Electronic Supplementary Material

Antimicrobial Poly(ionic liquid)-induced Bacterial Nanotube Formation and Drug-resistance Spread

Hailei Mao,[a] Jiangna Guo,[b] Jiamei Zhou,[b] Jie Shi,[b] Hengqing Cui,[c] Rongwei Shi,[d] Jieran Yao,[a] Xia Fang,[b] Bin Wang,*[c] Feng Yan*[b]*

Materials.

Vinyl imidazole, 1-bromohexane acrylonitrile, styrene, 1,4-divinylbenzene (DVB), 1-hydroxycyclohexyl phenyl ketone (HCPK), diethyl ether, ethyl acetate, acetonitrile, azobis-(isobutyronitrile) (AIBN), ampicillin, kanamycin, anhydrous ethanol (EtOH), dimethyl sulfoxide (DMSO), 25 wt % glutaraldehyde, deoxyribonuclease I (DNase I), sodium dodecyl sulfate (SDS), blood agar plate, Luria-Bertani broth medium (LB) were purchased from Biosharp Co. (Hefei, China).

Strains of *S. aureus* (ATCC 6538) and *E. coli* (8099), and clinical strain ampicillin-resistant *S. aureus* were provided by Dr. Shengwen Shao (Huzhou University School of Medicine, China). Two strains of *E. coli* with unconjugated plasmids: DH5α (pKK233-2 plasmid, carrying red mCherry gene and Amp^R) and BL21 (pET28a plasmid, carrying green GFP gene and Kan^R) were provided by Dr. Yanran Chen (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, China). *Vibrio fischeri* (CICC 10483) and its special culture medium were purchased from China Center of Industrial Culture Collection, Beijing, China. All reagents were analytic grade and used as received without further purification. The deionized water was used throughout the experiments.

Characterization.

^{1}H NMR spectra of the synthesized substances were recorded on a Varian 400 MHz spectrometer using d_6-DMSO or D_2O as the deuterated solvent. Fourier transform infrared (FT-IR) spectroscopy was carried out on a Nicolet 5200 model spectrometer in the range of 400-4000 cm^{-1}. The field emission scanning electron microscope (FE-SEM) was examined by hitachi S-4700. The transmission electron microscopy (TEM) was supplied by Tecnai G220. Fluorescence images were obtained using a fluorescence microscope (Leica DM2500) with a 100 × objective. The optical density (OD) values of the bacterial suspension were obtained by a Multiskan GO microplate reader (Thermo Fisher Scientific Co.).

Synthesis of imidazolium-type ionic liquid monomer [HVIm][Br].

3-Hexyl-1-vinylimidazolium bromide ([HVIm][Br]) was synthesized via stirring a mixture containing 1-vinylimidazolium (4.00 g, 42 mmol) and 1-bromohexane (7.01 g, 42 mmol) in 30 mL acetonitrile solution at room temperature for 72 h. After the evaporation of acetonitrile, the product was washed with ethyl acetate three times and then dried at 30 °C overnight (**Scheme S1A**). ^{1}H NMR (400 MHz, D_2O, δ): 7.75 (d, J = 1.2 Hz, 1H), 7.56 (s, 1H), 7.21-7.03 (m, 1H), 5.78 (dd, J = 15.6, 2.5 Hz, 1H), 5.47-5.34 (m, 1H), 4.22 (t, J = 7.1 Hz, 2H), 1.93-1.82 (m, 2H), 1.28 (s, 6H), 0.84 (d, J = 6.7 Hz, 3H) (**Fig. S1A**).

Preparation of poly(3-hexyl-1-vinylimidazolium bromide) (PIL) homopolymer.

3-Hexyl-1-vinylimidazolium bromide ([HVIm][Br]) (1.5 g, 5.79 mmol), AIBN (0.03 g,

0.18 mmol) and DMSO (2 mL) were mixed and polymerized at 60 °C under N_2 for 8h. After the raw polymer was dropped into ethyl alcohol for precipitation, the resultant product was further centrifuged and washed with acetone solution three times to remove the unreacted monomer. The obtained polymer was dried under vacuum at 50 °C for 24 h (yield 54.10 %) (**Scheme S1B**). ^{1}H NMR (400 MHz, d_6-DMSO, δ): 9.68 (s, 1H), 7.64 (d, 2H), 4.08 (s, 2H), 1.85 (s, 2H), 1.30 (s, 6H), 0.87 (s, 3H) (**Fig. S1B**).

Preparation of imidazolium-type PIL membranes.

A series of PIL membranes were prepared via photo-crosslinking of [HVIm][Br] monomer with acrylonitrile and styrene using DVB as a crosslinker and HCPK as a photo-initiator (**Scheme S1C**). A mixture containing IL monomer (molar ratio of 20 %, 30 %, 50 %, respectively), acrylonitrile/styrene (3:1 molar ratio), together with DVB (2 wt % to the formulation based on the weight of monomer), and 1 wt % of HCPK was ultrasonicated to obtain a homogeneous solution, which was further photo-cross-linked in a glass mold by irradiation with UV light (λ=250 nm) at room temperature. The thickness of the PIL membranes was controlled by two standard spacer bars (about 40 μm in diameter). The unreacted monomer residues were removed by immersing the polymer membranes in ethanol and ultrasonicated at room temperature. Then the depurated membranes were thoroughly washed with deionized water for 24 h before use.

Bacteria culture.

Prior to the antibacterial experiments, all strains of *S. aureus* and *E. coli* were grown in a Luria−Bertani broth medium (LB) at 37 °C for 24 h, while *V. fischeri* was grown in a special medium (LB) at 22 °C for more than 72 h. The concentration of bacteria used in the experiments was controlled by the optical density at a wavelength of 600 nm (OD_{600}). The drug-resistant bacteria in this study were screened on the plates with ampicillin (50 mg mL^{-1}) and/or kanamycin (30 mg mL^{-1}), respectively.

Minimum inhibitory concentration (MIC) test.

MIC value was measured to investigate the antibacterial activities of imidazolium-type PIL in the way of broth microdilution format in accordance with Clinical & Laboratory Standards Institute guidelines. The 96-well plates were incubated at 37°C and tested the optical density (OD) at set intervals with a microplate reader. MIC50 is the minimum PIL concentration that can inhibit 50% of bacterial growth after incubating for 24h at 37°C.)

Using the similar method, all intra/interspecies bacteria before and after obtaining antibiotic resistance genes (ARGs) under the stress of PIL were tested at different concentrations by gradient dilution of Amp (0.125-256 μg/mL), Kan (0.125-256 μg/mL) and PIL (1.0-2048.0 μg/mL) solutions for their inhibition rates.

Hemolysis and erythrocyte morphology assays.

The fresh human blood samples from healthy volunteers were centrifuged at 1000 rpm for 10 min to harvest red blood cells (RBC), and then the erythrocyte samples were carefully

washed with PBS at room temperature until the supernatant was transparent and then diluted to 4 vol% in PBS for further usage. After the gradient diluted PIL solutions (1-2048 μg/mL, 0.75 mL for each) were added into equal volume of diluted RBC suspension and incubated at 37 °C for 30 min, the treated blood samples were centrifuged at 1000 rpm for 10 min, and then aliquots of 100 μL supernatant were transferred to a 96-well plate for measure of the absorbance at 576 nm on the Eon microplate spectrophotometer (Bio-Tek instruments, Inc). The diluted RBCs in 2 % Triton and in PBS were applied as the positive and negative controls, respectively. The hemolysis rate was calculated by the following formula. The independent experiments were performed in triplicate. Meanwhile, the centrifuged blood cell pellets were collected and visualized by a Nikon Eclipse 80i microscope for the erythrocyte morphology. The counting of echinocytes was based on 50 blood cells.

$$\text{Hemolysis rate (\%)} = \frac{OD_{sample} - OD_{negative\ control}}{OD_{positive\ control} - OD_{negative\ control}} \times 100\%$$

Antibacterial activities of synthesized PIL membranes.

Two microbes of *S. aureus* (ATCC 6538) and *E. coli* (8099) were used to detect the antimicrobial ability of PIL membranes. Firstly, the bacteria were inoculated in Luria−Bertani (LB) medium, and cultured overnight at 37 °C, with 150 rpm of shaking, until the exponential growth phase reached.

