Stepwise Co-Delivery of Enzyme and Prodrug based on Multi-Responsive Nanoplatform for Accurate Tumor Therapy

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1. Materials

Doxorubicin (DOX), 3-Methacryloxypropyltrimethoxysilane (MPS), bis[3-(triethoxysilyl)propyl]disulfide (TESPD), tetraethyl orthosilicate (TEOS), hyaluronic acid (HA, M_w = 1000kDa), methacrylic acrylate, esterase and hyaluronidase were purchased from Sigma-Aldrich, USA. All the chemicals were analytical grade and used without further treatment. The glutathione (GSH) was obtained from Shanghai Macklin Biochemical Co., Ltd. The deionized water was prepared by a Millipore NanoPure purification system (resistivity higher than 18.2 MΩ/cm).

2. Synthesis of HAase encapsulated silica nanoparticles (HAase@SiO2)

The HAase@SiO_2 core-shell hybrid was fabricated according to our previous work. Triton X-100 (35.4 mL) and n-hexanol (36.0 mL) were dissolved in cyclohexane (150.0 mL) with magnetic stir (Solution i). Separately, 6.0 mL of a 2.5 $mg \cdot mL^{-1}$ aqueous solution of HAase were mixed with 0.6 mL of tetraethyl orthosilicate (TEOS) and 2.1 mL of bis[3-(triethoxysilyl)propyl]disulfide (TESPD) (Solution ii). After vigorous stiring for 5 minutes, this solution ii was added to the solution i. Then 1.0 mL of 25 % aqueous ammonia solution was added and the mixed water–oil emulsion was stirred for 12h at room temperature. After that 400 mL of pure acetone was subsequently added in order to precipitate the HAase@SiO_2. The resulting material was lyophilized after twice wash with ethanol and five times wash with water.

3. Surface modification of HAase@SiO2

The HAase@SiO2 powder was washed with copious anhydrous ethanol, and then re-dispersed in 50 mL anhydrous ethanol. Subsequently, 3-Methacryloxypropyltrimethoxysilane (MPS, 1 mL, 50% in anhydrous ethanol) was added into the suspension and stirred for another 2 h. After that, the resulting HAase@SiO2-C=C was further centrifuged, washed several times with ethanol and dried through lyophilization.

4. Synthesis of methacrylic acrylate and doxorubicin modified hyaluronic acid (MA-HA-DOX, prodrug)

2.0 g of HA was dissolved in 100 mL of DI water at 4 ^oC, to which 1.6 mL of methacrylic anhydride (MA) was dropwise added. The reaction solution was adjusted to pH 8-9 by the addition of 5 M NaOH and stir at 4^oC for 24 h. The resulting polymer was obtained by precipitation in acetone, followed by washing with ethanol for 3 times. The product was re-dissolved in DI water and the solution was dialyzed against DI water for 2 days to get methacrylic acrylate modified hyaluronic acid (MA-HA). After that, 50 mg of the resulting MA-HA was mixed with 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS) (117 mg/81 mg) for the activation of carbonyl groups on MA-HA in a pH 5.0 sodium

acetic buffer for 30 min at RT, and the unreacted EDC and NHS were removed using a centrifugal filter (100 kDa MWCO, Millipore). Then 10 mg DOX was added to react with the activated MA-HA at RT for overnight. Free DOX were removed using a centrifugal filter (100 kDa MWCO).

5. Formation of HAase@SiO2@Prodrug by copolymerization

30 mg as-sunthesized HAase@SiO2-C=C, 10 mg glycerol dimethacrylate and 30 mg MA-HA-DOX prodrug were dissolved well in 30 ml deoxygenated and deionized water containing 2 mg ammonium persulphate and 4 ml N,N,N',N'-tetramethylethylenediamine. The reaction was allowed to proceed for 2 h in a nitrogen atmosphere. The resulting mixture was centrifuged, washed several times with water to remove monomers and initiators, and then dried through lyophilization. The amount of loaded drug for the HAase@SiO2@Prodrug was determined by absorption peak at 500 nm in UV-Vis spectrophotometer (Hitachi U-2910, Japan).

The drug loading efficiency (weight of cargos in carriers/weight of cargos loaded carriers) of the DOX and HAase in HAase@SiO2@prodrug were 8 wt% and 13 wt%, and the encapsulation efficiency (weight of cargos in carriers/initial used weight of cargos) of the DOX and HAase in HAase@SiO2@prodrug were 87 wt% and 33 wt%, which were all measured by the fluorescence spectroscopy at 590 nm for DOX and 530 nm for FITC labeled HAase.

