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Assembly of Catalase-Based Bioconjugates for Enhanced Anticancer Efficiency of Photodynamic Therapy *in Vitro*

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Experimental section

Materials

9,10-Diphenylanthracene (DPA) was from Alfa Aesar China (Tianjin) Co., Ltd. 3-(4, 5-Dimethylthiazolyl-2)-2, 5-diphenyl tetrazolium bromide (MTT) was purchased from Amresco. Medium dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) were obtained from Invitrogen. Other materials were purchased from Sigma-Aldrich, Inc. The water used in the experiments was ultrapure water with a resistivity of 18.2 MΩ cm.

Preparation of $MnCO_3$ and RB Loaded Porous $CaCO_3$ Composites

$MnCO_3$ microparticles with an average diameter of 4 μm were prepared by mixing $MnSO_4$ (0.016 M) and NH_4HCO_3 (0.16 M) solutions according to the previously reported method.[1,2] Porous $CaCO_3$ particles with an average diameter of 500 nm were prepared by mixing Na_2CO_3, PEI, and $CaCl_2$ solutions according to improved literature procedures.[3] In a typical experiment, 1 mL of Na_2CO_3 solution (0.2 M) was firstly mixed with 3 mL of PEI solution (1 mg mL^{-1}) and agitated for 10 s. Then, 1 mL of $CaCl_2$ solution (0.2 M) was quickly added to the mixture under vigorously stirring for 45 s. After rinsed three times by H_2O, the synthesized porous $CaCO_3$ particles were dispersed in aqueous solution containing RB to adsorb overnight (at least 10 h), followed by centrifugation and rinse with pure water for 3 times.

Preparation of Hollow $(CAT/ADA)_6$ Microcapsules and $CaCO_3$@RB-$(CAT/ADA)_6$ Composites

Both of the microstructures mentioned above were fabricated via the LbL technique. ADA was prepared by the method reported previously.[4] $MnCO_3$ microparticles or RB loaded porous $CaCO_3$ microspheres were immersed into 1 mg·mL^{-1} PEI solution in 0.5 M NaCl for 20 min, followed by three times centrifugation and washing. Then the particles were dispersed into ADA solution (1 mg mL^{-1}) in (PBS, 0.01 M, pH 7.4) and the adsorption process was allowed to proceed for 6 h. After being washed three times with PBS solution, the particles were redispersed into CAT solution (4 mg mL^{-1}) in PBS (0.01 M, pH 7.4) and stayed for 6 h at 4 ^{o}C, followed by the same washing protocol. After desired number of CAT/ADA layers was deposited, hollow microcapsules were obtained by dissolving the $MnCO_3$ cores in 0.01 M Na_2EDTA solution (pH 7.4). As in the case of $CaCO_3$@RB-$(CAT/ADA)_6$ composites, the particles were rinsed and stored in PBS solution at 4 ^{o}C just after the LbL assembly process.

Measurement of Enzymatic Activity of $(CAT/ADA)_6$ Microcapsules

A certain number of hollow $(CAT/ADA)_6$ microcapsules was incubated in PBS solution for a desired amount of time at 37 ^{o}C and 4 ^{o}C separately. After that, appropriate amount of H_2O_2 was added into the microcapsule suspension and the reaction time was lasted for 1 min, then the reaction was stopped by the addition of H_2SO_4 (25%) containing $Ti(SO_4)_2$ (0.6 %). The activity of $(CAT/ADA)_6$ microcapsules was determined by monitoring the yellow complex that formed with unreacted H_2O_2

via UV/Vis absorbance reduction at 410 nm. One unit of catalase is defined as the amount needed to convert 1.0 micormole of H_2O_2 to O_2 and H_2O per minute at pH 7.0.

Detection of $^1O_2$ and the Relationship between the Concentration of $^1O_2$ and H_2O_2

DPA was chosen to measure $^1O_2$ generated during the PDT process. The sample was prepared by mixing 400 μL of $CaCO_3$@RB-$(CAT/ADA)_6$ composites suspension (the concentration of RB is 0.05 mg mL^{-1}) in PBS solution and 10 μL of DPA stock solution in DMSO. After irradiating with 561 nm laser for a desired amount of time, the sample was centrifuged, and UV/Vis spectra of the supernatant were recorded. In order to detect the relationship between the concentration of H_2O_2 and $^1O_2$, 10 μL of H_2O_2 with a concentration of 0.3 % (w/w) was added into the sample, and UV/Vis spectra of the sample were recorded after irradiating the sample for a desired amount of time and centrifugation. Both of the procedures mentioned above were repeated until there were no changes could be observed by UV/Vis analysis.

