Supporting information

Bacterial biofilm destruction by size/surface charge-adaptive micelles

Maohua Chen, Jiaojun Wei, Songzhi Xie, Xinyan Tao, Zhanlin Zhang, Pan Ran, Xiaohong Li*

Key Laboratory of Advanced Technologies of Materials, Ministry of Education, School of Materials Science and Engineering, Southwest Jiaotong University, Chengdu 610031, P.R. China

* Corresponding author. School of Materials Science and Engineering, Southwest Jiaotong University, Chengdu 610031, P.R. China. Phone: +8628-87634068; fax: +8628-87634649.
E-mail Address: xhli@swjtu.edu.cn

S1. Materials

ε-Benzyloxycarbonyl L-aspartic acid N-carboxyanhydride (BLA-NCA), diethylenetriamine (DET), 2-(diisopropylamino)ethylamine (DIP), succinic anhydride, ethylenediamine, *cis*-aconitic anhydride, triphosgene, octadecanamine, D-tyrosine and poly(ethylene glycol) (PEG) diamine (*M*w: 2 and 6 kDa) were procured from Sigma (St. Louis, MO). 4-Dimethylaminopyridine (DMAP), N,N'-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), deuterated dimethyl sulfoxide (DMSO-*d6*), chloroform ($CDCl_3$) and D_2O were purchased from Aladdin (Beijing, China), and azithromycin was from Heowns Biochem LLC (Tianjing, China). All other chemicals were analytical reagent and received from Changzhen Regents Company (Chengdu, China), unless otherwise indicated. Dichloromethane was dried with 4 Å molecular sieves and redistilled before use. Dimethyl sulfoxide (DMSO), N,N-dimethyl formamide (DMF), and ε-caprolactone was distilled under reduced pressure after dried by CaH_2 overnight.

S2. Synthesis of PEG- PAsp(DIP)

PEG-PAsp(DIP) was prepared by ring-opening polymerization of BLA-NCA initiated by PEG diamine, followed by DIP aminolysis (Fig. S1a). Briefly, 0.6 g of PEG diamine (*M*w: 6 kDa) was dissolved in 40 mL of dry THF under argon, followed by the addition of BLA-NCA (1.9 g, 7.6 mmol) in dry THF (3 mL). The mixture was stirred at 35 ℃ for 48 h under argon, followed by precipitation into excessive diethyl ether and vacuum drying to obtain PEG-PBLA (yield: 87%).[1] The ^{1}H NMR spectrum was recorded on a Bruker AM 400 apparatus, using tetramethylsilane as the internal reference. ^{1}H NMR (DMSO-*d6*, ppm, δ): 8.18 (-CO-N<u>H</u>-), 7.29 (-C_6<u>H_5</u>), 5.02 (-C<u>H_2</u>-C_6H_5), 4.59 (-NHC<u>H</u>(CH_2-)CO-), 4.12 (-COC<u>H_2</u>C<u>H_2</u>O-), 3.49 (-OC<u>H_2</u>C<u>H_2</u>O-), 2.32–2.91 (-CO(C<u>H_2</u>)$_2$NH- and -COC<u>H_2</u>C<u>H_2</u>CO-) (Fig. S1b). The molecular weight and polydispersity index of polymers were measured by gel permeation chromatography (GPC, waters 2695 and 2414, Milford, MA) using tetrahydrofuran as the eluent at a flow rate of 1.0 mL/min (Fig. S2).

The aminolysis of PEG-PBLA with DIP was performed by mixing PEG-PBLA (0.3 g) in DMF (10 mL) with DIP (4 mL).[2] The mixture was stirred for 24 h at 35 ℃, followed by precipitation into diethyl ether and vacuum drying to obtain PEG-PAsp(DIP) (yield: 90%). ^{1}H NMR (DMSO-*d6*, ppm, δ): 4.69 (-NHC<u>H</u>(CH_2-)CO-), 4.17 (-COC<u>H_2</u>C<u>H_2</u>O-), 3.52 (-OC<u>H_2</u>C<u>H_2</u>O-), 2.96 (-C<u>H</u>(CH_3)), 2.59–2.91

(-CO(C$\underline{H}_2$)$_2$NH- and -COC$\underline{H}_2$C$\underline{H}_2$CO-), 2.47 (-NHC$\underline{H}_2$C$\underline{H}_2$CH-), 0.91 (-C$\underline{H}_3$) (Fig. S1c).

S3. Synthesis of OA-PAsp(DET/DET-Az)

OA-PAsp(DET/DET-sa-Az) was synthesized by conjugation of azithromycin with DET-grafted poly(aspartamide) via succinic acid linkages (Fig. S3a). DET-grafted poly(aspartamide) was obtained by ring-opening polymerization of BLA-NCA initiated by octadecylamine, followed by aminolysis by DET. Briefly, octadecylamine (0.135 g, 0.5 mmol) was mixed with BLA-NCA (3.7 g, 30 mmol) in 50 mL of dry THF at 35 ℃ for 48 h under argon,[1] followed by precipitation into excessive diethyl ether and vacuum drying to obtain OA-PBLA (yield: 85%). ^{1}H NMR (DMSO-*d6*, ppm, δ): 8.18 (-CO-N$\underline{H}$-), 7.29 (-C$_6\underline{H}_5$), 5.02 (-C$\underline{H}_2$-C$_6$H$_5$), 4.59 (-NH-C$\underline{H}$(CH$_2$-)CO-), 2.32–2.91 (-CO(C$\underline{H}_2$)$_2$NH-), 1.13 (-(CH$_2$)$_{16}$CH$_3$), 0.76 (-CH$_3$) (Fig. S3b).

