PAD modified Fe_3O_4@PEI as a selective removal of cadmium ion from blood

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1. Materials and methods

1.1. Materials.

Ferric chloride hexahydrate ($FeCl_3$ $6H_2O$, >99%) as a single iron source, sodium acetate anhydrous, ethylene glycol, 1,6-hexanediamine, triethylamine, N,N-dimethylformamide, acetone, ethanol, polyethyleneimine, (branched, M.W. 10000, 99%) were purchased from the Alfa Aesar Reagent Company and metal ions are standard for AAS. All chemicals were analytical grade and used as received without further purification. Ultrapure water was used throughout the experiment.

1.2 Preparation of amine-functionalized magnetic nanoparticles(Fe_3O_4-NH_2)[1].

Amine-functionalized magnetite nanoparticleswere synthesized via a versatile solvothermal reaction reported by Li with a slight modification. Typically, 1.0 g of $FeCl_3$ • $6H_2O$, 6.0 g of 1,6-hexanediamine, and 2.0 g of anhydrous sodium acetate were added to 40mL of ethylene glycol to give a transparent solution via vigorous stirring. This mixture was then transferred to a Teflon-lined autoclave (50 mL) for treated at 200 ° C for 6 h. The products were obtained with the help of a magnet and washed with deionized water. Finally, the nanoparticles were stored in distilled water

for further processing.

1.3 Synthesis of Polyethyleneimine (PEI) coated magnetite nanoparticles (Fe_3O_4@PEI).

The core-shell Fe_3O_4-NH_2@PEI nanospheres were produced according to the previously reported literature.[2] Briefly, 45 ml of N,N-dimethylformamide(DMF), which containing 3g of Polyethyleneimine (PEI), was added to the 256.2 mg iron oxide nanoparticles. After ultrasonic radiation, dispersion 30min, the suspension was stirred violently at room temperature for 24 h. Fe_3O_4 @PEI were separated from solution by a permanent magnet and washed several times with DMF subsequently.

1.4 Synthesis of 2，2'-(phenylazanediyl）diacetic acid(PAD)

PAD was synthesized according to the previously reported literature.[3] Briefly, phenylamine(5.4 g), chloroacetic acid(13.1 g) and sodium carbonate(16.6 g) were mixed in a 1:2:2 molar ration in aqueous solution. The mixture was stirred and refluxed for 4 h, giving a light brown lucid solution. After cooling to room temperature, the solution was acidified with hydrochloric acid till pH≈1. The resulting precipitate was collected and re-crystallized with boil water. PAD was finally obtained after dried in air. ^{1}H NMR (300 MHz, D_2O): δ=7.39(t, 2H, Ph), 6.92(t, 1H,Ph), 6.71(d, 1H,Ph), 4.29(s, 4H, $PhCH_2COOH$)

1.5 Synthesis of PAD-PEG-NH_2 [4]

PAD (420 mg, 2 mmol) was dissolved in 12 mL of dry Tetrahydrofuran (THF) to which 275 mg (1.33 mmol) of DCC and 230 mg (2 mmol) of NHS were added. The reaction was left overnight at room temperature, in the dark. The DCU precipitate (a

side product of the reaction) was filtered out, and the filtrate was used immediately in the next step of synthesis.

NH_2-PEG-NH_2 (Mol MW=2000, 4g, 2 mmol) was dissolved in 100 mL of dry pyridine. Then PAD-NHS Ester obtained from the activation of 420 mg (2 mmol) of PAD (see previous paragraph) was added to the solution and the reaction was left overnight stirring in the dark. The product was crystallized from Et2O. 1HNMR (300MHz, chloform-d6): δ 7.18(d, 2H, Ph), 6.70(d, 2H, Ph), 6.62(d, H, Ph), 4.02(s, H, -CH_2-COOH), 3.64-3.68 (234H, -O-CH_2-CH_2-O-).

