Supplementary Information

For

Prolonged Control of Patterned Biofilm Formation by Bio-inert Surface Chemistry

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MATERIAL AND METHODS

Bacterial strains and growth media. Three different strains were used to form biofilms in this study including *Escherichia coli* K12 strain RP437 (t*hr*(Am)-1 *leu*B6 *his*-4 *metF*(Am)159 *eda*-50 *rpsL*136 *thi*-1 *ara*-14 *lacY*1 *mtl*-1 *xyl*-5 *tonA*31 *tsx*-78),[1, 2] *Pseudomonas aeruginosa* PA01, and *Candida albicans* SC5314 (a clinical isolate [3]). To visualize the cells, *E. coli* RP437 was engineered to express the DsRed-Express fluorescent protein by transformation of the plasmid pRSH103, which was derived from pDsRed-Express (BD, Franklin Lakes, NJ) [4] by replacing the ampicillin resistant marker with the tetracycline (Tet) resistant marker tet^R. Tet was added at a concentration of 10 μg/mL in all the cultures to maintain the plasmid pRSH103. *E. coli* and *P. aeruginosa* were grown in Luria-Bertani (LB) medium [5] containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L sodium chloride. *C. albicans* were grown in YPD medium [5] containing 10 g/L Bacto-yeast extract, 20 g/L Bacto-peptone, and 20 g/L dextrose. The long-term biofilm experiments were conducted in M63 minimal medium [6] containing 13.6 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 0.5 mg/L $FeSO_4{\cdot}7H_2O$, 0.25 g/L $MgSO_4{\cdot}7H_2O$, 0.5% vitamin-free casamino acids, and 0.2% glucose. The pH was adjusted to 7.0 using solid sodium hydroxide.

Synthesis of D-mannitol-terminated alkanethiols. D-mannitol-terminated alkanethiol was synthesized in 5 steps. Briefly, D-mannose was treated with acetone and iodine to afford 2,3:5,6-Di-O-isopropylidenemannopyranose. Reduction of the hemiacetal by sodium borohydride resulted in a primary alcohol that was further coupled with 11-undecenyl bromide to afford an alkene conjugated with protected mannitol. Photoaddition of thiolacetic acid to the alkene afforded a terminus thiol ester. Hydrolysis of the thiol ester and deprotection of the O-isopropylidene groups in one step afforded the desired mannitol-terminated alkanethiol.

Preparation of self-assembled monolayers (SAMs) of alkanethiols. The method to prepare SAMs of alkanethiols on gold surfaces has been described in detail previously.[7] The microscope glass slides coated with 46 Å titanium followed by 243 Å gold were prepared and cut into 2.5 × 0.6 cm slices by a glass cutter (Fisher Scientific Company LLC, Suwanee, GA, USA). The coated slides were soaked in 2 mM alkanethiol solution in ethanol overnight at room temperature to allow the SAMs to form. The slices were then taken out and washed with absolute (200 proof) ethanol to sterilize before use.

To form the patterned SAMs, the gold slides were cut into 7.6 x 1.25 cm pieces, washed with absolute ethanol and dried with a stream of nitrogen. The microcontact printing was conducted with a slight modification from a previously reported procedure.[8] A solution of 1 mM pentadecanethiol in 200 proof ethanol was used for microcontact printing with polydimethylsiloxane (PDMS) stamps. The PDMS stamps dabbed with pentadecanethiol were placed in contact with the gold slides for 15 seconds. The pentadecanethiol-printed slides were then washed with ethanol, dried with nitrogen and soaked in 1 mM solutions of D-mannitol- or TEG-terminated alkanethiols in ethanol for 10 hours. The slides were then taken out of the solution, washed with absolute ethanol, and dried with nitrogen.

Biofilm formation in batch cultures. *E. coli* RP437/pRSH103, *P. aeruginosa* PA01, and *C. albicans* SC5314 were used to form biofilms on bare gold surfaces and those modified with D-mannitol-terminated SAMs. To grow biofilms, an overnight culture was grown for each strain, with shaking at 200 rpm, using LB medium supplemented with 10 μg/mL Tet at 37^{o}C for *E. coli* RP437/pRSH103, LB medium at 37^{o}C for *P. aeruginosa* PAO1, and YPD medium at 30^{o}C for *C. albicans* SC5314. The overnight cultures were used to inoculate biofilm cultures in the same

medium to an optical density at 600 nm (OD_{600}) of 0.05 as measured with a Genesis 5 spectrophotometer (Spectronic Instruments, Rochester, NY, USA).