6. **Stimulated DOX release**

In the GSH and esterase triggered DOX release experiment, a certain amount of HAase@SiO2@Prodrug powder was dispersed in 50 mL of 3 different type of PBS buffer (i: pH 7.4 in the absence of 10 mM of GSH and 4 mg/L of esterase, ii: pH 7.4 in the presence of 10 mM of GSH and iii: pH 7.4 in the presence of 10 mM of GSH and 4 mg/L of esterase) at 25 °C in a dialysis bag. Subsequently, 2 mL of supernatant was taken periodically from the suspension in and out of the dialysis bag at 25 °C, followed by centrifugation (15000 rpm, 5 min). The release of HA-DOX and free

DOX from the HAase@SiO2@Prodrug to the buffer solution was determined by measuring the absorption peak of DOX at 500 nm using UV-Vis spectrophotometer.

7. Fluorescence microscopy analysis of tumor targeting and selective drug delivery

To observe the selective tumor targeting drug delivery of the HAase@SiO2@Prodrug, SCC cells (cancer cells) and HaCaT cells (normal cells) were employed. The cells were seeded at 2.5×10^4 per well onto 24-well plates containing glass coverslips, and were cultured in Dulbecco's modification of Eagle's medium (DMEM, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Gibico, USA) and penicillin-streptomycin (100 U/mL and 100 μg/mL, Gibico, USA), and incubated at 37 ^{o}C in 5% CO_2. On the following day, HAase@SiO2@Prodrug containing 0.1 μg/mL DOX and/or BSA@SiO2@Prodrug containing 0.1 μg/mL DOX were added to these cells, respectively. After 6 h and 12 h, the cells were rinsed with PBS, fixed with 4% paraformaldehyde for 20 min, permeabilized in 0.1% Triton X-100 for 5 min, stained with 4′, 6-diamidino-2-phenylindole (DAPI, Life Technologies, USA). Afterwards, the cells were rinsed, mounted and the fluorescence was observed under a fluorescence microscope (Olympus BX51, Olympus, Japan).

8. *In vitro* cytotoxicity analysis

SCC cells and HaCaT cells were seeded at 3×10^3 per well in 96-well plate for 24h before treatment, respectively. The cells were exposed to HAase@SiO2@Prodrug containing 0.1 μg/mL DOX, BSA@SiO2@Prodrug containing 0.1 μg/mL DOX and 100 ug/ml HAase@SiO2@HA-PE for 6 h and 12 h. Cell viability was measured by using the Cell Counting Kit 8 (CCK-8, Dojindo Co., Ltd. Japan) proliferation assay according to the manufacture's protocol. The absorbance of the wells was read at 450 nm by using Varioskan Flash multimode reader (Thermo Fisher Scientific, USA).

9. *In vivo* Antitumor Efficacy and Safety Evaluations

All experimental protocols were approved by the Ethics Committee of the Peking University Health Science Center, Beijing, China. To set up the tumor xenograft model,

BALB/c female nude mice (5 weeks old) were prepared. A total of 3×10^6 SCC cells were injected subcutaneously into the back, and permitted the tumor to reach a size of approximately 100 mm^3 in volume.

To show the antitumor therapy effect *in vivo*, twenty-four tumor-bearing mice were randomly divided into four groups (n=6). Then 100 μL of (1) PBS (2) aqueous solution of HAase@SiO2@PE-HA, (3) aqueous solution of free DOX, and (4) aqueous solution of HAase@SiO2@Prodrug were injected to these mice *via* the tail vein, respectively. Tumor size was measured and the tumor-bearing mice were weighed every two days in the following two weeks. The tumor size was monitored by a vernier caliper and the volume was calculated as $V=W^2 \times L/2$, where W and L were the width and length of the tumor, respectively.

10. Characterization

Transmission electron microscopy (TEM) images were recorded on a Philips CM200 transmission electron microscope operated at 200 kV. For the TEM observation, samples were obtained by dropping 5 μL of solution onto carbon-coated copper grids. All the TEM images were visualized without staining. The infrared (IR) spectra were measured by AVATAR 320 FT-IR using KBr pellets. The ultraviolet-visible (UV-Vis) spectra were measured with dilute aqueous solution in a 2 mm thick quartz cell using a Hitachi U-2910 spectrophotometer. All pH value measurements were carried out on a Sartorius BECKMAN F 34 pH meter. The intracellular DOX release was monitored by fluorescence microscopy using a Olympus BX51 microscope equipped with a fluorescent lamp; ex = 488 nm, em = 590 nm for DOX.

