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Multicharge β-cyclodextrin Supramolecular Assembly for ATP Capture and Drug Release

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Contents

1. Materials and methods

All the reagents and solvents were commercially available and used as received unless otherwise specified purification. All aqueous solutions were prepared in distilled water. 1-hexylimidazole, 1-methylimidazole and adamantyl amine were purchased from Sigma-Aldrich. Hyaluronic acid and chlorambucil were purchased from Macklin. NMR spectra were recorded on a Bruker AV400 instrument. UV/vis spectra were recorded on a Shimadzu UV-3600 spectrophotometer in a quartz cell (light path 10 mm) at 25 ℃. TEM images were acquired by a high-resolution transmission electron microscope (Philips Tecnai G2 20 S-TWIN microscope) operating at an accelerating voltage of 200 keV. The samples were prepared by placing a drop of solution onto a carbon-coated copper grid and air-dried. The zeta potential was recorded on NanoBrook 173 Plus (Brookhaven company) at 25 ℃. The sample solutions for dynamic light scattering measurements (DLS) were examined on a laser light scattering spectrometer (BI-200SM) equipped with a digital correlator (Turbo Corr.) at 636 nm at a scattering angle of 90°. The confocal images were carried out on Olympus FV1000 confocal laser scanning microscope. Isothermal titration calorimetry (ITC) experiments were carried out on a thermostatted and fully computer-operated isothermal calorimetry (VP-ITC) instrument, purchased from Microcal Inc., Northampton.

Preparation of Cbl@HAAD-AMCD. Cbl (5 mg) was added to $CHCl_3$ (2 mL) , followed by mixing for 2 h. AMCD (29.7 mg, 10 μmol) and HAAD (2.2 mg, 1 μmol) were dissolved in distilled water (10 mL). The $CHCl_3$ solution of Cbl was added to the aqueous solution under vigorously stirring. The mixture was stirred under reduced pressure, allowing slow evaporation of $CHCl_3$. The solution was then dialyzed for 3 hours to remove the unloaded Cbl. Then drug-loaded solution was lyophilized for future use.

The Cbl released experiment. The Cbl released experiment was carried out by adding HAase to the solution of Cbl@HAAD-AMCD co-assembly and kept system in 37 ℃ for 6 hours. Then the solution was dialyzed for 3 hours to remove the released Cbl.

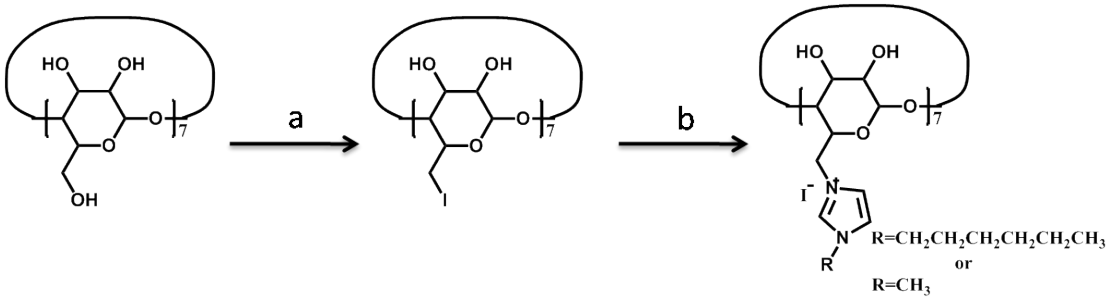
Cell Culture. The human embryonic kidney normal cell line 293T cells and human lung cancer cell line A549 cells were all purchased from Cell Resource Center, Chinese Academy of Medical Science Beijing. A549 cancer cells were incubated by using Ham's F12 nutrient medium supplemented with 10 % FBS and 1 % penicillin/streptomycin in a humidified incubator with 5 % CO_2 atmosphere at 37 ℃. 293T normal cells were cultured with DMEM high glucose nutrient medium containing 10 % FBS and 1 % penicillin/streptomycin in a humidified incubator with 5 % CO_2 atmosphere at 37 ℃. Before being used in experiments, all cells were pre-cultured to achieve confluence.

Cell cytotoxicity. A549 cells were seeded into a 96-well plate and then treated with different concentrations of HAAD/HAAD-Cbl/HAAD-AMCD/Cbl@HAAD-AMCD. The cell viability was evaluated by CCK8 assay according to the kit instruction. The plate was then read by a microplate reader at a wavelength of 450 nm. All the data were presented as the mean ± standard deviation.

Confocal laser scanning microscopy Experiments. A549 cells were seeded in a 96-well cell culture plate at a density of 1.00×10^4 cells per well and were allowed to attach for 24 h. To load calcein, firstly, A549 cells were incubated with its hydrophobic precursor, calcein acetoxymethyl ester (calcein-AM) (1 mg/mL stock solution in DMSO), at the concentration of 20 μg/mL in DMEM. After incubation at 37 ℃ for 30 min, cells were washed twice with PBS. HAAD or HAAD-AMCD assembly in different concentration were added. The control experiments were conducted in the absence of HAAD and AMCD. The medium was removed for fluorescence determination. The cells were washed with PBS and then visualized under a Olympus FV1000 confocal laser scanning microscope.

