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Electronic Supplementary Information

Beyond "turn-on" readout: from zero background to signal amplification by combination of magnetic separation and plasmon enhanced fluorescence

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1. Experimental Section

1.1 Reagents

Hyaluronidase (HAase, 300 U mg^{-1}), poly(vinylpyrrolidone) (PVP, Mw=40000), tetraethylorthosilicate (TEOS), (3-aminopropyl)triethoxysilane (APTES), urea, uric acid, creatinine, thrombin, lysozyme, ribonuclease, trypsin, glucose and dopamine were obtained from Sigma-Aldrich. Hyaluronan (HA) (Mw=403.3) was purchased from Shanghai Macklin Biochemical Co. Te powder was purchased from Alfa Aesar. Sodium borohydride ($NaBH_4$), ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), ethylene glycol (EG), diethylene glycol (DEG), silver nitrate ($AgNO_3$) and ascorbic acid were acquired from Sinopharm. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and various amino acids were obtained from Aladdin. Glutathione, bovine serum albumin (BSA) and human serum albumin (HSA) were acquired from BBI life sciences Co. Cadmium chloride ($CdCl_2{\cdot}2.5H_2O$), sodium hydroxide (NaOH) and thioacetamide were acquired from Shanghai Reagent Co. Trisodium citrate dihydrate and sodium acetate (CH_3COONa, NaAc) were purchased from Shanghai Lingfeng Chemical Reagent Co. Serum samples were donated from school infirmary. Urine samples were donated from our lab. Enzyme-linked immunosorbent assay (ELISA) kit for HAase was purchased from Shanghai Yanxin Biological Technology Co. All solutions were prepared with deionized water (18.25 MΩ cm).

1.2 Apparatus

Fluorescence spectra were recorded by a Hitachi F-4600 fluorescence spectrophotometer with excitation wavelength being 380 nm. A Hitachi U-2910 spectrometer was used to record the UV−visible spectra. Transmission electron microscopy (TEM) photographs were taken with a HT-7700 Hitachi microscope at an accelerating voltage of 100 kV. High-resolution TEM (HRTEM) characterizations were carried out by Tecnai G2 20 ST (FEI) under the accelerating voltage of 200 kV. The solutions were analyzed for particle sizes and zeta-potential values using dynamic light scattering (DLS, Zetasizer Nano ZS series, Malvern Instruments) with 633 nm

laser wavelength and a measurement angle of 173° (backscatter detection) at 25°C. Fourier transform infrared (FT-IR) spectra were recorded from a KBr window on a PerkinElmer PE-983 FT-IR spectrophotometer. X-ray powder diffraction (XRD) patterns of the products were determined using powder XRD (Bruker D8 Advance X-ray diffractometer with Cu K_α X-ray source). The magnetic properties (M–H curve) were measured at 300 K on a Lakeshore 7300 magnetometer. X-ray photoelectron spectroscopy (XPS) measurements were carried out on a Perkin Elmer PHI 5600 spectrometer operating at 10^{-7} Pa. The absorbance for ELISA analysis was recorded on a microplate reader (BIO-TEK Synergy HT, U.S.A.) at 450 nm.

1.3 Synthesis, modification and functionalization of various nanoparticles (NPs)

1.3.1 Synthesis and purification of CdTe and CdTe@CdS core@shell quantum dots (QDs)

Cysteine-capped CdTe QDs were prepared in aqueous solution using the method described previously.[1] Briefly, 1.25×10^{-3} mol of $CdCl_2\cdot2.5H_2O$ was dissolved in 100 mL of deionized water, and 3.0×10^{-3} mol of cysteine was added under stirring, whose pH value was adjusted to 11.2 by adding dropwise 1.0 M NaOH. The solution was deaerated by N_2 bubbling for 0.5 h. Under vigorous stirring, 1.6×10^{-4} mol of freshly prepared oxygen-free NaHTe, which was fabricated by mixing Te powder and $NaHB_4$ in 0.3 mL of deionized water and reacting 9 h, was injected into the above solution. Afterward, the solution mixture was heated to 100°C and refluxed for 2 h to obtain highly emitting CdTe QDs ($\lambda_{ex/em}$=380/600 nm). We then used previous reports for the synthesis of CdTe@CdS core@shell QDs.[2] 50 mL of the crude CdTe solution was cooled to room temperature and deaerated N_2 bubbling for 0.5 h. Under vigorous stirring, 8.0×10^{-5} mol of thioacetamide, which acted as sulfur source, was added to the QD solution. Next, the mixture was heated and maintained at 100°C in an oil bath for 3 h to form near-infrared CdTe@CdS core@shell QDs ($\lambda_{ex/em}$=380/690 nm). For purification of the CdTe@CdS core@shell QDs, 5 mL of ethanol was added to 5 mL of the QD solution, then the mixture was centrifuged (7500 rpm, 10

min). The purified CdTe@CdS core@shell QDs were redispersed in 5 mL of deionized water as stock solution. The concentration of the CdTe@CdS QDs was estimated according to the concentration of CdTe core using the method reported previously.[3]

