Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary information for:

Polyacrylamide hydrogels as substrates for studying bacteria

Hannah H. Tuson[a], Lars D. Renner[a], and Douglas B. Weibel[a,b]*

a. Department of Biochemistry, University of Wisconsin – Madison

433 Babcock Dr., Madison, WI 53706 U.S.A.

b. Department of Biomedical Engineering, University of Wisconsin – Madison

1550 Engineering Dr., Madison, WI 53706 U.S.A.

* Author to whom correspondence should be addressed

Experimental Procedures:

Synthesis of polyacrylamide hydrogels. Acrylamide (**1**) was dissolved in ddH_2O to a concentration of 5.0 M and filtered through a 0.2-μm filter. N,N′-methylenebisacrylamide (**2**) was dissolved in ddH_2O to a concentration of 0.2 M and filtered through a 0.2-μm filter. The appropriate volumes of solutions of **1** and **2** were combined with water to give the desired final concentrations of monomer (varied) and crosslinker (typically, 0.02 M). TEMED was added to obtain a final concentration of 0.1% v/v. Argon was bubbled through the solution for 1 min. The solution was placed in a glove bag under nitrogen. In the glove bag, 10% w/v APS was added to the solution to obtain a final concentration of 0.1% w/v. The solution was mixed and poured into Petri dishes to yield polymers with a height of ~3.4 mm (e.g., 10 mL of the polymerization mixture was poured into 60-mm diameter Petri dishes). The Petri dishes were placed on a hot plate at 45 °C and the gels were polymerized in a glove bag for 60 min. The hydrogels were removed from the glove bag and immersed in 4 L of ddH_2O to extract unpolymerized monomers and oligomers. After 24 h, the wash water was discarded and replaced. After an additional 24 h, the hydrogels were removed from the wash water and overlaid with a volume of ddH_2O that was approximately half of the volume of the gel and incubated for 18 h. This liquid phase was removed and saved for HPLC analysis to monitor the extraction of unpolymerized monomers. The hydrogels were overlaid with a volume of Luria-Bertani (LB) broth (1% w/v Bacto tryptone, 0.5% w/v yeast extract, 1% w/v NaCl) that was approximately half of the volume of the gel

and incubated for 1 h at 25 °C; the process was repeated four times. The fifth overlay step was performed overnight at 25 °C. The liquid was decanted, excess liquid on the gels was removed by incubating the gels for 2 h with the lid removed, and the substrates were sterilized for 15 min by exposure to UV light in a laminar flow hood.

Synthesis of polyacrylamide substrates incorporating monomer or crosslinker analogs. Acrylamide analogs **3**-**7** (Fig. 1) were dissolved in ddH_2O to a concentration of 5.0 M. N,N′-dihydroxyethylene-bis-acrylamide (**6**; Promega, Madison, WI) was dissolved in ddH_2O to a concentration of 0.2 M. N,N′-Bis(acryloyl)cystamine (**7**; Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO to a concentration of 0.7 M and filtered through a 0.2-μm filter. PA substrates incorporating these analogs were prepared as described above.

Preparation of agar substrates. Agar was dissolved in ddH_2O to the desired concentration and autoclaved. Agar solutions were pipetted into either a Petri dish or a well of a 24-well plate and solidified for 30 min at 25 °C. The hydrogels were dried and sterilized by exposure to UV light for 15 min in a laminar flow hood, then overlaid with a volume of LB that was approximately half of the volume of the gel and incubated for 1 h at 25 °C; the process was repeated four times. The fifth overlay step was performed overnight at 25 °C. The liquid was decanted, excess liquid on the gels was removed by incubating the gels for 2 h with the lid removed, and the substrates were sterilized for 15 min by exposure to UV light in a laminar flow hood.