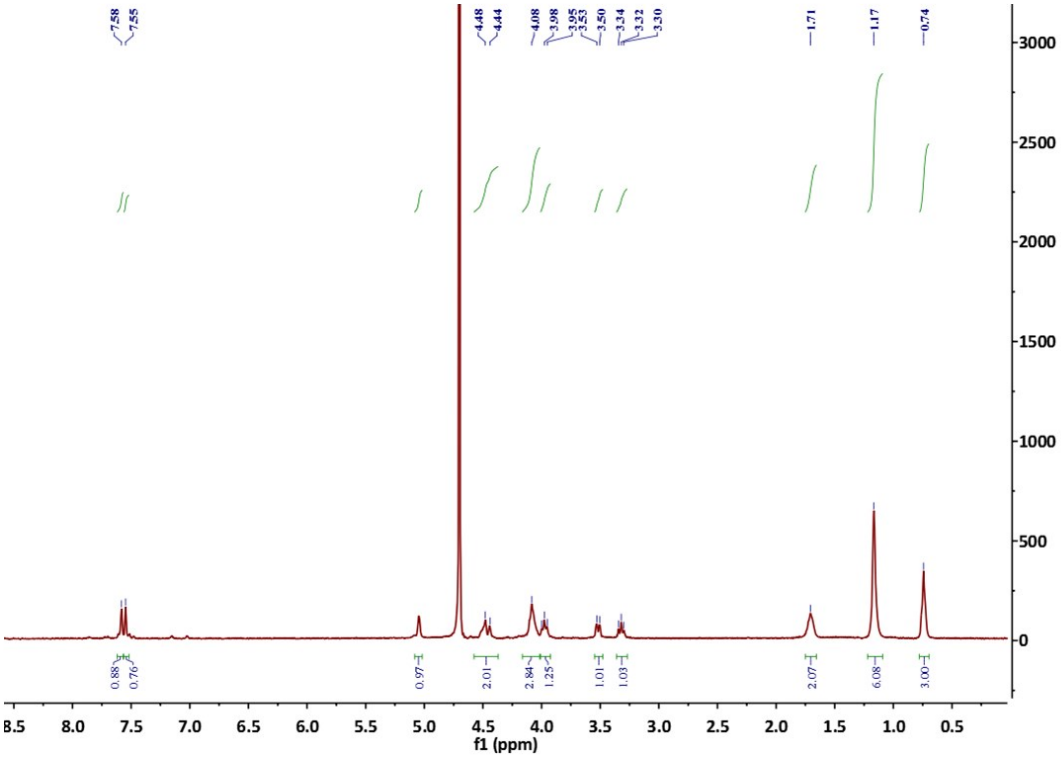
The concentration is calculated according to HAAD.

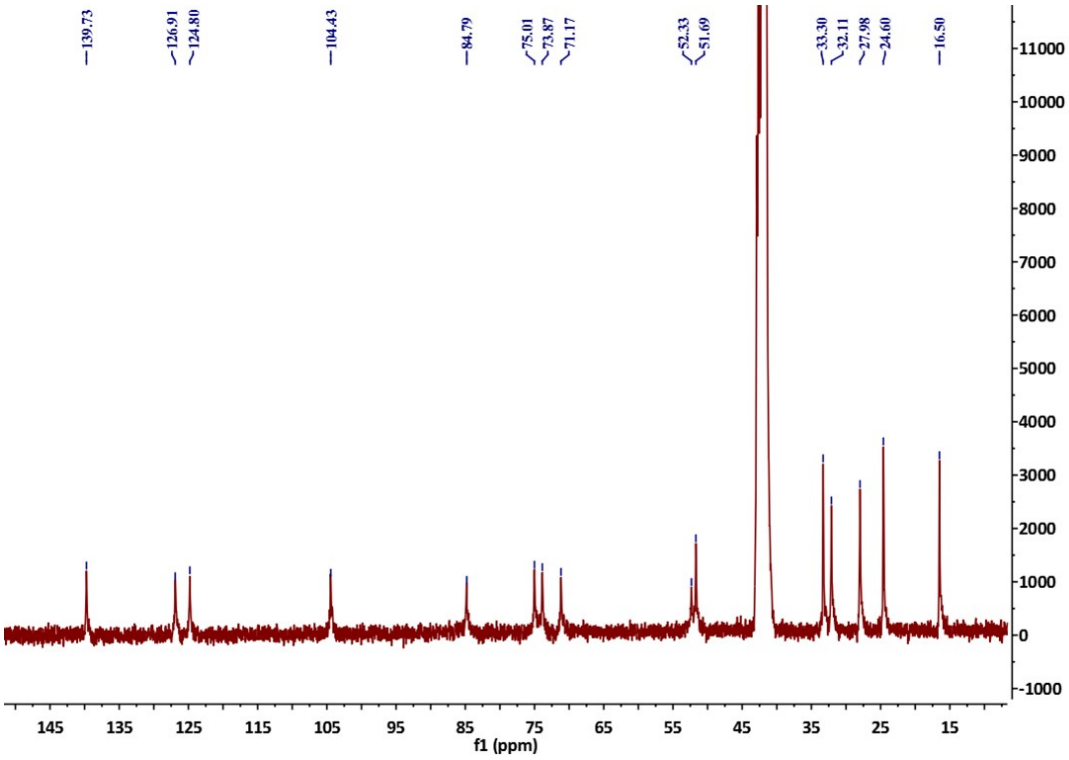
2. Synthesis and characterization of AMCD.



