

Rapid Identification of Biofilms Using a Robust Multichannel Polymer Sensor Array

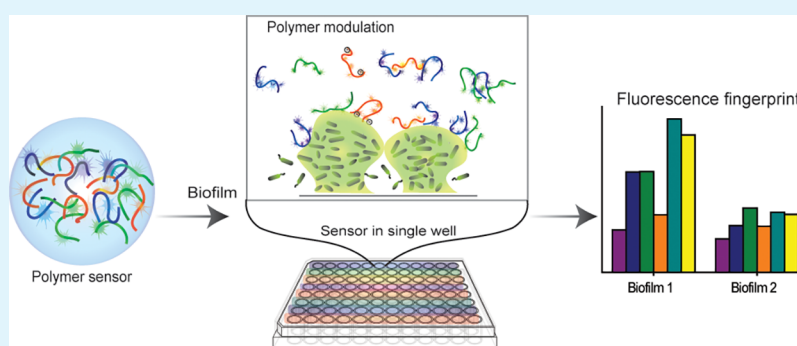
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S Supporting Information



ABSTRACT: Infections caused by bacterial biofilms are challenging to diagnose because of the complexity of both the bacteria and the heterogeneous biofilm matrix. We report here a robust polymer-based sensor array that uses selective interactions between polymer sensor elements and the biofilm matrix to identify bacteria species. In this array, an appropriate choice of fluorophore enabled excimer formation and interpolymer FRET, generating six output channels from three polymers. Selective multivalent interactions of these polymers with the biofilm matrices caused differential changes in fluorescent patterns, providing a species-based signature of the biofilm. The real-world potential of the platform was further validated through identification of mixed-species bacterial biofilms and discrimination of biofilms in a mammalian cell-biofilm co-culture wound model.

KEYWORDS: biofilm infections, biosensor design, fluorescent polymers, bacterial pathogens, multichannel sensor

INTRODUCTION

Infectious microbial biofilms on wounds and synthetic implants can cause persistent inflammation, as well as tissue and foreign body infections.^{1,2} Without effective treatment, these chronic infections can lead to amputations, organ failure, and even death.³ Infectious biofilms are difficult to treat because of the complexity of the biofilm matrix that protects bacteria from antimicrobial treatment, heightening the therapeutic challenge presented by multidrug resistant species.^{4,5} The rapid and accurate identification of biofilm infections at an early stage can provide crucial information for effective treatment, in particular, the choice of therapeutic agents and strategies.^{6,7} The heterogeneity of biofilm matrices, however, makes it challenging to identify the bacterial species present.⁸ Conventional biofilm detection and identification methods such as plating and culturing require long processing time and have low sensitivity.⁹ Polymerase chain reaction,¹⁰ target-specific immunoassays,¹¹ and fluorescence in situ hybridization¹² require expensive instrumentation, clinical

expertise, and are susceptible to false positive/negative responses because of the species/strain-specific nature of these biomarkers.¹³

The biofilm matrix itself provides a target for diagnosis of bacterial infections. Biofilms consist of bacteria encased in biomaterials, with up to 90% of dry mass found in the matrix. This matrix provides a three-dimensional scaffold that offers protection and cohesion to the bacteria in the biofilm,^{14,15} controlling the physical and functional properties of the biofilm.¹⁶ The extracellular polymeric substances (EPS) of biofilms are made of a complex of moieties including polysaccharides, proteins, lipids, and nucleic acids. Significantly, the EPS differs substantially depending on the species of bacteria.^{17,18} For example, *Pseudomonas aeruginosa* secretes mostly neutral and polycationic polysaccharides,¹⁹ whereas

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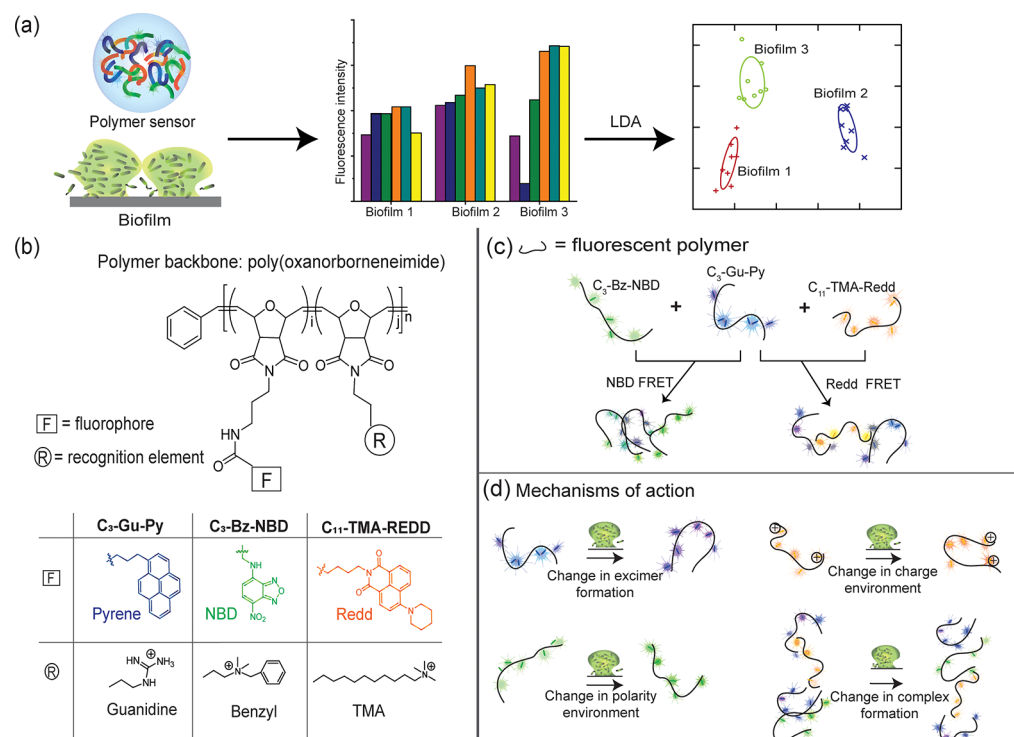