1.3.2 Synthesis and purification of HA functionalized QDs

HA functionalized fluorescent QDs were prepared in aqueous solution by the modified method reported previously.[4] In brief, 2.5×10^{-4} mol of HA was dissolved in 100 mL of PBS buffer (10 mM, pH 5.0) to give an HA solution. 1.0×10^{-4} mol of EDC and 2.6×10^{-4} mol of NHS were added to this solution. The solution was stirred for 30 min to modify the carboxyl ligands of HA. Then 5 mL (2×10^{-6} M) of the cysteine-capped CdTe@CdS core@shell QD solution was added to the activated HA solution, which was stirred for 6 h. After reaction, the solution was transferred to dialysis tubing (MW=3500) and dialyzed for 12 h to remove excess EDC and NHS. The resulting solution was centrifuged (10000 rpm, 10 min) to get the HA functionalized fluorescent QDs. And the product was redispersed in 5 mL of deionized water as stock solution.

1.3.3 Synthesis of superparamagnetic Fe_3O_4 (Fe_3O_4) NPs

Fe_3O_4 NPs were synthesized according to the literature method.[5] 2.0×10^{-3} mol of $FeCl_3{\cdot}6H_2O$ was dissolved in a mixture of EG and DEG (V_{EG}/V_{DEG}=19/1, and the total volume is 20 mL) in a beaker under magnetic stirring. After 30 min, 5.0×10^{-5} mol of PVP was added to the above solution and the suspension was heated at 120°C. After an hour, 1.8×10^{-2} mol of NaAc was added into the above solution and heating stopped. After vigorous stirring for a further 30 min, the obtained homogeneous solution was transferred to a Teflon-lined stainless-steel autoclave (50 mL volume) and heated at 200°C. After a 12 h reaction, the autoclave was cooled to room temperature. The obtained superparamagnetic Fe_3O_4 NPs were isolated by magnet and were washed three times by ethanol and deionized water.

1.3.4 Synthesis and surface modification of Fe_3O_4@SiO_2 core@shell NPs

Fe_3O_4@SiO_2 NPs were prepared through the hydrolysis of TEOS, using sol-gel method.[6] The details were follows: all of the obtained Fe_3O_4 NPs were redispersed in a solution containing 20 mL of ethanol and 1.0 mL of deionized water. The mixture was ultrasonicated for 15 min, prior to the addition of 1.0 mL of ammonia hydroxide solution (25 wt%). After ultrasonic treatment for another 15 min, an ethanolic solution of TEOS (0.1 mL) was added into the above dispersion, the reaction was performed for another 1.5 h ultrasonic treatment to get Fe_3O_4@SiO_2 NPs. Then the products were collected with the help of a magnet and washed three times with ethanol and deionized water.

The APTES as silane coupling agent was used to covalently couple with the silica surface of the Fe_3O_4@SiO_2 NPs to get Fe_3O_4@SiO_2@NH_2 NPs. At first, all of the obtained Fe_3O_4@SiO_2 NPs were redispersed in 10 mL of ethanol. After ultrasonic treatment for 15 min, 1.0 mL of ammonia solution (25 wt%) was added into the reaction mixture. After ultrasonic treatment for another 15 min, 0.2 mL of APTES was added to the mixture. And then the solution was subjected to ultrasonic treatment for 1h. The Fe_3O_4@SiO_2@NH_2 precipitant was separated with a magnet and washed with ethanol three times in order to remove the excessive coupling agent. Finally, the obtained Fe_3O_4@SiO_2@NH_2 precipitant was redispersed in 10 mL of deionized water for further usage.

1.3.5 Synthesis of Ag NPs

Ag NPs were synthesized according to the literature.[7] All glassware used in the following procedures were cleaned in a bath of freshly prepared 3:1 HCl: HNO_3 (aquaregia) and rinsed thoroughly in deionized water prior to use. In a typical synthesis, 5.0×10^{-5} mol of $AgNO_3$ was dissolved in 100 mL of deionized water and brought to boiling under vigorously stirred. 6 mL of 1% (wt%) sodium citrate solution and 4 mL of 0.1 M ascorbic acid were rapidly added, respectively. The solution was kept on boiling for 5 min and then cooled to room temperature.

1.3.6 Synthesis and surface modification of Ag@SiO_2 core@shell NPs

The freshly prepared Ag NPs (10 mL) were centrifuged at 10000 rpm for 20 min. The precipitate was redispersed

in 2.5 mL of an ethanolic solution of TEOS (15μL). Then 25 μL of a 0.1 M NaOH solution was added under vigorous stirring. Subsequently, it was allowed to gentle stir for 3 h to get $Ag@SiO_2$ NPs.

The as-prepared $Ag@SiO_2$ NPs (4 mL) were centrifuged at 8000 rpm for 20 min, and the precipitate was redispersed in 5 mL of ethanol. Then 20 μL of 0.50 vol% APTES in ethanol was added under vigorous stirring. The mixture was allowed to react for 3 h. Then the resulting solution was centrifuged (8000 rpm, 10 min) to get $Ag@SiO_2@NH_2$ NPs. And the product was redispersed in 1 mL of deionized water as stock solution.

