Electronic Supplementary Material (ESI) for Nanoscale.

Targeted Therapy of Atherosclerosis by pH-Sensitive Hyaluronic Acid Nanoparticles Co-Delivering All Trans Retinal and Rapamycin

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Table S1. Primer Sequences for Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Gene	Primer sequence (5′−3′)
TNF-α	forward: GCGACGTGGAACTGGCAGAA
	reverse: CAGTAGACAGAAGAGCGTGGTG
IL-6	forward: GTTGCCTTCTTGGGACTGAT
	reverse: CATTTCCACGATTTCCCAGA
GAPDH	forward: TGACCACAGTCCATGCCATC
	reverse: GACGGACACATTGGGGGTAG

Table S2. Characteristics of a series of HR nanoparticles.

Sample	Feed ratio[a]	DS (%)[b]	CMC (mg/L)	Size (nm)[c]	PDI[c]	Zeta potential (mV)[c]
HR_7[d]	0.5	6.80	234.8	299.6 ± 14.7	0.496 ± 0.23	-27.6 ± 2.7
HR_8[d]	0.8	8.01	200.9	193.3 ± 0.6	0.284 ± 0.04	-23.1 ± 1.4
HR_{12}[d]	1	12.54	196.2	188.3 ± 11.1	0.430 ± 0.05	-26.2 ± 2.0
HR_{13}[d]	2	12.80	184.3	182.2 ± 8.4	0.264 ± 0.11	-21.4 ± 1.9
HR_{17}[d]	4	16.76	72.1	171.3 ±13.4	0.225 ± 0.08	-21.5 ± 3.2

[a] Molar feed ratio of ATR to HA-ADH modified polymer. [b] Degree of substitution of ATR defined as ATR molecules per 100 sugar residues of HA. [c] Data are represented as mean ±S.D. (n=3). [d] The number represents the degree of substitution of ATR.

Table S3. Characteristics of a series of HR_{RAP}NPs.

Sample	EE (%)[a,c]	DL (%)[b,c]	Size (nm)[c]	PDI[c]	Zeta potential (mV)
$HR_{7\ RAP}$ NPs[d]	65.9 ± 3.2	24.8 ± 0.6	180.2 ± 5.4	0.073 ± 0.01	-29.5 ± 5.9
$HR_{8\ RAP}$ NPs[d]	64.8 ± 0.9	24.4 ± 0.2	173.9 ± 4.7	0.081 ± 0.01	-21.9 ± 2.2
$HR_{12\ RAP}$ NPs[d]	73.6 ± 1.3	26.9 ± 0.7	160.7 ± 4.6	0.089 ± 0.02	-28.0 ± 6.8
$HR_{13\ RAP}$ NPs[d]	72.7 ± 1.7	26.6 ± 0.2	155.3 ± 4.4	0.089 ± 0.03	-18.5 ± 2.2
$HR_{17\ RAP}$ NPs[d]	98.5 ± 1.3	30.9 ± 0.5	140.2 ± 4.7	0.061 ± 0.02	-21.1 ± 1.5

[a, b]The encapsulation efficiency of RAP and the loading capacity of RAP, respectively. [c] Data are represented as mean ±S.D. (n=3). [d] The number represents the degree of substitution of ATR.

Table S4. Pharmacokinetic parameters of RAP and HR_{RAP}NPs after IV injection into BALB/c mice at 10 mg/kg (mean ± SD, n=3).

Pharmacokinetic parameters	RAP solution	HR_{RAP}NPs
AUC_{inf} (ug. h /mL)	32.5 ± 9.7	50.2 ± 18.1*
C_{max} (ug/mL)	7.0 ± 2.1	16.1 ± 7.3
$t_{1/2}$ (h)	6.5 ± 0.5	6.8 ± 1.8
Cl (L/h/kg)	6.1 ± 0.7	4.9 ± 1.2*

AUC_{inf}: Area under the curve from time zero to infinity; C_{max}: Maximum concentration; $t_{½}$: Half-life; Cl: Clearance. * Statistically significant difference between RAP and HR_{RAP}NPs groups (*P < 0.05).

