

1 Stepwise Co-Delivery of Enzyme and Prodrug based on 2 Multi-Responsive Nanoplatfrom for Accurate Tumor 3 Therapy

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24 25 1. Materials

26 Doxorubicin (DOX), 3-Methacryloxypropyltrimethoxysilane (MPS),
27 bis[3-(triethoxysilyl)propyl]disulfide (TESPD), tetraethyl orthosilicate (TEOS),
28 hyaluronic acid (HA, $M_w = 1000\text{kDa}$), methacrylic acrylate, esterase and
29 hyaluronidase were purchased from Sigma-Aldrich, USA. All the chemicals were
30 analytical grade and used without further treatment. The glutathione (GSH) was
31 obtained from Shanghai Macklin Biochemical Co., Ltd. The deionized water was
32 prepared by a Millipore NanoPure purification system (resistivity higher than 18.2
33 $\text{M}\Omega/\text{cm}$).

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

1 The HAase@SiO₂ core-shell hybrid was fabricated according to our previous work.
2 Triton X-100 (35.4 mL) and n-hexanol (36.0 mL) were dissolved in cyclohexane
3 (150.0 mL) with magnetic stir (Solution i). Separately, 6.0 mL of a 2.5 mg·mL⁻¹
4 aqueous solution of HAase were mixed with 0.6 mL of tetraethyl orthosilicate (TEOS)
5 and 2.1 mL of bis[3-(triethoxysilyl)propyl]disulfide (TESPD) (Solution ii). After
6 vigorous stirring for 5 minutes, this solution ii was added to the solution i. Then 1.0
7 mL of 25 % aqueous ammonia solution was added and the mixed water–oil emulsion
8 was stirred for 12h at room temperature. After that 400 mL of pure acetone was
9 subsequently added in order to precipitate the HAase@SiO₂. The resulting material
10 was lyophilized after twice wash with ethanol and five times wash with water.

11 **3. Surface modification of HAase@SiO₂**

12 The HAase@SiO₂ powder was washed with copious anhydrous ethanol, and then
13 re-dispersed in 50 mL anhydrous ethanol. Subsequently,
14 3-Methacryloxypropyltrimethoxysilane (MPS, 1 mL, 50% in anhydrous ethanol) was
15 added into the suspension and stirred for another 2 h. After that, the resulting
16 HAase@SiO₂-C=C was further centrifuged, washed several times with ethanol and
17 dried through lyophilization.

18 **4. Synthesis of methacrylic acrylate and doxorubicin modified hyaluronic acid** 19 **(MA-HA-DOX, prodrug)**

20 2.0 g of HA was dissolved in 100 mL of DI water at 4 °C, to which 1.6 mL of
21 methacrylic anhydride (MA) was dropwise added. The reaction solution was adjusted
22 to pH 8-9 by the addition of 5 M NaOH and stir at 4°C for 24 h. The resulting
23 polymer was obtained by precipitation in acetone, followed by washing with ethanol
24 for 3 times. The product was re-dissolved in DI water and the solution was dialyzed
25 against DI water for 2 days to get methacrylic acrylate modified hyaluronic acid
26 (MA-HA). After that, 50 mg of the resulting MA-HA was mixed with
27 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS)
28 (117 mg/81 mg) for the activation of carbonyl groups on MA-HA in a pH 5.0 sodium

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

5 **5. Formation of HAase@SiO₂@Prodrug by copolymerization**

6 30 mg as-synthesized HAase@SiO₂-C=C, 10 mg glycerol dimethacrylate and 30 mg
7 MA-HA-DOX prodrug were dissolved well in 30 ml deoxygenated and deionized
8 water containing 2 mg ammonium persulphate and 4 ml
9 N,N,N',N'-tetramethylethylenediamine. The reaction was allowed to proceed for 2 h
10 in a nitrogen atmosphere. The resulting mixture was centrifuged, washed several
11 times with water to remove monomers and initiators, and then dried through
12 lyophilization. The amount of loaded drug for the HAase@SiO₂@Prodrug was
13 determined by absorption peak at 500 nm in UV-Vis spectrophotometer (Hitachi
14 U-2910, Japan).

