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Enzyme-responsive sulfatocyclodextrin/prodrug supramolecular assembly for controlled release of anti-cancer drug chlorambucil†

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Supramolecular drug delivery systems are becoming an increasingly important part in controlled drug release. In this work, we report a novel enzyme-responsive supramolecular assembly directly constructed using biocompatible sulfato-β-cyclodextrin (SCD) and an anti-cancer prodrug, *i.e.* choline modified anti-cancer drug chlorambucil (QA-Cbl). The supramolecular assembly acts as an effective drug delivery system *via* the controlled drug loading and enzyme-responsive drug release, because the butyrylcholinesterase (BChE) can cleave the ester bond of QA-Cbl prodrug, resulting in the release of anti-cancer drug chlorambucil (Cbl). Compared to other sophisticated drug delivery systems, the present system provides a feasible and functional approach for achievement of controlled drug release.

Cancer is currently one of the most challenging health problems to overcome in the world, and therefore many scientists are working tirelessly to explore ways of cancer therapy. Among various anti-cancer treatments, chemotherapy is currently an indispensable therapeutic tool for most oncological treatments. Unfortunately, traditional chemotherapy still faces several limitations in clinical application; for example, the chemotherapy drugs used to cause cancer cell apoptosis can lead to toxicity to human body and severe multi-drug resistance (MDR).[1] In order to overcome these limitations, novel and efficient drug delivery systems (DDSs), which allow the release of the drugs only at specific sites *via* external stimuli, have gradually aroused widespread concerns.[2] Despite the tremendous development of DSSs to date, there still exist several challenges to overcome. Firstly, many DDSs rely mainly on some nano-carriers (micelles,[3] liposomes,[4] water-soluble polymers,[5] *etc.*) to encapsulate drugs, and then deliver them to the targeted sites, which often leads to low drug loading and therefore, this becomes a major drawback in clinical applications.[6] Secondly, in order to solve the problems of poor water solubility and low blood circulation of the anti-cancer drugs, a number of sophisticated DDSs have been designed, but shelved in practical application, because of their high cost and tedious covalent synthesis.[7] Thirdly, in the process of controlling drug release, some DDSs may introduce external stimuli that are harmful to the human body, such as ultraviolet light[8] and electroporation,[9] causing other undesirable side effects. In this regard, compared with other external stimuli, the enzyme-responsive drug release typifies an elegant biocompatible method with high sensitivity and specific selectivity.[10]

Since Albert introduced the concept of "prodrug" in 1958,[11] employing a prodrug has become an effective method of integrating chemotherapy and diagnosis. A prodrug itself has no biological activity or has low activity, and becomes an active drug after being metabolized in the body. It can be imagined that using supramolecular chemistry as a bridge, DDSs that combine prodrugs with macrocyclic molecules can provide new ideas for therapeutics in the field of medicine.[12] Recently, combinations of prodrugs with macrocyclic compounds *via* host–guest complexation have been reported, which have essential influence in the supramolecular biochemistry field. Wang *et al.* reported a supramolecular assembly constructed by DOX-based prodrug with water-soluble pillar[6]arene (WP6) and its self-catalyzed rapid drug release. Therein, WP6 effectively catalyzed the cleavage of hydrazone bond of the prodrug *via* a favored intramolecular process, under acidic conditions.[13] Huang *et al.* reported that water-soluble pillar[6]arene modified by carboxylate anionic groups on both rims was utilized as a supramolecular container to enhance the solubility of the prodrug *via* hydrophobic interactions.[14] Liu *et al.* presented an enzyme-responsive supramolecular vesicle by employing *p*-sulfonatocalix[4]arene and natural myristoylcholine as the macrocyclic host and enzyme-cleavable guest, respectively. The operational targeted DDS could be dissipated by cholinesterase with high specificity and efficiency.[15]

Herein, we report the construction of an enzyme-responsive small molecule prodrug/macrocyclic supramolecular assembly for anti-cancer drug release from sulfato-β-cyclodextrin (SCD)

