Electronic Supplementary Material (ESI) for Nanoscale.
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Supporting Information for

Construction of antibody-like nanoparticles for selective protein sequestration in living cells

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Materials and methods

Materials

Amino-functionalized superparamagnetic Fe_3O_4 nanoparticles were purchased from Beijing Nachen S&T Ltd (Beijing, China). 4,4'-Azobis-(4-cyanopentanoic acid) (ACPA), N-isopropyl acrylamide (NIPAm), methacrylamide (AAm), Streptolysin O (SLO), poly(ethylene glycol) methyl ether (2-methyl-2-propionic acid dodecyl trithiocarbonate)(PEG Macro-CTAs, average Mn 10 kDa, n~227) and Tris base were purchased from Sigma-Aldrich (Steinheim, Germany). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), Fluorescein O-methacrylate(FMA) and N-[3-(dimethylamino)propyl]methacrylamide (DMAPMA)were obtained from Alfa-Aesar (MA, USA). N-Hydroxysuccinimide (NHS) and N, N'-methylene bisacrylamide (BIS) were bought from Fluka (Buchs, Switzerland). Acrylicacid (AAc) and dimethyl sulfoxide (DMSO) were purchased from Beijing Chemical Works (Beijing, China). The fluorescent oligonucleotide probes and deoxyribonuclease I (DNase I, from bovine pancreas) were supplied by China Sangon Company (Shanghai, China). Exonuclease III (Exo III) was purchased from New England Biolabs (London, UK). 1× DNase I buffer consists of 10 mM Tris-HCl (pH 7.6), 2.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$. Hoechst 33342 and cell-counting kit (CCK-8) were obtained from Dojindo Laboratories (Kumamoto, Japan).Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's phosphate buffer solution without calcium & magnesium (DPBS) were purchased from Corning (Manassas, VA, USA). Human cervical carcinoma cell line（HeLa)was purchased from ATCC (Manassas, VA, USA).

Synthesis of surface-imprinted Fe_3O_4@MIP NPs(ANP1)without the addition of PEG Macro-CTAs

Amino-functionalized superparamagnetic Fe_3O_4NPs (100mg) were first dispersed in 50 mL of DMSO by ultrasonication for 10 min. To attach ACPA to the surface of Fe_3O_4 NPs, 100 mg of ACPA (0.357 mmol) were mixed with 41.0 mg of NHS (0.357mmol)and 171 mg of EDC (0.893 mmol) (molar ratio, ACPA: NHS: EDC = 1:1:2.5) in 50 mL of DMSO by stirring for 0.5 h, and then added to the above DMSO suspension containing Fe_3O_4NPs. The mixture was further stirred in dark for12 h at room temperature. The resultant Fe_3O_4@ACPANPs were collected by magnetic separation, washed twice with ethanol and dried overnight under vacuum.

To synthesize the surface-imprintedFe_3O_4@MIP NPs,6.2 mg (0.2 μmol)of the template DNase I, 182 μL (0.96 mmol)DMPMA, 226 mg (2.0 mmol)NIPAm, 66 μL (0.96 mmol) AAc, 12.3 mg (0.08 mmol) BISand 0.2 mL isopropanol were mixed in 100 mLof PB (10 mM, pH=7.6), to which 100 mg of Fe_3O_4@ACPA NPswere added and the resultant solution was degassed for 20 min. The polymerization

was initiated with an ultra-high intensity UV lamp (MAXIMA ML 3500C/F)at room temperature under stirring at 300 rpm for 30 min. The distance between the lamp and the quartz flasks was fixed at 5 cm. After the reaction, the magnetic NPs were isolated from the mixture by using a magnet and washed with 1 M NaCl at 30 °C under ultrasonication.

The corresponding non-imprinted control NPs (CP1)were synthesized following the same procedures as above but without addition of the DNase I template.

Synthesis of surface-imprinted Fe_3O_4@MIP NPs (ANP2)with the addition of PEG Macro-CTAs

To synthesize the MIP layer via RAFT polymerization method,80 mg (0.08 mmol) PEG Macro-CTAs was added to 100 mL of afore-mentioned mixture solution containing 6.2 mg DNase I, 182 μL DMPMA, 226 mg NIPAm, 66 μL AAc, 12.3 mg BIS, and 0.2 mL isopropanol in PB (10 mM, pH=7.6). Then 100 mg of Fe_3O_4@ACPA NPswere added and the resultant solution was degassed for 20 min. The polymerization was performed following the same procedures as above. The corresponding non-imprinted control NPs (CP2)were synthesized in the same way without addition of the DNase I template.

Synthesis of surface-imprinted Fe_3O_4@SiO_2@MIP NPs (ANP3 and ANP4)with the addition of PEG Macro-CTAs

Synthesis of Fe_3O_4@ SiO_2magnetic nanoparticles

90 mg of dried magnetic Fe_3O_4 powder was first dispersed in 20 mL of deionized water under ultrasonication and then added dropwisely with intense stirring to a mixture solution containing 462 mL cyclohexane, 120 g triton X-100 and 96 mL hexanol. To themicroemulsion,0.3 mL of TEOS and 0.15 mL of APTS were added. After 0.5 h, 3.6 mL of aqueous ammonia was added to the solution dropwisely. After 12 h, acetone was added to destabilize the microemulsion system. The resultantFe_3O_4@SiO_2NPs were isolated using a magnet and washed by acetone and deionized water alternately, each for 3 times, to remove the surfactant and unreacted reactants. The product was dried overnight under vacuum.

The thickness of the SiO_2layer was optimized by varying the amount of TEOS and APTS in the reaction solution. Typically, 0.3 mL of TEOS and 0.15 mL of APTS would result in a SiO_2 layer of about 10 nm thickness, while 0.9 mL of TEOS and 0.45 mL of APTS would form a SiO_2 layer of about 20 nm thickness.