Cell growth. The following strains were used for these studies: *Escherichia coli* BW25113, *Proteus mirabilis* HI4320, *Pseudomonas aeruginosa* PAO1, *Salmonella enterica* serovar Typhimurium, *Serratia marcescens* ATCC 274, *Bacillus subtilis* 168, and *Staphylococcus epidermidis* 3004. Cells of each strain were grown in LB broth at 37 °C with shaking at 200 rpm for 18 h. The culture was diluted 1:100 in fresh LB and grown at 37 °C with shaking at 200 rpm to an absorbance of 0.60 (λ=600 nm).

Inoculation of PA hydrogels. PA hydrogels and cell cultures were grown to an absorbance of 0.60 (λ=600 nm) as described above. A volume of culture that was ~20% of the hydrogel volume was pipetted onto the PA surface and incubated for 60 sec. Unabsorbed liquid was removed using a pipette, and excess liquid was removed by incubating the hydrogel for 20 min with the lid removed in a laminar flow hood; this step forced cells to grow in contact with the surface, rather than in a thin film of liquid suspended on the polymer surfaces. Hydrogels were incubated at 37 °C for a desired period of amount of time. When viewed by microscopy, cells were short (undifferentiated) and non-motile, indicating that they were in direct contact with the polymer surface. No cells were observed growing within the hydrogel, indicating that they were confined to the gel surface.

Harvesting of cells from PA surfaces. We reduced the disulfide bonds of crosslinker **7** that was incorporated into PA gels by adding 1 mL of a solution of TCEP (10 mM) and

Tris base (30 mM) in 1X PBS to the hydrogel surface. After incubating the gels for 20 min at 25 °C, we collected the solution in a Falcon tube by decanting the liquid. We washed the hydrogel surface with 5 x 1 mL of 1X PBS, and collected the liquid in the Falcon tube containing the TCEP solution. The collected cells were either used directly in experiments or fixed or frozen for later analysis. A comparison of the collection of *E. coli* BW25113 cells from PA hydrogels with dissolvable crosslinker **7**, non-dissolvable crosslinker **2**, or agar is shown in Fig. S3. We found that the retrieval of cells using TCEP was quantitative.

Viability testing of cells removed from hydrogels surfaces. To ensure that cells were viable following TCEP treatment, we prepared PA hydrogels, inoculated them with cells, incubated, and harvested the cells using TCEP as described above. We centrifuged the suspension of cells for 5 min at 2000 rpm. Cell pellets were resuspended in 1 mL 1X PBS and diluted $1:10^7$ and $1:10^8$ in 1X PBS. An aliquot (50 µL) of each dilution was plated on a 1.5% LB/agar plate. Plates were incubated overnight at 37 °C, and colonies were counted to determine colony forming units (CFU)/mL. In parallel, the number of cells/mL was determined using a haemacytometer (Hausser Scientific, Horsham, PA), and CFU/mL was divided by cells/mL to determine the percentage of cells that were viable following removal from the PA hydrogel surface. All of the cells collected from the plates were viable.

Plate reader growth curves. PA or agar hydrogels (620 μL) were prepared in the wells of a 24-well Costar culture dish (Corning Incorporated, Corning, NY) as described above. Wells 1 and 2 in each row were uninoculated and used as controls; wells 3-6 in each row were inoculated as described above. Immediately following inoculation, the plate was inserted into a Tecan Infinite M500 plate reader (Tecan Group, Ltd., Männedorf, Switzerland) at 37 °C, and the absorbance at λ=595 nm was read at 12 different positions in each well every 5 min for 20 h. Prior to data analysis, the average absorbance from the two uninoculated wells was subtracted from the absorbance for each inoculated well.

Measurement of Young's Modulus of hydrogels. Polyacrylamide (0.5 – 2.0 M **1**, 0.02 M **2**) and agar (1.0 – 2.5% w/v) gels were prepared immediately prior to tensile testing and stored in ddH_2O to keep them hydrated. The Type IV sample described in ASTM D 638-03 was scaled up to yield a sample thickness of ~3.2 mm, which better approximated the hydrogels used for our experiments. We used an Instron Model 5548 MicroTester (Instron, Norwood, MA, USA) equipped with a 10-N load cell to measure the tensile properties of the gels. For each concentration, we measured the mechanical properties of 3 duplicate hydrogels by elongating them at a constant rate of 5 mm/min until fracture. The Young's Modulus of the gel was determined by calculating the slope of the linear response region of the stress versus strain curve.