15 The drug loading efficiency (weight of cargos in carriers/weight of cargos loaded
16 carriers) of the DOX and HAase in HAase@SiO₂@prodrug were 8 wt% and 13 wt%,
17 and the encapsulation efficiency (weight of cargos in carriers/initial used weight of
18 cargos) of the DOX and HAase in HAase@SiO₂@prodrug were 87 wt% and 33 wt%,
19 which were all measured by the fluorescence spectroscopy at 590 nm for DOX and
20 530 nm for FITC labeled HAase.

21 **6. Stimulated DOX release**

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

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

3 **7. Fluorescence microscopy analysis of tumor targeting and selective drug** 4 **delivery**

5 To observe the selective tumor targeting drug delivery of the HAase@SiO₂@Prodrug,
6 SCC cells (cancer cells) and HaCaT cells (normal cells) were employed. The cells
7 were seeded at 2.5×10^4 per well onto 24-well plates containing glass coverslips, and
8 were cultured in Dulbecco's modification of Eagle's medium (DMEM, Invitrogen,
9 USA) supplemented with 10% fetal bovine serum (FBS, Gibico, USA) and
10 penicillin-streptomycin (100 U/mL and 100 µg/mL, Gibico, USA), and incubated at
11 37 °C in 5% CO₂. On the following day, HAase@SiO₂@Prodrug containing 0.1
12 µg/mL DOX and/or BSA@SiO₂@Prodrug containing 0.1 µg/mL DOX were added to
13 these cells, respectively. After 6 h and 12 h, the cells were rinsed with PBS, fixed with
14 4% paraformaldehyde for 20 min, permeabilized in 0.1% Triton X-100 for 5 min,
15 stained with 4', 6-diamidino-2-phenylindole (DAPI, Life Technologies, USA).
16 Afterwards, the cells were rinsed, mounted and the fluorescence was observed under a
17 fluorescence microscope (Olympus BX51, Olympus, Japan).

18 **8. *In vitro* cytotoxicity analysis**

19 SCC cells and HaCaT cells were seeded at 3×10^3 per well in 96-well plate for 24h
20 before treatment, respectively. The cells were exposed to HAase@SiO₂@Prodrug
21 containing 0.1 µg/mL DOX, BSA@SiO₂@Prodrug containing 0.1 µg/mL DOX and
22 100 µg/ml HAase@SiO₂@HA-PE for 6 h and 12 h. Cell viability was measured by
23 using the Cell Counting Kit 8 (CCK-8, Dojindo Co., Ltd. Japan) proliferation assay
24 according to the manufacture's protocol. The absorbance of the wells was read at 450
25 nm by using Varioskan Flash multimode reader (Thermo Fisher Scientific, USA).

26 **9. *In vivo* Antitumor Efficacy and Safety Evaluations**

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

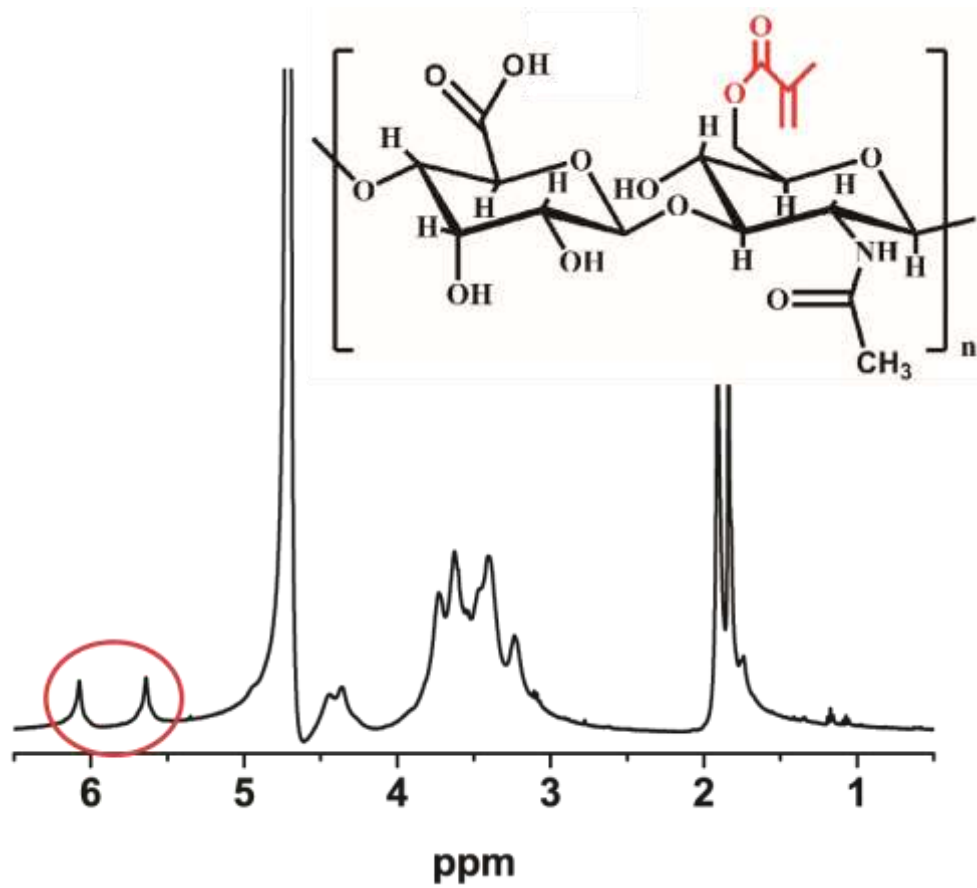
1 BALB/c female nude mice (5 weeks old) were prepared. A total of 3×10^6 SCC cells
2 were injected subcutaneously into the back, and permitted the tumor to reach a size of
3 approximately 100 mm^3 in volume.

