Published in final edited form as:

ACS Sens. 2018 March 23; 3(3): 700–708. doi:10.1021/acssensors.8b00025.

A ratiometric fluorescent sensor array as a versatile tool for bacterial pathogen identification and analysis

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Abstract

Rapid and reliable identification of pathogenic microorganisms is of great importance for human and animal health. Most conventional approaches are time-consuming and require expensive reagents, sophisticated equipment, trained personnel, and special storage and handling conditions. Sensor arrays based on small molecules offer a chemically stable and cost-effective alternative. Here we present a ratiometric fluorescent sensor array based on the derivatives of 2-(4'-N,N-dimethylamino)-3-hydroxyflavone, and investigate its ability to provide a dual-channel ratiometric response. We demonstrate that by using discriminant analysis of the sensor array responses, it is possible to effectively distinguish between eight bacterial species and recognize their Gram status. Thus, multiple parameters can be derived from the same dataset. Moreover, the predictive potential of this sensor array is discussed, and its ability to analyze unknown samples beyond the list of species used for the training matrix is demonstrated. The proposed sensor array and analysis strategies open new avenues for the development of advanced ratiometric sensors for multiparametric analysis.

Graphical Abstract

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Competing Interests Disclosure

The authors declare no competing financial interests.

ASSOCIATED CONTENT

Supporting Information. Description of the synthetic procedures for the reporter dyes, their structure confirmation, size and dispersity characterization of the dye-loaded HA nanoparticles, TEM images, fluorescence spectra of the reporter dyes upon their interaction with bacteria, 3D canonical plot for the dual-channel sensor response analysis, results of the sensor validation using unknown samples, results of sensor's predictive potential assessment on classification of Gram status and "one against the rest" discrimination for the bacterial species outside of the training subset. This information is available free of charge.

Keywords

multiparametric sensing; chemical nose; 3-hydroxyflavone; ESIPT; discriminant analysis; Gram status; pattern analysis; predictive analysis

Timely and efficient identification of bacterial pathogens is of high importance for public health and safety. Traditional identification methods rely on phenotypic characterization of the bacterial species by Gram staining and culturing in combination with other biochemical methods and PCR. These methods, however, are sometimes inconclusive, time-consuming and reliant on expensive equipment, which delays treatment and limits their application to laboratory settings. The development and use of molecular sensing techniques has the potential to overcome the limitations of the traditional phenotypic approaches. The majority of the current sensing methods are based on optical and/or electrochemical techniques for analysis of biomarkers¹ or whole bacteria², where selectivity is usually achieved by using antibodies,³ their fragments, natural or engineered peptides,⁴ or aptamers.^{5–7} However, the important limitations for using these elements as a part of the sensor are cost, stability and adaptability: synthesis of antibody-based reporters is usually quite challenging since these molecules often require special handling and they are tailored to be specific to only a narrow range of analytes.

Alternatively, a combination of multiple cross-reactive reporters may be used instead of a single selective indicator. Despite the fact that such reporters are not intrinsically selective to a particular analyte, each of them provides a different response to every analyte. Hence, the combination of the signals of these reporters will be unique for each analyzed sample, thus leading to acquired specificity.⁸ Multivariate analysis using sensor arrays has been actively developed with the introduction of the concept of the *chemical nose*.^{9,10} Such arrays have been developed for various applications, including detection and analysis of small molecules and ions,^{11–14} proteins,¹⁵ and even whole microorganisms.¹⁶

The majority of the fluorescent sensor arrays reported to date are based on either quenching (“turn-off”) or activation (“turn-on”) of emission. While the latter usually yields better performance due to improved contrast, each technique requires an external standard to be used as a reference signal. In terms of further real-life applications, the need for a standard reference adds additional steps and makes the procedure more complex. Ratiometric dyes have been long explored as promising alternatives as they have several bands in their emission spectra which provide a self-referenced analytical signal independent of the

concentration of the probe. With the advantages of ratiometric probes being largely used for single-analyte sensing, relatively few sensor arrays that leverage this principle have been explored. The few examples that exist include using a combination of two independent fluorophores acting independently,¹⁷ or being part of a covalently bound FRET dyad.¹⁸

Here, we describe a new ratiometric fluorescent sensor array that is based on derivatives of 3-hydroxyflavone loaded into polymeric nanoparticles for efficient delivery and staining. This sensor array demonstrates the ability to distinguish between eight bacterial species of different Gram status with high accuracy. We discuss using the increased number of data-collecting channels from a single molecule as a way to improve the differentiation of the signals originating from different bacterial species. Manipulating data from a single dataset allows answering multiple questions without additional measurements. Additionally, the predictive potential of the sensor array is demonstrated for determination of the Gram status and discrimination of bacterial species that did not belong to the training dataset.