Figure 1. Molecular design and working principle of the polymer sensor platform. (a) Schematic illustration of the sensor platform. The sensor is composed of three fluorescent polymers. Interaction of the polymers with the biofilm causes a disruption or conformational change of the complex that leads to variation in fluorescence outputs, resulting in distinct fluorescence patterns for biofilm identification. (b) Molecular structures of cationic fluorescent polymers with multiple types of recognition elements and fluorophores. (c) Generation of two FRET pairs: C₃-Gu-Py with C₃-Bz-NBD (NBD FRET) and C₃-Gu-Py with C₁₁-TMA-Redd (Redd FRET). (d) Possible structural changes of the polymer complex in response to the biofilm environment lead to distinct fluorescence fingerprints.

Enterobacter coli secretes a predominately polyanionic matrix.²⁰ These differences occur between both species and strains, for example, polysaccharides from different *Streptococcus thermophilus* biofilms have different monomer compositions, monomer ratios, and molecular masses.²¹

The heterogeneity and phenotypic diversity of biofilm matrices make it challenging to apply strategies based on specific biomarkers.^{22,23} However, selective “chemical nose” strategies provide a complementary approach to discriminate complex chemical and biological analytes by generating unique “fingerprints” for analytes of interests.^{24–26} Array-based sensors have been used to differentiate a variety of species and strains of dispersed planktonic bacteria with high classification accuracy,^{27–29} Discrimination of biofilms using array-based strategies, however, has only been achieved using fluorescent protein-based sensors, with the relative instability of the biomolecular components preventing their translation to clinical and point-of-care diagnosis.³⁰

Here, we report a robust and fully synthetic sensor array for biofilm identification. The sensor consists of three cationic fluorescent polymers with multiple types of recognition elements. These polymers feature environmentally responsive fluorophores that are chosen to form excimers and interpolymer Förster resonance energy transfer (FRET) partners, generating six distinct ratiometric channels from only three polymer sensor elements. These outputs change reproducibly upon interacting with biofilm matrices, resulting in a distinct fluorescent pattern for each type of biofilm (Figure 1a). Using this sensor platform, we have successfully discriminated mono-species biofilms formed by five different

bacterial strains, including three pathogenic clinical isolates. The robust nature of this sensor system likewise enables identification of biofilm bacteria in more complex milieus relevant to real-world diagnostics, including dual-species biofilms, as well as wound biofilm models generated through co-culture of biofilm-forming bacteria with fibroblast cells.

RESULTS AND DISCUSSION

In this study, we chose poly(oxanorborneneimide) (PONI) polymers as the scaffold for our sensor because of their ease of synthesis, well-controlled molecular weight, and high biocompatibility.^{31,32} These PONI polymers were fabricated to incorporate one cationic recognition element and one environmentally sensitive transducer: C₃-guanidine-pyrene (C₃-Gu-Py), C₃-benzyl-nitrobenzoxadiazole (C₃-Bz-NBD), and C₁₁-trimethylammonium-Redd (C₁₁-TMA-Redd) (Figure 1b). The careful selection of transducers offers two donor–acceptor FRET pairs: C₃-Gu-Py with C₃-Bz-NBD (NBD FRET) and C₃-Gu-Py with C₁₁-TMA-Redd (Redd FRET) (Figure 1c). These three polymers were added together with the biofilm sample in a single microwell and fluorescence emission of each polymer was recorded, offering a total of six output channels. The cationic recognition elements were chosen to show selective binding toward biofilm matrices, concomitantly generating binding patterns (Figure 1d).^{33,34} The generated patterns were then used for data analysis to identify and classify each type of biofilm. Taken together, this six-channel system provides high content “one well” sensing of biofilms.

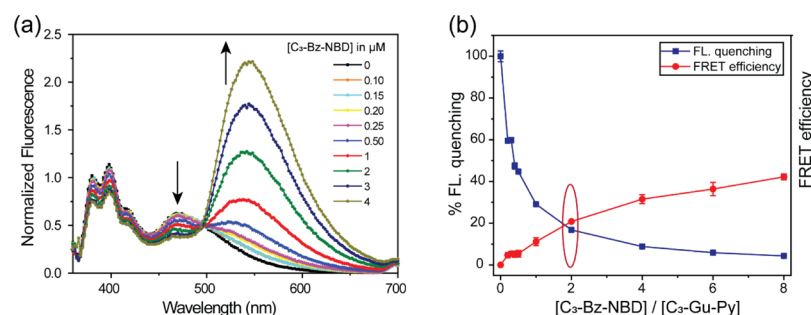


Figure 2. FRET efficiency between C_3 -Gu-Py- C_3 -Bz-NBD. (a) Fluorescence titration of $0.5 \mu\text{M}$ C_3 -Gu-Py with varying concentrations of C_3 -Bz-NBD. (b) Quenching of C_3 -Gu-Py fluorescence (blue squares) and the corresponding FRET efficiency (red circles) as a function of increasing C_3 -Bz-NBD concentration. Each value is an average of three data points, and the error bars are standard deviations.