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

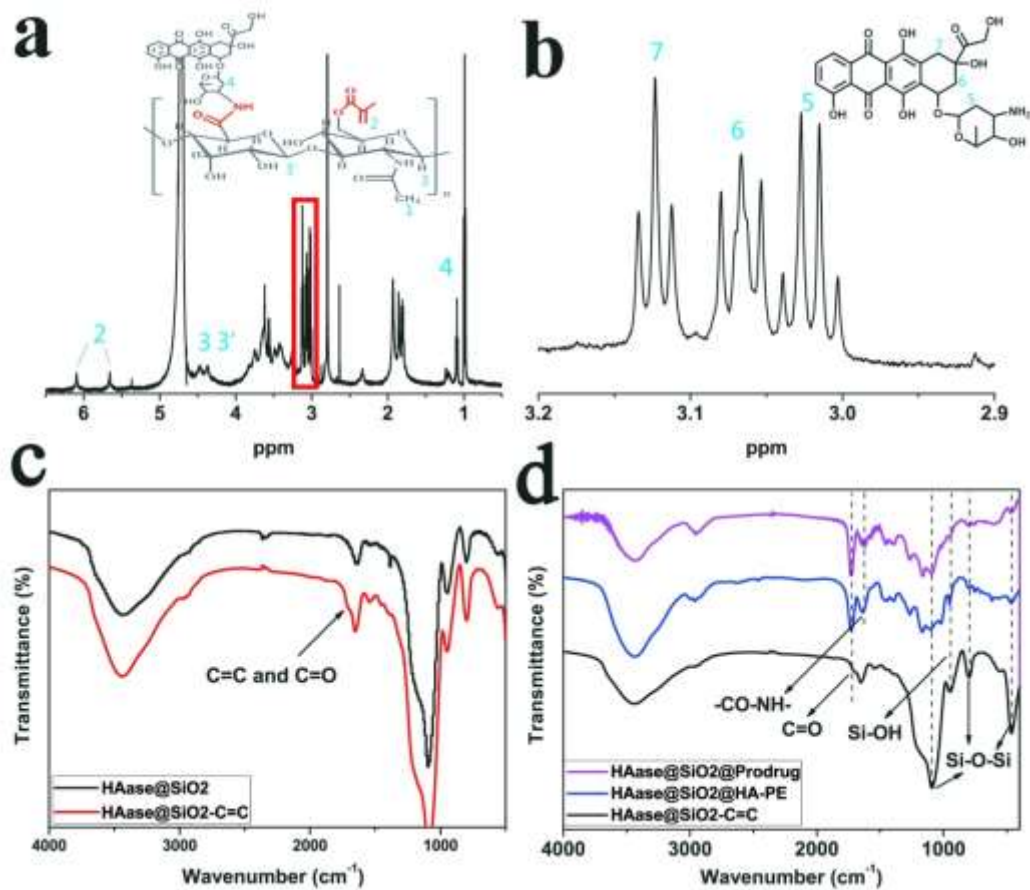
12 **10. Characterization**

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

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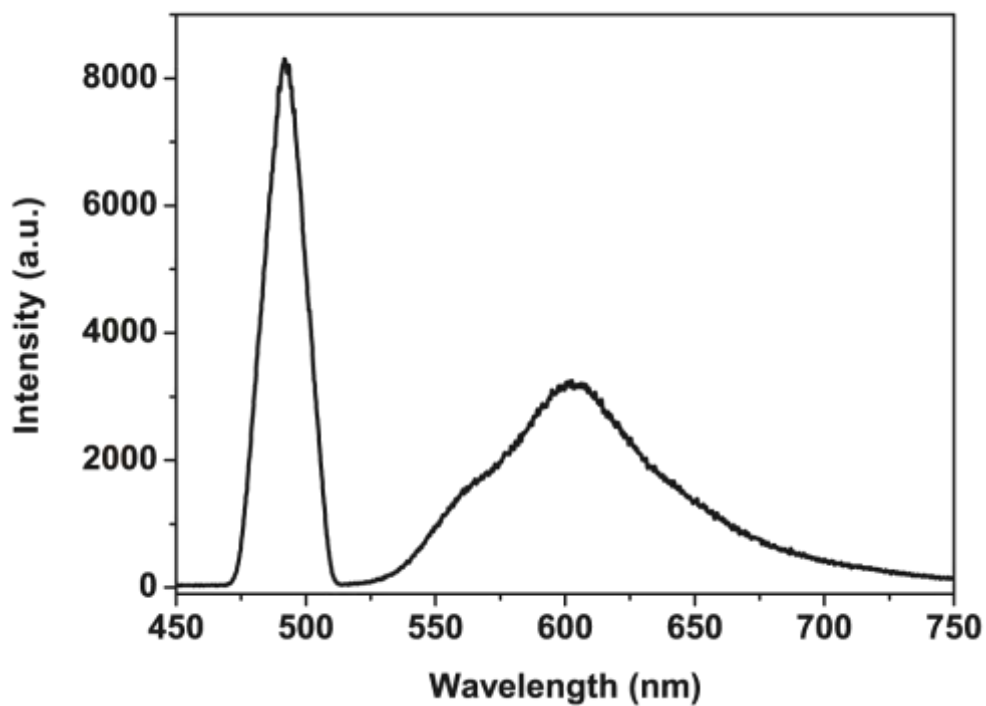


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2 **Figure S1.** The ¹H NMR spectra of the MA-HA