Supplementary figures

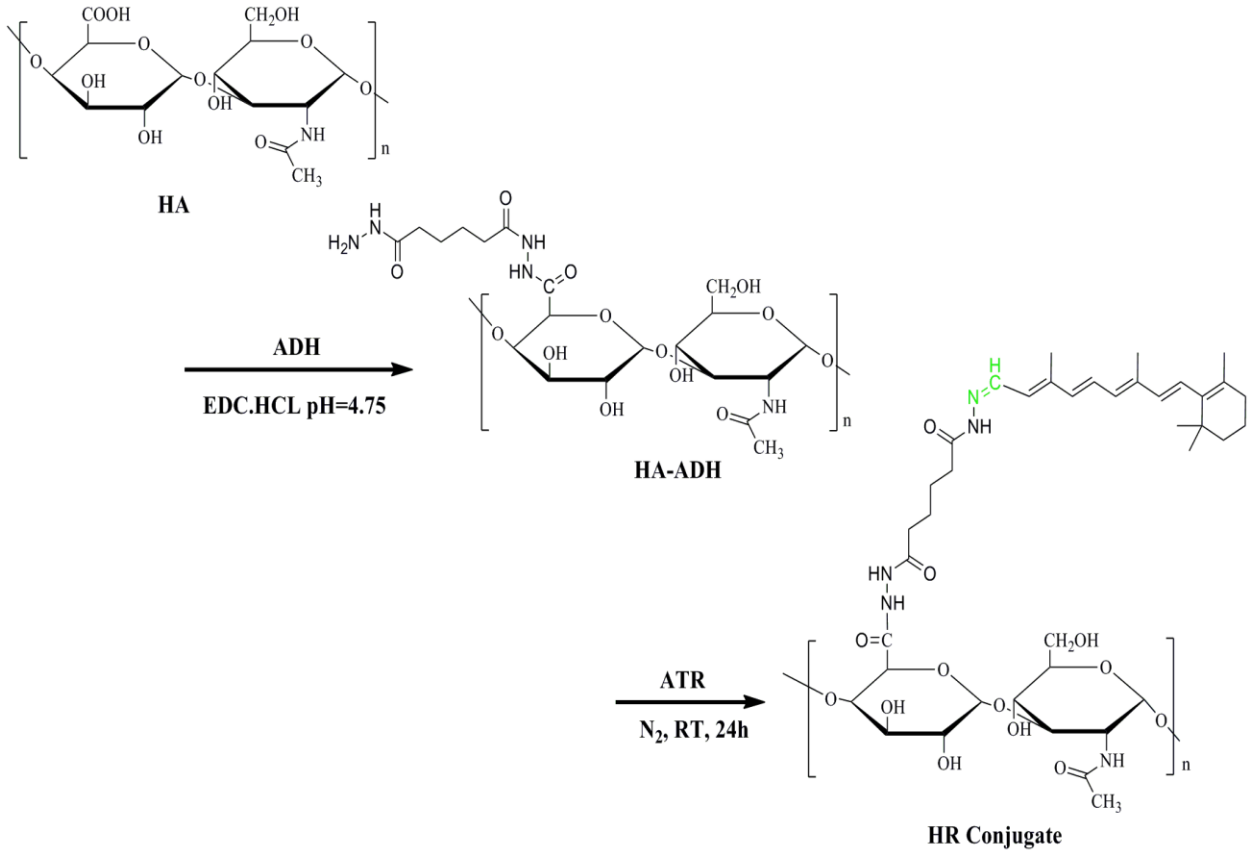

Figure S1. Scheme of the synthetic pathway of HR conjugate.

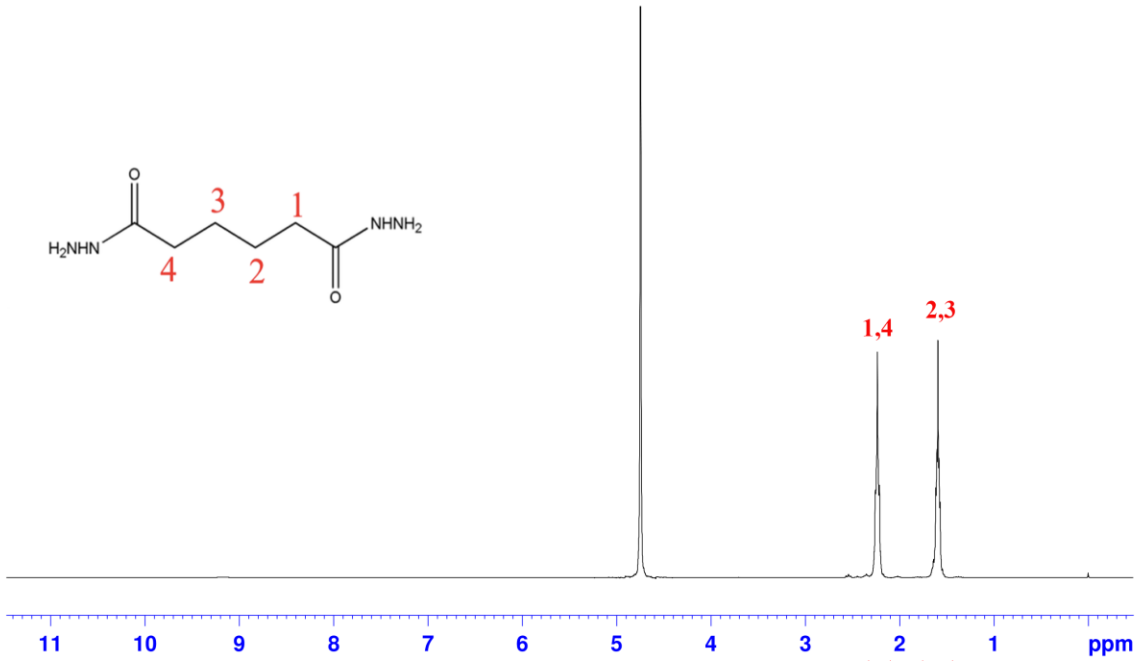

Figure S2. ^{1}H NMR spectrum of adipic dihydrazide in D2O.

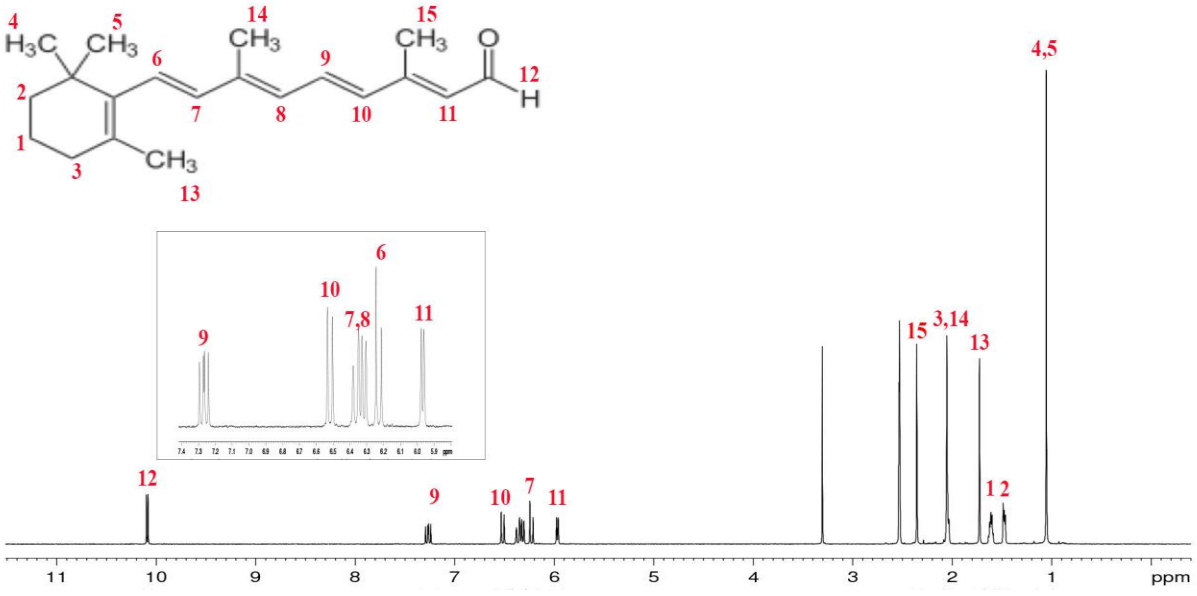

Figure S3. ^{1}H NMR spectrum of all trans retinal (ATR) in d-DMSO.

