Electronic Supplementary Material (ESI) for Chemical Communications.
This journal is © The Royal Society of Chemistry 2021

Slippery Nanoemulsion-Infused Porous Surfaces (SNIPS): Anti-Fouling Coatings that Can Host and Sustain the Release of Water-Soluble Agents

Harshit Agarwal,[1] Thomas J. Polaske,[2] Gabriel Sánchez-Velázquez,[1] Helen E. Blackwell,[2] and David M. Lynn [1,2,*]

[1]Department of Chemical & Biological Engineering, University of Wisconsin-Madison, 1415 Engineering Dr., Madison, WI 53706, USA; [2]Department of Chemistry, University of Wisconsin-Madison, 1101 University Ave., Madison, WI 53706, USA. E-Mail: (D.M.L) dlynn@engr.wisc.edu

Supporting Information

Materials and Methods

Materials. Unlaminated PTFE membrane filters (pore size = 5 μm, thickness ~200 μm) were purchased from Sterlitech Corporation (Kent, WA). *n*-Hexadecane (≥99%), fluorescein isothiocyanate-dextran (average molecular weight = 3000-5000 g/mol), sorbitane monooleate (Span 80), polyoxyethylene sorbitan monooleate (Tween 80), glycerol, calcein, and D-(+)-glucose were purchased from Millipore Sigma (Milwaukee, WI). Albumin from bovine serum, tetramethylrhodamine conjugate (BSA-TMRC) was purchased from Thermo Fisher Scientific (Waltham, MA). Tetramethylrhodamine (TMR)-labelled plasmid DNA (pDNA) was prepared using procedures described previously.[1] A model antifungal β-peptide (coumarin-linker-(ACHCβ^3hVal-β^3hLys)$_3$) was synthesized using previously reported methods.[2,3] Lake water was locally sourced from Lake Mendota, Madison, WI. Nature's Touch skim milk was purchased from Kwik Trip (Madison, WI). Pooled human urine was purchased from Innovative Research Inc. (Novi, MI). Ethanol (EtOH, 200 proof) was obtained from Decon Laboratories (King of Prussia, PA). Soy sauce (Kroger) was purchased from Pick 'n Save (Madison, WI). Fresh porcine blood was collected in a 50 mL conical centrifuge tube with 3.4% sodium citrate in PBS

at a ratio of 9:1 (blood:citrate) from the Meat Plant located in the Meat Science & Animal Biologics Discovery Building (UW-Madison, Madison, WI) and stored in a refrigerator until use. BacTiter-Glo microbial cell viability assay kits were purchased from Promega Corporation (Fitchburg, WI). Brain heart infusion (BHI) medium was purchased from Teknova (Hollister, CA). LB medium (Lennox L Broth) was purchased from Research Products International (Mt. Prospect, IL).

General Considerations. Dynamic light scattering measurements were performed using a Malvern Zetasizer ZS Nano. Aliquots (1 mL) of nanoemulsions were transferred to a 1 cm × 1 cm plastic cuvette. The cuvette was then placed in the Zetasizer for 2 min at 24 °C, and the scattered light intensity was measured by the detector placed at an angle of 173° from the 632.8 nm incident laser. The correlator measured the intensity correlation function for delay times ranging from 2 μs to 200 ms. Sliding times on oil- and nanoemulsion-infused materials were measured by placing a desired volume of water (containing a dye to aid visual inspection) on the surface of SLIPS and SNIPS held at an angle of 30°. The time required by a droplet to slide over a defined length (e.g., 3 cm) was measured using a digital timer. Measurements of the fluorescence of solutions used to characterize the release of FITC-dextran from SNIPS were made using a NanoDrop3300 (Thermo Scientific). Fluorescence microscopy was performed using an Olympus IX71 inverted microscope and images were obtained using the MetaMorph Advanced version 7.7.8.0 software package (Universal Imaging Corporation). Images were processed using NIH Image J software and Microsoft Powerpoint for Office 365. Laser-scanning confocal microscopy (LSCM) images were acquired using a Nikon A1-R high-speed confocal microscope and processed using Nikon Instruments Software. Scanning electron micrographs

