Tocopherol polyethylene glycol succinate modified hollow silver nanoparticles for combating bacteria-resistance

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Experimental Section

Materials

D-α-Tocopherol polyethylene glycol 1000 succinate (TPGS1000) and sodium borohydride ($NaBH_4$) were purchased from Sigma Aldrich Co., Ltd (USA). Sodium citrate (SC) was purchased from ACROS ORGANICS (New Jersey, USA). Silver nitrate was purchased from Sinopharm Chemical Reagent Co., Ltd. Tigecycline (TIG) was purchased from Nanjing Chia Tai Tianqing Co., Ltd (Nanjing, China). Nutrient Agar and LB Broth were purchased from Hangzhou Microbial Reagent Co., Ltd (Hangzhou, China). Dulbecco's minimum essential medium (DMEM) was purchased from Corning (USA). 3-(4, 5-dimethyl- thiazol-2-yl)-2, 5-diph-enyltetrazoli-nbromide (MTT) and Hoechst 33342 were purchased from Sigma Chem. Co., Ltd (St. Louis，USA). All of the other chemicals were of analytical or chromatographic grade.

Cell culture

LO2 (human normal liver cell) cell lines from Institute of Biochemistry and Cell Biology (Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM, high-glucose) supplemented with 10% (v/v) fetal calf serum (FBS), penicillin (100 U/mL), streptomycin (100 U/mL), and 1% nonessential amino acids (NEAA) with

the environmental condition maintained at 37 °C in an atmosphere of 5% CO_2/95% O_2 with 90% relative humidity. The cells were sub-cultured regularly using trypsin/EDTA.

The standard *Escherichia coli* ATCC 25922 (S-*E.coli*) was obtained and clinical isolates of ofloxacin non-susceptible *Escherichia coli* (N-*E.coli*), tigecycline non-susceptible *Acinetobacter baumannii* 7865 (TNAB) and tigecycline susceptible *Acinetobacter baumannii* 8010 (TSAB) were isolated and collected in the First Affiliated Hospital, School of Medicine, Zhejiang University in China. Bacterial identification was performed by the Vitek 32 system (bioMérieux, France).

The isolates were cultivated on nutrient agar plates, then harvested and cultured in LB medium or MH medium at 37 °C, 180 rpm under aerobic conditions grown to an OD600 of 0.5 ~ 0.6 that corresponds to the exponential phase.

Animals

All experiments were performed in compliance with guidelines set by the Zhejiang University Institutional Animal Care and Use Committee. All animal procedures were conducted in accordance with national regulations and approved by the local animal experiments ethical committee.

Synthesis of AgNPs

The silver nanoparticles using TPGS as a surfactant (TPGS/AgNPs) were synthesized based on the fast reaction-diffusion method. Briefly, aqueous solutions of silver nitrate (900 μL, 10 mM) and TPGS (120 μL, 40 mM) were added into 15.6 mL of water, followed by the addition of sodium hydroxide (3 mL, 0.1M) under the ultrasonication in an ice-cold water bath. Then, 1.08 mL of freshly prepared $NaBH_4$

solution (1.08 mL, 10 mM) was immediately introduced into the mixture. After 1 h, the resultant was centrifuged and the precipitate was collected and lyophilized. The reaction was also performed by replacing the surfactant into sodium citrate (aqueous, 300 μL, 10 mM) to fabricate the AgNPs (SC/AgNPs) under the similar protocol.

Material characterization

The hydrodynamic particle size distribution (PSD) of TPGS/AgNPs and SC/AgNPs were detected by dynamic light scattering (DLS) using a Zetasizer (S90, Malvern Co., UK) at room temperature. Further, TPGS/AgNPs and SC/AgNPs were suspended in deionized water and deposited on carbon coated 250-mesh Cu grids overnight, dried, and then the morphologies of TPGS/AgNPs and SC/AgNPs were observed using transmission electron microscopy (TEM; JEM-1200EX, JEOL, Japan) at an accelerating voltage of 80 kV and 200 kV. UV-visible adsorption spectres of TPGS/AgNPs and SC/AgNPs were obtained by using a UV-vis spectrophotometer (TU-1080, Beijing Purkinje General Instrument Co., Ltd., China) from 300 nm to 800 nm. FT-IR spectra of TPGS/AgNPs was recorded with a FT-IR spectrometer (VECTOR22, Bruker) using KBr pellets.