Bare gold surfaces (as control surfaces) and the gold surfaces modified with D-mannitol-terminated SAMs were sterilized by rinsing with 100% ethanol three times and then air dried. All biofilms were cultured in plastic Petri dishes (O.D. 100 mm ×15 mm) at 37°C without shaking. We chose 37°C rather than 30°C for *C. albicans* biofilm formation because high temperature can stimulate its filamentous growth, and consequently increase biofilm formation.[9] To minimize the variation in sample preparation, bare gold surfaces and SAM-modified surfaces were incubated in the same Petri dish containing 20 mL medium for each strain. Duplicate samples were included in each experiment, and the experiments were repeated once. Consistent results were obtained from the total four replicates for each condition.

Biofilm staining methods. *E. coli* RP437/pRSH103 expresses red fluorescence constitutively; thus, its biofilms were visualized directly without staining. *P. aeruginosa* PAO1 biofilm cells were labeled with LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen Co., Carlsbad, CA, USA).[10, 11] The biofilms were stained in 0.85% (w/v) NaCl buffer containing 0.15% (v/v) component A and 0.15% (v/v) component B for 15 mins in dark. *C. albicans* biofilm cells were stained with 200 μg/mL acridine orange (Sigma-Aldrich Co., St. Louis, MO, USA) [12, 13] in 0.1 mol/L phosphate buffer (12.49 g/L K_2HPO_4, 3.85 g/L KH_2PO_4, pH 7.2) for 5 mins in dark. All of the biofilm samples were visualized with a confocal laser scanning microscope (LSM5 PASCAL, Carl Zeiss, Inc., Berlin, Germany).

Confocal imaging of biofilms. Cell adhesion and biofilm development in the batch cultures were followed at 2, 6 and 24 hours after inoculation. To remove the suspension cells and analyze the bacterial and fungal biofilms using confocal laser scanning microscopy (CLSM), each

surface with biofilm was washed gently by dipping it vertically in 0.85% NaCl buffer three times (changed to fresh buffer after each dipping step). The *E. coli* biofilms that express DsRed-Express constitutively was visualized by excitation with a HeNe laser at 543 nm. Emission of fluorescence was detected with a LP 560 nm emission filter. The surfaces were gently put upside down on a microscope cover glass (24×60 mm, No. 2, VWR International LLC, West Chester, PA, USA) and analyzed with CLSM. A series of images (around 20) were obtained for each position with 1 μm intervals in Z-section for a three-dimensional view of the biofilm (from the substratum to the top of biofilm). The surface coverage (%), mean thickness (μm), and biomass ($\mu m^3/\mu m^2$) were calculated using the COMSTAT software written on the Matlab platform.[14] Surfaces with *P. aeruginosa* PAO1 or *C. albicans* SC5314 biofilms were stained with Live/Dead staining and acridine orange respectively by following manufactures' protocols. The green fluorescence was generated by excitation with an argon laser at 488 nm and detected using a BP505-530 nm filter. The red fluorescence was generated by excitation with a HeNe laser at 543 nm and detected using a LP 560 nm filter. At least five spots were examined and totally 100 images were analyzed for each biofilm sample.

Microplate-based biofilm assay. The 96-well-plate-based biofilm assay was used to study the effects of D-mannitol as free molecules on biofilm formation of *E. coli* RP437/pRSH103. This assay is based on previously reported protocols [15, 16] with slight modifications. Briefly, the overnight culture of *E. coli* was used to inoculate LB medium supplemented with 10 μg/mL Tet to OD_{600} of 0.05 in a 96 well plate. D-mannitol was added as free molecules to final concentrations of 0, 0.001%, 0.01%, 0.1%, and 1% (wt/vol). Four replicates were tested for each concentration. The 96 well plate was incubated at 37°C without shaking. OD_{600} was measured after 24-hour incubation to record the total growth. The plates with biofilms were then washed

three times with DI H_2O and dried by gently patting on a piece of paper towel. To stain the biofilms, 300 μL of 0.1% crystal violet was added in each well. After incubation at room temperature for 20 minutes, the plates were washed three times with DI H_2O to remove the extra dye. Then, 300 μL of 95% ethanol was added into each well and OD_{540} was measured to quantify the biofilm using a microplate reader (EL808; BioTek instruments, Inc, Winooski, VT, USA).

