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Supramolecular Assemblies

Enzyme-Responsive Supramolecular Nanoparticles Based on Carboxyl-Modified Cyclodextrins for Dual Substrate Loading

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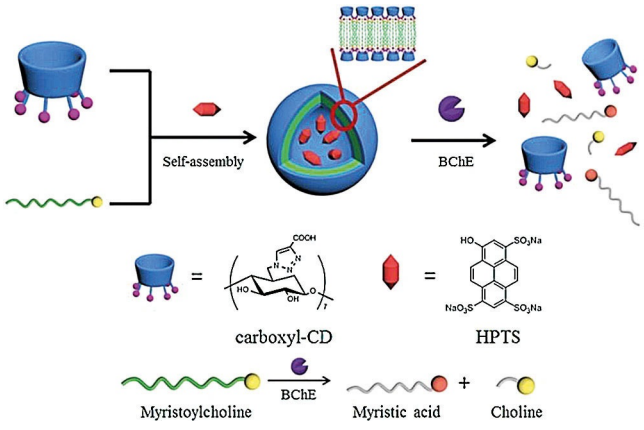
Abstract: Enzyme-responsive supramolecular assemblies have recently attracted more and more attention in biomaterial fields because of their controlled drug release at specific sites where the target enzyme is located. In this work, an enzyme-responsive nanoparticle (NP) was successfully constructed through the molecular-induced aggregation of hepta-carboxyl-modified cyclodextrins (carboxyl-CDs) towards the enzyme-responsive small molecule myristoylcholine, and the resulting spherical NP from carboxyl-CD and myristoylcholine was fully characterized. Significantly, this NP can load two different substrates, that is, amantadine and anionic dye HPTS, and disassemble when treated by butyrylcholinesterase (BChE), an enzyme that can break the ester linkage of myristoylcholine chloride. Therefore, this NP may find applications in dual drug delivery.

Stimuli-responsive assemblies that can disassemble and release their encapsulated cargo upon external stimuli represent one of the extraordinary fascinating topics due to their wide uses in fields of biotechnology, diagnostic, and drug delivery systems.[1] Compared to other external stimuli, such as temperature,[2] pH,[3] light[4] or their combination, the enzyme-responsive approach has attracted more and more interest because of its biocompatibility and high selectivity/sensitivity in drug delivery. Generally, many diseases are characterized by imbalances in the expression and activity of specific enzymes in the diseased tissue, and at this site the enzyme-responsive assemblies can disassemble and release the encapsulated cargo. Among the various enzymes, cholinesterases (ChEs) are a family of serine enzymes that show three distinct activities: entrase, aryl acylamidase, and peptidase. In all vertebrate species, butyrylcholinesterase (BChE) acts as not only a detoxification enzyme to scavenge anticholinesterase compounds but also an activator enzyme that converts prodrugs into their active forms.

On the other hand, some water-soluble macrocyclic compounds, such as cyclodextrins, sulfonatocalixarenes, pillararenes, and cucurbiturils, have been widely employed in constructing stimuli-responsive supramolecular assemblies.[5] Liu et al. reported a series of supramolecular vesicle/nanoparticles based on the complexation of anionic macrocyclic hosts and cationic molecules as carriers for drug delivery.[6] Zhang et al. reported the enzyme responsive polymeric supra-amphiphile for drug delivery.[7] Kim et al. reported the α-amylase and lipase responsive release of guest molecules from Si-MPs with cyclodextrin-gatekeepers.[8] Schalleyet al. reported the water-soluble pillar[5]arene-based polyethyleneglycol-substituted amphiphile which self-assembled to micelles responsive to enzyme catalysis.[9] Wang et al. reported a multistimuli-responsive supramolecular assembly based on water-soluble pillararenes and its drug release.[10] In this work, we constructed a BChE-responsive supramolecular nanoparticle employing biocompatible hepa-carboxyl-modified cyclodextrins (carboxyl-CDs) as the macrocyclic host to induce the molecular aggregation of cationic enzyme-cleavable guest myristoylcholine (Scheme 1). There are some advantages of this carboxyl-CD/myristoylcholine system: (1) The molecular induced aggregation of carboxyl-CDs greatly lowers the critical concentration (CAC) of myristoylcholine. (2) CDs are water-soluble, nontoxic, commercially available at low cost, and can include various in-



Scheme 1. Schematic illustration of carboxyl-CD/myristoylcholine supramolecular nanoparticles.