Covalent immobilization of ACPA on the surface of Fe_3O_4@ SiO_2NPs

To attach ACPA to the surface of Fe_3O_4@SiO_2NPs, 100 mg of ACPA (0.357 mmol), 41.0 mg of NHS (0.357 mmol)and 171 mg of EDC (0.893 mmol) (molar ratio, ACPA: NHS: EDC = 1:1:2.5) were mixed

in 50 mL of DMSO and stirred for 0.5 h. Then the mixture solution was added to a separate flask containing 100 mg of Fe_3O_4@SiO_2NPs in 50 mL of DMSO suspension. The mixture was stirred in dark for12 h at room temperature. The resultant Fe_3O_4@SiO_2@ACPANPs were collected by magnetic separation, washed twice with ethanol and dried overnight under vacuum.

Synthesis of surface-imprinted Fe_3O_4@SiO_2@MIP NPs using DNase I as the template

To synthesize the MIP layer over the surface of Fe_3O_4@SiO_2 NPs, 6.2 mg (0.2 μmol) of the template DNase I, 182 μL (0.96 mmol) DMPMA, 226 mg (2.0 mmol) NIPAm, 66 μL (0.96 mmol) AAc, 12.3 mg (0.08 mmol) BIS, 80 mg (0.08 mmol) PEG Macro-CTAs and 0.2 mL isopropanol were mixed in 100 mL of PB (10 mM, pH=7.6), to which 100 mg of Fe_3O_4@SiO_2@ACPA NPs were added and the resultant solution was degassed for 20 min. The polymerization conditions were the same as above. The corresponding non-imprinted control NPs (CP3 andCP4) were synthesized in the same way in the absence of the template DNase I.

Dynamic light scattering (DLS) and zeta potential measurements

The average hydrodynamic sizes of the obtained Fe_3O_4@SiO_2@MIP NPs were determined by DLS measurements using a particle-size analyzer from Brookhaven Instruments Ltd. (U.S.A) at 25 °C and a scattering angle of 90°. The NPs were dispersed in 10 mM PB (pH=7.6) by ultrasonic treatment for 10 min. Approximately 2.5 mL of the obtained homogeneous solution (0.1 mg/mL) was transferred to a 4.5 cm^3 disposable polystyrene cuvette for DLS measurements. Zeta potential of the Fe_3O_4@SiO_2@MIP NPs were measured using a zeta PALS (Brookhaven Instruments, U.S.A.). The measurements were performed for at least three times.

Transmission electron microscopy (TEM) imaging

Samples were dissolved in water at a concentration of 0.1 mg/mL and ultrasonicated for 10 min. Then a drop of the sample solution was placed on a carbon-coated copper grid and dried in air for at least 24 h. TEM measurements were performed using a Tecnai G2 T20 Transmission Electron Microscope at an accelerating voltage of 100 kV.

Enzyme activity assay

Fluorescent oligonucleotide probes (see Table S2) were employed for determination of the enzyme activity. All in-vitro enzymatic reactions were carried out in 50 μL sealed tubes and monitored by real-time PCR (Stratagene Mx3000P, USA).

For DNase I detection, 5 μL of 10×DNase I buffer, 2 μL of 2 μM TAMRA-*Probe-D*solution and a proper amount of the tested enzyme solution were added to the tube, and the total volume of the solution was brought up to 50 μL by distilled water. The thermal program was 250 cycles at 37°C with 5 s per cycle, and fluorescence was measured at the end of each cycle with excitation and emission wavelengths at 556 and 580 nm, respectively. All the experiments were performed in triplicate.

For 3'-Exo detection, 2 μL of 2 μM FAM-*Probe-E* was used and the fluorescence intensity was measured with excitation and emission wavelengths at 492 and 516 nm, respectively. Other experimental conditions are the same as above.

Binding of enzymes by ANPs or CPs

48.0 μL of the tested ANPs (1 mg/mL) were incubated with 2 μL of 100 μg/mL DNase I or 3'-Exo at 25°C for 15 min. After isolation of the ANPs with an external magnet, the enzyme activity in the supernatant was determined by using the corresponding fluorescent oligonucleotide probes. Similar experiments were also carried out by using CPs for comparison.

Measurement of the non-specific adsorption of fluorescent probes on the ANPs

100 pmol of TAMRA-*Probe-D* was incubated with each tested ANPs solutions atdifferent concentrations(0.1-1.0 mg/mL) in 10 mM PB (pH=7.6) at 25°Cfor 15 min. After isolation of the ANPs with an external magnet, the amount of probe remaining in the supernatant was determined. A proper amount of the supernatant was mixed with 1 μL of 10 μg/mL DNase I and 5 μL of 10×DNase I buffer in the tube, and then the total volume of the solution was brought up to 50 μL by distilled water. The thermal program was 250 cycles at 37°C with 5 s per cycle, and fluorescence was measured at the end of each cycle with excitation and emission wavelengths at 556 and 580 nm, respectively.

Direct detection of the enzyme activity in the solutions in the presence of ANPs/CPs

For DNase I detection, 5 μL of 1 mg/mL ANP4 or CP4, 5 μL of 10×DNase I buffer and 1 μL of 10 μg/mL DNase I were added to the tube, and the total volume of the solution was brought up to 48 μL by distilled water. The mixture solution was allowed to stand for 15 min at 25°C, and then 2 μL of 2 μM TAMRA-*Probe-D* was added. Then the fluorescence was measured under the same conditions as above.

For 3'-Exo detection, 1μL of 10 μg/mL Exo III and 2 μL of 2 μM FAM-*Probe-E* were used, respectively.