Determination of doubling time. To determine the doubling time for each sample, we used MATLAB (Mathworks, Naticks, MA, USA) to fit the growth curve data obtained from the plate reader to the logistic equation:

$$m = \frac{K}{1 + ce^{-rt}}$$

where t is time, m is the biomass, K is the carrying capacity or saturation point, r is the growth rate, and c is an arbitrary positive constant. Because the growth rate during the exponential phase of growth can be described by the equation:

$$2m = me^{r(t_1 - t_0)}$$

from which the doubling time $T = t_1 - t_0$ can be calculated by dividing ln(2) by r. See Table S1 for the doubling times of various strains on PA and agar substrates. Statistical significance was determined using a Student's t-test.

Measurement of diffusion through hydrogels. To compare the diffusion of nutrients through PA and agar hydrogels, we prepared 10-mL solutions of PA (0.5, 1.0, 1.5, or 2.0 M **1**, all 0.02 M **2**) in 15-mL Falcon tubes and polymerized as described above. Similarly, we pipetted 10 mL of a solution of agar (1.0, 1.5, 2.0, or 2.5% w/v in water) into 15-mL Falcon tubes and solidified it for 30 min at 25 °C. We pipetted 200 µL of a solution of fluorescein (100 mM) in water onto the top of each hydrogel and photographed the gels every 10 min for 2 h. We measured the distance the dye had traveled at each time point as an indication of the diffusion rate of small molecules through the hydrogel (Figure S2).

Motility measurements of *Proteus mirabilis* communities spreading across surfaces.

In order to examine the effect of PA surface chemistry on community spreading on surfaces, we measured the diameter of *P. mirabilis* communities inoculated on hydrogels consisting of **1** or **4** (1 M and 2 M) and crosslinked with **2** (0.02 M). An overnight culture of *P. mirabilis* was diluted 1:100 in fresh LB and grown at 37 °C with shaking at 200 rpm to an absorbance of 0.6 (λ=600 nm). *P. mirabilis* culture (3 μL) was pipetted onto the centre of the PA surface. The inoculated plates were dried for 15 min in a laminar flow hood and incubated at 37 °C for 72 h. An image of each plate was taken at 24, 48 and 72 h (Fig. S4). We performed the experiment in triplicate and measured the diameter of each bacterial community (Fig. S5).

Fig. S1. Growth of *E. coli* BW25113 on agar hydrogels of different stiffness. The rate of cell growth on agar is independent of surface stiffness. Hydrogels containing various concentrations of agar were inoculated with *E. coli* BW25113 and incubated at 37 °C for 20 h. The absorbance at 595 nm was measured in 5 min intervals. Data is shown as the mean (n=4). Red line indicates 1.0% (w/v) agar; blue line indicates 1.5% (w/v) agar; green line indicates 2.0% (w/v) agar; black line indicates 2.5% (w/v) agar.