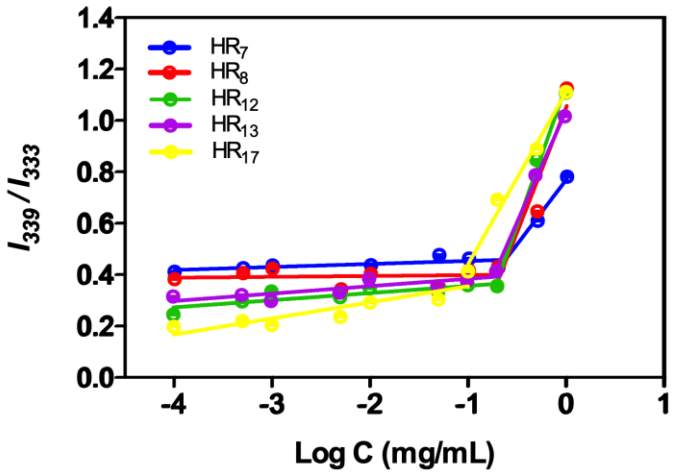

Figure S4. Critical micelle concentration (CMC) of a series of HR conjugates with different DS using pyrene as fluorescent probe.

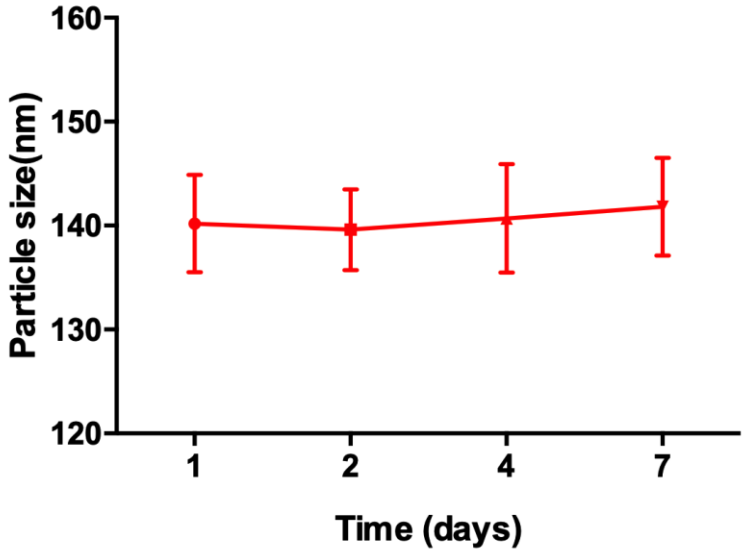

Figure S5. Changes in the particle size of HR_{RAP} NPs during storage at 4°C for 7 days. Bars represent mean ± SD (n=3).

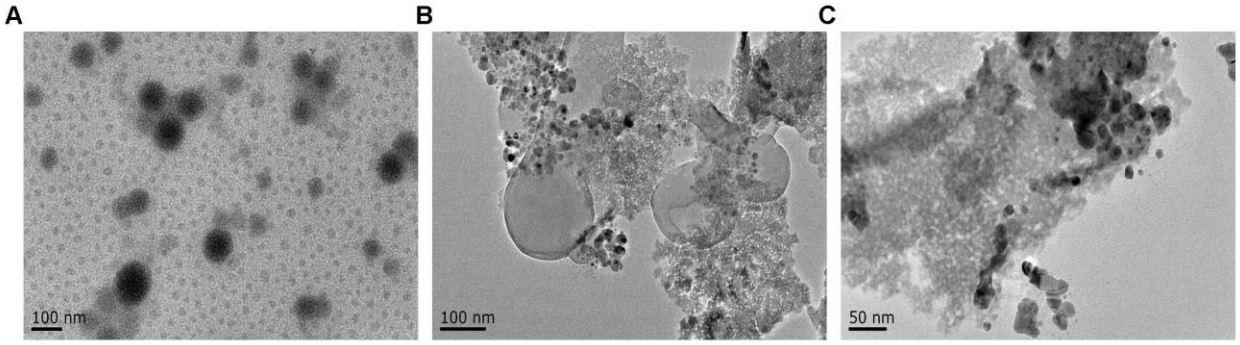

Figure S6. The morphological changes of HR_{RAP} NPs monitored by TEM. (A) Representative TEM image of HR_{RAP} NPs after incubation at pH 7.4 for 48 h at 37°C. (B) Representative TEM image of HR_{RAP} NPs after incubation at pH 5.2 for 24 h at 37°C. (C) Representative TEM image of HR_{RAP} NPs after incubation at pH 5.2 for 48 h at 37°C.