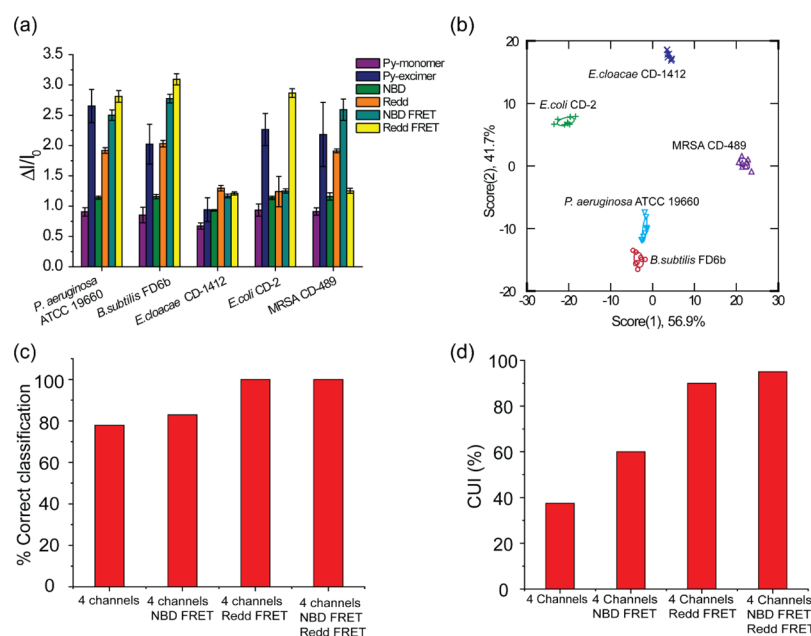


Figure 3. Discrimination of single-species biofilms. (a) Normalized fluorescence intensity against five types of biofilms. ΔI is the sensor response of biofilms after removing biofilm autofluorescence in water. I_0 is the sensor-only response. Each value is the average of eight independent measurements. (b) Canonical score plot for the first two factors of fluorescence patterns was obtained with the polymer sensor against five single-species biofilms. The scores were generated through LDA with 95% confidence ellipses ($n = 8$). (c) Correct classification percentage of biofilms using different combinations of sensor channels. (d) Correct unknown identification of biofilms using different combinations of sensor channels.

Initial studies focused on sensor outputs. We first validated the two FRET pairs by fluorescence titration. Results showed a decrease of the C_3 -Gu-Py monomer at 392 nm and excimer at 470 nm, while an increase of C_3 -Bz-NBD emission at 545 nm and C_{11} -TMA-Redd emission at 528 nm were observed, confirming the presence of NBD and Redd FRET pairs (Figure 2a, Figure S2). To evaluate the dynamic changes in output, we quantified fluorescence quenching and FRET efficiency from the titration curve. Fitting of titration data revealed differential interactions between C_3 -Gu-Py to C_3 -Bz-NBD and C_{11} -TMA-Redd, indicating a selective interaction. A ratio of 2:1 between C_3 -Bz-NBD and C_3 -Gu-Py was used to provide a quenching efficiency of $\sim 80\%$ and FRET efficiency of $\sim 20\%$ (Figure 2b). For the Redd FRET pair, the ratio of 0.5 was selected with quenching and FRET efficiencies of ~ 60 and $\sim 10\%$, respectively (Figure S2). The ratio was selected so that four characteristic excitation/emission peaks are present on the spectrum and these peaks have a dynamic range to either increase or decrease when interacting with biofilm matrices. After determining the optimal ratio of the three polymers, we

prepared the sensor by mixing the three PONI polymers in one microwell and determined the fluorescent output. Each of the three PONI polymers showed four characteristic excitation/emission peaks and two efficient FRET signals (Figure S3), altogether generating six-channel information from a single-well measurement.

The biofilm life cycle comprises four stages: adhesion, early biofilm development, biofilm maturation, and dispersal. During the second stage, EPS production is stimulated, further enhancing bacterial adhesion to the surface and forming the matrix that surrounds the cell. The EPS matrix continues to develop at maturation, enabling the formation of diverse physicochemical microenvironments.³⁵ In this study, biofilms used were 3-day old and the biomass formation was quantified using crystal violet staining experiment (Figure S4). Biofilm at this stage is at the early biofilm development to biofilm maturation. We tested the sensor against five mono-species biofilms to validate the ability of the sensor to discriminate bacterial species: *P. aeruginosa* (ATCC 19660), *B. subtilis* (FD6b), *E. coli* (CD-2), *Enterobacter cloacae* complex (CD-

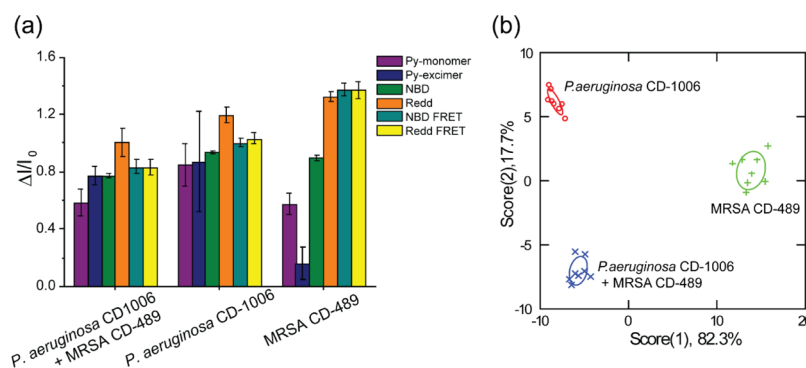


Figure 4. Discrimination of the mixed-species biofilms. (a) Normalized fluorescence patterns of the polymer sensor against three types of mixed-species biofilms. (b) Canonical score plot for the first two factors of fluorescence patterns were generated and plotted through LDA with 95% confidence ellipses ($n = 8$).

