Supporting Information

GSH/pH Dual Responsive Chitosan Nanoparticles for Reprogramming M2 Macrophages and Overcoming Cancer Chemoresistance

Xinzhi Zhao [a], Chunxiong Zheng [a,c], Ying Wang [a], Jialei Hao [a] and Yang Liu [a,*]

[a] State Key Laboratory of Medicinal Chemical Biology, Key Laboratory of Functional Polymer Materials of Ministry of Education, College of Chemistry, Nankai University, Tianjin 300071, China.

[c] Laboratory of Biomaterials and Translational Medicine, Center for Nanomedicine, The Third Affiliated Hospital, Sun Yat-sen University, Guangzhou 510630, China.

* Correspondence and request for materials should be addressed to Yang Liu (yliu@nankai.edu.cn).

Table of contents

Materials

Chitosan (molecular weight 50 kDa; deacetylation degree 85%) was purchased from Meilun Biotechnology Company (Dalian, China). Doxorubicin (hydrochloride, 98%) was obtained from Shanghai Aladdin Bio-Chem Technology Co., Ltd. Resiquimod (R848) was purchased from Sigma-Aldrich (Shanghai, China). N-succinimidyl3-(2-pyridyldithio) propionate (SPDP) was purchased from SangonBiotech Co., Ltd (Shanghai, China). 4-carboxyphenylboronic acid, thioglycolic acid (TGA), trimethylamine (TEA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC•HCl), N-hydroxysuccinimide (NHS), and sodium triphosphate (TPP) were purchased from J&K and used without further purification. Cell counting kit-8 (CCK-8) was purchased from ZOMANBIO Biotech (Beijing, China). Methanol and other chemical reagents were purchased from local chemical reagent companies. Griess Reagent kits were obtained from Beyotime Biotechnology (Shanghai, China). Human permeability glycoprotein, P-gp/ABCB1 ELISA Kit was obtained from Cusabio (Wuhan, China). Antibodies for flow cytometry were obtained from Biolegend. Female BALB/c mice at 6-8 weeks were purchased from Vital River Laboratory Animal Technology (Beijing, China). All animal studies were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (Tianjin, revised in June 2004) and adhered to the Guiding Principles in the Care and Use of Animals of the American Physiological Society, and were approved by the Animal Ethics Committee of Nankai University (Tianjin, China) (Approved number 2023-SYDWLL-000235). All protocols within the study involving animals were approved by the Institutional Animal Care and Use Committee, Nankai University.

Instruments

UV-visible spectra were acquired with a NanoDrop OneC (Thermo Scientific, USA). Dynamic light scattering (DLS) and zeta potential studies were performed on a Malvern Zetasizer Nano ZS (UK). Transmission electron microscopy (TEM) observations were performed on a Talos F200C electron microscope at an acceleration voltage of 120 kV. Fluorescence spectra were acquired on a Hitachi F4600. Ultraviolet absorption of the microplate was reported on a Tecan Spark plate reader. Flow cytometry analysis was performed on a BD LSR Fortessa flow cytometry. CLSM images were captured on TCS SP8 (Leica, Germany).

Cell culture

The MCF-7/ADR cell line was cultured in DMEM with 10% (v:v) fetal bovine serum (FBS), 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin in an incubator (Thermo Scientific) at 37 °C under an atmosphere of 5% CO_2 and 90% relative humidity. Human monocyte cell line THP-1 cells were cultured in similar conditions using RAPI 1640. Bone marrow-derived macrophages (BMDM) were isolated from the bone marrow of 6-week-old BALB/c mice according to an established method.

Preparation of CS-PBA

CS-PBA was synthesized through an amide reaction between the carboxyl group of 4-carboxyphenylboronic acid and amino groups of chitosan.[1] Briefly, a certain amount of 4-carboxyphenylboronic acid (1 equiv), EDC (1.2 equiv) and NHS (1.2 equiv) were added to 2 mL of DMSO and reacted for one hour at room temperature. Meanwhile, chitosan (100 mg) was dissolved in 20 mL of 0.1 M diluted hydrochloric acid (HCL), followed by adjusting pH to 6.0. Then, the solution containing 4-carboxyphenylboronic acid was added to 20 mL of chitosan (100 mg) solution, and the mixture was reacted at room temperature for 24 h. The resulting crude product was dialyzed against distilled water for 48 hours. Subsequently, the resulting solution was freeze-dried to obtain CS-PBA.

Synthesis of DOX-Conjugated CS-PBA (DOX-S-S-CS-PBA) and DOX-Conjugated CS (DOX-S-S-CS).

