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COMMUNICATION

Enzyme-Responsive Fluorescent Camptothecin Prodrug/Polysaccharide Supramolecular Assembly for Targeted Cellular Imaging and in Situ Controlled Drug Release

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A novel enzyme-responsive supramolecular polysaccharide assembly composed of disulfide linked adamantane-naphthalimide fluorescent camptothecin prodrug (AdaCPT) and β-CD modified hyaluronic acid (HACD) was constructed, which possessing low cellular cytotoxicity and exhibiting targeted cellular imaging and controlled drug release at specific sites while providing a concurrent means for the real-time tracking of drug delivery.

Conventional chemotherapy utilizing small molecular anticancer drugs is a leading therapeutic approach in clinical cancer treatment but usually suffers from a number of drawbacks, such as lack of specificity, high toxicity, and poor water solubility.[1] Typically, the construction of targeted theranostic drug delivery system, which could selectively target cancer cells and deliver therapeutic agents while producing a readily detectable signal under endogenous stimuli to monitor the release process, has drawn tremendous attention as a promising approach to enhancing the basic chemotherapy in recent years.[2] In this regard, the incorporation of cell-specific diagnostic and therapeutic functions into a self-assembled molecular vehicle may confer several practical superiorities in pharmacotherapy. Firstly, the targeting ligands could selectively deliver the system to tumor cells *via* specific binding with its receptor that over expressed on the cell surface, which improved therapeutic efficiency and decreased side effects.[3] Secondly, by introducing stimuli-responsive fluorophore, which would allow the visualization of drug delivery process.[4] Thirdly, anticancer drug could be integrated into water-soluble carriers, which could increase the solubility and biocompatibility.[5]

Although some theranostic drug delivery systems based on mesoporous silica nanoparticles, inorganic nanoparticles, liposomes, and vesicles were constructed and exhibited effective therapeutic activities to cancer cells,[6] there are still exist several challenges in building these systems such as poor aqueous solubility, nonselectivity and high toxicity, insufficient drug release, harmful external stimuli, and lack of real-time therapy monitoring. To this end, supramolecular chemistry provides an alternative and even a more powerful strategy by combining prodrug with macrocyclic molecules to construct drug delivery system to address these limitations.[7] For instance, an enzyme-responsive supramolecular assembly for controlled drug release was successfully constructed based on the host-guest complexation between sulfato-β-CD and choline modified chlorambucil prodrug in our group,[8] demonstrating that supramolecular methodology provides a feasible and functional approach for therapeutics in the field of medicine.

Herein, we report the construction of an enzyme-responsive fluorescent supramolecular polysaccharide assembly capable of targeted cellular imaging and controlled drug release at specific sites from disulfide linked adamantane-naphthalimide camptothecin prodrug (AdaCPT)[9] and β-CD modified hyaluronic acid (HACD)[10] (Scheme 1). The enzymatically degradable prodrug molecule AdaCPT, bearing a stimuli-responsive fluorophore adamantane-naphthalimide on one side and an anticancer drug camptothecin on the other side, was connected by a disulfide bond linker. HACD as a biocompatible polysaccharide, integrates the advantages of both β-CD's cavity bind various hydrophobic molecules and HA's polymeric skeleton selectively recognize HA receptor that overexpressed on the surface of cancer cells.[11] There are some inherent features of such a system: (1) In our case, the water solubility, biocompatibility and targeting ability of CPT could be concurrently enhanced in the constructed assembly; (2) with the goal of imaging-guided targeted delivery of anticancer drug CPT to cancer cells and then stimuli-responsive release, disulfide linkage was introducing as a stimulus-responsive site because of its fast responsiveness to redox environment, which allow the release of drug only at specific sites *via* endogenous stimuli;[12] (3) naphthalimide, a useful fluorescent response