The aminolysis was performed by mixing OA-PBLA (300 mg) with DET (4.0 g, 50 equiv to benzyl group of PBLA segment) in dry DMF (10 mL) for 24 h at room temperature. The mixture was added dropwise into 40 mL of acetic acid solution (10%, v/v) and dialyzed (3.5 kDa cutoff) in 0.01 M HCl solution, followed by freeze-drying to obtain OA-PAsp(DET) (yield: 88%). ^{1}H NMR (DMSO-*d6*, ppm, δ): 4.59 (-NH-C$\underline{H}$(CH$_2$-)CO-), 3.67 (NH$_2$-C$\underline{H}_2$-), 3.48 (-C$\underline{H}_2$-NH-C$\underline{H}_2$-), 3.25 (-CO-NH-C$\underline{H}_2$-), 2.81 (-CO(C$\underline{H}_2$)$_2$NH-), 1.19 (-(C$\underline{H}_2$)$_{16}$CH$_3$), 0.84 (-C$\underline{H}_3$) (Fig. S3c).

Azithromycin was conjugated onto OA-PAsp(DET) via succinic acid linkages. Briefly, succinic anhydride (35 mg, 0.267 mmol), DCC (55 mg, 0.267 mmol) and DMAP (32 mg, 0.267 mmol) were dissolved in dry chloroform (20 mL) under argon, followed by the addition of azithromycin (200 mg, 0.267 mmol) in DMF (5 mL).[3] After reaction for 24 h, the mixture was evaporated under reduced pressure and precipitated by excessive diethyl ether. The crude product was purified by column chromatography on silica gel using methanol/ethyl acetate/hexane (4/2/2) as elute to obtain Az-sa (yield: 76%). ^{1}H NMR (CDCl$_3$, ppm, δ): 5.06–4.58 (-C$\underline{H}$O-), 3.35 (C$\underline{H}_3$-O-), 2.51 (-COC$\underline{H}_2$C$\underline{H}_2$CO-), 2.33 (C$\underline{H}_3$N-), 1.34–0.81 (CH$_3$- of Az) (Fig. S3d).

Az-sa was conjugated with OA-PAsp(DET) by DCC/NHS chemistry.[4] Briefly, Az-sa (61 mg, 0.11 mmol) was dissolved in 10 mL of DMSO, followed by the addition of DCC (35 mg, 0.17 mmol), NHS (2.0 mg, 0.17 mmol) and triethylamine (0.05 mL). After kept stirring for 24 h at room temperature, the product was filtered to remove insoluble dicyclohexylurea. The solution of NHS ester of Az-sa was

added to OA-PAsp(DET) (300 mg) in DMSO, followed by stirring for 48 h at room temperature. The product was dialyzed (3.5 kDa cutoff) against distilled water for 3 days, followed by freeze-drying to obtain OA-PAsp(DET/DET-sa-Az) (yield: 86%). ^{1}H NMR (DMSO-*d6*, ppm, δ): 4.59 (-NH-C<u>H</u>(CH_2-CO)CO-), 3.67 (NH_2-C<u>H_2</u>-), 3.48 (-C<u>H_2</u>-NH-C<u>H_2</u>-), 3.25 (-CO-NH-C<u>H_2</u>) 2.82 (-NH-CH(C<u>H_2</u>-CO-)CO-), 2.61 (-COC<u>H_2</u>C<u>H_2</u>CO-), 2.34 (-N(C<u>H_3</u>)$_2$, 1.36–0.80 (-(C<u>H_2</u>)$_{16}$C<u>H_3</u> and protons of azithromycin) (Fig. S3e).

S4. Synthesis of azithromycin-conjugated PEG (PEG-sa-Az)

PEG-sa-Az was synthesized by conjugation of Az-sa with PEG catalyzed by DCC/NHS as described above (Fig. S4a). The solution of NHS ester of Az-sa was added to 200 mg of PEG diamine (*M*w: 2 kDa). After reaction for 48 h at room temperature, the product was dialyzed (1 kDa cutoff) against distilled water for 3 days, followed by freeze-drying to obtain PEG-sa-Az (yield: 85%). ^{1}H NMR ($CDCl_3$, ppm, δ): 4.31 (-COOC<u>H_2</u>C<u>H_2</u>O-), 3.51 (-OC<u>H_2</u>C<u>H_2</u>O-), 2.53 (-COC<u>H_2</u>C<u>H_2</u>CO-), 1.48–0.85 (protons of azithromycin) (Fig. S4b).

S5. Synthesis of CA-Tyr

Fig. S5a shows the conjugation process of D-tyrosine with *cis*-aconitic anhydride. Briefly, D-tyrosine (36 mg, 0.2 mmol) was dissolved in 10 mL of NaOH solution (pH 9) and cooled on ice, followed by the addition of 5 mL of p-dioxane (5 mL) containing *cis*-aconitic anhydride (38 mg, 0.24 mmol). The reaction mixture was stirred at pH 9.0 for 20 min in an ice bath and then slowly warmed to room temperature. After stirring for another 30 min, the mixture was cooled to 0 ℃ again and acidified with 1 M HCl solution until precipitate formation. The precipitate was collected by centrifugation and purified by column chromatography on silica-gel with chloroform/methanol (10/1) as elute to obtain CA-Tyr (yield: 64%).[5] ^{1}H NMR (D_2O, ppm, δ): 6.79 (-C<u>H</u>=CH-), 6.50 (CO-C<u>H</u>=CH-), 6.43 (-CH=C<u>H</u>-), 4.41 (-CH_2C<u>H</u>(NH)COOH), 3.56 (-C<u>H_2</u>COOH), 2.45–2.72 (-C<u>H_2</u>CH(NH)COOH) (Fig. S5b).

