Bacterium-Induced Fluorescence-Enhancement Kinetics: Breaking 100-Year Old Traditions of Staining Bioanalyses

Elizabeth Zielins, Valentine I. Vullev
Graduate Student Assistant: Marlon Thomas
Department of Bioengineering
University of California, Riverside

A B S T R A C T

The virulence and increasing antibiotic resistance of certain bacterial strains creates a need for efficient and timely detection of environmental pathogens. We evaluate the kinetics of the fluorescence enhancement of cationic dyes as an assay for differentiation between bacterial species. For several benzothiazole cationic dyes, such as 3-3'-diethylthiacyanine, we observed fluorescence enhancement in the presence of vegetative bacteria and bacterial spores. Different bacterial species manifested different rates of emission enhancement. Although staining, particularly fluorescence staining, has been a broadly used technique for the identification of bacterial species, the kinetics of the staining process has not been examined in detail. Analyses of the kinetics of emission enhancement for a series of fluorophores in the presence of one species of bacteria (or spore) can be used to create a set of kinetic parameters specific to that bacterial type. We hypothesized that these kinetic parameters can be utilized as “fingerprints” for detection and identification of bacterial species. We used three different vegetative bacteria and three different bacterial spores as model organisms for the collection of preliminary data that demonstrated the feasibility of our hypothesis. Conducting kinetic emission assays with various concentrations of bacteria and fluorophores allowed us to determine the first order time constants of the kinetics of emission enhancement. These time constants reflect the migration of the dye from the surrounding media to the fluorogenic microenvironment within the bacterial cell wall. Furthermore, we observed that the time constants were concentration independent and species specific.

F A C U L T Y M E N T O R

Valentine I. Vullev
Department of Engineering
The research in my laboratory is cross-disciplinary and involves students with different backgrounds and interests. Utilizing our expertise in spectroscopy for addressing fundamental issues and developing tools for microbiology resulted in the project described in this particular publication. Due to her experience and strong background in cell and microbiology, Elizabeth Zielins was a perfect fit for this project. Under the close supervision and mentoring from Marlon S. Thomas (a second-year graduate student and coauthor of this paper) and Duoduo Bao (a first year-graduate student), Elizabeth expediently expanded her skills and knowledge into the areas of spectroscopy and photophysics. Her talent, intelligence and perseverance, indeed, helped Elizabeth rise to the occasion. With Marlon Thomas, Elizabeth Zielins formed a “power team” that brought this project to its current stage in about six months.
Introduction

This paper describes kinetic studies on the fluorescence enhancement of 3,3'-diethylthiacyanine (THIA) in the presence of six different bacterial species. Currently, one quarter of human deaths worldwide are caused by bacterial infections. In the United States in the early 2000s, 33 million illnesses were caused by foodborne bacteria alone. Of these illnesses, 10,000 were fatal\textsuperscript{1-2}. In the meantime, patients are often treated with inappropriate antibiotics.\textsuperscript{5} Besides having a neutral effect on the patient’s health, such treatments may lead to the buildup of antibiotic-resistant bacterial strains. For example, the percentage of healthcare-associated \textit{Staphylococcus aureus} (staph) infections caused by Methicillin-resistant \textit{Staphylococcus aureus} (MRSA) has risen from 2\% in 1974, to 64\% in 2008.\textsuperscript{1,2,6-8}

In the modern healthcare setting, techniques used to identify unknown bacterial strains range from biochemically-based methods such as real-time PCR, DNA sequencing, and immunostaining, to methods in cell staining.\textsuperscript{9,10} While cell staining is perhaps the most economical and likely to produce expedient results, current methods in cell staining (including fluorescence assays) are inherently limited: (1) they yield only a Boolean outcome; and (2) they detect only species that are sought for.\textsuperscript{3,11}

Ever since the development of the Gram stains (more than 100 years ago),\textsuperscript{12,13} the identification of bacterial species using staining techniques has been based solely on the initial and final appearance of the cells (i.e., before and after the staining process). Hence, the staining analyses produce only Boolean outcomes: i.e., the reagents either stain (positive) or do not stain (negative) the analyzed bacteria. Gram staining may identify a bacterial species as gram positive. Further testing, however, is required to determine which gram positive species the sample belongs to. The general (Gram) staining tests dictate the types of further analyses (with increased specificity) which can be used for identification of the bacterial species. Due to insufficient specificity of the initial staining tests, however, the choices for the set of analyses strongly depend on the pathologist’s intuition. As a result, only species for which one is looking are finally identified, leaving key determining factors of the diagnosis quite susceptible to human error.

Herein we present the development of cost-efficient assays for expedient analysis of bacterial samples, utilizing the kinetics of fluorescence enhancement upon staining. Certain cationic dyes manifest enhanced fluorescence upon binding to bacterial spores or vegetative bacteria.\textsuperscript{14} Our findings revealed that for six different bacterial species, each had different kinetic signatures. Moreover, with the method we describe, the presence of unknown species can be detected even if their kinetic signatures are not associated with any of the previously investigated bacteria (whose kinetics have been characterized).