Tigecycline loading

For TIG loading, TIG aqueous solution (0.5 mg/mL) was added dropwise to AgNPs aqueous solution (0.44 mg/mL) at 28.5% (w/w) feeding ratios with constant shaking for 24 h at 37 °C at 100 r/min. The unloaded drug and TIG loaded AgNPs were separated by centrifugation of 13000 rpm for 10 min. The obtained TIG loaded AgNPs prepared by using TPGS and SC as surfactants, were termed as TPGS/AgNPs/TIG and

SC/AgNPs/TIG, respectively. Drug encapsulation was determined by measuring the amount of unencapsulated drug in the dispersion medium via detecting the UV-vis absorbance at a wavelength of 245 nm relative to standard curve. The TIG loading capacity and encapsulated efficiency were then calculated.

The *in vitro* drug release profiles were measured by suspending TPGS/AgNPs/TIG and SC/AgNPs/TIG in 15 mL of release medium (phosphate buffer saline, pH 5.5 and 7.4) under horizontal shaking (75 rpm) at 37 °C, and TIG solution was applied as a control. The released TIG was separated by centrifugation and collected at predetermined time intervals in triplicate, and then detected by High Performance Liquid Chromatography (HPLC). Then the cytocompatibility of these formulations were evaluated on human hepatic cells LO2 by MTT method. LO2 cells were incubated with TPGS/AgNPs, SC/AgNPs, TPGS/AgNPs/TIG and SC/AgNPs/TIG for 48 h respectively, then the cell viabilities were determined.

Antibacterial studies

After the TIG encapsulation, the *in vitro* antibacterial efficacy of TPGS/AgNPs/TIG was evaluated and compared to that of SC/AgNPs/TIG and TIG solution. S-*E.coli*, N-*E.coli*, TSAB and TNAB were selected as model bacteria for the minimal inhibitory concentration (MIC) tests. The bacteria were cultured in LB (Luria-Bertani) and MH (Müller-Hinton) medium at 250 rpm and 37 ℃, and ultimately harvested in the mid-exponential growth phase (absorbance at 600 nm is of 0.5 ~ 0.6) (BioPhotometer plus, eppendorf, Germany). The MICs of formulations were determined semiquantitatively using the microplate broth dilution method. In brief,

TIG, TPGS/AgNPs, SC/AgNPs, TPGS/AgNPs/TIG and SC/AgNPs/TIG were prepared and added into 96-well plates containing 100 μL bacterial cultures in exponential phase, then serially diluted into a final volume of 200 μL (absorbance at 600 nm is of 0.001) in triplicate. After incubation in a shaker at 37 ℃ for 20 h, the MIC results were interpreted as the smallest concentration leads to complete inhibition in broth. Afterwards, bacterial inoculum with series concentrations of formulations from the MIC studies were drawn onto the agar plates and incubated at 37 °C. And the minimum bactericidal concentration (MBC) could be determined as the lowest concentration leads to no colonies observed within 24 h.

Fluorescence confocal imaging

The bacteria were labelled with Hoechst 33342. Then, cultures of bacteria harvested in the mid-exponential growth phase were incubated with TPGS/AgNPs/DOX and SC/AgNPs/DOX (equivalent DOX concentration of 0.21 μg/mL) in an incubated shaker for 4 h at 37 °C and 4 °C. The fluorescent signals of labelled bacteria and DOX were observed using a confocal fluorescence microscope (Olympus IX81-FV1000, Olympus, Tokyo, Japan and then semi-quantitative analysed with Image J software. This study was further conducted after bacteria were treated with β-Ga_2O_3/DOX (141.67 ± 4.51 nm) and TPGS/DOX (11.37 ± 0.67 nm) for 4 h, where DOX concentrations (0.21 μg/mL) were equivalent. β-Ga_2O_3/DOX nanoparticles were prepared by the method described in the previous study,[16] and TPGS/DOX micelles were prepared by dialysis method. The particle size of β-

Ga_2O_3/DOX nanoparticles and TPGS/DOX micelles were determined by DLS using a Zetasizer (Figures S2a&b).

Transmission Electron Microscopy Experiments

Cultures of TNAB and TSAB harvested in the mid-exponential growth phase were treated with free TIG, TPGS/AgNPs, SC/AgNPs, TPGS/AgNPs/TIG and SC/AgNPs/TIG (the TIG, AgNPs content used were approximately 0.18 μg/mL and 10 μg/mL, respectively) in an incubated shaker for 4 h at 37 ℃. After incubation, the suspensions were centrifuged at 5000 rpm for 10 min to pellet bacteria. All treated bacteria samples were fixed, sectioned, stained and then observed by a TEM (Hitachi Model H-7650). The EDS qualitative analysis was further conducted on sections of TPGS/AgNPs and SC/AgNPs treated samples obtained from the TEM experiments mentioned above.

