

Supplementary Information

Inherently Self-Sterilizing Charged Multiblock Polymers that Kill Drug-Resistant Microbes in Minutes

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In this document, we provide experimental details responsible for the results reported in the main narrative.

A. Polymeric materials

Material and chemical details

Two sulfonated grades of the commercial (NEXAR[®]) poly[*tert*-butylstyrene-*b*-(ethylene-*alt*-propylene)-*b*-styrenesulfonate-*b*-(ethylene-*alt*-propylene)-*b*-*tert*-butylstyrene) (TESET) block polymers were kindly provided in sheet form by Kraton Polymers (Houston, TX). The block weights of the parent (unsulfonated) polymers were supplied by the manufacturer: 15 (T), 10 (E) and 28 (S) kDa. As described in detail elsewhere,³⁸ the chemically comparable poly(*tert*-butylstyrene-*b*-styrene-*b*-*tert*-butylstyrene) (TST) triblock copolymer was synthesized by living anionic polymerization initiated by *sec*-butyllithium in cyclohexane at 25-30 °C. According to size exclusion chromatography and proton nuclear magnetic resonance (¹H NMR) spectroscopy, the T and S block weights were 21 and 80 kDa, respectively, and the polydispersity was 1.01. Dichloroethane (DCE, 99.8% pure), sulfuric acid (H₂SO₄, 98% pure), acetic anhydride (AA, 99% pure), and tetrahydrofuran (THF, 99.6% pure), as well as buffer salts, were all purchased from Fisher Scientific (Pittsburgh, PA), whereas silver nitrate (AgNO₃), copper sulfate (CuSO₄), zinc chloride (ZnCl₂), and aluminum chloride (AlCl₃) were all acquired from Sigma-Aldrich (St.

Louis, MO). The LB broth (Miller) was obtained from EMD Chemicals (Billerica, MA), while Tryptic Soy Broth and Nutrient Broth #234000 were procured from Teknova (Hollister, CA) and BD Difco (Franklin Lakes, NJ), respectively. Deionized (DI) water was used to prepare all salt, media and buffer solutions.

Sulfonation of the TST polymer

Sulfonation of the TST polymer was conducted according to the functionalization procedure previously reported.³⁸ The parent (unsulfonated) TST copolymer was dissolved at 5 wt% in an 18/1 v/v DCE/AA mixture. Upon TST dissolution, the solution was heated to 50 °C and the sulfonating agent, acetyl sulfate generated from a H₂SO₄/AA mixture at a molar ratio of 1.00/1.15, was added dropwise. The reaction continued at 50 °C for 6 h, after which time the reaction products were precipitated upon addition of DI water and purified by repeated dialysis against DI water. Complete removal of the starting reactants was ensured by measuring the pH of the solution. The degree of midblock sulfonation was quantified by ¹H NMR spectroscopy according to the protocol described elsewhere.³⁸ Three grades of sulfonated TST copolymers were prepared and designated as TST_x, where *x* represents the degree of sulfonation (in mol%).

Preparation of polymer films

Films were prepared by first adding 10 wt% polymer to THF at ambient temperature. After complete polymer dissolution under continuous agitation for ~30 min, each solution was transferred to a Teflon mold and subsequently dried for 24 h in a laminar fume hood. Resultant films produced in this fashion, measuring about 250-300 μm thick, were further dried under vacuum at 40°C for an additional 24 h to help remove entrapped solvent and stored in a desiccator until used. Although small concentrations of solvent likely remained entrapped in the

polymers due to their glass-forming styrenic blocks, small quantities of THF purposefully added to the microbial suspensions to discern the effect of solvent on microbial proliferation did not promote any discernible level of inactivation. To promote neutralization of the sulfonic acid groups in the TESET52 polymer, salt solutions were first prepared at 1.0 M in DI water, and polymer films were subsequently immersed in each resulting salt solution, stirred for 24 h at ambient temperature and dried under quiescent conditions.

Measurement of pH level

All the pH measurements reported in the main narrative were performed in triplicate with a Mettler Toledo FiveEasy Plus FP20 pH probe calibrated with respect to standard buffer solution.

