A pH-sensitive polymer based tumor precise targeting strategy with reduced uptake of nanoparticles by non-cancerous cells

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Materials and Methods

Materials

$FeCl_3 \cdot 6H_2O$ (99%), $FeCl_2 \cdot 4H_2O$ (99%), NaOH (99.99%), diethylene glycol (DEG, 99%), glutaraldehyde (50 wt%), methoxypolyethylene glycol amine (mPEG, Mw = 2, 5, and 10 kDa), 2,3-dimethylmaleic anhydride (DCA) and dimethyl sulfoxide (DMSO) were purchased from Aladdin Industrial Inc. (Shanghai, China). Bovine Serum Albumin (BSA) was purchased from Sigma (USA). Folic acid (FA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCl), N-hydroxysuccinimide (NHS), L-lysine (Lys) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Cell culture

Human breast cancer cell line (MCF-7) was cultured in the dulbecco's modified eafle's medium (DMEM) supplemented with 10 wt% of fetal bovine serum (FBS), 100 units/mL of penicillin and 100 mg/mL of streptomycin.

Cellular uptake of the nanoparticles

The cellular uptake of SPION-AN-FA@mPEG4 pretreated in pH 7.4 or 5.5 of PBS by MCF-7 cells was tested using a laser scanning confocal microscope (LSCM). Typically, 2.0 mL 4×10^4 cells mL^{-1} of the MCF-7 cells were seeded into a glass bottom cell culture dish and allowed to adhere at 37 ^{0}C under 5 % CO_2 atmosphere overnight, respectively. Then DMEM medium was replaced with fresh one containing SPION-AN-FA@mPEG4 (0.25 mg/mL), which were pre-incubated in pH 7.4 or 5.5 of PBS for 12 h. After 2.0 h incubation, the cells were washed thrice with PBS, fixed with 4 % formaldehyde for 30 min, permeabilized with 0.1 % triton for 5.0 min,

blocked with 1.0 % BSA for 30 min and then stained with Hoechst (5.0 μg/mL) for 30 min at room temperature. Finally, the sample were simultaneously excited at 405 and 488 nm and the fluorescence images at emission wavelengths of 420-480 and 510-540 nm were observed using a LSCM (TCS SP5 II, Leica, Germany).

Flow cytometry analysis

We investigated the cellular uptake of SPION-AN-FA@mPEG1-6 nanoparticles by MCF-7 cells. In a typical experiment, 2.0 mL 5×10^4 cells/mL MCF-7 cells were seeded into a 6-well plate. After 48 h of incubation at 37°C, the growth medium was replaced with 2.0 mL of fresh one (without FBS) containing SPION-AN-FA@mPEG1-6 (0.25 mg/mL), which were pre-incubated in pH 7.4, 6.5 or 5.5 of PBS for 12 h. After 2.0 h incubation, the cells were washed thrice with PBS to remove the extracellular nanoparticles. The cells were then treated with trypsin for 3.0 min, centrifuged (500 × g, 5 min), and then subjected to flow cytometry analysis on a BD LSRfortessa instrument (BD biosciences COE, Shanghai, China).

Cytotoxicity of the nanoparticles

To evaluate the cytotoxicity of the SPION-AN-FA@mPEG, experiments were carried out with MCF-7 cells by MTT assay. Typically, 100 μL 1×10^5 cells/mL were seeded into 96-well plate and allowed to adhere overnight. After 24 h, the medium was replaced with 100 μL of fresh one containing various concentrations of the nanoparticles (C_{Fe} 0~1 mM). After 48 h, 10 μL of MTT (5.0 mg/mL in PBS) was added into each well for another 4 h incubation. Then the growth medium was replaced with 100 μL of DMSO to dissolve the fromazan crystal. Finally, the absorbance was measured at a wavelength of 550 nm by an automated plate reader (iMark 168-

1130, Bio-rad, USA) to calculate the cell viability.

Tumor model

All animal experiments were approved by the animal care and use committee of the Ningbo University, China. Female BALB/c mice (4 weeks) were purchased from the Card Vince Laboratory Animal Co. Limited (Changzhou, China). MCF-7 cells were inoculated by subcutaneous injection to obtain tumor-bearing mice.