Real-time RT-PCR

Oligonucleotide primers used for the real-time RT-PCR were designed and purchased from Sangon Biotech (Shanghai) Co., Ltd. The expression levels of the adeB and adeJ genes were measured using real-time reverse transcription-PCR (real-time RT-PCR) method. Cultures of TNAB harvested in the mid-exponential growth phase were incubated with different formulations (TIG, TPGS, TPGS/AgNPs, SC/AgNPs, TPGS/AgNPs/TIG, SC/AgNPs/TIG) for 20 h at 37 ℃.Additional, TNAB was firstly treated with TIG solution and subsequently incubated with TPGS solution for 20 h at 37 ℃ in another group. The final concentrations of TIG, TPGS, and AgNPs content in corresponding samples were approximate as 0.15 μg/mL, 27.60 μg/mL, and 30 μg/mL,

respectively. After centrifugation (8000 rpm) for 5min at 4 ℃, DNase-treated RNA templates were extracted from the pelleted bacterial cultures by RNAiso Plus, and the concentrations of RNA were quantified using a Nanodrop spectrophotometer (Thermo scientific, MA, USA). Then cDNA was synthesized using a PrimeScript RT-PCR kit (TaKaRa Bio) afterwards. Real-time PCR was performed by using a SYBR Premix Ex Taq kit (TaKaRa Bio) on an Applied Biosystems StepOnePlus Real-Time PCR system and each sample was performed in duplicate. The 16S rRNA gene was served as a reference gene to normalize the expression of target genes. The relative expression of the target mRNA compared with that of TNAB were determined and the expression analysis was performed subsequently using the $2^{-\Delta\Delta Cq}$ method.

EB accumulation

Ethidium bromide (EB) was used as an efflux pump substrate, and carbonyl cyanide m-chlorophenylhydrazone (CCCP) was employed in the positive control group. After 1 h of incubation with TPGS with various amount (20 μM, 66 μM and 100 μM) and CCCP (100 μM[17]) , the TNAB cultures were subsequently incubated with EB (2 μg/mL[18]) for another 3 h to process the EB accumulation. The EB incubated TNAB without pre-treatment of CCCP or TPGS was applied as a negative control group. After incubation, the suspensions were centrifuged at 5000 rpm for 10 min to pellet bacteria. The bacteria were harvested and observed by the confocal fluorescence microscope, then semi-quantitatively analysed by Image J. The EB content detected in the supernatant was the total EB amount subtracted those accumulated in bacteria. For the EB accumulation quantification, the fluorescence intensity of EB content was measured

using a spectrofluorometer (λex=530 nm; λem=600 nm). Compared with the negative control group, the increased percentage of EB accumulation after TPGS or CCCP exposure were then calculated.

Acute peritonitis mouse model

After these mice (male, 23 ± 2g) were immunodepressed by peritoneal injection of cyclophosphamide (150 mg/kg/d) for three days, the acute peritonitis mouse model was generated by peritoneal injection of TNAB and TSAB bacterial suspension (200 μL). After 2 h of the TNAB and TSAB injection, these mice showed acute peritonitis symptoms, including low spirits, rolling up and excreting mucous stool. The concentration of TNAB injected was from 4×10^7 to 6×10^7 CFU/mL. While the concentration of TSAB injected was from 3×10^7 CFU/mL to 5×10^7 CFU/mL. The mice were observed for 5 days to evaluate the survival rates. The lowest concentration of TNAB and TSAB that killed 100% of the mice was utilized to build the acute peritonitis mouse model.

In vivo treatment effects

After the acute TNAB and TSAB peritonitis mouse models were successfully established, the TNAB and TSAB models were divided into 5 groups (n=6 per group) respectively. The mice in groups 1-5 were peritoneal injected with Saline, TIG (1.5 mg/kg), TIG (30 mg/kg), TPGS/AgNPs/TIG (TIG 1.5 mg/kg), SC/AgNPs/TIG (TIG 1.5 mg/kg) and TPGS/TIG (TIG 30 mg/kg) respectively. Survival rates were observed within 5 days.