References:

[1] A. Koide, A. Kishimura, K. Osada, W.D. Jang, Y. Yamasaki, K. Kataoka, Semipermeable polymer

vesicle (PICsome) self-assembled in aqueous medium from a pair of oppositely charged block copolymers: physiologically stable micro-/nanocontainers of water-soluble macromolecules, J. Am. Chem. Soc. 128 (2006) 5988–5989.

[2] J. Dai, S.D. Lin, D. Cheng, S.Y. Zou, X.T. Shuai, Interlayer-crosslinked micelle with partially hydrated core showing reduction and pH dual sensitivity for pinpointed intracellular drug release. Angew. Chem. Int. Ed. 50 (2011) 9404–9408.

[3] M.K. Mishra, K. Kotta, M. Hali, S. Wykes, H.C. Gerard, A.P. Hudson, J.A. Whittum-Hudson, R.M. Kannan, PAMAM dendrimer-azithromycin conjugate nanodevices for the treatment of chlamydia trachomatis infections. Nanomedicine 7 (2011) 935–944.

[4] M.H. Chen, Y. Zhang, Z.J. Chen, S.Z. Xie, X.M. Luo, X.H. Li, Synergistic antitumor efficacy of redox and pH dually responsive micelleplexes for co-delivery of camptothecin and genes. Acta Biomater. 49 (2016) 444–455.

[5] A. Sakai, D.F. Xiang, C. Xu, L. Song, W.S. Yew, F.M. Raushel, J.A. Gerlt, Evolution of enzymatic activities in the enolase superfamily: N-succinylamino acid racemase and a new pathway for the irreversible conversion of D-to L-amino acids. Biochemistry 45 (2006) 4455–4462.

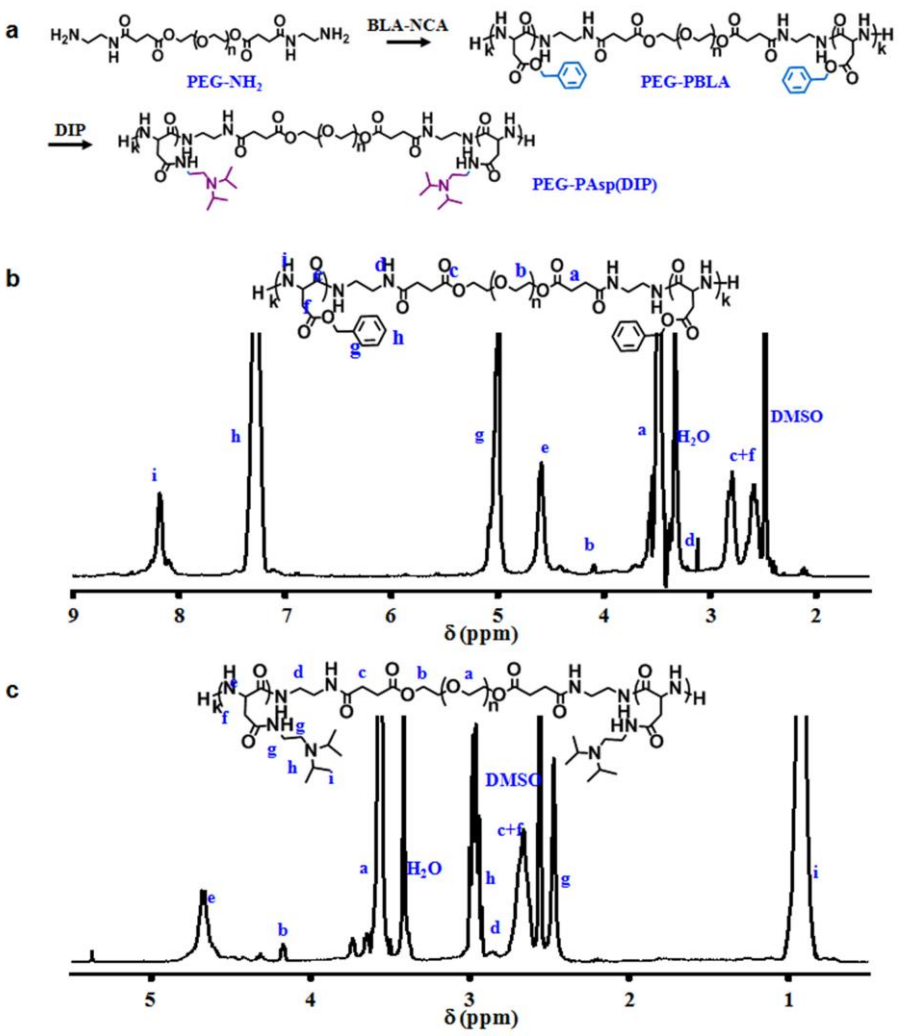
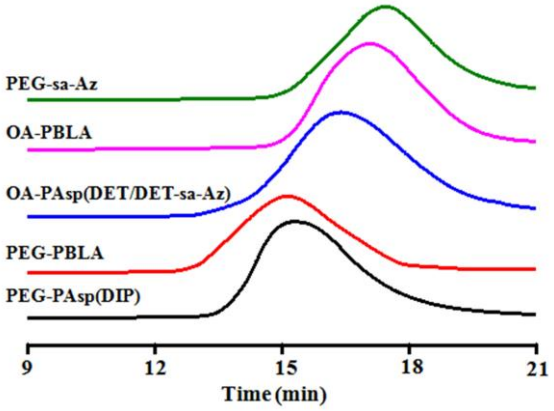