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organic/organic/biological molecules in their cavity. (3) Because carboxyl-CD possesses 7 negative charges on the primary rim, but sulfonato-calix[4]arene has only 4 negative charges. Therefore, the negative charge density of carboxyl-CD is higher than that of sulfonato-calix[4]arene. (4) Myristoylcholine can be cleaved to myristic acid and choline in the presence of BChE. It is our special interest to provide an applicable dual substrate carrier model based on the molecular induced aggregation approach and enzyme-responsive control release.

Construction of supramolecular nanoparticles. Myristoylcholine was an enzyme-responsive antimicrobial agent and could be used in the drug delivery system. However, it could not form the enzyme-responsive self-assembly because the CACs of the substrate (2.5 mM) and product (4.5 mM) were similar.[11] Surprisingly, a simple mixture of carboxyl-CD and myristoylcholine in aqueous solution showed the obvious Tyndall effect, indicating the formation of large aggregates. Moreover, the critical aggregation concentrations (CAC) of myristoylcholine with and without carboxyl-CDs were measured by monitoring the dependence of the optical transmittance of myristoylcholine. In the absence of carboxyl-CDs, the optical transmittance of myristoylcholine showed no apparent change as the concentration increases from 0.01 mM to 0.06 mM. With the addition of carboxyl-CDs, the optical transmittance of myristoylcholine at 450 nm decreased gradually with increasing the myristoylcholine concentration. In addition, an inflection point at 0.074 mM was observed on the plot of optical transmittance at 450 nm versus the concentration of myristoylcholine (Figure S1, Supporting Information). This means that myristoylcholine gave a carboxyl-CD-induced CAC value as 0.074 mM. Moreover, the preferable mixing ratio of the assembly was further measured. By gradually adding carboxyl-CD to a myristoylcholine solution at a fixed concentration of 0.14 mM, the optical transmittance at 450 nm decreased rapidly with the addition of carboxyl-CDs, and then gradually increased to a stable value. The minimum was reached at a carboxyl-CD/myristoylcholine ratio of 1:7, i. e. a ratio of carboxyl anion/myristoylcholinecation of 1:1 (Figure 1). This result indicated that the multiple charge of carboxyl-CD played an important role in the molecular induced aggregation of myristoylcholine. Control experiments show that, when replacing the carboxyl-CD by *para*-hydroxybenzoic acid, there was no transmittance change at 450 nm (Figure S2, Supporting Information).

Based on previous research on host-induced aggregation,[6] the addition of carboxyl-CDs could decrease the CAC of myristoylcholine. Herein, a possible assembly mode would be deduced as illustrated in Scheme 1. Without carboxyl-CD, free myristoylcholine molecules could not form a large self-aggregate. Upon the addition of carboxyl-CD, one carboxyl-CD and several myristoylcholine would form a complex. Through the hydrophobic interactions among the aliphatic tails of myristoylcholine, many complexes subsequently integrated together to form a large aggregate that curved to a spherical nanostructure. Dynamic light scattering (DLS), zeta potential, transmission electron microscopy (TEM), and scanning electron microscopy (SEM) were employed to investigate the molecular induced aggregation behaviors of carboxyl-CD/myristoylcholine system. DLS results gave a hydrodynamic diameter of 288 nm at a scanning angle of 90° with a narrow size distribution (Figure S3, Supporting Information). TEM images showed a number of spherical nanosturctures with an average diameter around 200 nm (Figure 2a). SEM images also showed many spherical nanostructures around 250 nm (Figure 2b), which was basically consistent with the corresponding values from DLS and TEM. Because there was no critical evidence to prove that such nanostructures were hollow or solid, thus we classified them as a kind of nanoparticle. Zeta potential measurements give an average zeta potential of carboxyl-CD/myristoylcholine system as +9.22 mV (Figure S4, Supporting Information), indicating that the resultant nanoparticles may have the capability of associating anionic substrates. Moreover, the car-

