Supporting information

A New Strategy for Hydrophobic Drugs Delivery Using Hydrophilic Polymer Equipped with Stacking Units

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Materials and methods

1.1 Materials

Agmatine dihydrochloride, Doxorubicin hydrochloride (DOX•HCl) and Cystamine dihydrochloride were purchased from J&K (Beijing, China). D,L-1,4-dithiothreitol (DTT) was purchased from Sigma-Aldrich (St. Louis, USA). Poly Acrylic Acid (PAA) was purchased from Acros Organics (Geel, Belgium). RPMI 1640, Hochest 33342 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from KeyGEN Biotech (Nanjing, China). Trypsin-EDTA solution (0.25 %) and fetal bovine serum (FBS) were obtained from Gibco (Burlington, Canada). All other chemicals and reagents were obtained from commercial sources and used without further purification.

1.2. Synthesis of N,N'-cystamine-bis-acrylamide (CBA)

Cystamine dihydrochloride (2.252 g, 10 mmol) was dissolved in 10 mL distilled water in a three-necked flask (100 mL). Equip the flask with two dripping funnels and a stirrer. Acryloyl chloride (1.810 g, 20 mmol) solution in dichloromethane (2 mL) and NaOH solution (1.2 g, 0.04 mol) were added simultaneously under stirring for 60 min at 0 $^{\circ}$C. The reaction was continued for another 6 h at room temperature. After that, the reaction mixture was extracted with dichloromethane 3 times, combined the organic phase and dried with anhydrous Na_2SO_4 (5 g) over night. The product was obtained by crystallization from ethyl acetate.

1.3. Synthesis of poly(agmatine) (PCA)

PCA was synthesized by michael addition according the following procedure. Briefly, equal molar CBA and agmatine dihydrochloride were both dissolved in methanol. The mixture was stirred for 24 h at 90 $^{\circ}$C under Ar atmosphere in dark after adding triethyamine. Then, pH of product was adjusted with 0.1 M HCl to 4 and dialyzed against distilled water with dialysis membrane (MWCO = 3000) for 48 h, followed by lyophilized to remove water. At last, the product was characterized with ^{1}H-NMR spectroscopy.

1.4. Molecular weight measurement

The molecular weight of PCA was measured by gel permeation chromatography equipped with RID-10A (GPC, LC-20 AB, Shimadzu, Japan). Polyethylene glycol was used as standard. PCA was dissolved using mobile phase to 5 mg/mL. An ultrahydrogel 250 column was used with 50 mM Sodium sulfate as an eluent at 1.0 mL/min.

1.5. Deprotonation of DOX·HCl

DOX•HCl (10 mg) was dissolved in 5 mL distilled water. NaOH (1 M) was slowly added into the aqueous solution to adjust pH to 9.6. The mixture was centrifuged at 10000 g for 10 min and washed

with distilled water three times. The product was lyophilized to receive hydrophobic doxorubicin (DOX). Every step was kept in dark during the process.

1.6. DOX loading capacity of PCA

PCA (2 mg) was dissolved in 2 mL of distilled water. Different content of DOX solution in DMSO (0, 0.2, 0.4, 0.5, 1, 3 and 6 mg, respectively) was dropped into distilled water under stirring. The mixture was dialyzed against ultrapure water with dialysis membrane (MWCO = 500) for 6 h and subsequently lyophilized after centrifugation (3000 g for 10 min). The whole procedure was performed in dark. The content of DOX was determined by UV-Vis measurement (483 nm) and the calibration curve was obtained from DOX/DMSO solutions with different DOX concentrations.

