Supplementary Information

Albumin-coated pH-responsive Dimeric Prodrug-based Nano-assemblies with High Biofilm Eradication Capacity

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1. Materials and methods

1.1 Materials

1.1.1 Reagents

The dimeric prodrug of azithromycin-citraconic amide-tobramycin (AZM-Cit-TOB) was purchased from Ruixi Biological Technology Co., Ltd (Xi'an, Shanxi, China). Albumin from human serum was purchased from Sigma-Aldrich Co. LLC (Shanghai, China). A LIVE/DEAD® BacLight™ Bacterial Viability Kit (L7012) was purchased from Thermo Fisher Scientific Inc (Shanghai, China).

1.1.2 Bacteria and Cells

PAO1 were purchased from Hangzhou BIOSCI Company (Hangzhou, China). RAW264.7 cells and Vero cells were purchased from American Type Culture Collection (Manassas, VA, USA), and cultured in DMEM media (Gibco BRL, Grand Island, NY) containing 10% FBS.

1.1.3 Animals

BALB/c nude mice (male) and BALB/c mice were purchased from Pengyue Laboratory Animal Breeding Co., Ltd (Jinan, Shandong, China). The New Zealand white rabbits were purchased from Qingdao Kangda Biotechnology Co., Ltd. The animal studies were conducted according to the experimental protocols by Institutional Animal Care and Use Committee of Binzhou Medical University.

1.2 Methods

1.2.1 Synthesis and characterization of azithromycin-citraconic amide-tobramycin

Azithromycin was dissolved in anhydrous DMSO with vigorous stirring. Followed the citraconic anhydride DMSO solution was drop-added and performed 72 h at room temperature. Next, EDC and NHS were introduced to active of carboxyl for 4 h. Finally, tobramycin DMSO solution was drop-added and kept the reaction going to another 72 h. The target product was obtained by precipitation in diethyl ether and washed with DCM. The molar ratio of azithromycin: citraconic anhydride: tobramycin was 1:1.2:1, and the molar ratio of azithromycin: EDC: NHS was 1:1.5:1.5. The detailed synthetic route of azithromycin-citraconic amide-tobramycin was shown in Fig. S1. The red dotted line

represents the chemical site for pH cleavage. NMR spectra were recorded at 400 MHz for ^{1}H NMR on a Bruker Avance III 400 NMR spectrometer in DMSO-*d6*.