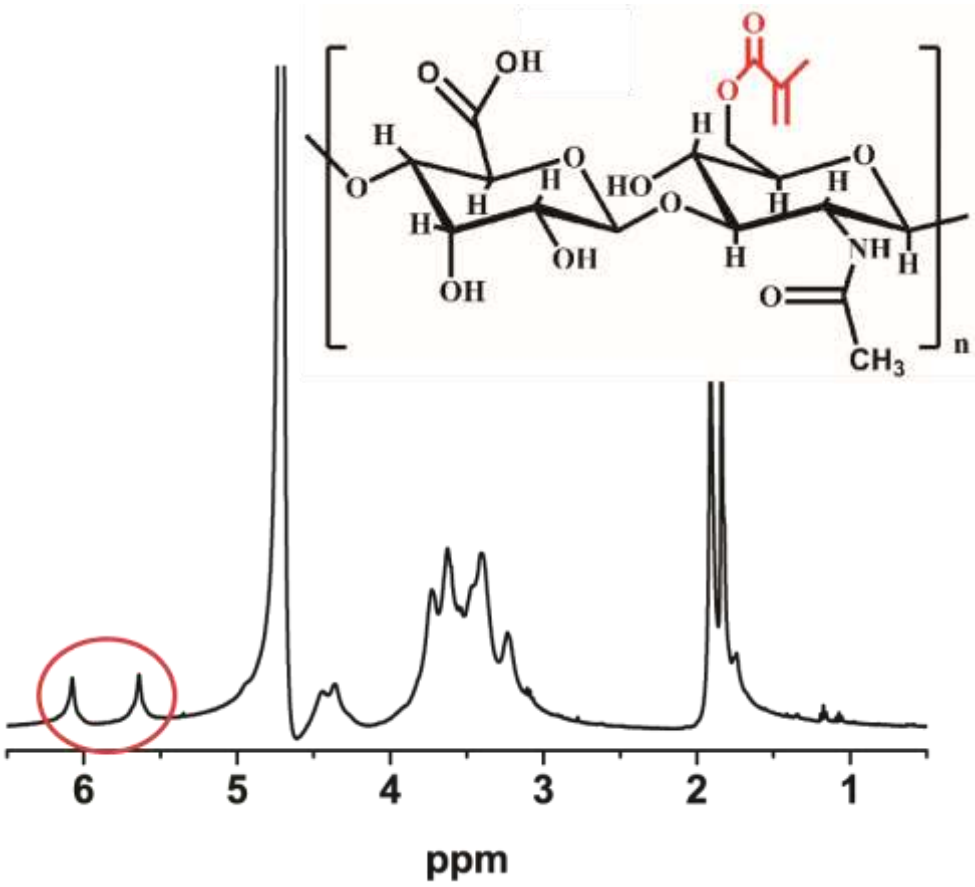

Figure S1. The 1H NMR spectra of the MA-HA

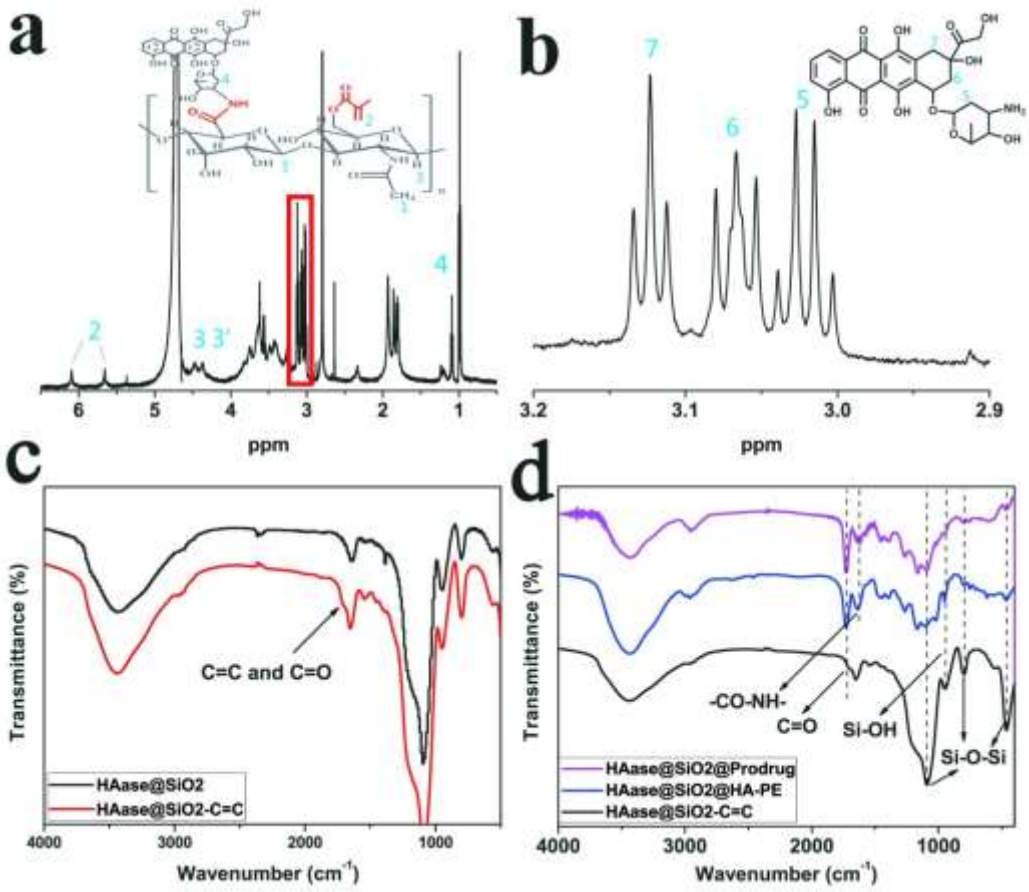

Figure S2. (a, b) The 1H NMR spectra of the MA-HA-DOX. (c, d) The infrared spectrum of HAase@SiO2, HAase@SiO2-C=C and HAase@SiO2@Prodrug.