1.7. Preparation of conjugate systems between PCA and hydrophobic drugs (Drug/G-CS)

The freeze-dried PCA (10 mg) was dissolved in 1 mL of DOX solution in DMSO (2.5 mg) under ultrasonic. Then the solution was dropped into 15 mL distilled water under stirring. The mixture was dialyzed against ultrapure water with dialysis membrane (MWCO = 2000) for 6 h and subsequently lyophilized after centrifugation (3000 g for 10 min). The content of DOX was determined as method mentioned before. Drug loading contents (DLC) was calculated according to the following formula:

DLC (%) = (weight of loaded drug / weight of drug loaded conjugate system) × 100 %

1.8. Simulation Methods

Materials Studio Program (Accelrys Inc.) was performed to calculate the solubility parameters and molar volumes of beads. MD simulations were performed using the dissipative particle dynamics (DPD) simulations. The molecular structure of doxorubicin is divided into three types of beads (I, J, and K). PCA is separated into four types of beads (NO, NC, SS and Gua). A cubic simulation box with periodic boundary condition was applied in all three directions. A box of 35 × 35 × 35 is sufficient to avoid the finite size effects, and the integration time step of 0.05 is small enough for our system to get thermodynamic equilibrium. The system comprised of 85 % water, 12.5 % PCA, and 2.5 % DOX.

1.9. Characterization of conjugate systems between PCA and DOX (DOX/G-CS)

The morphology of DOX/G-CS was observed by high-resolution transmission electron microscopy (HRTEM) (FEI Tecnai G2 F20, USA). For TEM analysis, DOX/G-CS was directly dropped onto copper grids followed by vacuum drying, and then observed at an accelerating voltage of 120 kV. The particle size of DOX/G-CS was determined by ZetaPlus particle size analyzer (Brookhaven Instruments, USA). Measured average sizes were presented as the average values of 3 runs. To visualize the co-localization of the DOX in the conjugate systems, high-resolution dark-field images and hyperspectral fluorescence signal images were obtained via CytoViva™ hyperspectral microscopy system (CytoViva, USA). The interaction mechanism between polymer PCA and hydrophobic DOX was explored by the UV-Vis measurement and fluorescence spectrophotometry. The hydrophilic

DOX•HCl was selected as control because free hydrophobic DOX would precipitate in the water, which were adverse to the UV-Vis measurement and fluorescence spectrophotometry.

1.10. MicroScale Thermophoresis (MST) assays

To assay affinity between PCA and DOX, PCA were prepared by 1:1 (v/v) serial dilution of the 7.5 mg/mL stock solution with ultrapure water till 16 steps and the final concentrations ranged from 3.75 to 0.0001 mg/mL. The addition of DOX in DMSO solution led to a final DOX concentration of 0.025 mg/mL in each sample. To minimize pipetting errors, the individual binding reactions was prepared with an optimal volume of 20 μL. Samples were incubated for 40 min and loaded into capillaries (MO-K005). Samples were measured with a Monolith NT.115 (Munich, Germany) using blue excitation and emission with 20, 40, 80 % MST power. In the initial capillary scan, the fluorescence signals of different samples were adjusted by LED strength (40 % here) to a minimum of 200 counts and a maximum of 1000 counts. Each measurement was repeated three times with 5 s equilibration, 30 s Laser-On and 5 s decay time. The data from 80 % MST power was fitted to obtain the dissociation constant (Kd). Agmatine was chosen as a control.

1.11. Cell culture

Michigan Cancer Foundation-7 (MCF-7) and Michigan Cancer Foundation-7/Adriamycin Resistance (MCF-7/ADR) were purchased from the Chinese Academy of Sciences Shanghai Institute of Cell Bank (Shanghai, China) and cultured in RPMI 1640 supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 80 units/mL of penicillin, and 80 μg/mL of streptomycin. Cells were incubated at 37 $^{\circ}$C in a humidified 5 % CO_2 atmosphere.

1.12. In vitro cytotoxicity assays

Cytotoxicity of the polymer PCA was measured by MTT assay in MCF-7 and MCF-7/ADR cells. Cells were seeded in 96-well cell culture plates at a density of 1×10^4 cells/well. When achieving 70 % of confluency after 12 h, the cells were incubated with 100 μL polymer solutions which concentrations from 5 μg/mL to 80 μg/mL in medium containing 10 % FBS and further incubated for 24 h. Then, the cells were treated with 20 μL MTT stock solution for 4 h at 37 $^{\circ}$C in dark. After removing medium carefully, 150 μL DMSO was added to dissolve the formazan crystal formed by proliferating cells. The absorbance at 570 nm in each well was recorded using a Microplate Reader (Thermo, USA). Cells without treatment were used as control. All experiments were conducted in thrice.