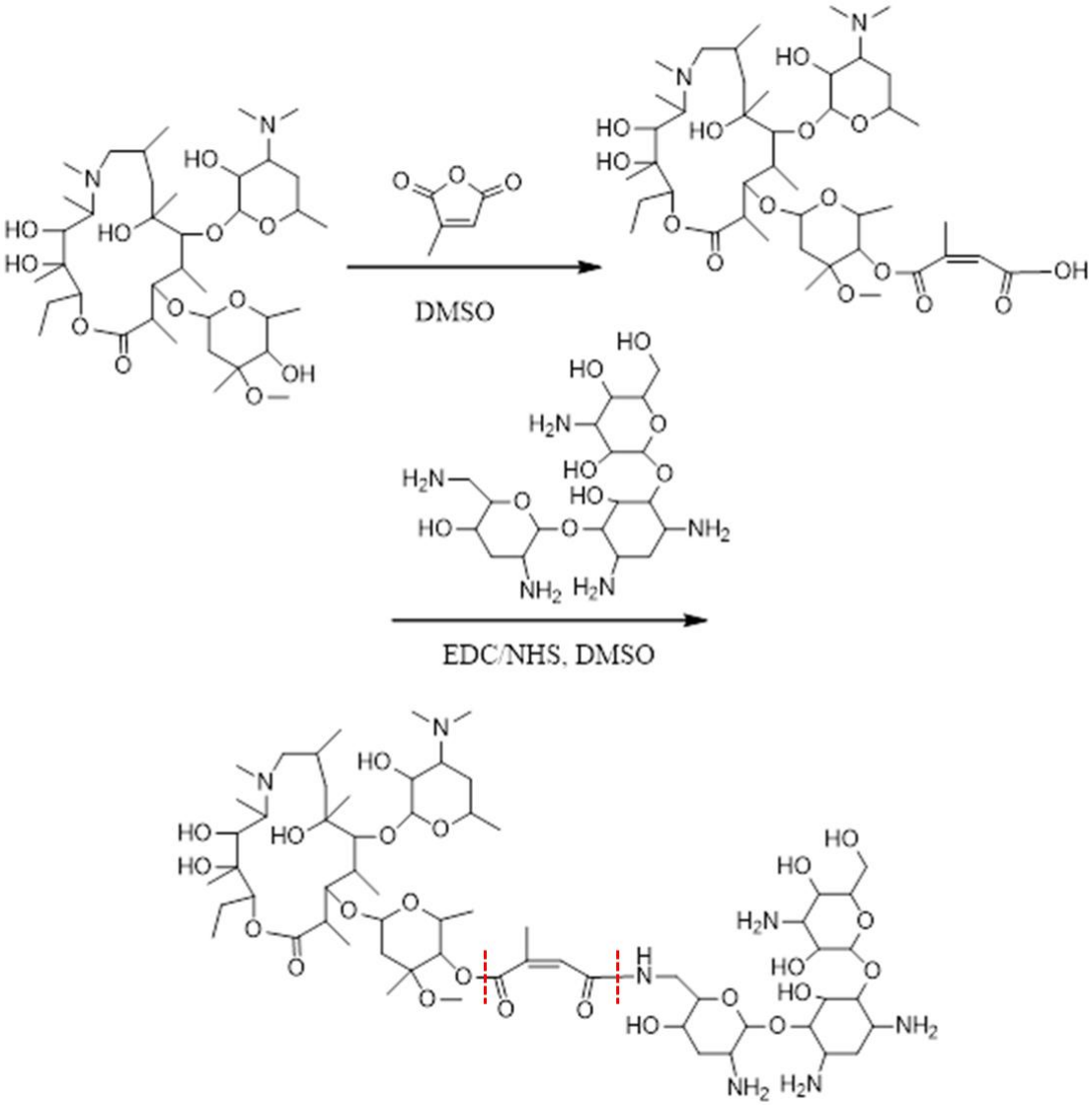

Fig. S1 The synthetic route of azithromycin-citraconic amide-tobramycin (AZM-Cit-TOB).

1.2.2 Characterization of Nano-assemblies

The morphology of HSA@DPNA and DPNA was observed by a JEOL JEM1400 transmission electron microscope (TEM, Japan). Briefly, a drop of nano-assemblies was placed on a TEM copper grid coated with carbon film and maintained for 8 min. After removing the excess solvent, the samples were negatively stained with 1% phosphotungstic acid and fully dried before investigation. The particle sizes and zeta potentials of both nano-assemblies were measured by a Malvern Zetasizer Nano ZS analyzer (UK) at 25 °C, respectively. Each test was reproduced at least three times.

For accurately investigating the *in vivo* stability of HSA@DPNA nanoparticles, plasma stability assay was performed. The HSA@DPNA solution was mixed with PBS (pH 7.4) containing 10% of bovine serum with shaken in 37 ℃. And then, samples were taken at 0, 2, 4, 8, 12, 24, 36 and 48 h to measure the particle sizes and PDI through a Malvern Zetasizer Nano ZS analyzer (UK) at 25 °C.

Next, a long-term stability study was taken for the assessment of the shelf life of HSA@DPNA nanoparticles. Briefly, the HSA@DPNA solution was placed at 4 ℃ and 25 ℃, respectively. And then, samples were taken at predicted intervals to measure the particle sizes and PDI through a Malvern Zetasizer Nano ZS analyzer (UK) at 25 °C.