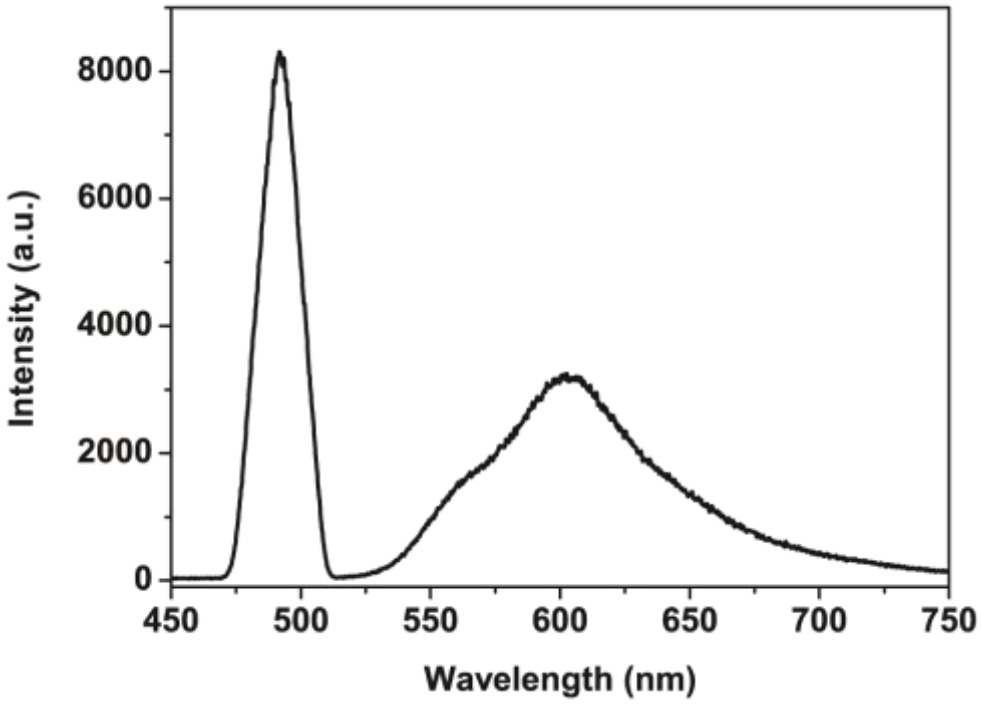

Figure S3. Fluorescence spectrum of the HAase@SiO2@Prodrug

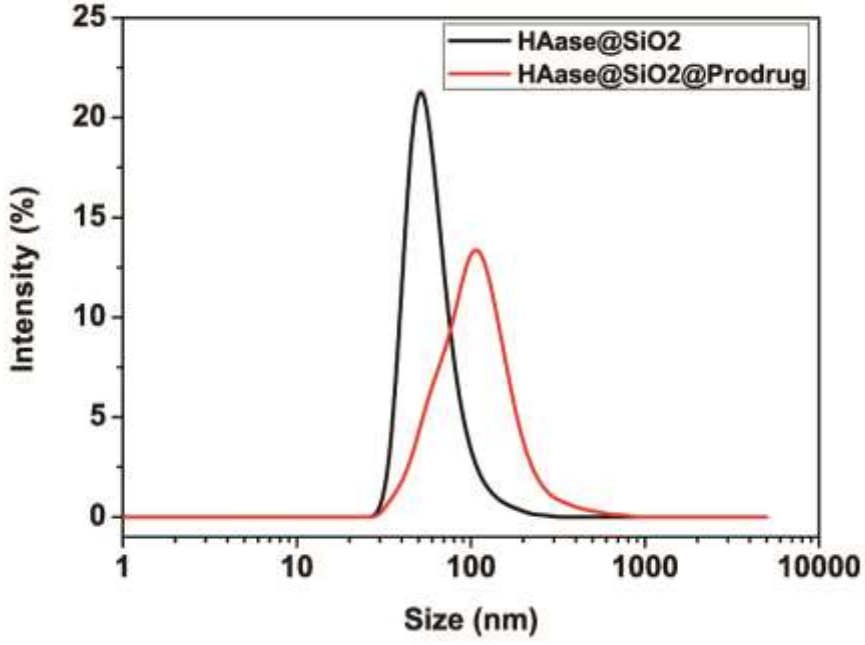

Figure S4. Dynamic light scattering analyses of HAase@SiO2 (Black curve) and HAase@SiO2@Prodrug (Red curve).

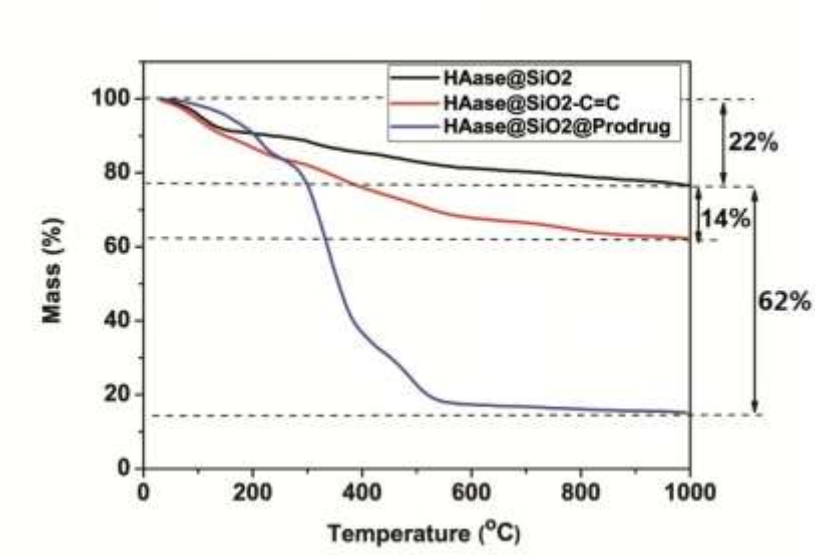

Figure S5. Thermogravimetric analysis (TGA) curves of HAase@SiO_2 (Black curve), HAase@SiO_2-C=C (Red curve) and HAase@SiO_2@Prodrug (Blue curve). Samples were run under O_2 atmosphere.