were acquired using a LEO 1550 SEM at an accelerating voltage of 3 kV using an in-lens SEM detector. For cross-sectional SEM images, porous matrices were dipped in liquid N_2 for 2 minutes and then fractured inside the liquid N_2 with scissors that were also cooled inside liquid N_2 for 2 minutes. The porous PTFE membranes were mounted on a SEM stub by conductive carbon tape, and the sides of the membranes were grounded to the stub using conductive carbon cement. Samples were coated with a thin layer of gold using a gold sputterer operating at 45 mA under a vacuum pressure of 50 mTorr for 2 min before imaging. Water with a resistivity of 18 MΩ was obtained using a Millipore filtration system. All experiments using bacterial cultures, including biofouling assays, were performed in a BSL 2 laboratory.

Preparation of Water-in-Oil Nanoemulsions. Polyoxyethylene (20) sorbitan monooleate (Tween 80; 7.5 parts by weight) and sorbitan monooleate (Span 80; 22.5 parts by weight) were dissolved in *n*-hexadecane (70 parts by weight). The surfactant solution in *n*-hexadecane was then vortexed for 1 minute and filtered through a 0.2 μm PTFE filter. Milli-Q water (100 μL; 5% v/v) was added to a glass vial (16 × 50 mm), and after that the surfactant mixture in *n*-hexadecane was gently added to the vial (at a rate of 200 μL per 20 s) under constant stirring using a magnetic stir plate. The resulting nanoemulsion was then left stirring at room temperature for 10 min and finally filtered through 1 μm PTFE filter. Nanoemulsions loaded with water-soluble agents were also prepared using this protocol, except that the aqueous phase varied depending on the loaded agent and either consisted of 5 mg/mL FITC-dextran solution in water, 1 mg/mL BSA-TMRC in water, ~0.8 mg/mL TMR-labelled plasmid DNA in 100 mM sodium acetate buffer (pH = 4), 1 mg/mL β-peptide (coumarin-linker) in water, or 1 mg/mL calcein in water (pH adjusted to 8).

Infusion of Nanoemulsion into Porous Substrates. Lubricating liquid (w/o nanoemulsion or *n*-hexadecane; 15 μL/cm^2) was added to the top surface of the porous PTFE membranes (pore size of 5 μm, thickness of 150-250 μm) using a pipette. The liquid was then spread using tweezers to form a uniform over-coated layer. After waiting a few minutes for the liquid to infuse in the porous PTFE membrane (evident by change in opacity of the membrane) through capillary wicking, the excess liquid was removed from the surface by dabbing with weighing paper. All nanoemulsion-infused surfaces (loaded or unloaded) were prepared using this protocol.

Release of FITC-Dextran. Characterization of the release of FITC-dextran from SNIPS was performed by incubating loaded SNIPS (1 × 3 cm) in 3 mL of PBS buffer at 37 °C. At predetermined time points, SNIPS were removed from the incubator for sliding time measurements, characterization of biofouling, and fluorescence imaging assays. The buffer was removed for analysis and the solution fluorescence was measured at an excitation of 490 nm and an emission of 525 nm, corresponding to the excitation and emission maxima of FITC-dextran. Fluorescence measurements resulting from these experiments were converted to FITC-dextran mass using a calibration curve generated using known concentrations of FITC-dextran. After each measurement, SNIPS were immersed in an aliquot of fresh PBS and returned to the incubator. Plots shown in the main text were made by cumulatively summing the concentration of FITC-dextran released into solution at each time point. Total FITC loading concentration was determined by measuring the mass of the infused nanoemulsion phase in the membranes and then multiplying it by the concentration of FITC-dextran in the nanoemulsion (0.12 mg FITC per gram of nanoemulsion).