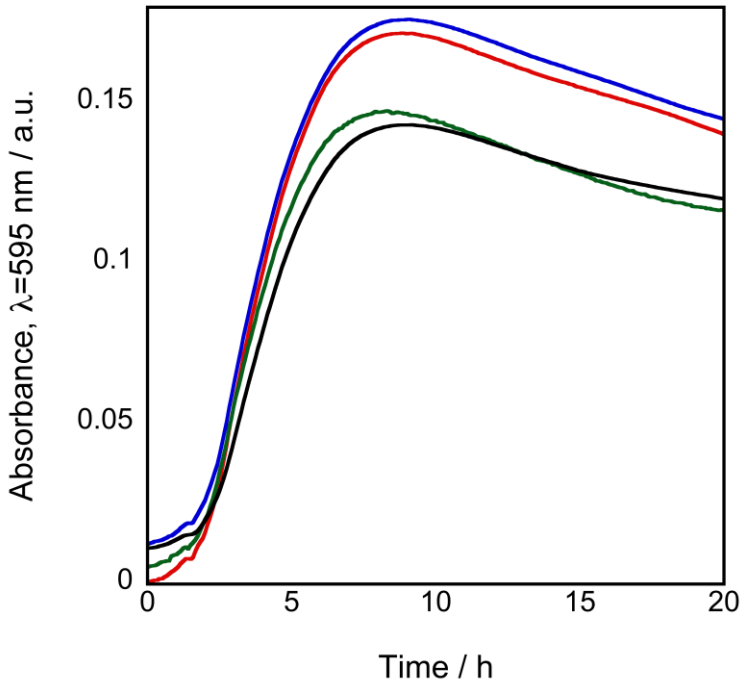

Fig. S1

Fig. S2. Rate of diffusion through hydrogels. Hydrogels of agar or PA were cast in 15-mL Falcon tubes, overlaid with a solution of fluorescein, and photographed every 10 min for 2 h. The distance that the dye front had migrated was measured at each time point and plotted as a function of time. Data is shown as the mean value determined from three independent experiments.

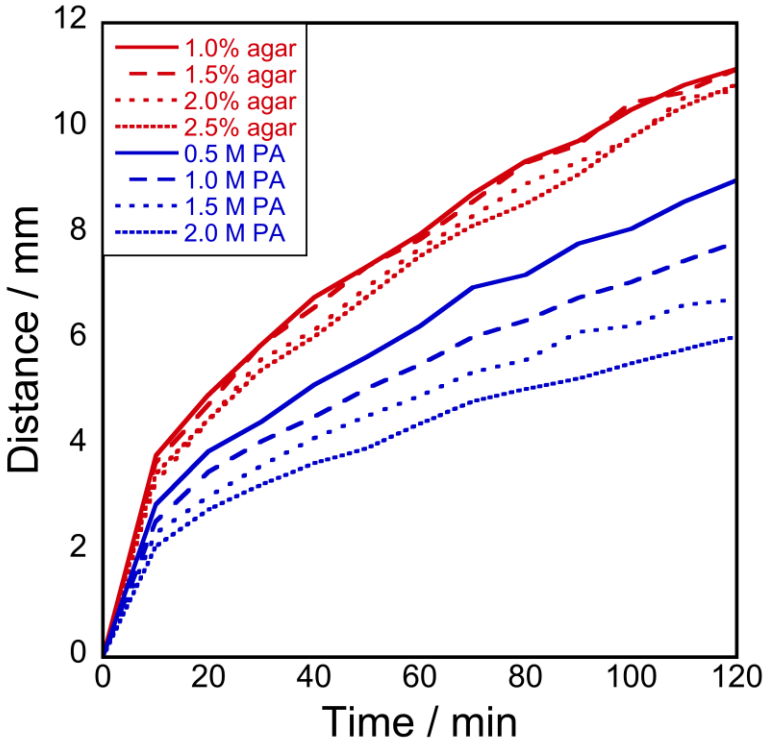

Fig. S2

Fig. S3. Retrieving *E. coli* BW25113 cells after growth on hydrogel surfaces. *E.* coli BW25113 was grown on hydrogel surfaces and imaged after 3 h of growth. Cells were then removed from hydrogels surfaces using TCEP (as described above) or 1X PBS, and the plates were imaged again. Cells were removed most efficiently using a TCEP wash on plates with a dissolvable crosslinker (1 M **1**, 0.02 M **7**). As a control, cells were also removed from 1% agar substrates with or without TCEP. Washing with and without TCEP removed almost all cells from agar substrates.