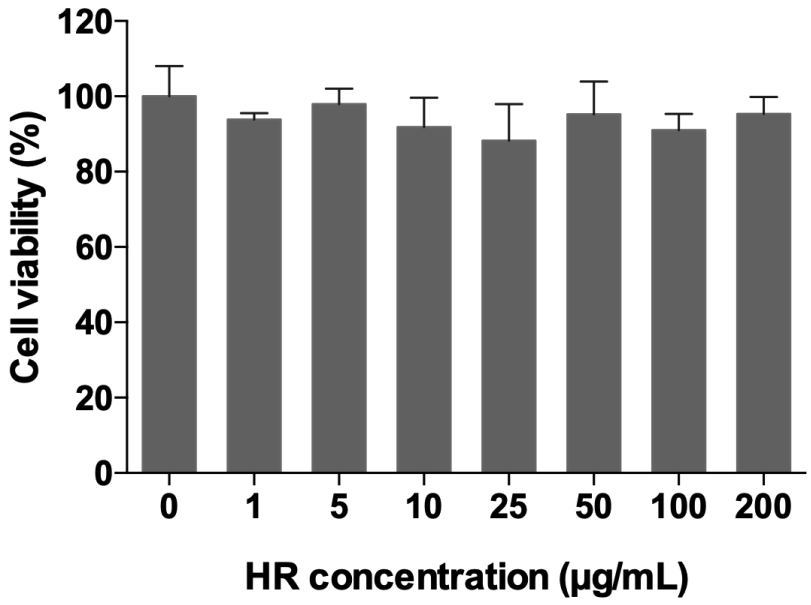

Figure S7. *In vitro* cytotoxicity evaluation of pH-sensitive HR conjugate. Cell viability values of human foreskin fibroblasts (HFF-1) after incubation with various concentrations of HR conjugate at 37 °C for 48 h. Cell viability were assessed by Cell Counting Kit-8 (CCK-8) assay. Bars represent mean ± SD (n=3).

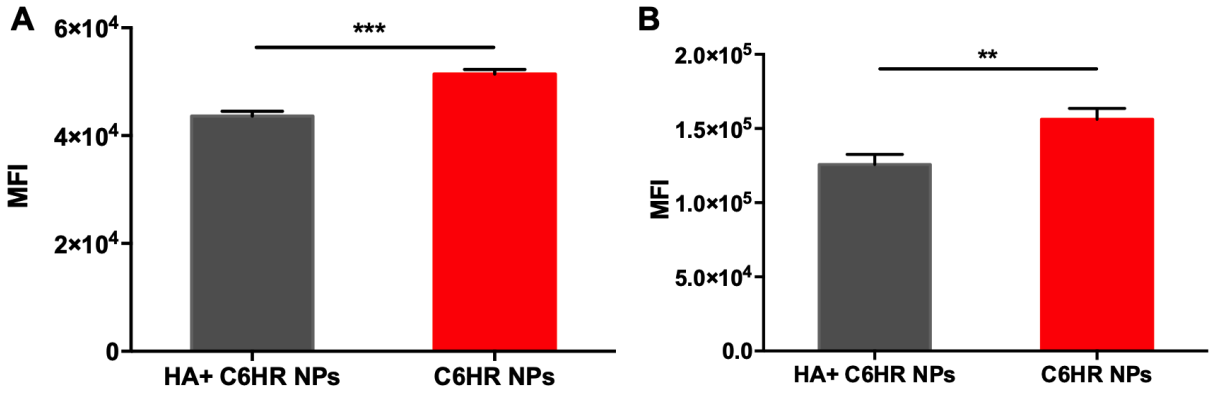

Figure S8. Cellular uptake of HR NPs after pre-treatment with HA. Quantified mean fluorescent intensity (MFI) of the uptake of C6 HR NPs (A) in LPS-activated macrophages RAW264.7 and (B) TNF-α-activated HUVECs after pre-treatment with HA (10 mg/mL) for 1 h prior incubation with 20 μg/mL C6 HR NPs for 2 h at 37 °C. Bars represent mean ± SD (n=3); $^{**}p < 0.01$; $^{***}p < 0.001$ vs. HA pre-treated group.

Figure S9. Schematic illustration of the treatment protocol of various formulations in ApoE-/- mice. ApoE-/- mice were fed a high fat diet (HFD) diet for 12 weeks. After the first 4 weeks, all mice received various treatments by i.v. injection every three days for additional 8 weeks.

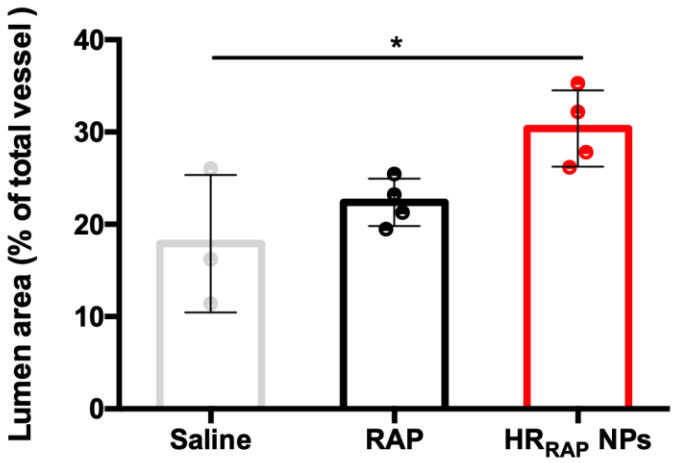

Figure S10. Quantitative analysis of the lumen area relative to the total vessel of the aortic sinus section from ApoE-/- mice subjected to various treatments. ApoE-/- mice were fed a high fat diet (HFD) diet for 12 weeks. After the first 4 weeks, all mice received various treatments by i.v. injection every three days for additional 8 weeks. Mice treated with saline represent the control group. Bars represent mean ± SD (n = 4-5); *: $p < 0.05$ vs. any other group.