Cell Cultivation and Photodynamic Activity

The breast cancer cell MCF-7 were cultured in DMEM supplemented with 10 % FBS, in the presence of 1 % penicillin and streptomycin. The cells were reseeded every 2-4 days, and all cultures were maintained at 37 ^oC in 5 % CO_2 with 70 % humidity.

For the cytotoxicity tests, exponential growing cells were seeded in a 96-well plate and incubated in culture medium for 24 h. Then cells were treated with bare $CaCO_3$ microparticles, $CaCO_3$-($CAT/ADA)_6$ spheres, $CaCO_3$@RB-$(CAT/ADA)_6$ composites, and free RB solution with the concentration of formulated RB at 0, 1.0, 2.0, and 3.0 μg mL^{-1} for 6 h. After the removal of nonefficient microspheres and the addition of fresh

growth medium, cells were irradiated with a Xe lamp, equipped with filters to isolate a narrow spectral region around 561 nm for 15 min. The cell viability was measured by MTT assay 12 h after the irradiation, as described previously.[5]

Interaction Profile Analysis between $CaCO_3$@RB-(CAT/ADA)$_6$ Composites and Cells

In order to visually monitor the interaction between $CaCO_3$@RB-(CAT/ADA)$_6$ composites and MCF-7 cells as a function of time, cells were seeded in a 35 mm glass-bottom Petri dish and incubated with $CaCO_3$@RB-(CAT/ADA)$_6$ composites suspension for 1, 3, 6, and 10 h, respectively. After incubation, the cells were washed twice with PBS solution to remove excess particles and dead cells. Then, the membrane and the nuclei of the cells were stained by incubating the cells Alexa fluor 488 and Hoechst 33342 solution (10 μL) for 10 min, followed by two washes with PBS. The cells were examined by CLSM.

The detached cells were seeded in a 6-well plate until logarithmic growth phase. Then 40 μL of dispersed $CaCO_3$@RB-(CAT/ADA)$_6$ composite suspension or free RB solution was added into dishes and incubated for 6 h. After that, the cells were harvested and resuspended in PBS solution for flow cytometry analysis.

Intracellular ROS and $^1O_2$ Determination

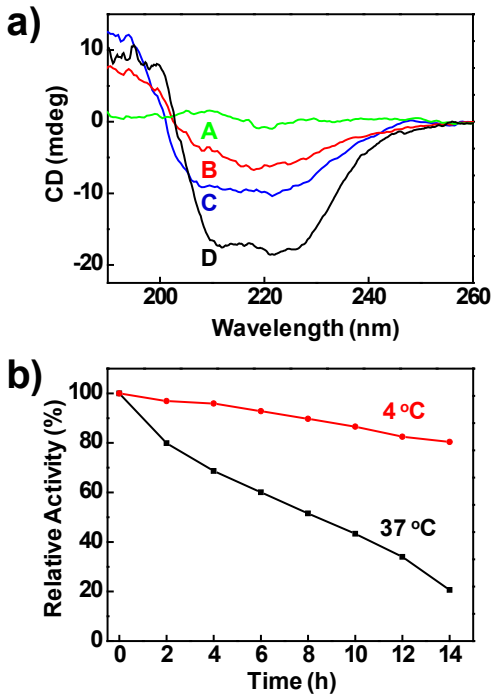
Exponential growing cells were seeded onto 96-well plate and incubated in culture medium for 24 h. 2,7-dichlorofluorescein diacetate (DCFH-DA) with a concentration of 10 mM was added into wells and loaded with cells at 37 ^oC for 30 min. After washing, cells were incubated with $CaCO_3$@RB-(CAT/ADA)$_6$ composites or RB solution for 6 h,

washed twice with HBSS, and irradiated with 561 nm laser for 15 min. The fluorescent intensity of DCF was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. For the $^1O_2$ detection, cells were incubated with $CaCO_3$@RB-$(CAT/ADA)_6$ composites or $CaCO_3$@RB-$(HSA/ADA)_5$ composites for 6 h, and washed twice to remove the unattached particles. Then, 3 μL of DPA stock solution in DMSO was added into wells, and UV/Vis spectrum was recorded after 15 min irradiation.