Scheme S1. Synthetic routes of AMCD or AMCD-1β. (a) triarylphosphines, iodine and DMF; (b) 1- hexylimidazole or 1-methylimidazole.

The AMCD was synthesized according to the following general procedure. Octakis (6-deoxy-6-iodo)-β-cyclodextrin (200 mg) was dissolved in 1-hexylimidazole (20.0 mL). The reaction mixture was stirred at 80 ℃ under argon atmosphere for 2 days. After that the resultant solution was poured into acetone (100 mL) and the precipitate formed was collected by filtration. The filter was recrystallized from water to give a light yellow solid. ^{1}H NMR (400MHz, D_2O) δ: 7.58 (s, 1H), 7.55 (s, 1H), 5.02 (s, 1H), 4.50-4.40 (m, 2H), 4.20-4.00 (m, 3H), 4.00-3.89 (t, 1H), 3.50 (t, 1H), 3.34 (t, 1H), 1.71 (m, 2H), 1.17 (m, 6H), 0.74 (t, 3H). ^{13}C NMR (101 MHz, DMSO) δ: 139.73, 126.91, 124.80, 104.43, 84.79, 75.01, 73.87, 71.17, 52.33, 51.69, 33.30, 32.11, 27.98, 24.60, 16.50. ESI-FTMS m/z: [M - I] +, found 2842.28.





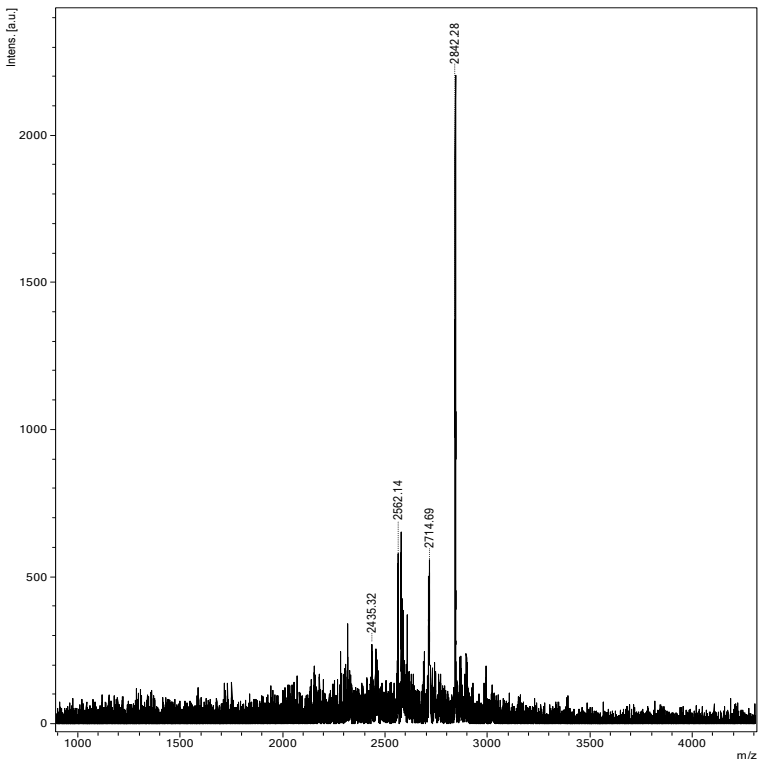
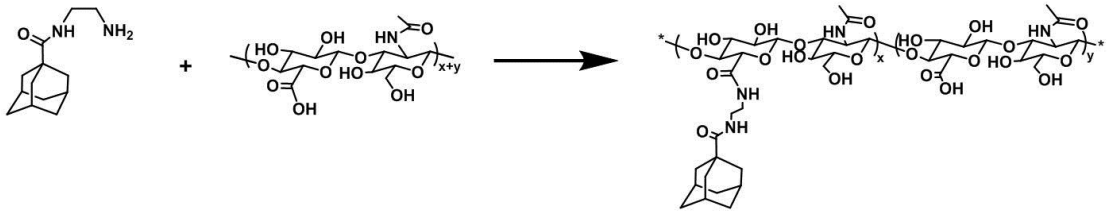


Fig. S1 (a) ^{1}H NMR spectrum of **AMCD** in D_2O (400 MHz, 25 ℃); **(b)** ^{13}C NMR spectrum of **AMCD** in DMSO (100 MHz, 25 ℃); (c) ESI-FTMS of **AMCD.**

3. **Synthesis and characterization of HAAD.**



Scheme S2. Synthetic routes of HAAD.

