Supplementary Information (SI) for Journal of Materials Chemistry B.

A pH-responsive fluorescence nanosystem for imaging lipid deposition in diseased aortic valves

Shufen Li, [a,b] Tianyi Qu,[a] Zheng Chai,[a,c] Dongxu Yang,[a] Ming Chen,[a,c] Ying Zhang,[a,c] Chengming Li,[d] Yongchao Yao,[e] Weihua Zhuang,*[a,b,e] Wenchuang (Walter) Hu[e] and Mao Chen[a,b,c]

[a] Laboratory of Cardiac Structure and Function, Institute of Cardiovascular Diseases, West China Hospital, Sichuan University, Chengdu, 610041, China.

[b] Cardiac Structure and Function Research Key Laboratory of Sichuan Province, West China Hospital, Sichuan University, Chengdu, 610041, China.

[c] Department of Cardiology, West China Hospital, Sichuan University, Chengdu, 610041, China.

[d] Department of Liver Transplantation Center & Institute of Organ Transplantation, West China Hospital, Sichuan University, Chengdu 610041, China.

[e] Precision Medicine Translational Research Center, West China Hospital, Sichuan University, Chengdu, 610041, China.

* Corresponding author, Email address: weihuaz@scu.edu.cn.

Experimental

Experimental

Materials and methods

Materials

2,2'-Azobis(2-methylpropionitrile) (AIBN), 4-Chloro-7-nitrobenzofurazan, (9-Ethyl-9H-carbazol-3-yl)boronic acid, poly(ethylene glycol) methyl ether (4-cyano-4-pentanoate dodecyl trithiocarbonate) (PEG-CPDT), cesium Fluoride (CsF), 2-phenylethyl methacrylate (PMA) and bis(tri-tert-butylphosphine)palladium(0) were obtained from Adamas Reagent, Ltd. (Shanghai, China). All other chemicals and solvents were used as received without further purification.

Characterization

NMR spectra were acquired on a Bruker AVANCE NEO 400 spectrometer. High-resolution mass spectra (HRMS) were obtained with a Shimadzu LCMS-IT-TOF (ESI). Absorption spectra were obtained on a HITACHI U-2910 spectrometer. Fluorescence emission spectra were obtained via a Hitachi 4700 fluorescence spectrometer. The particle size and Zeta potential were measured on a Zetasizer Pro (ZSU3200). The observation of cells and tissues was performed on a Leica Stellaris 5 confocal laser scanning microscope (CLSM).

Synthesis of the lipid-specific probe ECNBD

Under an argon (Ar) atmosphere, bis(tri-tert-butylphosphine)palladium(0) (42.76 mg, 0.084 mmol), CsF (1.14 g, 7.52 mmol), 4-Chloro-7- (9-Ethyl-9H-carbazol-3-yl)boronic acid (0.63 g, 2.63 mmol) and nitrobenzofurazan (0.50 g, 2.51 mmol) were added to a 120 mL Schlenk flask with a magnetic stirrer. A mixed solution of 15 mL tetrahydrofuran (THF) and 1.5 mL H_2O was added, and the resulting solution was stirred at 75 ^oC for 24 h under an Ar atmosphere. After cooling to room temperature, the solution was removed via rotary evaporation and redissolved in dichloromethane (DCM), followed by filtration and concentration. The purified product ECNBD was obtained via flash column chromatography (DCM : n-hexane = 1:1, V:V), with a yield of 86.5% (reddish brown). ^1H NMR (400 MHz, DMSO-d_6): δ (ppm) 9.05 (s, 1H), 8.77-8.89 (d, 1H), 8.29-8.34 (m, 2H), 8.15-8.17 (d, 1H), 7.87-7.89 (d, 1H), 7.70-7.72 (d, 1H), 7.53-7.57 (t, 1H), 7.29-7.33 (t, 1H), 4.52-4.57 (m, 2H), 1.36-1.39 (t, 3H). ^{13}C NMR (DMSO-d_6, 100 MHz): δ (ppm) 150.39, 144.27, 141.56, 140.75, 138.71, 133.87, 133.39, 127.50, 127.18, 126.41, 124.81, 123.43, 122.75, 122.32, 121.30, 120.33,

110.55, 110.30, 37.79, 14.26. HRMS (ESI+): calcd for $C_{20}H_{15}N_4O_3^+$ $(M+H)^+$ 359.11387, found 359.11383.

Optical properties of ECNBD

The absorption spectra and emission spectra of ECNBD in different solutions were studied at an ECNBD concentration of 10 μM.

Synthesis of the amphiphilic polymer PEG-P (AEMA-*co*-PMA)

AEMA was prepared according to previous work [39]. The amphiphilic PEG-P (AEMA-*co*-PMA) block copolymer was prepared via reversible addition-fragmentation chain transfer (RAFT) polymerization with PEG-RAFT (item number: 751626, Sigma-Aldrich, Mn = 5400 g/mol) as the RAFT agent and AEMA and PMA as monomers. Typically, PEG-CPDT (1.00 g, 0.185 mmol), AEMA (312.60 mg, 1.48 mmol), AIBN (10.619 mg, 0.0647 mmol) and PMA (351.9 mg, 1.85 mmol) were added to a 120 mL Schlenk flask, and 5 mL N, N-Dimethylformamide (DMF) was added. The resulting solution was evacuated and backfilled with Ar five times. After stirring at 65 °C for 24 h, the solution was added dropwise to cold diethyl ether, filtered and dried via vacuum (82.5% yield).