Characterization

The morphology of $CaCO_3$@RB-$(CAT/ADA)_6$ composites and $(CAT/ADA)_6$ microcapsules were examined using SEM (Hitachi S-4800, 10 kV), CLSM (Olympus FV500 with a 60×oil-immersion objective and a numerical aperture of 1.4), and AFM (Digital Instrument Nanoscopy III) in the tapping mode at room temperature. FTIR spectra were recorded by using a Tensor 27 instrument (Bruker). XPS (VG Escalab 220i-XL) was performed to investigate the component of the microcapsules. UV/Vis spectra were taken with a Hitachi U-3010 UV/Visible spectrophotometer. The fluorescent property of the microcapsules was measured by Spectrofluorometer (F-4500, Hitachi).

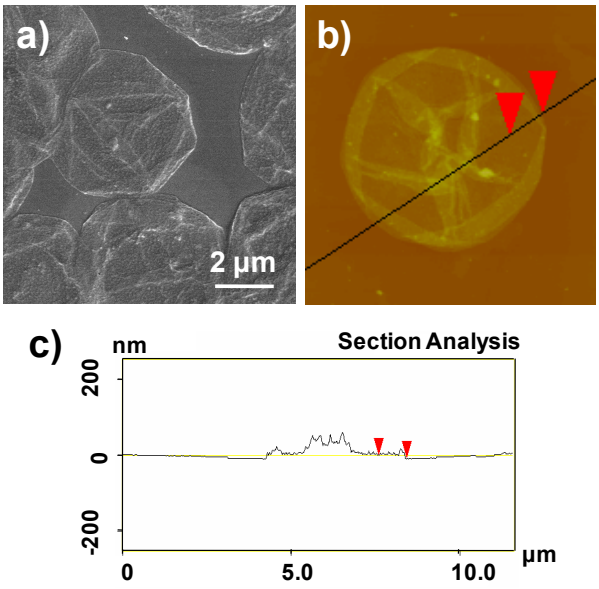
Figure S1.



a) CD spectra of (CAT/ADA)n multilayer thin films assembled onto quartz slides in which n=(A) 3, (B) 6, (C) 9, and (D) 12; b) The relative activity of the assembled CAT stored at 4 ^{o}C and 37 ^{o}C in PBS solution.

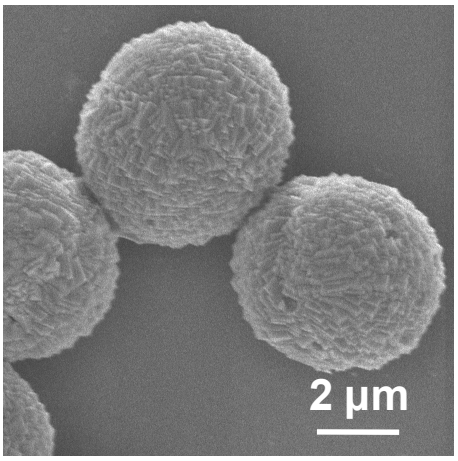
For the further evaluate the long-term stability of the multilayers, $(CAT/ADA)_6$ microcapsules were stored at different temperatures. As shown in Figure S1b, for the microcapsules stored in phosphate-buffered saline (PBS) solution at 4 ^{o}C, there was 96% of the activity remained for 14 h. By contrast, the activity of the enzyme reduced to 20% when incubated at 37 ^{o}C. The results indicated that the suitable temperature for the preparation of CAT/ADA films is 4 ^{o}C. CAT/ADA films kept 60% of the initial activity when incubated at 37 ^{o}C for 6 h, which means that the shells assembled can decompose intracellular H_2O_2 during the PDT process.

Figure S2



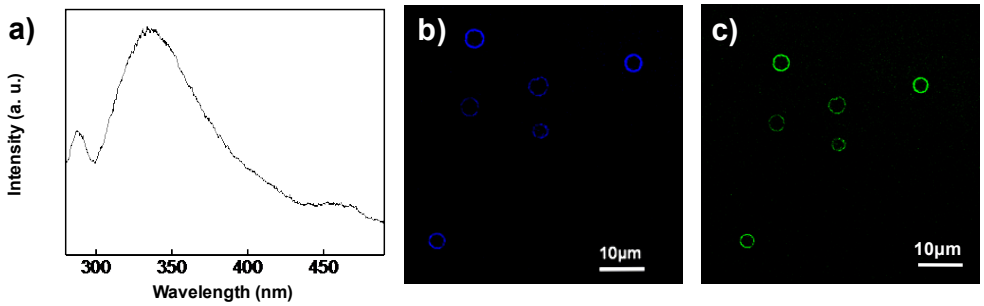
a) SEM image of $(CAT/ADA)_6$ microcapsules; b) AFM image of a single $(CAT/ADA)_6$ microcapsule and c) relevant section analysis.

