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Supporting information for

Hybrid polymer micelles capable of cRGD targeting and pH-triggered surface charge conversion for tumor selective accumulation and promoted uptake

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Experimental details

Materials. Monomethoxyl poly(ethylene glycol) (mPEG, average Mn=5000, Aldrich), N-(3-Aminopropyl)-imidazole (API, Alfa Aesar, 99%), dicyclohexylcarbodiimide (DCC, Aldrich, 99%), and N-hydroxysuccinimide (NHS, Aldrich, 99%) were used as received. Doxrobicin (Dox) was purchased from Zhejiang Hisun Pharmaceutical Co. Ltd. Cyclic (RGDfK) peptide was customized from Shanghai China peptides Ltd. Ultrapure water was prepared from a Milli-Q system (Millipore, USA). α-Allyloxyl poly(ethylene glycol)-b-poly(lactic acid) (allyl-PEG-b-PLA) and its post-functional copolymer with a carboxyl group at the end of PEG segment (HOOC-PEG-b-PLA) has been reported by our group previously,[1] so it is not described here. The polymerization degree of PEG and PLA block used in this study was 113 and 24, respectively. All other chemicals were analytical grade or above.

Preparation of micelles. The micelles were prepared using a solvent displacement method. Briefly, copolymer allyl-PEG-b-PLA (100 mg) was first dissolved in tetrahydrofuran (THF) (5 mL) and the solution was injected to 10 mL of deionized water with gentle agitation, followed by removing THF by dialysis (cutoff=3500) against deionized water for 10 h.

Measurement of CMC. The critical micelle concentration of the micelles was measured by a fluorescence technique using pyrene as a probe reported before.[2] A predetermined amount of pyrene solution in acetone was added to a series volumetric flasks to ensure that the pyrene concentration in the final solution was 6×10^{-7}mol/L, the saturation solubility of pyrene in water at 22 °C. Acetone was evaporated

completely. The micelle solutions of allyl-PEG-b-PLA with various concentrations from 10^{-4} to 1.0 g/L were added to each of the flasks. The flasks were thermostated at 25 °C for about 2 h to equilibrate pyrene partition between water and micelles. For fluorescence-excitation spectra, the emission wavelength was set to 391 nm and the excitation bandwidth was 5 nm. The spectra were recorded at a scan rate of 240 nm/min.

Synthesis of c(RGDfK)-PEG-b-PLA. 0.2 g of HOOC-PEG-b-PLA (25 μmol) was dissolved in 4 mL DMSO, then 7.74 mg DCC (37.5 μmol) and 4.32 mg NHS (37.5 μmol) were added. The reaction was conducted at room temperature for 4 hours and then 15 mg c(RGDfK) peptide (25 μmol) was added. After dialysis against distilled water for 12 hours (MWCO=3500), the final suspension in dialysis bag was freeze-dried. The c(RGDfK) peptide content of the polymer was determined by measuring the arginine content according to the reference. [3]

Synthesis of API-PEG-b-PLA. API-PEG-b-PLA was prepared by similar method above just replacing c(RGDfK) peptide with N-(3-aminopropyl)-imidazole (API). The API-PEG-b-PLA lyophilized powder obtained was characterized by ^{1}H NMR.

Characterization of copolymers. ^{1}H NMR spectra were recorded on a Bruker AV400 M in $CDCl_3$ at 25 °C. Chemical shifts were given in parts per million from that of tetramethylsilane (TMS) as an internal reference.

Preparation of Dox loaded micelles. The Dox loaded micelles were prepared as follows: 5 mg of Dox·HCl and 10 μL of triethylamine (TEA) were dissolved in 2 mL of DMSO and stirred for half an hour. Then 45 mg of mPEG-b-PLA dissolved in 4

mL of DMSO was added. After 2 minutes of sonication, the polymer and drug solution was added dropwise to 10 mL of deionized water. Unloaded Dox and DMSO was removed by dialysis against distilled water for 12 hours (MWCO=3500). The lyophilized micelles powder was abbreviated as M(Dox).