This compound was synthesized through the amidation reaction using a previously reported method.[1] The acid form of HA (MW=10000, 0.5 g, 1.32 mmol) was dissolved in 50 mL DMSO at 60 ℃. After the polymer was completely dissolved, the solution was cooled to room temperature. Then, triethylamine (0.92 mL, 6.6 mmol) was added and the reaction solution was stirred for another 10 min. At this moment, ethyl chloroformate (0.38 mL, 4.0 mmol) was added and the mixture was continued to stir at room temperature for 1 h. After the corresponding adamantyl amine (146.6 mg, 0.66 mmol) was added, the reaction mixture was allowed to stir at room temperature for 24 h. Afterwards, the solution was diluted with 50 mL water and dialyzed (molecular weight cutoff 7000D) against 0.1 M of NaCl for 24 h, and then dialyzed against deionized water for 7 days. After dialysis, the sample was freeze-dried as white powder. Using the single-point method from the integrated peak area of adamantyl moiety and HA backbone in NMR spectra, the degree of substitution (DS) was determined as 18%, indicating that adamantane was introduced every 5.6 repeating sugar units on average. ^{1}HNMR (400 MHz, D_2O, ppm): δ 4.24–4.63 (m, 2H), 3.05–4.15 (m, 10.95H), 2.0 (s, 3.79H), 1.57–1.75 (m, 2.15H). MW_{HAAD} = N × 18% × MW_1 + N ×（1-18%） × MW_2 = 10933 (N: the number of repeating units in HA with a molecular weight of 10000; MW_1: molecular weight of HA repeating units bearing the adamantyl groups, MW_2: molecular weight of HA repeating units without the adamantly groups).

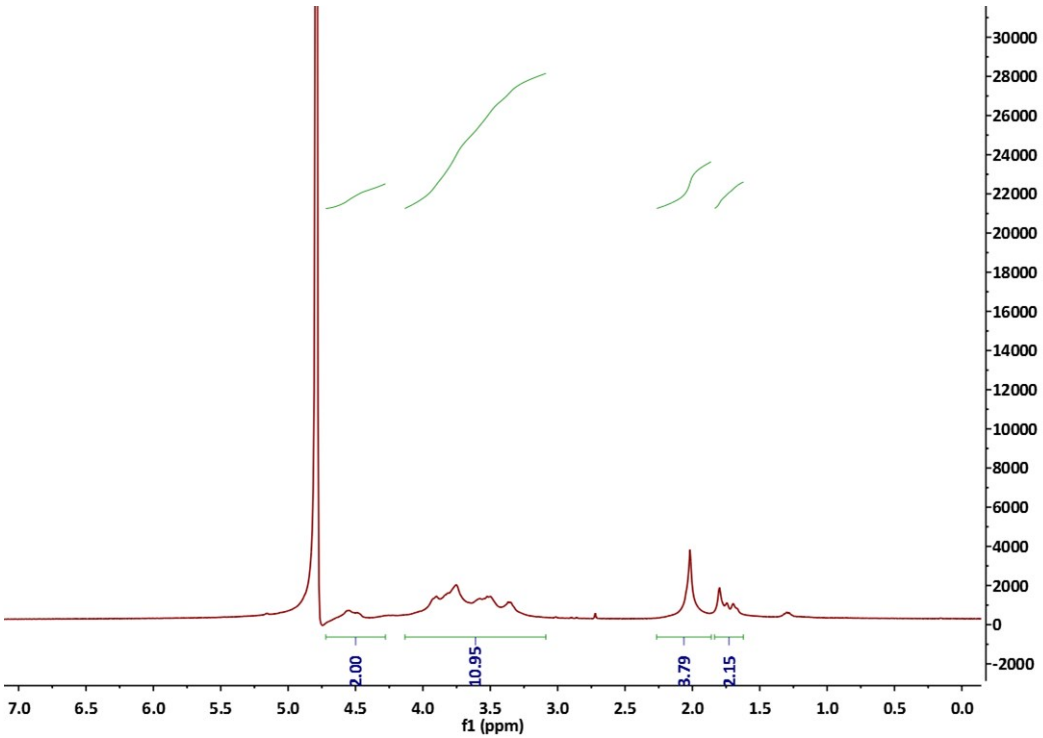


Fig. S2 ^{1}H NMR spectrum of HAAD in D_2O (400 MHz, 25 ℃). The degree of substitution was determined as 18 %, the integral ratio of ethyl protons in adamantane (*δ* 1.57–1.75, 12H) relative to the protons in HA backbone (*δ*

4.24–4.63, 2H).