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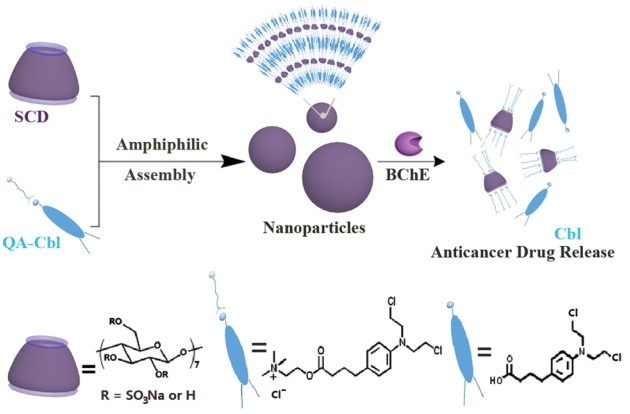
and a water-soluble prodrug, *i.e.* choline-modified chlorambucil (QA-Cbl). Sulfato-β-cyclodextrin, a negatively charged macrocyclic receptor, is water-soluble, non-toxic, inexpensive, and more importantly, can greatly lower the critical aggregation concentration (CAC) of the prodrug QA-Cbl *via* electrostatic interactions.[16–18] The enzymatically degradable prodrug molecule QA-Cbl, containing a quaternary ammonium group and an anti-cancer drug chlorambucil moiety, was designed and synthesized by introducing a cleavable ester bond spacer. It is noteworthy that chlorambucil (Cbl), a water-insoluble DNA-alkylating anti-cancer drug, has been approved by the Food and Drug Administration (FDA).[19] Importantly, butyrylcholinesterase (BChE) can cleave QA-Cbl into two parts, the anti-cancer drug chlorambucil (Cbl) and choline, leading to the SCD/QA-Cbl supramolecular assembly being dissipated. Our constructed prodrug/sulfatocyclodextrin nano-assembly possesses the following four advantages: (1) free Cbl can be released to kill the cancer cells owing to enzyme-responsive cleavage of the ester bond in QA-Cbl; (2) high drug loading efficiency by directly using prodrug QA-Cbl molecules as building blocks of the supramolecular assembly, compared to traditional drug encapsulation method; (3) due to their amphiphilic structures, SCD and QA-Cbl can assemble to form nanoparticles that have enhanced permeability and retention (EPR) effect, as well as the reverse cancer multidrug resistance; (4) the system is not only simple, efficient, safe and non-toxic, but also has good biocompatibility (Scheme 1).

Since the QA-Cbl molecule contains a hydrophilic choline moiety and a hydrophobic Cbl moiety, its amphiphilic framework can be aggregated by SCD to form large assemblies *via* host–guest interactions. The critical aggregation concentration (CAC) of QA-Cbl in the presence of SCD was measured by monitoring the dependence of the optical transmittance at 460 nm on the concentration of QA-Cbl. Without SCD, the optical transmittance of QA-Cbl at 460 nm showed no obvious change as the concentration increased from 0.10 to 1.00 mM (Fig. S4, ESI†), indicating that QA-Cbl alone cannot aggregate in this concentration range. Upon addition of SCD, the optical transmittance decreased gradually with increasing concentration of QA-Cbl, owing to the formation of large aggregates.



Scheme 1 Schematic illustration of the cholinesterase-responsive SCD/QA-Cbl drug delivery system.

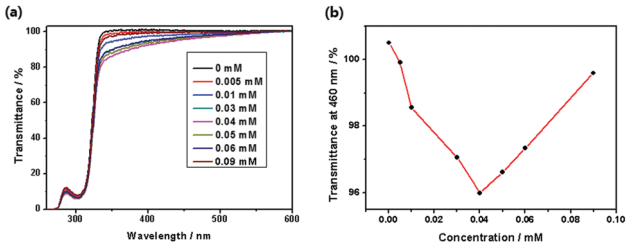


Fig. 1 (a) Optical transmittance of QA-Cbl (0.70 mM) upon increasing concentration of SCD (0–0.09 mM) at 25 °C in PBS. (b) Dependence of the optical transmittance at 460 nm on the SCD concentration (0–0.09 mM) with a fixed QA-Cbl concentration (0.70 mM) in PBS at 25 °C.