100 μL of bacterial suspension (OD_{600}=0.1) was added into the sterilized PIL membranes (1.0 cm^2 for each), and incubated at 37 °C with a relative humidity higher than 90 %. Microbial suspension (10 μL of each strain) was streaked onto LB agar plates. The number of the colony-forming units (CFUs) was counted after incubation at 37 ℃ for 24 h. PET

membranes with the same size were used as negative controls. Bacterial viabilities after contacting with PIL membrane surface for various time were compared with the number of colonies from PET control. The antibacterial rate calculation complied with the following equation:

$$\text{Antibacterial activities (\%)} = \frac{N_{\text{negative control}} - N_{\text{sample}}}{N_{\text{negative control}}} \times 100\%$$

Computational simulations for the interaction of PILs with bacterial membranes.

The simulations were carried out with the GROMACS package version 5.1.2, using CHARMM36 force field for the outer membrane of Gram-negative bacteria *E. coli*. For modeling cationic PILs and PET polymers, the corresponding parameters were generated from the Antechamber and ACPYPE tools, and the RESP charges were derived from ab initio calculations performed at B3LYP/6-31g(d) level using Gaussian03 software. According to the molecular structure with different components of IL monomer (x), acrylonitrile (y) and styrene (z) shown in **Table S1** and **Scheme S1C**, three simulated PIL models (PIL_{20}, PIL_{30} and PIL_{50}, with x=1, 2, 4, y=3, and z=1) were constructed by saturating terminals with H atoms, while PET model was built by adding H atom and OH group at two terminals, respectively. Finally, four interaction models of *E. coli* outer membrane with PILs and PET were set up: PIL system consists of 25 PIL_{20} structures with 50 positive charges or 14 PIL_{30} structures with 56 positive charges or 7 PIL_{50} structures with 56 positive charges, and the PET system contains 100 PET units with no charges, and both PILs and PET were randomly placed about 0.8 nm above the surface of the pre-equilibrated *E. coli* outer membrane, which

was composed of 5 LPS (lipopolysaccharide) molecules and 146 POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine) lipids. It is noted that PIL_{20}, PIL_{30}, PIL_{50}, and LPS molecules were modelled with 2, 4, 8 positive and 10 negative charges, respectively. In order to maintain the system's electrical neutrality approximately and save the cost, 5 LPS were selected with 25 PIL_{20}, 14 PIL_{30}, and 7 PIL_{50} molecules, respectively. Nevertheless, 100 PET molecules were randomly chose to fully cover the top surface of *E. coli* outer membrane.

The detailed simulations were performed as follows: the particle-mesh Ewald method was used to calculate the long-range electrostatic interactions, the Lennard-Jones potential and forces were truncated at 1.2 nm, and the bond lengths were constrained by PLINCS with 2 fs time step. Each system was first equilibrated using an isochoric-isothermal (NVT) ensemble for 8 ns with the Berendsen weak coupling algorithm and a coupling constant of 0.1 ps, and then equilibrated under an isobaric-isothermal (NPT) ensemble for 10 ns with the coupling constants for temperature and pressure of 0.2 ps and 2.0 ps, respectively. After that, the production simulations were performed for 600 ns using a Nose-Hoover thermostat and a Parrinello-Rahman barostat. During all simulations, the temperature was maintained at 310 K.

Bacterial nanotubes of *E coli* induced by antimicrobial PILs.

According to MIC50 against wild-type *E. coli* (8099), PIL solutions with different concentrations (0.1 MIC, 0.3 MIC and 1.0 MIC) were prepared, and then mixed onto PET

membranes (1.0 cm^2) surfaces with equal volume of *E. coli* suspension (100 μL, OD_{600} = 0.1), respectively. After 4 h incubation at 37 °C, the PET membranes were totally immersed in 2.5 wt % glutaraldehyde solution for 2 h. Then, the membranes were dehydrated stepwise by 10 vol %, 30 vol %, 50 vol %, 70 vol %, 80 vol %, 90 vol %, and 100 vol % ethanol solution (10 min for each step). The morphological changes of bacteria were then observed by SEM.

Similarly, *E. coli* suspensions were dropped onto three cationic PIL membranes with different IL contents (PIL_{20}, PIL_{30} and PIL_{50}) for the morphological observation, including SEM and TEM images, and fluorescence microscopy for the signal of a lipophilic dye (DiO). PET membranes were used as controls.

Intra/inter-species bacterial nanotubes and drug-resistance spread induced by PILs.

Two *E. coli* strains of DH5α (pKK233-2 plasmid, carrying red mCherry gene and Amp^R) and BL21 (pET28a plasmid, carrying green GFP gene and Kan^R) were used for intraspecies assays of bacterial nanotubes and drug-resistance spread. 100 μL of bacterial suspension with equal volume of DH5α and BL21 (OD_{600}=0.1 for each) was mixed on the sterilized PIL_{20} membrane (1.0 cm^2), and incubated at 37 °C for 4h with a relative humidity higher than 90 %. To observe the formation of bacterial nanotubes and the spread of drug resistance, the bacteria on the membrane were detected by SEM, colony and fluorescence assays without or with two antibiotics of ampicillin (50 mg mL^{-1}) and kanamycin (30 mg mL^{-1}), respectively. Furthermore, the double antibiotics screened colonies were analyzed by PCR for examination of the transferred ARGs. PET membranes were used as controls.

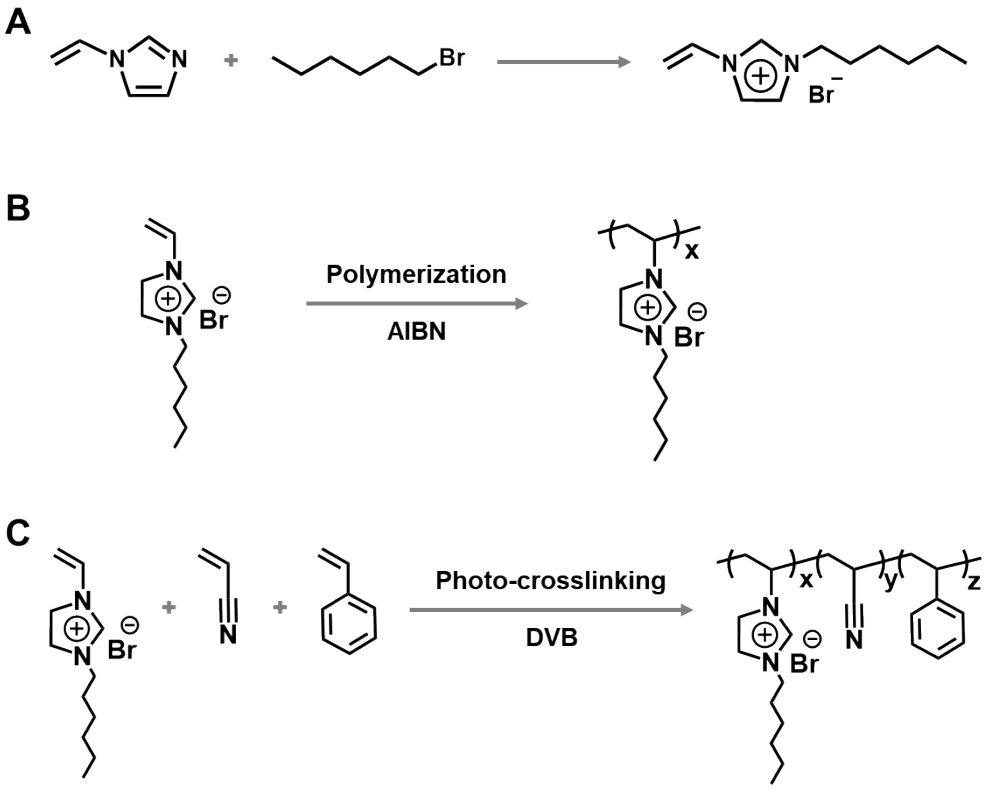
Parallel experiments of *E. coli* were conducted on PIL_{20} membranes in the presence of DNaseI (100 μg/mL) or SDS (0.009%) for 4 h, respectively, and then screened on the plates with two antibiotics of ampicillin (50 mg mL^{-1}) and kanamycin (30 mg mL^{-1}) for colony assay. Finally, the transfer frequencies of plasmid in four tested groups, including PIL_{20}, PIL_{20}+DNaseI, PIL_{20}+ SDS, and PET, were calculated.