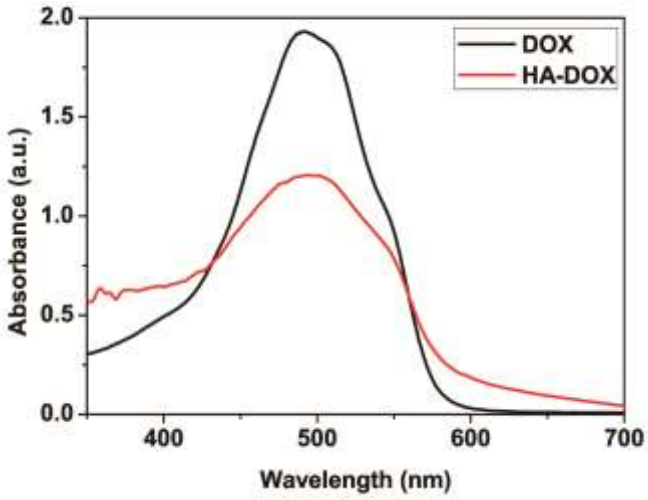

Figure S6. UV-vis spectrometry of DOX and HA-DOX in water solution

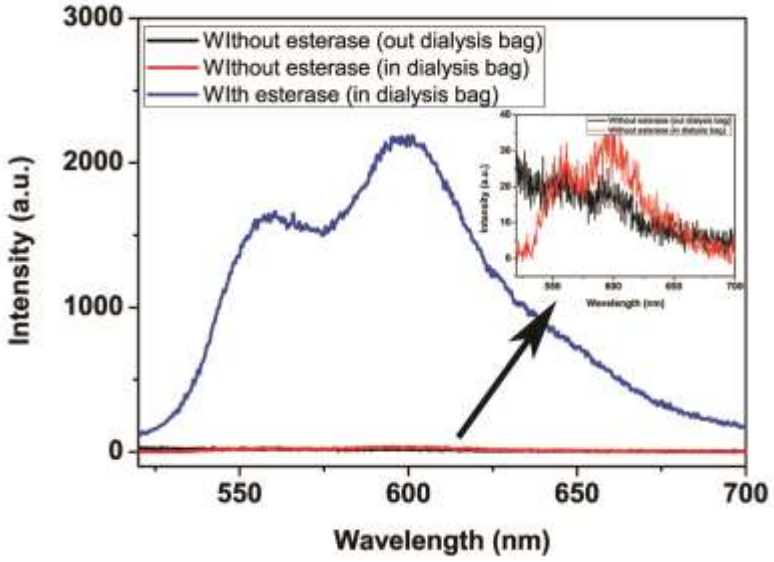

Figure S7. Fluorescence spectroscopy of the HAase@SiO2@prodrug solution in and out of the dialysis bag before and after adding the esterase.

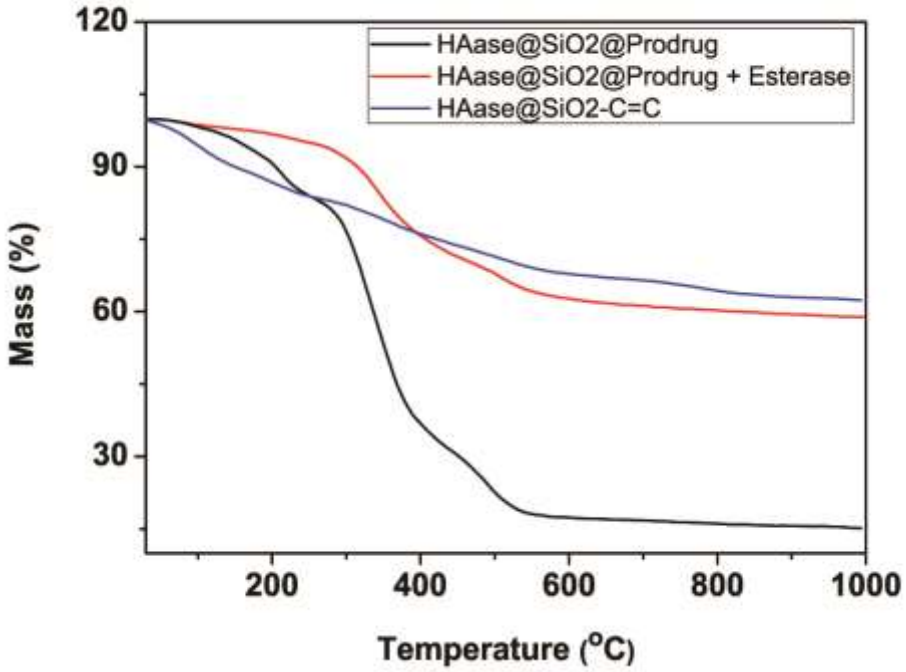

Figure S8. Thermogravimetric analysis (TGA) curves of HAase@SiO2@prodrug (Black curve), HAase@SiO2@prodrug after the treatment of esterase (Red curve), and HAase@SiO2-C=C (Blue curve). Samples were run under O2 atmosphere.

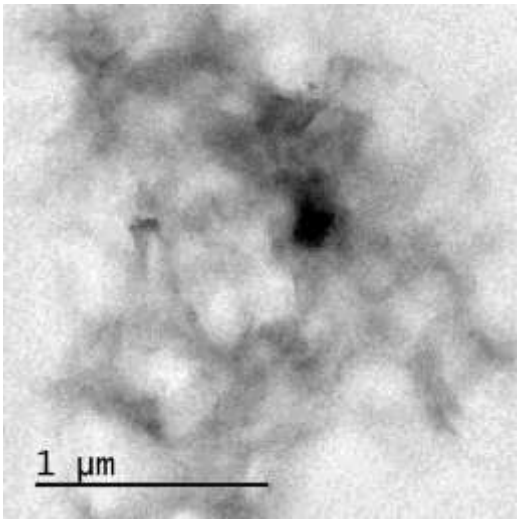

Figure S9. Transmission electronic microscopic images of (a) HAase@SiO2 after 5 days incubation in GSH solution (10 mM)