MRI studies *in vivo*

T_2-weighted MRI of tumor-bearing mice pre- and post-injection of SPION, SPION-AN-FA and SPION-AN-FA@mPEG nanoparticles were conducted on a MRI instrument (MesoMR23-060H-1, TR = 1600 ms, TE = 40 ms). The nanoparticles (30 mg/kg) was injected *via* tail vein on the MCF-7 tumor bearing nude mice. The T_2 images were observed at 0, 1, 4, 12, 24, and 48 h post-injection.

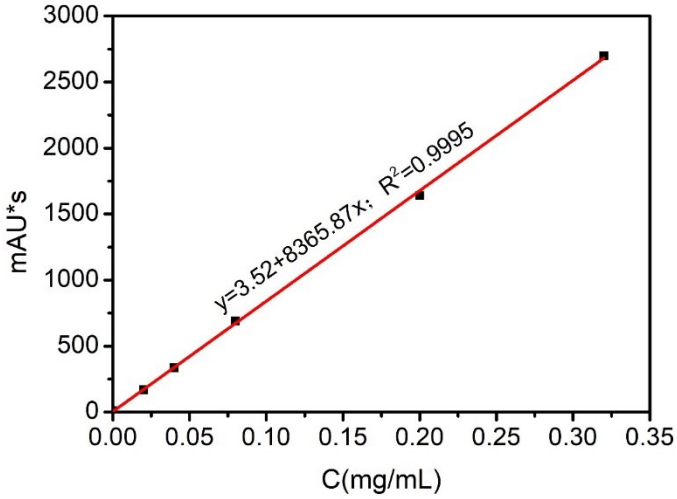
Histopathology assessment of healthy mice treated with SPION-AN-FA@mPEG

In vivo toxicity of SPION-AN-FA@mPEG nanoparticles was evaluated on healthy mice. Typically, the mice were intravenously injected with PBS (control) or SPION-AN-FA@mPEG (30 mg/kg). The mice were sacrificed at 7 days post-injection. The main organs, including heart, liver, spleen, lung, and kidney, were preserved in aqueous buffered zinc formalin fixative (Z-FIX) and stained with hematoxylin and eosin for histological analysis.

Table S1. Synthesis of the SPION-AN-FA@mPEG nanoparticles

nanoparticles	Molecular weight of mPEG (kDa)	[mPEG]/[SPION-AN-FA] (mass ratio) [a]
SPION-AN-FA@mPEG1	2	2
SPION-AN-FA@mPEG2	5	2
SPION-AN-FA@mPEG3	10	2
SPION-AN-FA@mPEG4	10	0.5
SPION-AN-FA@mPEG5	10	1
SPION-AN-FA@mPEG6	10	2

[a] Mass ratio of total polymers of mPEG to nanoparticles of SPION-AN-FA



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94 **Figure S1.** Standard curve of DCA measured by HPLC.