Cell culture and intracellular uptake of the Fe_3O_4@SiO_2@MIP ANPs

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% Penn/Strep, 10%fetal bovine serum and incubated at 37 °C in a humidified atmosphere of 5% CO_2 /95%air. The cells were grown to 70-80% confluency, treated with 0.25% trypsin for 4 min at37 °C. The attached cells were trypsinized and moved to a 96-well, flat-bottomed plates (Costar, USA) for fluorescence imaging. To each well (0.3 mm^3), 0.1 mL solution containing about 10^4 cells was added. The plates were incubated at 37 °C for 24 h to allow the cells to adhere to the bottom of the well. Subsequently, after washing with Dulbecco's phosphate buffer solution without calcium & magnesium (DPBS), the cells were incubated in 100 μL of 10 mM PB (pH=7.6) containing 100 μg/mL ANPs at37 °C for30 min under 5% CO_2 on MagnetoFACTOR-96 plate (Chemicell, Berlin, Germany). Then the plate was removed and the cells were washed with DPBS sufficiently to remove excess NPs. Fluorescent labeled NPs uptaken by HeLa cells was observed with fluorescence microscope.

Delivery of the fluorescent DNA probes into HeLa cells

Fluorescent DNA probes were delivered into HeLa cells by using a reversible permeabilization approach with streptolysin O (SLO). Adherent cells grown in 96-well plates were incubated at 37 °C with a mixture of 1.6 U/mL SLO (about 0.09 U SLO per 10^4cells), 1.0 μM TAMRA-*Probe-D* and 1.0 μM Cy5-*Probe-E* in 100 μL of DPBS for 5 min. After incubation, the cells were washed with DPBS once and cultured in regular growth medium then incubated at 37 °C for 30 min prior to fluorescence imaging.

Cell viability assay

Cytotoxicity of the ANPs/CPs was assayed by using a Cell Counting Kit-8 (CCK-8) which contains a highly water-soluble tetrazolium salt, WST-8[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5- (2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]. The cells were plated at a density of 10^4 cells per well in a 96-well plate and incubated with fresh serum-free medium containing 0.5 mg/mL WST-8 at 37 °C for 1 h. The assays were carried out according to the manufacturer's instructions. The optical density(OD) value at450 nm of each well, with background subtraction at 690 nm, was measured by microplate reader (GENios, Tecan, Switzerland). Untreated wells were used as control.

To further confirm that the cells are alive after internalization of the ANP5, the cells were stained with Hoechst33342 (25 μg/mL) at 37°C for 20 min and then imaged by fluorescence microscopy.

Fluorescence Imaging

An Olympus IX 71 inverted fluorescence microscope (Japan), equipped with an Evolve-EMCCD

camera (Photometrics, USA), was used for cell imaging. The fluorescence signals were detected by using different filters (see Table S3). The cells in the 96-well plate were observed by a 100× objective of the microscopy. Images were acquired by EMCCD (exposure time 10 ms and EM gain100) and analyzed by ImageJ software.

Enzyme recovery study

To evaluate the recovery capability of the ANP5, 0.5 μg of DNase I was incubated with 0.4 mg of ANP5/CPs in 6 mL of DMEM cell culture solution. Then the mixture solution was tip-sonicated for 4 min (sonicated for 3 s and paused for 5 s repeatedly)to simulate the cell lysis treatment using a JY92-II ultrasonic homogenizer (Ningbo Scientz Biotechnology Co., Ltd., China). The ANP5/CP5 were collected with a magnet and the bound enzymes were released with 10×DNase I buffer. The recoveries of DNase I activity from ANP5/CP5 were measured by using TAMRA-*Probe-D*.

To confirm the intracellular internalization of ANP5 and in-situ binding to target enzymes within the cells, HeLa cells were cultured in T7 cell culture flask for 2 days and grown to 70-80% confluency. The activities of DNase I and 3'-Exo in the culture medium were determined by using the TAMRA-*Probe-D*and the FAM-*Probe-E*, respectively. After washing the cells with DPBS buffer for 3 times, the enzyme activity in the washing buffer was tested again to confirm that no enzymes are present. Then the cells were incubated in 4 mL of 10 mM PB (pH=7.6) containing 100 μg/mL ANP5 at37 °C under 5% CO_2 on MagnetoFACTOR-96 plate. After 30 min, the plate was removed and the cells were washed with DPBS for at least three times to remove excess ANP5 remained in the extracellular medium. The extracellular ANP5 were isolated from the washing buffer by using a magnet and eluted with10×DNase I buffer to test if any enzymes were captured. The attached cells were then trypsinized, and moved to a 10 mL centrifuge tube containing 6 mL of DMEM cell culture solution. The mixture was tip-sonicated for 4 min under the same conditions as above. Microscopy observation was performed to confirm all cells were cracked.ANP5in the cell lysates were isolated by using a magnet and eluted with 10×DNase I buffer. The released enzyme activity was determined by using TAMRA-*Probe-D*and the FAM-*Probe-E*, respectively.

Table S1. DLS diameter and zeta potential of different NPs.

NPs	Particle size (nm)[a]	Polydispersity	Zeta Potential (mV)
Fe_3O_4	571.4±30.5	0.322±0.014	35.49±1.42
Fe_3O_4@ACPA			-11.75±2.08
Fe_3O_4@10 nm SiO_2	195.5±2.8	0.186±0.016	22.71±0.62
Fe_3O_4@10 nm SiO_2@ACPA			-12.36±1.75
Fe_3O_4@20 nm SiO_2	251.1±4.9	0.144±0.070	32.37±1.55
Fe_3O_4@20 nm SiO_2@ACPA			-12.36±2.89
ANP 1	373.5±15.3	0.305±0.010	12.28±0.52
ANP 2	79.0±3.4	0.220±0.017	-25.50±3.14
ANP 3	99.5±1.5	0.152±0.016	-44.36±8.32
ANP 4	93.0±2.6	0.135±0.008	-38.11±9.92

[a]Hydrodynamic size

Table S2. Sequences of the fluorescent oligonucleotide probes used in this work[a].