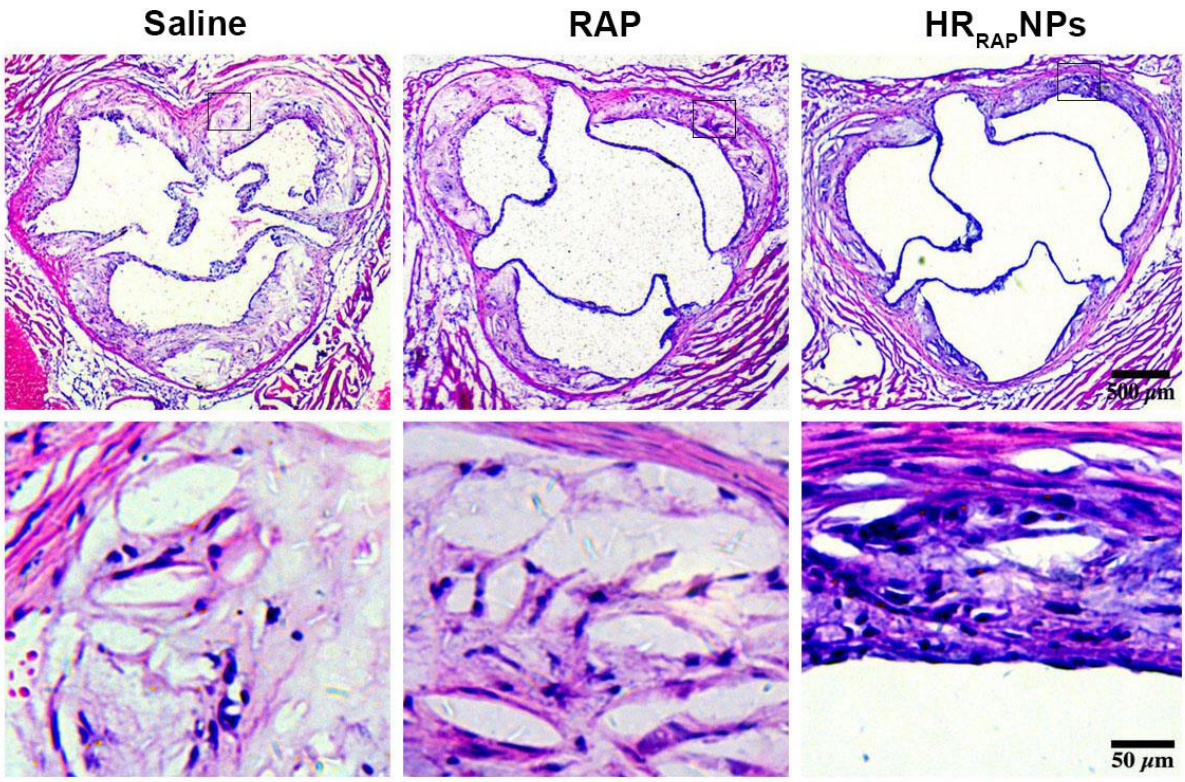

Figure S11. H&E stained sections of the aortic roots from ApoE−/− mice subjected to various treatments. ApoE-/- mice were fed a high fat diet (HFD) diet for 12 weeks. After the first 4 weeks, all mice received various treatments by i.v. injection every three days for additional 8 weeks. Mice treated with saline represent the control group. The black boxes indicate the magnified locations, while images in the second row show the magnified plaques. Scale bar: 500 μm for the first row. Scale bar: 50 μm for the second row.

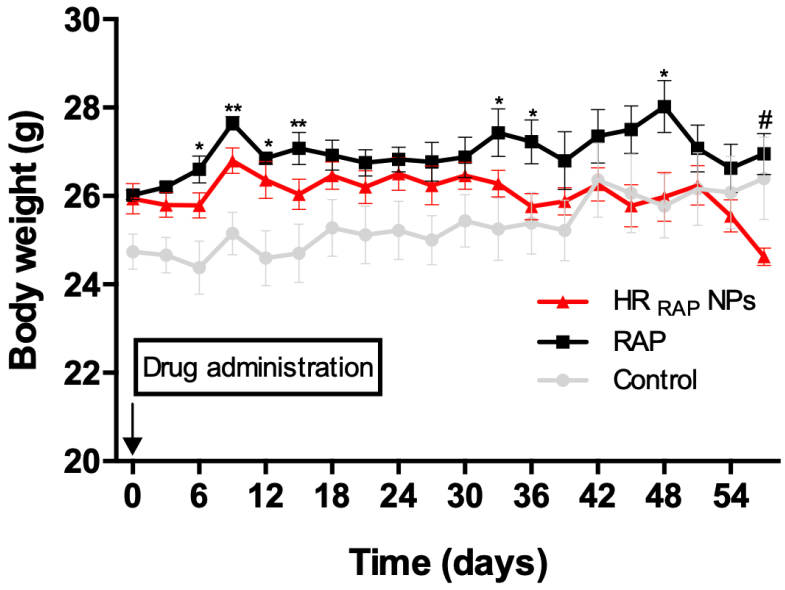

Figure S12. Body weight changes of ApoE mice-/- during the treatment. ApoE-/- mice were fed a high fat diet (HFD) diet for 12 weeks. After the first 4 weeks, all mice received various treatments by i.v. injection every three days for additional 8 weeks. Mice treated with saline represent the control group. Bars represent mean ± SD (n=3-4); $^{*}p < 0.05$; $^{**}p < 0.01$ vs. control group; $^{\#}p < 0.05$ vs HR_{RAP}NPs treated group.

