Electronic Supplementary Material (ESI) for Analytical Methods.

Supporting Information

Whole-Cell Biosensing by Siderophore-Based Molecular Recognition and Localized Surface Plasmon Resonance

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Materials and Methods

Materials. PEG thiol (MW 550 Da) was purchased from Creative PEGWorks. Biotin PEG thiol (MW 1000 Da) was purchased from Nanocs. NeutrAvidin Protein and formaldehyde (16% w/v aq soln, methanol-free) were purchased from Thermo Fisher Scientific. HOOK™ Biotin-PEG3-Amine was purchased from GBiosciences. Iron (III) acetylacetonate ($Fe(acac)_3$, 99.9% trace metals basis), 2,2′-bipyridyl, tris(2-carboxyethyl)phosphine hydrochloride powder, isobutyl chloroformate ($ClCO_2i$-Bu), tetrahydrofuran (THF), polystyrene beads (0.6 μm mean particle size), Mueller Hinton Broth, sodium phosphate monobasic monohydrate, sodium phosphate dibasic, ammonium phosphate dibasic, sulfuric acid (95.0 to 98.0%), hydrogen peroxide (30% w/w in H_2O), dimethyl sulfoxide (DMSO), ethyl alcohol (200 proof), and *N*-methylmorpholine were all purchased from Sigma-Aldrich. Glass microscope slides (3 × 1") were purchased from Ted Pella. Micro cover glass (No. 1, 18 mm × 18 mm) was purchased from VWR. Lysogeny broth and granulated agar were purchased from BD Difco. Deionized (DI) water was generated using a Milli-Q Gradient water purification system (Millipore, 18.2 MΩ·cm at 25 °C). Sterile 10 mM sodium phosphate buffer (pH 7.5) was used in all experiments. Tetrahydrofuran (THF) was freshly distilled over sodium before use. All other chemicals were used as received.

Siderophore Synthesis. All reactions were conducted under an atmosphere of dry argon unless otherwise stated. The mixed-ligand siderophore **1** (**Scheme 1**, main text) was prepared following a previously established procedure.[1] Specifically, to a cooled (0 °C, ice-salt bath) solution of the penta-acetylated siderophore **1** (73 mg, 0.078 mmol) in anhydrous THF (1.5 mL) was added *N*-methylmorpholine (10.30 μL, 0.094 mmol, 1.2 equiv.) followed by isobutyl chloroformate (12.2 μL, 0.094 mmol, 1.2 equiv.). A white precipitate formed gradually after

addition of the chloroformate (*N*-methylmorpholine hydrochloride salt). The reaction mixture was stirred at 0 °C and the progress of the isobutyl chloroformate ester formation was monitored by thin-layer chromatography (TLC) analysis. After 1 h, TLC showed complete consumption of starting siderophore **1**. The reaction mixture was filtered through a plug of cotton and rinsed with anhydrous THF (0.5 mL) to obtain a clear solution of the mixed anhydride of **1**.

To conjugate PEG and biotin to the siderophore **1**, the following procedures were carried out. In a separate round-bottom flask, biotin-PEG-amine (25 mg, 0.060 mmol, 0.77 equiv.) was added into a cooled (0 °C, ice-salt bath) solution of sodium bicarbonate (15.7 mg, 0.187 mmol, 2.4 equiv.) in distilled DI water (2 mL). The mixed anhydride **1** was added to the resulting solution in a dropwise fashion over a period of 5 min. The reaction mixture was then stirred for 3 h at 0 °C (ice-salt bath). The coupling reaction progress, as indicated by the consumption of the biotin-PEG-amine, was monitored by reverse phase TLC and LC-MS analysis. The organic layer was separated, washed with brine, and concentrated to obtain the desired PEG-biotinylated siderophore as a mixture of products. The mixture was then purified by reverse phase chromatography (C18) using a gradient of CH_3CN/H_2O (20% to 70%) to obtain the desired biotinylated siderophore **2** (**Scheme 1**) as the major component. The minor components resulted from the loss of one (M-OAc), two (M-2OAc), three (M-3OAc) or four (M-4OAc) acetyl groups, and were also characterized by LC-MS analysis (**SI**).