Inhibitory effects of DOX/G-C systems in cancer cell proliferation were performed in MCF-7/ADR cells. The concentrations of samples were at indicated concentrations, respectively. The concentration of PCA at each group was same with that in DOX/G-CS, which was confirmed by method described in 2.7. After further incubated for 24 h, the supernatant was removed and the cells were washing three times with 100 μL PBS. Subsequently, 20 μL of MTT solution was added into

each well, and then the solution was replaced by 150 μL DMSO after 4 h of incubation at 37 °C in the dark. The absorbance at 570 nm of the solution in each well was recorded using a Microplater Reader (Thermo, USA). All experiments were conducted in thrice.

1.13. Cellular internalization assays

The cellular internalization behaviors of DOX/G-CS were studied in MCF-7 and MCF/ADR cells using flow cytometry and confocal laser scanning microcopy (CLSM). For flow cytometer assay, cells were seeded in 24-well plates at a density of 1.5×10^4 per well in 1.0 mL of RPMI 1640 medium containing 10 % FBS and cultured overnight. Cells were treated with DOX•HCl, DOX/G-CS for 4 h (10 μM). Subsequently, the supernatant was removed and the cells were washing three times with PBS. After that, the cells were harvested and suspended in 400 μL serum-free medium. Cell uptake was examined by using flow cytometer (BD AccuriC6, USA) at a minimum of 1×10^4 cells gated per sample.

For the CLSM assay, cells were seeded in 3.5 cm-confocal dishes at a density of 1.0×10^5 per well in 2.0 mL of RPMI 1640 medium containing 10% FBS and cultured overnight. Followed by removing culture medium and adding DOX/G-CS and free DOX•HCl solutions at the concentration of 5 μM. After incubation at 37 °C for 1, 4 and 10 h, culture medium was removed, and cells were washed with PBS for three times. Afterwards, cells were stained with Hoechst 33342 (100 μM in PBS) for 30 min and fixed using 4 % (w/v) formaldehyde solution for 20 min. After staining, the solution was removed, and the cells were rinsed with PBS three times and subjected to CLSM (Leica, Germany).

1.14. Drug efflux assays of DOX/G-CS

To determine the potential drug efflux effect of DOX/G-CS, MCF-7 cells and MCF-7/ADR cells were seeded in 24-well plates at a density of 1.5×10^5 per well in 2.0 mL of RPMI 1640 medium containing 10 % FBS and cultured overnight. The cells were first incubated with either 10 μM free DOX or DOX/G-CS solution for 4 h, then the medium was removed and the cells were washed three times with PBS and subsequently incubated with fresh RPMI 1640 medium for different intervals (1 h, 4 h). At the end of incubation time, the cells were harvested and analyzed using a flow cytometer.

1.15. Animals and tumor model

Animal care and handing procedures were in agreement with the guidelines evaluated and approved by the ethics committee of China Pharmaceutical University. 6 weeks female BALB/c nude mice (18-20 g) were purchased from Shanghai Laboratory Animal Limited Company. The mice were maintained in a pathogen-free environment (23 ± 2 °C and 55 ± 5 % humidity) under a light/dark cycle (12 h/12 h) with food and water supplied ad libitum throughout the period. Three days before the injection of MCF-7/ADR cells, 1.5 mg/kg estradiol was injected into leg muscles of mice. Then tumor cells (1×10^7 cells in 100 μL serum-free 1640 medium) were subcutaneously injected into the back of mice. After inoculation of tumor cells, estradiol was continuously injected into mice weekly until they

were sacrificed. The tumor sizes were measured by a caliper, and the tumor volume was calculated as (tumor length) × (tumor width)^2/2.

1.16. In vivo efficacy

When the tumor sizes reached ~100 mm^3, 100 μL of free DOX•HCl and DOX/G-CS at a DOX dose of 2.5 mg/kg were intratumorally injected into female nude mice bearing MCF-7/ADR tumors (three times per week for 2 weeks, n = 6 per group). Saline and PCA were treated as control groups (n = 6 per group). Tumor volumes were determined during the treatment. In the end, organs including heart, liver, spleen, lung, kidney, and tumor were harvested and the tissue samples were embedded in paraffin blocks, sectioned into 5 mm slices and mounted onto the glass slides. After hematoxylin-eosin (H&E) staining for organs, the sections were examined by a digital microscope (Olympus, DP 73, Japan).