Preparation of ECNBD-loaded nanoparticles

ECNBD-loaded nanoparticles were prepared via a dialysis method. Typically, 20 mg of PEG-P (AEMA-*co*-PMA) and 2 mg of ECNBD were dissolved in a mixed solution of 2 mL of DMF and 1 mL of THF, and the mixed solution was quickly added to 9 mL of stirred H_2O (pH 7.4). The resulting solution was stirred for 15 min, followed by dialysis against deionized water (pH 7.4, MWCO = 2 000) for 24 h. The concentration of the polymer was set to 1 mg/mL, and the mixture was filtered via a 0.22 μm filter membrane. The loading content and loading efficacy of the probe were measured via UV-visible spectroscopy and calculated according to the following formulas, respectively. Blank nanoparticles were prepared without the addition of ECNBD.

$$loading\ content = \frac{wieght\ of\ probe\ loaded}{eight\ of\ polymer\ +\ weight\ of\ probe\ loaded} \quad ①$$

$$loading\ efficacy = \frac{wieght\ of\ probe\ loaded}{weight\ of\ probe\ added} \quad ②$$

Optical properties of ECNBD-loaded nanoparticles

The optical properties of the ECNBD-loaded nanoparticles were studied via UV-visible spectroscopy and fluorescence spectrometery at different pH values.

Stability studies of ECNBD-loaded nanoparticles

The stability of the ECNBD-loaded nanoparticles was studied via dynamic light scattering (DLS, Malvern, Zetasizer Pro) at different storage time intervals. The morphology of nanoparticles was observed via transmission electron microscopy (TEM). The changes in the particle size and Zeta potential of the nanoparticles at different pH values were studied via DLS.

***In vitro* ECNBD release study**

The release behavior of ECNBD from ECNBD-loaded nanoparticles was studied via a dialysis method and measured via UV-visible spectroscopy. Typically, 2 mL of ECNBD-loaded nanoparticles (1 mg/mL) were transferred to dialysis bags (MWCO = 2000) and the bags were immersed into 30 mL of release medium with different pH values (7.4 and 6.5). The release system was shaken in the dark, and 2 mL of release medium was collected at preselected time intervals and 2 mL of fresh medium was added. The released of the probe was measured via UV-visible spectroscopy.

Cytotoxicity of ECNBD and ECNBD-loaded nanoparticles

The potential cytotoxicity of ECNBD, blank nanoparticles and ECNBD-loaded nanoparticles was studied via an MTT assay (Cat No. 40206ES80, Yeasen, Shanghai, China) against mouse valve interstitial cells (VICs) and HeLa cells. VICs were obtained according to our previous work. A total of 5 000 cells per well were seeded in 96-well plates and incubated for 24 h. Then, the culture media were removed, and fresh culture media containing different concentrations of ECNBD, blank nanoparticles or ECNBD-loaded nanoparticles were added. After incubation for 24 h, the relative viability of cells was measured via the MTT assay.

Cellular imaging of ECNBD and ECNBD-loaded nanoparticles

The lipid droplet (LDs) specific imaging ability of ECNBD was studied by co-staining HeLa cells with BODIPY 493/503 Green. Oleic acid (20 μM) was used to pretreated HeLa cells for 2 h, and the cells were stained with ECNBD (5 μM) for 1 h. Then, HeLa cells were co-stained with BODIPY 493/503 Green (100 nM) for 30 min. Before being observed via CLSM, the cells were washed with PBS three times. Moreover, HeLa cells were stained with different concentrations of ECNBD to further study its imaging ability.

To study the cell imaging ability of the ECNBD-loaded nanoparticles, the cells were incubated with the nanoparticles (ECNBD concentration of 5 μM) for different time intervals (0.5 h, 1 h and 2 h), followed by staining with LysoTracker Green (1 μM) or BODIPY 493/503 Green (100 nM) for 30 min. Before being observed via CLSM, the cells were washed with PBS three times.

Tissular imaging of ECNBD and ECNBD-loaded nanoparticles

The study of diseased human heart valves was approved by the Ethical Research Committee of West China Hospital, Sichuan University. To study the tissue LD imaging of ECNBD, a diseased human aortic valve was stained with ECNBD (1 μM) and BODIPY 493/503 Green (100 nM) for 1 h. The valve was observed via CLSM after being washed with PBS three times. To further study the *in vivo* heart valve-targeted imaging ability of ECNBD-loaded nanoparticles, genetically engineered apo-lipoprotein E-deficient ApoE$^{-/-}$ (male) mice were used to construct an animal model. All animal experiments were approved by Ethical Research Committee of the West China Hospital, Sichuan University (20220303071), and complied with relevant national regulations strictly. ApoE$^{-/-}$ (male) mice were supplied by Beijing Weitong Lihua Experimental Animal Technology Co., Ltd., and housed in an SPF class animal facility and fed a high-fat diet. After 6 months, the mice were treated with ECNBD-loaded nanoparticles (at a ECNBD dosage of 1 mg/kg) via tail vein injection. After 24 h, the mice were sacrificed, the hears were carefully isolated, and the aortic valves were carefully exposed under a microscope. The aortic valves were imaged via CLSM.

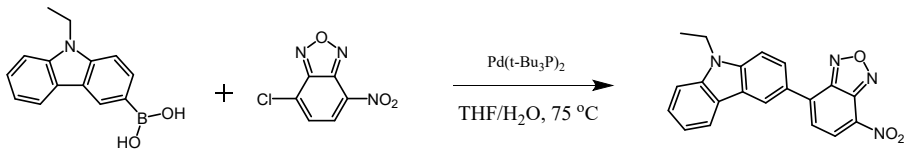

Fig. S1. Synthetic route of ECNBD.