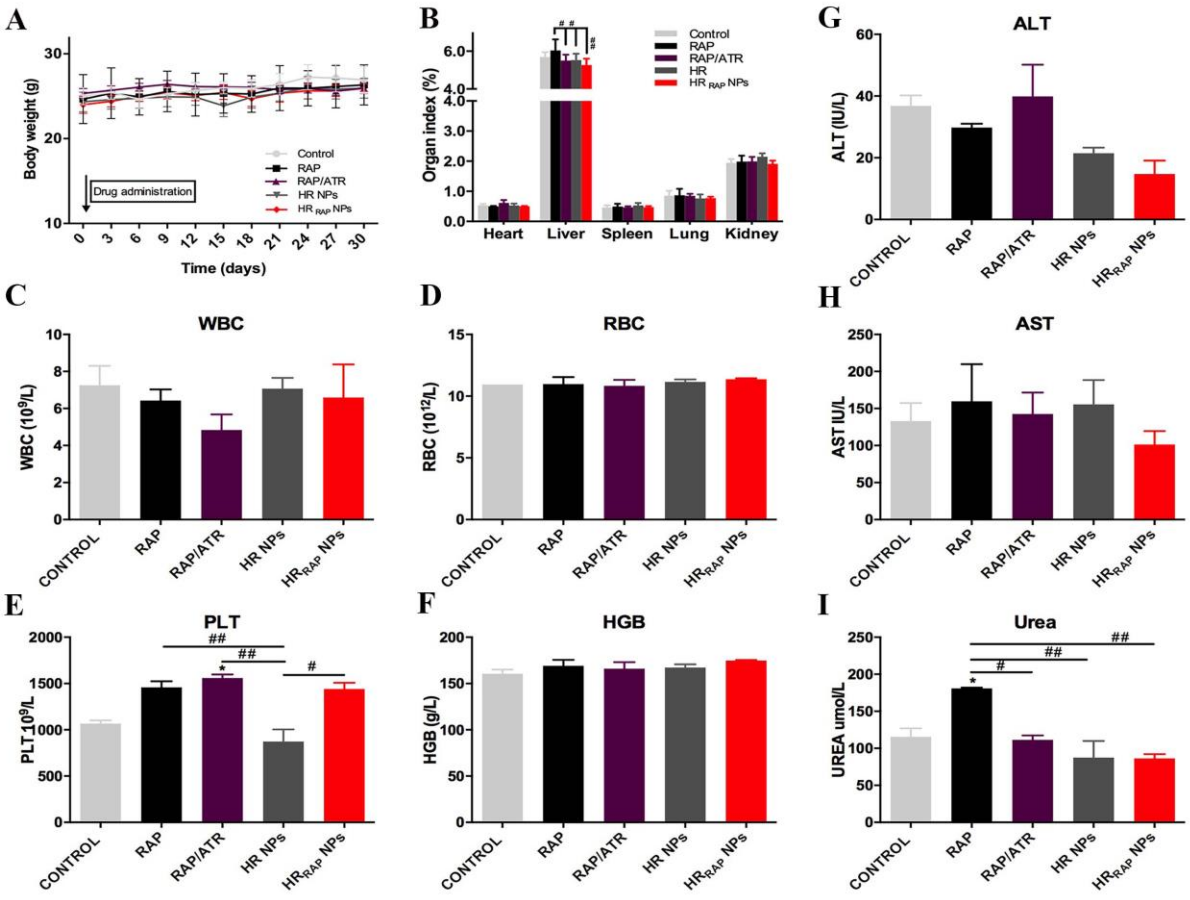

Figure S13. Biosafety evaluation in BALB/c mice subjected to various treatments. (A) Body weight changes of BALB/c mice subjected to various treatment during one month. (B) Organ indexes. (C-F) Hematological parameters: WBC, red blood cell; WBC, white blood cell; PLT, platelet and HGB, hemoglobin. (G-I) Biochemical markers relevant to hepatic and kidney functions: ALT, alanine aminotransferase; AST, aspartate aminotransferase; UREA. BALB/c mice were fed a normal diet for one month. Bars represent mean ± SD (n=3); $^{*}p < 0.05$ vs. control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs treated group.

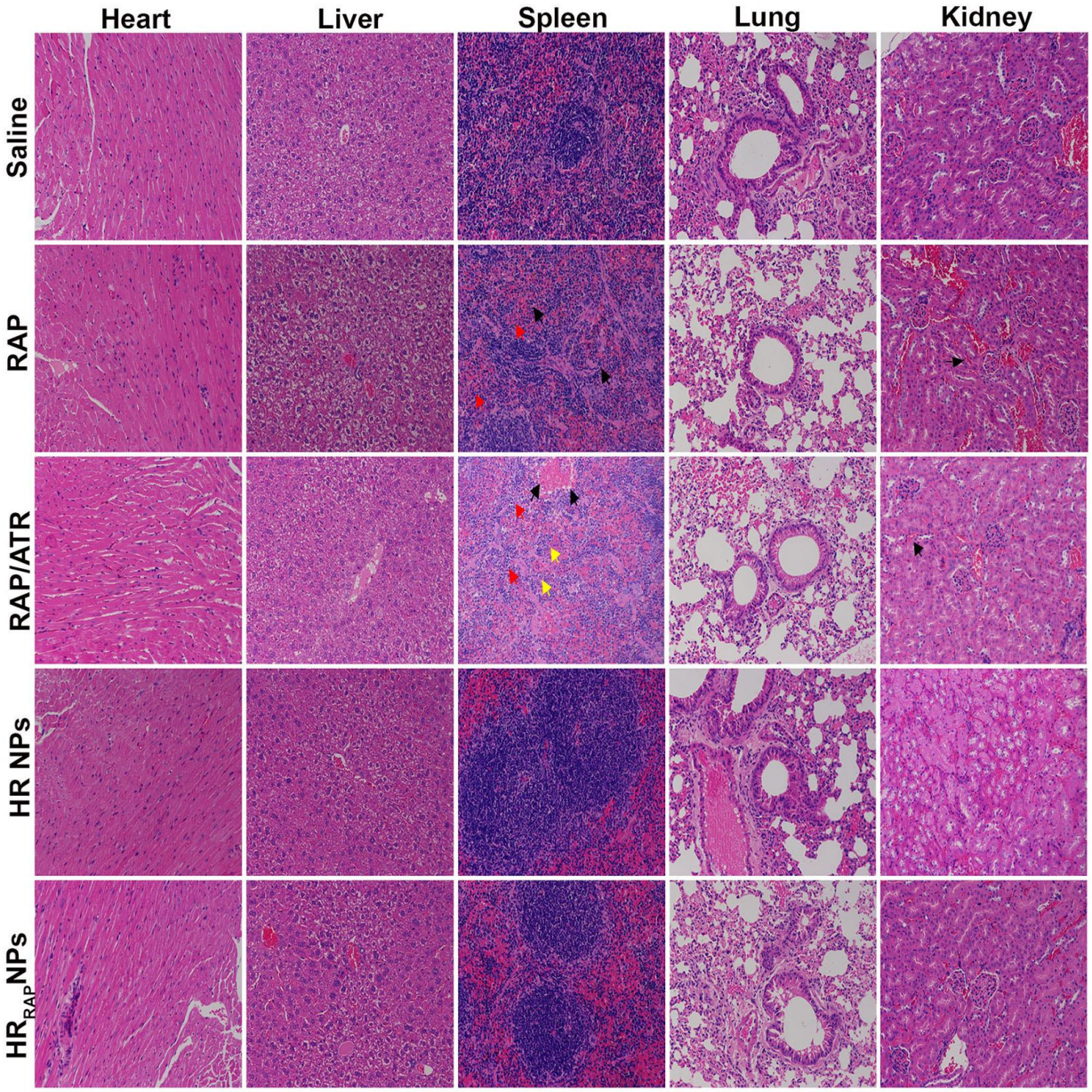

Figure S14. Representative images of H&E stained major organs resected from BALB/c mice subjected to various treatment for one month. BALB/c mice were fed a normal diet for one month. Organs including heart, liver, spleen, lung, and kidney were resected after one month of various treatments. All the micrographs were acquired at ×200 magnification.