1.17. Ex vivo drug distribution and tumor accumulation

When tumor volume reached approximately 500 mm^3, free DOX•HCl, DOX/G-CS were intratumoral injected at an equivalent DOX dose of 2.5 mg/kg body weight. After 1, 2, 4, 6 and 12 h, mice were sacrificed. Then, organs including heart, liver, spleen, lung, kidney, thyroid and tumor were harvested and immediately scanned by an *in vivo* Imaging System (FXPRO, Kodak, USA).

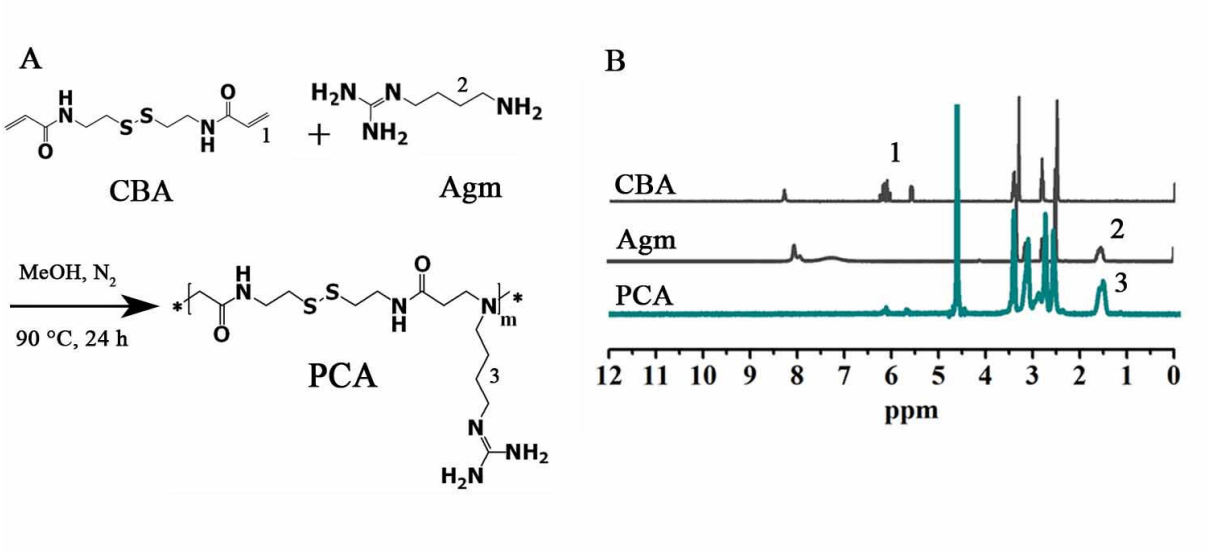

Figure S1. (A) Synthesis scheme of PCA and (B) ^{1}H NMR spectra of PCA, Agm and CBA (Deuterium Oxide).

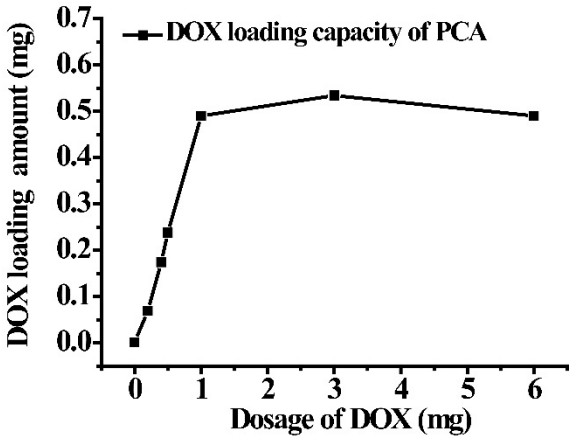

Figure S2. DOX loading capacity of PCA.