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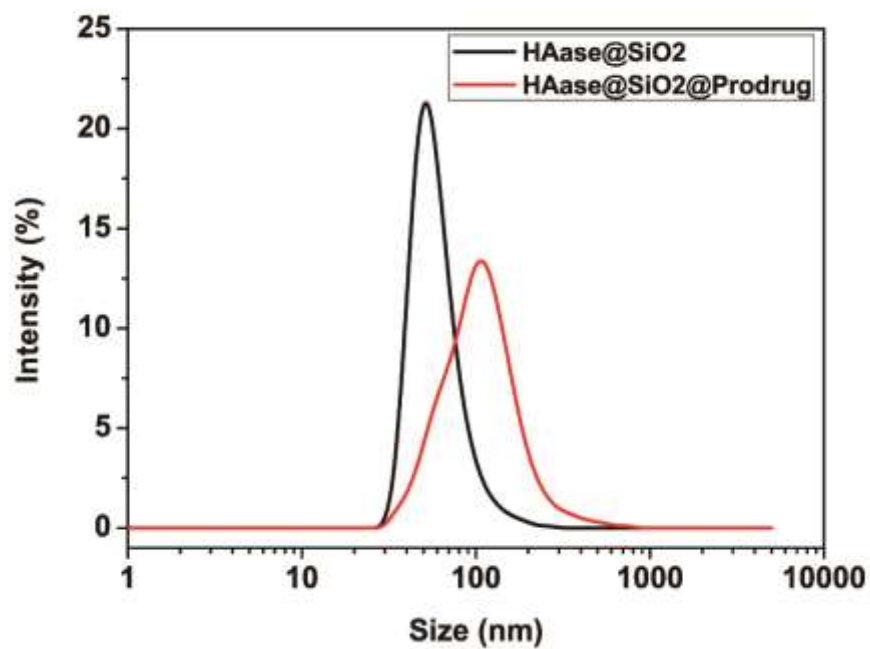
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Figure S2. (a, b) The ¹H NMR spectra of the MA-HA-DOX. (c, d) The infrared spectrum of HAase@SiO₂, HAase@SiO₂-C=C and HAase@SiO₂@Prodrug.