DOX-S-S-CS-PBA was synthesized by conjugating DOX with CS-PBA using N-succinimidyl3-(2-pyridyldithio)propionate (SPDP).[2] Briefly, TGA-modified CS-PBA was obtained by the covalent attachment of TGA to the primary amino groups of the polymer CS-PBA as described previously. To obtain DOX-SPDP, DOX·HCl (17.4 mg, 30 μmol, in 1 mL DMSO) was mixed with the SPDP solution (11.2 mg, 36 μmol, in 1 mL DMSO). A catalytic amount of triethylamine (3 μL) was added to the reaction mixture, which was stirred for 4 hours at 50 °C to produce DOX-SPDP intermediates.

Subsequently, a specific weight of TGA-modified CS-PBA was dissolved in 0.1 M HCL, and the pH was adjusted to 6.0. Drops of DOX-SPDP solution (10 mg mL^{-1}) were added to the chitosan

solution. The mixture was reacted at 25 °C for 12 h solution, followed by dialysis against deionized water for 48 hours and freeze-drying to obtain the crude product. Afterwards, the lyophilized product was washed thrice with DMSO to remove unreacted DOX-SPDP molecules, and DOX-S-S-CS-PBA was obtained by vacuum drying overnight.

To prepare DOX-conjugated CS, TGA-modified CS was obtained as described previously. Then, DOX-SPDP was added to the TGA-modified CS solution, and the mixture was stirred for 12 h. Afterward, it was dialyzed against deionized water for 48 hours and freeze-dried to yield the crude product. Afterwards, the lyophilized product was washed thrice with DMSO to remove unreacted DOX-SPDP molecules, and DOX-S-S-CS was obtained by vacuum drying overnight.

Preparation of CS(DOX)-PBA and CS(DOX)

CS(DOX)-PBA was synthesized using an ion-induced crosslinking method. To achieve this, 20 mg of DOX-S-S-CS-PBA was dissolved in 20 mL of 0.1 M HCL under stirring. The pH was adjusted to 6.0, and 1.0 mL of TPP solution (2 mg mL^{-1}) was added to the DOX-S-S-CS-PBA solution. After 12 hours, the nanoparticles were dialyzed against phosphate buffer saline (PBS) overnight to obtain CS(DOX)-PBA. CS(DOX) were prepared in similar conditions.

Preparation of GPNP

To synthesize CS(DOX + R848)-PBA, R848 solution was mixed with CS(DOX)-PBA solution and incubated overnight at 25 °C. Unloaded R848 was removed through ultrafiltration to yield CS(DOX+R848)-PBA. Subsequently, GPNP was fabricated by mixing the PMPC-*b*-PApm/Glu and CS(DOX+R848)-PBA at the mass ratio of 20:1, followed by dialyzing in PBS for 48 h.

Preparation of Lipo(DOX+R848)

Lectin and cholesterol were dissolved in chloroform to get stock solutions with concentrations of 5 mg mL^{-1} and stored at −20 °C. Next, lipid films containing lectin:cholesterol:R848 at mole ratios of 3:1:1 were prepared on the inner wall of a flask. This was achieved by mixing the stock solutions and evaporating the solvent using a rotary evaporator for 30 minutes. To remove any residual solvent, the film was further dried under vacuum conditions for 6 hours. The lipid film was

then hydrated with 2 mL of 0.3 mol L^{-1} citric buffer, vortex 5 min, and then shaking 1 h at 65◦C to achieve liposome vesicles of 1 mg mL^{-1}. The resulting liposome vesicles were extruded ten times through polycarbonate filters with a pore diameter of 0.22 μm. Transmembrane pH gradients were established by titrating the exterior pH to 7.5 with sodium carbonate. Subsequently, the proper volume of DOX was incubated with the vesicles at 60°C for 10 min with intermittent vortex mixing. Then, the mixture was dialyzed against PBS buffer for 2 days with a dialysis membrane (MWCO = 10 kDa). After dialysis, Lipo(DOX+R848) solution was condensed by centrifugal filtration and stored at 4°C for further characterizations.

The drug-loading content of GPNP

DOX and GPNP were dispersed in a mixture of DMSO and H_2O (5:95, v/v), and the DOX content was determined using a UV spectrophotometer at 480 nm. Free R848 in the tested nanoparticles was separated via ultrafiltration (30 KDa, Millipore) at a speed of 2000 rpm for 15 min, and the R848 level in the ultrafiltrates was subsequently analyzed using a UV-visible spectrometer at 320 nm. The drug content was calculated as the difference between the total amount of added drugs in the solution and the amount of free drugs in the ultrafiltrate. The drug loading content (DLC) and drug loading efficiency (DLE) were calculated using the following equation:

DLC(%)=(weight of drugs in NPs)/(weight of drug-loaded NPs)×100 %

DLE(%)=(weight of drug in NPs)/(weight of the feeding drug)×100 %

Drug release behavior of different nanoparticles

To determine the release kinetics of R848, drug-loaded nanoparticles were suspended in 1 mL of PBS with different pHs. These suspensions were then enclosed within a dialysis bag (MWCO 10000) and dialyzed against 5 mL of buffer solution with different pHs at 37 ºC under gentle shaking. At predefined time intervals, 500 μL of the sample was extracted from the dialysis buffer for subsequent analysis. The released R848 was quantified using a UV-visible spectrometer with a wavelength of 320 nm. The drug release behavior of DOX under different concentrations of GSH was similarly investigated. Released DOX was assessed using a UV-visible spectrometer with a wavelength of 480 nm.