cRGD and/or imidazole decorated micelles were prepared similarly by using desired ratios of c(RGDfK)-PEG-PLA, API-PEG-PLA, and mPEG-PLA as the starting materials. Typically, two cRGD micelles were prepared by using a mixture of c(RGDfK)-PEG-PLA and mPEG-PLA with weight ratio of 10/90 and 20/80, respectively. The obtained Dox-loaded micelles were abbreviated as RGD_{10}-M(Dox) and RGD_{20}-M(Dox), respectively. Similarly, two imidazole micelles were prepared by using a mixture of API-PEG-PLA and mPEG-PLA with weight ratio of 20/80 and 50/50, respectively, and the obtained Dox-loaded micelles were abbreviated as API_{20}-M(Dox) and API_{50}-M(Dox), respectively. cRGD and imidazole dual-targeted micelles were prepared by using a mixture of c(RGDfK)-PEG-b-PLA, API-PEG-b-PLA, and mPEG-b-PLA with a weight ratio of 20/50/30 and they were abbreviated as RGD_{20}/API_{50}-M(Dox) or simply RGD/API-M(Dox).

The loading content of Dox was determined by the UV absorbance at 480 nm for Dox, according to the standard calibration curve of free Dox in DMSO. The drug loading efficiency (DLE) was calculated according to the following equation:

DLE%= (weight of Dox in the micelle/weight of Dox added) ×100%

Micelle characterization. Size distribution of the micelles was determined by dynamic light scattering (DLS) with a vertically polarized He-Ne laser (DAWN EOS,

Wyatt Technology, U.S.A.). The scattering angle was fixed at 90° and the measurement was carried out at 25 °C.

In vitro drug release. Freeze-dried micelle samples (M(Dox), RGD_{20}-M(Dox) and APT_{50}-M(Dox), 20 mg each) were re-suspended in phosphate buffered saline (PBS, pH 7.4 and pH 6.5) solutions (5 mL), respectively, sealed in a dialysis bag (Mw cutoff: 3.5 kDa) and incubated in the release medium (25 mL) at 37 °C under oscillation at 90 r/min. At selected time intervals, 1 mL buffer solution outside the dialysis bag was removed for UV-vis analysis and replaced with 1 mL fresh buffer solution. The released amount of Dox was determined from the absorbance at 480 nm with the help of a calibration curve of Dox in the same buffer. Then the accumulative weight and relative percentage of the released Dox were calculated as a function of incubation time.

Cell culture. The mouse mammary tumor cell line EMT6 was routinely grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) containing 10% FBS (Hyclone). Cells were cultured in a humidified incubator at 37 °C with 5% CO_2.

Confocal laser scanning microscopy (CLSM). EMT6 cells were seeded on the coverslip in 6-well plates at a density of 5×10^5 cells per well in 1 mL of DMEM medium and incubated in a humidified 5% CO_2 atmosphere for 24 h. For cellular internalization observation, cells were incubated with free Dox and Dox-loaded micelles at an equivalent Dox concentration of 10 μg mL^{-1} in fresh culture medium. After incubation for 0.5 h at 37 °C, cells were washed twice with ice-cold PBS and fixed with fresh 4% (w/v) paraformaldehyde for 10 min at room temperature. The

cells were counterstained with 4, 6-diamidino-2-phenylindole (DAPI) for cell nucleus. The cellular localization was visualized under a confocal laser scanning microscope (Carl Zeiss LSM 780).

Cellular uptake measured by flow cytometry. EMT6 cells were seeded in six-well plates (2×10^5 cells/well) and cultured for 24 h. The medium was replaced with free Dox and Dox-loaded micelles at an equivalent Dox concentration of 10 μg mL^{-1} in fresh culture medium. After incubation for 0.5 h at 37 °C, the cells were washed three times with cold PBS and treated with trypsin. The harvested cells were suspended in PBS and centrifuged at 1000 rpm for 5 min. The supernatants were discarded and the cell pellets were re-suspended in 0.5 mL PBS. Flow cytometry analysis was performed by a flow cytometer (Beckman, USA) which collected 1×10^4 gated events for each sample.