4. The critical aggregation concentration (CAC) of AMCD.

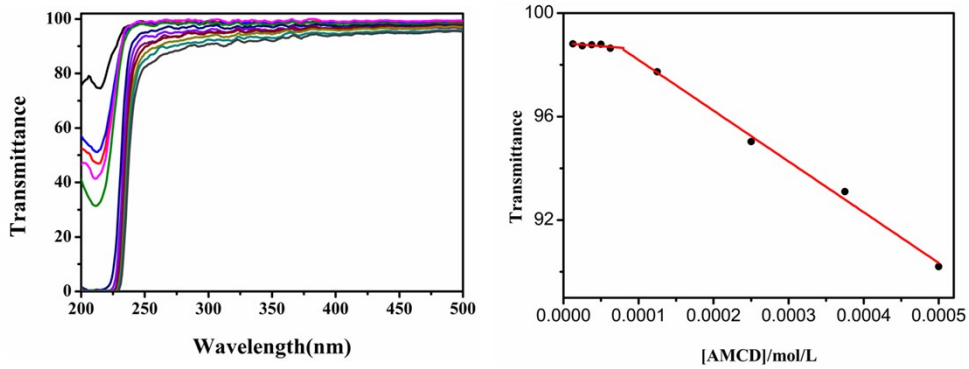


Fig. S3 (a) Optical transmittance spectra of AMCD; (b) the transmittance change at λ = 275 nm. [AMCD] = 0 to 500 μM.

5. The preparation of HAAD-AMCD assembly.

The preferable mixing ratio between HAAD and AMCD was determined by gradually adding HAAD to a solution of AMCD (10 μM). As shown in Fig. S4a and b, the minimum of the transmittance was reached at [HAAD] = 2.5 μM. The decrease of the optical transmittance may indicate the formation of a large aggregate. According to these results, the preferable mixing ratio for the formation of the HAAD-AMCD aggregate was [HAAD]= 2.5 μM and [AMCD] = 10 μM (**$H_{2.5}$ A_{10}**). However, the HAase response of HA-AMCD is lower than HAAD-AMCD. As shown in Fig. S4c and d, the system still has a low transmittance and Tyndall effect after 12 hours of hyaluronidase action, indicated that the system still exists in the form of an assembly. Therefore, we choose (**H_1 A_{10}**) which have two advantages as (1) the enzyme response of the system is more sensitive, (2) less AMCD cavity is occupied by adamantyl groups.

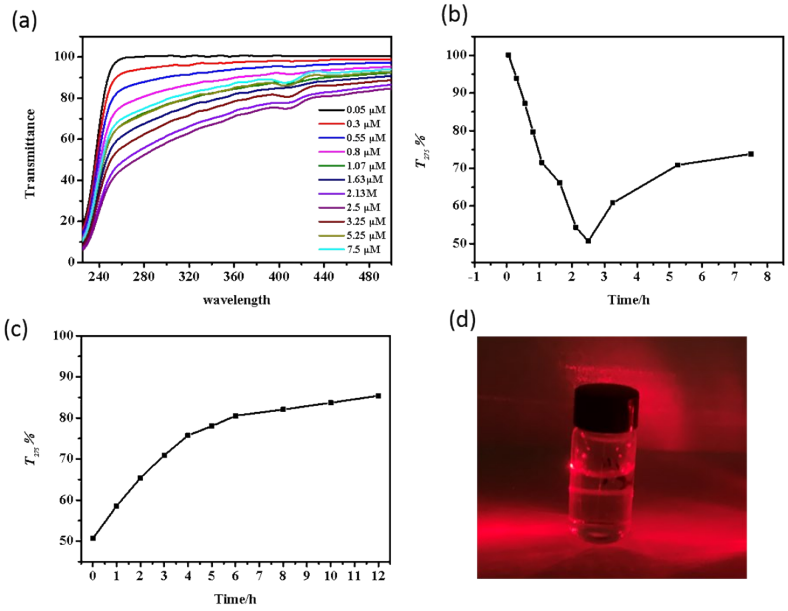


Fig. S4 (a) Optical transmittance of aqueous solutions of HAAD at different concentrations (0–7.5 μM) in the presence of AMCD (10 μM); (b) Dependence of T_{275} % on HAAD concentration in the presence of AMCD (1 μM); (c) transmittance at 275 nm of HAAD-AMCD assembly with HAase from 0 to 12 h. Conditions: [AMCD] = 10 μM, [HAAD] =2.5 μM, [HAase]= 10 U•ml^{-1} in water, T=37 ℃; (d) Tyndall effect of solution with HAase after stayed at 37 ℃ for six hours. Conditions: [AMCD] = 10 μM, [HAAD] =2.5 μM, [HAase]= 10 U•ml^{-1} in water.