Biofilm formation in flow cell. To study long-term biofilm formation on patterned SAMs, *E. coli* RP437/pRSH103 biofilms were cultured in a flow cell (Model FC81, BioSurface Technologies Corp., Bozeman, MT, USA) using M63 minimal medium supplemented with 10 μg/mL Tet. The flow cell consisted of a No.2 microscope cover glass (24 mm × 60 mm, VWR International, LLC, West Chester, PA, USA) and a regular microscope slide with gold film and SAMs (25 mm × 75 mm × 1mm, Fisher Scientific, Pittsburgh, PA, USA). The microscope slide serving as the bottom of the flow cell was modified with microcontact-printed methyl-terminated SAMs surrounded by D-mannitol- or tri(ethylene glycol) (TEG)-terminated SAMs. To inoculate the flow cell, the *E. coli* RP437/pRSH103 cells from an overnight culture were washed twice with 0.85% NaCl buffer and then resuspended to an OD_{600} of 0.05 in M63 minimal medium supplemented with 10 μg/mL Tet. The flow cell was inoculated by pumping the diluted *E. coli* cells through at 10 mL/h using a Cole-Parmer Masterflex peristaltic pump (Cole-Parmer Instrument Company, Vernon Hills, IL, USA). After inoculation, the flow of M63 minimal medium supplemented with 10 μg/mL Tet was arrested for 1 hour to allow the cells to attach, and then resumed with the same flow rate. The development of biofilm was followed using an Axio Imager M1 microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

Motility assay. To determine the best agar concentration for the motility assay, the overnight culture of *E. coli* RP437/pRSH103 grown in LB medium supplemented with 10 μg/mL Tet at 37

^{o}C was used to inoculate agar plates of the same medium with agar concentrations of 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, and 1.2% using toothpicks. The inoculated plates were incubated at 37^{o}C. The agar concentration that supports the highest motility was chosen to study the effects of D-mannitol, which was added into motility plates to different concentrations (0, 0.01, 0.1, 1, and 3%). The diameter of each swarming colony was measured over time and the swarming rates (averaged diameter of colonies / incubation time) were calculated.

RESULTS

Microbial biofilm formation on bare gold surfaces and D-mannitol-terminated SAMs. The confocal images of surfaces at different time points after inoculation are shown in Fig. S1. The biofilm parameters were calculated using the COMSTAT software and shown in Fig. S2. Consistent results were obtained in surface coverage, mean thickness and biomass.