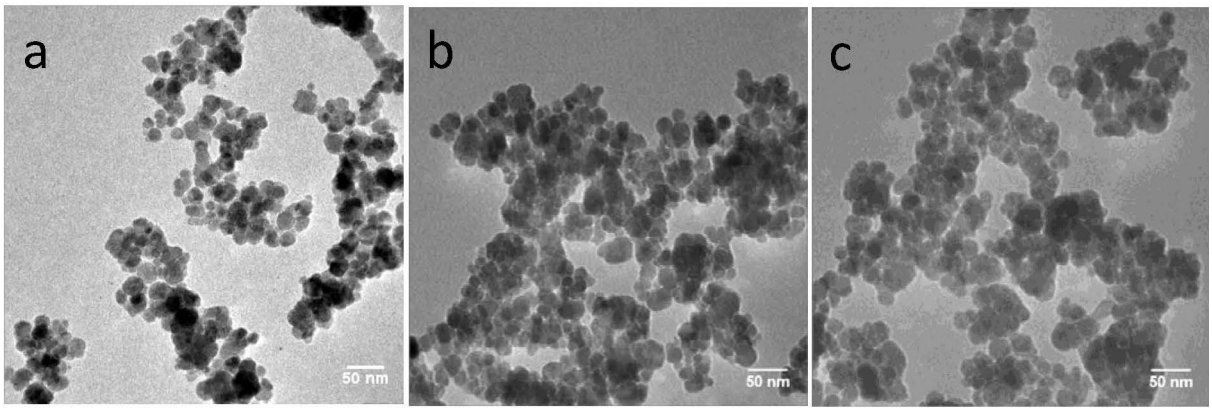
Probe Name	Sequence (5' to 3')
TAMRA-*Probe-D*	*C*A*A*C*(dT*-TAMRA)**ACATC*ACTCG*GATG(dT-BHQ2)*A*G*T*T*G*
FAM-*Probe-E*	*A*T*C*A*T*C*T*T*T*T**A*C*G*C**A*A*G*A*(dT-BHQ1)*G*A*T*-FAM
Cy5-*Probe-E*	*A*T*C*A*T*C*T*T*T*T**A*C*G*C**A*A*G*A*(dT-BHQ2)*G*A*T*-Cy5

[a]The self-complementary parts are underlined and shown in italic. The phosphorothioated nucleotides (at 3′ side) are indicated with an asterisk after the nucleotides. TAMRA is tetramethylrhodamine. FAM is fluorescein. Cy5 is cyanine dye 5. BHQ1 is Black Hole Quencher 1 and BHQ2 is Black Hole Quencher 2.

Table S3. Filters usedin fluorescence microscopy forlive-cell imaging.

Filter Name	Dye	Ex (nm)	Em (nm)	Dm (nm)
NUA	Hoechst 33342	360-370	420-460	400
WIBA	FAM	460-495	510-550	505
WG	TAMRA	510-550	590	570
CY5	Cy5	660	628	692

Supplementary results



Enlarged Fig. 1(a) I and II:

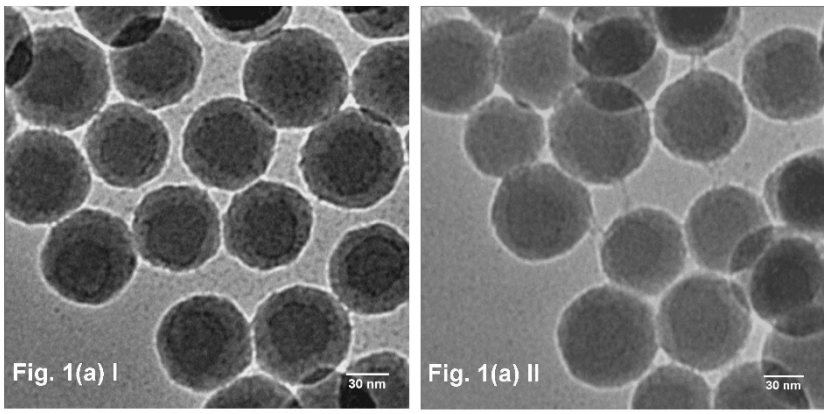


Fig. S1. TEM images of (a) Fe_3O_4; (b) ANP1; (c) ANP2 and enlarged images of Fig. 1(a) I and II.

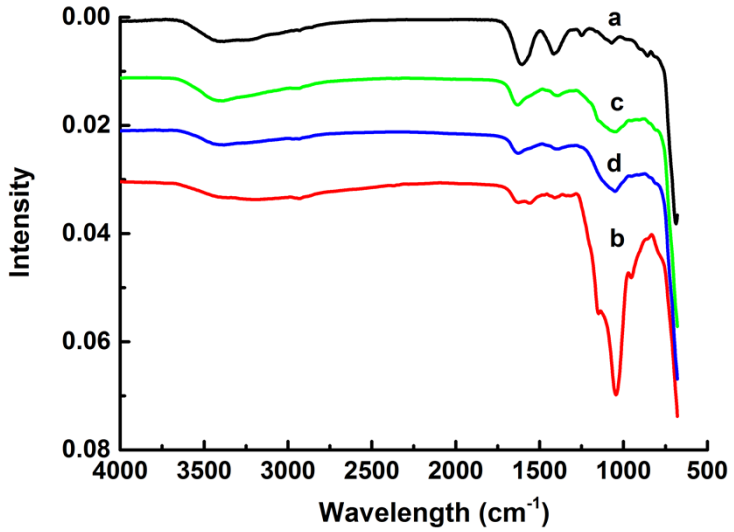


Fig. S2. FT-IR spectra of (a) Fe_3O_4; (b)Fe_3O_4@20 nm SiO_2; (c)ANP4; and (d)CP4.