Fig. S1. (a) Synthesis process of amphiphilic copolymer PEG-PAsp(DIP). (b) ^{1}H NMR spectra of PEG-PBLA and (c) PEG-PAsp(DIP).



Sample	Mw	PDI
PEG-PBLA	15490	1.47
PEG-PAsp(DIP)	14000	1.45
OA-PBLA	4310	1.44
OA-PAsp(DET/DET-sa-Az)	7160	1.40
PEG-sa-Az	3790	1.38

Fig. S2. GPC elution profiles, the molecular weights and polydispersity indices (PDI) of PEG-PBLA, PEG-PAsp(DIP), OA-PBLA, OA-PAsp(DET/DET-sa-Az) and PEG-sa-Az.

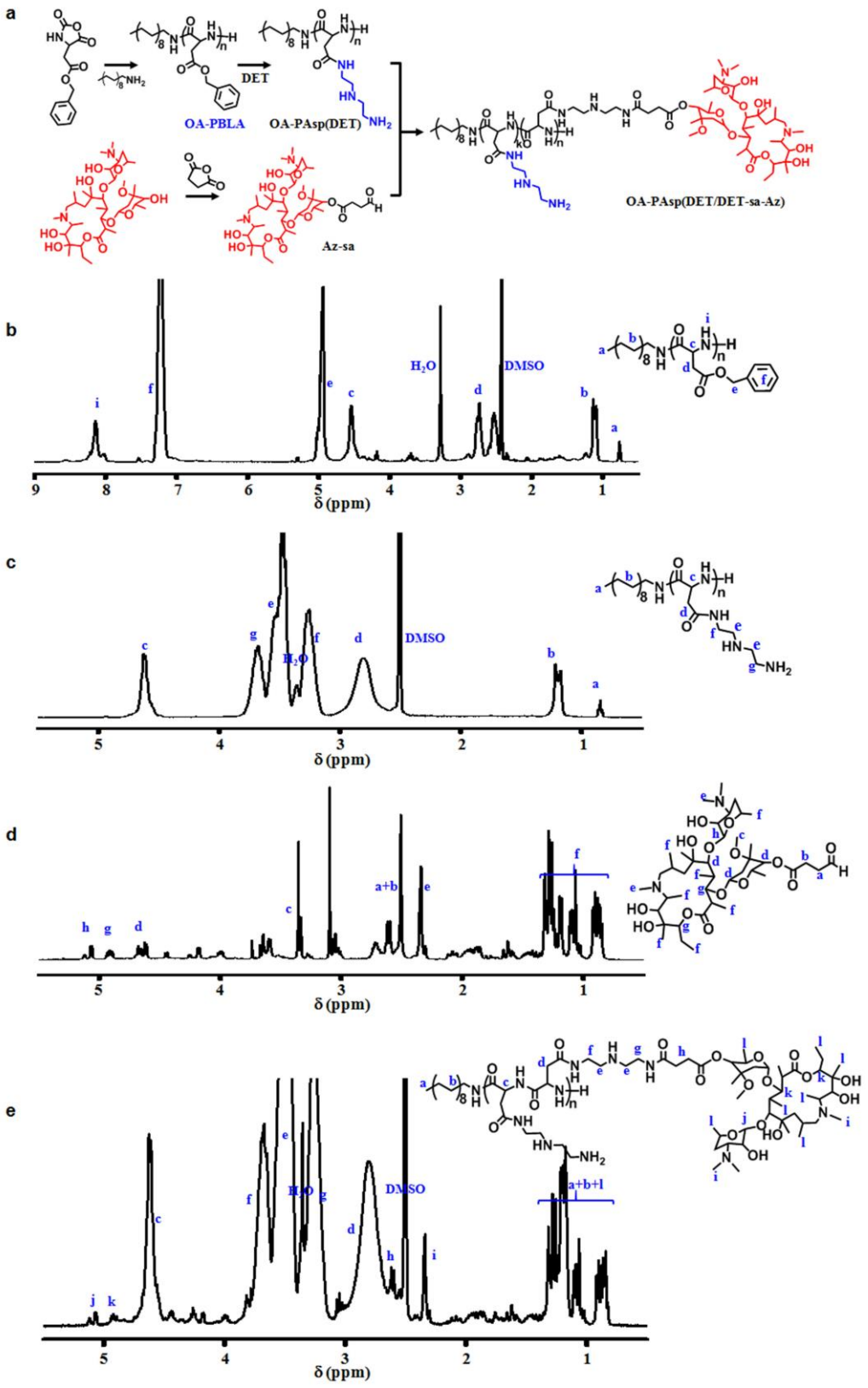


Fig. S3. (a) Synthesis process of amphiphilic copolymers OA-PAsp(DET/DET-sa-Az). (b) ^{1}H NMR spectra of OA-PBLA, (c) OA-PAsp(DET), (d) Az-sa and (e) OA-PAsp(DET/DET-sa-Az).