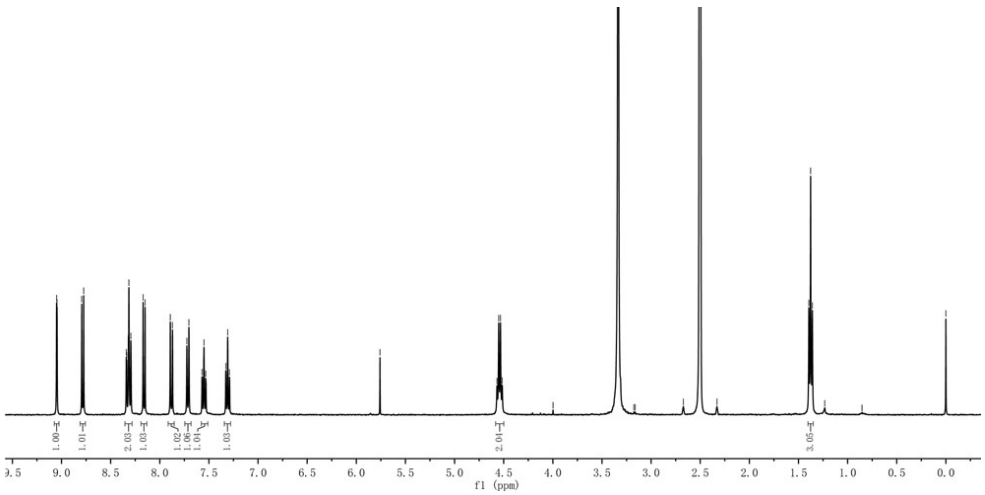

Fig. S2. ^{1}H NMR spectrum of ECNBD in DMSO-d_6.

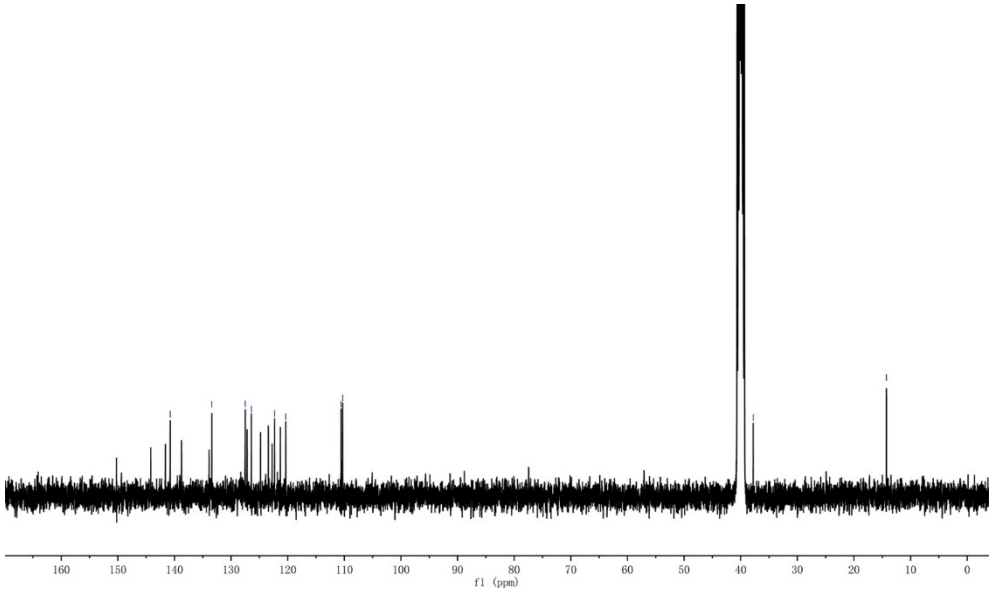

Fig. S3. ^{13}C NMR spectrum of ECNBD in DMSO-d_6.

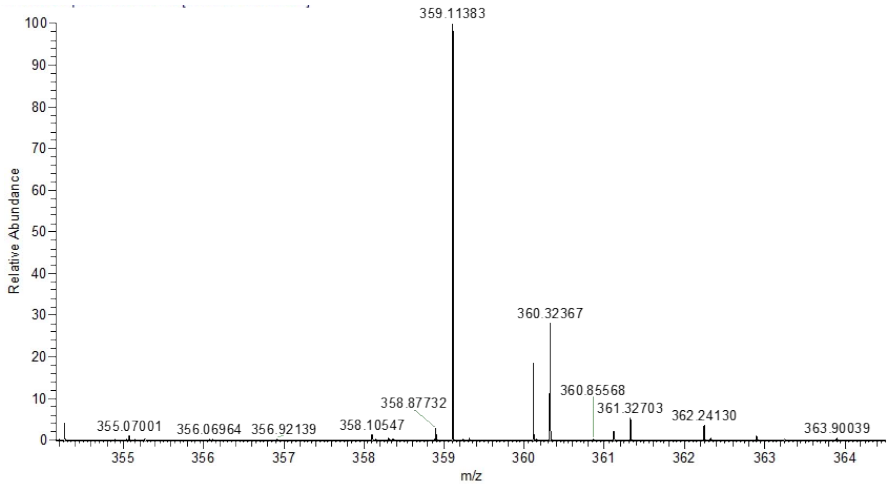

Fig. S4. HRMS spectrum of ECNBD.