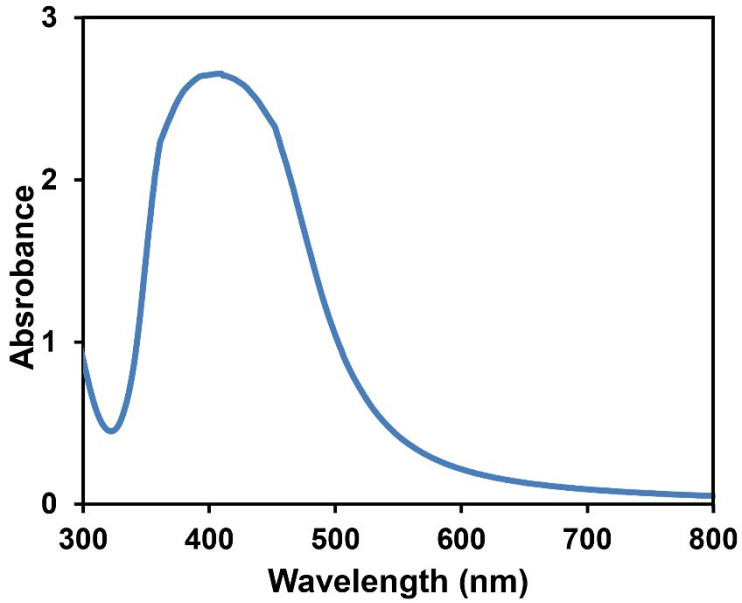
To evaluate the *in vivo* antibacterial effect, the acute peritonitis model was applied to 5 groups (n≥6 per group) according to the method mentioned above. Three

unmodeled immunosuppressed mice were used as a control group. After 48 h of the injection, three surviving mice in each group were sacrificed to obtain peritoneal fluid for bacterial counts analysis. Serial dilution of these peritoneal fluids were incubated in MH agar plates for 24 h at 37 ℃, then the number of CFU in MH agar plates was counted. Meanwhile, the tissues of lung, spleen and peritoneum of these sacrificed mice were also collected for H&E staining and microscopy observation.

Statistics

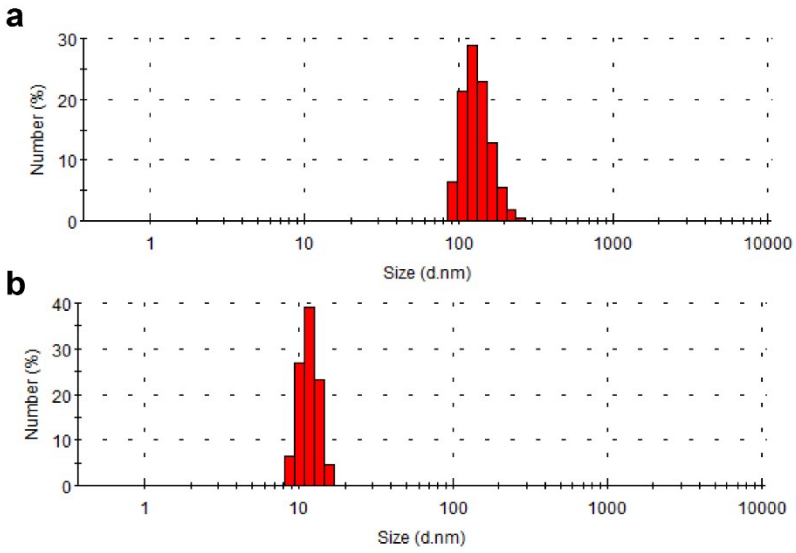
Each experiment was carried out in triplicate at least. Data are presented as mean ± standard deviation (SD). Differences between groups were analysed with two-tailed Student's t-test and ANOVA tests, and P-values < 0.05 were considered statistically significant. All the statistical analyses were performed on Graphic Pad 7.0 and Microsoft Excel software.

Figure S1. Absorption spectra of regular AgNPs.