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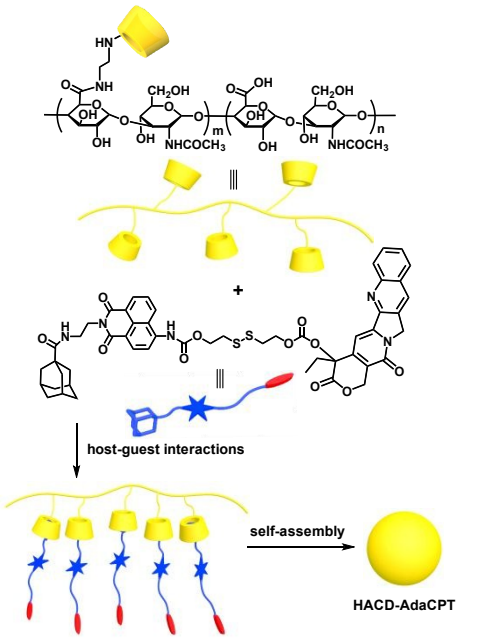
element, whose emission could be red-shifted from blue to green after the disulfide bond cleavage by endogenous thiols, was utilized as the fluorescence-responsive reporter to monitor drug release.[13] Consequently, the obtained supramolecular polysaccharide assembly may provide a new therapeutic options for simultaneous targeted cellular imaging and controlled drug release at specific sites.



Scheme 1 Construction of HACD-AdaCPT supramolecular polysaccharide assembly.

Taking advantage of the strong host-guest interaction between adamantane and β-CD, the targeted supramolecular theranostic assembly (HACD-AdaCPT) was constructed by simply mixing HACD and AdaCPT together. The critical aggregation concentration of the assembly was measured by monitoring the dependence of optical transmittance at 625 nm upon increasing assembly concentration from 0.025 to 10 μM. As shown in Fig. S1, a significant decrease was observed at the concentration of 2 μM due to the formation of large-sized assembly. The morphological and structural information of the assembly come from the high-resolution transmission electron microscopy (TEM), scanning electron microscopy (SEM), dynamic light scattering (DLS), and zeta potential experiments. The TEM image in Fig. 1a showed that the assembly existed as homogeneous spherical nanoparticles with an average diameter of ca. 270 nm, and the SEM image (Fig. 1b) gave similar morphological information. Moreover, the result of DLS experiment (Fig. 1c) indicated the hydrodynamic diameter of HACD-AdaCPT assembly as ca. 296 nm with a narrow distribution, which was very close to the results of electron microscopy. Besides, the zeta potential (Fig. 1d) of the assembly was measured to be ca. –23.66 mV due to the ionization of carboxyl groups on the HA skeleton, and this negatively charged surface would facilitate the stability and biocompatibility of the assembly in biological environments and extend its circulation time *in vivo*.[14] The loading efficiency of AdaCPT in assembly after dialysis was calculated as high as 99.2% by UV/vis standard curve of AdaCPT with absorption at 375 nm (Fig. S2). In addition, HACD and AdaCPT were combined completely and HACD-AdaCPT assembly was well-dispersed in aqueous for several months (Fig. S3).

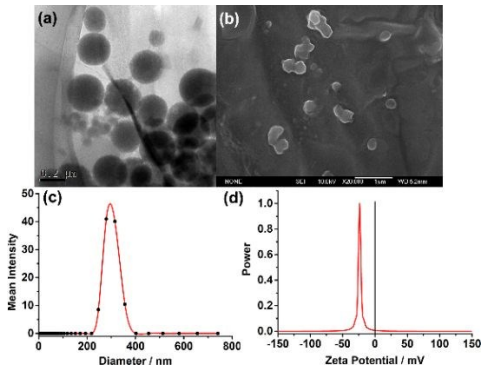


Fig. 1 Typical (a) TEM image, (b) SEM image, (c) DLS and (d) zeta potential of HACD-AdaCPT supramolecular polysaccharide assembly.