MATERIALS AND METHODS

Materials

N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylamino)propylcarbodiimide (EDC), pyrenebutyric acid, 1,3-propyldiamine, 2-hydroxyacetophenone and 2-hydroxynaphthophenone were purchased from Sigma-Aldrich (St. Louis, MO). N,N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), sodium methylate, hydrogen peroxide (30%), N,N-dimethylaminobenzaldehyde, N,N-diphenylaminobenzaldehyde, and hydrochloric acid (Certified ACS Plus, 36–38%) were purchased from Fisher Scientific (Pittsburgh, PA). Ethanol was purchased from UNMC internal supply. Sodium hyaluronate (HA) was purchased from Lifecore Biomedical (Chaska, MN).

Synthesis of ratiometric dyes

All four derivatives of 3-hydroxychromone were synthesized following a two-stage Algar-Flynn-Oyamada procedure modified for better performance with electron donor-substituted aldehydes.¹⁹ To perform this technique, a corresponding aldehyde is reacted with 2-hydroxyacetophenone in DMF with addition of sodium methoxide to obtain a chalcone, which is subsequently subjected to an oxidative cyclization in ethanol with excess of sodium methoxide and 30% aqueous H₂O₂. Here, structures of the obtained compounds were confirmed by ¹H NMR spectroscopy on a Bruker Advance 500 MHz NMR spectrometer at 25°C. Samples were analyzed in DMSO-d₆ (99.9% D, Cambridge Isotope Laboratories). Data was processed in Mnova NMR (Escondido, CA). The synthetic details for each dye, as well the structure confirmation data are described in the Supplementary Information.

Polymer synthesis and nanoparticles loading

Amphiphilic hyaluronic acid (HA) polymers were synthesized as described in previous reports.^{20,21} Briefly, 40–45 mg HA (M_N = 10–20 kDa) was dissolved in 1:1 ultrapure water and DMF along with 30 mg of NHS and 30 mg of EDC. After mixing for 30 minutes to activate the HA carboxylic acid groups, 10 weight percent of aminopropyl-pyrenebutanamide was added to the HA solution and allowed to react for 24 hours. The

reaction mixture was then removed and placed in 3500 MWCO dialysis tubing and dialyzed against 1:1 water and ethanol for 4 exchanges over 24 h, then against pure water for 8 exchanges over 48 h to remove any impurities. Finally, the product was frozen and freeze dried for later use.

Stock solutions containing 60 mg of pyHA in 40 mL of ultrapure water, as well as 3 mg of each dye (in 10 mL DMSO) were prepared. Solutions of the polymer and dyes were mixed together (10 mL of the modified HA solution + 10 mL of the dye solution) to obtain 4 systems containing every dye mixed with the polymer. The final solutions were thoroughly mixed on a vortex mixer and loaded into the 3500 MWCO dialysis bags. Samples were then dialyzed against ultrapure water with 8 exchanges over 48 hours. After dialysis, the samples were purified using PD-10 columns, then frozen and freeze-dried for further storage.

Hydrodynamic radii of the nanoparticles were measured with Malvern Zetasizer Nano ZS90 in 0.5 mg/mL solutions, and the corresponding data is presented in the Table S1.

Transmission electron microscopy imaging was performed on the FEI Tecnai G2 Spirit microscope (2% aqueous methylamine vanadate, pH 8, was used for negative staining).