1.4 The fabrication of the magnetic-semiconductor-metal NPs' assemblies based system and HAase assay

1.4.1 Preparation of the hybrid (SMNP-QDs) composites

The hybrid (SMNP-QDs) composites, in which, SMNP refer to superparamagnetic Fe_3O_4 NPs, were fabricated by two-step conjunction. First step is using the method described above to get the HA functionalized fluorescent QDs.[4] And the second step is followed by: HA functionalized fluorescent QDs (1 mL) were added to 1mL of PBS buffer solution (10 mM, pH 6.2). Then 2.0×10^{-5} mol of EDC and 8.0×10^{-5} mol of NHS were added to the above solution, and the mixture was incubated at room temperature for 1 h to activate the surface carboxyl groups. Afterwards, 20 μL of the SMNP solution was added. After reaction at 37°C for 1 h, the obtained hybrid composites were isolated by external magnetic field and were washed three times with deionized water.

1.4.2 HAase assay

HAase dissolved in 1.0 mL of a phosphate buffered saline solution (PBS, 10 mM, 0.15 M NaCl, pH 6.2) of varying concentrations (0-1.0 mg mL^{-1}) were incubated with the hybrid (SMNP-QDs) composites at 37°C for 55 min. Then, the fluorescence spectra of the reaction solutions were measured after magnetic separation at room temperature. For plasmon enhanced fluorescence, 50 μL of $Ag@SiO_2@NH_2$ NPs were added to the solution above, and the fluorescence spectra of the solutions were measured after 5 min incubation.

1.4.3 Determination of HAase in human serum and urine samples by our method

The serum samples were obtained from school infirmary and urine samples were obtained from four healthy people from our lab. The determination of HAase level in human biological fluids (serum and urine) was performed as follows:

For urine samples, 200 μL of each urine sample was mixed properly with 30 mg of chitosan and then centrifuged at 3000 rpm for 10 min (according to the literatures,[8] chitosan was used to agglomerate all negatively charged moieties in urine samples without adsorbing HAase molecules). And then, 0.1 mL of urine supernatant was dissolved in 999.9 mL of deionized water. Then, 0.1 mL of the diluted urine was dissolved in 0.9 mL of a phosphate buffered saline solution (PBS, 10 mM, 0.15 M NaCl, pH 6.2), and then incubated with the (SMNP-QDs) composites at 37°C for 55 min. The fluorescence spectra were measured after magnetic separation at room temperature. And then 50 μL of $Ag@SiO_2@NH_2$ NPs were added to the solution above. The fluorescence of the reaction solution was measured after 5 min. For recovery experiments, was used to the detection of HAase in human urine samples. 50 μL of 0.55 ng mL^{-1} standard HAase was added to reaction solution above with a 55 min incubation time. The fluorescence of the reaction solution was measured. The final concentration of the added standard HAase was 0.5 ng mL^{-1}.

For each serum sample, which was centrifuged at 3000 rpm for 10 min, and then, 0.1 mL of serum supernatant was dissolved in 599.9 mL of deionized water. Then, 0.1 mL of diluted serum was dissolved in 0.9 mL of a phosphate buffered saline solution (PBS, 10 mM, 0.15 M NaCl, pH 6.2), and then incubated with the (SMNP-QDs) composites at 37°C for 55 min. The fluorescence spectra were measured after magnetic separation at room temperature. And then 50 μL of $Ag@SiO_2@NH_2$ NPs were added to the solution above. The fluorescence of the reaction solution was measured after 5 min. For recovery experiments, was also used to the detection of HAase in human serum samples. 50 μL of 0.55 ng mL^{-1} standard HAase was added to reaction solution above with a 55

min incubation time. The fluorescence of the reaction solution was measured. The final concentration of the added standard HAase was 0.5 ng mL^{-1}.

1.5 Determination of HAase in human serum and urine samples by ELISA

The concentrations of HAase in human biological fluids (serum and urine) were also determined by measuring the absorbance values at 450 nm using a commercial ELISA kit.[9] First, the standard curve (OD=4.04×[HAase]+0.11, R=0.995) was obtained following the direction of the kit in the concentration range of HAase from 0 to 1600 U mL^{-1}. Then, a human sample (50 μL) and horseradish peroxidase-conjugate reagent (100 μL) were added to the ELISA kit wells. After incubation at 37°C for 60 min, all the samples were washed five times with 400 μL of wash solution, followed by the addition of 50 μL of chromogen solution A and 50 μL of chromogen solution B to each well. The reaction mixture was incubated at 37°C for 15 min in the dark. Finally, 50 μL of the stop solution was added to each well to stop the reaction, and the optical density (OD) was read immediately on the microtiter plate reader within 15 minutes (BIO-TEK Synergy HT, U.S.A.) at 450 nm.