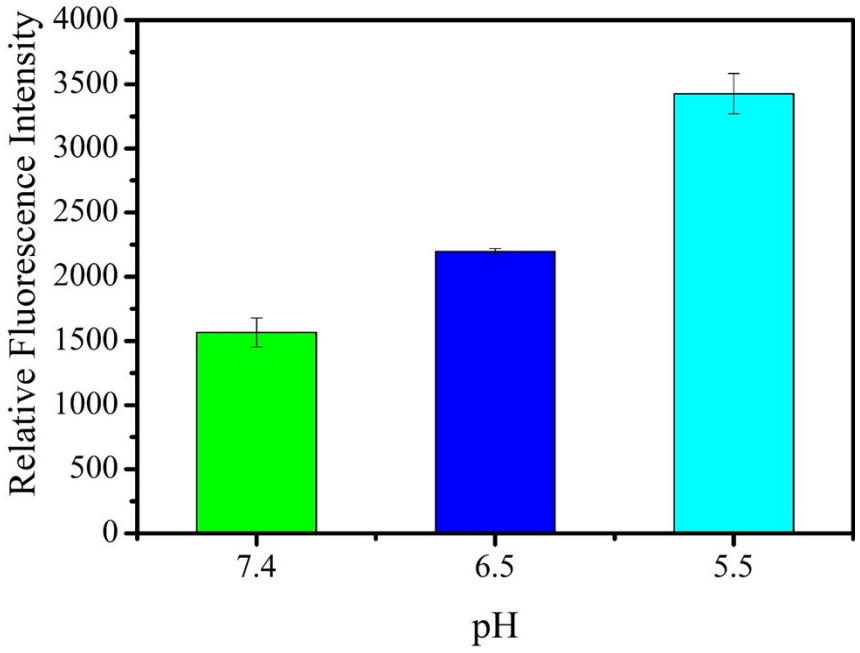


Figure S2. The ratio of relative fluorescence intensity of MCF-7 cells treated with SPION-AN-FA@mPEG4 nanoparticles, which were pretreated in pH 7.4, 6.5 and 5.5 of PBS for 12 h, to untreated cells (*i.e.*, relative fluorescence intensity).

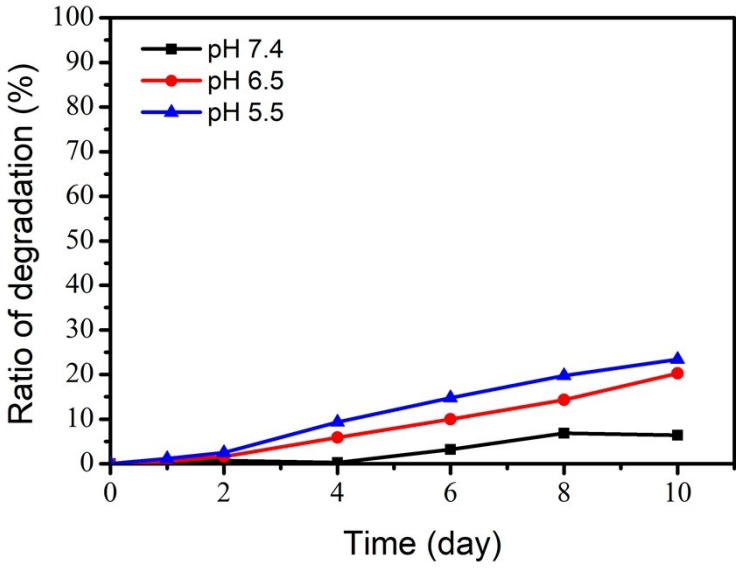
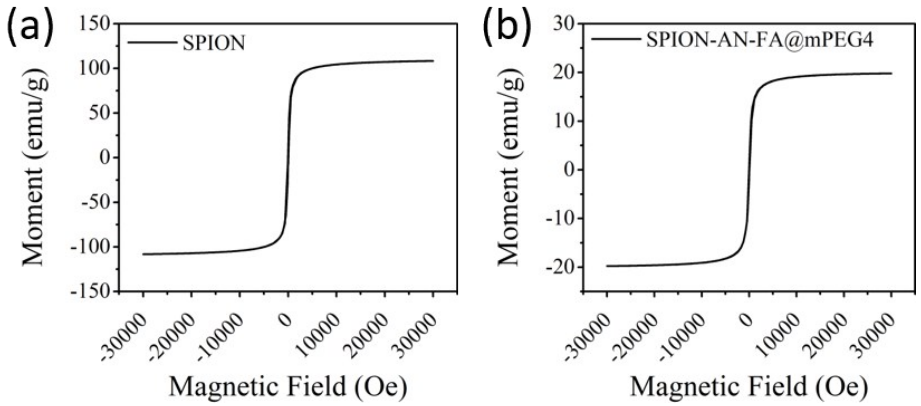


Figure S3. The degradation of albumin nanoparticles in different pH conditions.



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106 **Figure S4.** M-H curves of SPION (a), or SPION-AN-FA@mPEG4 nanoparticles (b).