In vitro cytotoxicity. The cytotoxicity of four kinds of Dox-loaded micelles (M(Dox), RGD_{20}-M(Dox), APT_{50}-M(Dox), RGD/APT-M(Dox)) and two copolymer micelles (Allyl-PEG-b-PLA and HOOC-PEG-b-PLA) was measured via MTT assay with free Dox as a control. Briefly, EMT6 cells harvested in a logarithmic growth phase were seeded in 96-well plates at a density of 2×10^3 cells/well and incubated in DMEM for 24 h. The medium was then replaced with micelles or free Dox at various Dox concentrations from 0.005 to 50 μg/mL or with blank polymer micelles at concentrations from 0.05 to 500 μg/mL. At the designated time intervals (48 h), 20 μL of MTT solution in PBS with the concentration of 5 mg/mL was added and the plate was incubated for another 4 h at 37 °C. After that, the medium containing MTT was

removed and 150 μL of DMSO was added to each well to dissolve the MTT formazan crystals. Finally, the plates were shaken for 10 min, and the absorbance of formazan product was measured at 490 nm by a microplate reader (BioTek, EXL808). Data are presented as means ±SD (n=4).

In vivo distribution. Balbc female mice were obtained from Jilin University, China (56-84 d, 15-20 g) and maintained under required conditions. All animal procedures were approved and controlled by the local ethics committee and carried out according to the guidelines of Chinese law concerning the protection of animal life. To develop the tumor xenografts, EMT 6 cells were injected into the lateral aspect of the anterior limb of the mice (5×10^6 cells in 0.1 mL PBS). After the tumor volume reached 50-100 mm^3, free Dox and Dox micelles were injected into the mice via tail veins with an equivalent Dox dose of 5 mg/kg body weight. At 3 h and 12 h post injection, the mice were sacrificed and the tumors and organs were excised and imaged by the Maestro in-vivo Imaging System (CRI Maestro™ 2.4, Cambridge Research & Instrumentation, Inc., USA), which consisted of a light-tight box equipped with a 150 W halogen lamp and an excitation filter (445–490 nm) to excite Dox. Fluorescence was detected by a CCD camera equipped with a C-mount lens and an emission filter (580 nm longpass).

Statistics. All experiments were performed at least three times and all results are expressed as mean ± (standard deviation). Student's t-test was used to demonstrate statistical significance ($P<0.05$).

Table S1. Properties of drug loaded micelles

Samples	Micelles	Diameter (nm)[a]	Zeta potential (mV)

1	M(Dox)	65±8	0±1.2
2	RGD_{10}-M(Dox)	50±15	0±0.5
3	RGD_{20}-M(Dox)	55±12	0±1.5
4	API_{20}-M(Dox)	60±12	0.96±4.0
5	API_{50}-M(Dox)	64±15	0.49±3.1
6	RGD/API-M(Dox)	55±10	0.18±3.6

[a]Determined by DLS.

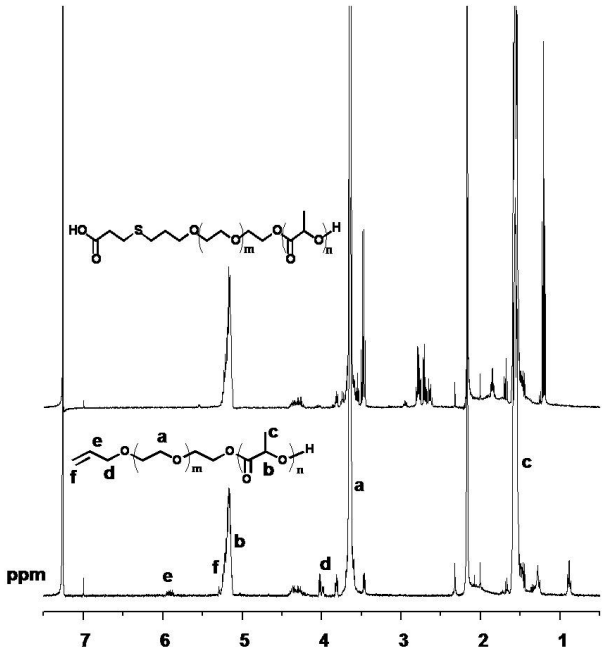

Figure S1. The ^{1}H NMR spectra of COOH-PEG-b-PLA and Allyl-PEG-b-PLA.

