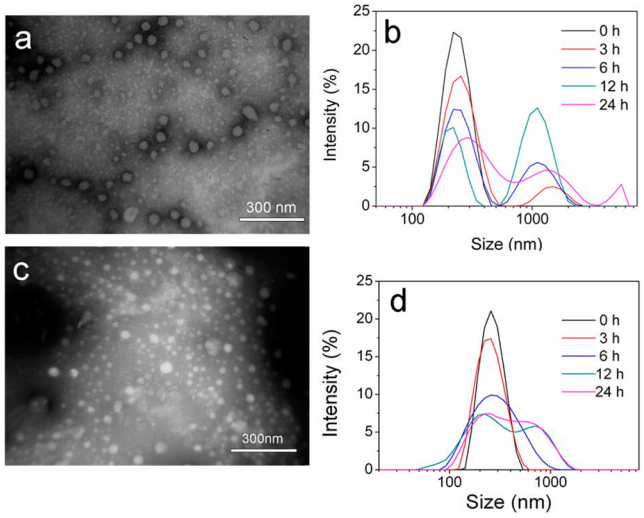

Figure 2. TEM image (a) and size distribution in response to β-CD (b) of P1 micelles in aqueous solution. TEM image (c) and size distribution in response to β-CD (d) of P2 micelles in aqueous solution.

the conformation of the copolymer, circular dichroism (CD) and FT-IR spectroscopy were used. In the FT-IR spectrum of the lyophilized micelles (Figure 3b), the amide I peak at 1652

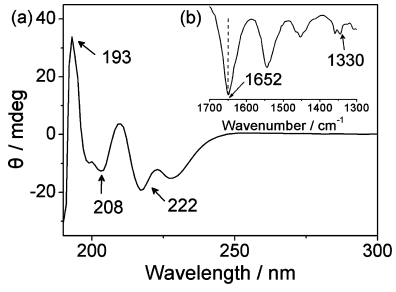

Figure 3. (a) CD spectrum of the micelles in aqueous solution (300 mg L^{-1}) and (b) FT-IR spectrum of the lyophilized micelles.

cm^{-1} and the amide III peak at 1330 cm^{-1} indicated that the conformation of the PLys segment in the micelles could adopt a random coil or an α-helix rather than a β-sheet (amide I peak at 1623 cm^{-1}). Figure 3a showed the CD spectrum of the micelles in aqueous solution, and the negative bands at 208 and 222 nm and the positive ellipticity at 193 nm were detectable (Figure 3a). The presence of these characteristics suggested the existence of α-helical conformation in the copolymer, which results in stable micelles.

In aqueous solution, the adamantane moieties of the copolymer could form the host−guest supermolecular inclusion with β-CD. Due to the solubility of β-CD, the hydrophilic/hydrophobic ratios of the copolymer would be disturbed with the complexing process carrying through. It is expected that the addition of β-CD would affect the assembly patterns of micelles or even disrupt the micelles. To investigate the stimuli-responsive property of the micelles, we monitored the size change of micelles in response to β-CD. When immersed in 20 μM β-CD solution, both the micelles showed a great decrease in scattered light intensity within 24 h. The P1 micelles (Figure 2b) began to aggregate, and the sizes gradually increased from 180 nm to 4 μm, while the P2 micelles (Figure 2d) did not show severe size change. After 24 h of incubation, the reliable measurements of particle sizes and polydispersity of P1 and P2 micelles could not be obtained one after another, which indicated the micelles were disassociated in succession. It is reasonable that the shorter hydrophobic chain of P1 micelles was more facile to be attacked by the trigger molecules, which made the more acute transformation of the micelles. In contrast, in the absence of β-CD, both the micelles were extraordinarily stable. In two weeks, no obvious size change was observed. The results testified our assumption that the micelles were sensitive to β-CD.

Since the micelles undergo triggered disassembly in the presence of β-CD, it is reasonable that the drug release behavior of micelles would be influenced by β-CD. The in vitro drug release from PLys$(Ad)_2$-*b*-PEG-*b*-PLys$(Ad)_2$ micelles under different concentrations of β-CD were investigated. Doxorubicin (DOX), an anticancer drug with red fluorescence, was employed as a hydrophobic model drug. DOX was loaded into the micelles by simple mixing of the copolymer and DOX, followed by dialysis against water. The whole drug release process was carried out in PBS. To P1, the loading content (LC) and encapsulation efficiency (EE) of DOX into the micelles were 2.4 wt % and 24.0%, respectively. To P2, they were 3.5 wt % and 35.0%. The values of EE and LC indicate that the P2 micelles were more stable and had higher loading capacity due to their higher hydrophobic content.