1412), and methicillin-resistant *Staphylococcus aureus* (MRSA, CD-489), of which three species are pathogenic clinical isolates (*E. coli* CD-2, MRSA CD-489, and *E. cloacae* CD-1412). Solutions of bacteria were incubated in M9 minimal media in a 96-well microplate for three days at room temperature to allow biofilm formation (early stage of biofilm development). After removing planktonic bacteria cells, the polymer sensor mixture was added to each well and incubated for 30 min prior to reading the fluorescence signals. A distinct fluorescence pattern was generated for each species of the biofilm (Figure 3a). The fluorescence response was further analyzed through linear discriminant analysis (LDA) to statistically classify these biofilms (5 mono-species bacterial biofilm $\times$ 8 replicates $\times$ 6 channels of three polymer complexes).³⁶ The LDA plot showed five nonoverlapping clusters with 100% accuracy classification, indicating a successful discrimination of the five biofilms (Figure 3b, Table S5). In addition, we validated the reliability of such sensor platform by carrying out unknown identification. Among the 40 blind samples, 38 were correctly predicted into the correct group giving an overall correct unknown identification of 95% (Table S8).

The components of the EPS across the biofilm matrix are heterogeneous and vary depending on bacterial species present. For instance, the following proteins are predominantly present in the biofilms of different bacterial species: TasA (*B. subtilis*), FapC (*Pseudomonas spp.*), and phenol-soluble modulins (*S. aureus*).¹⁷ The essence of our array-based sensing or selective sensing is that the sensor is not specific for a particular component of the biofilms. Instead, the sensor selectively interacts with EPS in the biofilm matrix, generating distinct response patterns for tested biofilms. Our previous sensing studies with mammalian cells suggest that glycosylation patterns on cell membrane are correlated with the sensing readout. However, biofilms are much more complex than the cell surface. The components of the EPS, commonly composed of lipids, DNA, extracellular proteins, and exopolysaccharides, are diverse and vary depending on the bacterial species present. We hypothesize that multiple parts of biofilm EPS could be interacting with our sensor elements. More mechanistic studies need to be conducted to elucidate which molecular interactions are important for biofilm classification.

Next, we performed LDA and correct unknown identification (CUI) with or without the two FRET channels to assess the contribution of each FRET pair in discriminating biofilms. As shown in Figure 3c–d, the six-channel combination provides the highest classification and unknown

identification accuracy when compared to other combinations. Interestingly, when using only the regular four channels from the three polymers as predictors, the classification dropped below 80% and correct unknown identification was very poor. To avoid the potential problem of overfitting the data, we combined two sets of sensing data (40 testing samples + 40 blind samples) and performed jackknife classification analysis. The results showed a similar trend where four channels had the lowest classification accuracy (79%) and the addition of two FRET channels demonstrated the highest accuracy (99%) (Figure S6).

These results indicated that efficient FRET from these polymers is essential for our sensor to discriminate the diversity of biofilms. We hypothesize that when the polymer complex interacts with biofilms, substrates in the biofilms alter the distance between the donor and acceptor fluorophore (either bringing them closer together or further apart). Because the efficiency of this energy transfer is dependent on the inverse sixth power of intermolecular separation, small changes in distance will result in a dramatic fluorescence changes.³⁷ In addition, it is also likely that changes in orientation happened on FRET pairs will further expose or hinder environment-sensitive dyes (pyrene, NBD, and Redd) to their local environment, enhancing the effects on the FRET output (Figure 1d).

Several biofilm-related chronic infections, such as otitis media, urinary tract infection, and osteomyelitis, are caused by the colonization of more than one pathogenic bacteria species. The presence of multispecies bacteria in the biofilm results in a greater diversity of matrices that is more challenging to diagnose and eradicate.^{38,39} We tested the platform on the mixed-species biofilm model to evaluate the ability of the sensor array to differentiate more complex biofilms. Two pathogenic bacteria, *P. aeruginosa* CD-1006 (Gram-negative) and MRSA CD-489 (Gram-positive), were chosen to form the mixed-species biofilm model because they are the opportunistic pathogens most often found together in chronic wound infections.^{40–42} The mixed-species biofilm showed distinct fluorescence patterns compared to their mono-species biofilms (Figure 4a). Furthermore, LDA-classified 24 samples into three distinct clusters with 100% accuracy (Figure 4b, Table S6). The results indicate a difference in physiological components between single and mixed-species bacterial biofilms. As mentioned above, we also investigated the reliability of the sensor by predicting unknown samples. A CUI of 100% was achieved with 24 samples (Table S9), demonstrating the

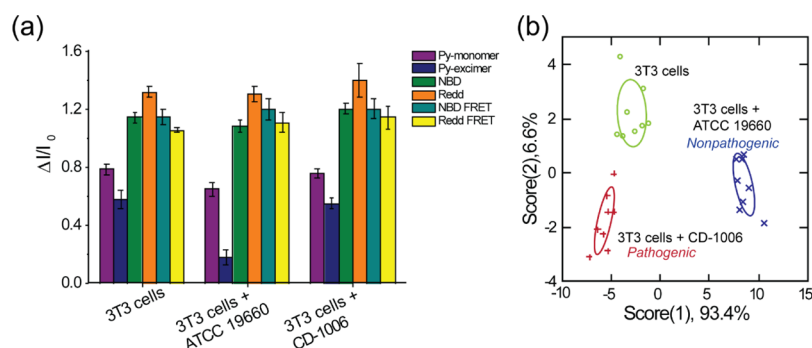


Figure 5. Detection and discrimination of biofilms grown on fibroblast cells. (a) Normalized fluorescence response patterns of polymer sensor against fibroblast cells with and without the biofilms. (b) Canonical score plot of the first two factors obtained from LDA with 95% confidence ($n = 8$).

efficiency of this polymer sensor in classifying complex biofilms.