FRET efficiency analysis

To investigate the FRET efficiency (E(FRET)) under different conditions, nCS-PBA and PMPC-b-PApm/Glu were pre-labeled with Cy5-NHS and Cy3-NHS. Cy5-nCS@PMPC-Cy3 was prepared by mixing Cy5-nCS-PBA and Cy3-PMPC-b-PApm/Glu and incubating them under different pH conditions at room temperature for 6 h. The fluorescent intensity of Cy5-nCS@PMPC-Cy3 was subsequently measured using a fluorescence spectrophotometer (Hitachi F4600) at the excitation of 515 nm. The E(FRET) was calculated as follows:

E(FRET)=I670/(I570+I670)

The E(FRET) of Cy5-nCS@PMPC-Cy3 in the presence of different concentrations of SA were similarly evaluated.

TEM analysis of CS(DOX+R848)-PBA and GPNP

The morphology of CS(DOX+R848)-PBA and GPNP was examined using transmission electron microscopy (TEM, FEI Talos F200C electron microscope). To prepare the TEM samples, CS(DOX+R848)-PBA and GPNP were initially diluted to a concentration of 500 μg mL^{-1}, followed by the drop-coating of 2 μL of samples onto carbon-coated copper grids (Beijing Zhongjingkeyi Technology Co., Ltd, China). A Droplet of the sample was contacted with the grids for 5~10 min, then the excess sample was removed. The grid was then rinsed and stained with 1 % phosphotungstic acid hydrate (5~10 μL) for 1.5 min.

Tumor-targeting capacity of GPNP

CLSM observation and FACS analysis were performed to study the tumor-targeting capacity of GPNP. For CLSM observation, MCF-7/ADR cells were seeded in a 35 mm confocal dish before their co-cultivation with these nanoparticles. Equivalent amounts of DOX, CS(DOX)-PBA, CS(DOX), and Lipo(DOX) (the dose of DOX was 2 μg mL^{-1}) was introduced and co-cultured with MCF-7/ADR cells for 4 h. To further study the cellular uptake mechanism, tumor cells were pre-incubated with free PBA (100 μg mL^{-1}) for 30 min, and then incubated with CS(DOX)-PBA for 4 h. After incubation, the cells were rinsed with PBS, fixed using 4% paraformaldehyde and subsequently stained with DAPI for cell nucleus. After the staining, images were captured using a

CLSM (TCS SP8, Leica, Germany). For FACS analysis, MCF-7/ADR cells were seeded in a 12-well plate one day prior to their co-cultivation with the nanoparticles. Then, cells were collected and washed with PBS for flow cytometry analysis (BD LSR Fortessa).

Study on the mechanism of cell internalization of GPNP

To study the cell internalization mechanism of GPNP (pH=6.5), MCF-7/ADR cells were seeded in 12-well plates and grown to approximately 70% confluence. MCF-7/ADR cells were then treated with endocytosis inhibitors including nystatin (15 μg mL^{-1}), amiloride (100 μg mL^{-1}), chlorpromazine (10 μg mL^{-1}) or PBS at 4 ℃ for 2 h before the endocytosis experiments. Cells that were treated without any inhibitors at 37 ℃ were served as a control. Later, tumor cells were exposed to 40 μg of GPNP (pH=6.5) for 1 h. Tumor cells were then collected by washing with PBS, digesting by using 0.25% trypsin and centrifugation. The cellular internalization of GPNP (pH=6.5) was analysed by using flow cytometry.

Lysosome escape of GPNP

CLSM observation were performed to study the Lysosome escape of GPNP (pH=6.5). MCF-7/ADR cells were seeded in a 35 mm confocal dish before their co-cultivation with these nanoparticles. Then, the nanoparticles were incubated with cells for 2 h at 37 °C. After that, the medium was removed, cells were washed with PBS twice and incubated with the fresh medium for 0 and 6 h. Afterwards, DAPI (10 μg mL^{-1}) and Lyso tracker Red (50 nM) were added for another incubation of 30 min and 15 min, respectively. Finally, cells were rinsed by fresh PBS and observed by CLSM (TCS SP8, Leica, Germany).