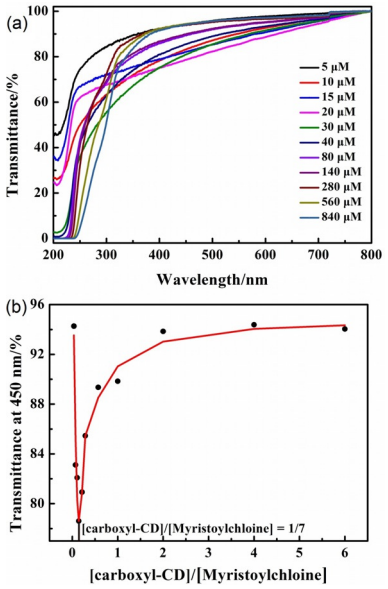

Figure 1. (a) Optical transmittance of aqueous solutions containing myristoylcholine (140 μM) and carboxyl-CD from 5 μM to 840 μM. (b) Dependence of the optical transmittance at 450 nm versus the ratio of carboxyl-CD to myristoylcholine, [myristoylcholine] = 140 μM.

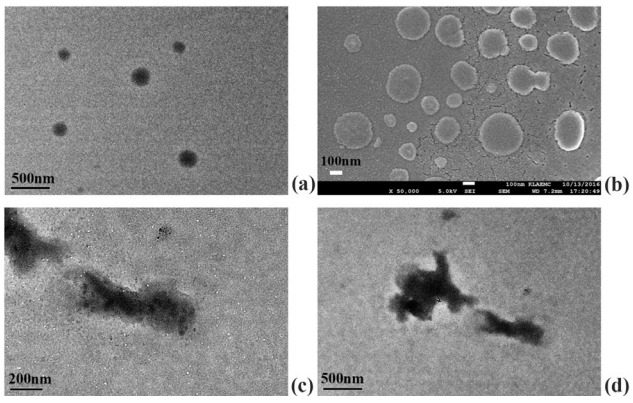

Figure 2. (a) TEM and (b) SEM images of carboxyl-CD/myristoylcholine system [carboxyl-CD] = 20 μM, [myristoylcholine] = 140 μM. (c–d) TEM images of carboxyl-CD/myristoylcholine system after addition of BChE for 3 h. [carboxyl-CD] = 20 μM, [myristoylcholine] = 140 μM, [BChE] = 0.1 U mL^{-1}.

boxyl-CD/myristoylcholine system was found to be very stable at room temperature in at least 6 h (Figure S5, Supporting Information).

Enzyme-responsive disassembly of carboxyl-CD/myristoylcholine nanoparticles. The enzyme-responsive disassembly process induced by BChE was monitored by the optical transmittance. As seen from Figure 3, the optical transmittance of

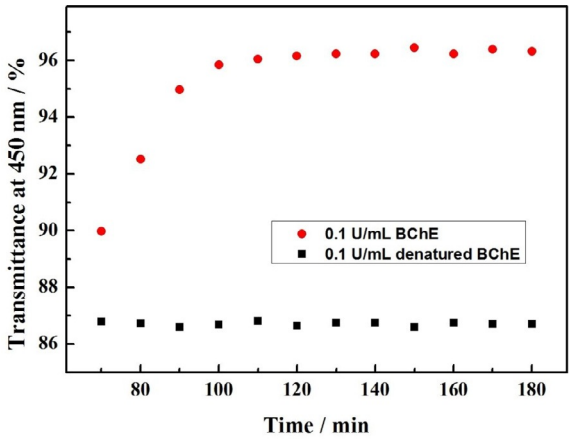

Figure 3. Dependence of the optical transmittance of carboxyl-CD/myristoylcholine system at 450 nm on time in the presence of 0.10 U mL^{-1} BChE and denatured BChE, [carboxyl-CD] = 20 μM, [myristoylcholine] = 140 μM.