2. Supplementary Data

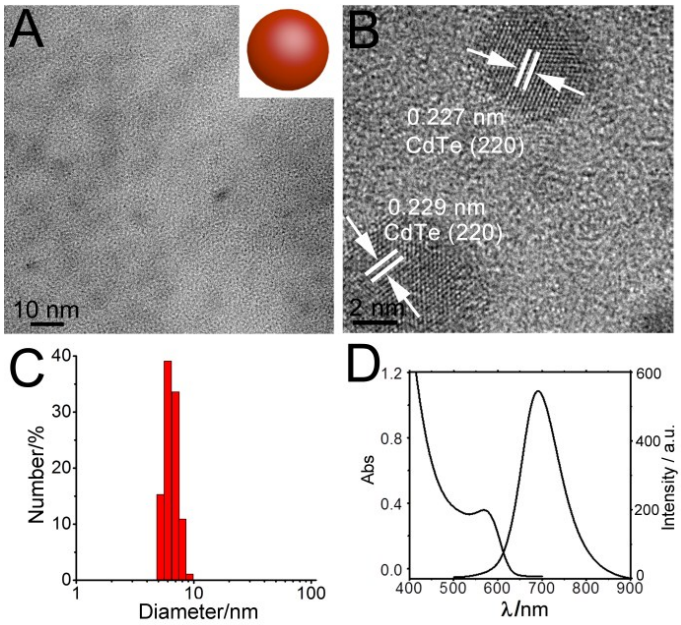

Fig. S1 TEM (A) and HRTEM (B) images, DLS size (C), absorption and fluorescence spectra (D) of the CdTe@CdS core@shell QDs ($\lambda_{ex/em}$=380/690 nm). The average diameter of the QDs is about 5-8 nm.

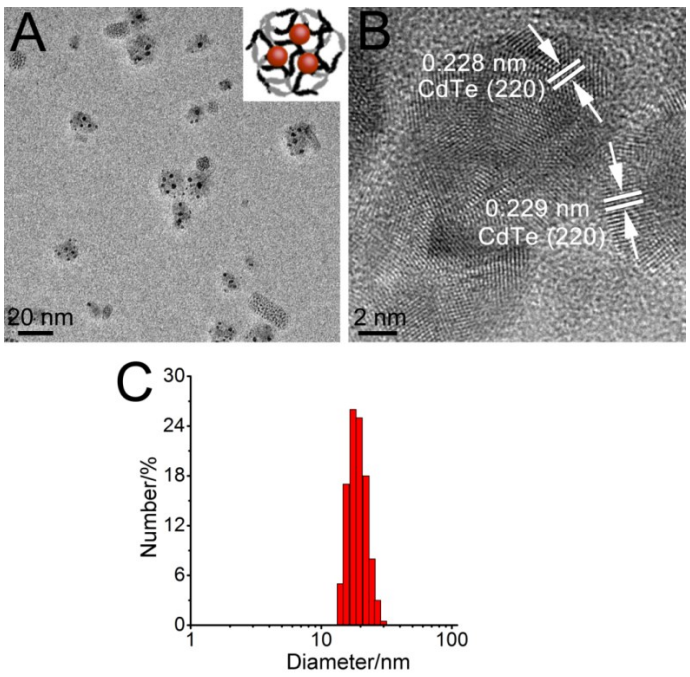

Fig. S2 TEM (A) and HRTEM (B) images and DLS size (C) of the HA functionalized fluorescent QDs. The average diameter of the HA functionalized fluorescent QDs is about 15-20 nm.

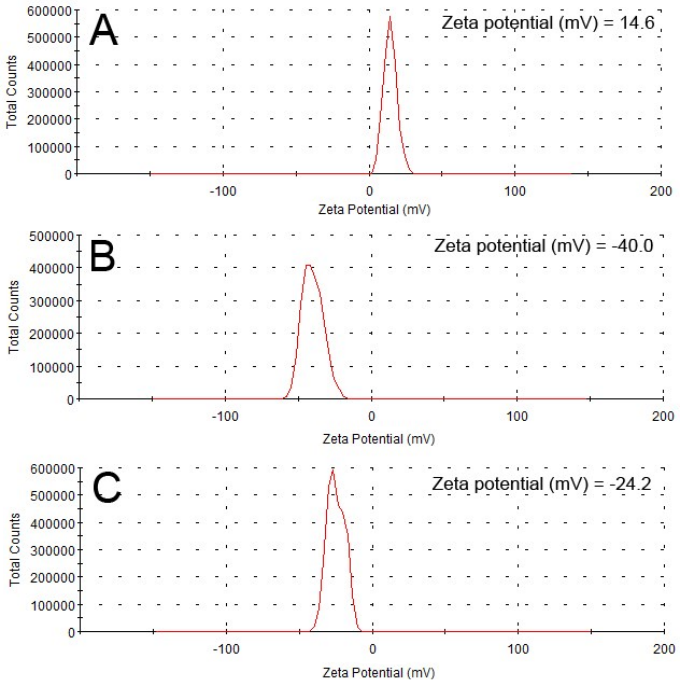

Fig. S3 Zeta potential values of the cysteine modified QDs (A), the HA functionalized fluorescent QDs (B) and the QDs broken away from the SMNPs' surface by 1.0 mg mL^{-1} HAase (C). The pH values are 5.0, 6.2, and 6.2, respectively. After modification and conjugation, the zeta potential values of the QDs change from +14.6 to -40.0 mV, and then change to -24.2 mV after addition of 1.0 mg mL^{-1} HAase.