Interspecies bacteria include three pairs of strains: *E. coli* BL21 (with green GFP gene and Kan^R) and *S. aureus* (with Amp^R), *E. coli* (no Amp^R) and *S. aureus* (Amp^R), and *V. fischeri* (without Kan^R) and *E. coli* BL21 (with green GFP gene and Kan^R), 100 μL of which were mixed on PIL_{20} membrane for 4h with the volume ratios of 1:5, 1:5 and 1:10 (OD_{600}=0.1 for each strain), respectively. Similarly, SEM, drug-resistant colony screening, fluorescence and PCR analyses were applied for the formation of inter-specific bacterial nanotubes and the spread of drug resistance.

PCR analyses of Kan^R and Amp^R genes.

The presence of drug-resistant genes was detected by PCR (polymerase chain reaction). Specific primers used in this study for Kan^R and Amp^R were listed in Table S3. KOD FX polymerase (TOYOBO, Osaka, Japan) was used for PCR amplification. The 25 μL volume of PCR mix included 12.5 μL of KOD FX buffer, 0.5 μL of KOD FX polymerase, 0.75 μL of each primer, 5 μL of dNTP, and 5.5 μL of deionized distilled water. The PCR amplification conditions were: predenaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 90 °C for 15 s, annealing at 55 °C for 30 s, and extension at 65 °C for 40 s, and then a final extension at 65 °C for 5 min. The PCR products were then analyzed by electrophoresis on a

1.5% agarose gel. Triplicate tests were conducted in each experiment.



Scheme S1. Syntheses of (A) imidazolium-type cationic IL monomer [HVIm][Br], (B) their corresponding PIL homopolymer and (C) membrane.

Table S1. Formulations for the preparation of three PIL membranes with different ratio of cationic imidazolium.

Samples	IL monomer (mol%)	Acrylonitrile (mol%)	Styrene (mol%)
PIL_{20}	20	60	20
PIL_{30}	30	52.5	17.5
PIL_{50}	50	37.5	12.5

Table S2. Mean interaction energies for LPS and POPE components of *E. coli* outer membrane with polymers during the whole 600 ns simulation.

Interaction components	Electrostatic interaction (Ele, kcal/mol)	Hydrophobic interaction (Hyd, kcal/mol)	Total interaction (kcal/mol)
PET-LPS	-6.92	-12.08	-19.00
PIL_{20}-LPS	-78.57	-191.00	-269.57
PIL_{30}-LPS	-118.63	-205.69	-324.32
PIL_{50}-LPS	-95.16	-188.01	-283.17
PET-POPE	-0.0013	-0.00084	-0.0021
PIL_{20}-POPE	-72.37	-101.55	-173.92
PIL_{30}-POPE	-130.61	-181.30	-311.91
PIL_{30}-POPE	-264.10	-345.50	-609.60

Table S3. Sequences of the primers used in polymerase chain reaction.

Gene Name	Primer	Sequence	Product size (bp)
Kan^R	Forward	5'-ATGAGCCATATTCAACGGGAAA-3'	816
	Reverse	5'-TTAGAAAAACTCATCGAGCATCAAATG-3'	
Amp^R	Forward	5'- TCCCCGTCGTGTAGATCACT-3'	449
	Reverse	5'- CGGATGGCATGACGGTAAGA-3'	

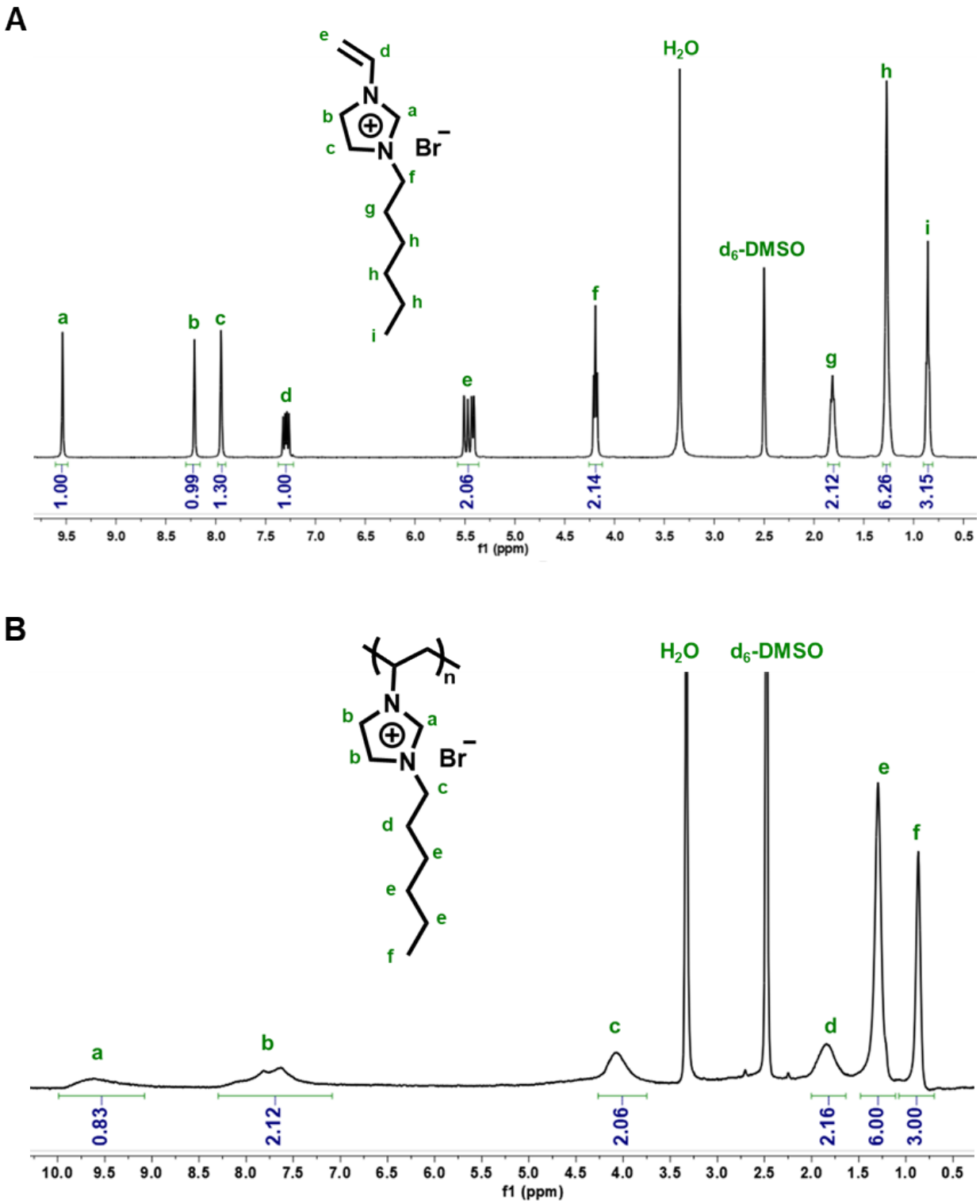


Fig. S1. ^{1}H NMR spectra of IL monomer [HVIm][Br] (A) and corresponding PIL homopolymer (B).

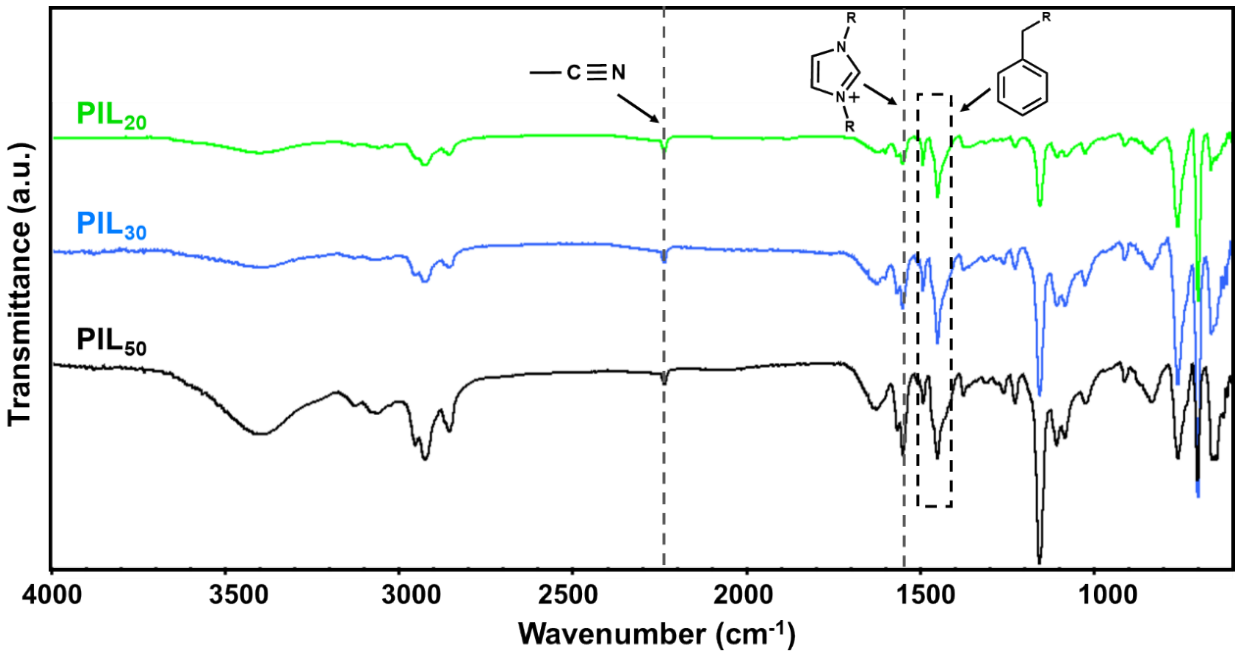


Fig. S2. FT-IR spectra of the synthesized PIL membranes.