***In vitro* cytotoxicity**

The cytotoxicity of DOX, CS(DOX)-PBA, Lipo(DOX+R848), CS(DOX)@PMPC (pH=6.5) and CS(DOX)@PMPC was determined by evaluating the cell viability after exposing MCF-7/ADR cells to these formulations. Briefly, MCF-7/ADR cells were seeded into 96-well plates at a density of 1×10^4 cells/well and allowed to grow to 70-80 % confluence. Subsequently, the culture medium was replaced with fresh medium containing various concentrations of DOX, specifically 0.5, 1, 2, 4, 6, 8, and 10 μg mL^{-1}, for each formulation. As a negative control, PBS was used. Following a 48-

hour incubation period, the cells were rinsed twice with PBS, followed by the addition of 100 μL of CCK-8 working solution (CCK-8 stock solution: culture media, 1:9, v/v) and another 1 h's incubation. Cell viability was quantified by measuring the absorbance using Tecan's Infinite M200 microplate reader (λ = 450 nm).

Measurement of CD80 and CD206 Levels

To obtain M2 macrophages, BMDM cells were initially subjected to pretreatment with interleukin-4 (IL-4).[3] These M2 macrophages were subsequently seeded into a 6-well plate (1 × 10^5 cells per well) for adherent growth. Then, cells were cultured with PBS, R848, CS(R848)@PMPC and CS(R848)@PMPC (pH=6.5) (the dose of R848 was 1 μg mL^{-1}) for 48 h. After being washed with PBS twice, the cells were harvested and stained with APC anti-mouse F4/80, FITC anti-mouse CD80 and PE anti-mouse CD206. Subsequently, the cells were fixed in 4% paraformaldehyde, and the levels of F4/80, CD80, and CD206 expression were assessed via flow cytometry measurements.

Repolarization of M2 macrophages

M2 macrophages were derived from THP-1 cells using a previously established protocol with slight adjustments.[4] Subsequently, these M2 macrophages were seeded within the upper chamber of a transwell cell culture plate, while MCF-7/ADR cells were cultivated in the lower chamber. Nanoparticles, containing DOX at a concentration of 2 μg mL^{-1} and R848 at 1 μg mL^{-1}, or equivalent concentrations of free drugs, were introduced into both the upper and lower chambers and incubated for an additional 48 hours. The changes in macrophage phenotypes after different treatments were investigated using FACS analysis after immuneostained with CD86 antibodies. Flow cytometry profiles of the cells were acquired on a BD FACS Calibu.

Measurement of P-gp expression, NO Levels and CD86 Levels

To determine the expression levels of P-gp, two distinct groups of MCF-7/ADR cells were utilized. Group one consisted of cells from the co-culture transwell system, while group two comprised MCF-7/ADR cells seeded in a 6-well plate without the influence of M2 tumor-associated macrophages (TAMs). These MCF-7/ADR cells were cultured for 24 hours before the

introduction of nanoparticles. After culturing for 48 h, the cells were washed three times with PBS to remove external nanoparticles. Subsequently, the cells were collected and lysed using lysis buffer. The resulting cell lysate was then subjected to centrifugation, and the supernatant was employed to quantify P-gp levels via ELISA, following the manufacturer's instructions. To determine the expression levels of NO within the MCF-7/ADR-M2 macrophages transwell system, the supernatants were collected and analyzed using Griess Reagent kits according to the manufacturer's instructions.

For analysis of the expression of CD86, THP-1 cells in the upper chamber of the transwell system were collected, washed with PBS and stained with PE anti-human CD86. The cells were then fixed in 4% paraformaldehyde, and the expression of CD86 was assessed through flow cytometry measurement.

Apoptosis assay

Apoptosis was conducted through Annexin V-FITC/PI dual-labeling. MCF-7/ADR cells in the lower chamber of the transwell system were collected using trypsin without EDTA, washed with PBS, and stained with Annexin V-FITC and PI according to the manufacturer's instructions. Finally, flow cytometry analysis was performed on a BD LSR Fortessa flow cytometry.

The pharmacokinetics study

To evaluate the pharmacokinetics of GPNP, CS(DOX+R848)-PBA was used as comparative group for better comparation. In details, CS(DOX+R848)-PBA (100 μg, 100 μL) and GPNP (100 μg, 100 μL) were first intravenous injected into BALB/c female mice. At different time points after injection (0, 1, 2, 3, 5, 6, 8, 12, 24 and 48 h), 50 μL of orbital venous blood was collected from mice in each group. Subsequently, the plasma samples were first incubated with 100 mM of DTT at 37 °C for 24 h to fully cleave the disulfide bonds. Next, the fluorescent intensity of DOX in the samples was tested by a Tecan's Infinite M200 microplate reader.