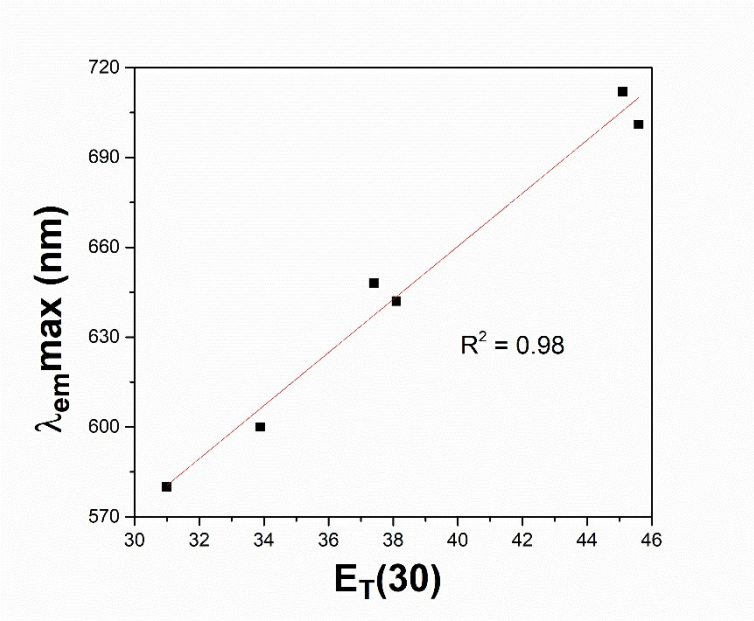

Fig. S5. Linear relationship between the maximum emission wavelength and the solvent's polarity. n-hexane: 31; toluene: 33.9; THF: 37.4; EA: 38.1; DMSO: 45.1; ACN: 45.6.

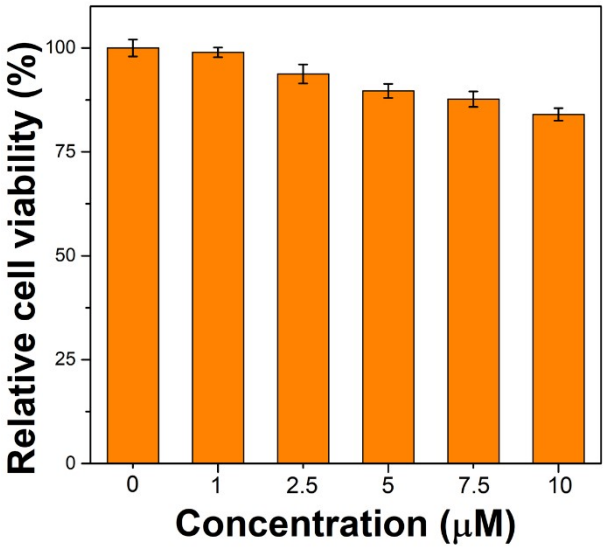

Fig. S6. Relative cell viability of HeLa cells co-cultured with different concentrations of ECNBD for 24 h.

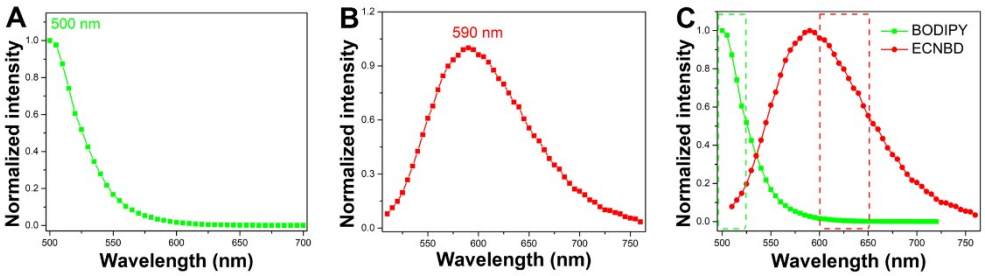

Fig. S7. Normalized fluorescence intensity of BODIPY 493/503 Green (A) and ECNBD (B) stained HeLa cells at different imaging wavelengths. (C) The green box represented the emission range of BODIPY 493/503 Green when taken fluorescence images, the red box represented the emission range of ECNBD when taken fluorescence images.

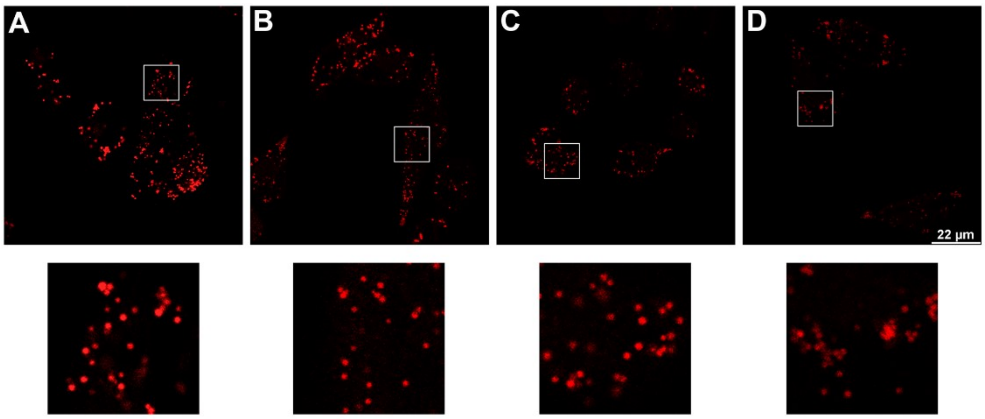

Fig. S8. CLSM images of ECNBD stained HeLa cells with different ECNBD concentrations: (A) 10 μM, (B) 1 μM, (C) 500 nM and (D) 200 nM. λ_{ex} = 485 nm. Scale bar: 22 μm.