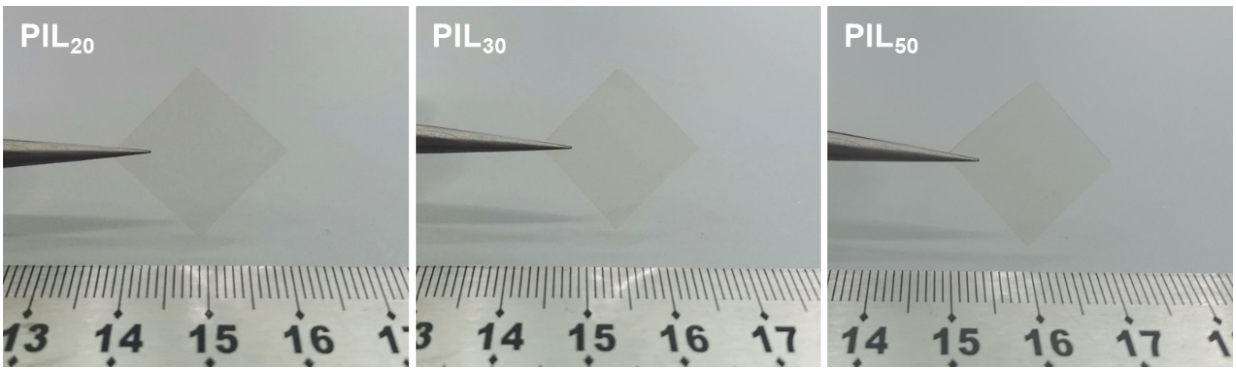


Fig. S3. Digital photographs of three PIL membranes.

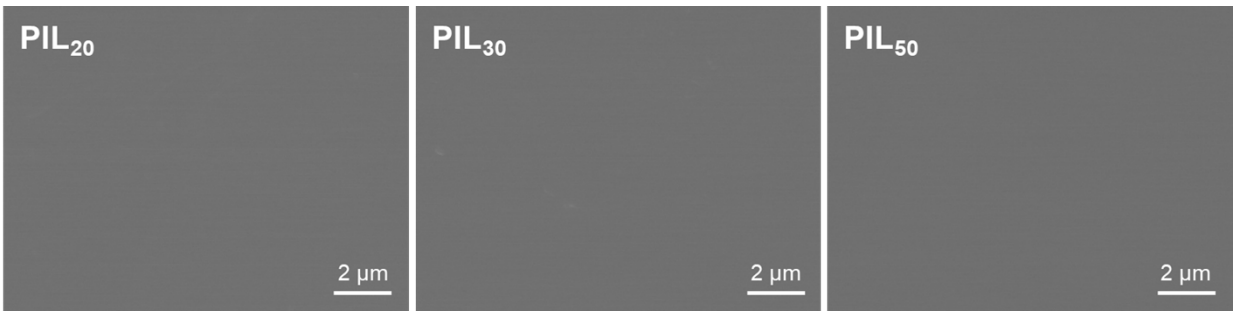


Fig. S4. SEM images of three PIL membranes. Scale bar: 2 μm.

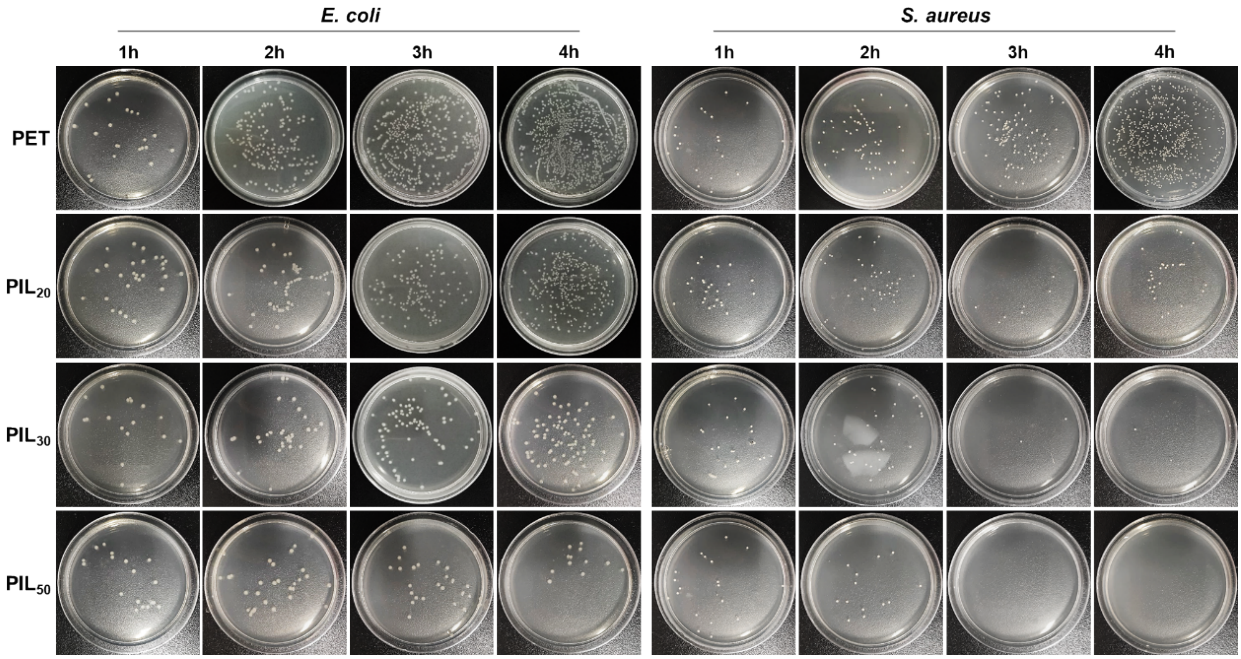


Fig. S5. Antimicrobial activities of PIL membranes against *E. coli* and *S. aureus* by CFU assay at various exposure times. The PET membrane was used as control.