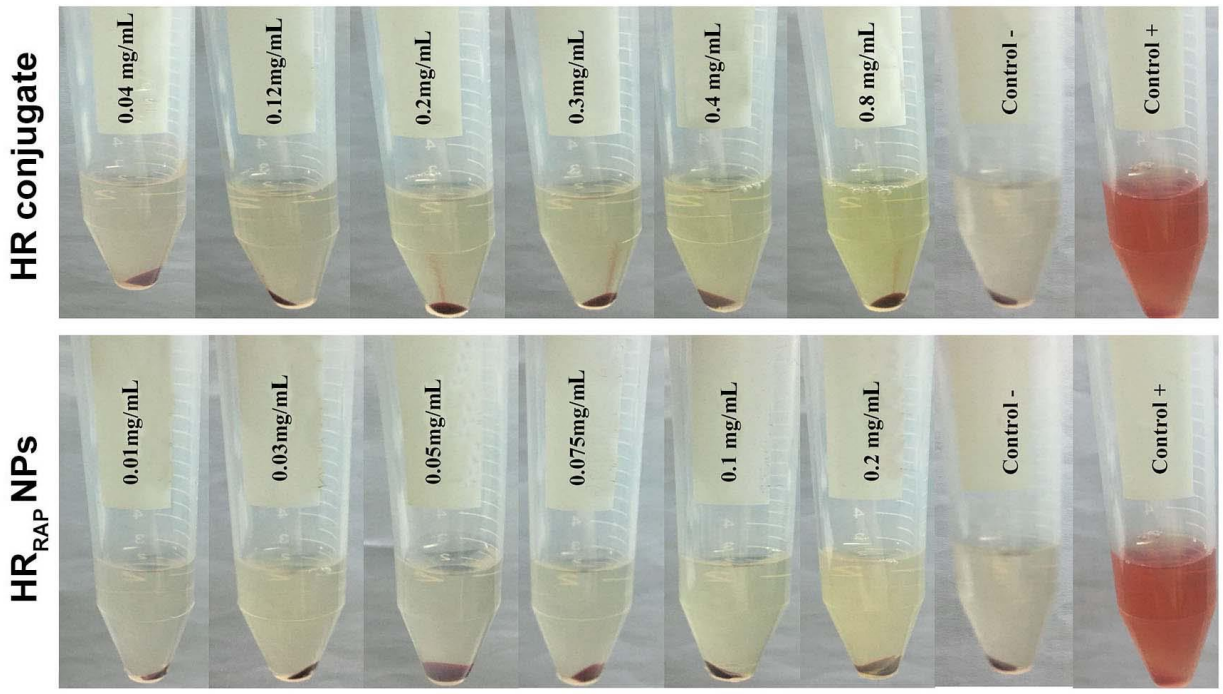

Figure S15. Images of hemolysis study of HR conjugate as vehicle and HR_{RAP} NPs after incubation with 2% of mice red blood cells (RBCs) suspension for 1 h at 37°C. Control + corresponds to 100% hemolysis. Control - corresponds to 0% hemolysis.

Supplementary methods

Materials. Hyaluronic Acid (HA, Mw 10 kDa) was purchased from Freda Biochem Co., Ltd. (Shandong, China). All Trans Retinal (ATR) was supplied by Shanghai Macklin Biochemical Co. Ltd (Shanghai, China). Adipic Dihydrazide (ADH), Coumarin-6 and Pyrene, were purchased from Alladin Co. Ltd (Shanghai, China), Rapamycin (RAP) were purchased from Wegene Inc. (Shanghai, China). 1-ethyl-3-(3-dimethylaminopropyl)-carbomiimide (EDC) was purchased from Sigma Chemical Co. Ltd (USA). DMEM and penicillin/streptomycin were supplied by Wisent Inc (Nanjing, China). Lysotracker Red, human TNF-α and ROS Assay Kit were supplied by Yeasen Biological and Technologies Co., Ltd. (Shanghai, China). Lipopolysaccharides (LPS) was obtained from Lianke Biotech, Co., Ltd. (Hangzhou, China). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies Inc. (Shanghai, China). Hoechst33342 was purchased from Sigma (USA). Red oil solution was purchased from Servicebio technology Co.Ltd (Wuhan, China). Masson trichrome solutions was obtained from (Sbjbio life science, Nanjing, China). Rat anti-CD68 (ab53444) was from Abcam (USA). Polyclonal rabbit anti-MMP-9 antibody (10375-2-AP) and Polyclonal rabbit α-SMA (55135-1-AP) were from proteintech (Wuhan, China). goat anti-rabbit IgG Alexa Fluor-488 and goat anti-rat IgG Alexa Fluor-594 were from abcam (USA). All other chemicals were of analytical grade and were used without further purification.