Characterization of Anti-Biofouling Performance. Freezer stocks of *Staphylococcus aureus* (RN6390b)[4] were maintained in 1:1 BHI:glycerol (50% v/v in Milli-Q water), and stocks of *Pseudomonas aeruginosa* (PAO1)[5] and *Escherichia coli* (K-12 MG1655; obtained from The Coli Genetic Stock Center, Yale University, New Haven, CT) were maintained in 1:1 LB:glycerol (50% v/v in Milli-Q water) at -80 °C. Overnight cultures of bacteria were grown in LB (*P. aeruginosa* and *E. coli*) or BHI medium (*S. aureus*) at 37 °C with shaking at 200 rpm. To prepare the inoculating subculture of *S. aureus*, the overnight cultures were washed 3 times with BHI + 1% (w/v) glucose. For washing, a desired volume of *S. aureus* suspensions was transferred to sterilized 1.5 mL microcentrifuge tubes, centrifuged at 16,100 × g for 5 min, and followed by resuspension of the cell pellet in an amount of fresh BHI + 1% (w/v) glucose equivalent to the original volume of cell suspension. The final *S. aureus* cell pellet after 3 washes was resuspended in BHI (+ 1% (w/v) glucose) in an amount equivalent to yield a starting inoculum OD_{600} of 0.23 (~ 10^8 CFU/mL). An inoculating subculture of *P. aeruginosa* was prepared by centrifugation of the overnight culture at 2,000 × g for 10 min followed by resuspension of the cell pellet in an amount of fresh M9+ medium, effecting a 1:10 dilution (v/v) of the overnight culture (M9+ medium consists of the M9 buffer supplemented with 0.4% arginine, 0.5% casamino acids, 0.2% glucose, 0.2% succinate, 0.2% citrate, 0.2% glutamate, 1×10^{-3} M $MgSO_4$, and 0.1×10^{-3} M $CaCl_2$, as described previously).[6] *E. coli* subcultures were prepared by diluting overnight cultures 1:100 into fresh LB medium.

For multiple challenge experiments, substrates were incubated with *S. aureus* inoculum, prepared as described above, in a 6-well plate at 37 °C. At the end of each 24 h period, three SNIPS substrates and controls were removed from their wells using forceps, gently dabbed on a

paper towel to remove excess liquid, and placed in the wells of a new 6-well plate to characterize the extent of biofouling on the surface using a BacTiter-Glo assay, as described below. The remaining SNIPS were then incubated in fresh *S. aureus* inoculum to perform the next challenge (new non-infused porous PTFE membranes were used in control experiments). Seven such 24 h challenges were performed, and at the end of the seventh challenge, along with characterization using a BacTiter-Glo assay, the biofilms on the substrates were stained with a green fluorescent nucleic acid stain (SYTO-9) according to the manufacturer's protocol. Excess staining solution was removed by dabbing on a paper towel and the substrates were then transferred to the wells of a 24-well plate and covered by 400 μL PBS. Biofilms were then imaged using an Olympus IX71 fluorescence microscope.

For the BacTiter-Glo assay, the BacTiter-Glo solution, prepared as described by the manufacturer's protocol, was diluted 2× in Milli-Q water and added to the wells of a 6-well plate containing SNIPS and control porous PTFE membranes. The 6-well plate was incubated for 5 min in the dark at room temperature. BacTiter-Glo solution from the plates (50 μL) was added to a clear-bottomed white 96-well microtiter plate. Luminescence was read in a Synergy 2 plate reader (Biotek) running Gen5 1.05 software. The luminescence values collected from the plate reader were normalized with respect to the control.