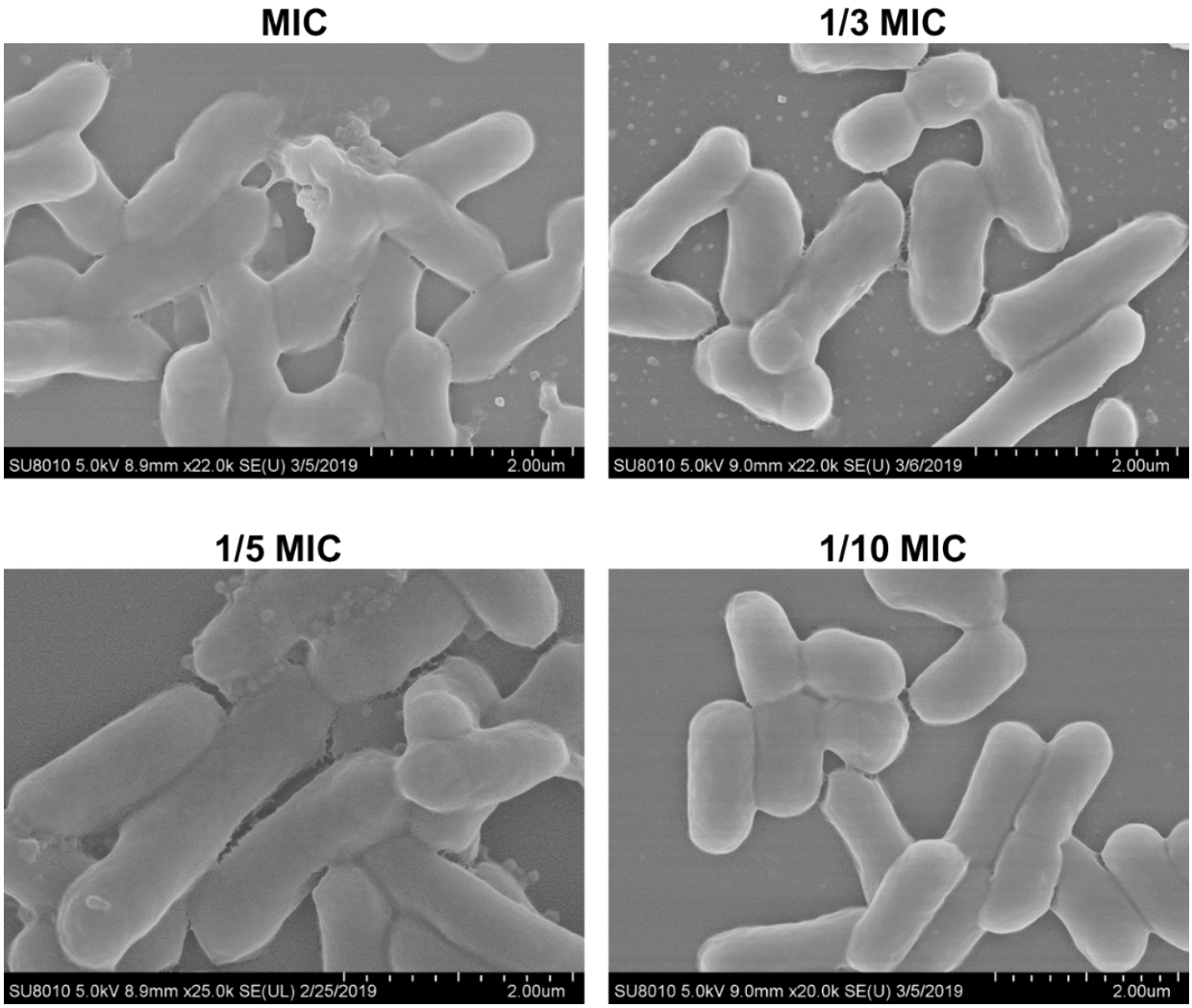


Fig. S6. Bacterial nanotubes formed in antimicrobial cationic PIL solution. Wild-type *E. coli* were cultured for 4h at different antimicrobial concentrations of Im cation based PIL solution, including minimum inhibitory concentration (MIC), and sub-MICs of 1/10, 1/5 and 1/3 MICs, and then visualized by SEM. Scale bar: 2 μm.

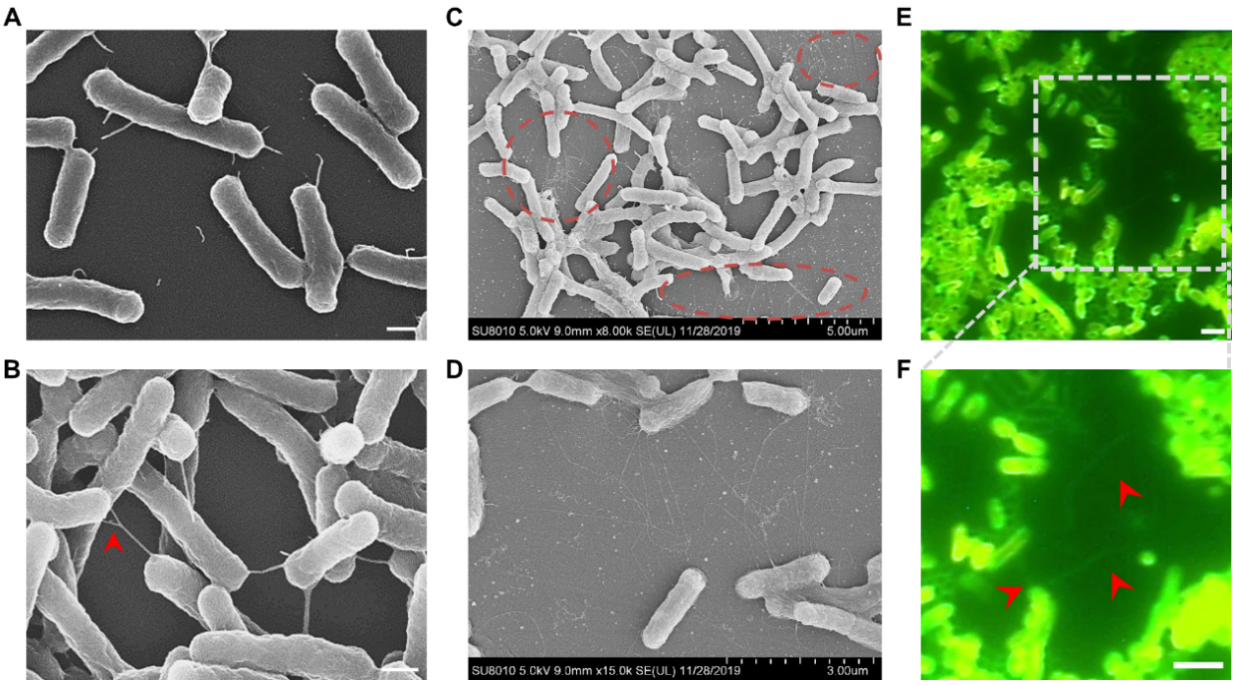


Fig. S7. Morphological characteristics of bacterial nanotubes on PIL membranes. Wild-type *E. coli* were cultured on PIL_{20} for 4h. Typical SEM fields for ENs (A), ICNs (B) and nanotube networks (C, D). The red arrow is the branched ICN, and the red circle represents the nanotube networks. Scale bar is 0.5μm in Fig. S7A-B. (E, F) Nanotubes of *E. coli* stained with a lipophilic dye (DiO) and observed in fluorescence microscopy. (F) An enlarged image of the boxed region in (E). The red arrows are ICNs. Scale bar: 3μm.

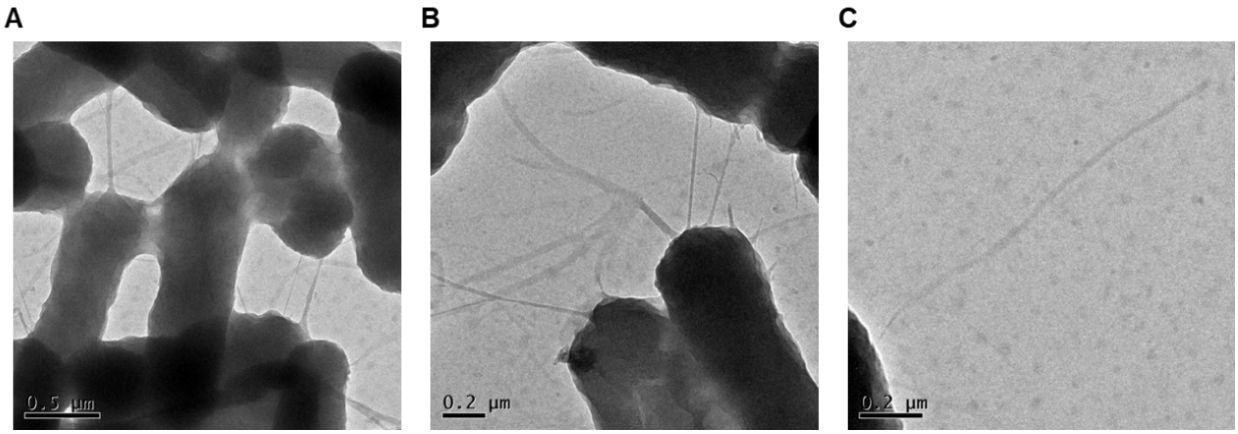


Fig. S8. Transmission electron microscopy (TEM) images of bacterial nanotubes. Wild-type *E. coli* were cultured on PIL_{20} for 4h, and then visualized by TEM.