Bacterial culture, staining and spectroscopy

Eight different bacterial species from our lab collection were used in this study, including four Gram-positive (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Enterococcus faecalis*) and four Gram-negative (*Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Citrobacter freundii*). Bacteria collected at the stationary phase of growth (15 hours) were washed twice with PBS, resuspended in fresh PBS, and diluted to $OD_{600} = 4$ for further use. Dye-loaded nanoparticles solutions were prepared with $OD_{400} = 0.3$. Bacteria and nanoparticles solutions were mixed 1:1, stirred on a vortex mixer, and incubated in dark for 15 minutes. All samples were subsequently centrifuged at 15000 rpm for 1 minute, washed once with PBS, and resuspended in the same volume of fresh PBS, keeping the original concentration. Samples of each bacterium with every dye were then plated on black 96-well plates (150 μ L/well, 12 replicates per sample). Fluorescence spectra of the samples were recorded at λ_{exc} 400 nm using Tecan infinite 200 spectrofluorometric plate reader. The spectra were used for visual analysis of spectral features. Subsequently, emission intensities at three channels (485, 515 and 575 nm) were recorded for each sample. Five independent measurements in each channel were averaged and used to calculate ratiometric responses for further discriminant analysis.

RESULTS AND DISCUSSION

Design of fluorescent reporters

Looking for factors that would lead to distinctively different, however non-selective, interactions of various bacteria with fluorescent dyes, we focused on the particularities of the bacterial cell envelope. Each bacterial species possesses unique traits of their cell envelope, i.e. the presence or absence of the outer membrane, composition of the peptidoglycan layer, etc.²² Thus, organic dyes sensitive to the polarity of their microenvironment, and to specific interactions, such as hydrogen bonding, would be ideal components for the proposed sensor array. Fluorescence was chosen as a signal, as it provides higher sensitivity than absorption.

Derivatives of 3-hydroxyflavone have been widely explored as powerful and sensitive tools to study different parameters of their microenvironment.^{23–26} Owing to the excited-state intramolecular proton transfer (ESIPT, Figure 1A) taking place in these molecules, they exhibit two distinct emission bands in their fluorescence spectra (Figure 1B). In addition to that, these molecules can participate in other protolytic equilibria. For example, in basic media or under the influence of strong external hydrogen bond acceptors (solvent or other molecules), 3-hydroxyflavones can form anions (Figure 1A) whose fluorescence is located between the emission bands of the normal and tautomer forms of the neutral molecule undergoing ESIPT (Figure 1B).

In particular, 2-(4-N,N-dimethylaminophenyl)-3-hydroxychromone, DMAF (Figure 1C), is known for its bright fluorescence and superior sensitivity to medium polarity due to the intramolecular charge transfer occurring in its excited state.^{25,27} Hence, this molecule was chosen as the basis reporter for the sensor array described in this study. To promote particular interactions of the probe with bacterial cell envelope features, the 3-hydroxyflavone core was modified by introducing various moieties (Figure 1C). For example, an additional benzene ring was annealed to the chromone core such that it would shield the 4-carbonyl group from external hydrogen bonding interactions in BfDMAF.¹⁹ Long octyl chains in DOAF were added to favor partitioning of the dye into hydrophobic parts of the bacterial cell envelope, whereas the phenyl moieties in DPAF were included to enhance the π - π interactions with aromatic components of the cell envelope.

Like other organic dyes commonly used for staining, the proposed sensor array components are hydrophobic. Application of such dyes usually requires organic media to be involved in the process, which may potentially harm the cells and/or alter processes in them, especially if live cells have to be imaged. Otherwise, a colloidal suspension of the dye can be prepared and used in aqueous medium. In this case, there is no harm from organic solvents, but colloidal aggregates of hydrophobic dyes are fairly unstable, and freshly prepared staining reagent is usually needed. To overcome this limitation, the dye structure can be modified,^{28,29} or it can be loaded into a colloiddally stable nanocarrier. Leveraging past experience of loading drugs and contrast agents into amphiphilic nanoparticles derived from hyaluronic acid,^{20,30} this approach was used to allow staining in aqueous media with a simple one-wash procedure described above in Materials and Methods. The resulting nanoparticles have a spherical shape and are fairly large (DLS data is presented in Table S1, and the TEM images are presented in Figures S1 and S2). One can notice that the nanoparticles appear significantly smaller on the TEM micrographs compared to their sized determined by DLS. This is a common discrepancy which is due to high hydration of the HA-derived nanoparticles in aqueous medium (they essentially are hydrogel particles), thus they significantly decrease in size under TEM imaging conditions.