Identifying the pathogen in biofilms infecting tissues or organs is critical and particularly challenging.^{43,44} We generated a biofilm-fibroblast co-culture model to test the ability of our sensor to discriminate biofilm species in a wound biofilm model.^{45,46} Different strains of *P. aeruginosa* were chosen as representative nonpathogenic (*P. aeruginosa* ATCC 19660) and pathogenic (*P. aeruginosa* CD-1006) bacteria in this study. Most strains of this species are opportunistic human pathogens commonly found in human wounds and often used in both in vitro and in vivo infection models.^{47,48} The bacteria were seeded to a fibroblast NIH-3T3 cell monolayer for 6 h to allow biofilm formation. Cytotoxicity assay confirmed that the biofilm was not toxic to 3T3 cells (Figure S5). Before adding the sensor, the co-culture models were washed to remove planktonic bacteria and nonadherent 3T3 cells. The fluorescence fingerprints of these two types of biofilm-infected 3T3 cells were different from the noninfected 3T3 cells (Figure 5a). The LDA plot showed nonoverlapping groups of co-cultures and 3T3 cells with 96% classification accuracy (Figure 5b, Table S10). A similar high percentage of CUI (92%) was achieved in this model, confirming the diagnostic potentials of our polymer sensor platform.

CONCLUSIONS

In summary, we have developed a rapid and effective three-polymer-six output multichannel sensor array for discrimination of bacterial biofilms. Using this “one well” sensor platform, we successfully classified and identified biofilms formed by nonpathogenic and pathogenic bacteria, as well as between pathogenic biofilms formed by single and mixed-species bacteria. This sensor array further showed its diagnostic potential by discriminating bacterial strain in a fibroblast-biofilm wound infection model. Taken together, this study demonstrated an effective sensor array to differentiate bacterial species in biofilms, providing a pathway to new rapid diagnostic methods for biofilm infections.

EXPERIMENTAL PROCEDURES

Synthesis of Fluorescent Polymers. Monomers and polymers were synthesized according to previous reports.^{49,50} Details are described in the Supporting Information.

Fluorescence Titration. Fluorescence titration of the C_3 -Gu-Py with C_3 -Bz-NBD and C_{11} -TMA-Redd were recorded in a Molecular Device Spectramax M2 plate reader. C_3 -Gu-Py solution (0.5 μ M) was titrated with either C_3 -Bz-NBD or C_{11} -TMA-Redd at a concentration

range from 0 to 4 μ M in a black 96-well microplate. The solutions were mixed and incubated for 30 min at room temperature followed by the recording of the fluorescence spectra with an excitation wavelength at 344 nm.

Single-Species Biofilm Model. A single colony of bacteria was inoculated into LB broth at 37 °C overnight to reach the stationary phase. Bacteria solution was then centrifuged, washed three times with 0.85% NaCl, and resuspended in phosphate buffer saline (PBS). Optical density (OD) of bacteria solution at 600 nm was measured to determine bacteria concentration. The bacteria solution was then diluted in M9 minimal media to 0.1 OD. Seed solution (100 μ L) was added to black 96-well microplate and incubated at room temperature under static condition for three days. Media was replaced every day. Prior to the sensing experiments, the plate was wash three times with PBS to remove planktonic bacteria.

Mixed-Species Biofilm Model. Solutions of bacteria were prepared as described earlier. For the mixed-species biofilm, 50 μ L of 0.1 OD₆₀₀ *P. aeruginosa* and 50 μ L of 0.1 OD₆₀₀ MRSA were added into 96-well microplate then mixed. Mixed-species biofilm was formed by incubating the bacterial solution on a 96-well plate at room temperature for 3 days with replacement of media every 24 h.

Biofilm-3T3 Fibroblast Cells Co-culture Model. A total of 10 000 NIH 3T3 cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% bovine calf serum at 37 °C in a humidified atmosphere of 5% CO₂ for 24 h. Bacteria were grown in DMEM media at 37 °C and 275 rpm up to the log phase (0.5 McFarland standard/ 1.5×10^8 CFU/mL). Then, 100 μ L of the bacterial solution was incubated with mammalian cells at 37 °C in a humidified atmosphere of 5% CO₂ for 6 h to allow biofilm formation. The co-culture was washed three times with PBS to remove planktonic bacteria and nonadherent 3T3 cells before addition of the sensor.

Sensor Complexation. The sensor solution was composed of 0.5 μ M C_3 -Gu-Py, 1 μ M C_3 -Bz-NBD, and 0.25 μ M C_{11} -TMA-Redd in Milli-Q (MQ) water. The polymer sensor was made fresh and incubated for 15 min before using. Each solution (150 μ L) was placed into each well of a 96-well microplate containing biofilms. After incubation for 30 min, the fluorescence intensity was recorded at optimal excitation/emission (Ex/Em) wavelengths: C_3 -Gu-Py at 344/392 nm and an excimer emission 344/470 nm; C_3 -Bz-NBD at Ex/Em 470/545 nm; C_{11} -TMA-Redd at Ex/Em 422/528 nm; FRET between C_3 -Gu-Py and C_3 -Bz-NBD at 344/545 nm; and FRET between C_3 -Gu-Py and C_{11} -TMA-Redd at 344/528 nm. The fluorescence intensity was monitored using Molecular Device Spectramax M2 plate reader. The auto-fluorescence of the biofilms in MQ water was also recorded.