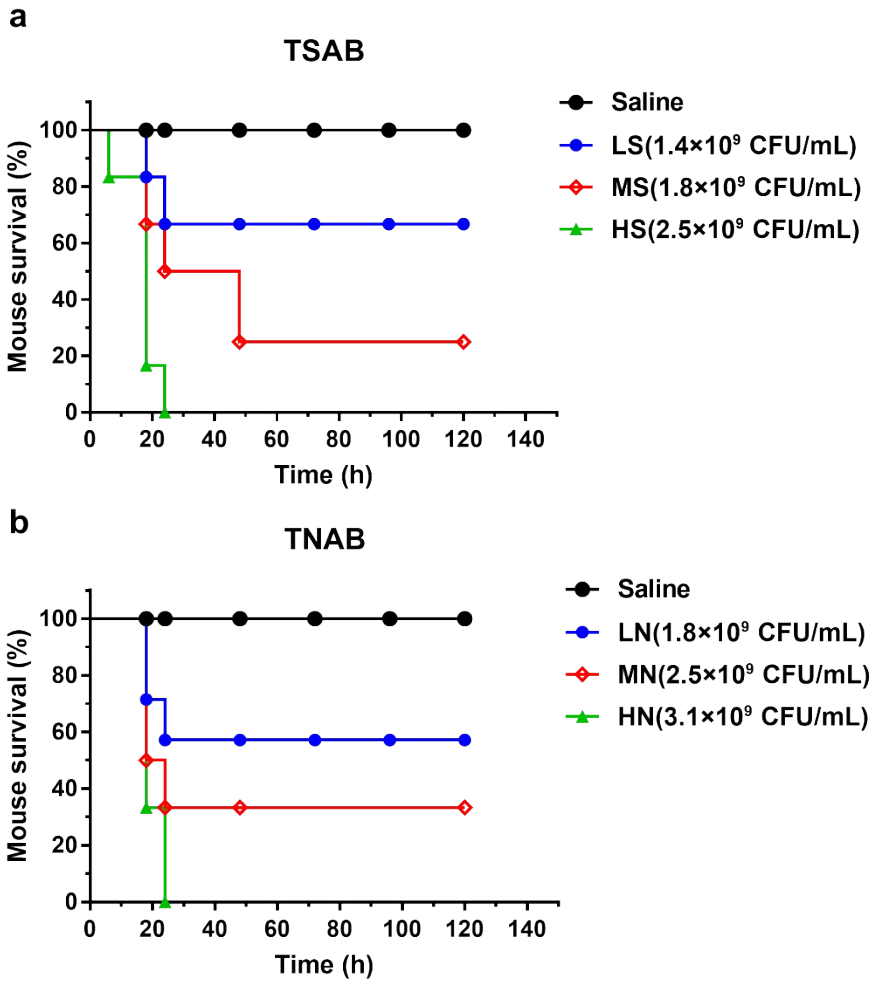
UV-visible adsorption spectra from 300 nm to 800 nm of regular AgNPs prepared by slow reduction using SC as reducing agent.

Figure S2. Size distribution of β-Ga_2O_3/DOX and TPGS/DOX.