Figure S3



SEM image of $MnCO_3$ microspheres with the diameter of about 4 µm.

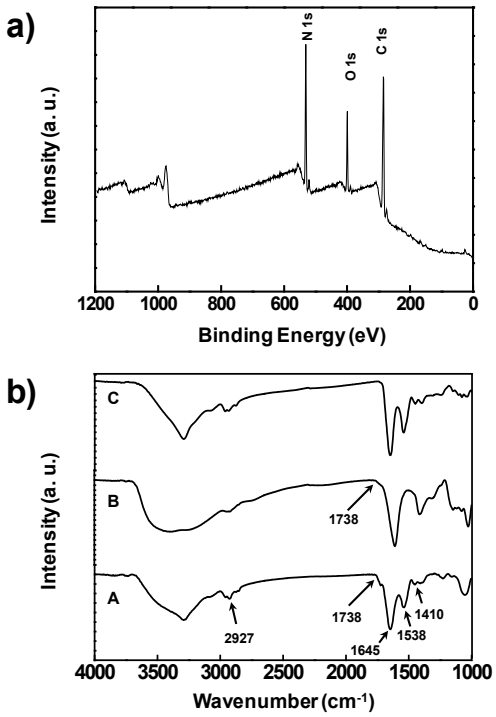
Figure S4



Fluorescent emission spectrum of $(CAT/ADA)_8$ excited at 260 nm (a); CLSM image of the microcapsules excited at 405 nm, collected at: b) 430-480 nm (blue) and c) 500-550 nm.

The optical excitation wavelength was at around 260 nm, and there are two emission bands in the fluorescent emission spectrum (Figure S4a). One is observed between 310 and 370 nm, and the other one is between 430 and 480 nm. CLSM was employed to further examine the autofluorescent nature of $(CAT/ADA)_6$ microcapsules (Figure S4b and c). When excited at 405 nm by laser, the microcapsules exhibit strong fluorescence in the range of 430-480 nm (blue channel), which is in accordance with the results of the emission spectrum; besides, a weak emission band could be observed between 500 and 550 nm (green channel).

Figure S5

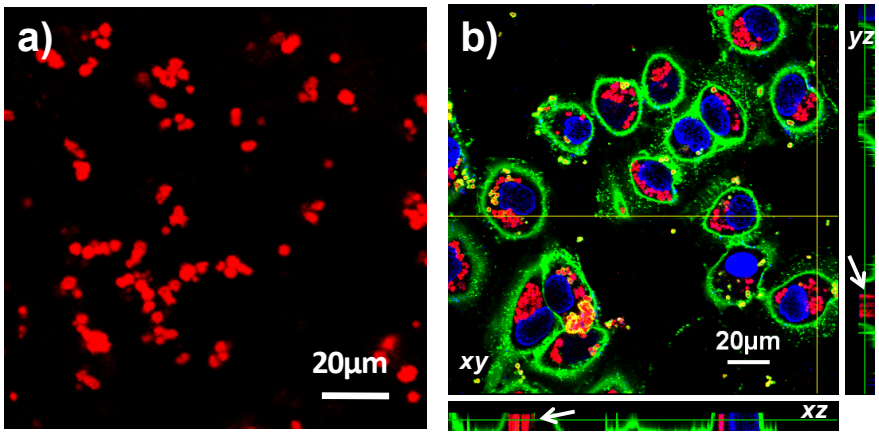


a) XPS spectrum of $(CAT/ADA)_6$ microcapsules; b) FTIR spectra of (A) $(CAT/ADA)_6$ microcapsules, (B) ADA, and (C) CAT.

In order to investigate the components of the shells and verify the formation of covalent bond between CAT and ADA, X-ray photoelectron spectroscopy (XPS) pattern and Fourier transform infrared (FTIR) spectra were introduced. XPS pattern confirms that CAT is successfully assembled onto microcapsules, as evidenced by the N 1s photoelectron line (Figure S5a). Because there is no nitrogen element in ADA, the N 1s line existing here comes from residual amido of CAT. In FTIR spectra (Figure S5b), the position and intensity of most absorption peaks of the microcapsules (pattern A) were similar to those of the superposition of ADA (pattern B) and CAT (pattern C), which implied that the microcapsules were composed of these two components. In the case of ADA, an aldehyde symmetric vibration band at around 1738 cm^{-1} could be observed.