carboxyl-CD/myristoylcholine system increased to >95% after the addition of BChE in 2 h, revealing that almost all the nanoparticles were disrupted. Similarly, no spherical particles could be observed in TEM images of carboxyl-CD/myristoylcholine + BChE system (Figures 2b–c). In the control experiment, no appreciable changes of optical transmittance were observed when using the denatured BChE (treated in boiling water for 1 h) instead of BChE, indicating the activity of BChE played an important role in the disassembly of carboxyl-CD/myristoylcholine nanoparticles. Furthermore, the disassembly rate increased with the addition of more BChE (Figure S6, Supporting Information). Moreover, the specificity of enzyme-responsive disassembly was also investigated. The results showed that the carboxyl-CD/myristoylcholine system gave no optical transmittance changes with the addition of other enzymes such as calf intestinal alkaline phosphatase (CIAP, 0.1 U mL^{-1}) (Figure S7, Supporting Information) and trypsin (0.1 U mL^{-1}) (Figure S8, Supporting Information), demonstrating the good enzyme specificity of carboxyl-CD/myristoylcholine system towards BChE.

Substrate loading and BChE-triggered release. It is reasonable to expect that the enzyme-responsive disassembly can trigger a concomitant release of substrates within the interior of nanoparticles. Herein, the trisodium salt of 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) was chosen as a model molecule to be loaded into the nanoparticles, and the loading/release behaviors were investigated in PBS buffer solution (pH 7.2). After loading the free HPTS to the carboxyl-CD/myristoylcholine system and removing the excess HPTS by dialysis, the fluorescence intensity quenched by 64% (Figure 4a), indicating the loading of HPTS into the carboxyl-CD/myristoylcholine

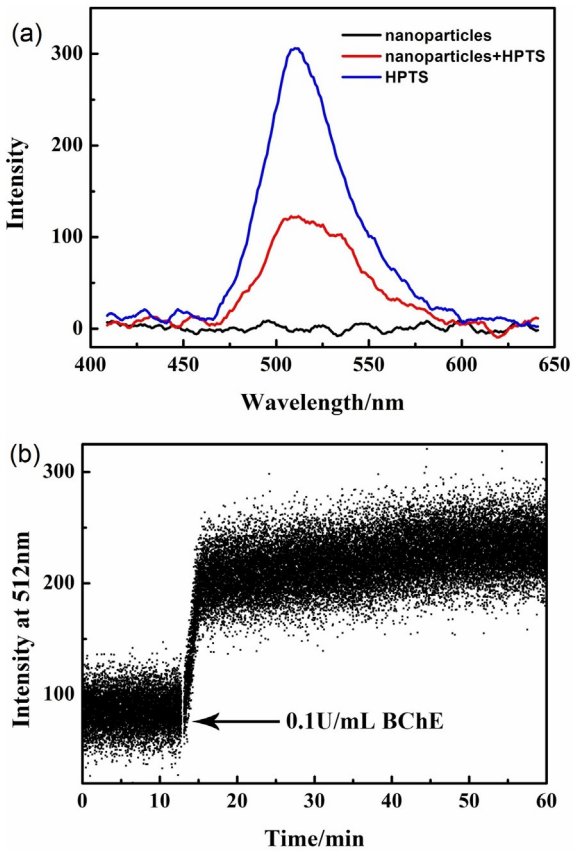

Figure 4. (a) Fluorescence emission spectra of HPTS-loaded NPs, free NPs, and HPTS. [carboxyl-CD] = 20 μM, [myristoylcholine] = 140 μM, [HPTS] = 10 μM. (b) Fluorescence emission kinetics spectra of HPTS-loaded nanoparticles at different time in the presence of BChE (0.1 U mL^{-1}), Ex = 339 nm, Em = 512 nm. pH 7.2.

system. Moreover, after the anionic dye HPTS was loaded, the zeta potential of carboxyl-CD/myristoylcholine system decreased to +4.16 mV (Figure S9, Supporting Information). The DLS of HPTS-loaded nanoparticles gave a hydrodynamic diameter of 307 nm, which was slightly larger than the corresponding value without HPTS (Figure S10, Supporting Information). Because the cavity of carboxyl-CD barely included HPTS, we deduced that HPTS should be encapsulated by the nanoparticles mainly through the electrostatic interactions between the positively charged nanoparticles and the anionic substrates. The enzyme-responsive release behaviors of HPTS with and without addition of BChE were studied through the fluorescence emission spectroscopy. Without BChE, the carboxyl-CD/myristoylcholine system showed a very low release of entrapped HPTS, indicating the nanoparticles were stable to the leakage at room temperature. However, the release rate was significantly enhanced when the nanoparticles were treated with BChE, and more than 70% of HPTS was released within 10 minutes (Figure 2b). In the control experiment, the fluorescence of HPTS unchanged with the addition of BChE only.