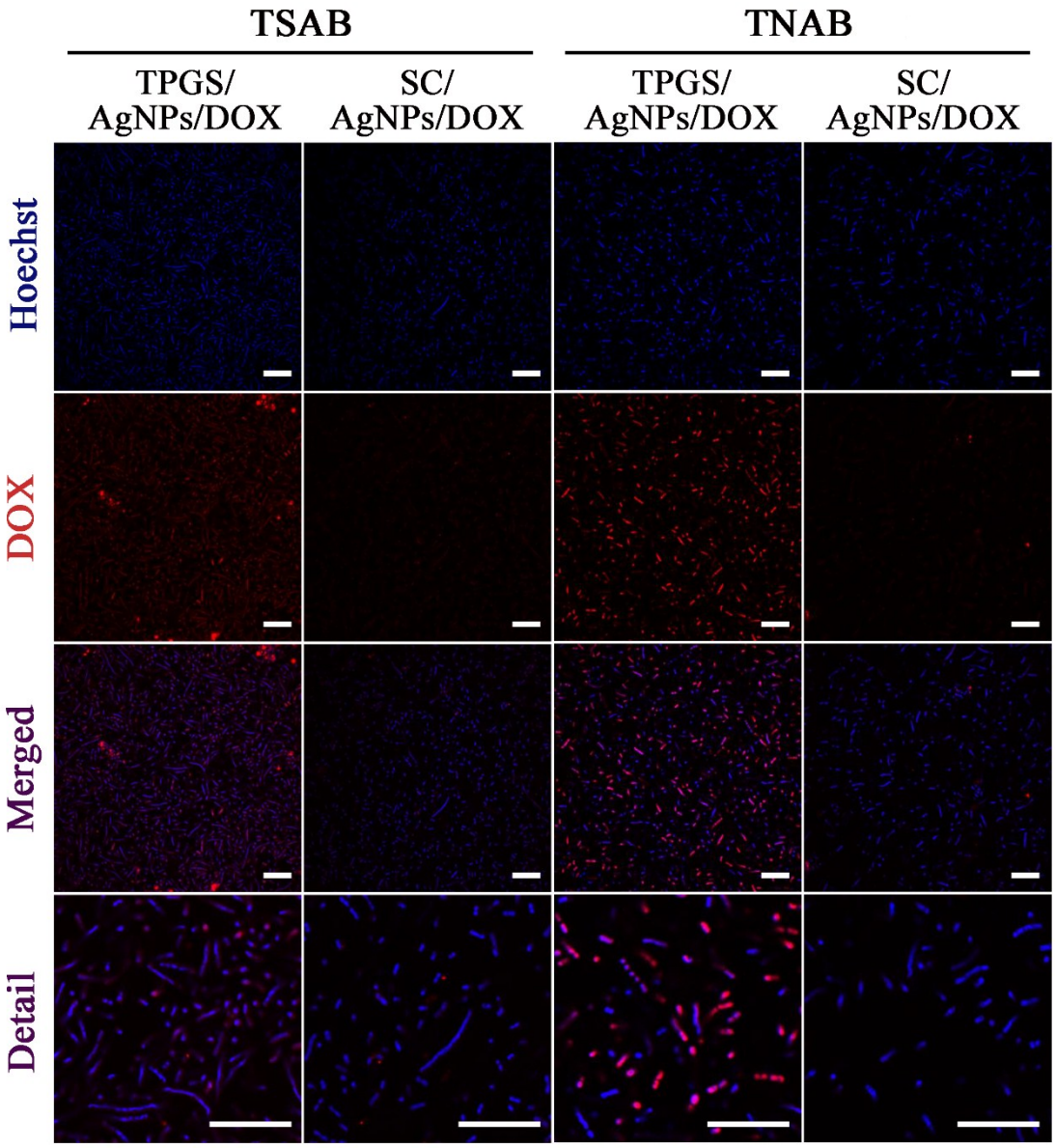
(**a**) The hydrodynamic diameters of β-Ga_2O_3/DOX in distilled water, determined by a Zetasizer analyzer (3000HS, Malvern Instruments Ltd., UK). (**b**) The hydrodynamic diameters of TPGS/DOX micelles in distilled water, determined by a Zetasizer analyzer (3000HS, Malvern Instruments Ltd., UK).

Figure S3. Survival rates of different acute peritonitis mouse model



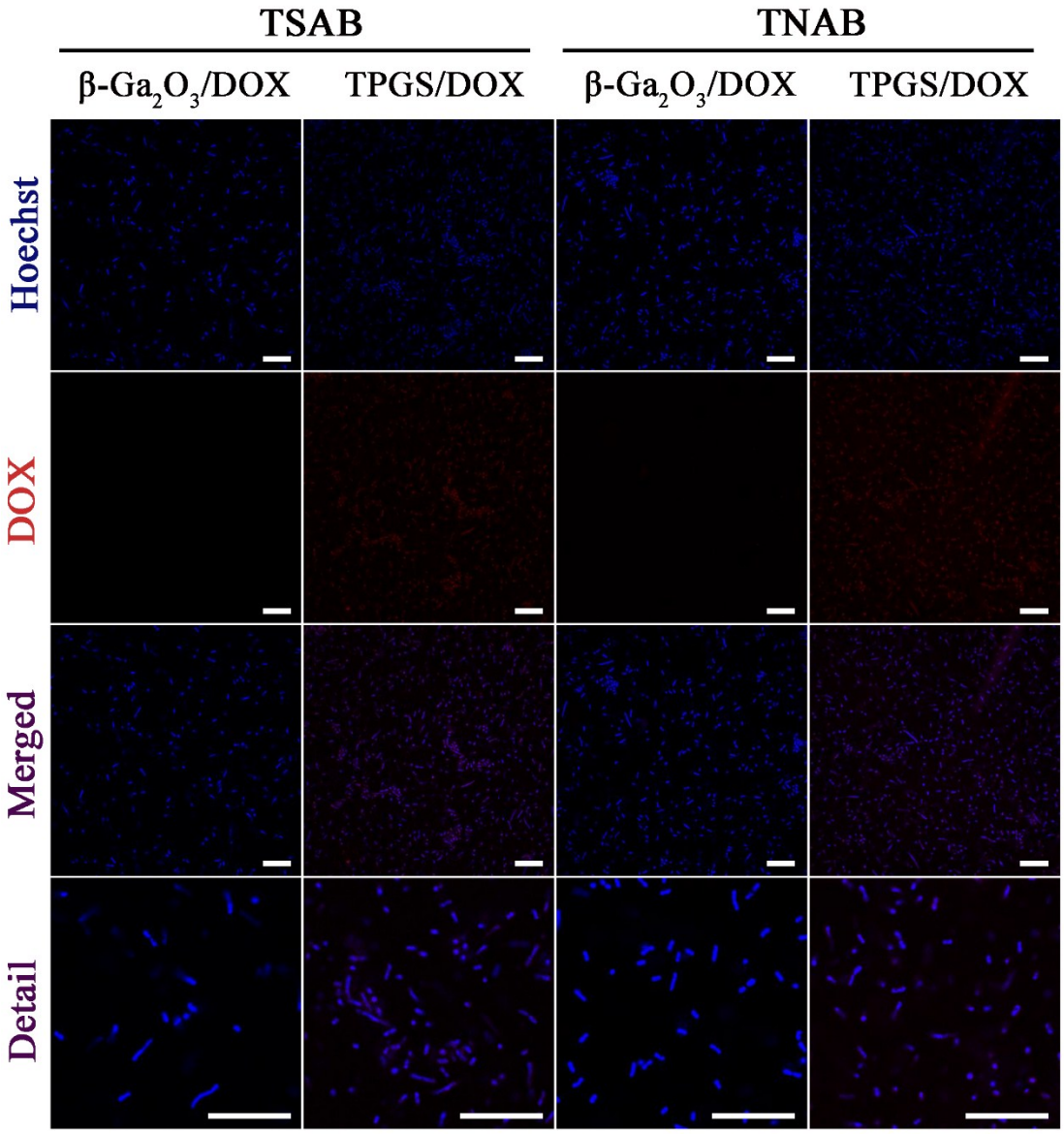
(a) Survival rates of acute peritonitis TSAB mouse mode after intraperitoneal injection of different concentrations of TSAB. (n=6).(b) Survival rates of acute peritonitis TNAB mouse model after intraperitoneal injection of different concentrations of TNAB. (n=6).