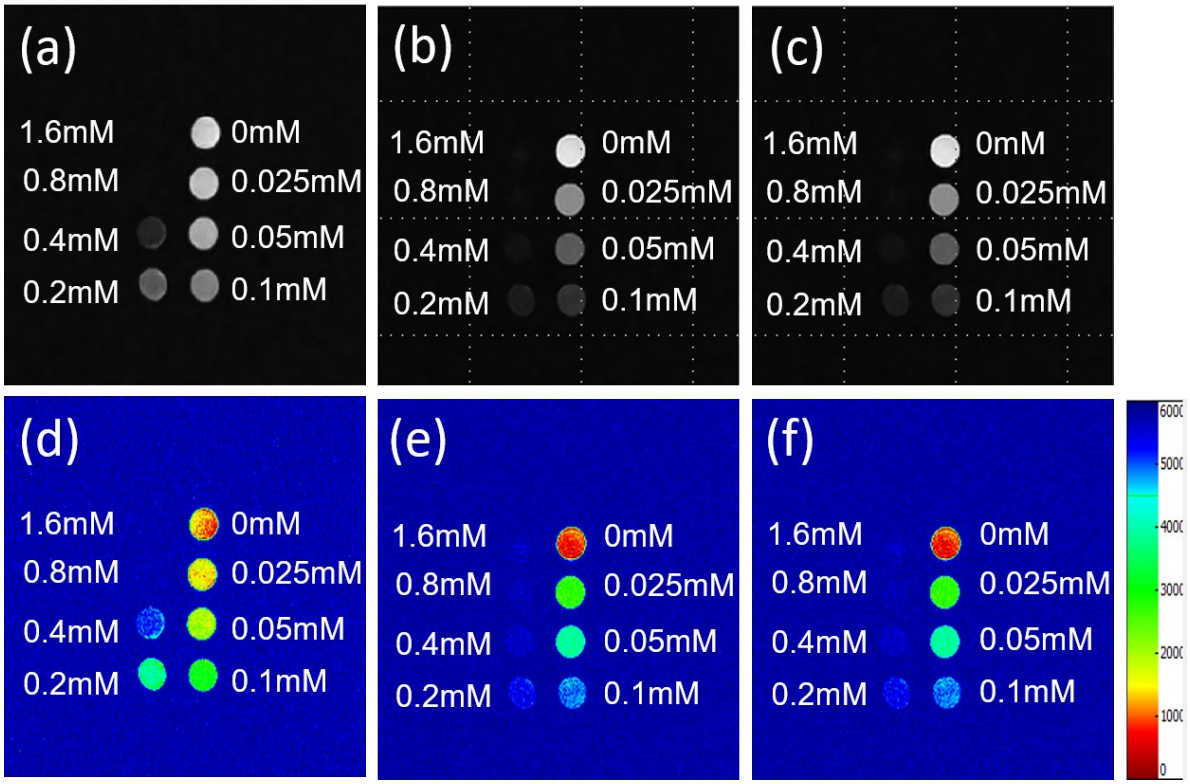


Figure S5. T_2-weighted MR images of (a) SPION, (b) SPION-AN-FA and (c) SPION-AN-FA@mPEG4 at different Fe concentrations. (d-f) are the corresponding pseudo color pictures of (a-c).