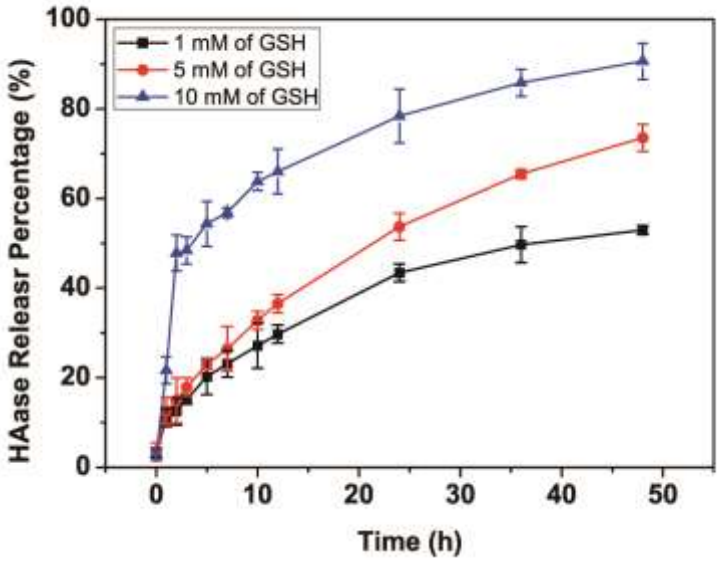

Figure S10. Glutathione (GSH) dependent release kinetics of HAase from HAase@SiO2 against incubation time.

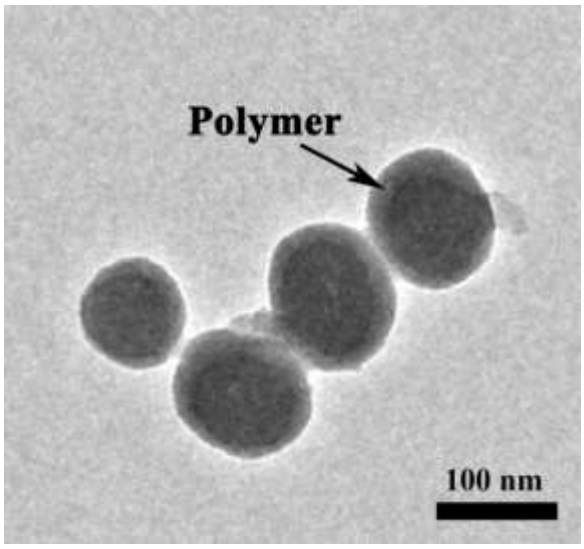

Figure S11. Transmission electronic microscopic images of HAase@SiO2@Prodrug after 48 h incubation in plasma.