Figure S4. Internalization of TPGS/AgNPs into TSAB and TNAB.



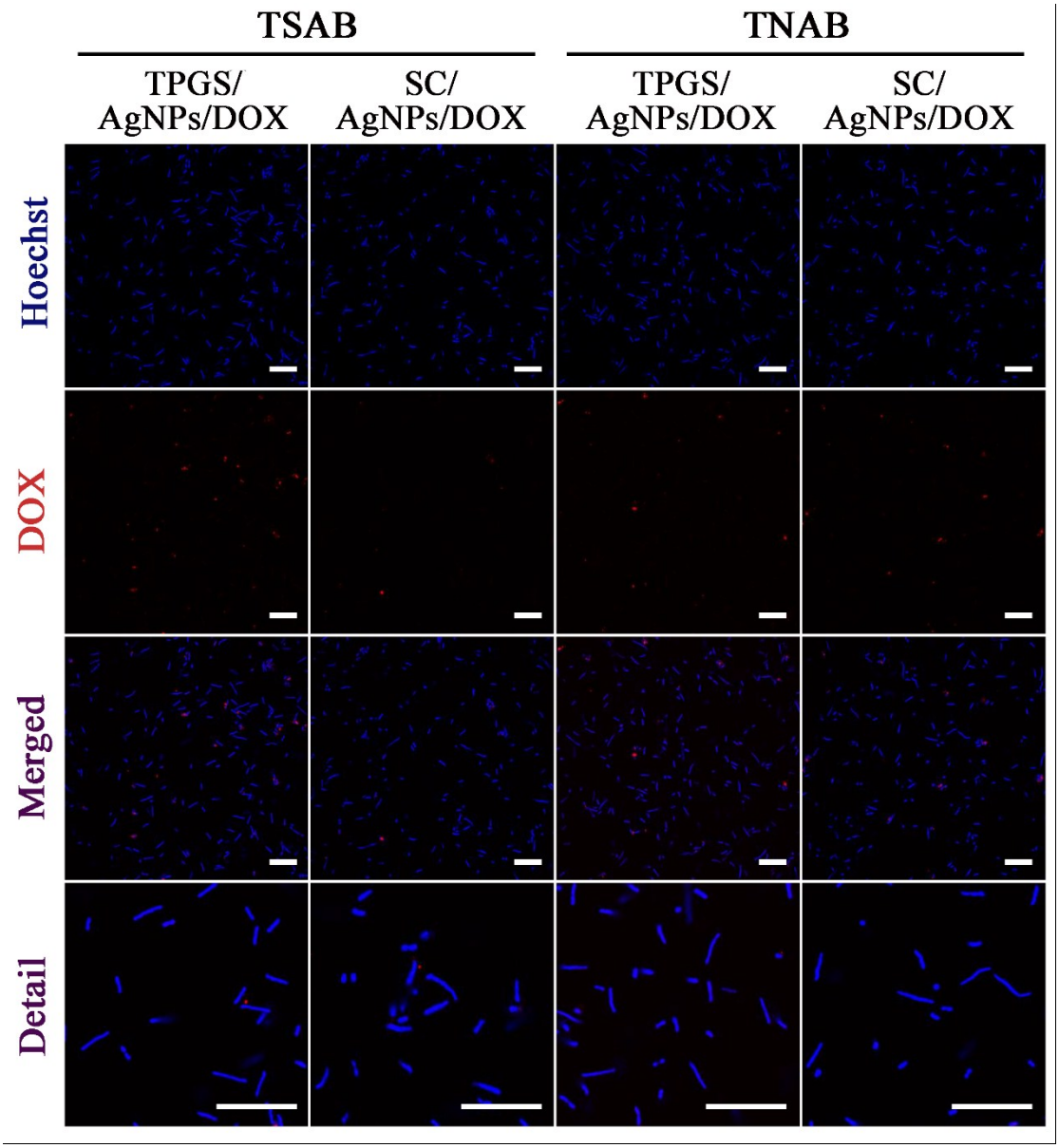
The fluorescent images after 4 h of exposure to DOX labelled TPGS/AgNPs (TPGS/AgNPs/DOX) and SC/AgNPs (SC/AgNPs/DOX) (red) at 37 °C, the bar is 5 μm.