1.6 Synthesis of (PAD-PEG-ClAc)

PAD-PEG-NH_2 (2.2g, 1mmol) was dissolved in 100 mL of dry THF. Then triethylamine (0.5mL, 3 mmol) was added dropwise, followed by addition of 0.2mL (2mmol) chloroacetyl chloride dropwise under nitrogen. The reaction was stirred overnight in the dark. The product was purified by precipitation in diethyl ether. After the product was dissolved in water, pH of the solution was adjusted to pH 6. The compound was then extracted three times with 20 mL of dichloromethane and precipitated out by addition of diethyl ether, the crude product was rinsed by diethyl ether. Then, compound 5 was obtained. ^{1}HNMR (300MHz, chloform-d6): δ 7.11(d, 2H, Ph), 6.70(d, 2H, Ph), 6.50(d, H, Ph), 4.31(s, 2H, -CO-CH_2-Cl), 4.19(s, H, -CH_2-COOH), 3.50-3.70(234H, -O-CH_2-CH_2-O-).

1.7 Preparation of PAD-PEG-Fe_3O_4@PEI [5]

Fe_3O_4@PEI (200 mg) was mixed with **5** (200 mg) in dry THF and 0.5ml of TEA and 10mg of KI was added to the mixture solution. The mixture was stirred to reflux

for 24hrs. The product was collected by a permanent magnet. After washed with THF 3 times and then dried in vacuum at 60°C for 6 h.

2. Cell Culture and 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) Viability Assay

Antiproliferative activity: The cytotoxicity of PAD-PEG-Fe_3O_4@PEI on HeLa cells was measured by the MTT method in vitro. A suspension of cells (4000/well in 100 μL) was plated in 96-well plates and cultured for 12 h before addition of PAD-PEG-Fe_3O_4@PEI. Then, PAD-PEG-Fe_3O_4@PEI ranging evenly from 12.5 to 400 μg/mL were added to the corresponding plates. The plates were subsequently incubated for 24 h, the culture medium was removed, and 100 μL of MTT solution (diluted in culture medium, 0.5 mg /mL) was added to each well. After 4 h of incubation at 37° C, the MTT/medium was removed carefully and DMSO (100 μL) was added to each well to dissolve the formazan crystal. The absorbance of the wells was read by a Victor Multilabel Reader with a test wavelength of 570 nm. Cell viability was expressed as a percentage of control. All experiments were performed in triplicate.

3. Hemolysis Assay[6]

Human blood was obtained from Gansu Blood Centers (Lanzhou). The whole blood was diluted to 1/10 of its original volume using calcium and magnesium-free Dulbecco's phosphatebuffered saline (DPBS) solution. Red blood cells (RBCs) were isolated by centrifugation at 1000 rpm for 10 min, washed, and resuspended five times with PBS solution. Then 0.2 mL of diluted RBCs suspension was added to 0.8

mL of nanoparticle solution at different concentration and mixed by vortexing. Incubation of deionized water and DPBS with RBCs were used as positive control and negative control, respectively. All the samples were kept in static conditions at room temperature for 3 h. Finally, the mixtures were centrifuged at 1000 rpm for 3 min. The absorbance values of supernatants at 570 nm were determined by using a microplate reader with absorbance at 655 nm as a reference. The hemolysis percentage of RBCs was calculated based on the formula shown below.

Hemolysis percentage=((sample absorbance - negative control absorbance)/(positive control absorbance - negative control absorbance)) ×100.

4. Investigation of the Removal Efficiency of PAD-PEG-Fe_3O_4 @PEI for metal cations

Typically, 1 mg of PAD-PEG-Fe_3O_4 @PEI was added to 1 mL of 1 ppm Cd^{2+} solution. Then this mixture was dispersed with the help of ultrasound radiation for 10 munites to ensure sufficient interaction between PAD-PEG-Fe_3O_4 @PEI and Cd^{2+}at room temperature. After PAD-PEG-Fe_3O_4 @PEI NPs were removed by magnet, the resulting solution was detected by ICP analysis. The other cations (Mg^{2+}, Cu^{2+},K^{+}, Fe^{3+}, Zn^{2+}, Pb^{2+}, Cu^{2+} and Hg^{2+}) were determined similar process as above Cd^{2+}.

$$R = \frac{C_i - C_f}{C_i} \times 100\%$$

This equation was written as where R was the removal efficiency; C_i and C_f were the initial concentration and final concentration of heavy metals, respectively.