Such specificity can be achieved due to the fact that the kinetics of emission enhancement reflects the migration of dye molecules from the aqueous solvent to the fluorogenic microenvironment within the bacterial cell walls. The increased fluorescence of the bacterium-bound dye could be due to either the polarity or the viscosity of the microenvironment. Our photophysical studies indicated that the observed emission enhancement is a result of migration of the dye from the relatively non-viscous aqueous media to the viscous environment of the cell wall.

Results and Discussion

A symmetric cyanine dye, 3,3'-diethylthiacyanine (THIA), manifests orders of magnitude increase in its fluorescence when in the presence of bacterial spores or vegetative bacteria (Figure 1). At the same time, the presence of bacteria does not cause wavelength shift in the
absorption maxima of THIA (data not shown), indicating that ground-state phenomena are not responsible for the observed fluorescence enhancement through alterations in $A$ (eq. 1).

We investigated the dependence of the fluorescence quantum yield, $\Phi_f$, of THIA in solvents with various polarities and viscosities (Table 1). The quantum yield did not exhibit dependence on the dielectric properties of the solvents: the correlation coefficient for $\Phi$ vs. $\varepsilon$ was $-7.5 \times 10^{-3}$ (Figure 2a). The correlation coefficient $r$ reflects the interdependence of the two quantities. A strong correlation (dependence) results in a value of $r$ close to 1 or -1. A lack of correlation results in $r$ having a value close to zero.

We did, however, observe a three orders of magnitude increase in $\Phi_f$ for THIA in glycerol compared with other solvents that have more than 100 times smaller viscosities. Indeed, the correlation coefficient for $\Phi_f$ vs. $\eta$ was close to unity (Figure 2b), indicating a strong dependence of the fluorescence properties of THIA on the viscosity of the surrounding media.

From these findings, we can therefore conclude that the reason for the observed fluorescence enhancement of THIA in the presence of bacterial species is due to an increase in the viscosity of the microenvironment of the dye. As the free dye in solution binds to the bacteria, it migrates from the relatively non-viscous aqueous media to the gel-like microenvironment of the cell wall—which has a significantly higher viscosity (Scheme 1). The fluorogenic effect of the viscous microenvironment could be due to suppression of the non-radiative decay processes resultant from molecular vibrational and rotational

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### Table 1.

<table>
<thead>
<tr>
<th>solvent</th>
<th>$\Phi_f \times 10^3$</th>
<th>$\varepsilon$</th>
<th>$\eta_0$/cP</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td>0.975</td>
<td>81</td>
<td>0.89</td>
</tr>
<tr>
<td>70% methanol</td>
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<td>1.3</td>
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<td>glycerol</td>
<td>141</td>
<td>43</td>
<td>930</td>
</tr>
<tr>
<td>methanol</td>
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<td>0.55</td>
</tr>
<tr>
<td>iso-propanol</td>
<td>0.634</td>
<td>20</td>
<td>2.3</td>
</tr>
</tbody>
</table>

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**Figure 2.** Linear correlation between the fluorescence quantum yield, $\Phi_f$, of THIA and (a) dielectric constant, $\varepsilon$, and (b) the viscosity, $\eta$, of the solvent media, with the corresponding correlation coefficients. The distortion of the linear fit is a result from the logarithmic representation, introduced for convenient visualization of the spread among the data points.

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**Scheme 1.** Interaction between THIA (structure shown) and a bacterial cell causing the emission enhancement.
The structure of THIA shows flexible bonds within the π-conjugation between the two ring systems (Scheme 1). Binding to a rigid microenvironment impedes molecular motions normally allowed by bond flexibility. Consequentially, radiative (fluorescence) processes become the predominant pathways for decay of the lowest singlet excited state.

A principal goal of our research was to investigate the dynamics of the fluorescence enhancement of THIA induced by bacterial species. The addition of bacteria to a solution of THIA produces a fluorescence increase in the time domain of seconds and minutes (Figure 3). Monoexponential fits of the kinetics data allowed us to extract the time constants, τ, of the emission-enhancement processes:

\[ F(t) = F_\infty \left( 1 - \exp\left( -\frac{(t - t_0)}{\tau} \right) \right) + F_0 \]  

Here, \( F \) is the measured fluorescence intensity over time, \( t \); \( F_\infty \) is the initial fluorescence intensity of the dye without bacteria; \( F_0 \) is the increase in the fluorescence intensity; \( t_0 \) is a delay time; and \( \tau \) is the time constant for the exponential decay of the fluorescence enhancement process.
intensity (due to the bacteria); and $t_0$ is the time of injection of the bacteria into the dye solution.

We investigated the interaction of THIA with six bacterial species: three vegetative bacteria ($B. sphaericus$, $B. subtilis$, and $E. coli$) and three bacterial spores ($B. globigii$, $B. pimulis$, and $B. thuringiensis$). Each vegetative bacterium species exhibited unique emission-enhancement time constants, regardless of its Gram-stain classification (Figure 4a). Bacterial spores manifested similar segregation in the values of their emission enhancement time constants (Figure 4b).