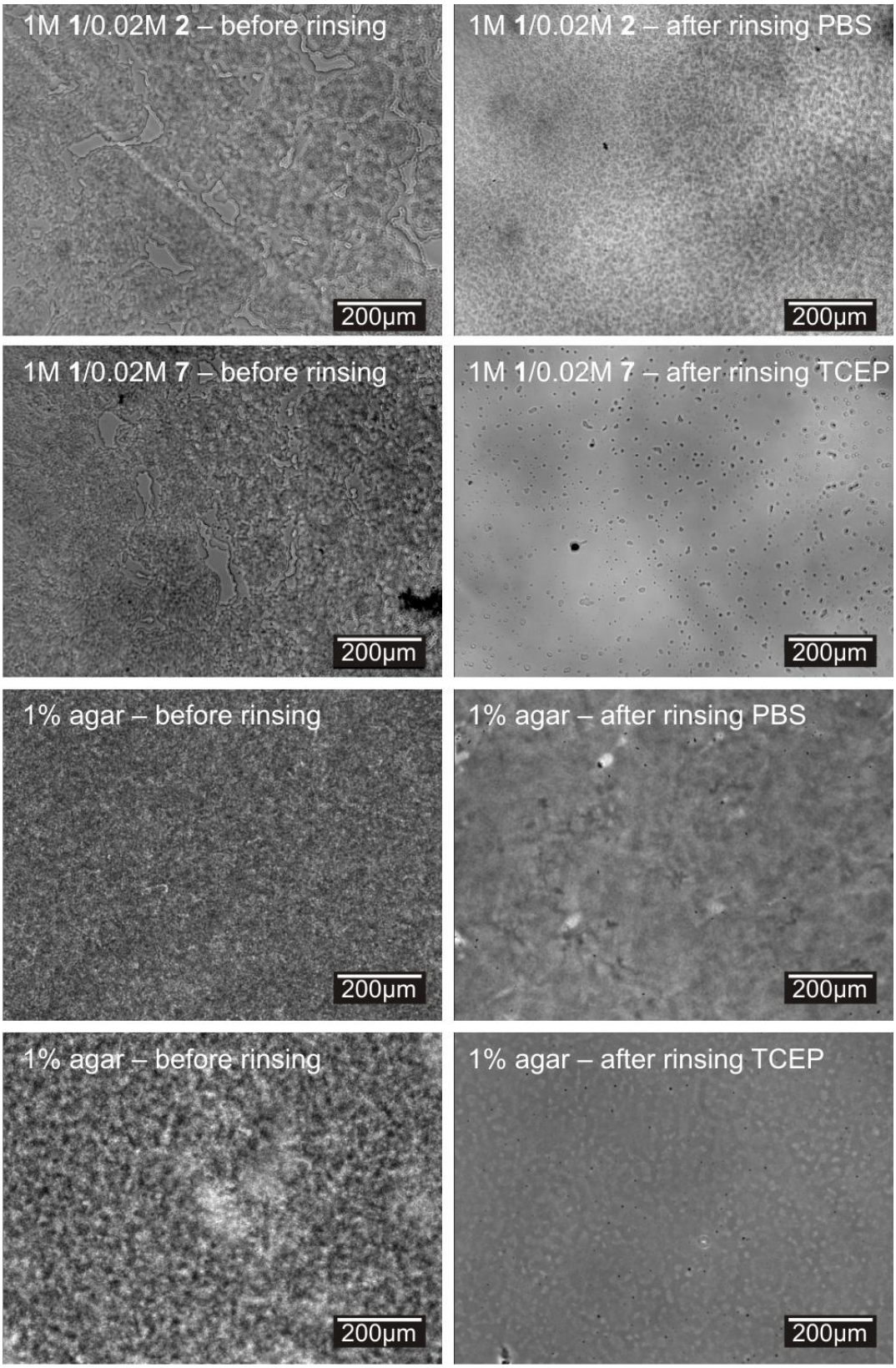

Fig. S3

Fig. S4. Motility of *Proteus mirabilis* on PA hydrogels. PA hydrogels were inoculated with *P. mirabilis*, incubated at 37 °C, and imaged every 24 h for 3 days. While cells of *P. mirabilis* migrate out from the central inoculation spot on gels made with **1** and **2**, they do not on gels made with **4** and **2**.