Preparation of 0.01 mg/mL Biotinylated Siderophore Solution. Biotinylated siderophore (Bt-siderophore) freezer stock (1 mg/mL) was prepared by dissolving 1.4 mg of synthesized Bt-siderophore into a mixture of 5% DMSO and 95% phosphate buffer saline solution to reach a final volume of 1.4 mL. Small aliquots of Bt-siderophore solution (0.01 mg/mL) were prepared by adding a 15 µL of Bt-siderophore (1 mg/mL) into a 74 µL of DMSO and a 1411 µL of 10

mM sodium phosphate solution. The small aliquots were mixed thoroughly and stored at -80 °C. Each aliquot of Bt-siderophore solution (0.01 mg/mL) was slowly thawed at room temperature prior to use.

Preparation of Mueller-Hinton (MH) Iron Deficient Media. Mueller-Hinton (MH) media was prepared by dissolving 10.5 g of MH powder in 0.5 L of DI water. The MH media was autoclaved and cooled to room temperature. 2,2′-bipyridyl solution (1 mg/mL, 1 mL) was added into a 40 mL of cooled MH media. The MH media containing 2,2′-bipyridyl (MH iron deficient media) was stored at room temperature for at least 2 weeks prior to use, allowing 2,2′-bipyridyl to completely chelate environmental Fe (III) in solution and resulting in an iron deficient media.

Bacterial Cell Culture and Counting. Acinetobacter baumannii ATCC 17961 was obtained from Professor M. Miller's laboratory (University of Notre Dame). *Pseudomonas aeruginosa* strain PAO1, *Escherichia coli* DH5α, *Bacillus cereus*, and *Staphylococcus aureus* RN4220 were obtained from Professor J. Shrout's laboratory (University of Notre Dame). All bacteria were streaked from -80 °C freezer stocks and grown on lysogeny broth (LB) agar plates in a 37 °C incubator for 17 h. Liquid cell cultures were grown in MH iron deficient media. Specifically, cell cultures were first grown in a glass tube containing 6 mL of MH iron deficient media for 9 h in a 37 °C incubator with shaking at 240 rpm. Nine-hour bacterial cultures (30 μL) were removed and added to glass tubes containing 6 mL of MH iron deficient media. Diluted bacterial samples were grown at 37 °C for 12 h with shaking at 240 rpm prior to harvest. Cell cultures were washed twice using 10 mM sodium phosphate solution by centrifuging at 1000 x G for 5 min each, and finally resuspended in 10 mM sodium phosphate solution. The optical density of the resuspended cell culture was measured at 600 nm (OD_{600}) and adjusted to 0.80.

Serial dilutions were then performed using 10 mM sodium phosphate buffer to achieve desired bacterial concentrations. Diluted bacterial samples were used immediately.

Standard microbiology plating and counting methods were used to correlate bacterial cell density (cfu mL^{-1}) to optical density (OD_{600}). Specifically, the optical density of all bacterial solution was adjusted to 0.80 after resuspending bacteria in a 10 mM sodium phosphate solution. *A. baumannii*, *P. aeruginosa*, and *E. coli* solutions were diluted 6 orders of magnitude in a 10 mM sodium phosphate solution, while *B. cereus* solution was diluted 5 orders of magnitude due to the difference in cell density according to our preliminary studies. Diluted bacterial solution (100 μL) was added onto an LB agar plate and spread using a metal spreader, which was sterilized by dipping in ethanol and flamed before and after each use. The bacterial containing LB agar plates were incubated at 37 °C overnight, and colonies were counted the next day. Final bacterial cell density was calculated by averaging the colony counts from 9 LB agar plates (3 plates were prepared in one experiment, and the same experiment was repeated 3 times on different days). At OD_{600} of 0.80, there are 0.4 x 10^9, 1.1 x 10^9, 0.5 x 10^9, and 0.1 x 10^9 cfu mL^{-1} of *A. baumannii*, *P. aeruginosa*, *E. coli*, and *B. cereus*, respectively.