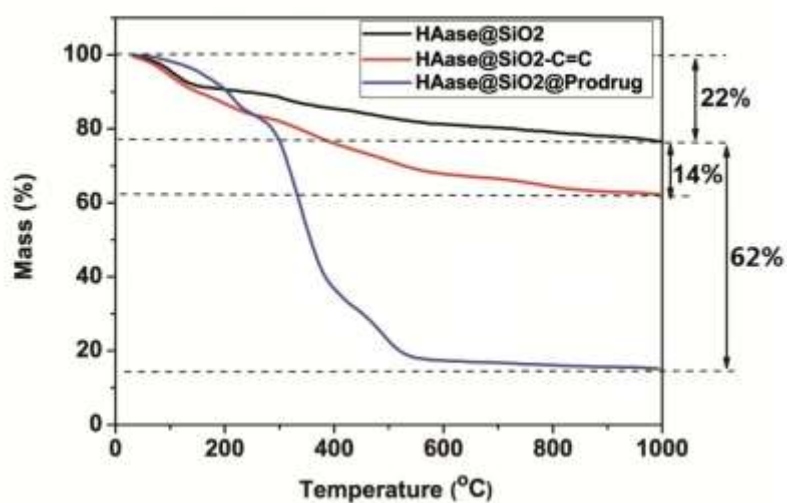
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2 **Figure S3.** Fluorescence spectrum of the HAase@SiO2@Prodrug



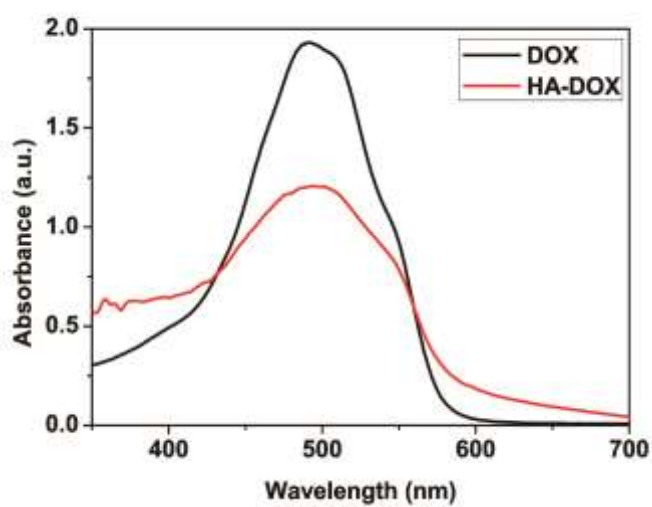
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4 **Figure S4.** Dynamic light scattering analyses of HAase@SiO2 (Black curve) and
5 HAase@SiO2@Prodrug (Red curve).



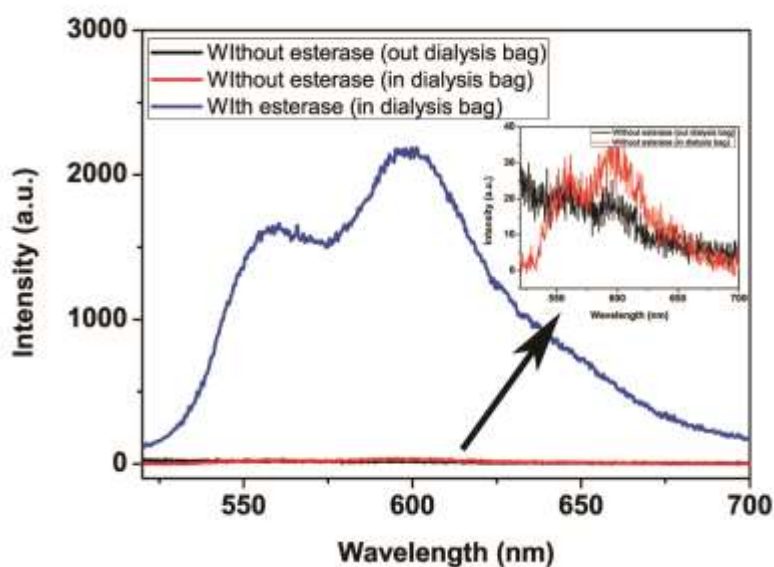
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Figure S5. Thermogravimetric analysis (TGA) curves of HAase@SiO₂ (Black curve), HAase@SiO₂-C=C (Red curve) and HAase@SiO₂@Prodrug (Blue curve). Samples were run under O₂ atmosphere.