5. Removing Cd^{2+} from blood

First, we added cadmium nitrate into 1.0 mL of blood (1 ppm) and applied 10

minutes' shake to make a homogeneous solution (I). Then we added 0.2 mg of PAD-PEG-Fe_3O_4@PEI into the solution and applied 10 minutes' shake to ensure the sufficient interaction between PAD-PEG-Fe_3O_4@PEI and Cd^{2+}. Afterward, we used a small magnet to attract and remove the magnetic nanoparticles from the blood and washed the nanoparticles with ultrapure water three times. We combined the blood and washings as part II and collected the magnetic nanoparticles as part III. Before ICP analysis, we added 5.0 mL of concentrated nitric acid to each sample first. Then we burned each sample to dryness by a gas stove and further burned the sample in muffle oven at 900 ^oC for 5 hours to remove remained organic compounds. Finally, the residue in each sample was dissolved in 5 mL of HNO_3 (2 N) for test.

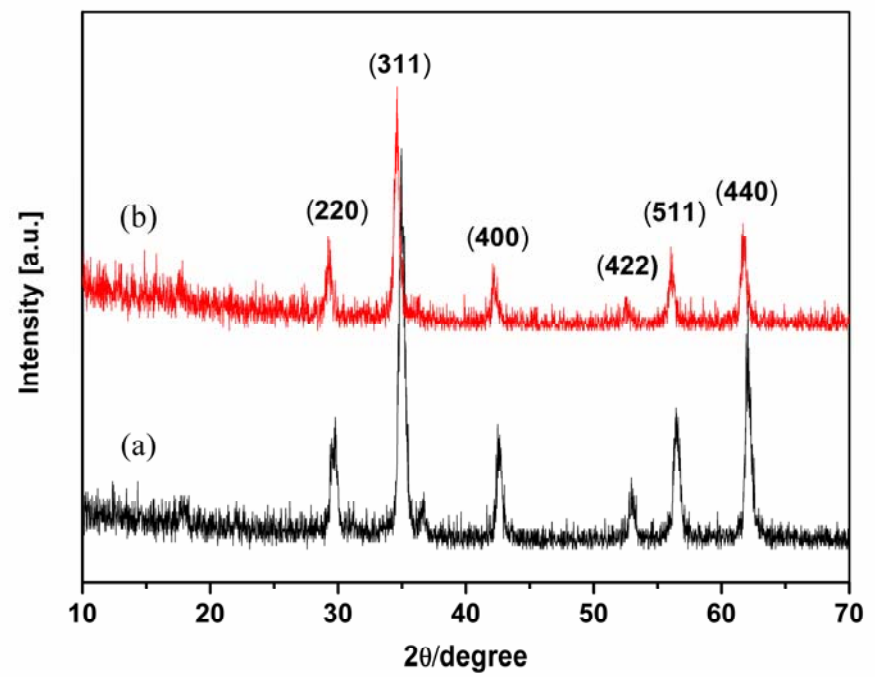

Figure 1S XRD of (a) Fe_3O_4-NH_2 and (b) PAD-PEG-Fe_3O_4@PEI.