LSPR Sensor Chip Fabrication. LSPR sensor chips were fabricated via NSL^2 using a fabrication process published previously.[3] Briefly, glass microscope slides were immersed in piranha solution (***Cation:*** *piranha, a strong oxidizer, contains sulfuric acid/hydrogen peroxide in a 3:1 v/v and should be handled with extreme caution!*) overnight, rinsed, and stored in DI water. Polystyrene (PS) beads were first diluted in ethanol (1:2 v/v) and well mixed. A monolayer of diluted PS beads was spread at an air-water interface and transferred onto a clean microscope slide by emersion through the interface. PS bead-coated glass slides were dried at 60 °C, and then coated with Cr (1 nm) and Au (50 nm) using an electron beam deposition (UNIVEX 450B,

Oerlikon). Metal coated glass slides were sonicated in chloroform for 15 min to remove the PS beads, resulting in a hexagonal nanoscale array of Au trigonal prisms on glass, and then rinsed with DI water, dried with N_2 gas, and stored under N_2. A dicing saw (Disco DAD3240) equipped with a diamond blade (Thermocarbon) was used to cut each Au-patterned glass slide (76.2 mm x 25.4 mm) into six individual sensor chips (10.16 mm x 15.24 mm). All sensor chips were subsequently sonicated in ethanol and DI water for 5 min each, rinsed with DI water, dried with N_2 gas, and stored under N_2 prior to use.

Sensor Surface Modification. Each sensor chip was modified with a mixture of Bt-PEG thiol (1 mM) and PEG thiol (1 mM) in a 1:3 v/v ratio for 16 h with mild shaking. A volume of 0.2 mL of NeutrAvidin (1 mg/mL), Bt-siderophore (0.01 mg/mL), and $Fe(acac)_3$ (1 mM) were subsequently incubated on the sensor surface for 0.5 h in a humidity controlled environment with mild shaking. Sensor chips were rinsed with DI water and dried with N_2 gas after each surface modification step. $Fe(acac)_3$ solution was freshly prepared in 1% of DMSO and 9% of 10 mM ammonium phosphate dibasic solution (pH 8) prior to each experiment.

Bacterial Binding and Fixation. Bacterial binding experiments were carried out by adding 0.2 mL of bacterial solution at the desired concentration onto the siderophore-Fe (III) modified sensor chips. The bacterial solution used for all experiments contained bacteria in 10 mM sodium phosphate buffer. Control chips were exposed to 0.2 mL of 10 mM sodium phosphate solution. These sensor chips were incubated in a 37 °C incubator for 1 h in a humidity controlled environment, allowing bacteria to recognize and bind to surface-confined siderophore-Fe (III) complexes. To remove unbound bacterial cells, the solution on the sensor chips was gently decanted, and the sensor chips were then washed three times in 10 mM sodium phosphate (5 min per wash). To inactivate surface bound bacterial cells, 200 μL of 6% formaldehyde

solution was added to the bacteria-containing sensor chips and incubated at 37 °C for 1 h in a humidity controlled environment. After 1 h cell fixation, the solution on the sensor chips was gently decanted, and the sensor chips were then washed three times in 10 mM sodium phosphate solution (5 min per wash), followed by a DI water wash for 30 s. Sensor chips containing bound inactivated bacteria were gently dried with N_2 gas for 5 min prior to characterization.