Figure S5. Internalization of TPGS/AgNPs into TSAB and TNAB.



The fluorescent images after 4 h of exposure to β-Ga_2O_3/DOX and TPGS/DOX (red) at 37 °C, the bar is 5 μm.

Figure S6. Internalization of TPGS/AgNPs into TSAB and TNAB.



The fluorescent images after 4 h of exposure to TPGS/AgNPs/DOX and SC/AgNPs/DOX (red) at 4 °C, the bar is 5 μm.

Table S1. MIC and MBC susceptibility semiquantitative profiles of TPGS/AgNPs/TIG and SC/AgNPs/TIG against S-*E.coli* and N-*E.coli* strains (n=3).

treatment		MIC		MBC	
		[NP](μg/mL)	TIG(μg/mL)	[NP](μg/mL)	TIG(μg/mL)
TPGS/AgNPs/TIG	N-*E.coli*	14.85	0.267	29.70	0.535
	S-*E.coli*	14.85	0.267	29.70	0.535
SC/AgNPs/TIG	N-*E.coli*	50.00	0.710	100.00	1.420
	S-*E.coli*	25.00	0.355	50.00	0.710
Free TIG	N-*E.coli*	N/A	0.750	N/A	1.500
	S-*E.coli*	N/A	0.375	N/A	0.750
TPGS/AgNPs	N-*E.coli*	200.00	N/A	400.00	N/A
	S-*E.coli*	100.00	N/A	200.00	N/A
SC/AgNPs	N-*E.coli*	＞500.00	N/A	＞500.00	N/A
	S-*E.coli*	＞500.00	N/A	＞500.00	N/A

Table S2. MIC and MBC susceptibility semiquantitative profiles of TPGS/AgNPs/TIG and SC/AgNPs/TIG against TSAB and TNAB strains (n=3).

treatment		MIC		MBC	
		[NP](μg/mL)	TIG(μg/mL)	[NP](μg/mL)	TIG(μg/mL)
TPGS/AgNPs/TIG	TNAB	32.00	0.576	64.00	1.152
	TSAB	16.00	0.288	32.00	0.576
SC/AgNPs/TIG	TNAB	120.00	1.704	160.00	2.432
	TSAB	30.00	0.426	80.00	1.216
Free TIG	TNAB	N/A	2.400	N/A	4.800
	TSAB	N/A	0.600	N/A	1.200
TPGS/AgNPs	TNAB	225.00	N/A	450	N/A
	TSAB	112.50	N/A	225	N/A
SC/AgNPs	TNAB	＞500.00	N/A	＞500.00	N/A
	TSAB	＞500.00	N/A	＞500.00	N/A