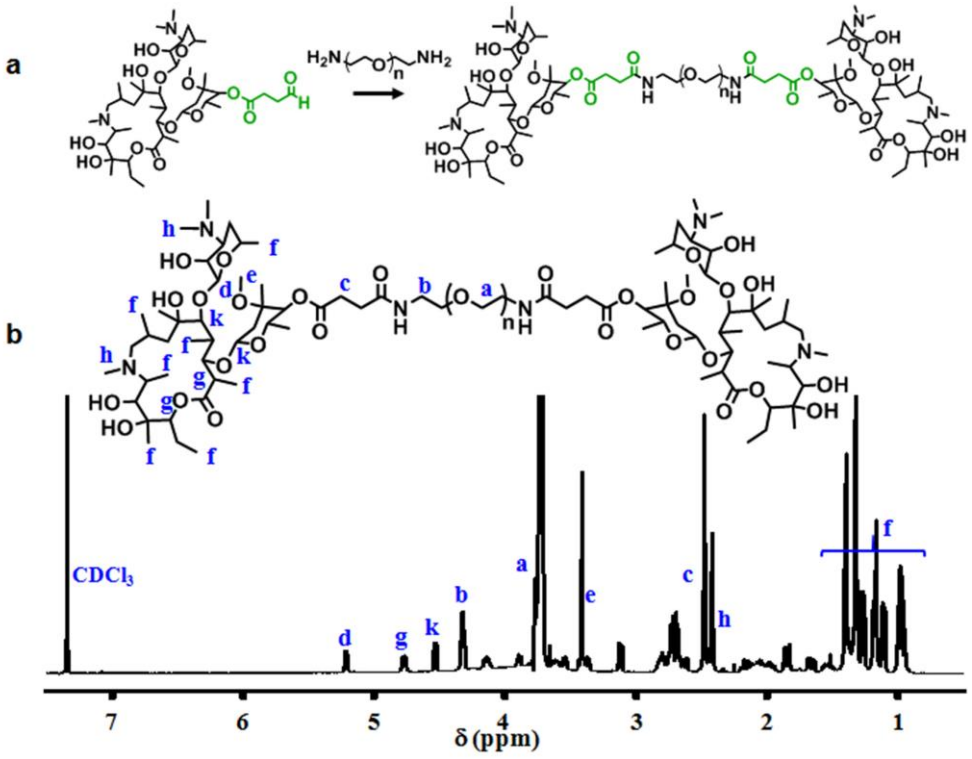


Fig. S4. (a) Synthesis process and (b) ^{1}H NMR spectrum of PEG-sa-Az.

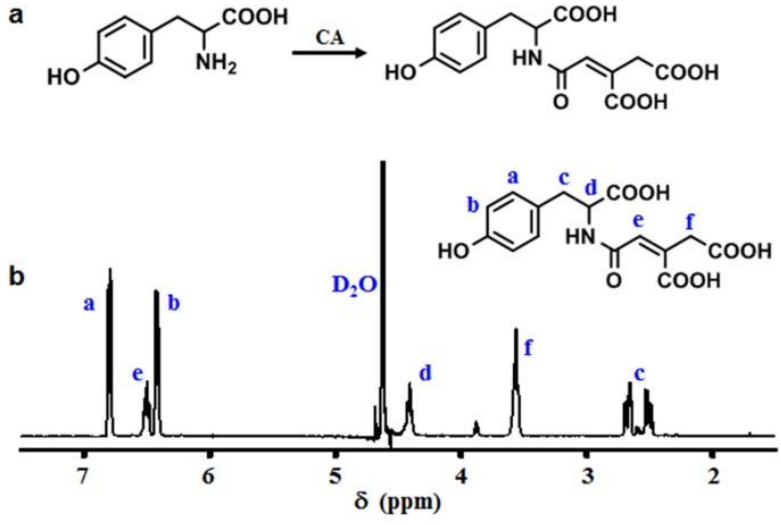


Fig. S5. (a) Synthesis process and (b) ^{1}H NMR spectrum of CA-Tyr.

S6. Stability of micelles in FBS

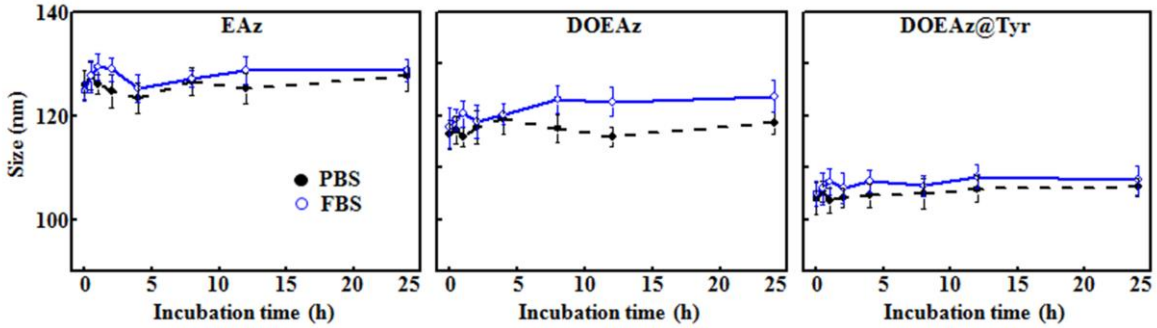


Fig. S6. Size changes of EAz, DOEAz and DOEAz@Tyr micelles after incubation in pH 7.4 buffers and in the absence or presence of FBS (n = 3).