B. Antibacterial studies

Culturing of bacteria

Methicillin-susceptible *S. aureus* (ATCC-2913) was cultured in antibiotic-free tryptic soy broth, while MRSA (ATCC-44) was grown in tryptic soy broth containing 5 µg/mL tetracycline. Vancomycin-resistant *E. faecium* (ATCC-2320) was cultured in the presence of 50 µg/mL ampicillin in DB Difco Bacto Brain Heart Infusion 237500. *E. coli* BL21-(Dε3)pLysS (Stratagene, San Diego, CA) and *A. baumannii* (ATCC-19606) were both grown in Miller LB media with 100 µg/mL ampicillin and without antibiotics, respectively. *K. pneumoniae* (ATCC-2146) was cultured in DB Difco Nutrient broth #234000 containing 100 µg/mL ampicillin. Each strain was grown in 5 mL of broth in a culture tube incubated at 37 °C and 250 rpm. All strains were grown to an optical density (OD₆₀₀) of ~0.4, which corresponded to $1-4 \times 10^8$ CFU/mL (where CFU refers to the number of colony-forming units). After centrifuging the broth at 3600 rpm for 5 min, the supernatant was removed, and the resultant bacterial cell pellet was re-

suspended in phosphate buffer saline (PBS) solution.

Inactivation of bacteria

The midblock-sulfonated polymer films were cut into discs with a hole punch so that each piece fit precisely into a well of a flat-bottom 24-well plate. To each well was pipetted 200 μL of bacterial suspension from the stock previously re-suspended in PBS solution. A control was used to check the growth of the bacterial stock solution and calculate the inactivation of suspended microbes exposed to the polymer surfaces. After a constant exposure time of 5 min (unless otherwise stated), 40 μL of bacterial suspension was withdrawn and added to an aliquot containing 360 μL of PBS. This procedure was repeated 5x so that each well contained 6 serial dilutions (from 10⁻¹-1,000,000x), and 10 μL from each aliquot were then added onto six-column square agar plates prepared with appropriate broth and agar for various bacteria. These plates were incubated overnight in an oven maintained at 37 °C. The CFUs were counted and the level of bacterial inactivation was calculated. In some cases, the exposure time was systematically varied (for MRSA on the TESET polymers) or increased beyond 5 min (for *S. aureus* on the TST17 polymer). Statistical significance was assessed using an unpaired Student's two-tailed *t*-test.

Confocal microscopy imaging

Confocal laser scanning microscopy (CLSM) was performed on three samples: a live control, a dead control and a bacterial suspension exposed to TESET52. All the fluorescence images were acquired on a Zeiss LSM 880 microscope in the Cellular and Molecular Imaging Facility at NC State University after staining the bacteria with a live/dead assay. A 1:1 SYTO9:propidium iodide mixture was used for this purpose, and the bacteria were stained for 30 min prior to

imaging. The CLSM images reported here were collected with a Zeiss C-Apochromat 40x water-immersion objective lens (1.2 numerical aperture). Laser wavelengths of 488 and 561 nm were used to excite green (for live bacteria) and red (for dead bacteria) fluorescence, respectively.

C. Antiviral studies

Vesicular stomatitis virus (VSV)

Polymer films were prepared in the same fashion as in the antibacterial tests, and a similar control was used as a reference to calculate the level of viral inactivation. All the tests were performed in at least triplicate unless otherwise noted. After exposing 25 μ L of VSV suspension to the TESET polymers for 5 min, 100 μ L of minimum essential medium (MEM) composed of 1% fetal bovine serum (FBS), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and antibiotics were added to remove adhered VSV from the polymer films. The viruses were subsequently titered after serial dilution (6 log units) using Vero mammalian cells (epithelial cells from the kidney of the African green monkey) that were incubated in a 24-well plate under isothermal conditions at 37 °C. The concentration of VSV was determined by plaque assay in which crystal violet was used to stain infected Vero mammalian cells after infecting them for 24 h. The level of VSV inactivation was determined by counting the number of plaques, and the MDL was 66.7 PFU/mL.

Human adenovirus-5 (HAd-5)

The procedure here is initially identical to that employed for VSV. After an exposure time of 5 min on the TESET26 and TESET52 polymers, 100 μ L of Dulbecco's Modified Eagle's medium (DMEM) containing 10% FBS and antibiotics were added to remove adhered HAd-5 from the films. The viruses were titered as above using A549 cells (from a human lung carcinoma cell

line) in 24-well plates at 37 °C. The A549 cells were infected for 120 h, after which time infected cells were stained with crystal violet. The level of HAd-5 inactivation was determined by counting the number of plaques, and the MDL was 66.7 PFU/mL.

Influenza A virus

The procedure here is initially identical to that employed for VSV and HAd-5. After an exposure time of 5 min on the TESET26 and TESET52 polymers, DMEM with 0.2% bovine serum albumin (BSA), 25 mM HEPES buffer, antibiotics, and 2 µg/mL TPCK (tosyl phenylalanyl chloromethyl ketone)-treated trypsin were used to remove adhered Influenza A viruses from the films. The viruses were titered as above using Madine-Darby canine kidney cells (MDCK), and the cells were infected for 48 h prior to staining with crystal violet. The level of Influenza A inactivation was determined by counting the number of plaques, and the MDL was 66.7 PFU/mL.