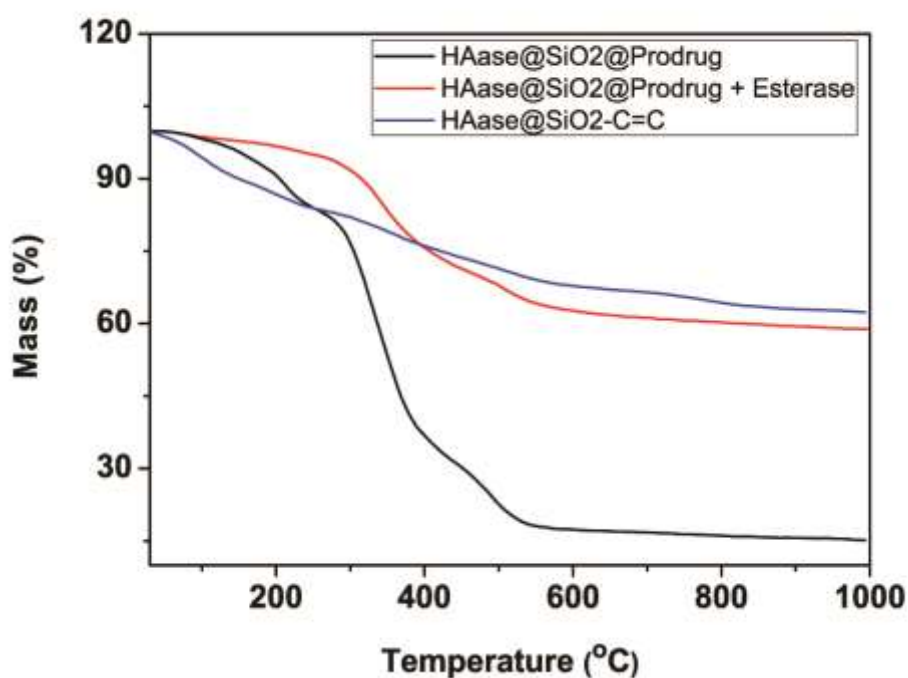
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Figure S6. UV-vis spectrometry of DOX and HA-DOX in water solution



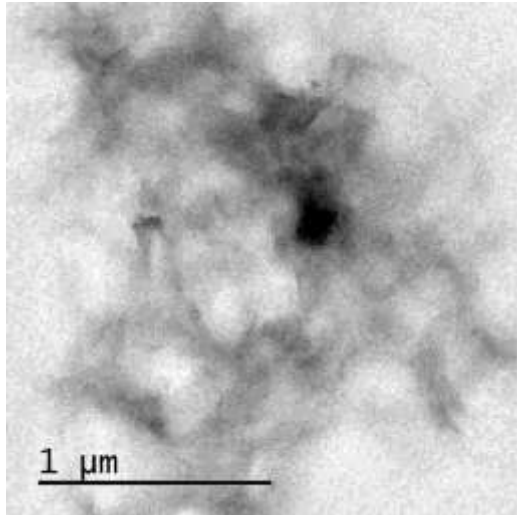
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Figure S7. Fluorescence spectroscopy of the HAase@SiO₂@prodrug solution in and out of the dialysis bag before and after adding the esterase.