Meanwhile, an inflection point at 0.50 mM was observed on the plot of optical transmittance at 460 nm *versus* the concentration of QA-Cbl (Fig. S5, ESI†), corresponding to a complexation-induced CAC value of QA-Cbl in the presence of SCD. There is no tendency for SCD to self-aggregate under the same conditions (Fig. S6, ESI†). To determine the best molar ratio of the intermolecular aggregation between SCD and QA-Cbl, the optical transmittance of QA-Cbl at a fixed concentration (0.70 mM) was monitored, while increasing the concentration of SCD. As can be seen from Fig. 1, at a fixed concentration of QA-Cbl (0.70 mM), the transmittance at 460 nm first gradually decreased until reaching a minimum at a SCD concentration of 0.04 mM, and then increased upon further addition of SCD. The sharp decrease indicates the formation of a higher order complex of SCD with QA-Cbl, eventually leading to a supramolecular assembly, which is disassembled upon extra addition of SCD to offer a simple inclusion complex. Accordingly, the optimum mixing ratio of SCD/QA-Cbl assemblies was measured as 0.04 mM SCD/0.70 mM QA-Cbl. The drug loading efficiency (DLE (wt%) = (weight of loaded drug/weight of feeding drug) × 100%) was calculated as 77.14% (see ESI† for details).

In addition, a simple mixture of SCD with QA-Cbl at optimum molar ratio in phosphate buffer solution exhibited a clear Tyndall effect (Fig. S6, ESI†), indicating the existence of abundant aggregates. In control experiments, neither SCD nor QA-Cbl exhibited Tyndall effect, revealing that both SCD and QA-Cbl did not form large self-aggregates under the same conditions. Furthermore, dynamic laser scattering (DLS) and transmission electron microscopy (TEM) were employed to identify the morphology and size distribution of the SCD/QA-Cbl assembly. The DLS results illustrated that the SCD/QA-Cbl assembly exhibited a narrow size distribution with an average hydrodynamic diameter around 251.93 nm, at a scattering angle of 90° (Fig. S7, ESI†). TEM images showed a number of spherical nanostructures with diameters ranging from 100 to 300 nm (Fig. 2a), indicating the formation of supramolecular aggregates. These results indicate that an assembly was formed *via* the electrostatic interactions between the polyanions on the outer edge of the SCD cavity and the quaternary ammonium cations of the guest molecules. Therein, the prodrug QA-Cbl directly serves as the building block, which significantly increases the ability to load chlorambucil. Since SCD exists as a mixture of CDs randomly substituted by sulfonate groups with an average degree of substitution of 13,

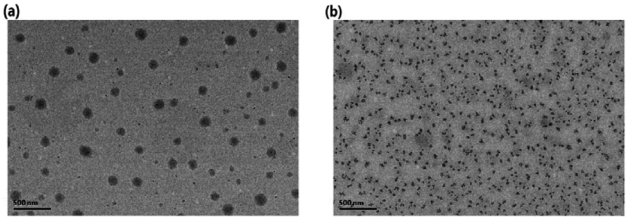


Fig. 2 TEM image of the SCD/QA-Cbl assembly (a), SCD/QA-Cbl assembly incubated with BChE 30 minutes (b), [BChE] = 0.2 U mL^{-1}.

the resultant SCD/QA-Cbl nanoparticles were inhomogeneous to some extent. In addition, we classified the spheres as a type of nanoparticle, since there was no definite evidence to prove whether they were hollow or solid. A probable principle of the formed multi-layer structure may be as follows: free QA-Cbl molecules cannot form a large self-aggregate. With the addition of SCD, one SCD and several QA-Cbls formed a complex, and these complexes then combined to form a large multi-layered aggregate. Subsequently, the aggregate curved to engender a multi-layered sphere with an alternating shell structure. The resulting assemblies were simultaneously stabilized by electrostatic, π–π, and hydrophobic interactions. The synergistic effect of these non-covalent interactions caused the QA-Cbls to accumulate tightly so as to form the multi-layered, high-ordered, and well-stabilized nanoparticles. Moreover, the optical transmittance of SCD/QA-Cbl assembly was nearly unchanged in PBS buffer within 6 h (Fig. S18 and S19, ESI†), indicating good stability of the supramolecular assembly.