In addition to the anionic substrate HPTS, the carboxyl-CD/myristoylcholine system could also load amantadine, an *anti*-Parkinson and antiviral drug, benefitting the strong association of β-CD cavity with amamantane derivatives. By fixing the con-

centration of carboxyl-CD/amantadine complex at 0.02 mM and varying the concentration of myristoylcholine, the minimum was observed at 0.12 mM at the plot of the transmittance at 450 nm vs. the concentration of myristoylcholine, referring to a molecular ratio of carboxyl-CD:amantadine:myristoylcholine as 1:1:6, that is, a ratio of anion/cation as 1:1 (Figure S11, Supporting Information). Interestingly, after loading the free HPTS to carboxyl-CD/amantadine/myristoylcholine ternary system, the fluorescence intensity of HPTS also quenched by 60% and nearly recovered when further treated with BChE (Figure S12, Supporting Information). This phenomenon implies that the present carboxyl-CD/myristoylcholine system is applicable as a potential drug carriermodel that can encapsulate two different substrates and realize the enzyme-responsive release.

In conclusion, we have successfully constructed an enzyme-responsive supramolecular nanoparticle through the molecular induced aggregation of negatively charged cyclodextrins towards positively charged small molecules. This nanoparticle can associate two different substrates and showed the good enzyme responsiveness. It is reasonable to assume that this kind of enzyme-responsive supramolecular nanoparticle could be explored as carries for drug delivery at specific sites where BChE is over-expressed.

Experimental Details

Material preparation. All of the reagents and solvents were commercially available and used without further purification. Carboxyl-CD was synthesized and purified according to the literature procedure[12] and indentified by ^{1}H NMR spectroscopy in D_2O, performed on a Varian 400 spectrometer. Column Chromatography was performed on silica gel.

HPTS-loaded nanoparticles. HPTS-loaded nanoparticles were prepared as follows. A certain amount of HPTS was added to a solution containing carboxyl-CD and myristoylcholine, and then some water was added until the volume of the solution reached 25 mL. The ultimate concentrations of HPTS, myristoylcholine, and carboxyl-CD were 0.01 mM, 0.14 mM, and 0.02 mM, respectively. Subsequently, the prepared HPTS-loaded nanoparticles were purified by dialysis (molecular weight cutoff 3500) in distilled water several times until the water outside the dialysis tube exhibited negligible HPTS fluorescence.

UV/Vis spectra. The optical transmittance of the aqueous solution was measured in a quartz cell (light path 10 mm) on a Shimadzn UV-3600 spectrophotometer equipped with PTC-348WI temperature controller.

Fluorescence spectra. Steady-state fluorescence spectra were recorded in a conventional quartz cell (light path 10 mm) on a Varian Cary Eclipse equipped with Cary single-cell Peltier accessory to control the temperature. The fluorescence was followed in the time-scan mode. (λ_{ex} = 339 nm, λ_{em} = 512 nm, band width (ex) 2.5 nm, band width (em) 5.0 nm)

DLS measurements. The sample solution for DLS measurements were prepared by filtering solution into a clean scintillation vial. The samples were examined on a laser light-scattering spectrometer (BI-200SM) equipped with a digital correlator (Turbbo-Corr) at 532 nm at a scattering angle of 90°.

Zeta potential. Zeta potential was measured by Zeta PALS + BI-90 instrument (Brookhaven Co. USA).

High-resolution TEM and SEM. High-resolution TEM images were acquired using a Tecnai 20 high-resolution transmission electron microscope operating at an accelerating voltage of 200 KeV. The sample for high-resolution TEM measurement was prepared by dropping the solution onto a copper grid. The grid was then air-dried. SEM images were recorded on a Hitachi S-3500N scanning electron microscope. The sample for SEM measurements was prepared by dropping the solution on to a coverslip, followed by evaporating the liquid in air.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: cyclodextrins · drug delivery · enzymes · nanoparticles

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