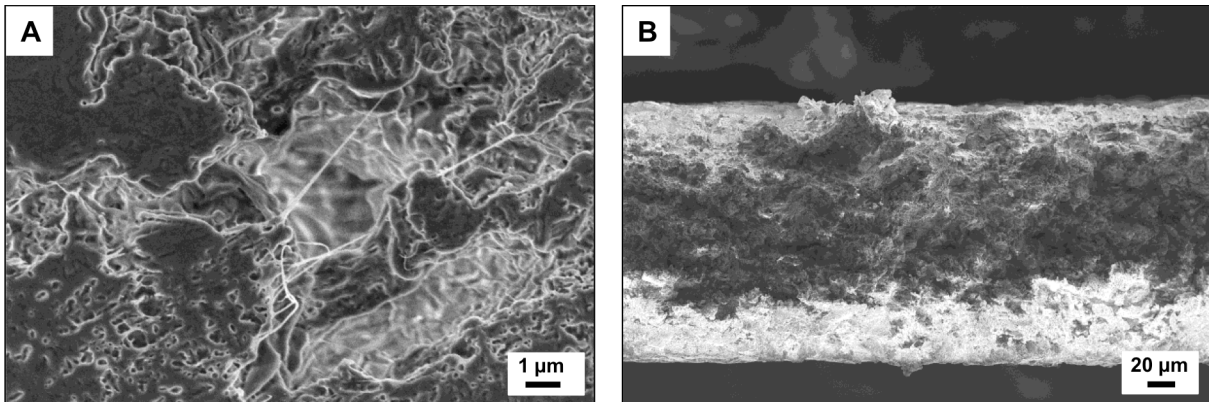

Figure S1. (A,B) Top-down (A) and cross-sectional (B) SEM images of the porous PTFE membranes (reported average pore size of 5 µm, thickness ~200 µm) used for fabrication of SNIPS in this study.

Table S1. Evaluation of the stability of SNIPS in the presence of water droplets.

Parameters	Values
$\Theta_{ws(a)}$	114 ± 1
$\Theta_{os(a)}$	41 ± 1
γ_{ow}	2.4 ± 0.4
γ_{oa}	27.2 ± 0.6
γ_{wa}	72.1 ± 0.2
$S_{os(w)}$	47.4 ± 2

Note: Unit of contact angle is in degrees. The contact angles are measured on a flat smooth PTFE surface using a 5 µL water droplet for $\Theta_{ws(a)}$ and a 5 µL w/o nanoemulsion for $\Theta_{os(a)}$. The unit of surface tension and interfacial tension is mN/m. Surface tension (γ_{oa}, γ_{wa}) and interfacial tension (γ_{ow}) measurements were performed by the pendant drop method at ambient conditions (temperature = 22 to 24 °C and relative humidity = 18 to 26 %). The density of water used for measurements was 0.997 gm/mL and the density of w/o nanoemulsions was 0.854 gm/mL. The values denote the mean of three independent measurements and error denotes standard deviation. $S_{os(w)} = \gamma_{oa} \cos \Theta_{os(a)} - \gamma_{wa} \cos \Theta_{ws(a)} - \gamma_{ow} \geq 0$ and the units of $S_{os(w)}$ is in mN/m. $\Theta_{os(a)} > 0$ suggests that the surface of PTFE membrane can emerge out of the nanoemulsion phase into air.