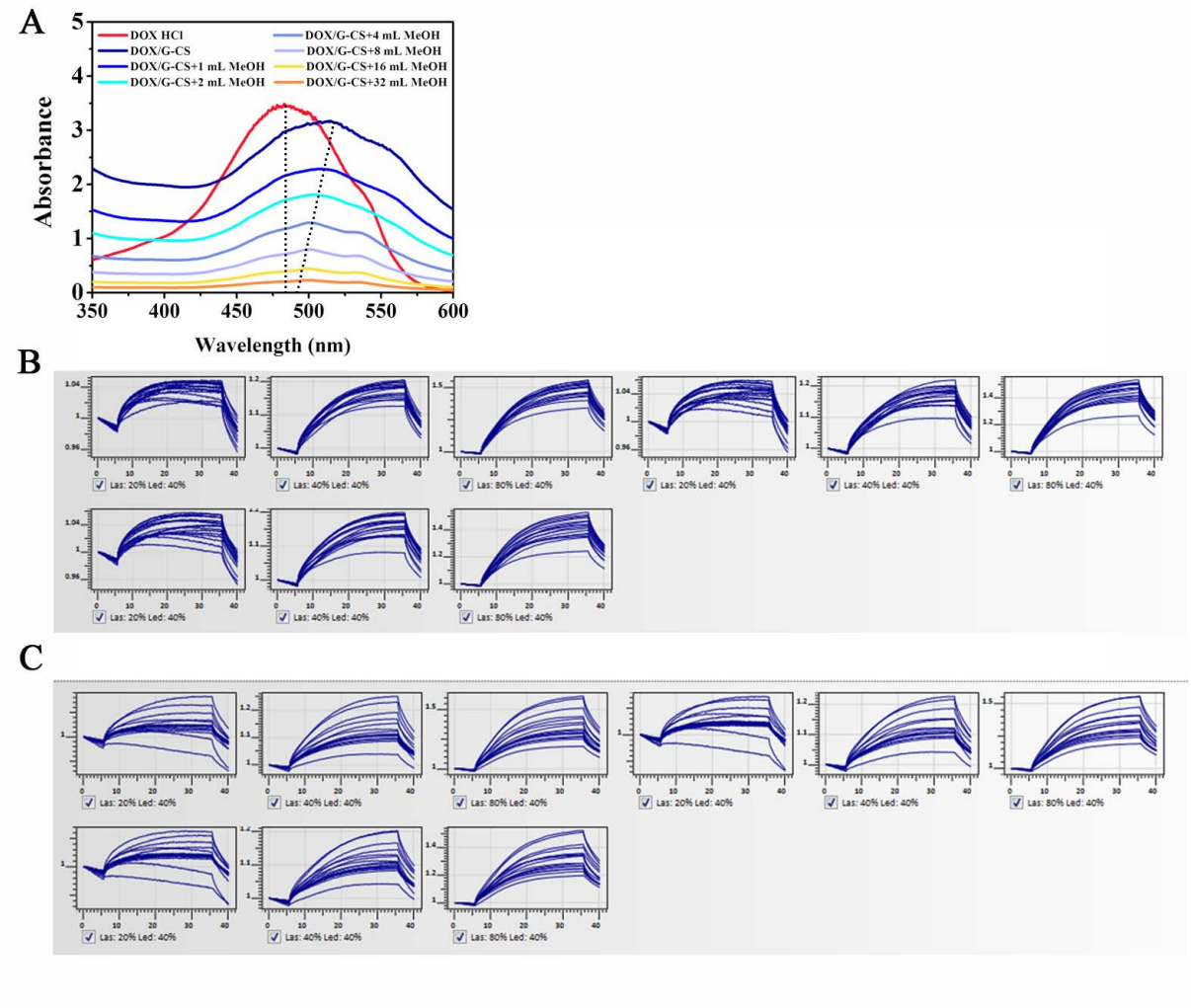

Figure S3. The determination of π-π interaction between PCA and DOX. A) The UV-vis spectra of DOX/G-CS in methanol (MeOH); B) The primary binding curves of nine measurement series of DOX/Agm with 20, 40, 80 % MST power; C) The primary binding curves of one measurement series of DOX/G-CS with 20, 40, 80 % MST power. All experiments were conducted in triplicate.