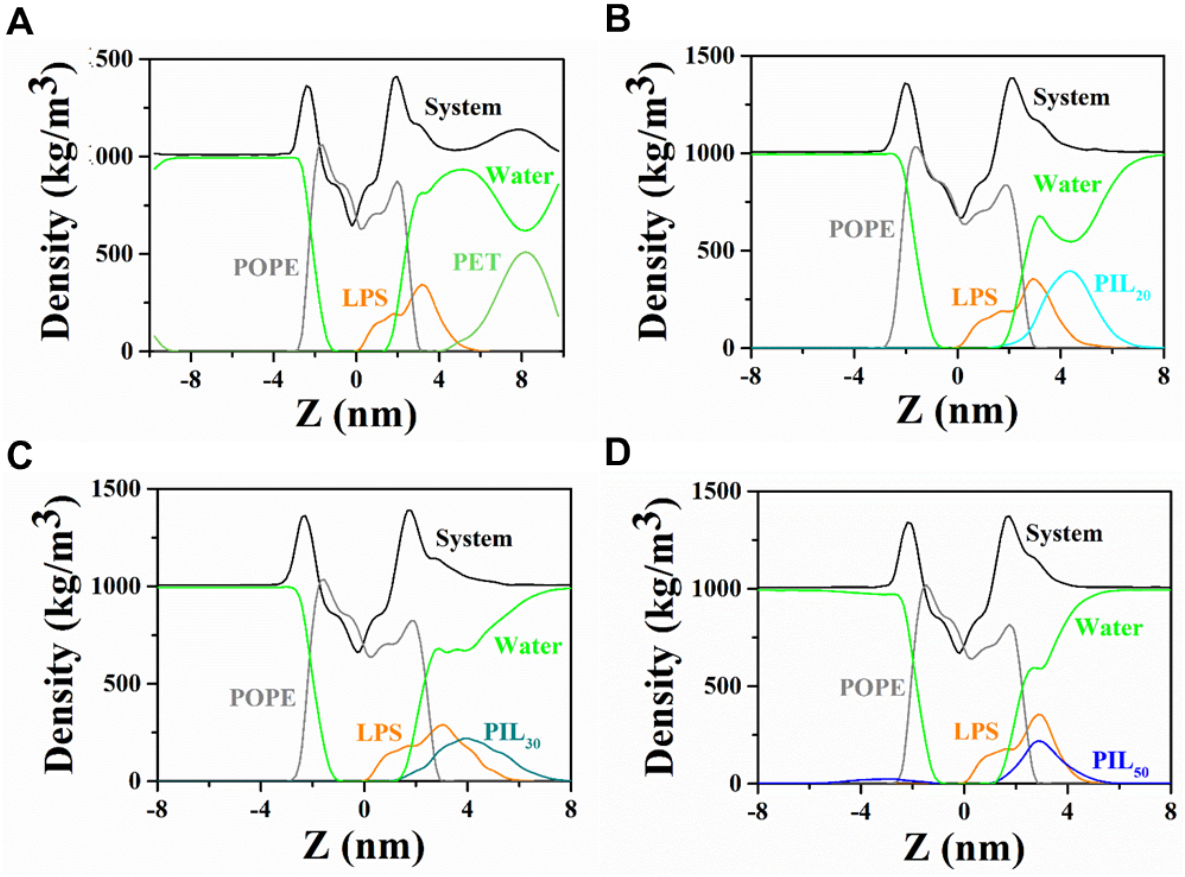


Fig. S9. Density distributions along Z axis for different components (System, Water, POPE, LPS, PET and PILs) in PET (A), PIL_{20} (B), PIL_{30} (C) and PIL_{50} (D) systems, respectively.

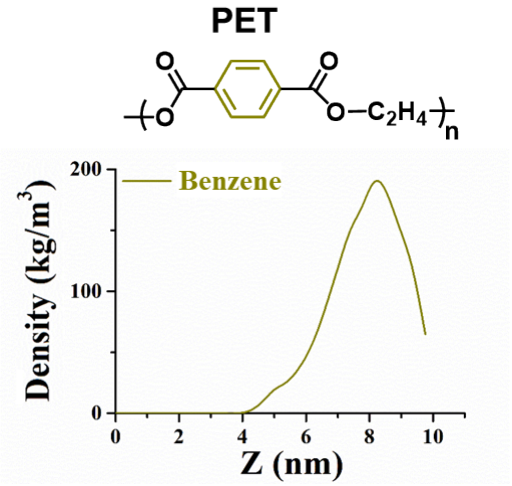


Fig. S10. Structure diagram of PET and the density distribution along Z axis for benzene group in PET.

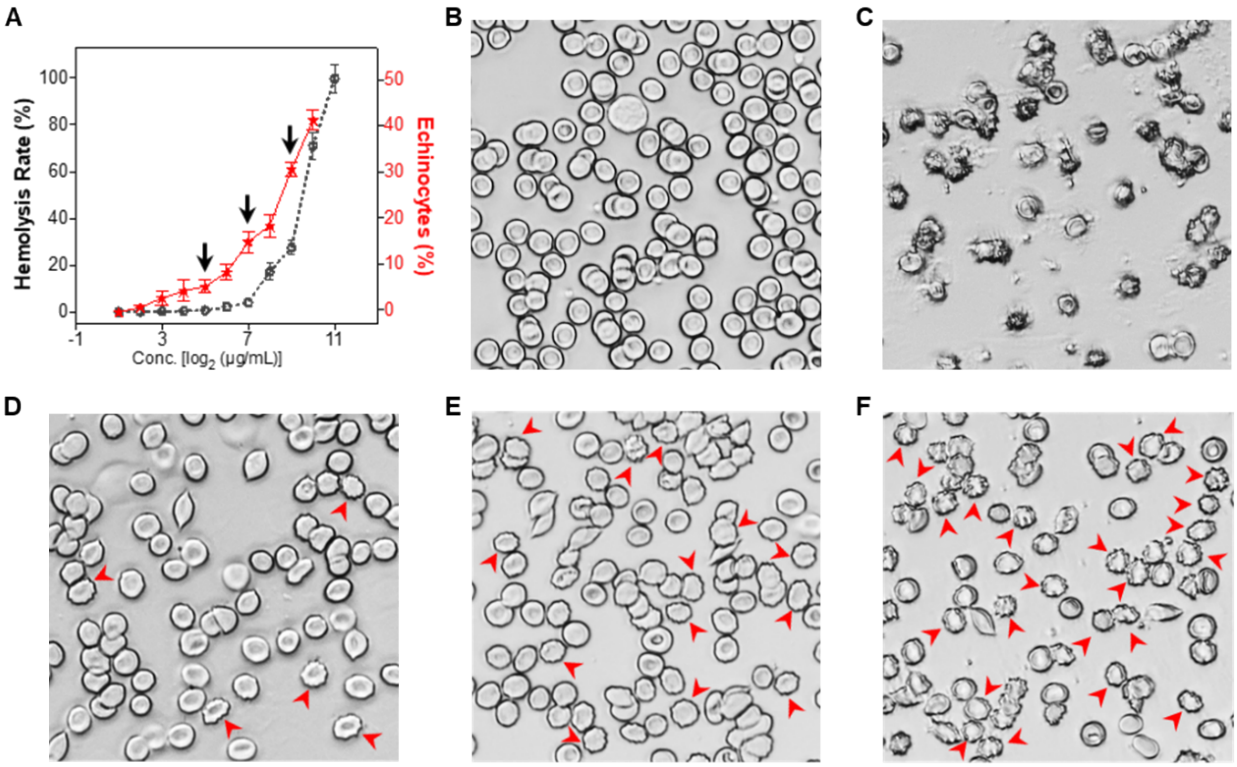


Fig. S11. (A) Hemolysis and echinocytes formed in antimicrobial cationic PIL solution. Fresh human red blood cells (RBCs) were cultured for 30min at different antimicrobial concentrations of Im cation based PIL solution, and then visualized by a microscope with a magnification of 40× (B-F). Three black arrows from left to right indicate the representative pictures for blood cells will be shown (see D-F in turn). The erythrocyte morphology in PBS (B) and PIL at the highest detection concentration (C) was shown as control. The echinocytes are marked with red arrows (D-F).