Next, in order to confirm the presumed concomitant release of the fluorophore and CPT, we monitored the responsive spectroscopic change of the assembly in the presence of thiols such as DL-dithiothreitol (DTT) with UV/vis and fluorescence spectrum.[13] As shown in Fig. 2a, the originally assembly displayed a wide absorption band from 310 nm to 500 nm centered at 375 nm, whereas a new absorption peak centered at 440 nm was observed after treated with DTT at 37 ℃ for 2 h. Subsequently, the time-dependent fluorescence emission change of the assembly was recorded after the addition of DTT. The HACD-AdaCPT assembly solution exhibited a rapid emission enhancement at 538 nm, meanwhile a gradually decreasing emission was observed at 480 nm accompanied with a solution color change from blue to green over 80 min (Fig. 2b and 2c). This obvious fluorescence red-shift endowed the assembly the ability for real-time tracking drug delivery and intracellular imaging, suggesting that the assembly might be of potential application value in cancer diagnosis. According to mass spectral analysis, the disulfide bond could be cleaved in the presence of thiols to form two unstable intermediates, followed by intramolecular cyclization to give Ada-naphthalimide and free CPT (Fig. S4–S5).

Moreover, the DTT-treated assembly solution was further characterized by TEM and DLS. As shown in Fig. S6, the DTT-treated assembly also exist as spherical nanoparticles, but their diameters increased from 270 nm to 350 nm, and a wide size distribution from 430 nm to 800 nm was confirmed by DLS result. That is, CPT was released after cleaving the disulfide bond, meanwhile a complex between Ada-linked naphthalimide and HACD still remained. Combining the enzyme-responsive spectroscopic change behaviors, this phenomenon further confirmed the enzyme-responsive release of CPT from HACD-AdaCPT assembly. This enzyme-responsive release would not improve the therapeutic efficiency of CPT but also reduce the toxicity to normal tissues, suggesting that the assembly possessed the ability of enzyme-responsive degradation, which could potentially be a controlled delivery system in cancer therapy.

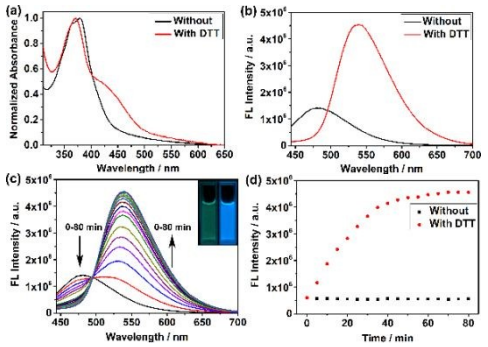


Fig. 2 (a) UV/vis, (b) fluorescence spectrum of HACD-AdaCPT (5 μM) assembly with and without DTT (5 mM). (c) Time-dependent fluorescence spectrum changes of assembly treated with DTT in PBS buffer (pH 7.4, 0.01 M) at 37 ℃. Inset: fluorescent color change of assembly before and after treated with DTT, left: with DTT, right: without DTT. (d) Time-dependent changes of fluorescence intensity at 540 nm for assembly treated with and without DTT.