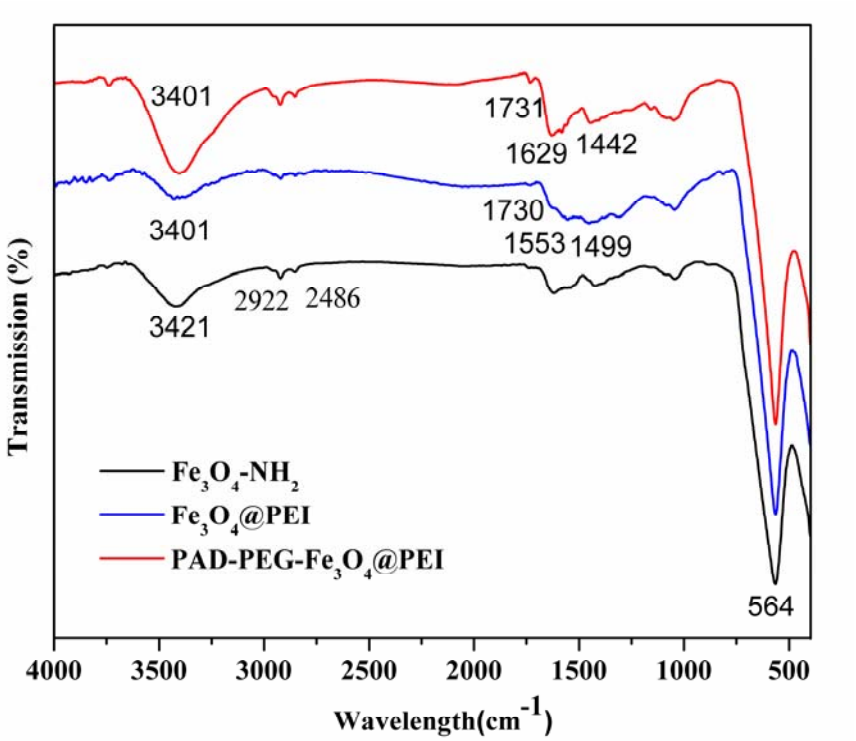

Figure S2 IR spectra of (a) Fe_3O_4-NH_2, (b) Fe_3O_4@PEI and (c) PAD-PEG-Fe_3O_4@PEI.

Figure S2 shows the IR spectrum of Fe_3O_4-NH_2, indicating that some 1, 6-hexanediamine molecules were immobilized on the surface of Fe_3O_4. The peaks at 3421 and around 2846-2922 cm^{-1} are ascribed to an N-H stretching vibration of free 1, 6-hexadiamine and the C-H stretching model of the alky chain. Only after close inspection, one can find there are still some differences between Fe_3O_4-NH_2 and PAD-PEG-Fe_3O_4@PEI. The band at 1629 cm^{-1} of PAD-PEG-Fe_3O_4@PEI indicates the formation of amide bond. Furthermore, a new peak appeared at 1731 cm^{-1} of PAD-PEG-Fe_3O_4@PEI demonstrated carboxyl group on the surface of Fe_3O_4@PEI compared with Fe_3O_4-NH_2 and Fe_3O_4@PEI. All of these confirmed PAD is successfully modified on the surface of Fe_3O_4@PEI via PEG.

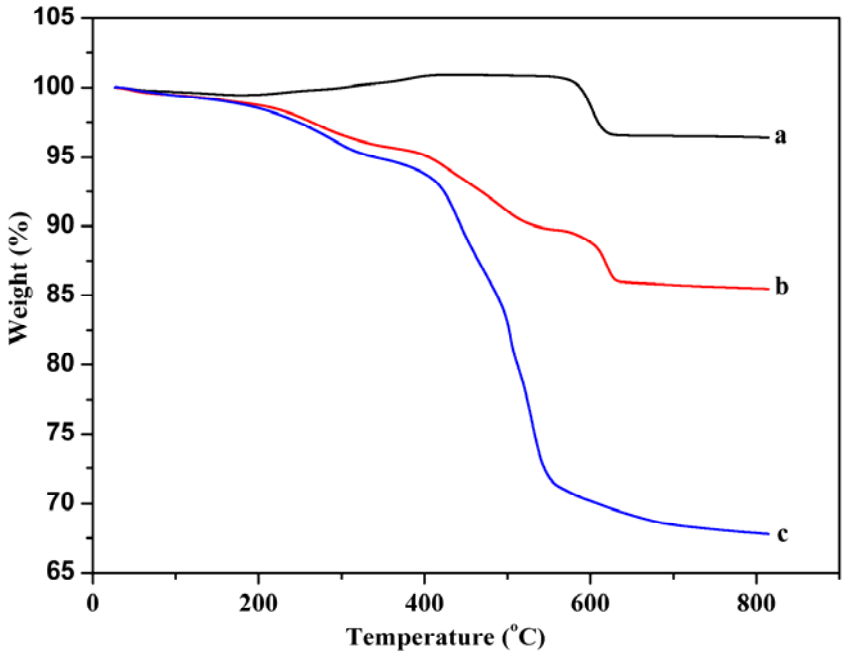

Figure S3 TGA curves of (a) Fe_3O_4-NH_2, (b) Fe_3O_4@PEI and (c) PAD-PEG-Fe_3O_4@PEI in nitrogen atmosphere

TGA curves of (a) Fe_3O_4-NH_2, (b) Fe_3O_4@PEI and (c) PAD-PEG-Fe_3O_4@PEI are shown in Figure S3. Figure S3 shows that the overall weight loss of Fe_3O_4-NH_2 is 3.6%. In contrast, the weight loss of Fe_3O_4@PEI is mainly divided into three temperatures regions: below 200 ℃, 200-680 ℃ and above 680 ℃. The weight loss during heating from 25 to 200 ℃ is assigned to the loss of loosely bound water (0.52%). The large weight loss of around 13.68% at temperature between 200 and 680 ℃ is due to the decomposition of coating organics on the surface of Fe_3O_4. Compare Fe_3O_4@PEI with PAD-PEG-Fe_3O_4@PEI, it can be calculated that the weight of PAD -PEG is 17.7%. The results from TGA imply that the Fe_3O_4@PEI nanoparticles have been successfully modified with PAD-PEG. Furthermore, this result is correspondence with FT-IR.