Upon incubation of bacterial cells with dye-containing nanoparticles, the dyes are released from the nanocarriers and are further uptaken by bacteria. The emission spectra were recorded and analyzed. Looking at common spectral features originating from bacteria of different Gram status, one can notice that T* emission of DMAF competes with short-wavelength fluorescence in Gram-positive bacteria (Figure S3). At the same time, the T* band is significantly more pronounced for BfDMAF, likely due to interaction of both dyes

with the rather polar peptidoglycan layer and the shielding effect of the annealed benzene ring in BfDMAF. Anionic emission is favored for DPAF, whereas T* fluorescence is more notable for DOAF, most likely due to its enhanced interaction with the apolar parts of the bacterial membrane. A different picture can be noted in the spectral behavior of the dyes upon interaction with Gram-negative bacterial cells (Figure S4). Specifically, a stronger prevalence of anionic and even normal form emission in DMAF and BfDMAF can be attributed to hydrogen bonding interactions with either polar parts of lipopolysaccharides on the surface of the outer membrane, or with polar environment of the periplasm or even cytoplasm. Generally, however, specifics of the spectral behavior (e.g. individual emission band intensities of all three protolytic forms) are strong for all dyes, and depend significantly on the specific features of the bacterial cell envelopes and the dye localization upon interaction with bacterial cells. This fact plays a major role in shaping the unique response patterns further used for analysis.

Pathogenic bacteria classification

The ratio of the normal (N*) and tautomeric (T*) form emissions is traditionally used as a ratiometric signal for ESIPT fluorophores, including the 3-hydroxyflavone derivatives used in this study. While the exact position of the maximum of each emission band depends on several factors, such as the polarity of the molecule's microenvironment, their characteristic location is known from previous studies.^{31,32} Thus, fluorescence intensities were recorded for all samples at 485 nm (N* emission) and 575 nm (T* emission), and the T*/N* ratio was used to generate the response patterns for all bacterial strands, with each sampling consisting of 12 replicates. The average response patterns are provided in Figure 2A and B for Gram-positive and Gram-negative bacteria, respectively.

The main advantage of the sensor array is its intrinsic multidimensionality, i.e. it gives enough information to distinguish between different samples without using highly selective indicators. However, a more rigorous approach is required to interpret the resulting data because the response datasets are multidimensional. To reduce the dimensionality and allow easier interpretation of the generated data, linear discriminant analysis (LDA) is often used. Linear discriminant analysis is a supervised statistical method of classification³³ that works towards maximizing the difference between within-class and between-class variations. The resulting scores are computed for each entry in the dataset. They are further used to group together the entries that belong to the same class (bacterial species in this particular case), while maximizing the distance between different classes. The scores computed for the entries that are part of the training matrix can be further used for classification of the unknown samples, whose response patterns are analyzed to place them in a certain part of the score plot.

Figure 2C represents the canonical score plot of the sensor array responses upon interaction with eight bacterial species using the first two discriminants. It can be seen from the plot that the first discriminant is responsible for almost 75% of the variability, while the second one contributes approximately 20%. In this particular case, it results in a fairly good separation of almost all groups of sensor responses originating from different bacterial strains. However, signals from *K. pneumoniae* and *B. subtilis* show a notable overlap, suggesting

that correct attribution of the unknown signal to either of these classes may be ambiguous. The cross-validation performed by jackknife resampling showed 96.8% accuracy of posterior classification.

To achieve better separation between classes, a third discriminant can be used that is responsible for an additional ~7% of between-class variability. The resulting 3D canonical plot (Figure 3) shows that full separation is observed between all classes. Thus, higher dimensional subspace can be used to improve the outcome of the discriminant analysis.

It is important to note that the system's response depends on the concentration of bacteria in the samples. Variations in the amount of the staining agents incubated with bacteria also influences the readout. Such dependency was reported before,³⁴ and in this particular case may be due to stoichiometry of the dyes interactions with bacteria. Further investigations will be needed to identify the causative factors and to overcome the concentration dependence of the signal. At this time, however, standardized concentrations of both the sample and the probe dye-loaded nanoparticles are required.