After verifying the responsive drug release and fluorescence change of the assembly, we continued to investigate the targeted cancer cell fluorescence imaging capability of the obtained assembly. HCT-116 human colon cancer cell line that over-express HA receptors on its surface was used as the HA receptor positive group,[15] and NIH3T3 mouse embryonic fibroblast cell line was used as the HA receptor negative group.[10] As shown in Fig. 3, HCT-116 cells exhibited bright blue and green fluorescence after incubation with HACD-AdaCPT for 24 h, but in contrast, only weak fluorescence was observed in NIH3T3 cells due to the lack of HA receptor on the cell surface. Besides, lysosomes were stained with Lysotracker Red for colocalizing polysaccharide assembly and high colocalization was observed (Fig. S7). Moreover, the cellular uptake of assembly was further studied with and without excess amount of HA. As shown in Fig. S8, weak fluorescence was observed in the presence of excess HA. These phenomena indicated that the assembly could be internalized *via* HA receptor-mediated endocytosis, and this phenomenon also indicated that other pathway may exist in the internalization process, such as energy-dependent process. Besides, green fluorescence also confirmed that disulfide bond could be efficiently cleaved by intracellular GSH to release the Ada-linked naphthalimide fluorophore reporter when internalized by the cancer cells, and the complex could disperse throughout the cytoplasm once the CPT was released, which endowed the assembly theranostic ability to monitor the distribution, accumulation, and real-time drug release. Moreover, the real-time intracellular fluorescence change and blue/green fluorescence ratio for HCT-116 cells treated with assembly were also evaluated during the incubation at 1h and 12 h. As shown in Fig. S9, green fluorescence could be observed at 1 h, and the green fluorescent intensities increased with time, indicating the dissociation and drug release process of the assembly. This phenomenon further validated that the assembly could simultaneous intracellular imaging and real-time monitoring of drug release.

Subsequently, cytotoxicity experiment were carried out by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay to evaluate the anticancer activities of HACD-AdaCPT assembly. As shown in Fig. 4a, CPT exhibited a satisfactory malignant cell inhibition effect toward HCT-116 cells. After the grafting of Ada-linked naphthalimide, the relative viability of HCT-116 cells was 30.6%, which was little higher than free drug (23.1%), this slight anticancer activity decline might be attributed to AdaCPT is activated under GSH to release CPT, which need response time for its reduction.[9] Nevertheless, after forming assembly with HACD, HACD-AdaCPT exhibited similar anticancer activity to that of free CPT, and the relative cellular viability for HCT-116 cells was measured as 24.8%. However, when the receptors on the HCT-116 cell surface were blocked by an excess amount of HA, the cancer cell inhibition of HACD-AdaCPT decreased distinctly, and the relative cellular viability was measured as 45.1%. Besides, ADACPT was synthesized without naphthalimide as reference compound (Scheme S1, Fig. S10-12). The relative cellular viabilities for ADACPT and HACD-ADACPT were measured as 27.5% and 23.9%, respectively, which were similar to AdaCPT and HACD-AdaCPT, indicating that the fluorescent unit as imaging agent was not an indispensable factor for cancer therapy (Fig. S13). Moreover, the complex of HACD with ADAND (the precursor compound of AdaCPT) was chosen as control (Fig. S14), the relative cellular viabilities with HACD-ADAND was 100% (Fig. S13). The enhanced anticancer activity indicated that the assembly were preferably internalized by HCT-116 cells *via* HA-receptor mediated endocytosis, followed by GSH-triggered release of CPT from assembly. Moreover, the similar anticancer activities between CPT and HACD-AdaCPT assembly were verified by corresponding half-maximal inhibitory concentration (IC50). As shown in Fig. S15, the IC50 of CPT, AdaCPT and HACD-AdaCPT toward HCT-116 cells were measured as 0.55 μM,[16] 2.33 μM and 0.77 μM, respectively, which would further indicate that HACD-AdaCPT assembly showed comparable anticancer activity to commercial CPT. Combining the confocal fluorescence images, these results further confirmed the interaction of HA and the HA receptor plays an important role in the internalization of HACD-AdaCPT assembly into cancer cells. Flow cytometry was performed to further confirm the anticancer activity of assembly. HCT-116 cells were treated with CPT, AdaCPT and HACD-AdaCPT, respectively, and then subjected to Annexin V-APC/PI staining. The flow cytometry analysis showed that the apoptosis rate was 45.53%, 32.75, 43.88% induced by CPT, AdaCPT and HACD-AdaCPT, respectively. This result further indicated that the polysaccharide assembly may hold great promise to be a promising candidate in cancer therapy (Fig. S16).