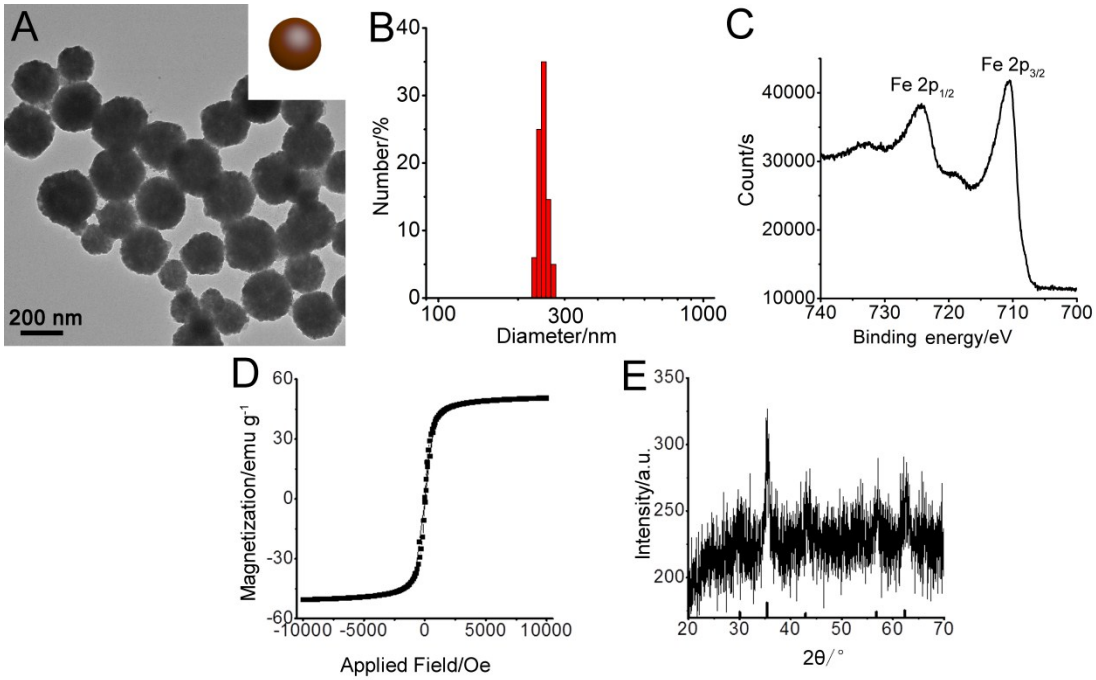

Fig. S4 TEM image (A), DLS size (B), XPS (C), magnetic hysteresis loop (D) and powder XRD pattern (E) of the Fe_3O_4 NPs. The XPS shows peaks at 711.8 and 724.8 eV, which are the characteristic of Fe_3O_4 and in good agreement with the known values of the $Fe2p_{3/2}$ and $Fe2p_{1/2}$ oxidation states, respectively; Magnetic hysteresis loop shows that the Fe_3O_4 NPs are superparamagnetic at room temperature and the saturation magnetization value of Fe_3O_4 NPs is 50.2 emu g^{-1}. The magnetic characterization was carried out at 300 K; The peaks at 30.09, 35.42, 43.05, 56.94, and 62.52° well match the (220), (311), (400), (511), and (440) reflections of Fe_3O_4. (JCPDS no. 19-0629). The average diameter of the Fe_3O_4 NPs is about 250-260 nm.