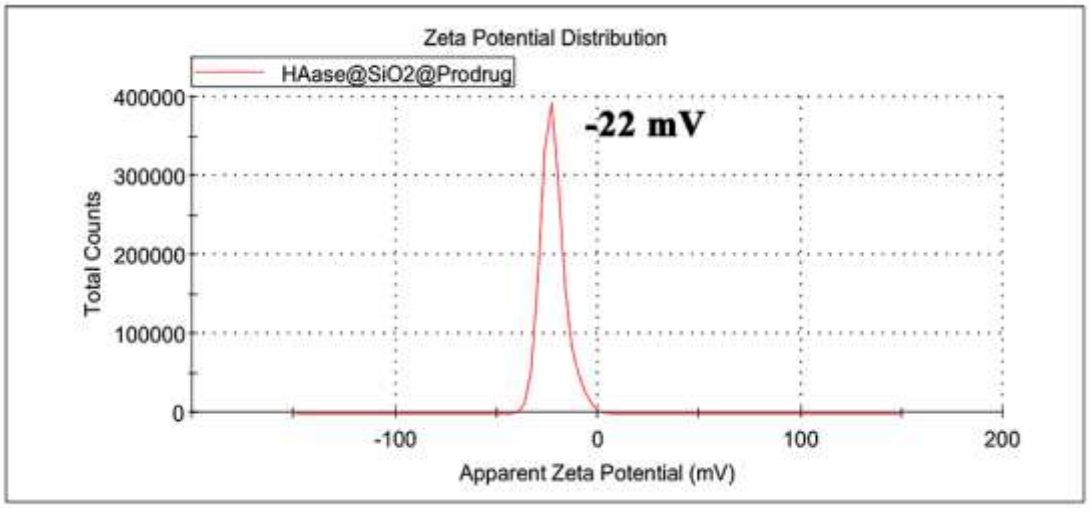

Figure S12. Zeta potential of the HAase@SiO2@prodrug

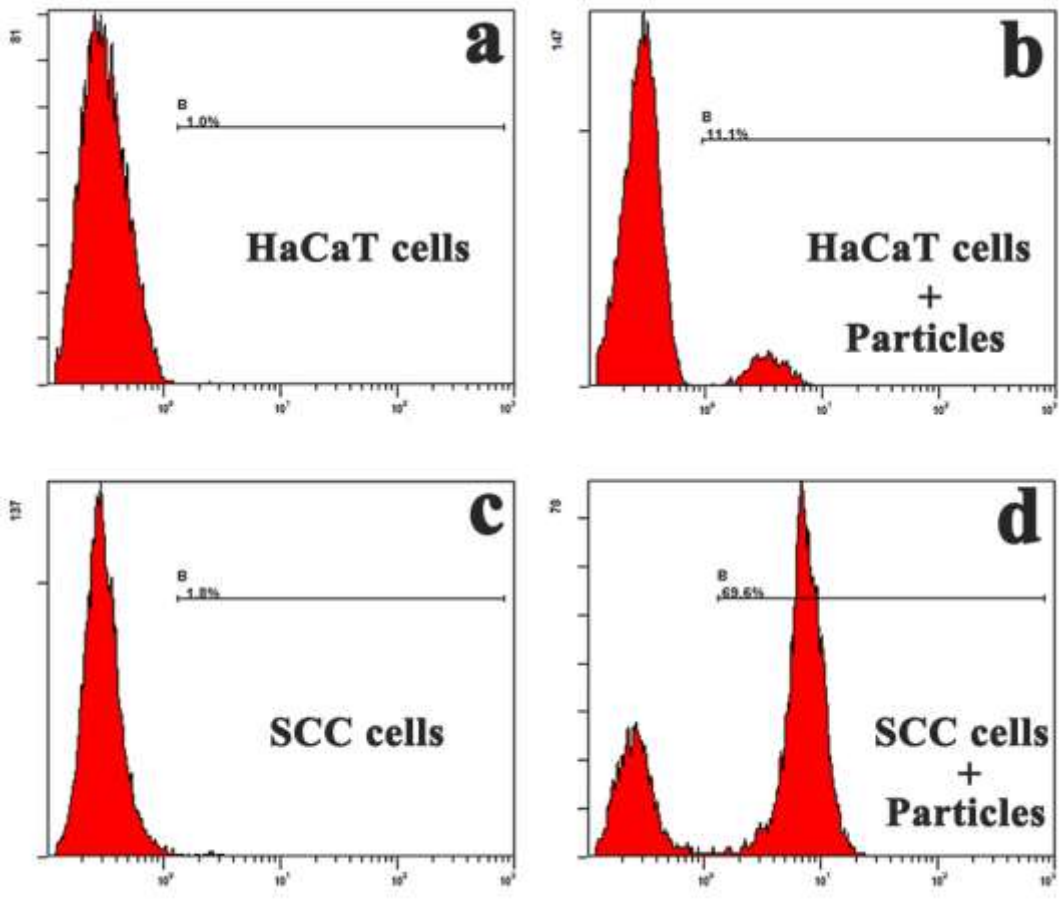

Figure S13. Quantitative analysis of the cellular uptake of HAase@SiO2@prodrug nanoparticles to SCC cells (tumor cells) and HaCaT cells (healthy cells), which were eveluated by flow cytometry.