Synthesis of pH-sensitive HR conjugates. pH sensitive HR conjugate material was synthesized by sequentially conjugating adipic dihydrazide (ADH) and all trans retinal (ATR) to hyaluronic acid (HA). HA functionalized with ADH (HA-ADH) was first synthesized. Briefly, 200 mg of HA (0.5 mmol) was dissolved in deionized (DI) water (4 mg/mL), then a 10-fold molar excess of ADH was added into the solution and stirred for 30 min. The pH of the reaction mixture was

adjusted to 4.75 by addition of 0.1 N HCl followed by the addition of 0.096 g EDC in solid form to initiate the reaction. The reaction was conducted under stirring for 1 h and the pH was maintained at 4.75 with 0.1 M HCl. To quench the reaction, the pH was adjusted to 7.0 by the addition of 0.1 M sodium hydroxide. The resulting solution was then transferred to pretreated membrane tubing (MWCO 3500) and dialyzed exhaustively against 0.1 M NaCl, then 25% EtOH/water and finally DI water. HA-ADH polymer solution was filtered through a 0.22 μm pore-sized microporous membrane then stored at 4ºC after being lyophilized.

ATR was subsequently conjugated to HA-ADH through pH-responsive hydrazone bond (Figure S1). Briefly, HA-ADH (100 mg, 0.2 mmol) was dissolved in 25 mL formamide by gentle heating then different amounts of ATR dissolved in DMF, in which the molar ratio of ATR to HA-ADH varied from 0.5 to 4, were added dropwise under magnetic stirring. The reaction was allowed to proceed for 24 h at room temperature under nitrogen atmosphere with light protection, in the presence of 3A° molecular sieve. The resulted solution was extensively dialyzed against excess amount of methanol (until no color change was observed) and DI water for 2 days with membrane tubing (MWCO 3500). Afterwards, the solution was centrifuged at 10000 rpm for 5 min prior to supernatant lyophilization. After being freeze-dried, the final products were obtained as a yellow powder and stored in light protected containers at 4ºC until further use.

Characterization of pH-sensitive HR conjugates. The chemical structure was characterized by proton nuclear magnetic resonance spectra (^{1}H NMR, 500 MHz, Bruker, Germany) and Fourrier transform infrared spectroscopy (FTIR) spectra (PerkinElmer, USA). The UV-vis absorption spectra of the conjugates were monitored at 250–500 nm (Shanghai Mapada Instruments, China). The degree of substitution (DS), defined as the number of ATR molecules per 100 sugar units of

HA polymer, was estimated using UV measurements based on a standard curve of ATR ranged from 1 to 10 μg/mL in 80% DMSO: Water 20% (λ =381nm).

Critical micelle concentration (CMC). CMC of HR conjugates was determined in water by fluorescence spectroscopy using pyrene as fluorophore[1]. Briefly, to a series of 10 mL volumetric flasks, 100 μL of pyrene acetone solution (6×10^{-5}M) was added, acetone was allowed to evaporate under vacuum. Then HR conjugates in aqueous solutions with serial concentrations ranging from 1×10^{-4} to 1 mg/mL were added into each vial and the final pyrene concentration was 6×10^{-7}M. The samples were sonicated for 30 min prior incubation at 50°C for 1 hour then left to equilibrate overnight at room temperature. The excitation spectra of pyrene were recorded by fluorescence spectrometer (Shimadzu, Kyoto, Japan) using an emission wavelength of 375 nm, and slit widths of both excitation and emission were 5 nm. The intensity ratios (I_{339}/I_{333}) were calculated and plotted against the logarithmic concentration values of the HR conjugates and the CMC was estimated as the cross-point of two linear regressions at low and high concentration regions.

pH-dependent disassembly of HR NPs. The pH sensitive propriety of HR NPs was examined by monitoring the size distribution changes in acidic pH over time. Briefly, 1 mL of HR NPs suspensions were dispersed in PBS buffers at pH 7.4, 6.5 and 5.2, and incubated at 37 °C. After 24 h, the size distribution was detected using DLS measurement. Furthermore, the morphology changes of HR NPs in acidic pH 5.2 was also observed by AFM (Bioscope resolve, USA) after incubation at different time points at 37°C.

Hemolysis study. To evaluate the safety of HR_{RAP}NPs i.v. injection, hemolysis study was conducted using BALB/c mice red blood cells (RBC) suspension. Briefly, Different volumes of

HR_{RAP}NPs were added to 2 % RBC suspension. Then normal saline was added to each tube to obtain different RAP concentrations. For comparison, RAP, RAP plus ATR and HR conjugate solutions were also prepared corresponding to their equivalent concentrations used in HR_{RAP}NPs samples. The positive (100% hemolysis) and negative (0% hemolysis) controls were prepared by mixing water and normal saline with 2% RBC suspension, respectively. Subsequently, all samples were incubated at 37°C for 1 h and centrifuged at 3000 rpm for 10 min. The percentage of hemolysis was determined by measuring the absorbance of supernatant at 540 nm according to following equation:

$$\text{Hemolysis}\ (\%) = (\text{A sample} - \text{A}\ 0\%)/(\text{A}100\% - \text{A}0\%) \times 100\%$$

Biosafety evaluation in BALB/c mice. To evaluate the acute toxicity of HR_{RAP}NPs, healthy BALB/c mice were randomly divided into 5 groups (n=3) and were injected intravenously with saline, RAP (3 mg/kg), RAP/ATR (3 mg/kg of RAP), HR_{RAP}NPs (3 mg/kg of RAP) and HR NPs (equivalent to HR in HR_{RAP}NPs) every 3 days for 30 days. Body weight and behaviors of the mice were monitored during the whole treatment period. After 24 h from the last injection, blood was collected in EDTA tubes to quantify hematological parameters and in normal tubes to evaluate serum levels of biochemical markers including alanine transaminase (ALT), aspartate transaminase (AST) and urea (UREA). The mice were scarified after three days from the last injection, and primary organs heart, liver, spleen, lung and kidney were collected, weighed and fixed in 4% paraformaldehyde for H&E staining. The organ index was calculated according to the equation:

$$\text{Organ Index}\ (\%) = \text{Organ weight}\ (\text{g})/\text{Body weight}\ (\text{g}) \times 100\%$$

Supplementary references

1. Liu, S.-Q.; Wiradharma, N.; Gao, S.-J.; Tong, Y. W.; Yang, Y.-Y., Bio-functional micelles self-assembled from a folate-conjugated block copolymer for targeted intracellular delivery of anticancer drugs. *Biomaterials* **2007,** *28* (7), 1423-1433.