The drug loading coefficient (DL%) was calculated using the following equations. Briefly, 20 mg of the freeze-dried DPNA or HSA@DPNA was dissolved in 10 mL of PBS (pH 4.5) and incubated overnight for complete hydrolysis of citraconic amide bonds, respectively. The AZM was firstly hydrolyzed by sulfuric acid at 60 °C and then measured by a Synergy H1 Hybrid Multi-Mode Reader (BIOTEK, USA) at 482 nm. The TOB amount was measured using high-performance liquid chromatography (HPLC) involving pre-column derivatization with 2,4-dinitrofluorobenzene. The results given were mean values of three independent determinations.

$$DL\% = \frac{\text{Weight of the drug in nano} - \text{assemblies}}{\text{Weight of freeze-dried nano} - \text{assemblies}} \times 100\%$$

1.2.3 *In vitro* cell viability assay

The *in vitro* cytotoxicity of DPNA and HSA@DPNA were evaluated against Vero cells using MTT method.[3] Briefly, Vero cells were planked in 96-well plated with a density of 3000 cells per well. After incubation overnight, the cells were treated with DPNA and HSA@DPNA at the concentration ranging from 32 to 1024 μg/mL for another 24 h. After incubation, the wells were washed twice with PBS, and the replaced with MTT solution (5 mg/mL). The plates were further incubated for 4 h at 37 °C, allowing the viable cells to reduce the yellow MTT into purple formazan crystals. Finally, the purple formazan crystal in each well was dissolved by 150 μL of DMSO, and the absorbance was measured at 570 nm using a BioTek Synergy H1 hybrid multi-mode microplate reader (USA). The experiment was verified by at least three independent trials, and data were averaged.

1.2.4 *In vitro* hemolytic test

Rabbit red blood cells (rRBCs) were used to investigate the hemolysis behavior of DPNA and HSA@DPNA. 200 μL of rRBCs (2%, v/v) was incubated with DPNA and HSA@DPNA at a concentration of 32 to 1024 μg/mL for 2 h and centrifuged at 1000 rpm for 10 min. The absorption of the supernatant was measured by a BioTek Synergy H1 hybrid multi-mode microplate reader (USA) at 570 nm. Saline and 0.5% Triton X-100 were set as the negative and positive control, respectively. Hemolysis was calculated using the following equation:

$$\text{Hemolysis (\%)} = \frac{\text{OD}_t - \text{OD}_0}{\text{OD}_{100} - \text{OD}_0} \times 100\%$$

Where, OD_t is the absorption of the rRBCs treated with DPNA and HSA@DPNA at the concentration of t, OD_0 is the absorption of rRBCs treated with saline, and the OD_{100} is the absorption of rRBCs treated with 0.5% of Triton X-100. The results given were the mean values of three independent determinations using six replicates.

2. Results

2.1 ^{1}H NMR spectra of AZM-Cit-TOB

The ^{1}H NMR spectra of AZM-Cit-TOB was shown in Figure S2. All the chemical shifts were expressed in parts per million (δ) relative to the solvent signal. The ^{1}H NMR spectrum (DMSO-*d6*) of AZM-Cit-TOB showed peaks at δ=0.78-1.24 ppm (-CH-C$\underline{H}_3$; NH_2-CH-C$\underline{H}_2$-CH-NH_2), δ=0.78-1.24 ppm (-O-C$\underline{H}$-; -O-CH-C$\underline{H}$OH-CH-O-; -CONH-C$\underline{H}_2$-), δ=1.31-1.56 ppm (-N-CH-C$\underline{H}_2$-CH-O-; -O-CH-C$\underline{H}_2$-CH_3-), δ=1.75-1.98 ppm (-O-CH-C$\underline{H}$-; -N-CH_2-C$\underline{H}$-CH_3-; NH_2-CH-C$\underline{H}_2$-CH-NH_2), δ=2.25-2.51 ppm (-N-C$\underline{H}$-CH_3; -N(C$\underline{H}_3$)$_2$; -N-CH-C$\underline{H}_2$-CH-O-; -OOC-C$\underline{H}$-CH_3; C$\underline{H}_3$-C=CH-), δ=2.67-2.94 ppm (-C$\underline{H}$-NH_2; -C$\underline{H}_2$-N-), δ=3.16-3.27 ppm (-C$\underline{H}_2$-OH; -C$\underline{H}$-OH;-O-C$\underline{H}$-), δ=3.39-3.52 ppm (-O-C$\underline{H}$-; -O-CH-C$\underline{H}$OH-CH-O-; -CONH-C$\underline{H}_2$-), δ=3.63-3.74 ppm (-O-C$\underline{H}$-; -O-CH-C$\underline{H}$OH-CH-O-; -CONH-C$\underline{H}_2$-), δ=4.01-4.10 ppm (-O-C$\underline{H}$-; -O-C$\underline{H}$-O-; -COO-C$\underline{H}$-), δ=4.37 ppm (-O-C$\underline{H}$(-CHOH-)-O-; -COO-C$\underline{H}$-), δ=4.58 ppm (-O-C$\underline{H}$(-CH_2-)-O-), δ=4.70 ppm (-O-C$\underline{H}$(-CH_2-)-O-), δ=5.75-5.78 ppm (-CO-C$\underline{H}$=) and δ=5.95-6.06 ppm (-CO-N$\underline{H}$-). The ^{1}H NMR spectrums (DMSO-*d6*) of AZM-Cit-TOB showed the characteristic peaks of both AZM and TOB suggesting AZM-Cit-TOB were obtained.