We observed the behavior of DOX-loaded micelles in PBS with different concentrations of β-CD. The DOX release profiles of P1 and P2 micelles were shown in Figure 4. In the

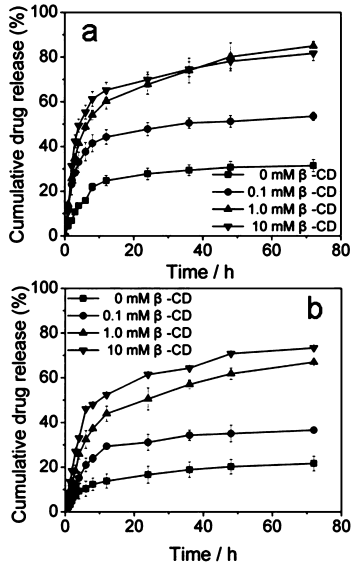

Figure 4. Drug release profiles of (a) P1 micelles and (b) P2 micelles.

absence of β-CD, the release of DOX from P2 and P1 micelles was retarded, and the release of DOX from P1 micelles was faster than P2 micelles. In the initial 24 h, about 27% DOX was released from P1 micelles and 16% DOX was released from P2 micelles. In the presence of 0.1 mM β-CD, the release rate from both the micelles was faster, but the drug release from the P1 micelles was still more convenient than that from the P2 micelles. As the β-CD concentration increases, a more rapid release was observed in the 1 mM β-CD solution. More than half of the drug molecules escaped from both the micelles in 24 h. However, in the 10 mM β-CD solution, the drug release from the micelles has no obvious increase compared to that in

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the 1 mM β-CD solution. Compared with P2 micelles, the release rate from P1 micelles was always faster and with a burst release, which was consistent with the size alteration of micelles in response to β-CD. We also investigated the behavior of DOX-loaded micelles in PBS with glucose, the analogue of β-CD. Results showed that the glucose had no effect to the drug release (Figure S3, Supporting Information). The results suggested that in the presence of β-CD the release of DOX could be accelerated due to the destabilization of micelles.

The biocompatibility of the PLys(Ad)$_2$-*b*-PEG-*b*-PLys(Ad)$_2$ copolymer was investigated. Here we have chosen HeLa cells to assess the cell cytotoxicity of the copolymer using the MTT assay. The effect of copolymer concentration on the proliferation of the cells was shown in Figure S2 (Supporting Information). At concentrations up to 500 mg L^{-1}, both the copolymers did not show apparent inhibition effects on cell viability and proliferation. The low cytotoxicity suggested that the copolymers were suitable for potential application in vivo.

To estimate the capacity of PLys(Ad)$_2$-*b*-PEG-*b*-PLys(Ad)$_2$ micelles for intracellular drug delivery, the internalization of the micelles into HeLa cells was monitored by confocal laser scanning microscopy (CLSM). The CLSM images of HeLa cells were shown in Figure 5. Figure 5b showed the cell nucleus

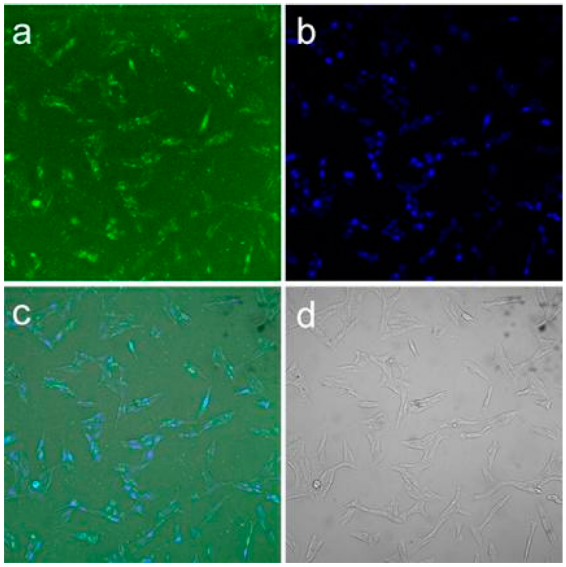

Figure 5. CLSM images of HeLa cells coincubated with PLys(Ad)2-b-PEG-b-PLys(Ad)2. (a) FITC fluorescence in cells (green); (b) cell nuclei stained by Hoechst 33258 (blue); (c) overlays of two fluorescence; (d) phase contrast.

location by coloration with Hoechst 33258. From the pictures of Figure 5a and 5c, we can observe that the green fluorescence from the FITC labeled PLys(Ad)$_2$-*b*-PEG-*b*-PLys(Ad)$_2$ appeared inside the whole cells, which indicated that the micelles were successfully uptaken by the cells.

In summary, we synthesized novel PEG−polypeptide copolymers PLys(Ad)$_2$-*b*-PEG-*b*-PLys(Ad)$_2$ via the ring-opening polymerization of amino acid *N*-carboxyanhydrides. The copolymers could spontaneously form core−shell micelles in aqueous solutions, which were highly sensitive to the β-CD due to the host−guest interaction between the adamantane moiety and β-CD. In the presence of β-CD, these micelles underwent triggered disassembly, inducing the accelerated release of the entrapped drug DOX from the micelles. The cytotoxicity studies showed that the copolymers were nontoxic. Cell internalization experiment confirmed that the micelles could be successfully uptaken by HeLa cells. These novel β-CD-sensitive micelles with great biocompatibility and biodegradablility open up a pathway for a new trigger mode.

■ ASSOCIATED CONTENT

Supporting Information

Materials, methods, instrumentation, and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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