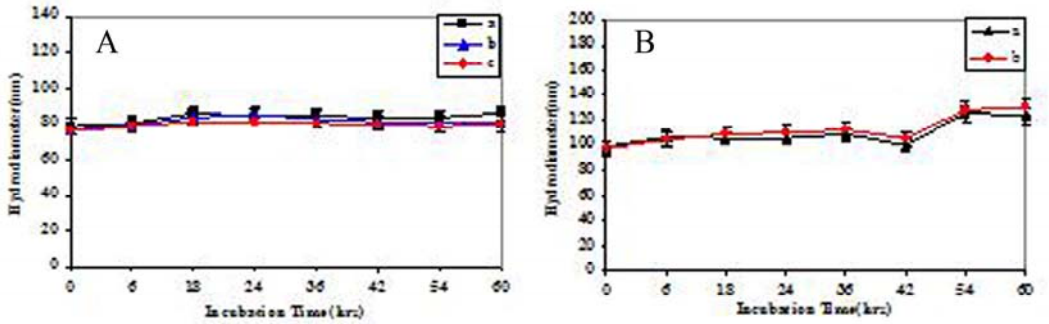

Figure S4 (A) Stability of PAD-PEG-Fe_3O_4@PEI in 1×PBS buffer under 37 °C with pH a) 5.0 b) 7.14 c) 8.0. (B) Stability of PAD-PEG-Fe_3O_4@PEI in 1×PBS buffer plus 10% FBS under 37 °C with pH=7.4 at different temperature a) 37°C b）25 °C.

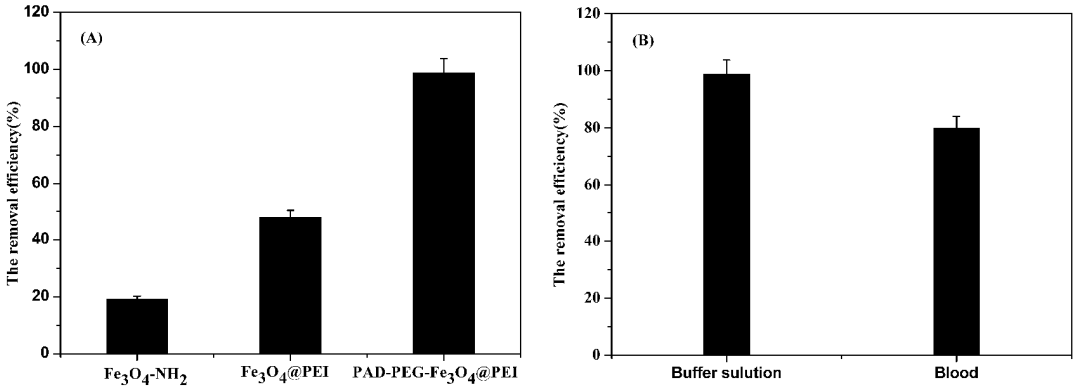

Figure S5 The removal efficiency of (A) Fe_3O_4 –NH_2, Fe_3O_4@PEI and PAD-PEG-Fe_3O_4@PEI for Cd^{2+} in water, respectively; (B) The removal efficiency of PAD-PEG-Fe_3O_4@PEI for Cd^{2+} in PBS buffer and blood.

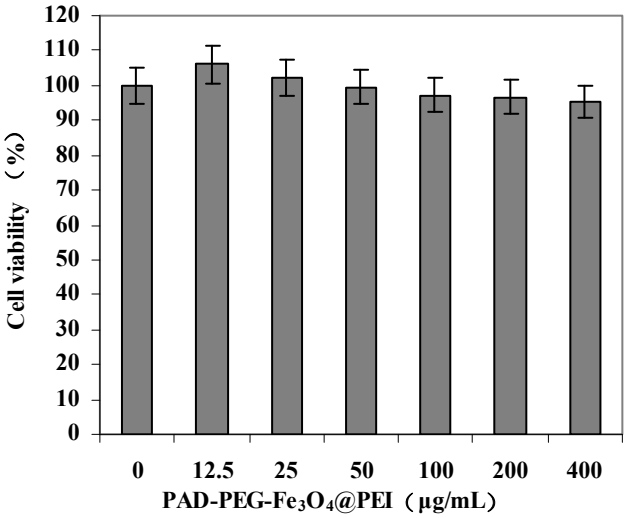

Figure S6 Cell viability of HeLa cells using the MTT assay after treatment with different concentrations of PAD-PEG-Fe_3O_4@PEI. Data represent to mean ±SD, n=3.