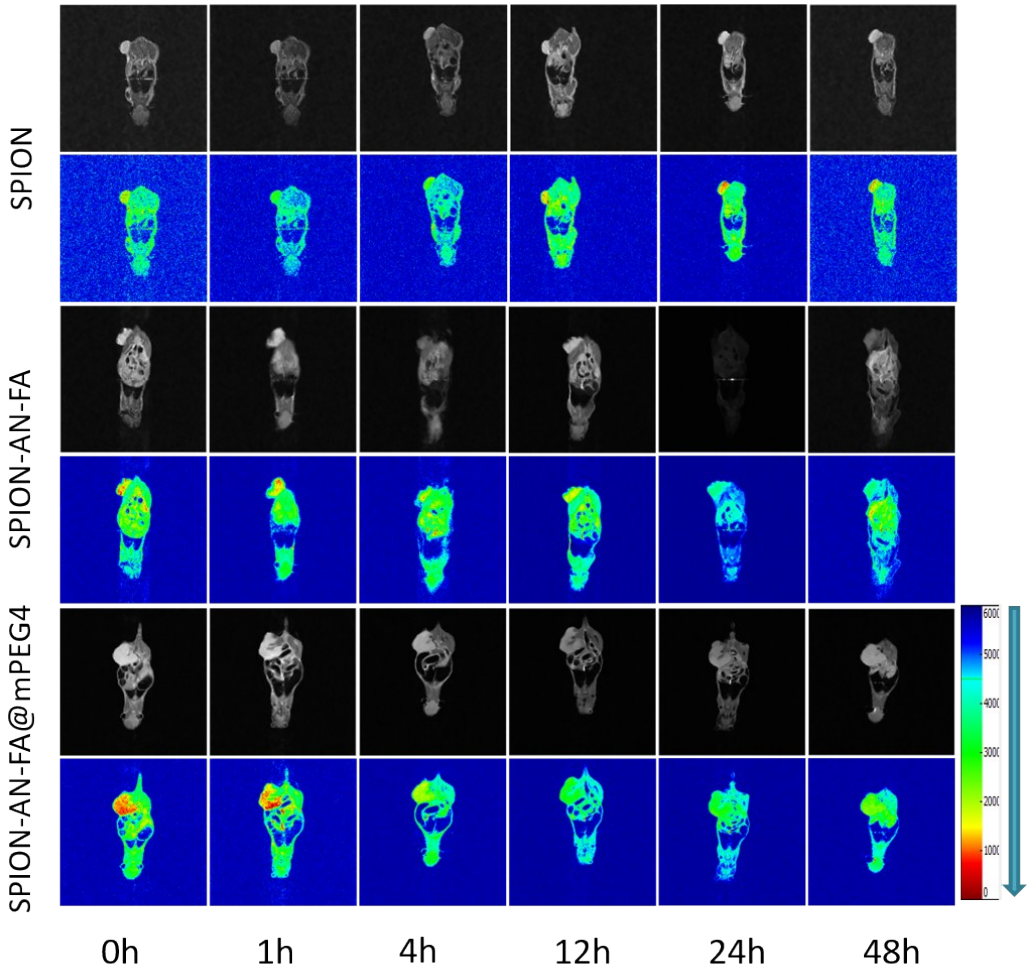


Figure S6. T_2-weighted MR images of tumor-bearing mice pre- or post-injection of SPION, SPION-AN-FA, or SPION-AN-FA@mPEG4 nanoparticles (TR = 1600 ms, TE = 40 ms).

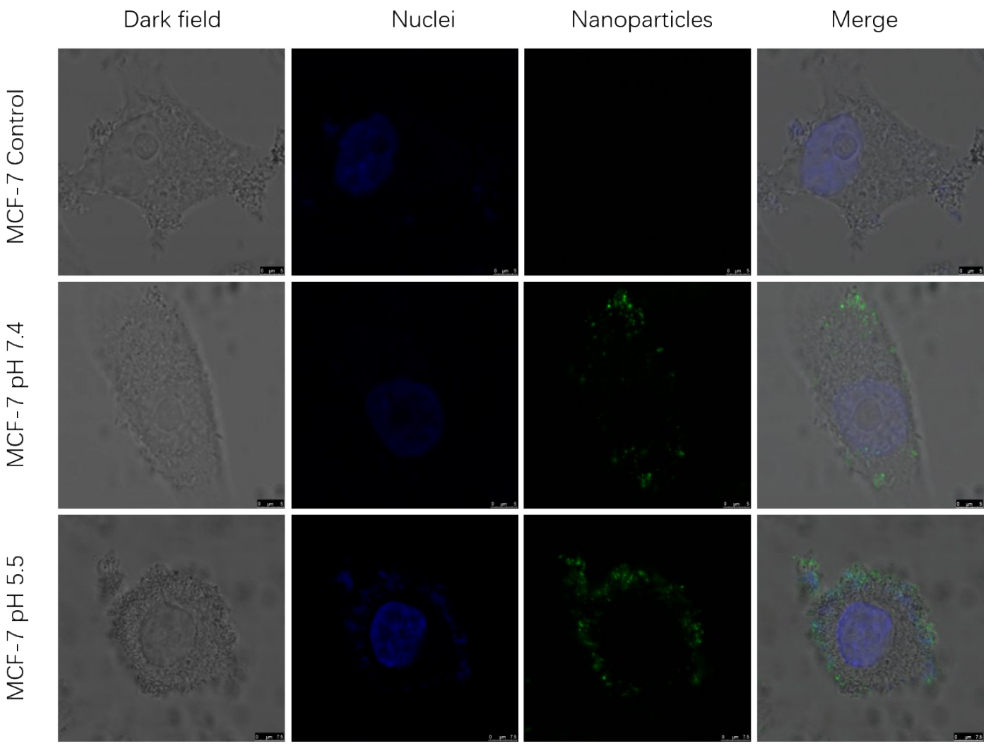


Figure S7. LSCM images of MCF-7 cells incubated with SPION-AN-FA@mPEG4 which was pre-treated in pH 5.5 or 7.4 of PBS for 12 h at 37 ^{0}C.