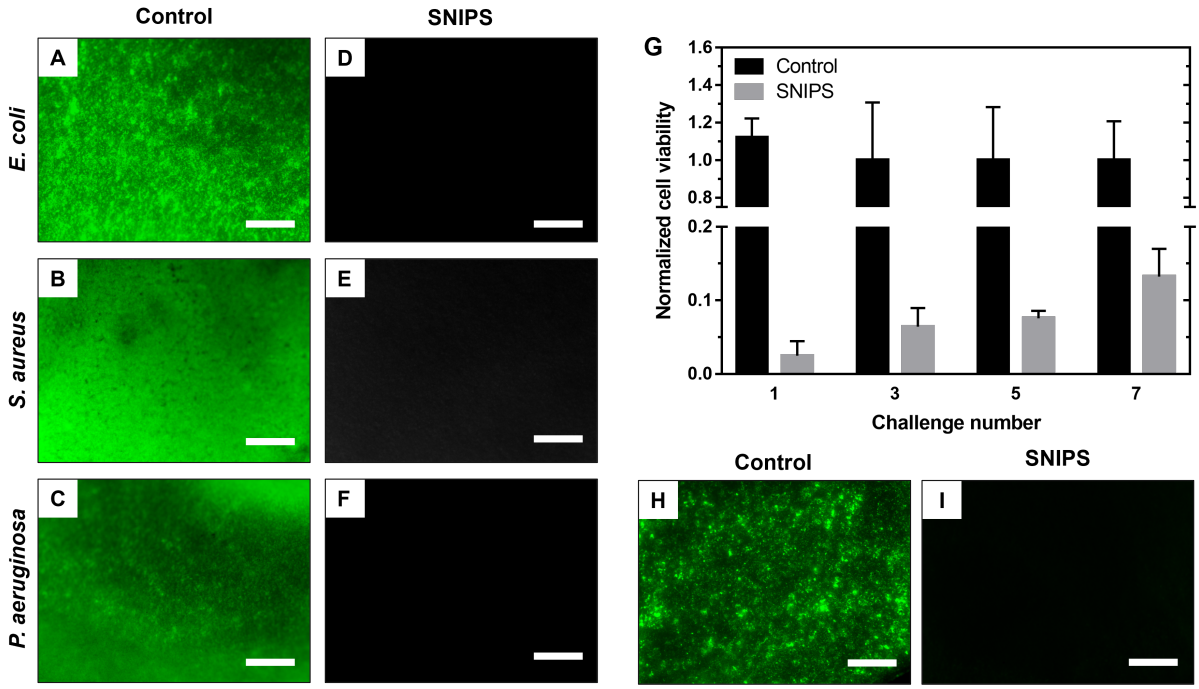

Figure S2. (A-F) Fluorescence microscopy images showing the surfaces of control PTFE membranes and SNIPS after incubation in suspensions of *E. coli* (A,D), *S. aureus* (B,E), and *P. aeruginosa* (C,F) for 24 h. The samples were stained with the green fluorescent nucleic acid stain SYTO-9 prior to imaging. Scale bars are 400 µm. (G) Plot showing the viability of *S. aureus* cells associated with the surfaces of control PTFE membranes (black bars) and SNIPS (gray bars) after each of seven consecutives 24 h challenges in *S. aureus* cultures (see main text and Methods section for details). Error bars represent standard deviation. (H,I) Fluorescence microscopy images showing the surfaces of (H) control and (I) SNIPS after the seventh challenge arising from the experiment performed in panel (G). Panels C, F, and G are reproduced from Figure 2.

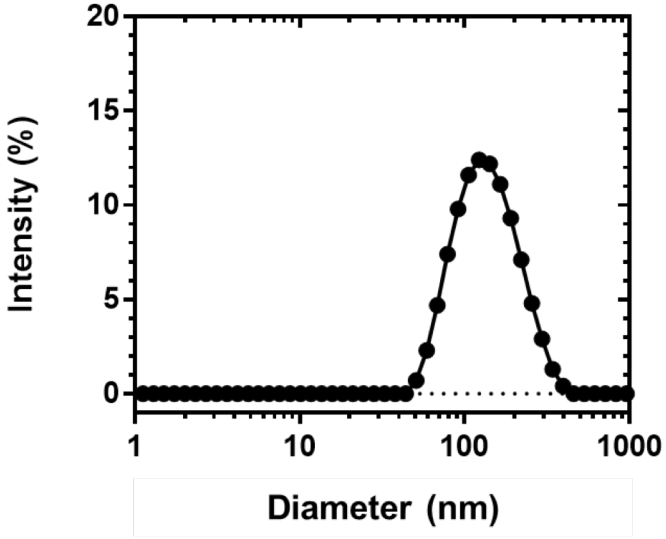

Figure S3. Plot showing the intensity-weighed particle size distribution of w/o nanoemulsion loaded with FITC-dextran. A unimodal distribution was obtained with a *z*-average size of 124 nm and a PDI of 0.153.