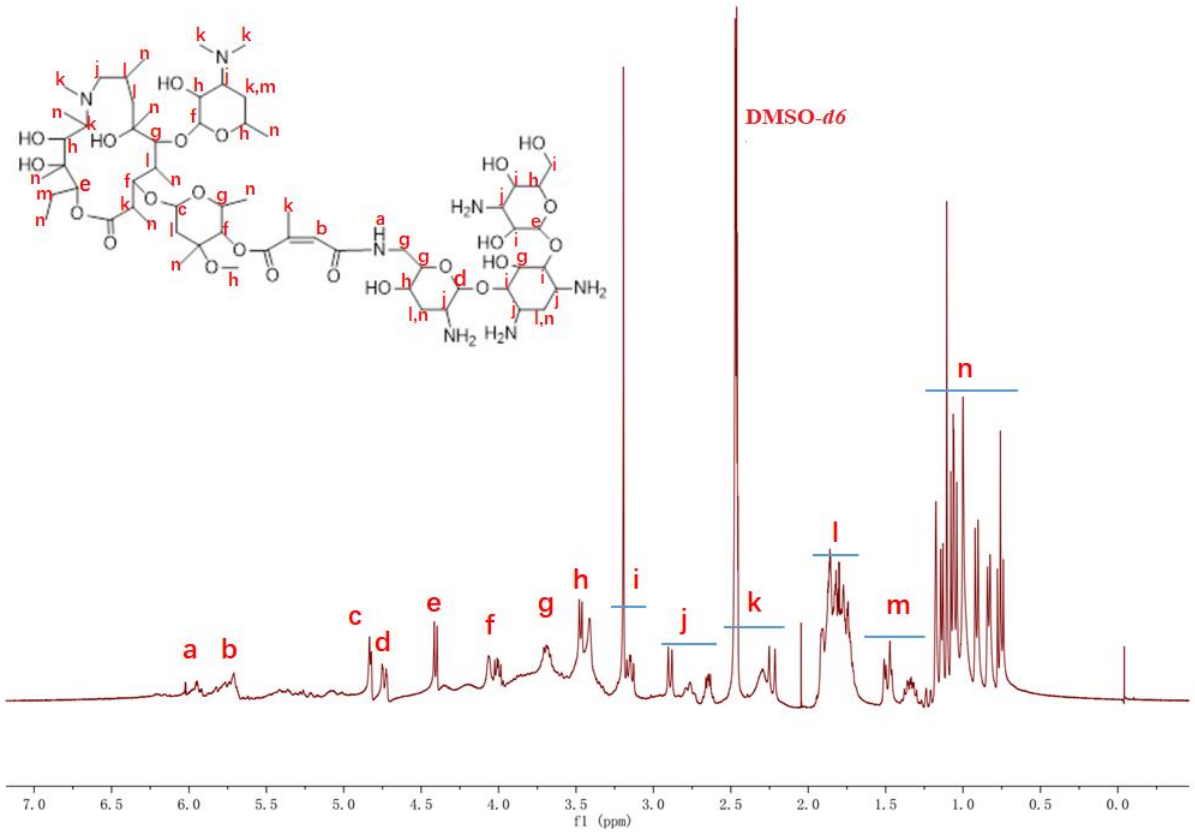

Fig. S2 ^{1}H NMR spectrum of AZM-Cit-TOB

2.2 Long-term stability of HSA@DPNA

The long-term stability study was taken for the assessment of the shelf life of HSA@DPNA. The corresponding results were illustrated in the Table S1 and Table S2. At 4 °C, the particle sizes and PDI of HSA@DPNA did not change significantly within 8 months, which verifying the favorable stability of HSA@DPNA under low temperature environment. However, the particle sizes and PDI of HSA@DPNA kept increasing over times at room temperature, even up to more than 400 nm after 8 months retention. The results suggested that HSA@DPNA showed better stabilities in the storage at low temperature. Next, we will optimize the formulation and preparation process of lyophilized HSA@DPNA. Moreover, the long-term stability study will be continued to determine the shelf-life of HSA@DPNA under low temperature.

Table S1 Long-term stability test of HSA@DPNA (4 °C)

Time (month)	0	1	2	3	4	6	8
Particle Size (nm)	151.2±3.12	152.4±2.33	154.5±3.45	158.9±2.98	161.2±2.27	165.4±1.89	169.3±3.01