The enzyme-responsive disassembly of the SCD/QA-Cbl system, induced by BChE was monitored by the optical transmittance. In Fig. S8 (ESI†), the optical transmittance at 460 nm gradually increased on increasing time and finally reached >97% after 10 min, revealing the disappearance of most nanoparticles. In contrast, no distinct changes in the optical transmittance were observed without the addition of BChE (Fig. S9, ESI†), showing that the hydrolysis of the ester bond is the main reason of disassembly. Furthermore, no large spherical aggregates can be observed in TEM images after treating the SCD/QA-Cbl assembly with BChE (Fig. 2b), and the remaining nanoparticles with a much smaller size than that of SCD/QA-Cbl assembly may be assigned to the aggregate of SCD with the enzymolysis product of QA-Cbl. The process of disassembly is accompanied by the visible disappearance of the Tyndall effect. In the control experiment, the same amount of denatured BChE (treated in boiling water for 1 h) was added to the SCD/QA-Cbl solution under the same conditions. However, no appreciable changes in the optical transmittance (Fig. S10, ESI†), turbidity or Tyndall effect were observed even after 10 minutes. Subsequently, the specificity of BChE-responsive disassembly was also investigated. The SCD/QA-Cbl solution showed no significant changes in optical transmittance at 460 nm with the addition of other enzymes, such as trypsin (Fig. S11, ESI†) and glucose oxide (GOx) (Fig. S12, ESI†), demonstrating that the SCD/QA-Cbl system exhibits a high specificity towards BChE.

In order to verify the release of Cbl from the SCD/QA-Cbl system upon the addition of BChE, high performance liquid chromatography (HPLC) was performed. In Fig. S13 (ESI†), it was found that Cbl can be released from the SCD/QA-Cbl system in the presence of BChE (0.2 U mL^{-1}) based on HPLC measurement, while no drug release can be detected in the absence of BChE. As expected, the peak intensity of Cbl (retention time = 4.90 min) increased gradually, whereas the peak strength of SCD/QA-Cbl assembly (retention time = 4.25 min) weakened with time in the HPLC chromatogram. It is worth noting that the anti-cancer drug Cbl itself is unstable in aqueous solution because the two chlorine atoms of nitrogen mustard can be hydrolyzed to two hydroxyl groups.[20] Therefore, the peak at retention time = 1.43 min in the HPLC chromatogram after adding BChE was speculated to denote the hydrolysates of QA-Cbl. In order to prove this surmise, we prepared the aqueous solution of the guest molecule QA-Cbl and placed it for three days at room temperature before HPLC and mass spectrometric analysis. Fig. S14 (ESI†) shows that there is a peak at retention time = 1.43 min in the HPLC chromatogram, which clearly verifies the peak assigned to the hydrolysates of QA-Cbl. In addition, the *m*/*z* peak at 352.40 in the mass spectrum strongly proved the degradation of the two chlorine atoms of QA-Cbl to two hydroxyl groups (Fig. S15, ESI†).