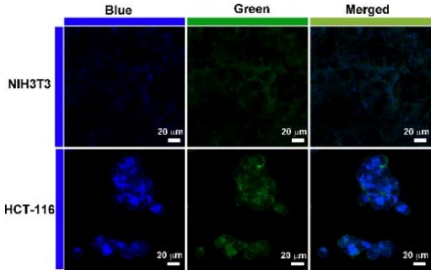


Fig. 3 Confocal fluorescence images of NIH3T3 and HCT-116 cells incubated with HACD-AdaCPT polysaccharide assembly for 24 h.

Besides, the cytotoxicity of HACD-AdaCPT assembly was investigated by measuring the relative cellular viability of normal fibroblasts NIH3T3 cells. As shown in Fig.4b, both CPT and AdaCPT exhibited side effects and gave relative cellular viabilities of 19.9% and 20.9%, respectively. Significantly, the relative cellular viability with HACD-AdaCPT was 60.2% due to the lack of HA receptor on the surface of normal cells. Moreover, the carrier HACD was nontoxic to both cancer cells and normal cells due to its satisfactory biocompatibility. These results jointly demonstrate that the obtained polysaccharide assembly displayed specific targeting ability with low cytotoxicity, which could facilitate the selective and rapid accumulation of CPT in cancer cells and hold great promise to be a safe and promising candidate for anticancer therapy.

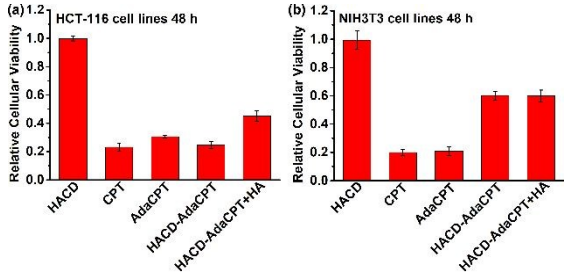


Fig. 4 Relative cellular viability of (a) HCT-116 and (b) NIH3T3 cell lines in 48 h ([CPT] = 15 μM).

In conclusion, an enzyme-responsive prodrug/macrocyclic supramolecular polysaccharide assembly for targeted cellular imaging and controlled drug release at specific sites was constructed by taking advantage of the strong supramolecular interaction between macrocyclic host HACD and fluorescent camptothecin prodrug AdaCPT, thereby displaying satisfactory stimulus-responsive drug release along with fluorescence change for real-time tracking of drug delivery and cellular imaging under intracellular GSH. Moreover, as investigated using cytotoxicity experiments, it can be seen that the assembly could be internalized into cancer cells by HA receptor mediated endocytosis and exhibited a similar cancer cell inhibition with commercial CPT but with much lower cytotoxicity than the free drug. In principle, this enzyme-responsive assembly successfully settle three key issues: (1) endowed anticancer drug CPT with water solubility and targeting ability; (2) controlled drug release at specific sites; (3) targeted cellular imaging and real-time tracking of drug release. We believe that this enzyme-responsive supramolecular polysaccharide assembly may provide a new therapeutic options to enhancing the basic chemotherapeutic approach.

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Conflicts of interest

The author declare no competing financial interest.

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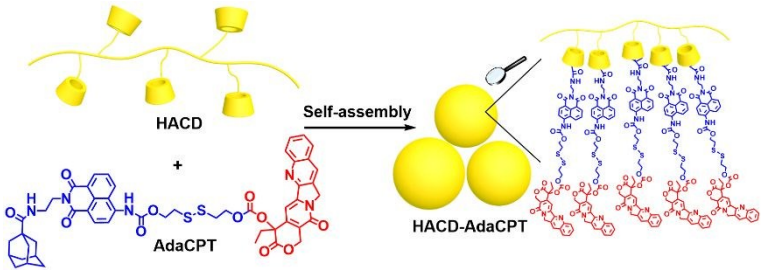

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