Statistical Analysis

Statistical comparisons were achieved by using one-way ANOVA with Tukey post-test with GraphPad Prism 7.0.

Supplementary Figures

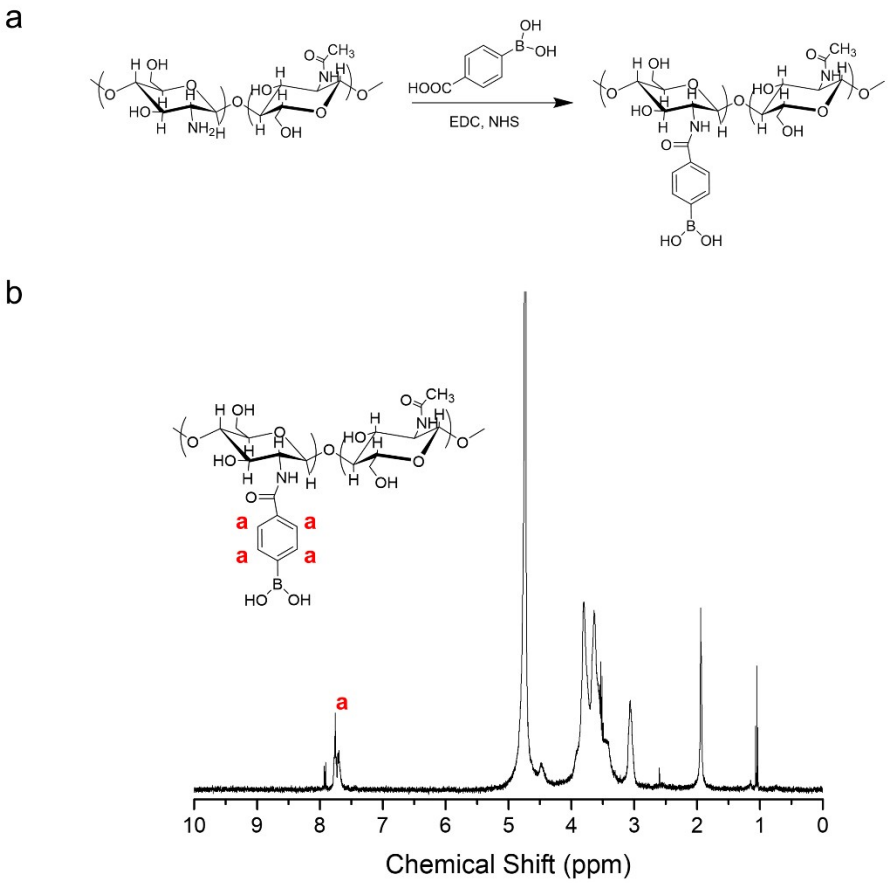

Fig. S1. Synthesis routes (**a**) and ^{1}H NMR spectrum (**b**) of CS-PBA.