Linear Discriminant Analysis. LDA was performed using the SYSTAT (version 13) program. For the single-species biofilm sensing, the raw fluorescence response data contained a matrix of 8 (replicates) $\times$ 5 (biofilms) $\times$ 6 (channels). For the mixed-species biofilm, a matrix of 8 (replicates) $\times$ 3 (mixed biofilm/biofilms) $\times$ 6 (channels) was generated. The biofilm-3T3 fibroblast cells co-culture

model provided a matrix of 8 (replicates) $\times$ 3 (biofilm-3T3 cells/3T3 cells only) $\times$ 6 (channels). The raw data were transferred to canonical score setting with bacterial species as the grouping variable. These canonical scores were plotted as a scatter plot with 95% confidence ellipse.

Unknown Identification. The identity of unknown samples was predicted by the Mahalanobis distance of the unknown data from the training groups.⁵¹ The LDA was performed on the raw fluorescence responses of the training set and the unknowns at the same time. The Mahalanobis distance of each unknown from the centroid of training groups was calculated. Sample is assigned to the group with the shortest Mahalanobis distance.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.9b00839.

Materials; synthesis and characterization of polymers; absorption and emission properties of three polymers; FRET efficiency data; quantification of biofilm formation; cytotoxicity test; normalized fluorescence responses; jackknifed analysis of the fluorescence responses; and identification of unknown samples (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

- (1) Zhao, G.; Usui, M. L.; Lippman, S. I.; James, G. A.; Stewart, P. S.; Fleckman, P.; Olerud, J. E. Biofilms and Inflammation in Chronic Wounds. *Adv. Wound Care* **2013**, *2*, 389–399.
- (2) Bjarnsholt, T.; Ciofu, O.; Molin, S.; Givskov, M.; Høiby, N. Applying insights from biofilm biology to drug development—can a new approach be developed? *Nat. Rev. Drug Discov.* **2013**, *12*, 791–808.
- (3) Hall-Stoodley, L.; Stoodley, P. Evolving Concepts in Biofilm Infections. *Cell. Microbiol.* **2009**, *11*, 1034–1043.
- (4) Lebeaux, D.; Ghigo, J.-M.; Beloin, C. Biofilm-Related Infections: Bridging the Gap between Clinical Management and Fundamental Aspects of Recalcitrance toward Antibiotics. *Microbiol. Mol. Biol. Rev.* **2014**, *78*, 510–543.
- (5) de la Fuente-Núñez, C.; Refuville, F.; Fernández, L.; Hancock, R. E. Bacterial Biofilm Development as a Multicellular Adaptation:

Antibiotic Resistance and New Therapeutic Strategies. *Curr. Opin. Microbiol.* **2013**, *16*, 580–589.

(6) Römling, U.; Balsalobre, C. Biofilm Infections, Their Resilience to Therapy and Innovative Treatment Strategies. *J. Intern. Med.* **2012**, *272*, 541–561.

(7) Hall-Stoodley, L.; Stoodley, P.; Kathju, S.; Høiby, N.; Moser, C.; William Costerton, J.; Moter, A.; Bjarnsholt, T. Towards Diagnostic Guidelines for Biofilm-Associated Infections. *FEMS Immunol. Med. Microbiol.* **2012**, *65*, 127–145.

(8) Parsek, M. R.; Fuqua, C. Biofilms 2003: Emerging Themes and Challenges in Studies of Surface-Associated Microbial Life. *J. Bacteriol.* **2004**, *186*, 4427.

(9) Tunney, M. M.; Klem, E. R.; Fodor, A. A.; Gilpin, D. F.; Moriarty, T. F.; McGrath, S. J.; Muhlebach, M. S.; Boucher, R. C.; Cardwell, C.; Doering, G.; Elborn, J. S.; Wolfgang, M. C. Use of Culture and Molecular Analysis to Determine the Effect of Antibiotic Treatment on Microbial Community Diversity and Abundance During Exacerbation in Patients with Cystic Fibrosis. *Thorax* **2011**, *66*, 579–584.

(10) Guembe, M.; Marín, M.; Martín-Rabadán, P.; Echenagusia, A.; Camúñez, F.; Rodríguez-Rosales, G.; Simó, G.; Echenagusia, M.; Bouza, E. Use of Universal 16S rRNA Gene PCR as a Diagnostic Tool for Venous Access Port-Related Bloodstream Infections. *J. Clin. Microbiol.* **2013**, *51*, 799–804.

(11) Delehanty, J. B.; Ligler, F. S. A Microarray Immunoassay for Simultaneous Detection of Proteins and Bacteria. *Anal. Chem.* **2002**, *74*, 5681–5687.

(12) Malic, S.; Hill, K. E.; Hayes, A.; Percival, S. L.; Thomas, D. W.; Williams, D. W. Detection and Identification of Specific Bacteria in Wound Biofilms Using Peptide Nucleic Acid Fluorescent *In Situ* Hybridization (PNA FISH). *Microbiology* **2009**, *155*, 2603–2611.