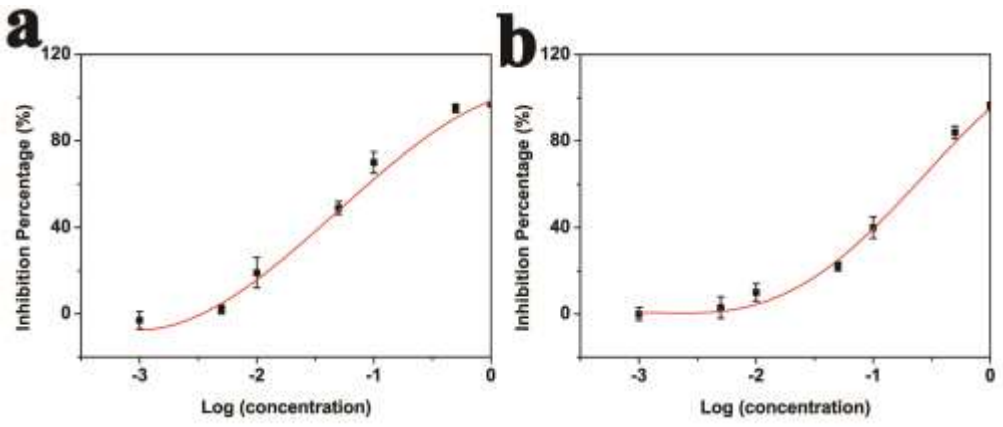

Figure S14. The normalized curves of the inhibition percentage to tumor cells (SCC cells) against the increasing amounts of DOX in the form of HAase@SiO2@Prodrug (a) and free DOX (b) for 72 h, which were obtained by GraphPad Prism.