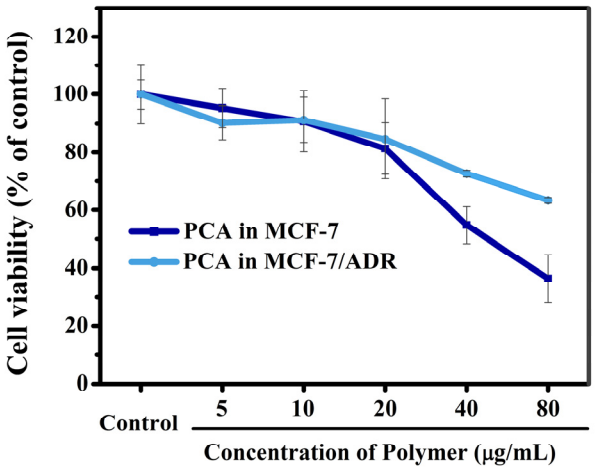

Figure S4. Cytotoxicity assay of PCA at various concentrations for 24 h in MCF-7 and MCF-7/ADR cells (mean SD, n = 5)

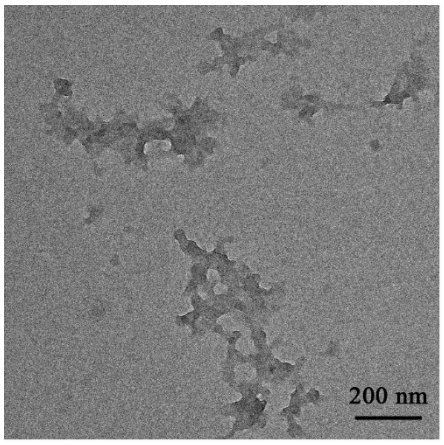

Figure S5. TEM image of DOX/G-CS after adding 25 μM DTT.

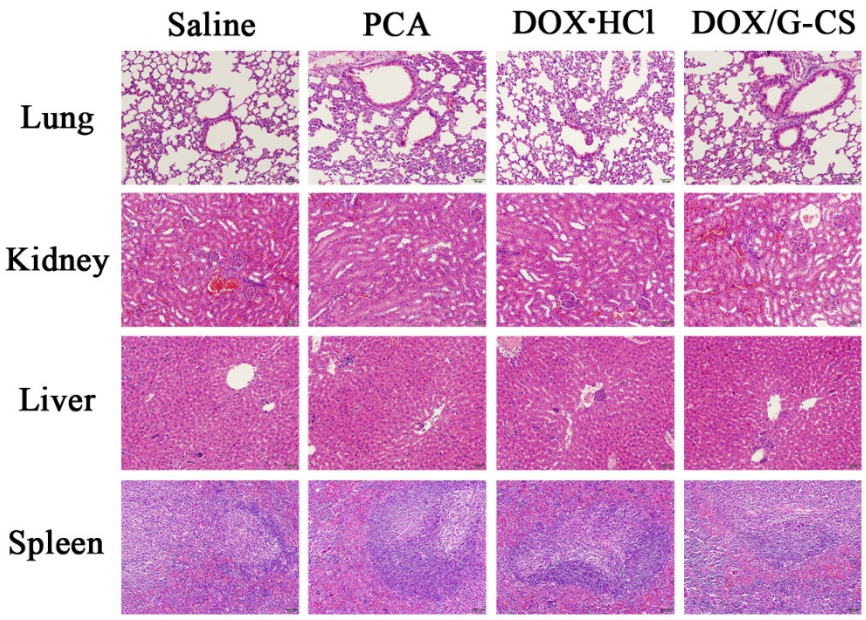

Figure S6. Histological analysis of lung, kidney, liver and spleen, was determined to evaluate the toxicity (scale bar = 50 μm).

Table S1. The molecular weight (Mw), degree of polymerization (m) and molecular weight distribution (PDI) of polymer PCA.

	Mw (kDa)	Mn (kDa)	PDI	Degree of polymerization (m)
PCA	3351	2043	1.64	8.59