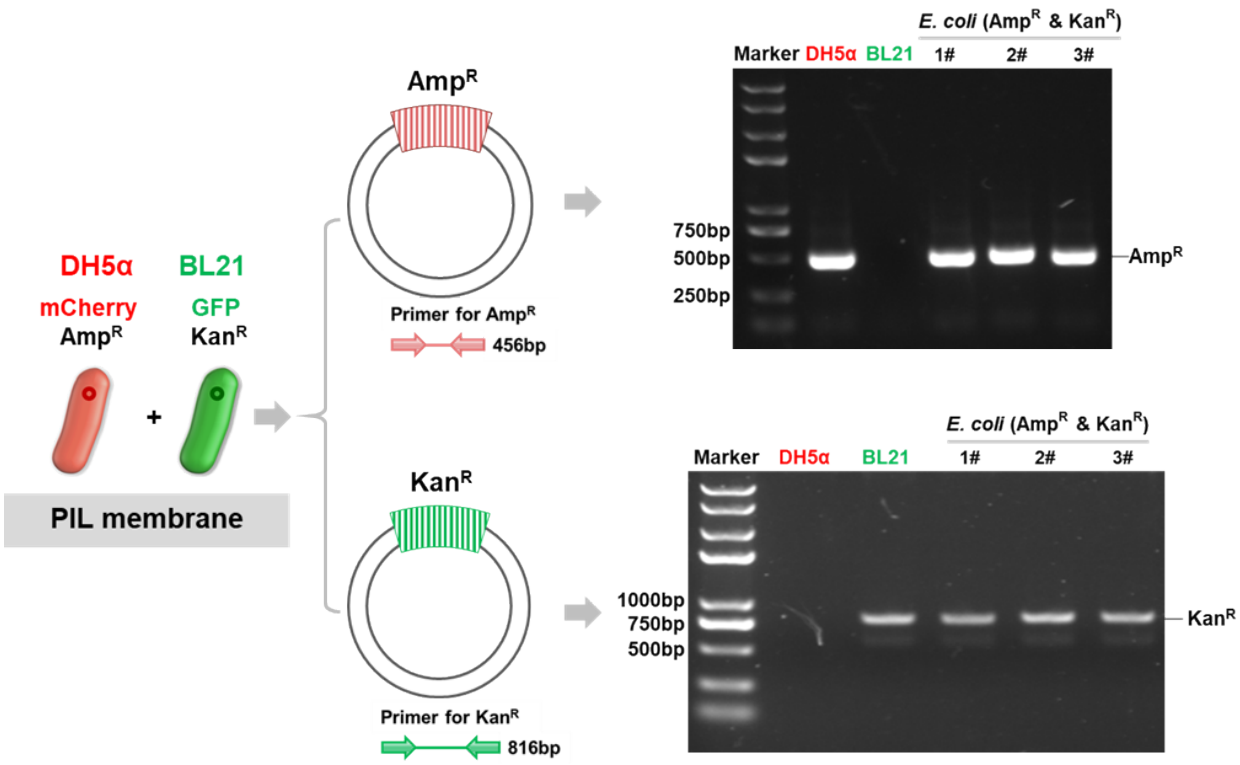
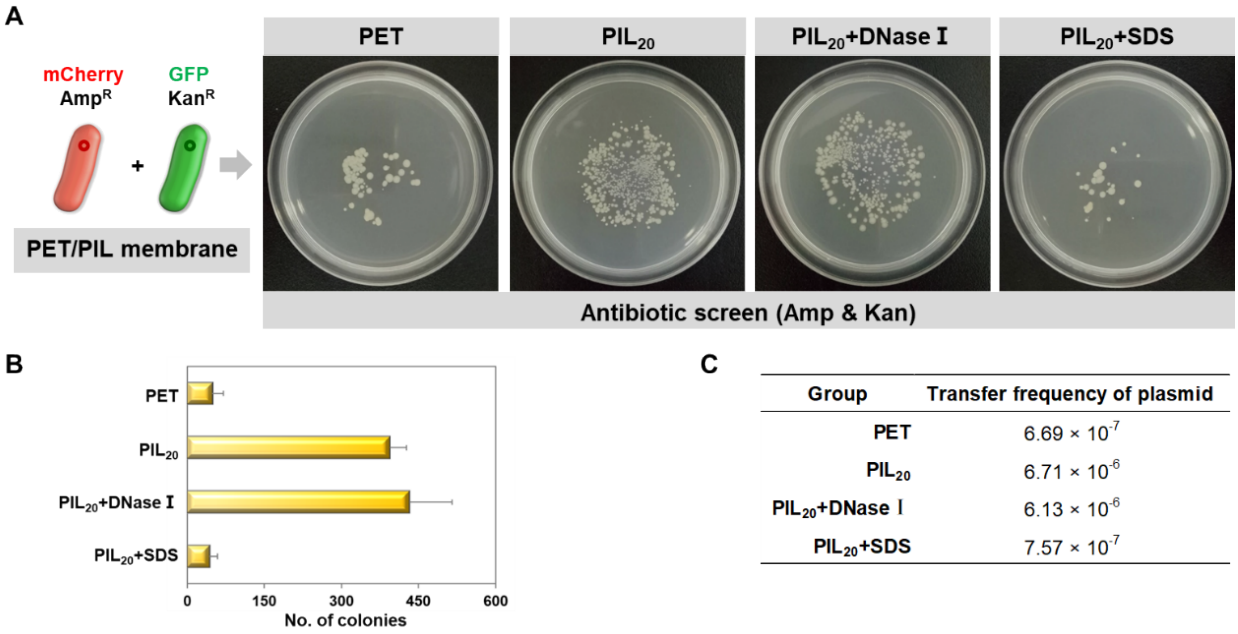


Fig. S12. Intraspecies transferred drug resistance genes of Kan^R and Amp^R in *E. coli* analyzed by PCR. Two strains of *E. coli*: DH5α (plasmid carrying red mCherry gene and Amp^R) and BL21 (plasmid with green GFP gene and Kan^R), were mixed equally on PIL_{20} membrane for 4h, and then screened with two antibiotics of Amp and Kan. The screened *E. coli* (Kan^R & Amp^R) were detected by PCR in the agarose gel. The left schematic diagram displays ARGs in *E. coli* plasmids for PCR assay.



C

Group	Transfer frequency of plasmid
PET	6.69×10^{-7}
PIL_{20}	6.71×10^{-6}
PIL_{20}+DNase I	6.13×10^{-6}
PIL_{20}+SDS	7.57×10^{-7}

Fig. S13. Transfer efficacies of ARGs between *E. coli* on PIL_{20} compared with those from three controls, including PET, PIL_{20} added with DNaseI (100μg/mL) and SDS (0.009%), respectively. Two strains of *E. coli*: DH5α (Amp^R) and BL21 (Kan^R), were mixed equally on membranes for 4h, and detected on the plates with two antibiotics of Amp and Kan for colony assay (A) and counting (B), and then the transfer frequencies of plasmid in four tested groups were calculated (C).