6. The HAase response of HAAD-AMCD assembly.

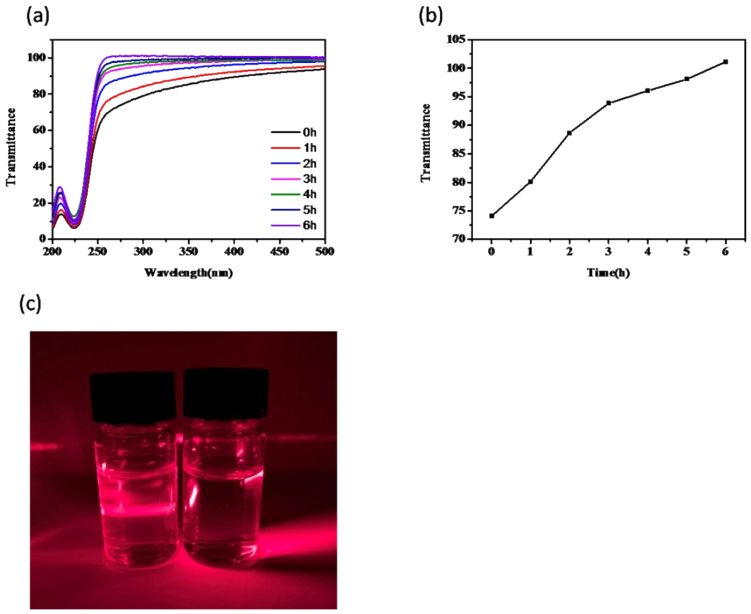


Fig. S5 (a) Transmittance spectra and (b) transmittance at 275 nm of HAAD-AMCD assembly with HAase from 0 to 6 h. Conditions: [AMCD] = 10 μM, [HAAD] =1 μM, [HAase]= 10 U•ml^{-1} in water, T=37 ℃; (c) Tyndall effect of solution with (right) and without HAase (left) after stayed at 37 ℃ for six hours.

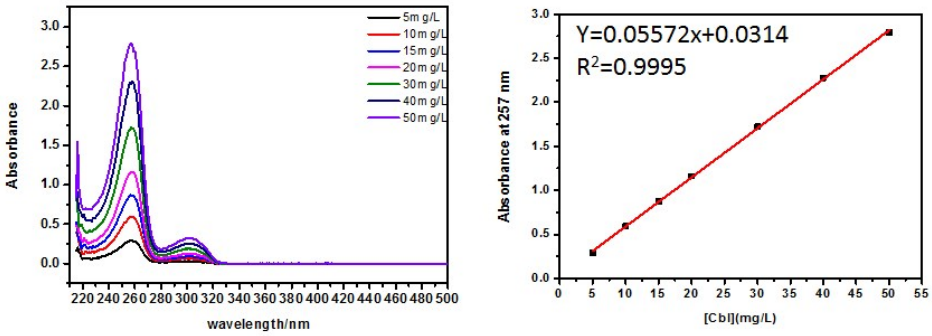


Fig. S6 The UV-vis absorption calibration curve line of Cbl at 257 nm.

7. The capability of HAAD-AMCD assembly on loading drugs.

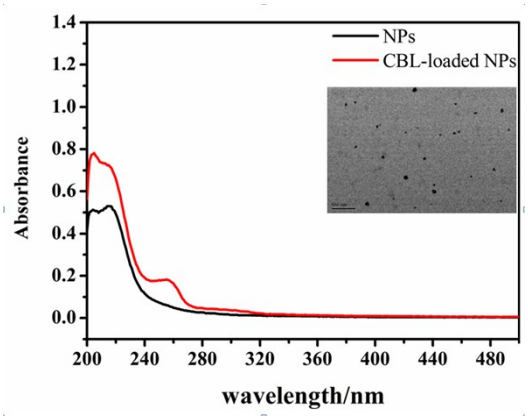


Fig. S7 The UV-vis absorption spectra of Cbl before (black line) and after (red line) loaded by HAAD-AMCD

assembly; Inset: The TEM images of Cbl@HAAD-AMCD.

The Cbl loading efficiency was calculated based on equation (1):

Loading efficiency (%) = ($m_{Cbl\text{-}loaded}/m_{Cbl\text{-}loaded+vesicles}$) × 100 (1)

where $m_{Cbl\text{-}loaded}$ and $m_{Cbl\text{-}loaded+vesicles}$ are mass of Cbl encapsulated into the vesicles and mass of Cbl-loaded vesicles, respectively.

The Cbl encapsulation efficiency was calculated by equation (2):

Encapsulation efficiency (%) = ($m_{Cbl\text{-}loaded}/m_{Cbl}$) × 100 (2)

where $m_{Cbl\text{-}loaded}$ and m_{Cbl} are mass of Cbl encapsulated into the vesicles and mass of Cbl added, respectively. The mass of Cbl was measured by a UV-vis absorption spectra at 257 nm.