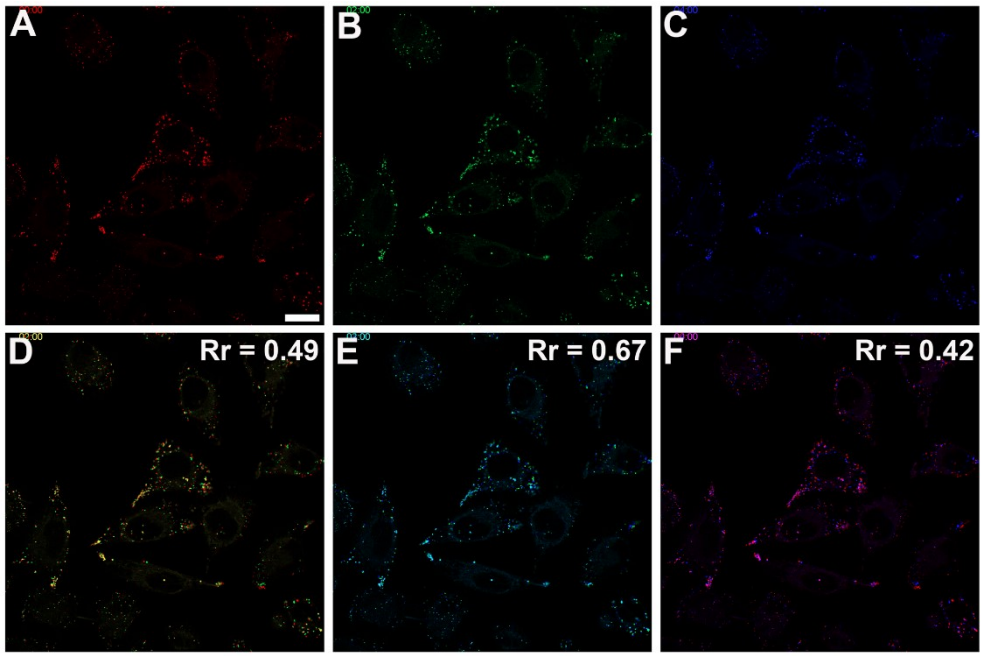

Fig. S9. CLSM images of ECNBD stained HeLa cells at different imaging time points. The fluorescence signals at 0 min, 2 min and 4 min were adjust to red (A), green (B)

and blue (C), respectively, to better understand the dynamic movement of LDs. (D) the merge image of A and B, (E) the merge image of B and C, (F) the merge image of A and C. Scale bar: 20 μm. HeLa cells were imaged every 5 seconds.

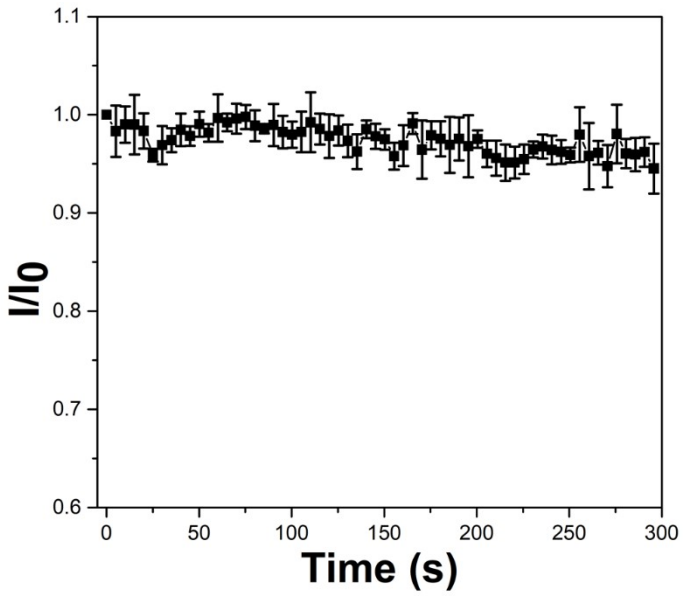

Fig. S10. Photostability of ECNBD: the ratio of the fluorescence intensity (I) of the images of ECNBD stained HeLa cells at different time points to the initial value (I_0).

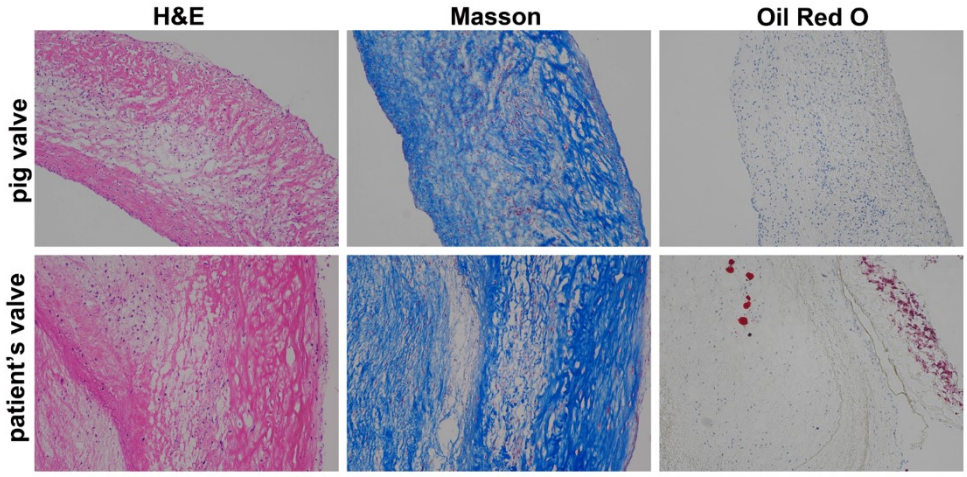

Fig. S11. Images of H&E, Masson and Oil Red O staining histologic sections of valve from a pig (top) and a patient (down). All sections 100X.