(13) Hassan, A.; Usman, J.; Kaleem, F.; Omair, M.; Khalid, A.; Iqbal, M. Evaluation of Different Detection Methods of Biofilm Formation in the Clinical Isolates. *Braz. J. Infect. Dis.* **2011**, *15*, 305–311.

(14) Sutherland, I. The biofilm matrix—an immobilized but dynamic microbial environment. *Trends Microbiol.* **2001**, *9*, 222–227.

(15) Gupta, P.; Sarkar, S.; Das, B.; Bhattacharjee, S.; Tribedi, P. Biofilm, Pathogenesis and Prevention—A Journey to Break the Wall: A Review. *Arch. Microbiol.* **2016**, *198*, 1–15.

(16) Branda, S. S.; Vik, Å.; Friedman, L.; Kolter, R. Biofilms: The Matrix Revisited. *Trends Microbiol.* **2005**, *13*, 20–26.

(17) Hobley, L.; Harkins, C.; MacPhee, C. E.; Stanley-Wall, N. R. Giving Structure to the Biofilm Matrix: An Overview of Individual Strategies and Emerging Common Themes. *FEMS Microbiol. Rev.* **2015**, *39*, 649–669.

(18) Flemming, H.-C.; Wingender, J. The Biofilm Matrix. *Nat. Revs. Microbiol.* **2010**, *8*, 623–633.

(19) Irie, Y.; Starkey, M.; Edwards, A. N.; Wozniak, D. J.; Romeo, T.; Parsek, M. R. *Pseudomonas aeruginosa* biofilm matrix polysaccharide Psl is regulated transcriptionally by RpoS and post-transcriptionally by RsmA. *Mol. Microbiol.* **2010**, *78*, 158–172.

(20) Limoli, D. H.; Jones, C. J.; Wozniak, D. J. Bacterial Extracellular Polysaccharides in Biofilm Formation and Function. *Microbiol. Spectr.* **2015**, *3*, 1–19.

(21) Sheppard, D. C.; Howell, P. L. Biofilm Exopolysaccharides of Pathogenic Fungi: Lessons from Bacteria. *J. Biol. Chem.* **2016**, *291*, 12529–12537.

(22) Pan, M.; Zhu, L.; Chen, L.; Qiu, Y.; Wang, J. Detection Techniques for Extracellular Polymeric Substances in Biofilms: A Review. *Bioresearch* **2016**, *11*, 8092–8115.

(23) Wimpenny, J.; Manz, W.; Szejczyk, U. Heterogeneity in Biofilms. *FEMS Microbiol. Rev.* **2000**, *24*, 661–671.

(24) Peveler, W. J.; Yazdani, M.; Rotello, V. M. Selectivity and Specificity: Pros and Cons in Sensing. *ACS Sens.* **2016**, *1*, 1282–1285.

(25) Turner, A. P. F.; Magan, N. Electronic Noses and Disease Diagnostics. *Nat. Rev. Microbiol.* **2004**, *2*, 161–166.

(26) Le, N. D. B.; Tonga, G. Y.; Mout, R.; Kim, S.-T.; Wille, M. E.; Rana, S.; Dunphy, K. A.; Jerry, D. J.; Yazdani, M.; Ramanathan, R.; Rotello, C. M.; Rotello, V. M. Cancer Cell Discrimination Using

Host-Guest “Doubled” Arrays. *J. Am. Chem. Soc.* **2017**, *139*, 8008–8012.

(27) Han, J.; Cheng, H.; Wang, B.; Braun, M. S.; Fan, X.; Bender, M.; Huang, W.; Domhan, C.; Mier, W.; Lindner, T.; Seehafer, K.; Wink, M.; Bunz, U. H. F. A Polymer/Peptide Complex-Based Sensor Array That Discriminates Bacteria in Urine. *Angew. Chem., Int. Ed.* **2017**, *56*, 15246–15251.

(28) Phillips, R. L.; Miranda, O. R.; You, C.-C.; Rotello, V. M.; Bunz, U. H. F. Rapid and Efficient Identification of Bacteria Using Gold-Nanoparticle-Poly(para-phenyleneethynylene) Constructs. *Angew. Chem., Int. Ed.* **2008**, *47*, 2590–2594.

(29) Liu, G.-j.; Tian, S.-n.; Li, C.-y.; Xing, G.-w.; Zhou, L. Aggregation-Induced-Emission Materials with Different Electric Charges as an Artificial Tongue: Design, Construction, and Assembly with Various Pathogenic Bacteria for Effective Bacterial Imaging and Discrimination. *ACS Appl. Mater. Interfaces* **2017**, *9*, 28331–28338.

(30) Li, X.; Kong, H.; Mout, R.; Saha, K.; Moyano, D. F.; Robinson, S. M.; Rana, S.; Zhang, X.; Riley, M. A.; Rotello, V. M. Rapid Identification of Bacterial Biofilms and Biofilm Wound Models Using a Multichannel Nanosensor. *ACS Nano* **2014**, *8*, 12014–12019.

(31) Matson, J. B.; Grubbs, R. H. Monotelechelic Poly(oxa)-norbornenes by Ring-Opening Metathesis Polymerization Using Direct End-Capping and Cross-Metathesis. *Macromolecules* **2010**, *43*, 213–221.

(32) AL-Badri, Z. M.; Tew, G. N. Well-Defined Acetylene-Functionalized Oxanorbornene Polymers and Block Copolymers. *Macromolecules* **2008**, *41*, 4173–4179.