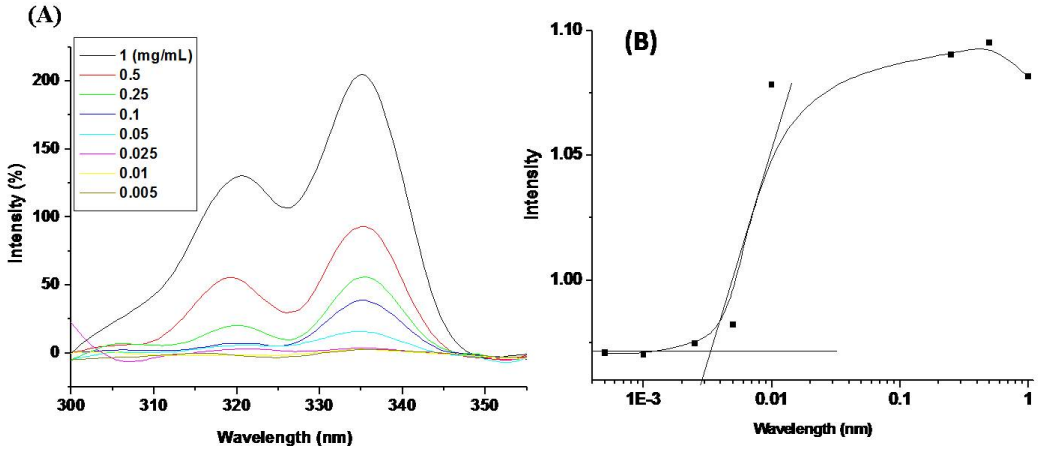

Figure S2. (A) Excitation spectra of pyrene at λ_{em}=391 nm in water solution of various concentrations and (B) plot of the intensity ratio I_{335}/I_{333} vs log C for allyl-PEG-b-PLA.

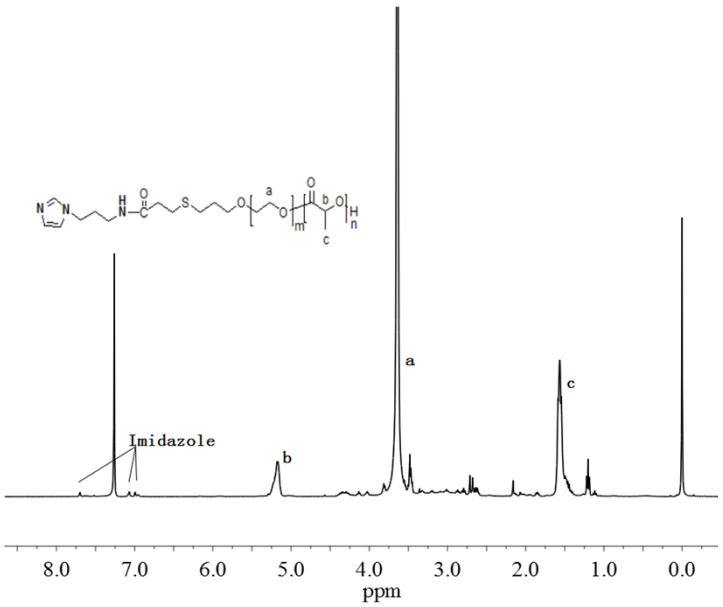

Figure S3. The ^{1}H NMR spectra of API-PEG-b-PLA

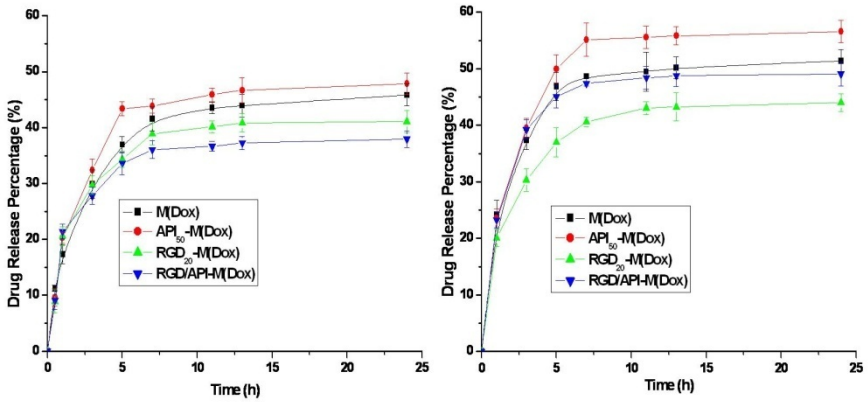

Figure S4. In vitro cumulative release profiles of Dox from M(Dox)s at different pHs at 37 ℃. (a) at pH 7.4; (b) at pH 6.5.

Reference

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