8. The Cbl release rate of the Cbl@HAAD-AMCD assembly.

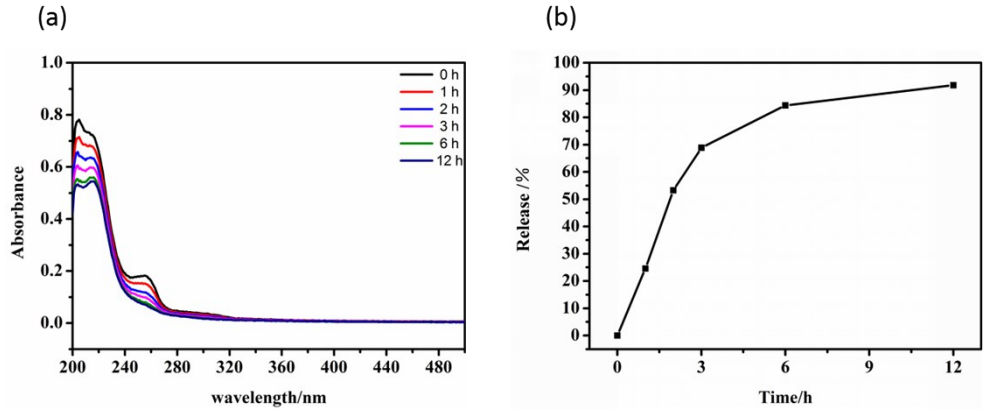


Fig. S8 (a) The UV-vis absorption spectra of Cbl @HAAD-AMCD assembly after the addition of HAase for six hours and the solution was dialyzed (molecular weight cutoff 1000) from 0 to 12 h; (b) Percentage of Cbl released from the disassembled Cbl@HAAD-AMCD assembly as a function of time.

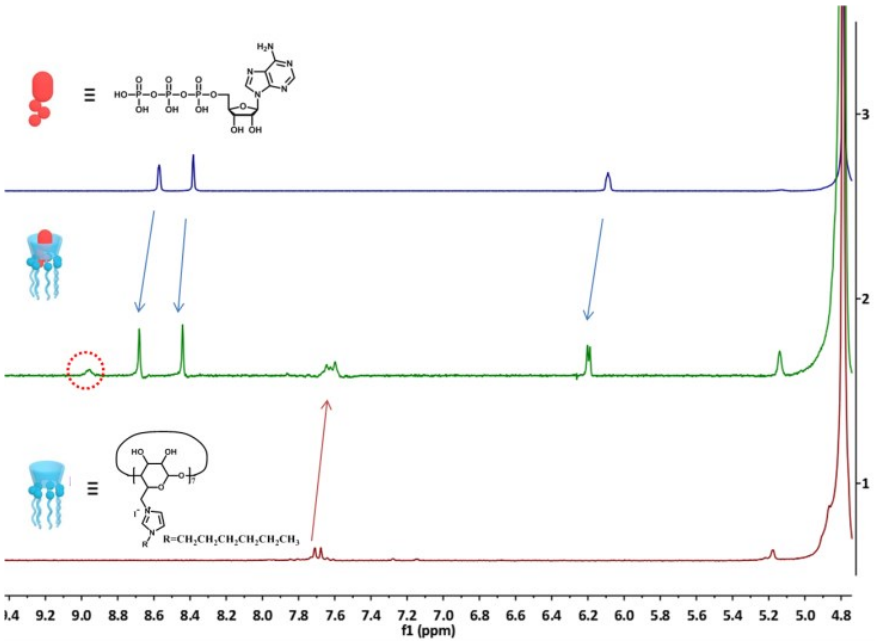


Fig. S9 Partial enlargement of 1H NMR spectrum of AMCD⊃ATP in D_2O (400 MHz, 298K).

9. Characterization and the stability of AMCD⊃ATP complex.

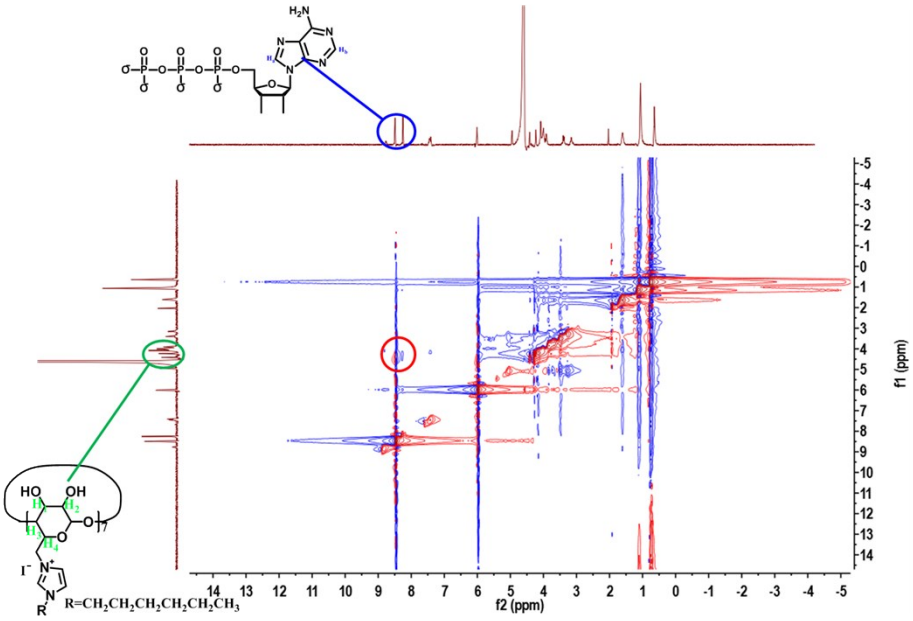


Fig. S10 2D ROESY spectrum of AMCD⊃ATP complex in D_2O (400 MHz, 25 ℃).