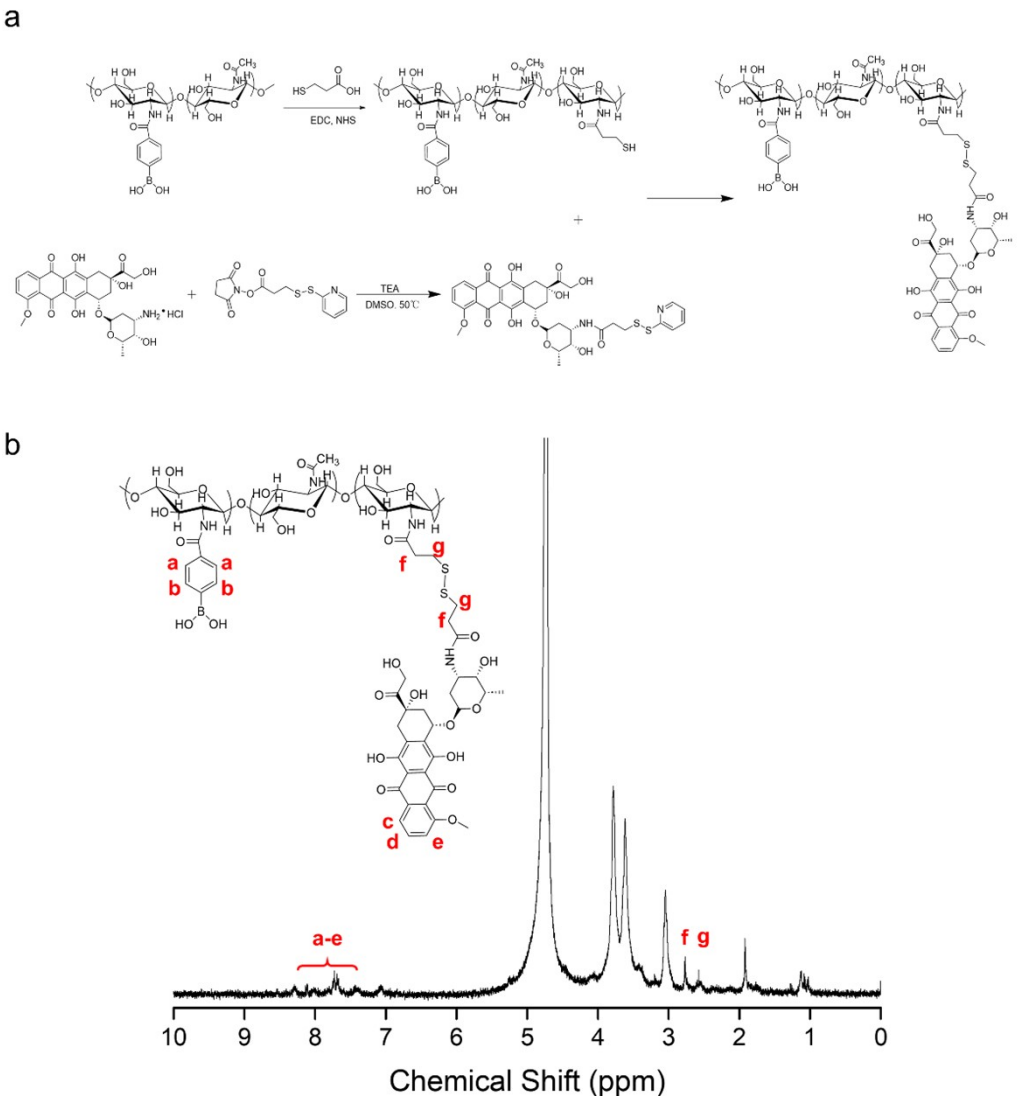

Fig. S2. Synthesis routes (**a**) and ^{1}H NMR spectrum (**b**) of DOX-S-S-CS-PBA.

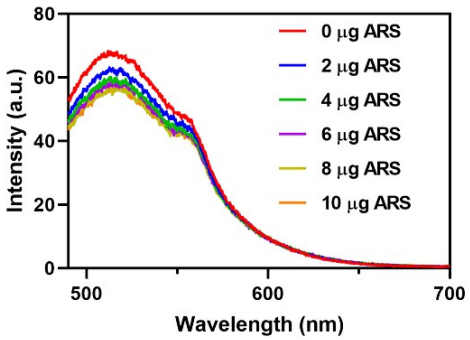

Fig. S3. Fluorescence spectra of nCS in the presence of ARS.

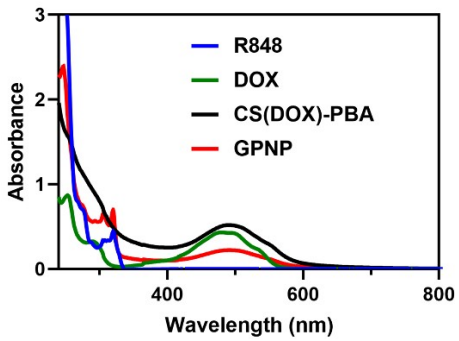

Fig. S4. UV–Vis absorption spectra of R848, DOX, CS(DOX)-PBA and GPNP.

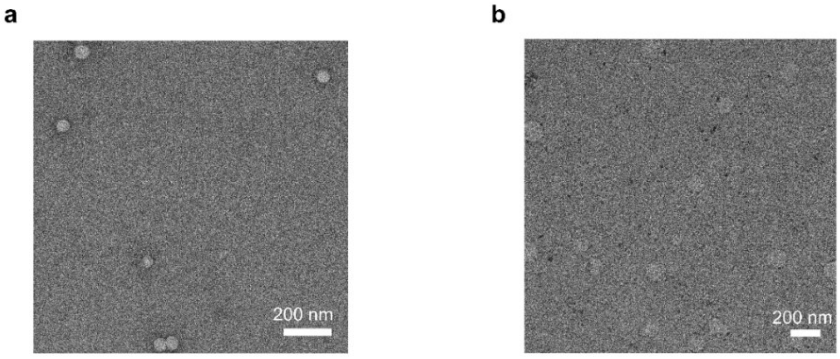

Fig. S5. Transmission electron microscopy (TEM) images of CS(DOX+R848)-PBA (**a**) and GPNP (**b**).