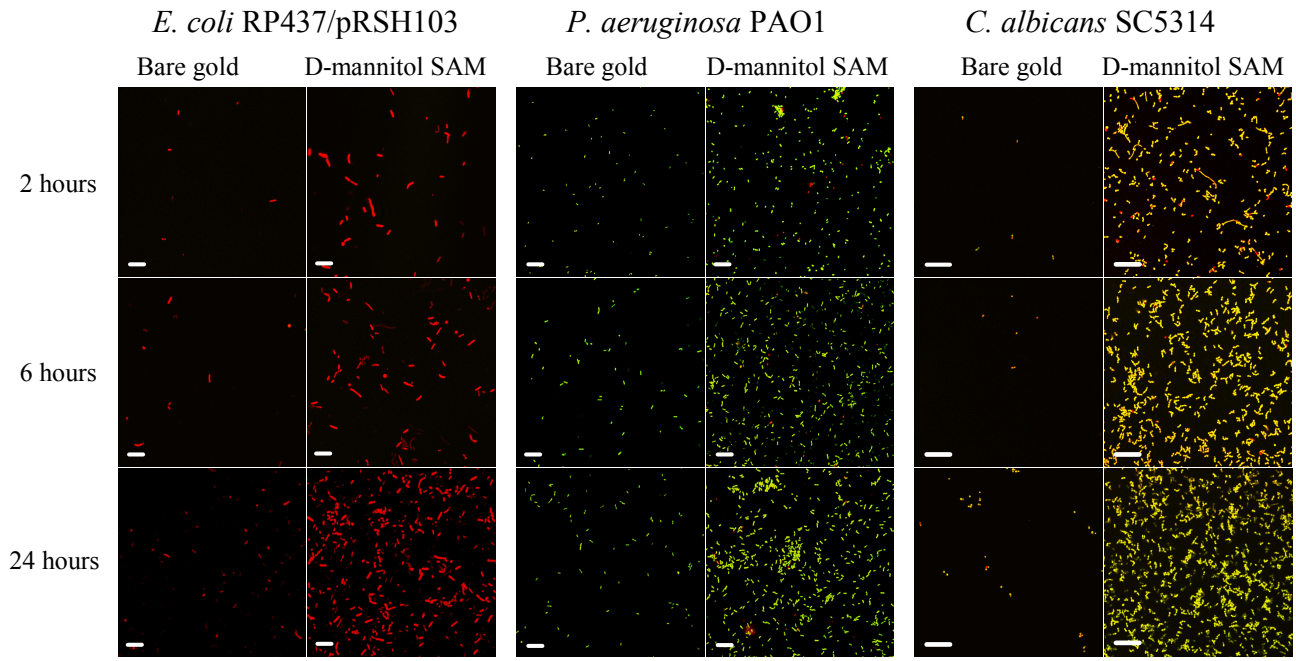
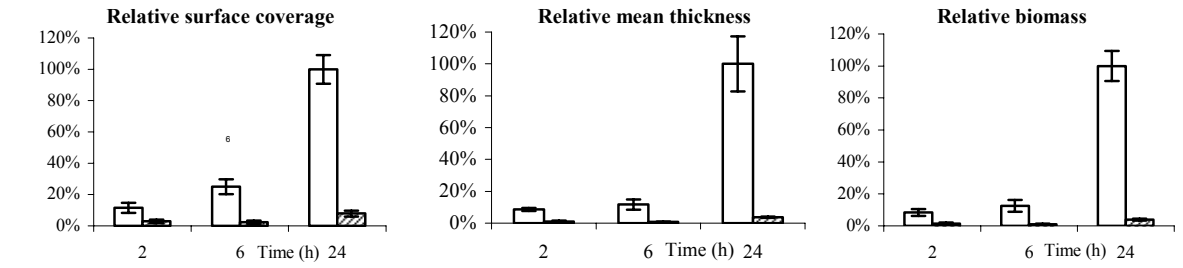
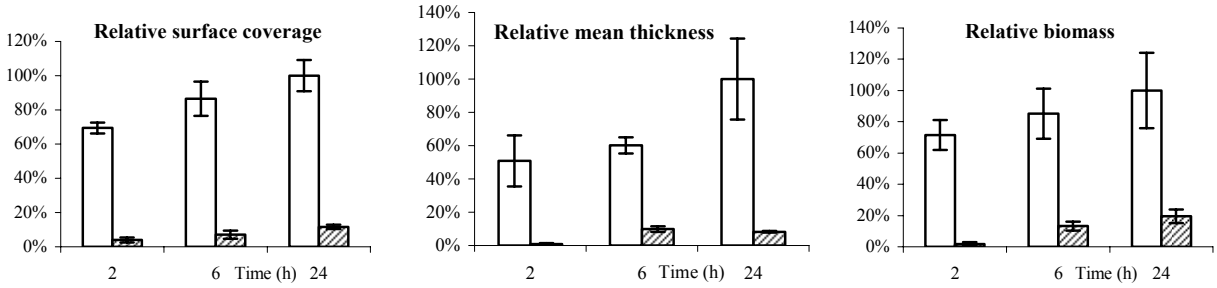