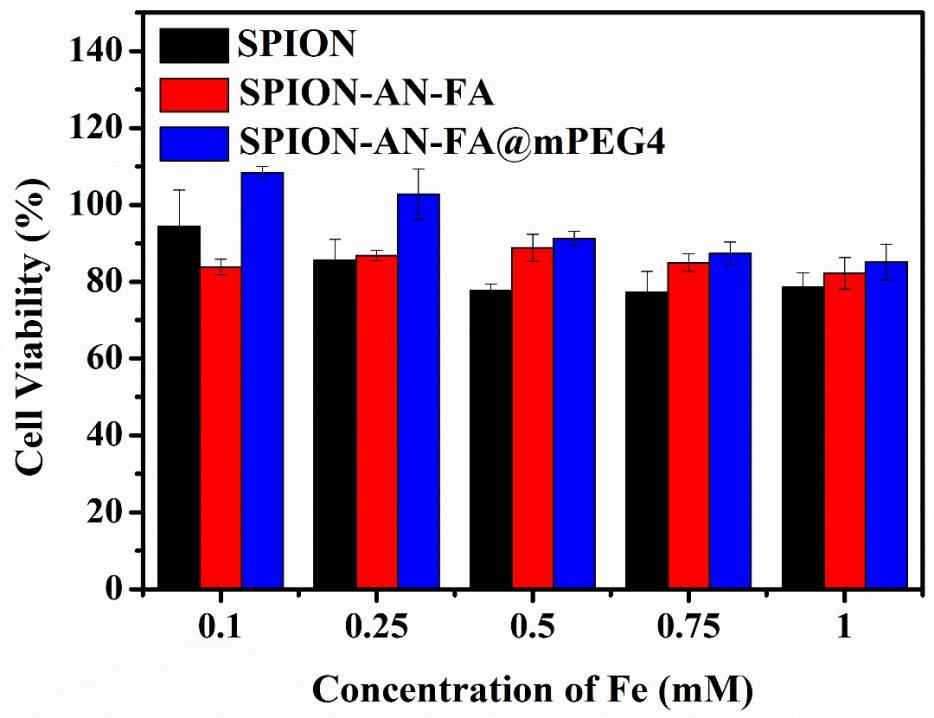
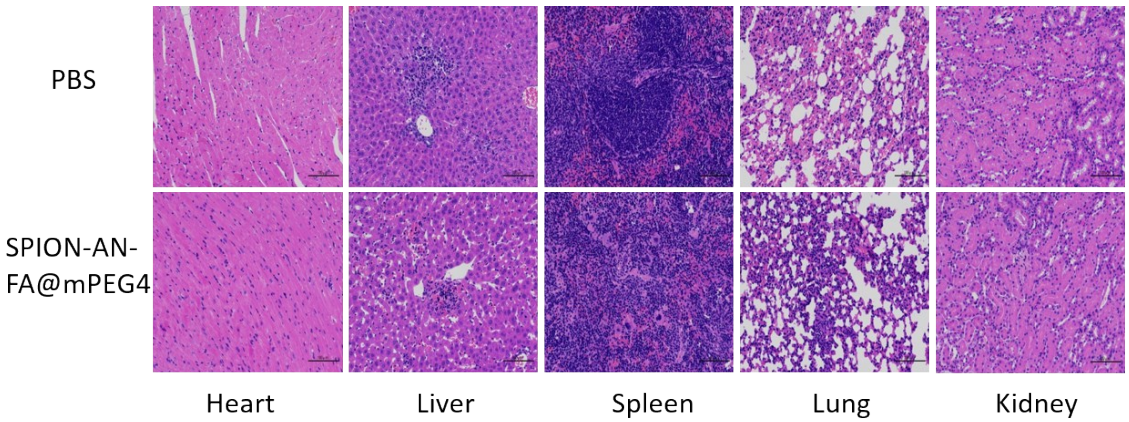


Figure S8. Cytotoxicity of SPION, SPION-AN-FA, or SPION-AN-FA@mPEG4 measured by MTT assay.



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127 **Figure S9.** Histological analysis of main organs from the mice injected with 200 μL of PBS for

128 control or SPION-AN-FA@mPEG4.

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