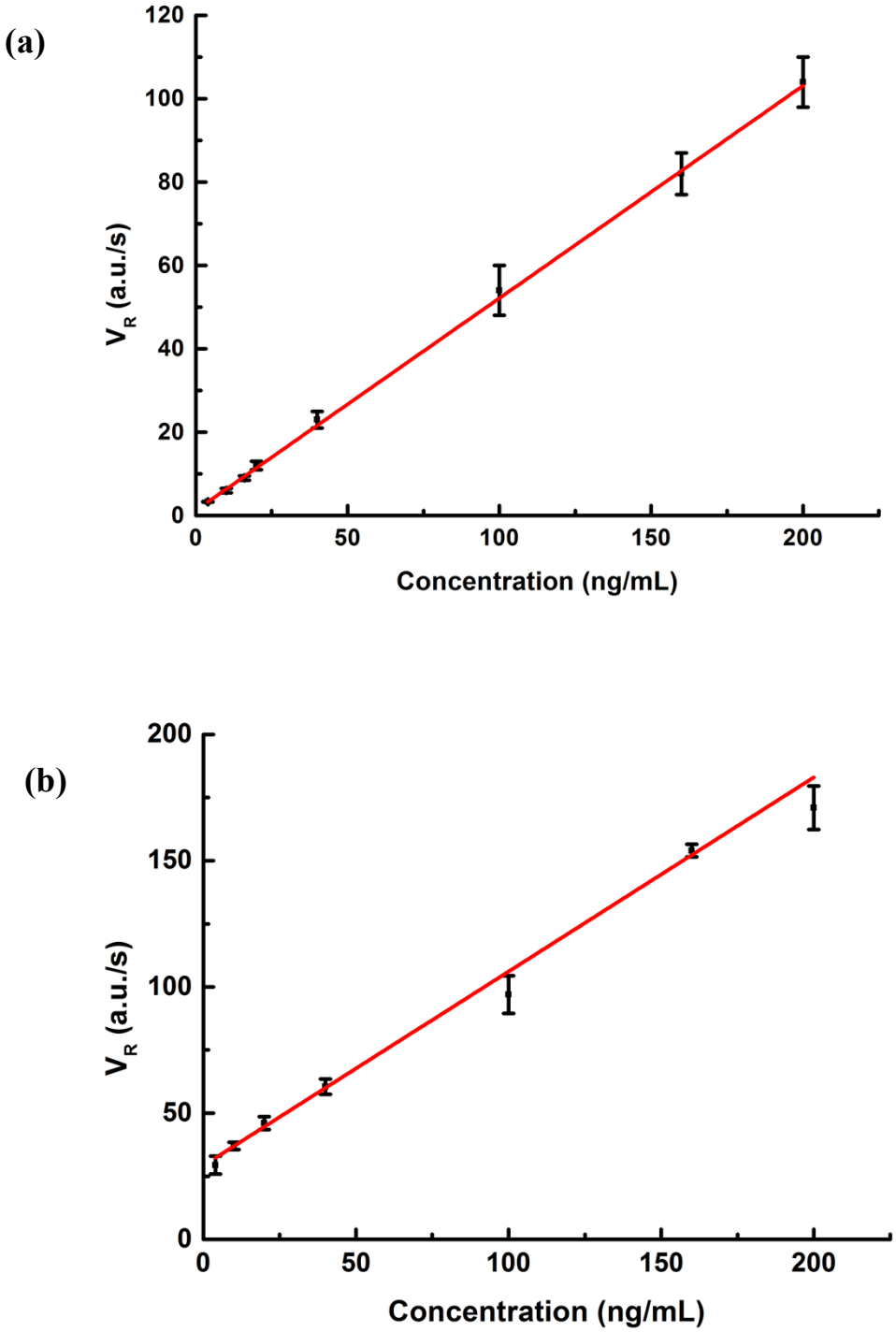


Fig. S3. (a) Calibration curve for quantification of the activity of DNase I. TAMRA-*Probe-D* listed in Table S2 was used for the detection. The linear working range is from 4.0-200 ng/mL. The linear regression equation is y=1.24+0.51x (R^2=0.997); (b)Calibration curve for quantification of the activity of Exo III. FAM-*Probe-E* listed in Table S2 was used for the detection. The linear working range is from 4.0-200 ng/mL. The linear regression equation is y = 0.730x + 29.3 (R^2=0.994).