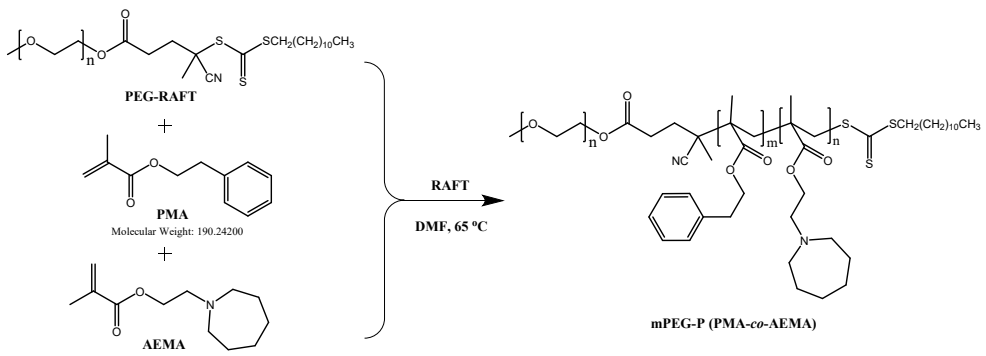

Fig. 12. Synthetic route of polymer mPEG-P (PMA-*co*-AEMA).

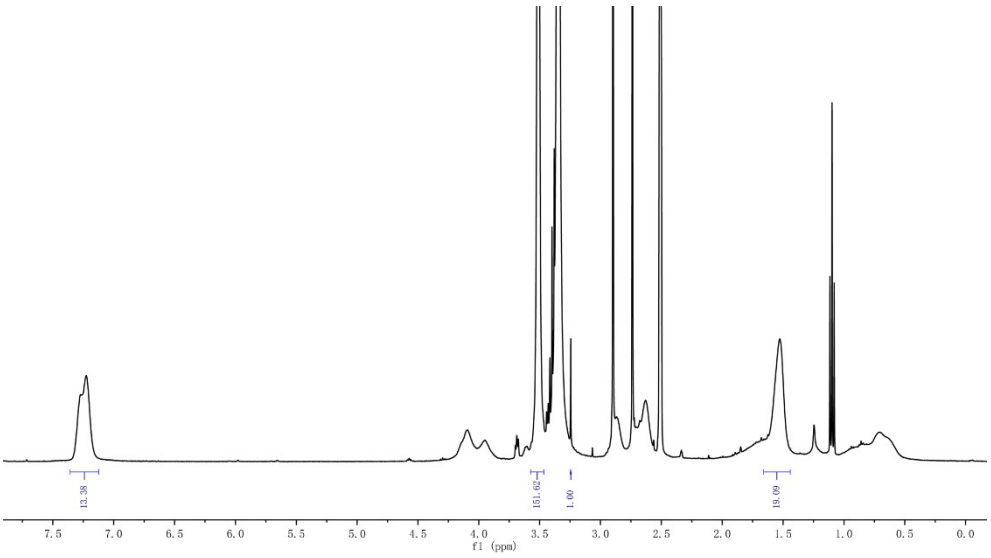

Fig. S13. ^{1}H NMR spectrum of PEG-P (AEMA-*co*-PMA) in DMSO-d_6.

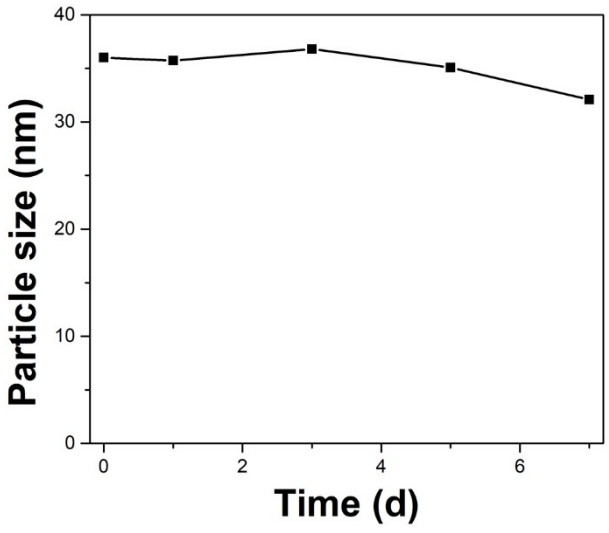

Fig. S14. Changes in particle size of ECNBD-loaded nanoparticles over time.

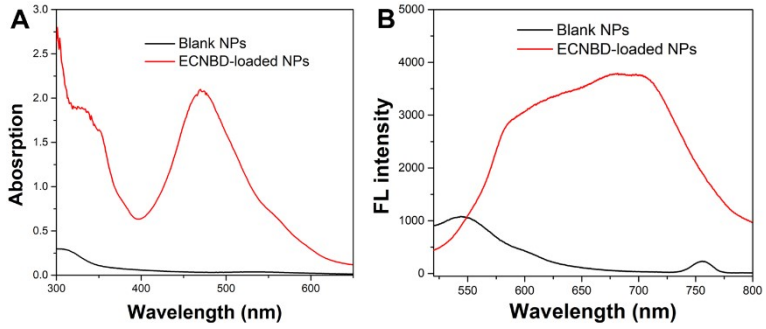

Fig. S15. Absorption spectra (A) and emission spectra of blank nanoparticles and ECNBD-loaded nanoparticles at pH 7.4.