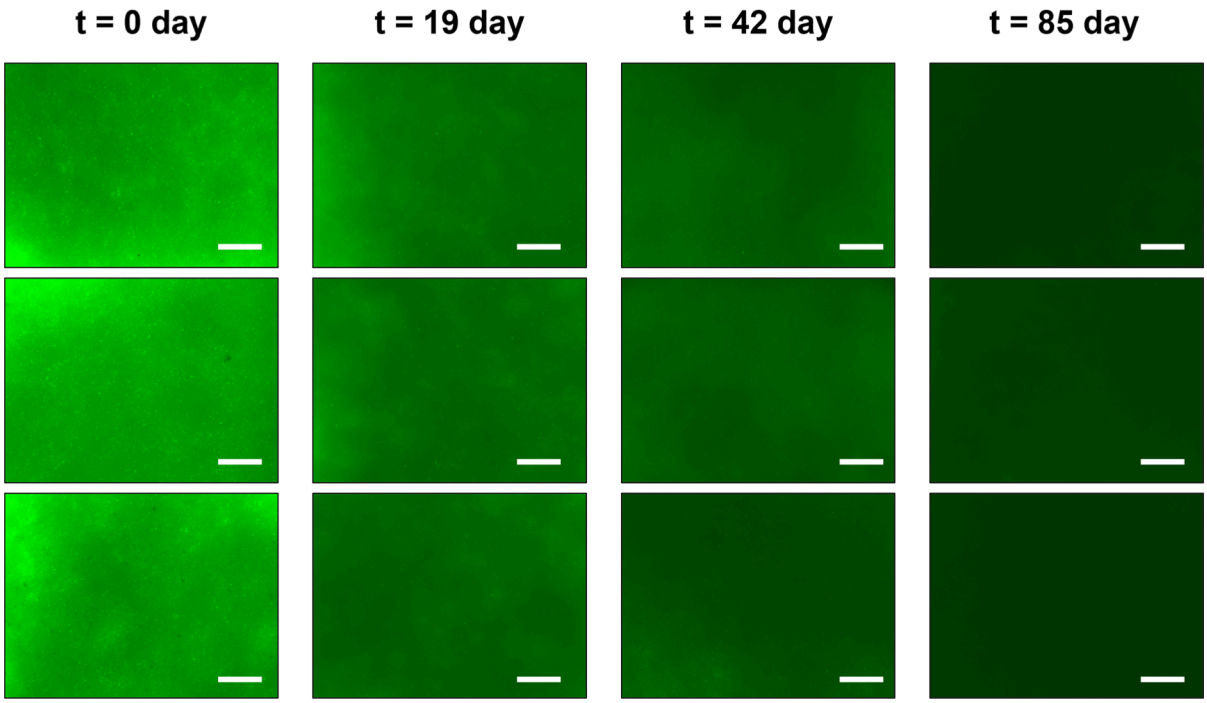

Figure S4. Top-down fluorescence microscopy images of FITC-dextran loaded SNIPS at different time points (t = 0, 19, 42, and 85 days) upon incubation in PBS buffer at 37 °C. Scale bars are 400 μm.