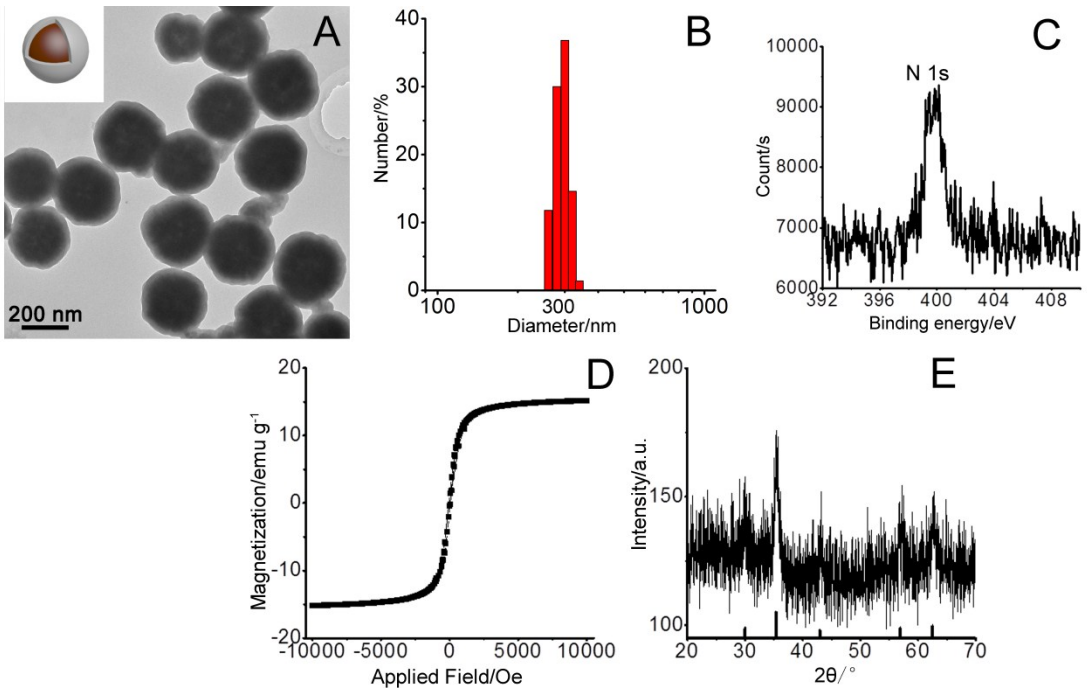

Fig. S5 TEM image (A), DLS size (B), XPS (C), magnetic hysteresis loop (D) and powder XRD pattern (E) of Fe_3O_4@SiO_2@NH_2 NPs. The XPS shows peak at 400.1 eV, which are in good agreement with the known values of the N1s. The result further confirms that the APTES have been successfully bound the surface of nanoparticles; The magnetic hysteresis loop clearly demonstrates that the nanoparticles show superparamagnetic behavior at room temperature and the saturation magnetization value of Fe_3O_4 NPs is 15.2 emu g^{-1}. The magnetic characterization was carried out at 300 K; Fe_3O_4@SiO_2@NH_2 exhibits similar diffraction patterns with Fe_3O_4. The average diameter of the Fe_3O_4@SiO_2@NH_2 NPs is about 290-310 nm.

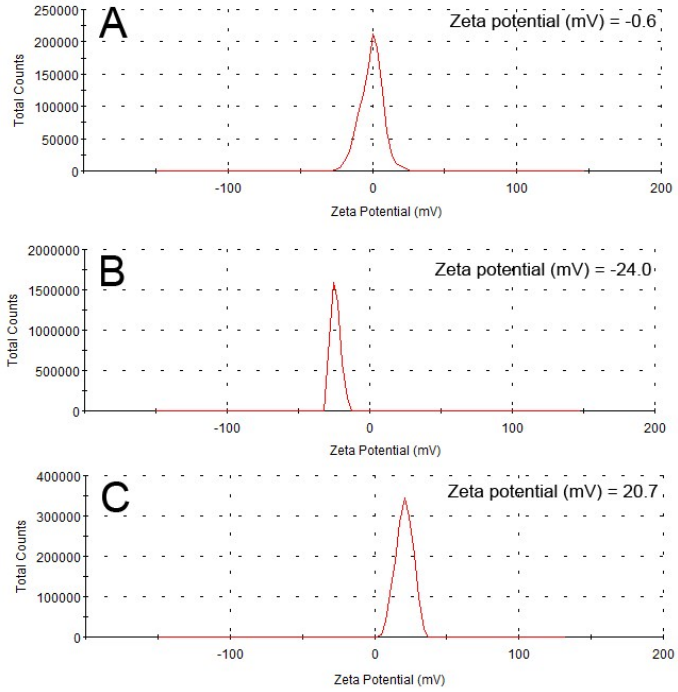

Fig. S6 Zeta potential values of the Fe_3O_4 NPs (A), Fe_3O_4@SiO_2 NPs (B) and Fe_3O_4@SiO_2@NH_2 NPs (C). After SiO_2 coating, the zeta potential values of the Fe_3O_4 NPs changed from -0.6 to -24.0 mV, which further reached to +20.7 mV after amine introduction.

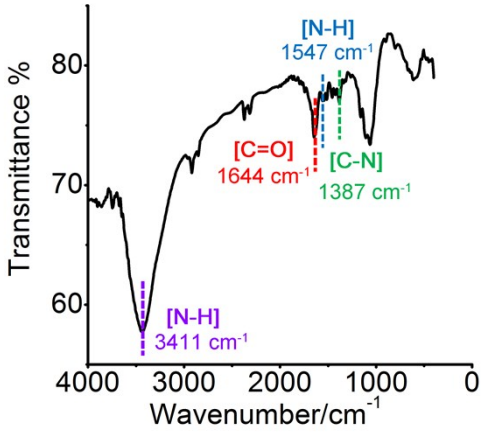

Fig. S7 The FT-IR spectrum shows the typical amide bond signals including the amide I band (1644 cm^{-1}) and amide II band (1547 cm^{-1}) as well as stronger N–H stretching vibration (3411 cm^{-1}). And the absorption band at 1387 cm^{-1} can be assigned to the C-N stretching vibration. The sample was mixed with KBr powder and then made into pellet under high pressure. Pure KBr acted as blank. The average diameter of the hybrid composites is about 310-330 nm.