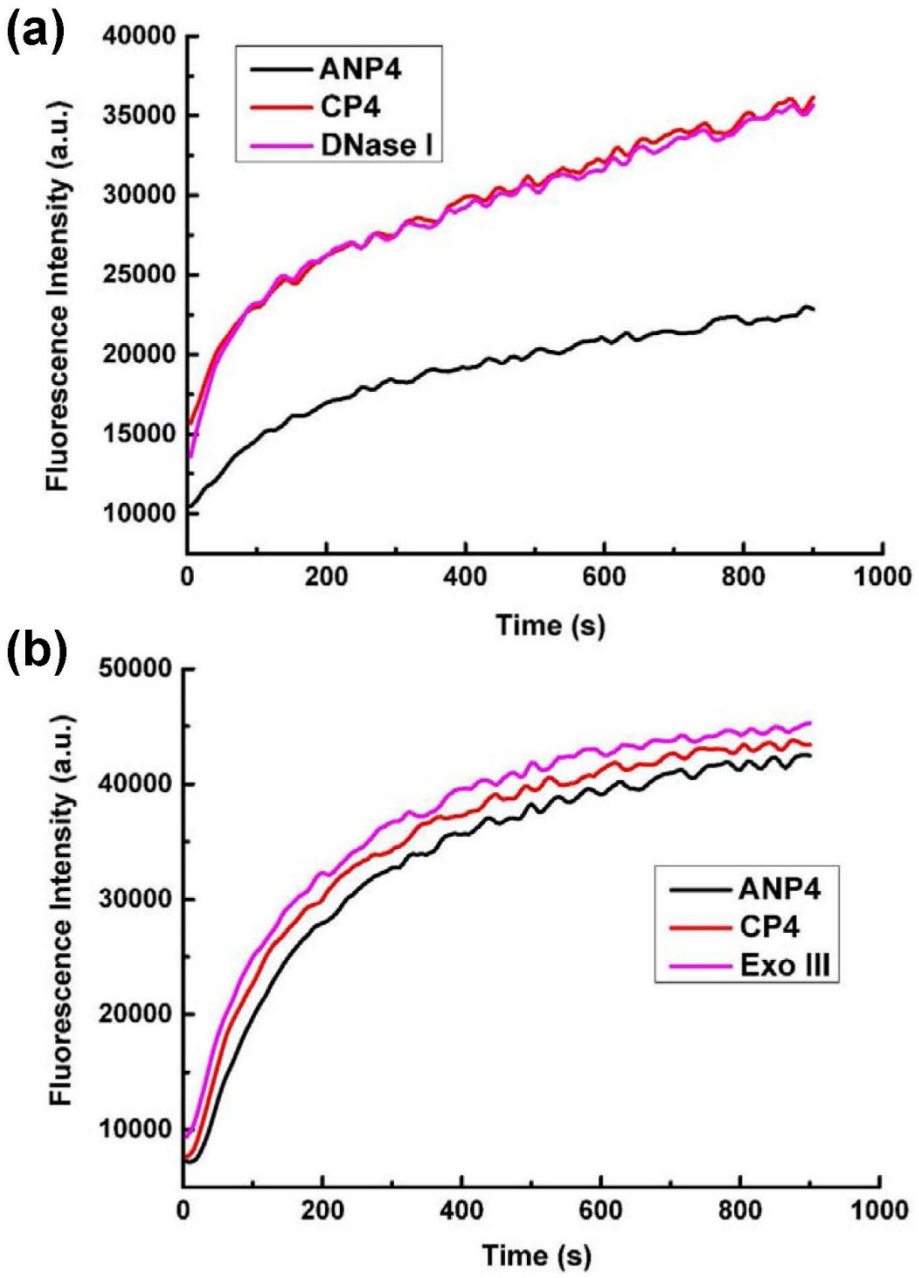


Fig. S4. Time curves for the detection of the activities of DNase I and ExoIII in the presence of ANP4 or CP4. (a) 5 μL of 1 mg/mL ANP4 or CP4, 5 μL of 10×DNase I buffer and 1 μL of 10 μg/mL DNase I were mixed for 15 min at 25°C, then 2 μL of 2 μM TAMRA-*Probe-D* was added. (b) 5 μL of 1 mg/mL ANP4 or CP4, 5 μL of 10×DNase I buffer and 1 μL of 10 μg/mL Exo III were mixed for 15 min at 25°C, then 2 μL of 2 μM FAM-*Probe-E* was added.

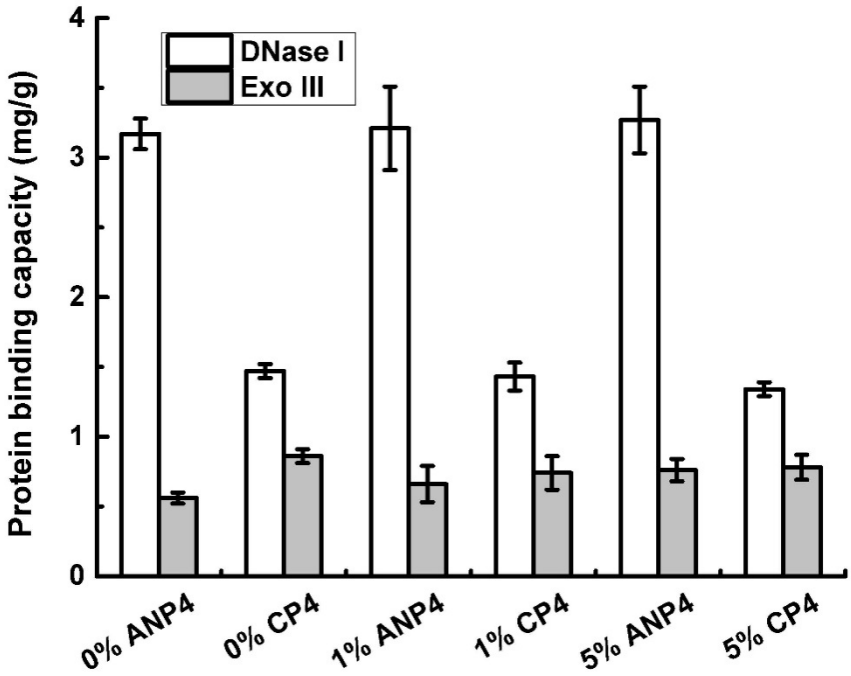


Figure S5. Binding capacities of DNase I and 3'-Exo onANP4 or CP4 containing different amounts of fluorescent functional monomers.

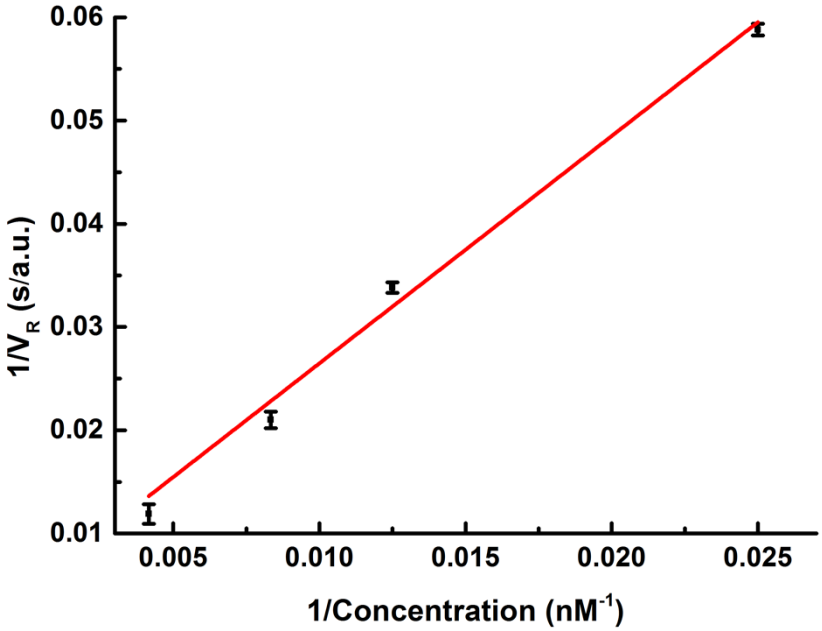


Fig. S6. Calibration curve for quantification of probe concentration. TAMRA-Probe-D listed in Table S2 was used for the test. The linear working range is from 40 nM-200 nM. The linear regression equation is $y = 2.2568x + 0.0032$ (R^2=0.994).