Moreover, mass-spectrometric detection is also carried out to validate the enzymatic cleavage of the ester bonds of QA-Cbl in the supramolecular nanoparticals. In Fig. S16 (ESI†), there was no ionic peak of Cbl observed in the mass spectrum without the addition of BChE to the SCD/QA-Cbl assembly. Fig. S17 (ESI†) shows mass spectra of the supramolecular nanoparticles at different time intervals, after the addition of BChE. The *m*/*z* peak of Cbl at 303.20 was clearly observed in the mass spectrum at different time intervals, after adding BChE. Furthermore, the peak at 388.30, assigned to $[QA\text{-}Cbl - Cl]^+$, was visibly weakened after 30 minutes and almost disappeared after 60 minutes, indicating that all of the ester bonds had been cleaved. These results jointly indicate that the anti-cancer drug Cbl is indeed released from the designed DDS through BChE-responsive breaking of the ester bond. The time taken for the complete disassembly of the supramolecular nanoparticles is shorter than that for the complete cleavage of the ester bonds. This is because although almost all the nanoparticles disappear, there are still some QA-Cbl molecules that are not enzymatically hydrolyzed. In addition, based on the previous reports, the rate of the cholinesterase-responsive disassembly increases with the increase in the enzyme concentration or the amount of enzyme added.[15,21] Besides, the hydrolysis rate of the SCD/QA-Cbl assembly by BChE is slower than that of free QA-Cbl. A possible reason may be that the QA-Cbl molecules are stacked closely in the assembly and some of them are being enveloped in the cavity of SCDs,[22] which hinders the attack of BChE to some extent. Finally, the Cbl release rate of the SCD/QA-Cbl assembly was examined by peak intensity integration by HPLC. The integral of the peak intensity of the Cbl is divided by the sum of the integrals of the two peak intensities of the SCD/QA-Cbl assembly and the Cbl, and then the percentage of the drug release is obtained. As shown in Fig. 3, the amount of released Cbl increases linearly with time. Nearly half of the Cbl was released in the first 30 min, reaching almost 100% in 60 min.

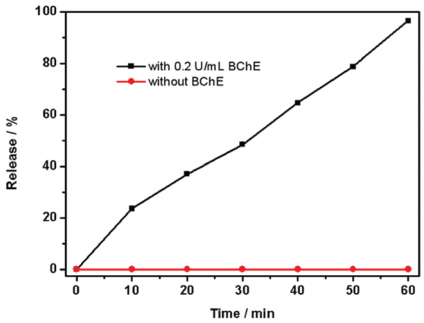


Fig. 3 Percentage of Cbl (as determined by HPLC) released from the SCD/QA-Cbl assembly as a function of time, with and without BChE (0.2 U mL^{-1}).

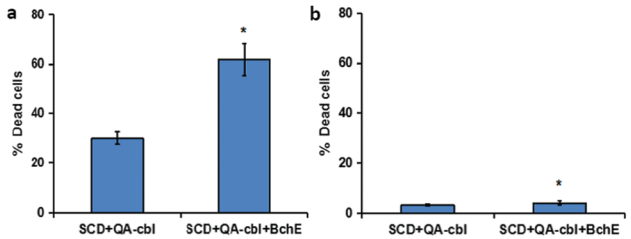


Fig. 4 % dead cells of A549 tumor cells (a) and 293T cells (b) in SCD/QA-Cbl assembly ([SCD] = 6.4 μM, [QA-Cbl] = 112 μM), with and without BChE (0.032 U mL^{-1}). A549 tumor cells and 293T cells were purchased from the Cell Resource Center, China Academy of Medical Science (Beijing, China).

BChE has been shown to be a biochemical marker for some cancers, so basic cell experiments were carried out to evaluate the anti-cancer effect before and after enzyme response of the SCD/QA-Cbl supramolecular assembly. The A549 tumor cells were incubated with and without BChE (0.032 U mL^{-1}). As shown in Fig. 4, the number of dead cells in the group after the addition of BChE was twice as high as that in the group without BChE, indicating that the SCD/QA-Cbl assembly had significant enzyme responsiveness and excellent anti-cancer activity. Moreover, SCD/QA-Cbl assembly is practically non-toxic toward normal cells, suggesting a good biocompatibility.

To summarize, we constructed a smart supramolecular assembly for controllable drug release, based on the host–guest complexation between a macrocyclic receptor SCD and a water-soluble prodrug QA-Cbl. Most importantly, butyrylcholinesterase could cleave the ester bond of the QA-Cbl molecule to cause the disintegration of the SCD/QA-Cbl assembly and the release of the anti-cancer drug Cbl. We believe that our designed supramolecular DDS may provide an applicable strategy for cancer therapy, because of its convenience and high specificity.

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Conflicts of interest

There are no conflicts to declare.

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