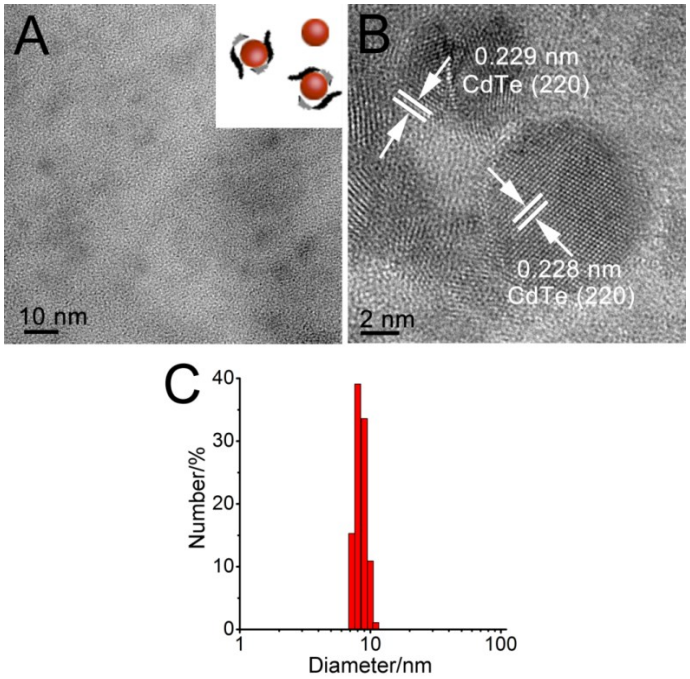

Fig. S8 TEM (A) and HRTEM (B) images and DLS size (C) of the QDs broken away from the SMNPs' surface by HAase. The diameter of the QDs is about 5-8 nm.

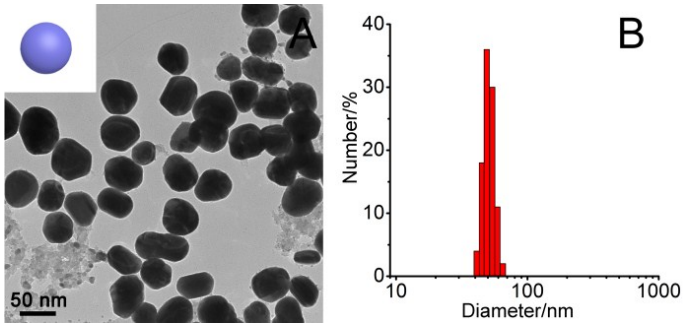

Fig. S9 TEM image (A) and DLS size (B) of the Ag NPs. The diameter of the Ag NPs is about 45-55 nm.

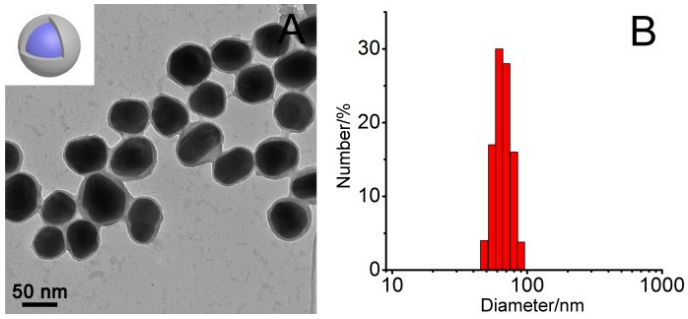

Fig. S10 TEM image (A) and DLS size (B) of the $Ag@SiO_2@NH_2$ NPs. The diameter of the $Ag@SiO_2@NH_2$ NPs is about 60-70 nm.

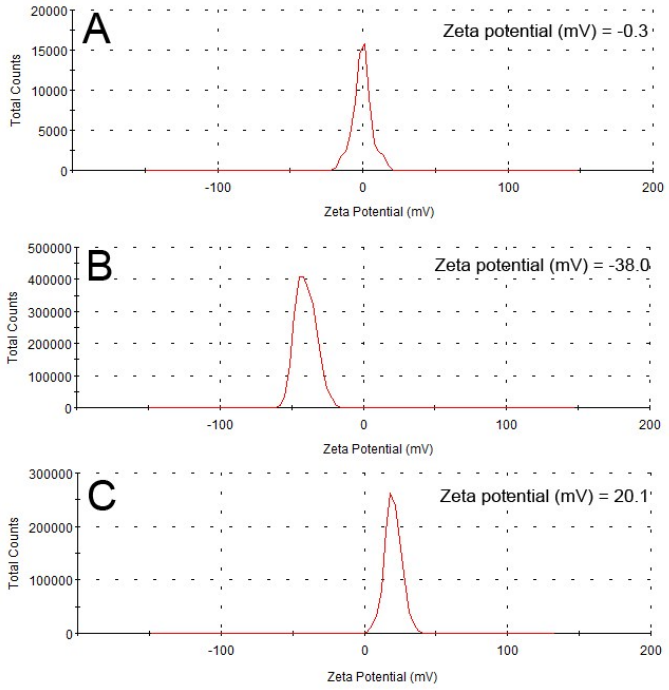

Fig. S11 Zeta potential values of the Ag NPs (A), Ag@SiO_2 NPs (B) and Ag@SiO_2@NH_2 NPs (C). After SiO_2 coating, the zeta potential values changed from -0.3 to -38.0 mV, which further changed to +20.1 mV after amine functionalization.