(33) Klymchenko, A. S. Solvatochromic and Fluorogenic Dyes as Environment-Sensitive Probes: Design and Biological Applications. *Acc. Chem. Res.* **2017**, *50*, 366–375.

(34) Beyazkılıç, P.; Yildirim, A.; Bayindir, M. Formation of Pyrene Excimers in Mesoporous Ormosil Thin Films for Visual Detection of Nitro-explosives. *ACS Appl. Mater. Interfaces* **2014**, *6*, 4997–5004.

(35) Koo, H.; Allan, R. N.; Howlin, R. P.; Stoodley, P.; Hall-Stoodley, L. Targeting Microbial Biofilms: Current and Prospective Therapeutic Strategies. *Nat. Rev. Microbiol.* **2017**, *15*, 740–755.

(36) Jurs, P. C.; Bakken, G. A.; McClelland, H. E. Computational Methods for the Analysis of Chemical Sensor Array Data from Volatile Analytes. *Chem. Rev.* **2000**, *100*, 2649–2678.

(37) Stryer, L.; Haugland, R. P. Energy Transfer: A Spectroscopic Ruler. *Proc. Natl. Acad. Sci. U.S.A.* **1967**, *58*, 719–726.

(38) Sanchez-Vizuet, P.; Orgaz, B.; Aymerich, S.; Le Coq, D.; Briand, R. Pathogens Protection Against the Action of Disinfectants in Multispecies Biofilms. *Front. Microbiol.* **2015**, *6*, 705.

(39) Thornton, R. B.; Rigby, P. J.; Wiertsema, S. P.; Filion, P.; Langlands, J.; Coates, H. L.; Vijayasekaran, S.; Keil, A. D.; Richmond, P. C. Multi-Species Bacterial Biofilm and Intracellular Infection in Otitis Media. *BMC Pediatr.* **2011**, *11*, 94.

(40) DeLeon, S.; Clinton, A.; Fowler, H.; Everett, J.; Horswill, A. R.; Rumbaugh, K. P. Synergistic Interactions of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in an In Vitro Wound Model. *Infect. Immun.* **2014**, *82*, 4718–4728.

(41) Mitchell, G.; Séguin, D. L.; Asselin, A. E.; Déziel, E.; Cantin, A. M.; Frost, E. H.; Michaud, S.; Malouin, F. *Staphylococcus aureus* Sigma B-dependent Emergence of Small-Colony Variants and Biofilm Production Following Exposure to *Pseudomonas aeruginosa* 4-hydroxy-2-heptylquinoline-N-oxide. *BMC Microbiol.* **2010**, *10*, 1–15.

(42) Pastar, I.; Nusbaum, A. G.; Gil, J.; Patel, S. B.; Chen, J.; Valdes, J.; Stojadinovic, O.; Plano, L. R.; Tomic-Canic, M.; Davis, S. C. Interactions of Methicillin Resistant *Staphylococcus aureus* USA300 and *Pseudomonas aeruginosa* in Polymicrobial Wound Infection. *PLoS One* **2013**, *8*, No. e56846.

(43) Gardner, S. E.; Frantz, R. A.; Doebbeling, B. N. The Validity of the Clinical Signs and Symptoms Used to Identify Localized Chronic Wound Infection. *Wound Repair Regen.* **2001**, *9*, 178–186.

(44) Percival, S. L.; Vuotto, C.; Donelli, G.; Lipsky, B. A. Biofilms and Wounds: An Identification Algorithm and Potential Treatment Options. *Adv. Wound Care* **2015**, *4*, 389–397.

(45) Bowler, L. L.; Ball, T. B.; Saward, L. L. A Novel In Vitro Co-Culture System Allows the Concurrent Analysis of Mature Biofilm and Planktonic Bacteria with Human Lung Epithelia. *J. Microbiol. Methods* **2014**, *101*, 49–55.

(46) Anderson, G. G.; Moreau-Marquis, S.; Stanton, B. A.; O'Toole, G. A. In Vitro Analysis of Tobramycin-Treated *Pseudomonas aeruginosa* Biofilms on Cystic Fibrosis-Derived Airway Epithelial Cells. *Infect. Immun.* **2008**, *76*, 1423–1433.

(47) Rybtke, M.; Hultqvist, L. D.; Givskov, M.; Tolker-Nielsen, T. *Pseudomonas aeruginosa* Biofilm Infections: Community Structure, Antimicrobial Tolerance and Immune Response. *J. Mol. Biol.* **2015**, *427*, 3628–3645.

(48) Lebeaux, D.; Chauhan, A.; Rendueles, O.; Beloin, C. From In Vitro to In Vivo Models of Bacterial Biofilm-Related Infections. *Pathogens* **2013**, *2*, 288–356.

(49) Landis, R. F.; Gupta, A.; Lee, Y.-W.; Wang, L.-S.; Golba, B.; Couillaud, B.; Ridolfo, R.; Das, R.; Rotello, V. M. Cross-Linked Polymer-Stabilized Nanocomposites for the Treatment of Bacterial Biofilms. *ACS Nano* **2017**, *11*, 946–952.

(50) Peveler, W. J.; Landis, R. F.; Yazdani, M.; Day, J. W.; Modi, R.; Carmalt, C. J.; Rosenberg, W. M.; Rotello, V. M. A Rapid and Robust Diagnostic for Liver Fibrosis Using a Multichannel Polymer Sensor Array. *Adv. Mater.* **2018**, *30*, 1800634–1800639.

(51) Mahalanobis, P. C. On the Generalized Distance in Statistics. *Proc. Natl. Inst. Sci. India* **1936**, *2*, 49–55.