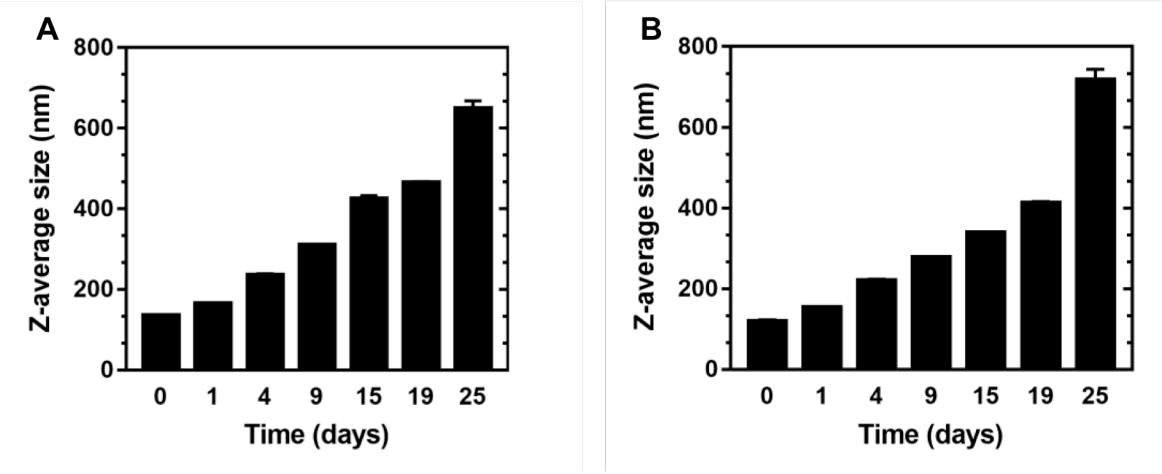

Figure S5. (A,B) Plot showing *z*-average size vs. time for (A) unloaded (no cargo) w/o nanoemulsion and (B) FITC-dextran-loaded w/o nanoemulsion incubated at 37 °C. Data points represent the mean of three independent DLS measurements. Error bars denote standard deviation.

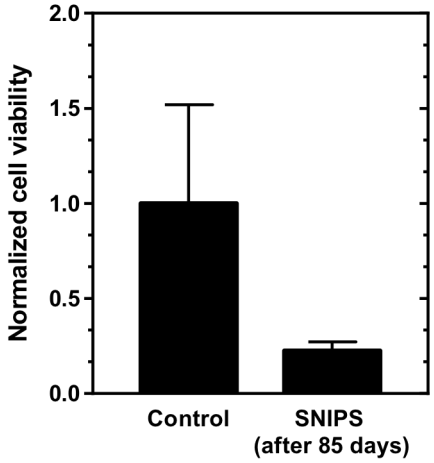

Figure S6. Plot showing the viability of *S. aureus* cells associated with the surfaces of control (uninfused porous PTFE) membranes and SNIPS after 85 days of incubation in PBS at 37 °C. The cell viability values are normalized to the control.