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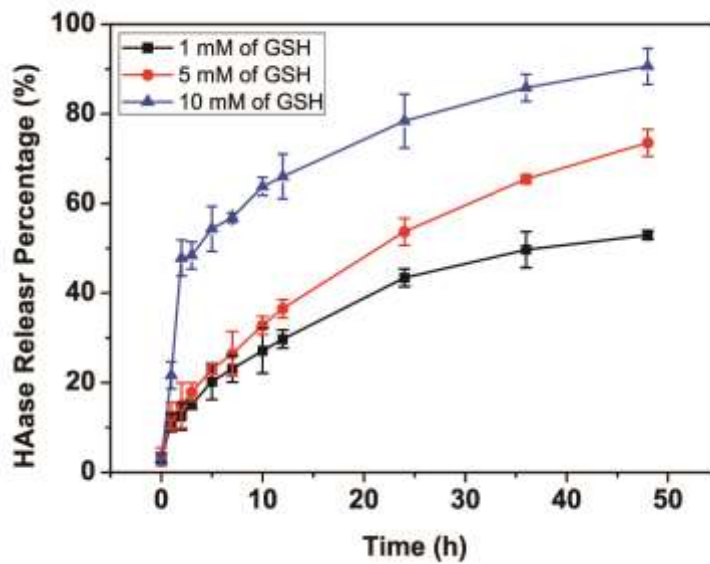
Figure S8. Thermogravimetric analysis (TGA) curves of HAase@SiO₂@prodrug (Black curve), HAase@SiO₂@prodrug after the treatment of esterase (Red curve), and HAase@SiO₂-C=C (Blue curve). Samples were run under O₂ atmosphere.



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

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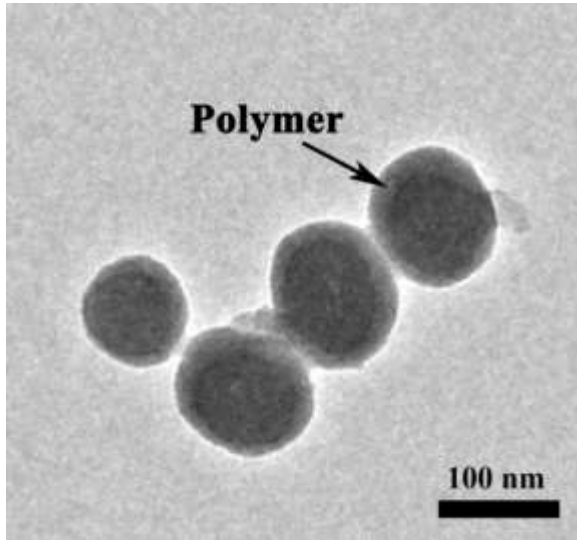


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6 **Figure S10.** Glutathione (GSH) dependent release kinetics of HAase from
7 HAase@SiO₂ against incubation time.