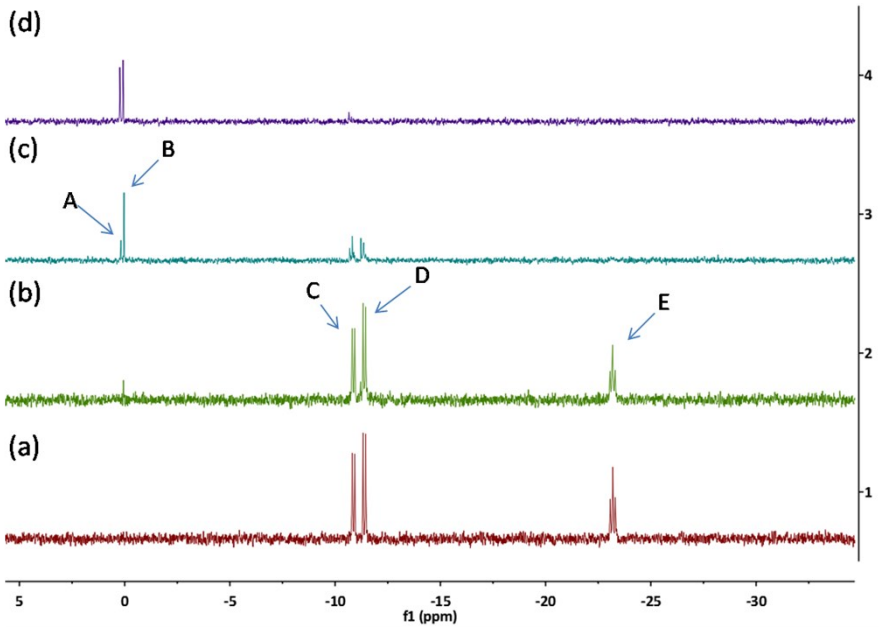


Fig. S11 ^{31}P NMR spectra: (a) ATP (2.00 mM); (b) ATP (2.00 mM) in the presence of alkaline phosphatase (CIAP) (10 U/mL) for 2 h; (c) ATP (2.00 mM) in the presence of CIAP (10 U/mL) for 6 h ; (d) ATP (2.00 mM) in the presence of CIAP (10 U/mL) for 12 h. The phosphorus signals in (b) and (c) are assigned as follows: A. α-AMP; B. phosphate; C. γ-ATP; D. α-ATP; E. β-ATP.

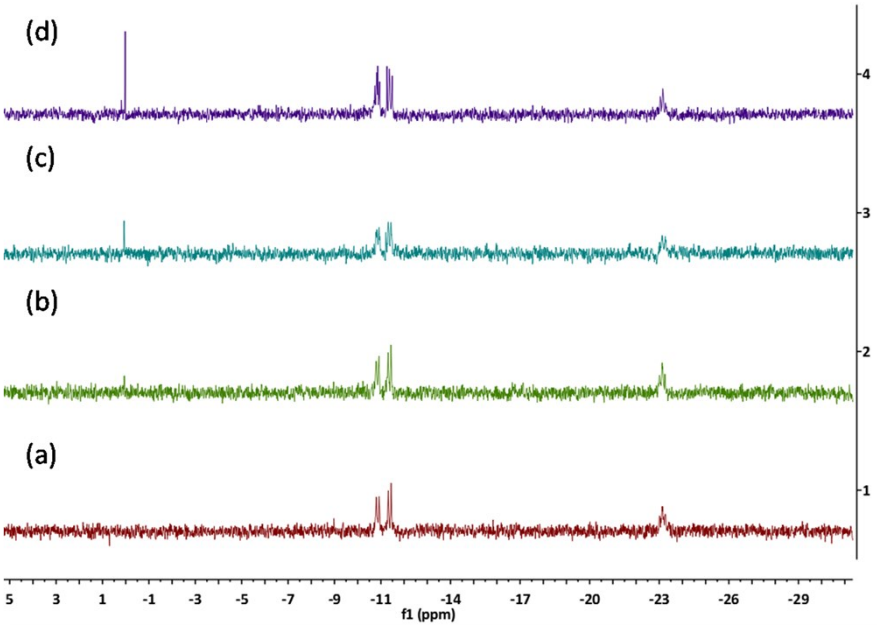


Fig. S12 ^{31}P NMR spectra: (a) AMCD (2.00 mM) and ATP (2.00 mM); (b) AMCD (2.00 mM) and ATP (2.00 mM) in the presence of alkaline phosphatase (CIAP) (10 U/mL) for 6 h; (c) AMCD (2.00 mM) and ATP (2.00 mM) in the presence of CIAP (10 U/mL) for 12 h; (d) AMCD (2.00 mM) and ATP (2.00 mM) in the presence of CIAP (10 U/mL) for 24 h; (e) AMCD (2.00 mM) and ATP (2.00 mM) in the presence of CIAP (10 U/mL) for 48 h.

Reference

(1) G. Huerta-Angeles, D. Šmejkalová, D. Chládková, T. Ehlová, R. Buffa and V. Velebný, Carbohydr. *Polym.*, 2011, **84**, 1293.