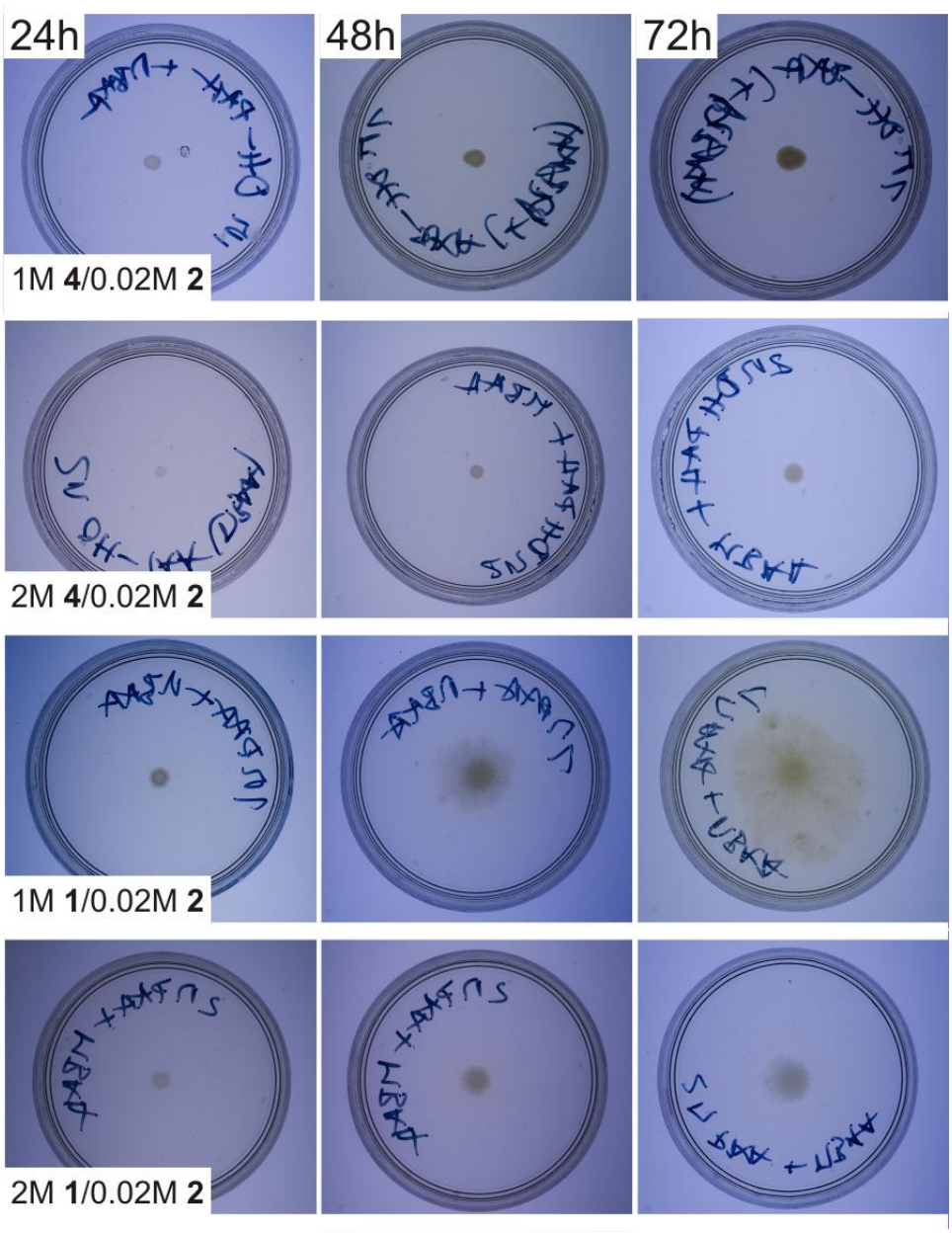

Fig. S4

Fig. S5. Diameter of spreading colonies of *Proteus mirabilis* on hydrogels with different surface chemistries. The diameter of the *P. mirabilis* colony was measured on the plates described in Fig. S4. Data is presented as the mean (n = 3) ± S.D.