Characterization. A UV-visible-NIR spectrometer (Jasco V-670) with a 60 mm integrating sphere (Jasco ISN-723) was used to acquire LSPR spectra. Each spectrum was an average of three spectral accumulations from 600 − 1400 at 0.5 nm interval. All extinction spectra were normalized. The probe beam size was ca. 8 mm x 9 mm. Scanning electron microscope images of a bare sensor chip (*i.e.,* nanoscale Au trigonal prism arrays) and a modified sensor chip with captured *A. baumannii* cells were acquired using a field-emission scanning electron microscope (FEI Magellan 400) at 5.00 kV. To avoid surface charging, a thin layer of iridium (2.0 nm) was sputtered on the substrate prior to imaging.

Liquid chromatography-mass spectrometry (LC-MS) was used to characterize both the freshly synthesized biotinylated siderophore (Bt-siderophore) and the Bt-siderophore in a buffer solution. In general, a UPLC system, autosampler, and photodiode array detector (Dionex Ultimate 3000) were coupled to a quadrupole time-of-flight hybrid mass spectrometer (Bruker MicrOTOF-Q II). Analytes were separated using a Thermo Scientific Acclaim™ RSLC 120 C18 column (2.2 μm particle size, 120 Å pore size, 2.1 mm inner diameter, 100 mm length) with a mobile phase composed of either A = 0.1% formic acid in water or B = 0.1% formic acid in acetonitrile. The samples were eluted at 0.4 mL/min with a mobile phase gradient of (*i*) 90% A/10% B for 2 min, (*ii*) 0% A/100% B for 18 min, (*iii*) 90% A/10% B for 2 min. For the freshly synthesized Bt-siderophore, the eluted samples were ionized in positive ion mode using a Bruker

electrospray ionization source (end plate offset voltage = -500 V, capillary voltage = 2200 V, N_2 gas as a nebulizer at 5 bar and a dry gas at 10.0 L/min flow rate at 220 °C). Mass spectra were acquired over a mass range of 50 − 3000 Da. For the Bt-siderophore in buffer, the eluted samples were ionized in positive ion mode using a Bruker electrospray ionization source (end plate offset voltage = -500 V, capillary voltage = 2400 V, N_2 gas as a nebulizer at 4 bar and a dry gas at 7.0 L/min flow rate at 180 °C). Mass spectra were acquired at 5000 scans/s over a mass range of 300 − 3000 Da. Hystar 3.2 software was used in both data analyses.

Thin layer chromatography of freshly synthesized biotinylated siderophore. Sorbent Technologies silica gel 60 (32 − 63 μm) was used for all silica gel column chromatography purifications. Reverse phase chromatographic purifications were performed on Teledyne Instruments' 30 gram RediSep Rf Gold® C18Aq reversed-phase columns (column volume: 26.4 mL, average particle size: 20 to 40 μm, average pore size: 100 Å) at a flow rate of 35 mL/min. Thin layer chromatography (TLC) was performed with Al-backed Merck 60-F254 or Al-backed Merck RP-C18 F256 silica gel plates using a 254 nm lamp and aqueous $FeCl_3$ for visualization**.**

LC-MS analysis of freshly synthesized biotinylated siderophore

The LC-MS analysis showed that there were a major component and four minor components in the synthesized biotinylated siderophore **2** (**Scheme 1**, main text). The major component, $[m+2H]^{2+}$ calculated for $C_{63}H_{91}N_9O_{21}S$, had a m/z value of 671.8110, which matched to its theoretical value (671.8098). The minor components resulted from the loss of one (M-OAc), two (M-2OAc), three (M-3OAc), and four (M-4OAc) acetyl groups. The experimental and theoretical m/z values of these minor components were: 650.8031 and 650.8045 for $[m+2H]^{2+}$ calculated for $C_{61}H_{89}N_9O_{20}S$ (M-OAc), 629.8050 and 629.7992 for

$[m+2H]^{2+}$ calculated for $C_{59}H_{87}N_9O_{19}S$ (M-2OAc), 608.7950 and 608.7939 for $[m+2H]^{2+}$ calculated for $C_{57}H_{85}N_9O_{18}S$ (M-3OAc), as well as 587.7968 and 587.7887 for $[m+2H]^{2+}$ calculated for $C_{55}H_{83}N_9O_{17}S$ (M-4OAc).