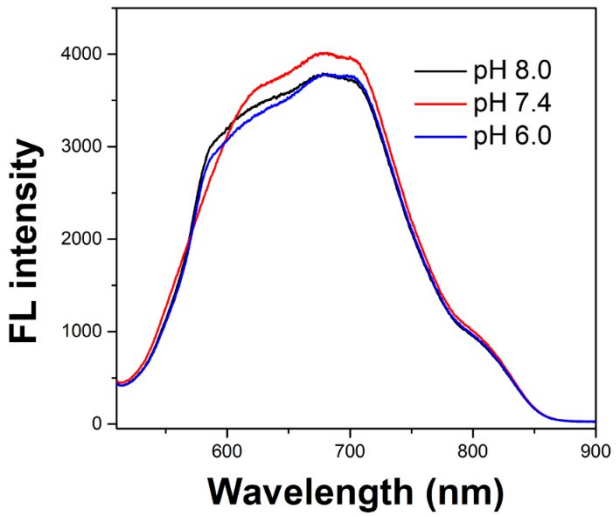

Fig. S16. Emission spectra of ECNBD-loaded nanoparticles at different pH.

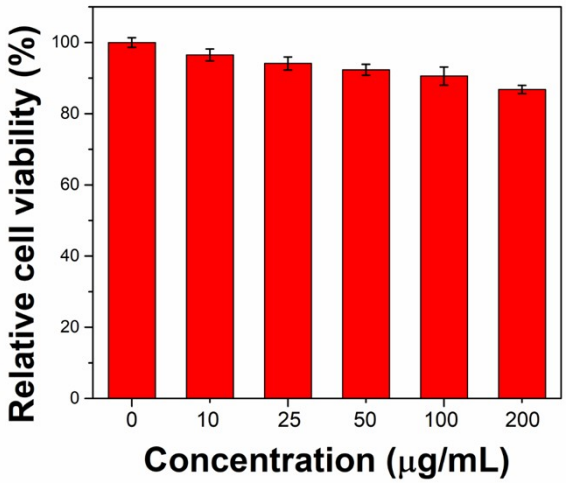

Fig. S17. Relative cell viability of blank nanoparticles against HeLa cells after co-incubation for 24 h.

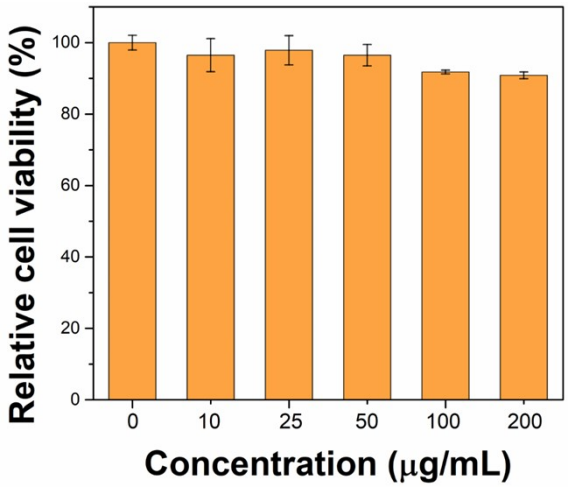

Fig. S18. Relative cell viability of ECNBD-loaded nanoparticles against HeLa cells after co-incubation for 24 h.

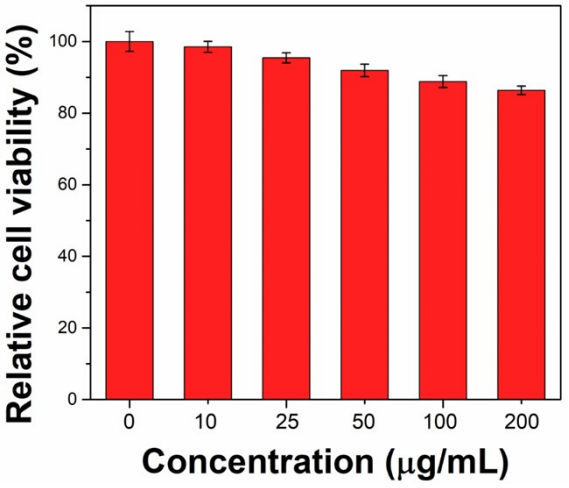

Fig. S19. Relative cell viability of blank nanoparticles against mouse VICs after co-incubation for 24 h.

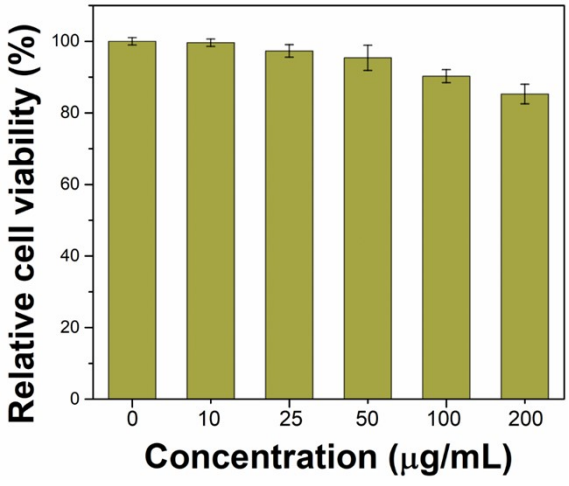

Fig. S20. Relative cell viability of ECNBD-loaded nanoparticles against mouse VICs after co-incubation for 24 h.