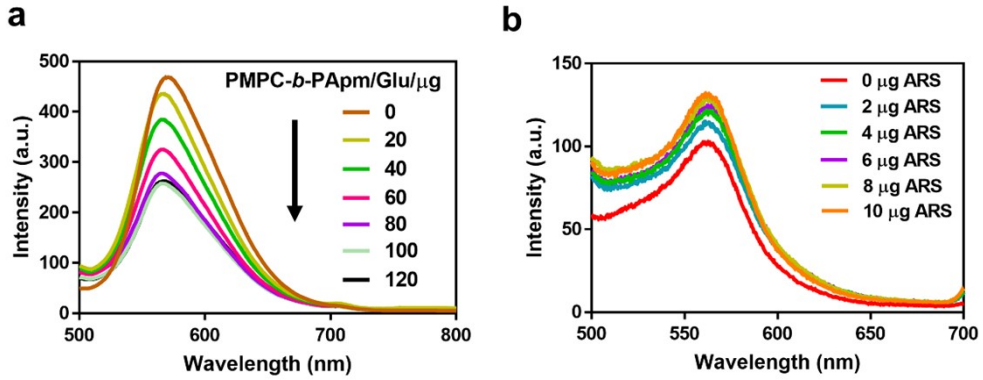

Fig. S6. (**a**) Fluorescence spectra of the mixture of nCS-PBA and ARS in the presence of PMPC-b-PApm/Glu. (**b**) Fluorescence spectra of GPNP in the presence of ARS**.**

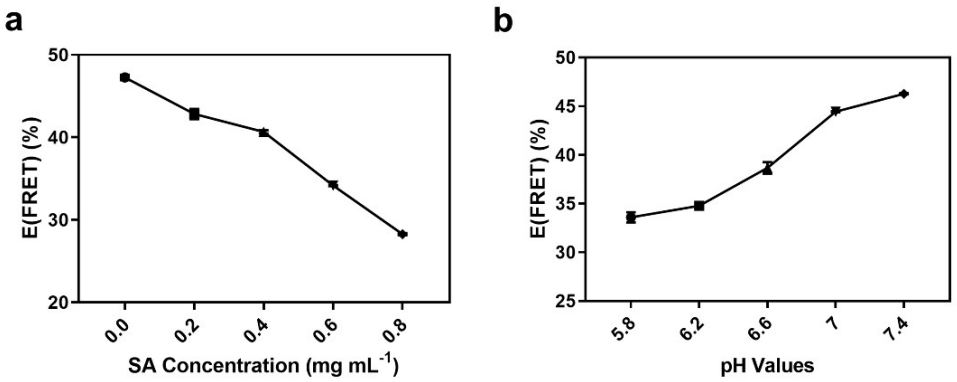

Fig. S7. FRET efficiency (E(FRET)) of Cy5-nCS@PMPC-Cy3 under different pH values (**a**) and various SA concentrations (**b**). Data in (**a**) and (**b**) are presented as mean ± SD (n = 3).

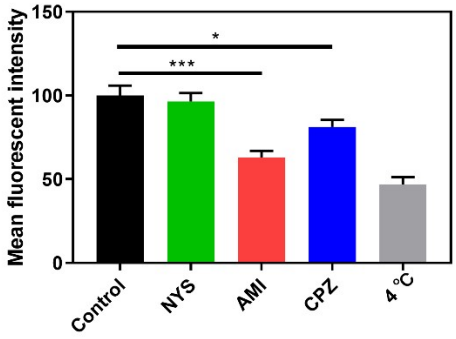

Fig. S8. Relative cellular uptake of GPNP (pH=6.5) by MCF-7/ADR cells in the presence of endocytosis inhibitors and when incubating at 4℃. Data are presented as mean ± SD (n = 3). Significant differences are expressed as asterisks: * $p < 0.05$ and *** $p<0.001$.

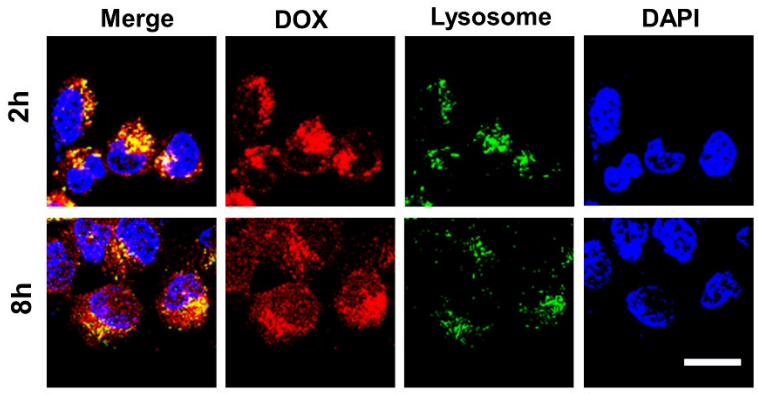

Fig. S9. CLSM images of GPNP (pH=6.5) co-cultured with MCF-7/ADR cells for different time intervals. Nuclei stained with DAPI were shown in blue, lysosomes stained with Lyso Tracker Red were shown in green. Scale bar, 20 μm.