3-hydroxyflavones as multichannel reporters

Expanding the dataset used for training purposes is the most direct way to improve the quality of between-class discrimination, and, consequently, the attributing of unknown sample responses to specific bacterial species. Increasing the number of independent measurements for each bacterial species in the training matrix is one potential way to address this need when it comes to proof-of-concept studies. However, this approach becomes less practical in real-life applications, where the training matrix may need to include response patterns from dozens of different bacterial species. Increasing the number of data-collecting channels by including additional reporters in the sensor array is an alternative way to improve the between-class separation and thus the overall specificity of the sensing system.

Importantly, 3-hydroxyflavones offer another opportunity to obtain more data from the same fluorescent reporters. Multiple efforts have been reported to design and explore the ESIPT molecules with an increased number of independent channels that provide information about their microenvironment. This is achieved by introducing a higher level of complexity into the molecule's excited-state behavior with adding another proton-donating or proton accepting center to promote concurrent proton transfer pathways,^{35,36} or design a molecule with more than one intramolecular hydrogen bond capable of excited-state proton transfer.^{37,38}

A combination of ground- and excited-state protolytic equilibria can be used to provide an additional ratiometric signal. As shown on Figure 1A, 3-hydroxyflavone's hydroxyl group can dissociate upon interaction with external proton acceptors, which disrupts the intramolecular hydrogen bond and makes the ESIPT process impossible. Thus, the ratio between the fluorescence of the anion, which is located between the emissions of the two ESIPT tautomers, and the fluorescence of the normal form can be considered as the second independent ratiometric output that is sensitive to the surrounding microenvironment. Recent studies showed that generation of the phototautomer from the anion in the excited state is

not possible,³⁹ thus confirming this assumption. One can see in the spectra (Figure S3 and S4) that distinct emission bands are present in the anion fluorescence range, especially for DMAF and DPAF.

Ratiometric fluorescent response combinations were derived from the primary data (Figure 4A and B), and one can note that anionic emission mostly prevails over the fluorescence of the ESIPT photoproduct in Gram-negative bacterial species. The obtained sensor response patterns were further subjected to discriminant analysis. It is evident from observing the results in Figure 4C that increasing the number of data-collecting channels led to significant improvement compared to the single-channel sensing. Specifically, improvements include decreased within-class variability (scattering of the experimental points belonging to the same bacterial species) and better between-class separation (mostly larger distances between groups representing various bacterial species and no overlap of the groups and/or confidence ellipses). The cross-validation resulted in 100% correct posterior classification of the sensor responses. The same effect can be visualized on the three-dimensional canonical plot (Figure S5).

The primary application of bacterial sensors is for diagnostic purposes. Thus, it is crucial to validate their ability to differentiate between bacterial species within unknown samples. Very much like the single-analyte, single-reporter sensors that need calibration before use, sensor arrays are able to recognize only those bacterial species that were part of the training dataset. To quantitatively assess the ability of the proposed ratiometric array to classify the unknown samples based on their response patterns, a randomized species-proportional subset of 75% of the obtained data was used as a training matrix, whereas the remaining data, unlabeled, were used as test entries.⁴⁰ As follows from the data in Table S2, the unknown samples were attributed to the correct bacterial special in 100% of cases.

Looking back to the Figure 4C, an interesting detail can be noted: signals from the Gram-positive bacteria tend to group on the left side of the canonical score plot, whereas those coming from the Gram-negative bacterial species cluster on the right side. This supports the earlier discussion about distinctions in the spectral features of each dye's fluorescence upon interaction with bacteria of different Gram statuses. Such behavior is similar to the clustering of signals originating from related analytes observed earlier in analysis of volatile gas samples.¹³

Inspired by the observation of Gram status-dependent clustering, the sensor array responses were processed in an alternative manner. This time, the sensor signals were assigned to only two classes: those originating from interaction with Gram-positive and Gram-negative bacteria. The discriminant analysis of these data revealed the ability of the sensor to accurately recognize the Gram status of the analyzed bacteria (Figure 5). Identifying the Gram status illustrates the versatility of the proposed sensor, since multiple types of analyses can be performed to answer a variety of different questions using the same dataset.