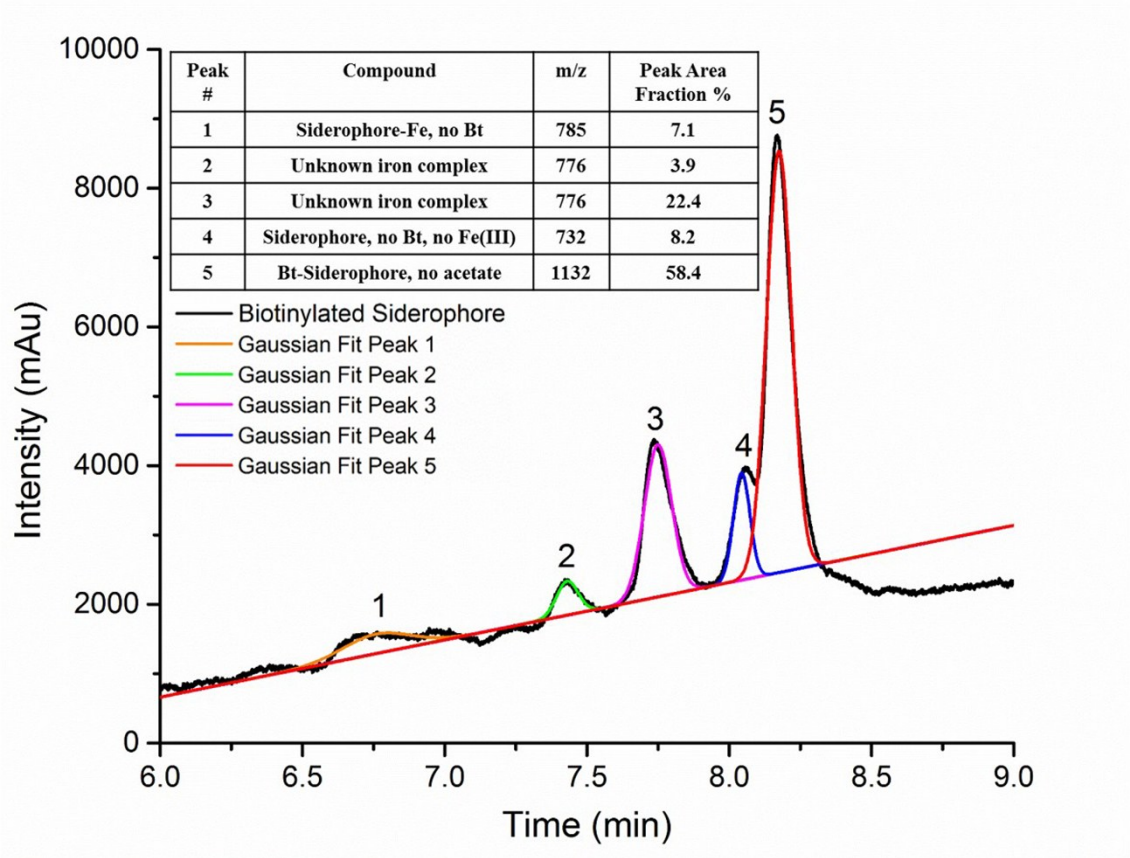

Figure S1. LC-MS characterization of biotinylated siderophore in buffer. UV chromatogram of the LC separated biotinylated siderophore (black trace). A number of components were found and the peaks were fit to Gaussians (peaks 1 to 5, colored traces) to estimate relative abundances of the components, which are tabulated in the *Inset*.