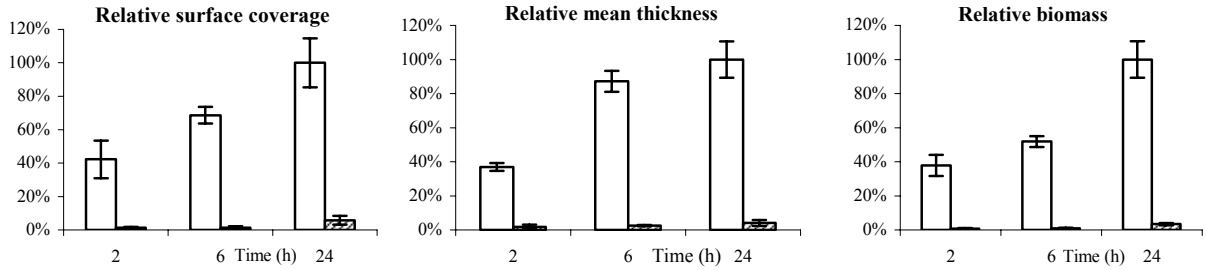
Figure S1. Representative confocal images of adherent and biofilm cells of *E. coli* RP437/pRSH103, *P. aeruginosa* PAO1 and *C. albicans* SC5314 labeled with DsRed-Express, Live/Dead BacLight Bacterial Viability Kit and acridine orange, respectively, on bare gold surfaces and D-mannitol terminated SAMs at different time points after inoculation. For *E. coli* and *P. aeruginosa* images: scale bar = 10 μm. For *C. albicans* images: scale bar =50 μm.



(A)



(B)



(C)

Figure S2. Biofilm parameters analyzed by using COMSTAT software: relative percentage (%) of surface coverage, mean thickness and biomass at 2, 6 and 24 hours after inoculation (□: bare gold surfaces; ▨: D-mannitol-terminated SAMs). The coefficients on bare gold surfaces at 24 hours after inoculation were normalized as 100% for comparison. (A) *E. coli* RP437/pRSH103; (B) *P. aeruginosa* PAO1; (C) *C. albicans* SC5314.

Effects of D-mannitol on the motility of *E. coli*. Motility has been found important to biofilm formation.[16] To determine if the reduction of *E. coli* RP437/pRSH103 biofilm formation on D-mannitol-terminated SAMs was due to inhibition of motility, the motility of *E. coli* RP437/pRSH103 was tested on 0.4% agar plates in the presence of different concentrations of D-mannitol (0, 0.01, 0.1, 1, and 3%) in triplicates. The agar concentration of 0.4% was chosen as it gave the highest motility among the agar concentrations tested (0.2, 0.3, 0.4, 0.6, 0.8, 1.0, and 1.2%). D-mannitol did not show any inhibition of motility. For example, the mean swarming rates were 3.20 ± 0.09 mm/h without D-mannitol, 3.47 ± 0.05 mm/h with 1% D-mannitol, and 3.50 ± 0.05 mm/h with 3% D-mannitol (Fig. S3). These results suggest that the inertness of D-mannitol-terminated SAMs to biofilm formation was not caused by inhibition of motility.

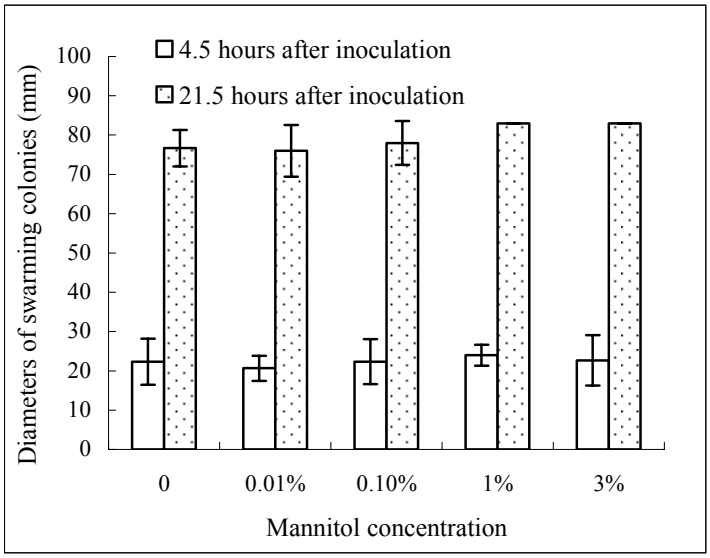


Figure S3. The development of *E. coli* RP437/pRSH103 swarming colonies on LB agar plates containing 0, 0.01, 0.1, 1.0, or 3.0% (wt/vol) D-mannitol.

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