Besides the classification of bacteria, discriminant analysis can be applied to determine whether or not the analyzed sample belongs to a particular bacterial species from the training dataset. For this, a "one against the rest" analysis was performed, where the species

of interest is assigned to one class, whereas all other species are grouped into the second class. As demonstrated in Figure 6, discriminant analysis of the same dataset as was used for classification purposes is capable of providing acceptable to excellent discrimination between particular bacteria and the rest of studied samples. The simplest application of this type of analysis is for the confirmation of the presence or absence of a particular bacterial species. On the other hand, it can also be used to support and verify the result of the species classification described above. For example, in the cases when a particular sample cannot be unambiguously attributed to a certain class from the training matrix, additional analyses may be performed based on “one against the rest” discrimination for two or more bacterial species. The obtained results, combined with probabilities for the given sample to belong to two or more classes in the classification analysis, will make the final attribution less ambiguous, thus increasing the robustness and reliability of such classification.

Predictive potential of the sensor array

Usually the analysis of unknown samples and their attribution to a particular class using discriminant analysis of the sensor array response pattern approach is limited to samples that belong to one of the classes used to construct the training matrix. Because the system has been “trained” to recognize the pattern of the eight bacterial species used in the experiments described above, it is intrinsically incapable of recognizing other bacterial species beyond this list. However, this limitation is not necessarily valid for the broader application of the obtained dataset, which was exemplified by Gram status classification and “one against the rest” analysis. In these cases, samples representing bacterial species not included in the training dataset will in fact still belong to one of the classes that are “known” to the system. For example, the training dataset is divided into two classes corresponding to Gram-positive or Gram-negative bacteria. Thus, even if an unknown sample contains a bacterial species that was not part of the training dataset, the analysis will still be able to attribute it to one of the two classes (i.e., there is no third option). Similarly, in “one against the rest” analysis, such an unknown sample would have to belong to the “other bacteria” class. Therefore, such logic expands the potential of the sensor array beyond its application to identify the bacterial species that are “known” to the system, i.e. that were used for sensor’s calibration.

To test this hypothesis for the case of Gram status determination, a truncated training dataset was used with a subset of responses originating from six bacterial species, whereas the data from two remaining bacterial species were used as the test subset. The results using the dual-channel readout are presented in the Table S3. They show that the system was able to successfully attribute the unknown samples to the correct Gram status in 100% of the cases, even taking into account that those samples represented bacterial species that were excluded from the training dataset.

A similar test was performed for the case of “one against the rest” discrimination. Here, a subset representing responses from a certain bacterial species was removed from the training set and used as a test one, whereas the remaining data were divided into two classes of the training matrix: bacterial species of interest and all other bacteria. As follows from the Table S4, in 89% of the cases the sensor is able to correctly determine that the unknown bacteria do not belong to any known tested bacterium of interest. Thus, the proposed sensor array not

only can be used for classification and multiparametric analysis of the samples, but is also capable of characterizing the unknown samples beyond the species used in training datasets.

CONCLUSIONS AND OUTLOOK

In this work, we described the design and synthesis of a ratiometric fluorescent sensor array that consists of hydrophobically-modified hyaluronic acid nanoparticles loaded with four derivatives of 3-hydroxyflavone. The dyes were substituted to promote various interactions with their microenvironment and favor localization to different parts of bacterial cell envelopes.

The sensor was tested on eight different bacterial species, half of which were Gram-positive and the other half were Gram-negative. Discriminant analysis of the sensor response patterns allows for the differentiation between different bacterial species, as well as classification of bacteria in unknown samples. Leveraging protolytic equilibria of the reporter dyes in both ground and excited states, two-channel ratiometric readout becomes possible from each dye, doubling the number of data-collecting channels compared to the number of sensor array components. Using the additional data for the discriminant analysis can improve the identification of bacterial species by reducing within-class variations, while maximizing between-class separation.