D. Rejuvenation studies

In the main narrative, we have demonstrated that repeated exposure to pathogens suspended in PBS results in a reduction in antimicrobial efficacy due to complexation of the sulfonic acid groups present along the midblock of the multiblock polymer and cations from PBS. It is important to recognize that, in practice, cations such as these will most likely not be present at high concentrations, if at all, as pathogens come in contact with the polymer surface. In other words, we have presented the worst case scenario wherein the self-sterilizing ability is progressively compromised as the polymer undergoes cyclic exposure to PBS. To discern the extent to which the sulfonic acid groups can be reactivated (and the antimicrobial performance rejuvenated), we have exposed the TESET52 material to 10 successive cycles of *S. aureus* in

PBS, after which time the surface was nearly completely deactivated (with a high bacterial survival of 87%). These specimens have been subsequently subjected to immersion in aqueous HCl solutions at different concentrations (0.01, 0.1 and 1.0 M) for different times, and their antimicrobial performance has been re-measured. The results, provided in **Figure S1**, clearly affirm that, even after deleterious complexation with cationic species from PBS, these charged multiblock polymers can be fully reactivated (to yield low *S. aureus* survival at the MDL) after relatively short rejuvenation times that vary inversely with acid strength: 5 min at 1.0 M, 15 min at 0.1 M and 30 min at 0.01 M. To put these results in perspective, the acidity of the 0.01 M HCl(aq) solution is comparable to that of either white distilled vinegar (pH \approx 2.5) or lemon juice (pH \approx 2-3).

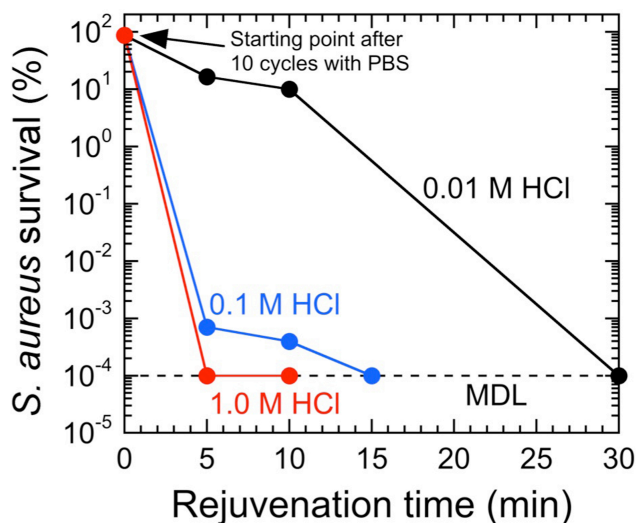


Figure S1. Survival of *S. aureus* as a function of rejuvenation time for the TESET52 polymer immersed in HCl(aq) solutions varying in concentration (labeled and color-coded) after 10 repeated exposures to PBS (which deactivates the polymer due to complexation of sulfonic acid groups on the polymer with cationic species in the PBS). The solid lines serve to connect the data.

E. Toxicity studies

The self-sterilizing TESET polymers discussed in this work display acute, fast-acting antimicrobial properties against several (drug-resistant) bacterial and viral strains. Here, we consider

their toxicity toward mammalian cells in case the materials come into contact with live cells in contemporary applications such as wound care dressings or transdermal drug delivery. While a full toxicity study is well beyond the scope of the present study, we examine here the effect of TESET26 and TESET52 on Vero mammalian cells, described above with regard to our VSV antiviral studies. The toxicity of the TESET materials was monitored by a Trypan Blue exclusion assay. Briefly, Vero cells were grown in 175 cm² flasks to confluency, trypsinized and adjusted to a cell suspension of 4 × 10⁶ cells/mL. Next, 25 μL of this suspension were added to discs of TESET26 or TESET52 in the bottom of a 96-well plate, and the cells were incubated for different time periods. Wells without TESET served as a negative control. At the end of the incubation, 25 μL of growth medium (Dulbecco's Modified Eagle Medium, supplemented with 6% fetal bovine serum and 1% penicillin/streptomycin) were added, and each cell suspension was transferred to a fresh well. Lastly, 50 μL of Hyclone Trypan Blue solution was added, and the cells were counted in a hemocytometer (Neubauer Improved) at 10x magnification on a Motic AE20 inverted brightfield microscope. As evinced by the data presented in **Figure S2**, both TESET materials are capable of killing Vero cells over the course of just a few minutes, with their effectiveness increasing with an increase in the degree of polymer sulfonation, thereby following the same trend observed with respect to antimicrobial performance. With these limited results in mind, we highly recommend that the TESET materials investigated here be restricted for use in conjunction with various environmental objects (*e.g.*, films, fibers and surfaces) that do not come into direct contact with live cells.