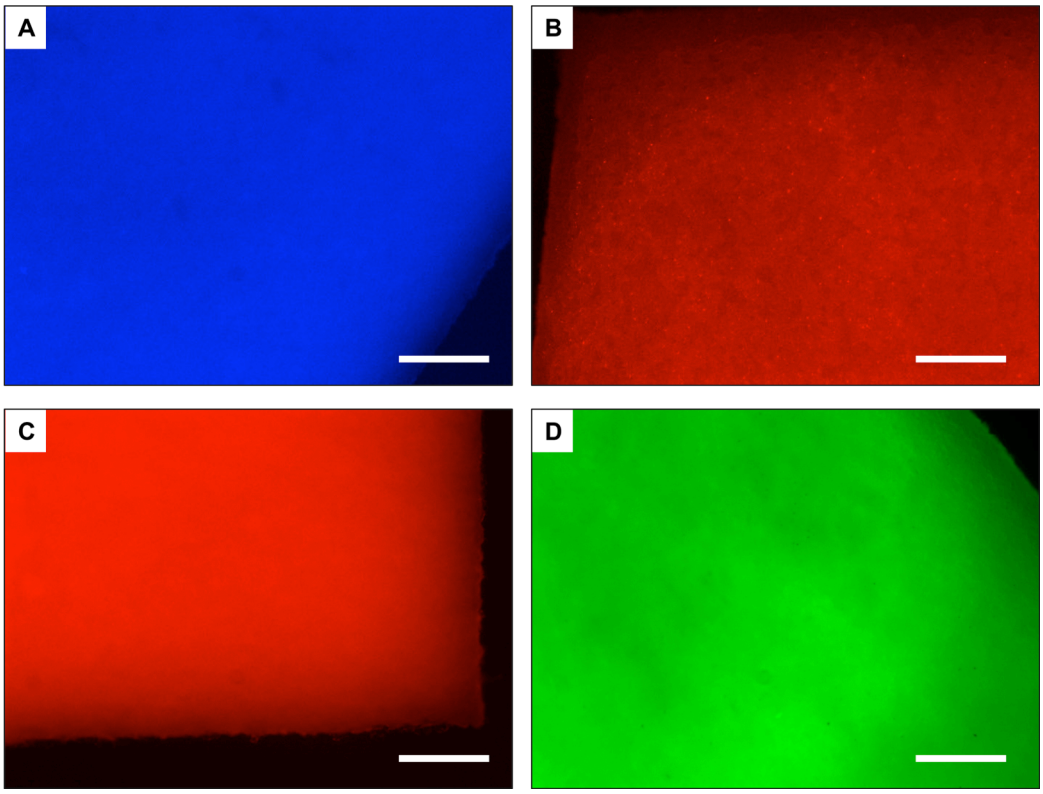

Figure S7. (A) Top-down fluorescence microscopy images of SNIPS fabricated by infusion of nanoemulsions loaded with various types of water-soluble cargo: (A) A model antifungal β-peptide (coumarin-linker), (B) tetramethylrhodamine-labeled DNA, (C) BSA conjugated to tetramethylrhodamine (BSA-TMR), and (D) calcein into porous PTFE membranes. Scale bars = 400 μm. The images are false colored. In each case, 20 μL droplets slid on nanoemulsion-infused substrates tilted at ~30°.

References

1. Yu, Y.; Si, Y.; Bechler, S. L.; Liu, B.; Lynn, D. M. Polymer Multilayers That Promote the Rapid Release and Contact Transfer of DNA. *Biomacromolecules* **2015,** *16,* 2998-3007.

2. Karlsson, A. J.; Pomerantz, W. C.; Neilsen, K. J.; Gellman, S. H.; Palecek, S. P. Effect of Sequence and Structural Properties on 14-Helical β-Peptide Activity against *Candida albicans* Planktonic Cells and Biofilms. *ACS Chemical Biology* **2009,** *4,* 567-579.

3. Karlsson, A. J.; Flessner, R. M.; Gellman, S. H.; Lynn, D. M.; Palecek, S. P. Polyelectrolyte Multilayers Fabricated from Antifungal β-Peptides: Design of Surfaces That Exhibit Antifungal Activity against *Candida albicans*. *Biomacromolecules* **2010,** *11,* 2321-2328.

4. Novick, R. P.; Ross, H. F.; Projan, S. J.; Kornblum, J.; Kreiswirth, B.; Moghazeh, S. Synthesis of Staphylococcal Virulence Factors Is Controlled by a Regulatory RNA Molecule. *The EMBO journal* **1993,** *12,* 3967-3975.

5. Holloway, B. Genetic Recombination in *Pseudomonas aeruginosa*. *Microbiology* **1955,** *13,* 572-581.

6. Frei, R.; Breitbach, A. S.; Blackwell, H. E. 2-Aminobenzimidazole Derivatives Strongly Inhibit and Disperse *Pseudomonas aeruginosa* Biofilms. *Angew. Chem. Int. Ed.* **2012,** *51,* 5226-5229.