PDI	0.189±0.034	0.191±0.029	0.187±0.025	0.193±0.031	0.195±0.022	0.194±0.029	0.201±0.023

Table S2 Long-term stability test of HSA@DPNA (25 °C)

Time (month)	0	1	2	3	4	6	8
Particle Size (nm)	151.2±3.12	162.3±2.89	191.2±4.55	243.7±9.88	292.9±12.15	343.5±17.22	403.5±22.68
PDI	0.189±0.034	0.201±0.027	0.293±0.096	0.335±0.131	0.391±0.113	0.443±0.101	0.642±0.129

2.3 Releasing behavior of HSA@DPNA

The release profiles of AZM and TOB from DPNA and HSA@DPNA were also determined at the upper (pH 6.5) and lower (pH 4.5) limit of biofilm microenvironment (Fig. S3). As shown in Fig. S3A, both DPNA and HSA@DPNA were relatively stable at pH 6.5, less than 25% of AZM and TOB were released within 3 h. However, when the environmental pH decreased to 4.5, the cumulative release of AZM and TOB from DPNA and HSA@DPNA was accelerated (Fig. S3B). More than 60% of AZM and TOB were released within 60 min, while nearly 90% of AZM and TOB were released after 180 min.

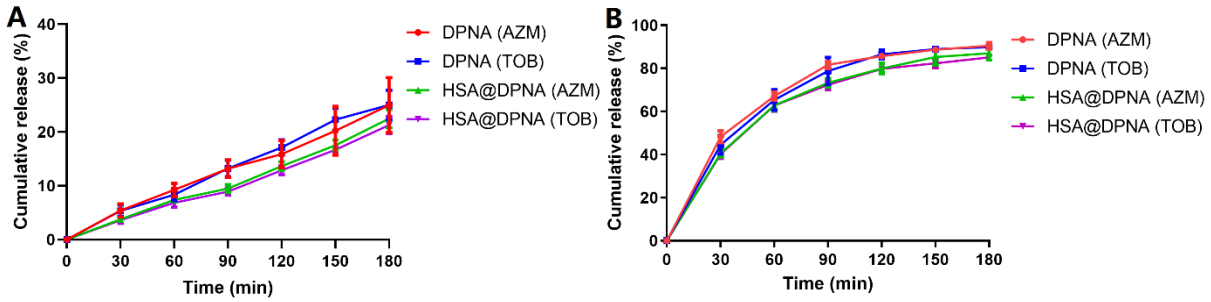

Fig. S3 pH-triggered drug release behaviors of DPNA and HSA@DPNA at pH 6.5 (A) and pH 4.5 (B), respectively.