The peak is weak and, in some cases, may not be detected (pattern B). This might be due to the hemiacetal formation.[6] In the spectrum of $(CAT/ADA)_6$ microcapsules (pattern A), the band at 1645 cm^{-1} represent the C=O stretching vibrations (amide I bands), and the band at 1538 cm^{-1} was attribute to N–H bending vibrations (amide II bands) of CAT.[7] The asymmetrical COO^- stretching peak at 1410 cm^{-1} in microcapsules was attributed to the carboxylic groups of ADA. The intensity of the peak at 2927 cm^{-1} belonging to C−H stretching of the cross-linked CAT increased substantially after fabrication, demonstrating the formation of Schiff's base.[8] All these evidences meant that aldehyde groups in ADA were successfully combined with amine groups in CAT by Schiff's base.

Figure S6

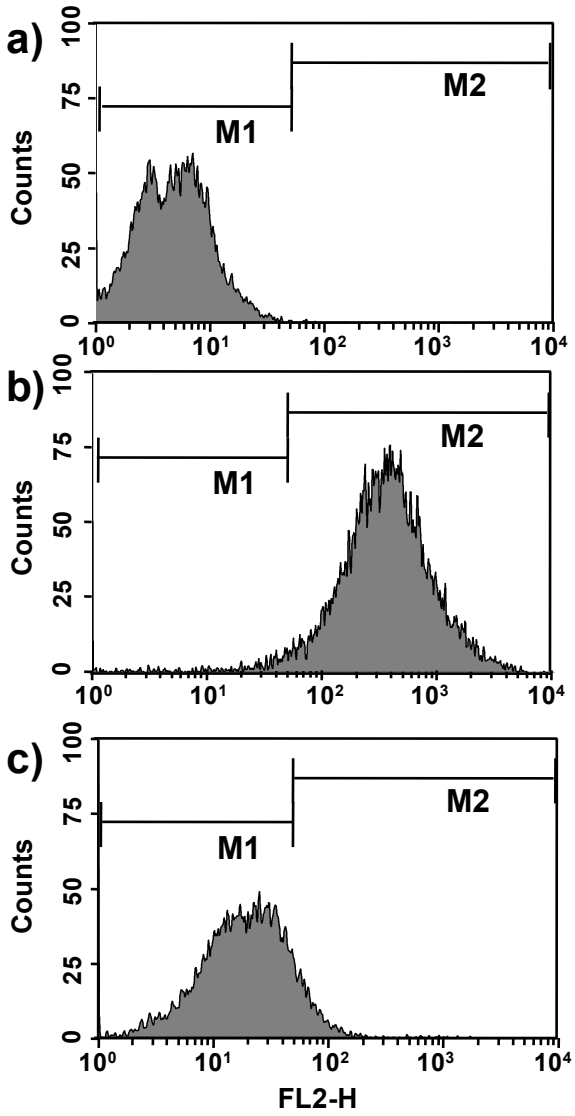


a) CLSM image of $CaCO_3$@RB-$(CAT/ADA)_6$ composites excited at 559 nm layser. b) 3D rebuilding image of MCF-7 cells, the main image is the area of xy section; the bottom and the right images represent the corresponding xz and yz orthogonal sections.

Because of the addsorption of RB, the $CaCO_3$@RB-$(CAT/ADA)_6$ composites have a fluorescent property. When excited at 559 nm by laser, the microcapsules exhibit strong fluorescence in the range of 580-610 nm (red channel), which is in accordance with the results of the emission spectrum.

The nuclei of MCF-7 cells were stained by Hoechst 33342, and the blue fluorescence was excited by 405 nm laser.

Figure S7



Flow cytometry diagrams of uptake of spheres in MCF-7 cells. a) untreated cells; b) cells cultured with $CaCO_3$@RB-$(CAT/ADA)_6$ composites; c) cells cultured with free RB solution. M1 corresponds to the cells that do not take up any composites, while M2 corresponds to the cells that ingested composites. Percentage of cells associated with composites was evaluated by the flow cytometrical analysis.

References

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