S7. Antibacterial activity of copolymers and micelles

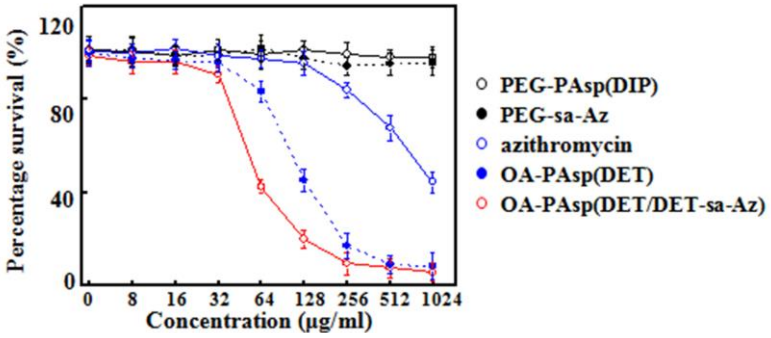


Fig. S7. Survival rates of *P. aeruginosa* after treatment with different concentrations of PEG-PAsp(DIP), OA-PAsp(DET/DET-sa-Az) and PEG-sa-Az micelles, OA-PAsp(DET) copolymers and azithromycin.

S8. Treatment efficacy against biofilms grown in TCPs

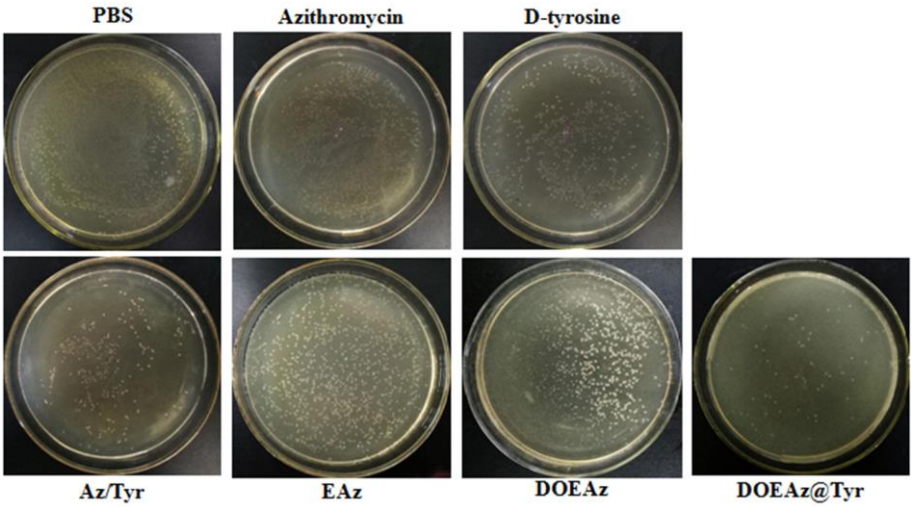


Fig. S8. Photographs of agar plates for CFU counting of viable *P. aeruginosa* in biofilms grown in TCPs after treatment with EAz, DOEAz and DOEAz@Tyr micelles, using azithromycin, D-tyrosine and Az/Tyr mixture and PBS treatment as control..

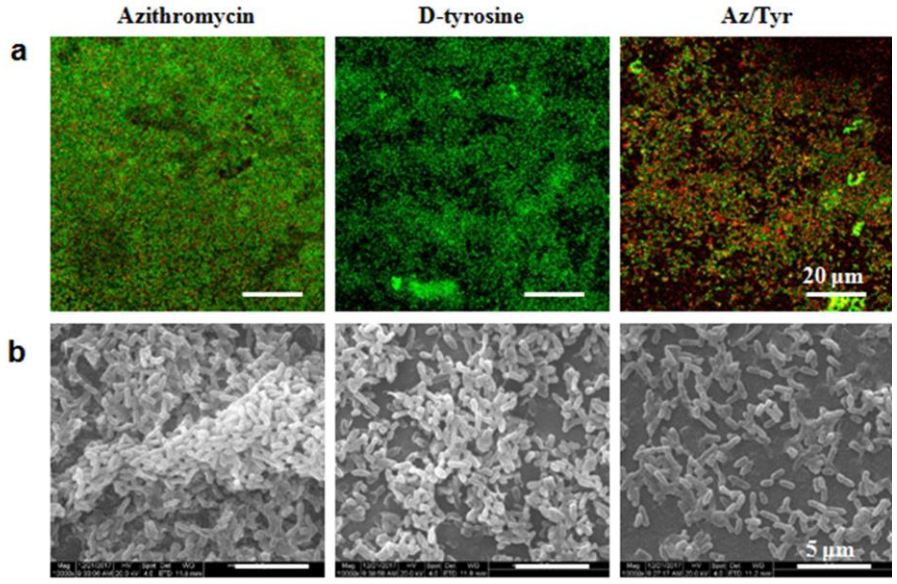


Fig. S9. (a) Typical fluorescent images of live (green) and dead (red) bacteria in biofilms and (b) SEM images of residual biofilms after treatment with free azithromycin, D-tyrosine and Az/Tyr mixtures.