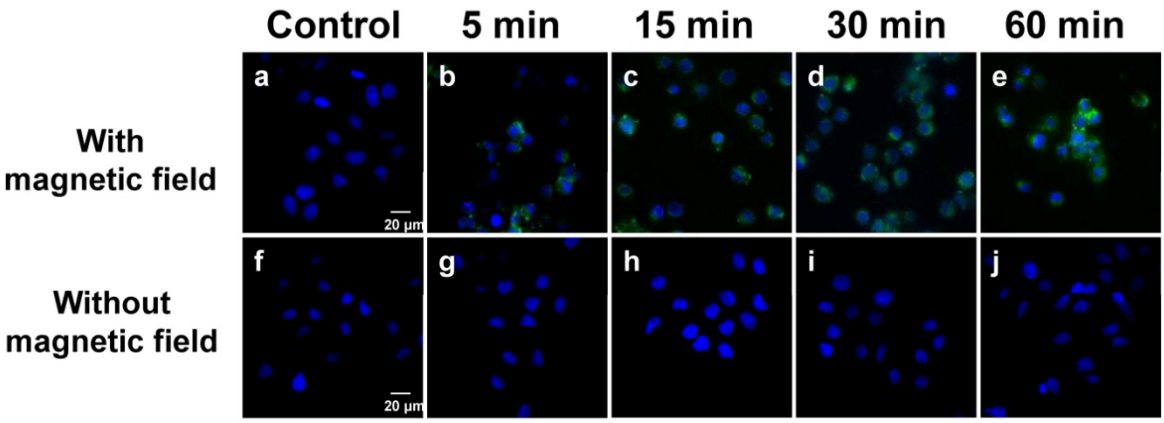


Fig. S7. Fluorescent images of Hela cells incubated with fluorescent ANP5 in the presence (a-e) and absence of a magnetic plate (f-j) for different time period (5 min to 1h). Cell nuclei were stained with Hoechst 33342. Scale bar = 20 μm.

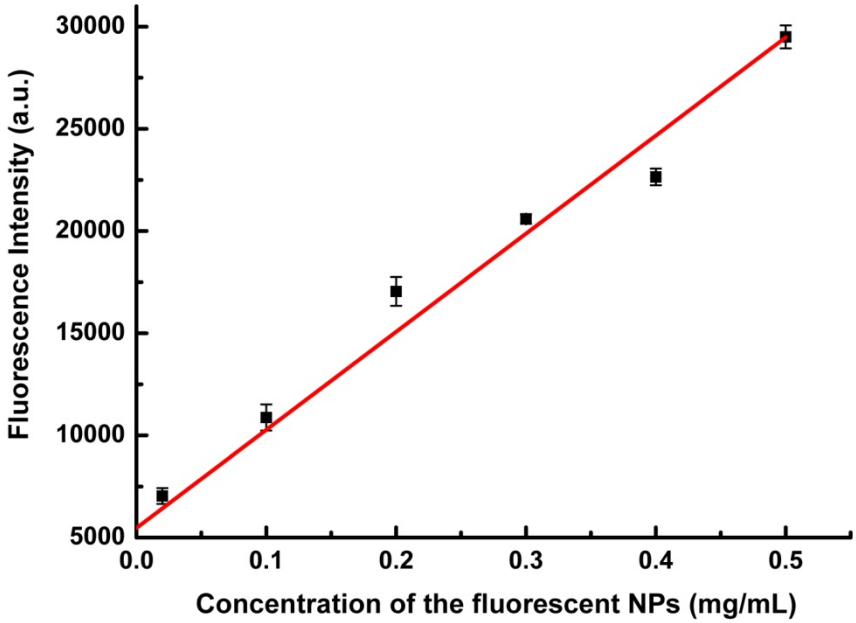


Fig. S8. Calibration curve for quantification of the amount of fluorescent ANP5. The linear working range is from 0.02-0.5mg/mL. The linear regression equation is y = 5469 + 48004x (R^2=0.980). According to the fluorescence intensity of the recovered NPs, about 3.6 μg of ANP5 was internalized by the tested cells.