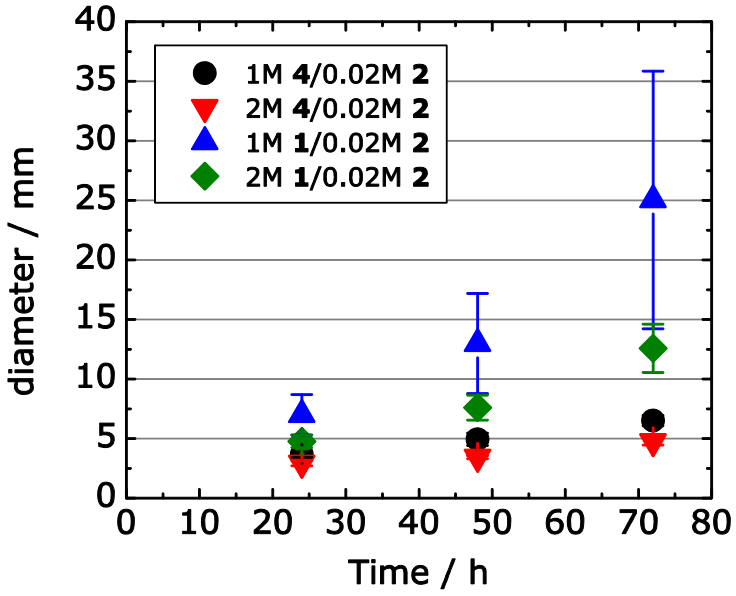
diameter / mm
Time / h
1M 4/0.02M 2
2M 4/0.02M 2
1M 1/0.02M 2
2M 1/0.02M 2
0
5
10
15
20
25
30
35
40
0
10
20
30
40
50
60
70
80

Fig. S5

Table S1. Summary of bacterial strains used to examine growth on PA hydrogels made up of different combinations of monomers and crosslinkers.

Strain	Agar %	Monomer Identity / Molarity	Crosslinker Identity / Molarity	Doubling Time (min)
BW25113	-	**1** / 0.5	**2** / 0.02	50 ± 2.9
	-	**1** / 1.0	**2** / 0.02	42 ± 4.5
	-		**6** / 0.02	47 ± 4.4
	-		**7** / 0.02	43 ± 3.2
	-	**1** / 1.5	**2** / 0.02	51 ± 3.3
	-	**1** / 2.0	**2** / 0.02	52 ± 3.8
	-	**3** / 1.0	**2** / 0.02	48 ± 6.7
	-	**4** / 1.0	**2** / 0.02	52 ± 5.2
	-	**5** / 1.0	**2** / 0.02	53 ± 4.9
	1.0	-	-	33 ± 0.5
	1.5	-	-	38 ± 1.4
	2.0	-	-	31 ± 6.9
	2.5	-	-	38 ± 4.9
P. mirabilis HI4320	-	**1** / 1.0	**2** / 0.02	56 ± 5.5
P. aeruginosa PAO1	-	**1** / 1.0	**2** / 0.02	88 ± 1.2
S. enterica serovar Typhimurium	-	**1** / 1.0	**2** / 0.02	38 ± 1.5
S. marcescens ATCC	-	**1** / 1.0	**2** / 0.02	49 ± 8.1
S. epidermidis 3004	-	**1** / 1.0	**2** / 0.02	72 ± 3.1
B. subtilis 168	-	**1** / 1.0	**2** / 0.02	40 ± 1.1