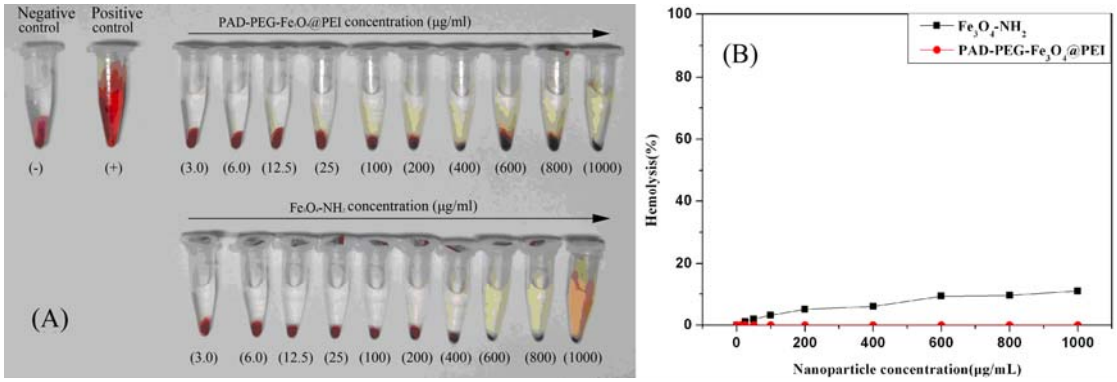

Figure S7. The photograph of RBCs incubated with Fe_3O_4–NH_2 and PAD-PEG-Fe_3O_4@PEI at different concentrations ranging from 12.5 to 1000 μg/mL for 3 hours(A).Concentration-dependent hemolysis of Fe_3O_4–NH_2 and PAD-PEG-Fe_3O_4@PEI(B).

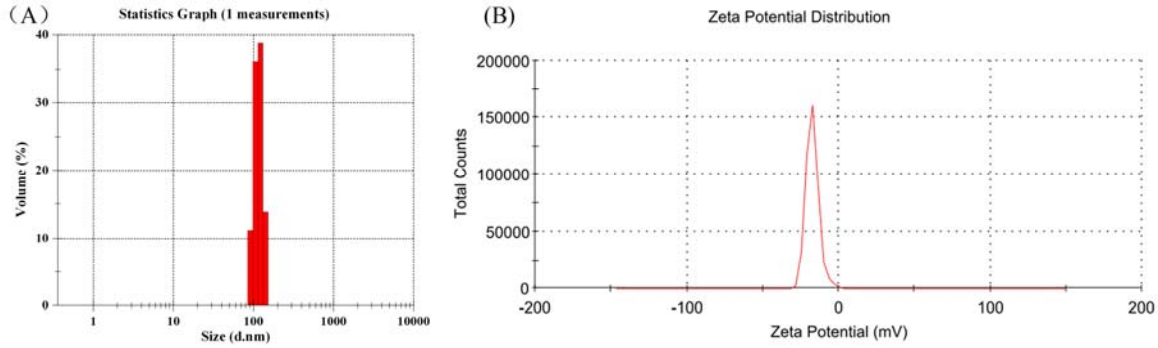

Figure S8 Hydrodynamic diameter distribution of PAD-PEG-Fe_3O_4@PEI nanoparticles in water(A), and Zeta potential of PAD-PEG-Fe_3O_4@PEI nanoparticles in water(B)**.**

The size distribution of nanoparticles is analyzed by Zetasizer in deionized water. Numeric statistics graph in Fig.S8(A) indicates that these nanoparticles have hydrodynamic diameters mainly in the range of 92-141 nm. As is shown in Fig.S8(B), when dispersed in deionized water, Zeta potential of PAD-PEG-Fe_3O_4@PEI is -22.8 mV.

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