F. Material summary

Due to the wide range of available polymeric materials that are capable of exhibiting antimicrobial properties, we have compiled a non-exhaustive list of some of these materials and

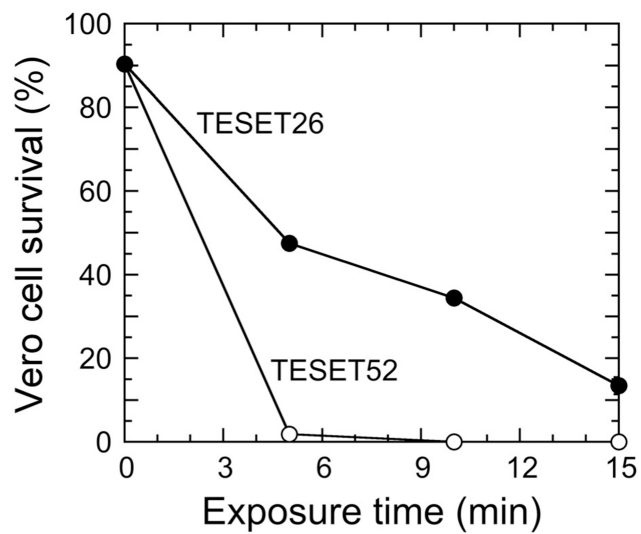


Figure S2. Survival of Vero mammalian cells after exposure to the TESET26 and TESET52 polymers (labeled) over the course of 15 min. The solid lines serve to connect the data.

the pathogens against which they are most effective. This list with associated references is provided in **Table S1**.

Table S1. Compilation of several effective antimicrobial polymeric materials.

Type	Polymer, Class or Functionality	Pathogen(s) examined	Ref.
Zwitterionic & cationic polymer brushes	Poly(<i>N,N</i> -dimethyl- <i>N</i> -(ethoxy carbonylmethyl)- <i>N</i> -[2'-(methacryloyloxy)ethyl]-ammonium bromide)	<i>E. coli</i>	23
Peptide-conjugated polymer brushes	Poly(<i>N,N</i> -dimethylacrylamide- <i>co-N</i> -(3-aminopropyl)-methacrylamide hydrochloride)	<i>P. aeruginosa</i>	24
Polycationic hydrogel	Chitosan/dextran-derived	<i>E. coli</i> , <i>P. aeruginosa</i> , <i>S. aureus</i> , <i>S. pyogenes</i> , <i>C. albicans</i> , <i>C. perfringens</i>	26
Zwitterionic hydrogel	Poly(2-(2-((2-(methacryloyloxy) ethyl) dimethylammonio) acetoxo) benzoate) with salicylic acid conjugated to carboxyl betaine	<i>S. epidermis</i>	27
Cationic bulk polymer	Guanidine-based polymers	<i>E. coli</i> , <i>P. aeruginosa</i> , <i>S. aureus</i> , <i>C. albicans</i>	28
Cationic bulk polymer	Polyethyleneimine derivatives	<i>E. coli</i> , <i>S. aureus</i>	29
Cationic bulk polymer	Random/alternating copolymers and homopolymers containing quaternary ammonium groups	<i>E. coli</i> , <i>S. aureus</i>	30
Aromatic self-assembling polymers	Peptide-based diphenylalanine and diglycine	<i>E. coli</i>	40

Self-assembling β -hairpin peptide hydrogels	Hydrophobic valine and hydrophilic lysine/arginine	MR <i>S. aureus</i>	41
Amphiphilic monodisperse microstructures	Polyethyleneimines	<i>E. coli</i> , <i>S. aureus</i> , <i>B. subtilis</i>	42
Peroxide-releasing microgels	Prepared from <i>N</i> -hydroxyethyl acrylamide, methylene bis-acrylamide and dopamine methacrylamide	<i>E. coli</i> , <i>S. epidermis</i> , porcine parovirus, bovine viral diarrhea virus	43
Amphiphilic peptide mimics	Guanylated polymethacrylates	<i>E. coli</i> , <i>S. epidermis</i> , <i>S. aureus</i> , <i>C. albicans</i>	44
Midblock-sulfonated block polymers	Poly[<i>tert</i> -butylstyrene- <i>b</i> -ethylene- <i>alt</i> -propylene- <i>b</i> -(styrenesulfonate)- <i>b</i> -ethylene- <i>alt</i> -propylene- <i>b</i> - <i>tert</i> -butylstyrene]	<i>E. coli</i> , (MR) <i>S. aureus</i> , VR <i>E. faecium</i> , <i>A. baumannii</i> , <i>K. pneumonia</i> , vesicular stomatitis virus, human adenovirus-5, influenza A virus	This work