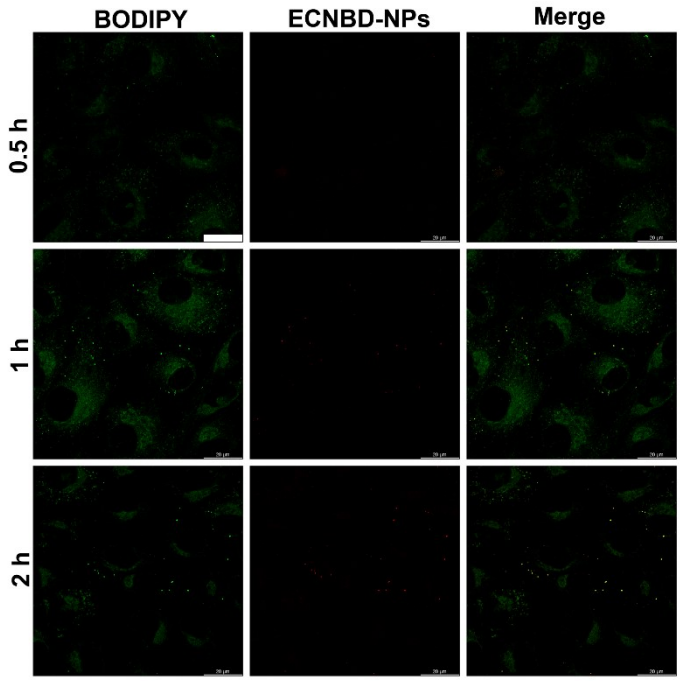

Fig. S21. CLSM images of mouse VICs without oleic acid pretreated co-staining with BODIPY 493/503 Green and ECNBD-loaded nanoparticles at different incubation time intervals. Scale bar: 20 μm.

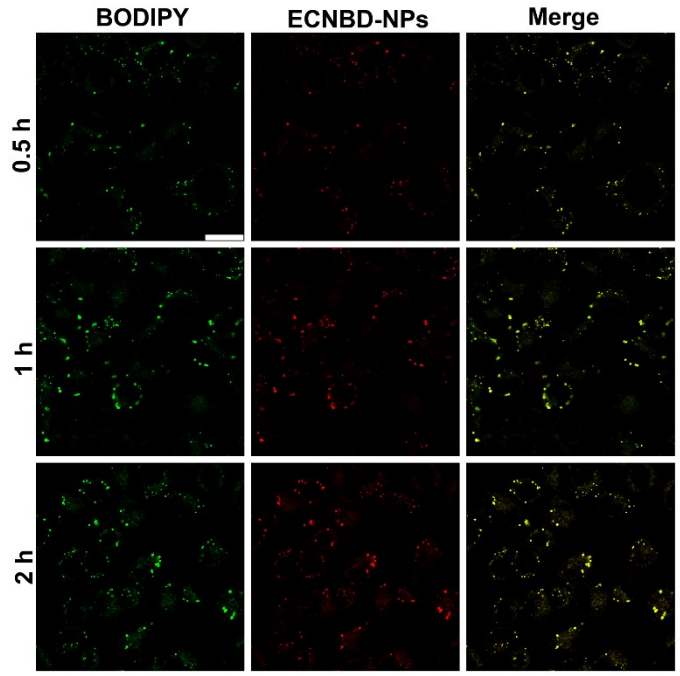

Fig. S22. CLSM images of 10 μM oleic acid pretreated (2 h) HeLa cells co-staining with BODIPY 493/503 Green and ECNBD-loaded nanoparticles at different incubation time intervals. Scale bar: 20 μm.

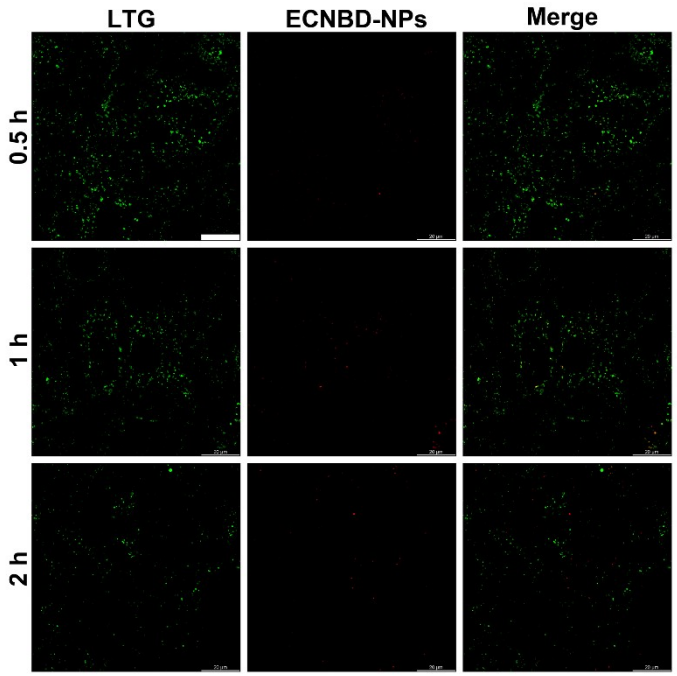

Fig. S23. CLSM images of mouse VICs without oleic acid pretreated co-staining with lysosome-tracker green (LTG) and ECNBD-loaded nanoparticles at different incubation time intervals. The imaging parameters of the channel of ECNBD-loaded nanoparticles were consistent with that of Figure 5, Figure S21 and Figure S22. Scale bar: 20 μm.