S9. In vitro cytotoxicity of micelles

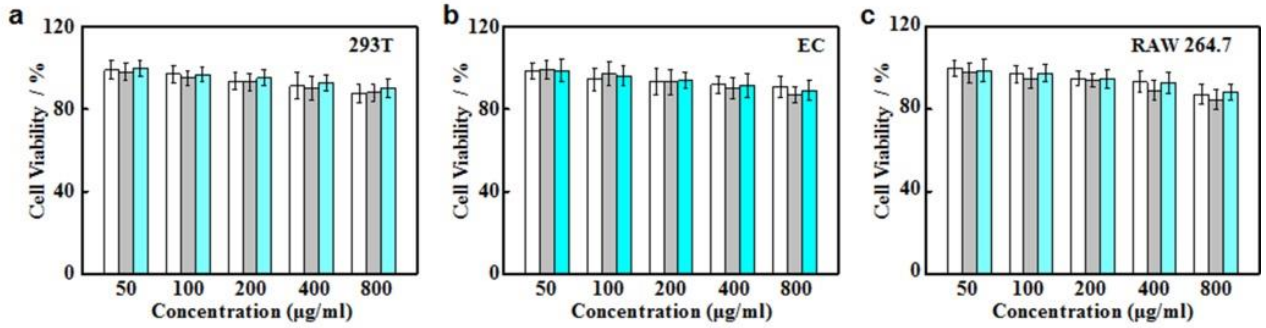


Fig. S10. Cell viability of 293T, (b) ECs and (c) RAW 264.7 cells after incubation for 24 h with EAz, DOEAz and DOEAz@Tyr micelles (n = 5).

S10. Pharmacokinetics analysis of hybrid micelles

Table S1. Pharmacokinetic parameters of micelles

Sample	$T_{1/2}$ (h)	$AUC_{0-\infty}$ (μg/mL·h)	MRT (h)	CL (μg/mL/h)	Cmax (μg/mL)
Azithromycin	0.96 ± 0.14	11.33 ± 2.23	1.39 ± 0.35	13.64 ± 3.79	5.87 ± 1.25
EAz	4.56 ± 0.73	56.59 ± 5.76	6.58 ± 0.97	3.53 ± 0.75	8.54 ± 0.95
DOEAz	3.47 ± 0.98	40.51 ± 4.64	5.01 ± 1.04	4.93 ± 0.89	7.98 ± 1.06
DOEAz@Tyr	6.84 ± 1.05	88.53 ± 7.93	9.86 ± 2.16	2.25 ± 0.41	9.04 ± 0.91

S11. Treatment efficacy against biofilms grown on subcutaneously implanted catheters

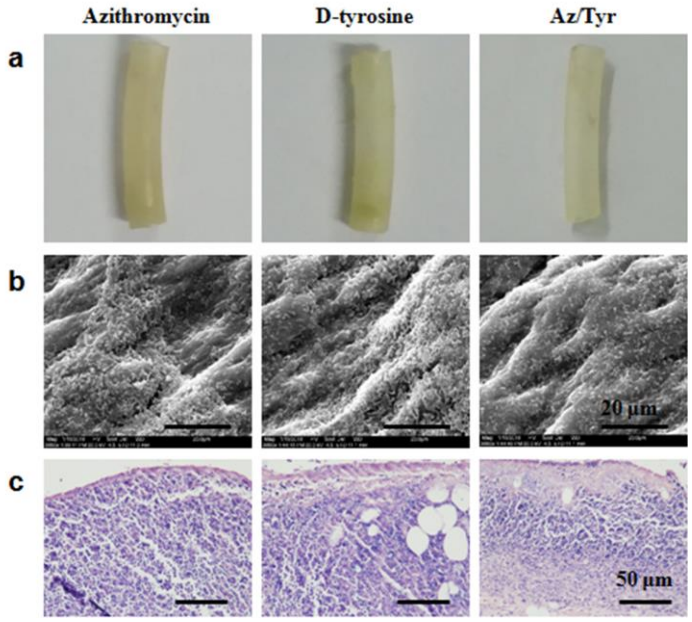


Fig. S11. *In vivo* treatment efficacy of free drugs against biofilms grown on subcutaneously implanted catheters. (a) Photographs of the implanted catheters, (b) typical SEM images of residual biofilms and bacteria on the catheters and (c) H&E staining images of tissues around the implanted catheters after treatment for 7 days with azithromycin, D-tyrosine and Az/Tyr mixtures.