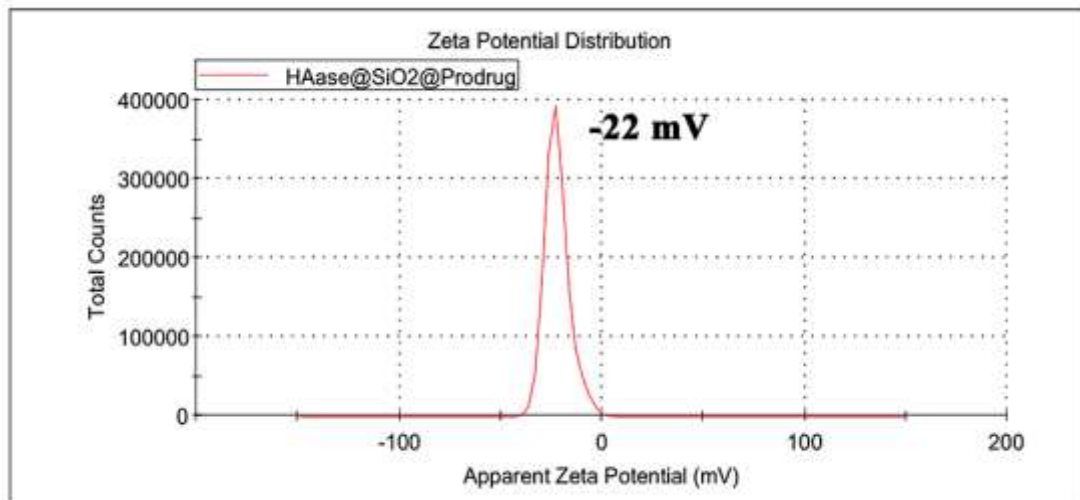
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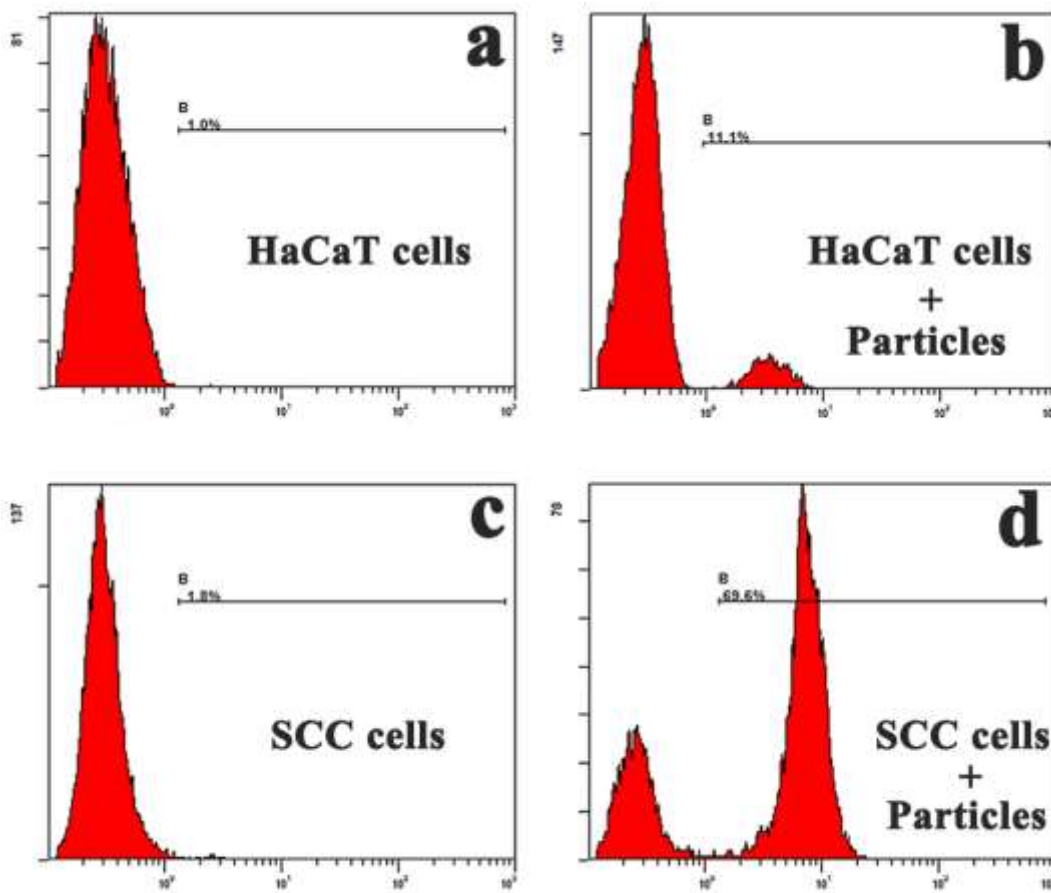
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Figure S11. Transmission electronic microscopic images of HAase@SiO₂@Prodrug after 48 h incubation in plasma.



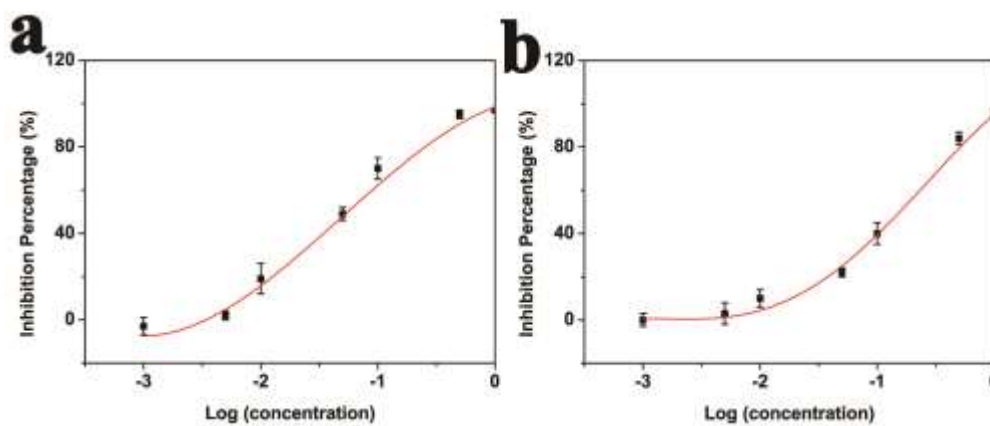
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Figure S12. Zeta potential of the HAase@SiO₂@prodrug



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

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

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