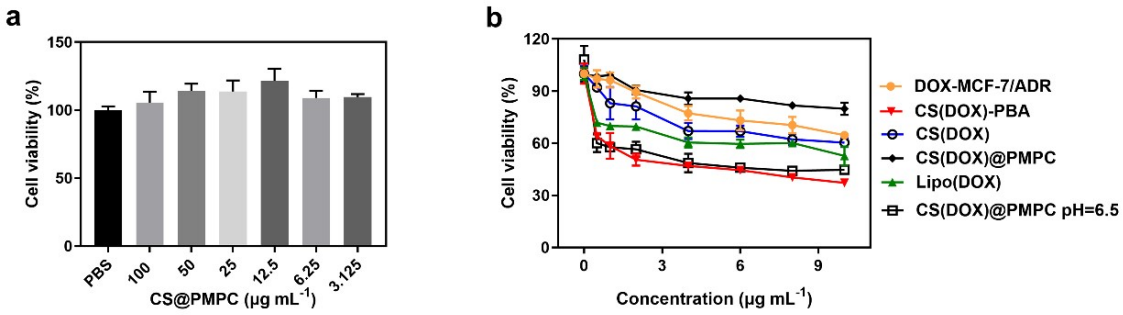

Fig. S10. (**a**) Cell viability of MCF-7/ADR cells after 48 h incubation with CS@PMPC. (**b**) Cell viability of MCF-7/ADR cells after 48 h incubation with DOX and CS(DOX)@PMPC with various concentrations at 37 ºC. Data in (**a**) and (**b**) are presented as mean ± SD (*n* = 3).

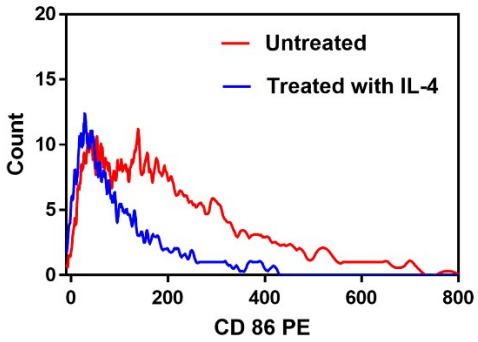

Fig. S11. Flow cytometry analysis of the CD86 expression in THP-1 cells before and after treated with IL-4 for 48 h.

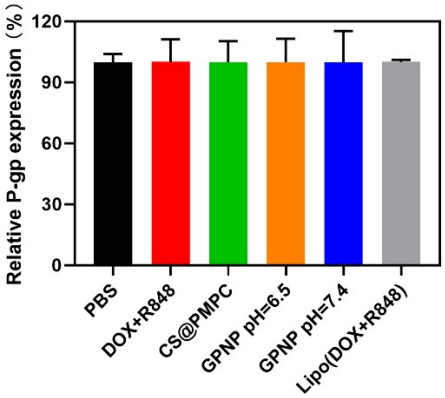

Fig. S12. P-gp expression of MCF-7/ADR cells treated with PBS, DOX+R848, CS@PMPC, GPNP (pH=6.5), GPNP (pH=7.4) and Lipo(DOX+R848). Data are presented as mean ± SD (*n* = 3).

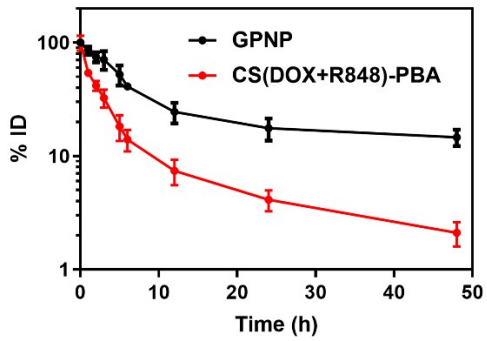

Fig. S13. Blood clearance profiles of CS(DOX+R848)-PBA and GPNP. Data are presented as mean ± SD (n = 3).

References

1. J. Wang, L. G. Liu, W. Q. Jiao, H. Yang, J. Liu and D. Liu, *Carbohydr Polym*, 2021, **256**, 117497.
2. Y. Su, Y. Hu, Y. Du, X. Huang, J. He, J. You, H. Yuan and F. Hu, *Mol Pharm*, 2015, **12**, 1193-1202.
3. Y. Wang, Z. Luan, C. Zhao, C. Bai and K. Yang, *Eur J Pharm Sci*, 2020, **142**, 105136.
4. X. Wu, J. Yan, X. Han, R. Zheng, P. Song, Y. Wang and H. Zhang, *Chemical Engineering Journal*, 2022, **429**.