Different approaches to the sensor response dataset analysis were used to validate the abilities of the system beyond identification of bacterial species. The sensor is shown to be capable of recognizing the Gram status of bacteria, which is particularly important when species identification is ambiguous. It can also perform discriminatory analysis to detect whether or not an unknown sample belongs to a particular bacterial species of interest. First examples of predictive potential of the sensor array are discussed, where the data was used to correctly recognize the Gram status and perform “one against the rest” discrimination using a subset of responses that originated from bacterial species completely excluded from the training dataset.

Additional investigations are necessary to eliminate the concentration dependence of the sensor response. Like in other reported analogous systems,^{34,41} this does not constitute a critical drawback, but overcoming it would be a significant step towards a simpler device for field applications. Lack of the ability of the sensor arrays to recognize components in mixed systems is one of their major disadvantages in comparison with selective single-analyte sensors, and new detection and signal processing approaches can potentially help solving this problem. Uncovering the details of the polymeric nanoparticles interaction with the bacterial cell walls constitutes a whole other research direction. It would not only help improving the sensor design, but will be a significant step forward in understanding of the dynamics at the bio-nano interface, which is important for development of improved antibiotic nanoformulations and other applications. Transitioning from sensing in bulk solution to microchannel or solid-based platforms will take additional steps to bring the proposed sensor closer to the actual application.

Overall, the multichannel ratiometric sensor array described in this paper is based on the environmentally-sensitive small-molecule fluorophores that do not require special handling and can be obtained through relatively simple synthetic procedures. Together with its ability to answer multiple questions beyond simple phenotyping using the same dataset, this approach provides new knowledge important for developing advanced, flexible and expandable sensor platforms for rapid and efficient detection of bacterial pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources

This work was funded in part by the Nebraska Research Initiative to AMM and research grants from the National Institutes of Health (P30 CA036727 (Fred & Pamela Buffett Cancer Center), 1S10RR17846, and 1S10RR027940) and the National Institute of Allergy and Infectious Diseases (P01 AI83211 and R01 AI125589 to KWB).

The authors would like to thank Nicholas Conoan of the Electron Microscopy Core Facility (EMCF) at the University of Nebraska Medical Center for technical assistance. The EMCF is supported by state funds from the Nebraska Research Initiative (NRI) and the University of Nebraska Foundation, and institutionally by the Office of the Vice Chancellor for Research. The authors acknowledge William Payne for technical assistance during preliminary experiments.

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Figure 1.

(A) Main protolytic equilibria and photochemical cycle of the 3-hydroxyflavone derivatives in ground and excited states; (B) experimental fluorescence spectrum of DMAF upon interaction with *S. aureus* (black line) and its computational deconvolution into components demonstrating emissions of normal (N*), anionic (A*) and tautomeric (T*) forms; (C) structures of 3-hydroxyflavone derivatives used as ratiometric reporters in the sensor array.

Figure 2.

Single-channel ratiometric response from the four sensor array components upon interaction with (A) Gram-positive and (B) Gram-negative bacteria. Each response signal is an average of 12 replicate measurements. (C) Canonical plot of the discriminant analysis of the sensor response using first two discriminants. Each class (bacterial species) contains 12 experimental points representing replicate measurements. Each experimental point is an average of 5 independent measurements. Ellipses represent 95% confidence areas.

Figure 3.
Canonical plot of the single-channel ratiometric response from the four sensor components in the subspace of the first three discriminants.

Figure 4.

Dual-channel ratiometric response (T*/N* channel – solid bars, A*/N* channel – dashed bars) from the four sensor array components upon interaction with (A) Gram-positive and (B) Gram-negative bacteria. Each response signal is an average of 12 replicate measurements. (C) Canonical plot of the discriminant analysis of the sensor response using first two discriminants. Each class (bacterial species) consists of 12 experimental points representing replicate measurements. Each experimental point is an average of 5 independent measurements. Ellipses represent 95% confidence areas.

Figure 5. Recognition of the Gram status of the bacterial species using the discriminant analysis of the sensor array dual-channel responses.

Figure 6. Discrimination of a single bacterial species against all other species using the discriminant analysis of the sensor array dual-channel responses. Results for the Gram-positive bacteria are presented in the top row, and for Gram-negative bacteria are presented in the bottom row.