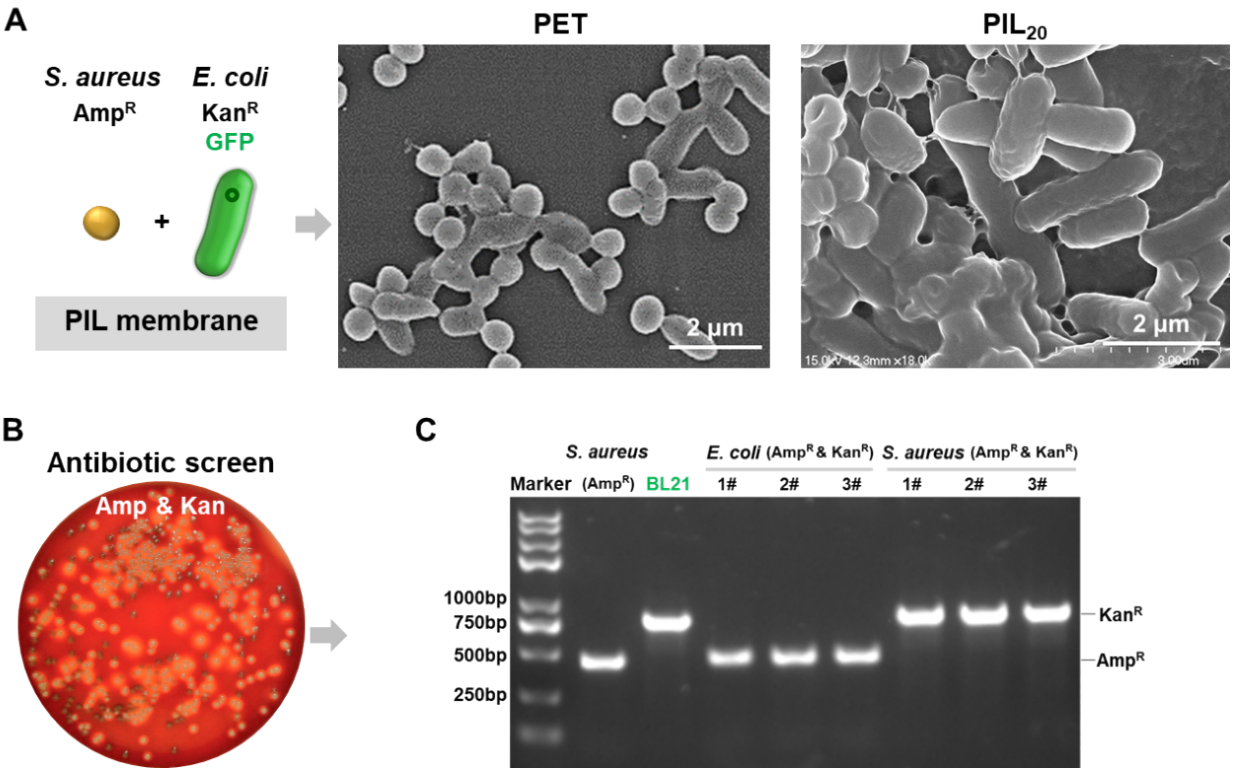


Fig. S14. Interspecies bacterial nanotubes and drug-resistance spread after contacting with PIL_{20} membrane. Gram-positive *S. aureus* (with Amp^R) and Gram-negative *E. coli* BL21 (with green GFP gene and Kan^R) were mixed on PIL_{20} membrane for 4h, and then detected by SEM (A), drug-resistant colony screening (B) and PCR analyses (C). Scale bar: 2μm. The schematic diagram for *E. coli* and *S. aureus* on PIL_{20} is drawn on the left of Fig. S14A. The screened colonies were displayed on the blood plate with Amp and Kan, and detected by PCR in the agarose gel.

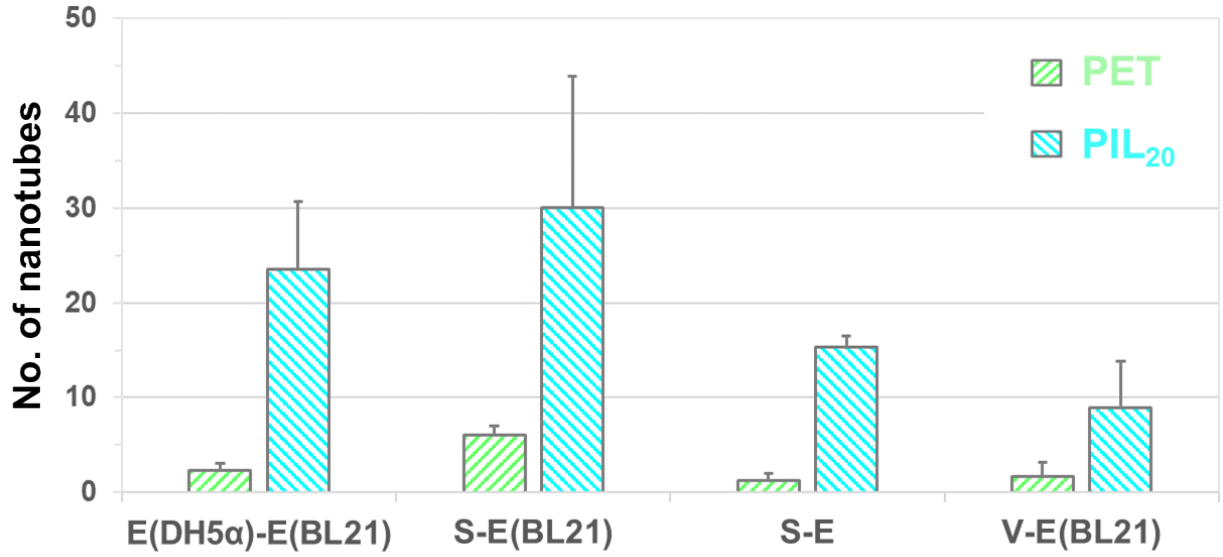


Fig. S15. Nanotube formation of all intra/interspecies bacteria upon contacting with antimicrobial PIL membranes. The quantity of bacterial nanotubes was based on SEM images from four pairs of mixed bacteria, including *E. coli* DH5α (Amp^R) and *E. coli* BL21 (Kan^R), *S. aureus* (Amp^R) and *E. coli* BL21, *S. aureus* (Amp^R) and wild type *E. coli* (no Amp^R), and *V. fischeri* (without Kan^R) and *E. coli* BL21, which corresponded to Fig. **3-5A** and **Fig. S14A**. The counting of bacterial nanotubes was based on 50 bacteria.

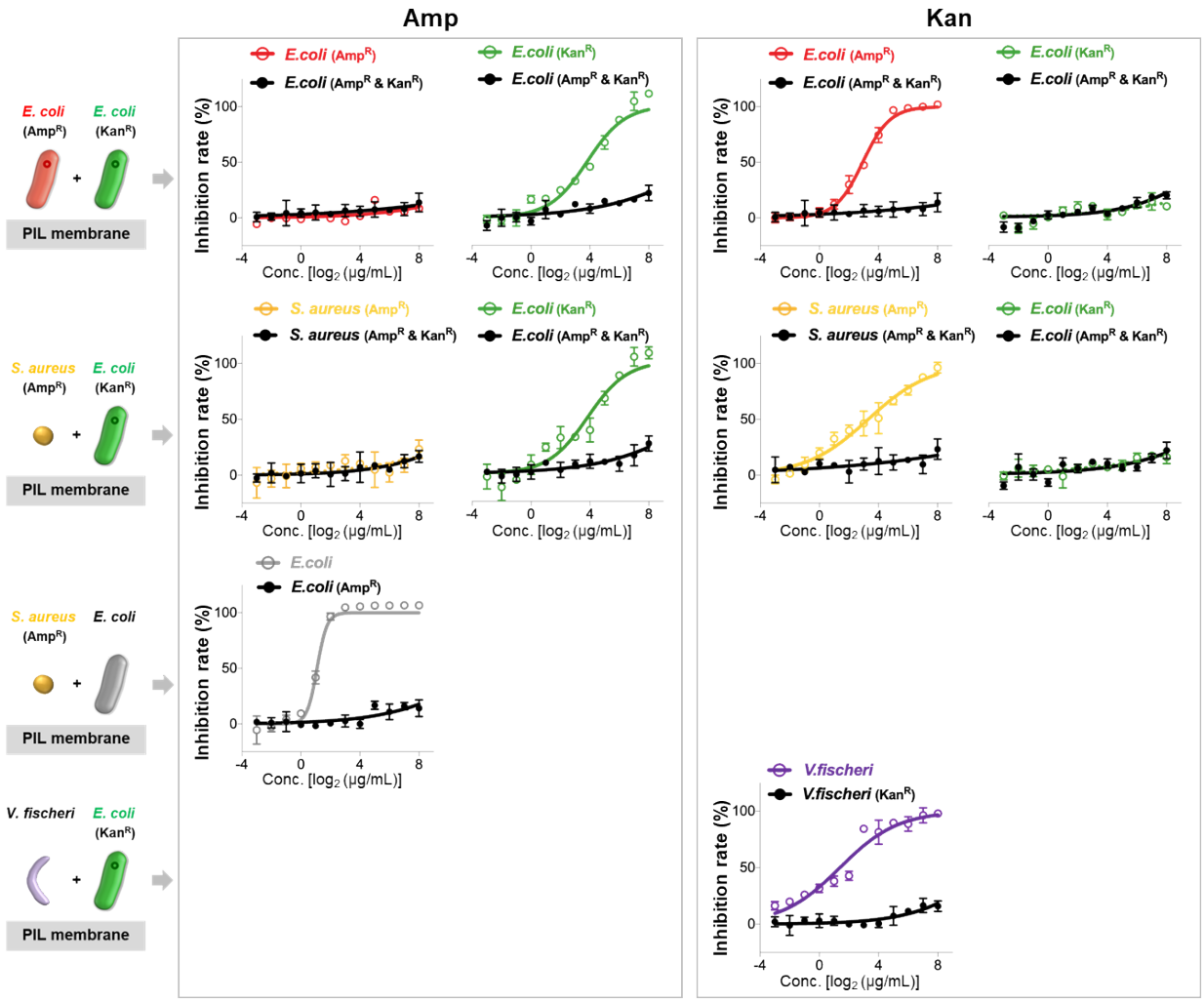


Fig. S16. Inhibition rates against all intra/interspecies bacteria before and after obtaining ARGs under the stress of PIL membrane at different concentrations of Amp and Kan solutions.