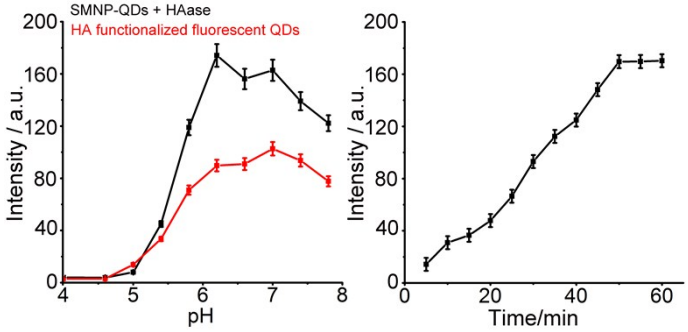

Fig. S12 (A) Effect of pH on the fluorescence of the HA functionalized fluorescent QDs and the hybrid (SMNP-QDs) composites with 1.0 mg mL^{-1} HAase in PBS buffer (pH 6.2, 0.01 M) at 37°C. (B) Effect of incubation time on the fluorescence of the hybrid (SMNP-QDs) composites reacting with 1.0 mg mL^{-1} HAase in PBS buffer (pH 6.2, 0.01 M) at 37°C.

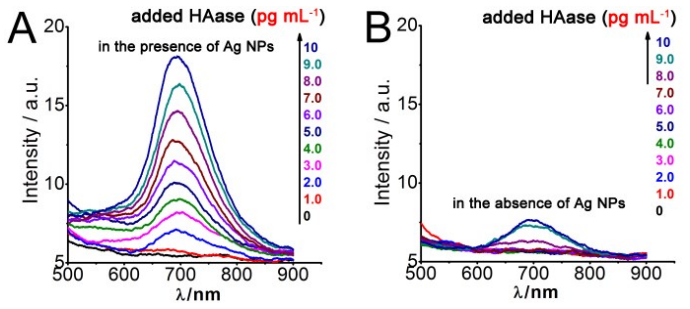

Fig. S13 Fluorescence emission spectra the hybrid (SMNP-QDs) composites with increasing HAase (0-10.0 pg mL^{-1}) in the presence (A) and absence (B) of the Ag NPs.

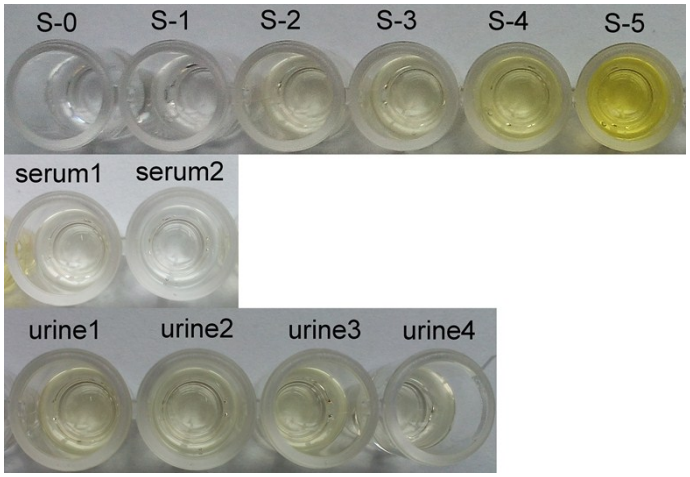

Fig. S14 The photograph of HAase assay with ELISA kit. The concentrations of standards (S-0, S-1, S-2, S-3, S-4, S-5) are followed by: 0, 100, 200, 400, 800, 1600 U L^{-1}.

Table S1 Comparison of analytical parameters between our method and some other fluorescent nanosystems for HAase sensing

Probes	LOD[a]	Linear range	Response mode	Real samples	Refs.
MoS_2 QDs/HA-AuNPs	1.75 ug mL^{-1}	2.5-125 ug mL^{-1}	off-on	serum and urine	10
CD/HA-AuNP	0.15 ug mL^{-1}	0.25-200 ug mL^{-1}	on	serum	11
AN-N^+/TPE-$2N^+$	0.81 mg mL^{-1}	2.38-95.2 mg mL^{-1}	/[b]	serum and urine	8*b*
AuNPs-HA-CV-porphyrin	1.5 ug mL^{-1}	20-120 ug mL^{-1}	off-on	/	12
HA/N-Py	14.7 ng mL^{-1}	/	/	/	13
SMNP-QDs-AgNPs	0.4 pg mL^{-1}	0-0.12 ng mL^{-1}	beyond turn-on	serum and urine	This work

[a] LOD, refer to limit of detection. [b] /, refer to not available.

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