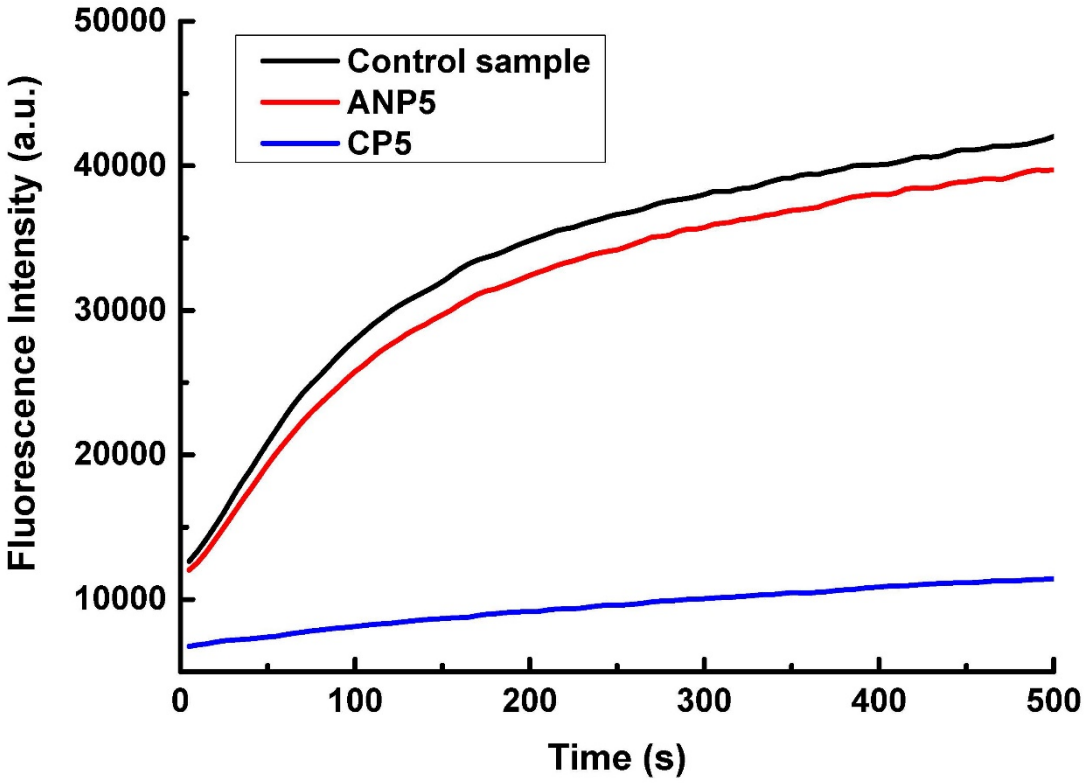


Fig. S9. Time curves for the detection of the activity of DNase I in different sample solutions for evaluation of the recovery capability of ANP5 and CP5. 0.5 μg of DNase I was incubated with 0.4 mg of ANP5/CP5 in 6 mL of DMEM cell culture solution. After tip-sonication for 4 min, the ANP5/CP5 were collected with a magnet and the bound enzymes were released with 10×DNase I buffer. The enzyme activity in the obtained solutions were quantified by using TAMRA-*Probe-D*.The recoveries of DNase activity by ANP5 and CP5 were found to be 89.8±2.7% and 8.5±0.2 %, respectively.

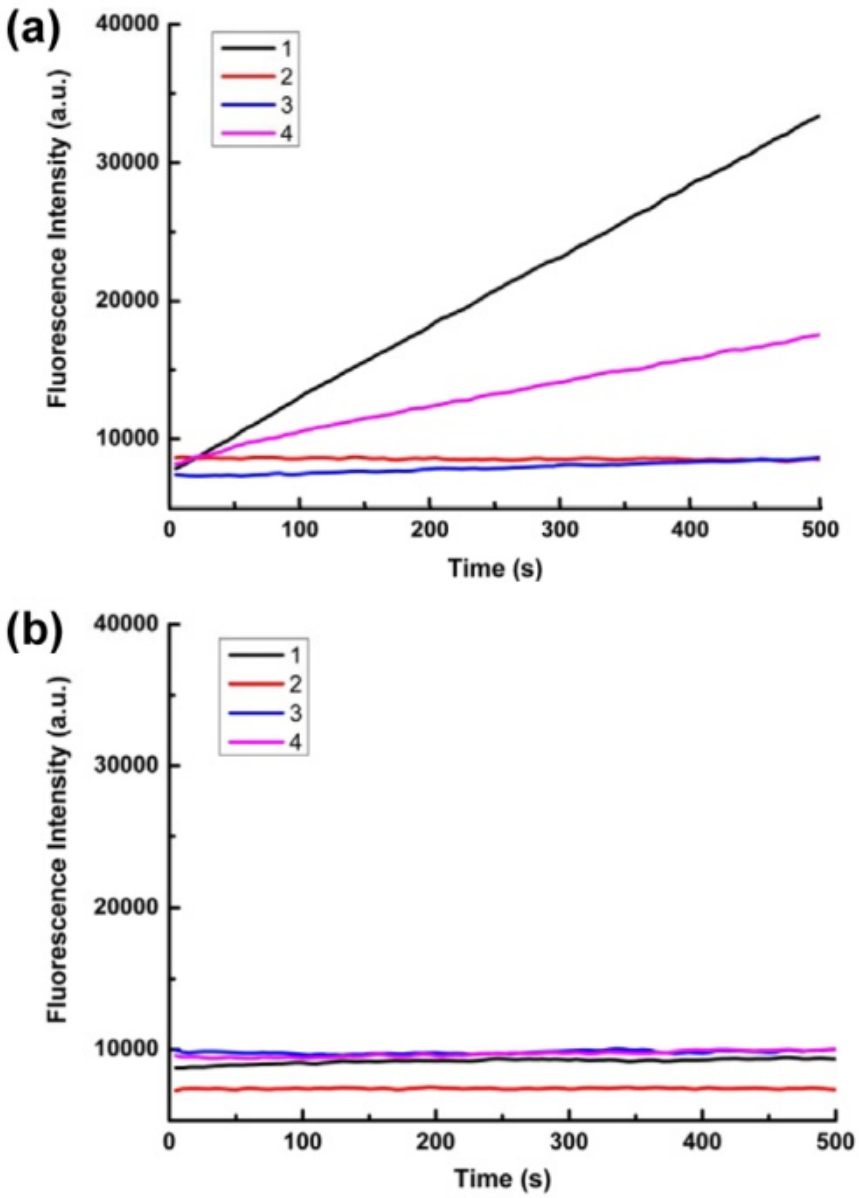


Fig. S10. Confirmation of the intracellular binding to target enzymes by the ANP5. (a) Time courses of the reaction ofTAMRA-*Probe-D* (80 nM) with DNase I in different sample solutions; (b) Time courses of the reaction of FAM-*Probe-E* (80 nM) with 3' exonuclease in different sample solutions.

Line 1: Normal cell culture medium;

Line 2: Washing DPBS buffer after the cells were washed for three times;

Line 3: Enzymes released from extracellularANP5;

Line 4: Enzymes released from intracellular ANP5.