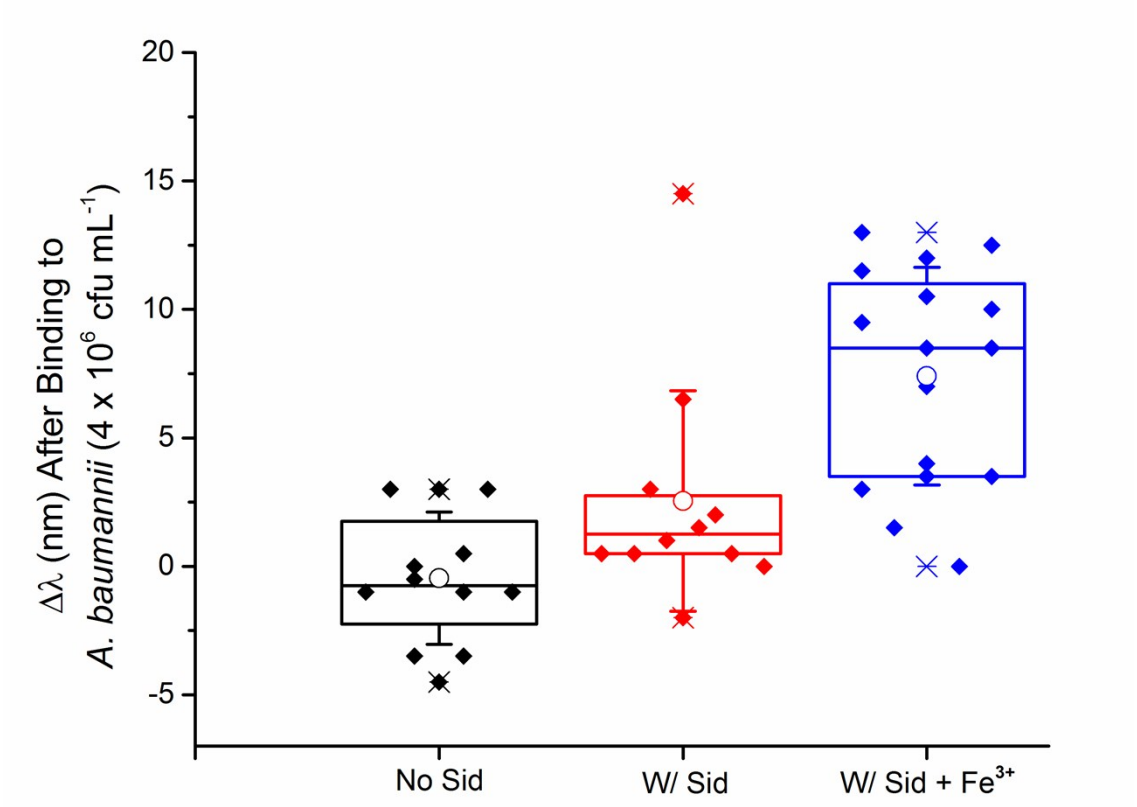

Figure S2. Box and whisker plot showing the LSPR wavelength shift after bacterial binding onto various sensor surfaces. *A. baumannii* (4 x 10^6 cfu mL^{-1}) was used in all experiments. Each color represents a different surface modification with 12 to 16 individual data points (solid diamonds are results of individual experiments). The circle represents the mean of all data points. The whisker extends to one standard deviation in each direction. The lower, central, and upper box lines represent 25th, 50th, and 75th percentiles, respectively. Diagonal crosses (x) represent the 1st and 99th percentiles.

Table S1. Student's *t*-test for siderophore selectivity.

	Control[b]	*P. aeruginosa*	*E. coli*	*B. cereus*
t_{exp} (*t*-values calculated against *A. baumannii*[a])	5.63	4.85	4.19	5.21
$t^{v}_{0.999}$	3.75	3.71	3.71	3.71

[a]*t*-value for all bacteria at a 99.9% confidence level; n = 16 (*A. baumannii*); 10 (control); 12 (*P. aeruginosa*, *E. coli*, and *B. cereus*). [b]Control sample presents the siderophore surface recognition agent, but has not been exposed to bacteria.

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