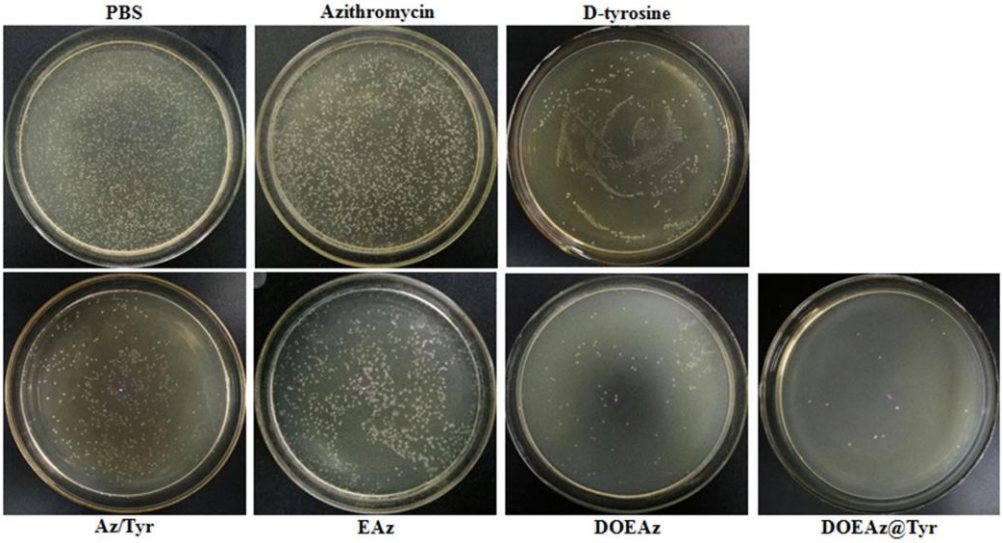


Fig. S12. Photographs of agar plates for CFU counting of residual *P. aeruginosa* in biofilms on catheters after treatment for 7 days with EAz, DOEAz and DOEAz@Tyr micelles, using azithromycin, D-tyrosine, Az/Tyr mixture and PBS treatment as control.

S12. Treatment efficacy against biofilms grown on subcutaneously implanted catheters

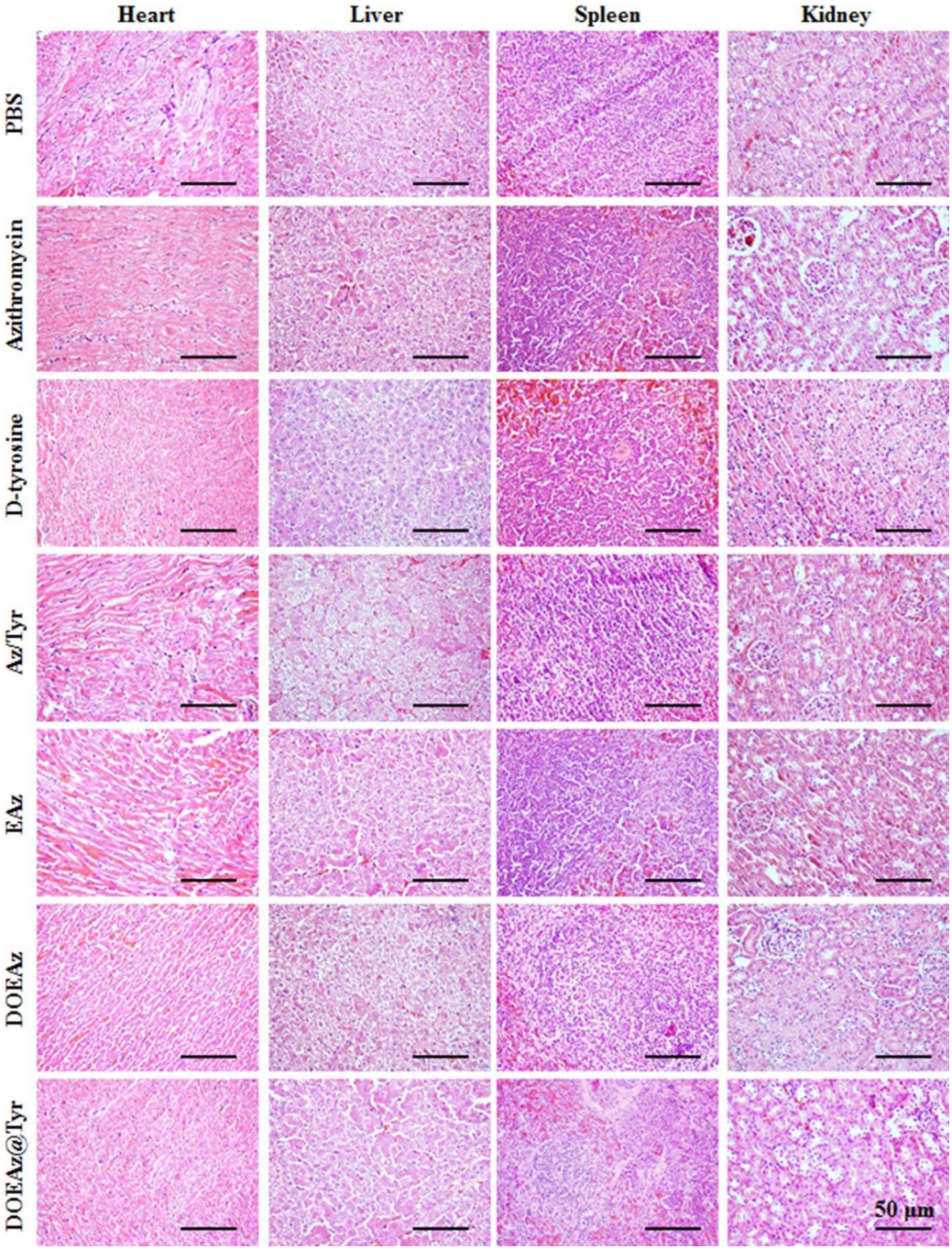


Fig. S13. H&E staining images of heart, liver, spleen and kidney after treatment for 7 days with EAz, DOEAz and DOEAz@Tyr micelles, using azithromycin, D-tyrosine, Az/Tyr mixture and PBS treatment as control.