Furthermore, the measured time constants did not show significant dependence on the dye concentration or cell density of the samples (Table 2), though the characteristics of fluorescence enhancement appear to be most discernable when 6.43µM THIA is used. It is also clear that, aside from limiting whether or not the data can be fit with a kinetic function, bacterial concentration does not significantly affect the values of the time constants.

An inspection of Figure 4 and Table 2 shows that the time constants for each vegetative bacterium species fall within distinct ranges: i.e., for $E. coli$, $15 < \tau < 20$ s; for $B. subtilis$, $5 < \tau < 11$ s; for $B. sphaericus$, $30 < \tau < 40$ s. We ascribe the observed differences in the time constants to the different composition of the cell walls of the vegetative bacteria.

Although the variations in the compositions of the spore coats for different species are not truly substantial,\textsuperscript{17-19} the values of the time constants for the bacterial spores still fell within different regions: i.e., for $B. globigii$, $75 < \tau < 85$ s; for $B. pimulis$, $15 < \tau < 20$ s; and for $B. thuringiensis$ $10 < \tau < 15$ s. It should be emphasized that the time constant values for $B. pimulis$ and $E. coli$ appear to be within the same region. This overlap, however, is most probably coincidental because there is no similarity between the $E. coli$ cell wall and the $B. pimulis$ spore coat.\textsuperscript{17-24}

An ANOVA analysis (two-factor with replication) was performed on the data in order to test whether the measured time constants have a significant dependence on the cell densities of the bacterial species, and the concentrations of dye. The first “null” hypothesis states that the time constants for the different bacterial species are the same, while the second “null” hypothesis states that the time constants for the different dye concentrations are the same. For our tests, we set a confidence level of 95% or $p=0.050$. The ANOVA analysis generated three p-values: the first and the second p-values are for the first and second null hypotheses, respectively. The third p-value represents the interaction between the first two parameters. The p-value for the first null hypothesis was $5.66 \times 10^{-46}$, which is considerably smaller than $p = 0.050$, indicating that the time constants for different bacteria are unique. The p-value for the second null hypothesis was 0.12, which is greater than $p=0.050$, suggesting that the time constants do not significantly depend on the dye concentration. The interaction between the two parameters, bacterial species and dye concentration, however, gives a p-value of 0.0020. This is an additional indication of the uniqueness of the time constants for each bacterial species, at a given dye concentration.

Conclusions

We demonstrated that the kinetics of fluorescence staining with a cyanine dye, THIA, is species-specific and does not show concentration dependence. We believe that the kinetics of the staining processes will produce “fingerprint” features for detection and identification of bacterial species. Our findings on the media-dependence of THIA fluorescence indicate that viscosity-sensitive dyes which stain bacteria will allow for the development and expansion of emission-enhancement methodology for detection and identification of microorganisms.

Materials and Methods

A solution of 643µM THIA was prepared by dissolving solid THIA in a 70% ethanol solution. The solutions for the bacterial analyses were prepared via 10x, 100x, and 1000x dilutions of the stock dye solution in 2mM Tris buffer (pH 8.5).

Bacterial cultures of two gram positive species, $Bacillus subtilis$ and $Bacillus sphaericus$, and one gram negative species, $Escherichia coli$, were prepared on solid, Luria broth agar media. Colonies from these cultures were transferred to liquid media and allowed to grow for no longer than 24 hours. When not in use, both liquid and solid media stock solutions were stored in a -4°C refrigerator. For use in experimental measurements, fractions of the liquid cultures were centrifuged, washed twice, and re-suspended in 2mM Tris buffer solution.

Bacterial spores $Bacillus globigii$, $Bacillus pimulis$, $Bacillus sphaericus$, $Bacillus thuringiensis$, $Bacillus globigii$, $Bacillus pimulis$, and $B. thuringiensis$. Each vegetative bacterium species exhibited unique emission-enhancement time constants, regardless of its Gram-stain classification (Figure 4a). Bacterial spores manifested similar segregation in the values of their emission enhancement time constants (Figure 4b).
and *Bacillus thuringiensis*, were donated by the U.S. Army Research Laboratory and treated as we have previously described. For stock solutions, bacterial spores were suspended in 2mM aqueous Tris buffer, stored at 4°C and used within 24 hours (for prevention of germination). Prior to making spectroscopic measurements, 2µL TWEEN 40 was added to each stock solution in order to reduce the aggregation of the spores (in order to increase the homogeneity of the solution).

In order to determine the cell and spore density of the suspensions of vegetative bacteria and bacterial endospores, cell counts were performed using a hemocytometer at 40x magnification (transmission optical microscope).

Fluorescence cuvettes (four polished sides) were filled with 3mL of aqueous THIA solution (64.3µM, 6.43µM, and 643nM in Tris buffer) for absorption and emission measurements. 3µL, 30µL, or 300µL of bacterial suspensions in Tris buffer were added to the 3mL of dye solution in each cuvette. Due to differences in the bacteria concentrations of the liquid culture stock solutions, the final cell densities of the dye-bacteria solutions ranged from $10^5$ to $10^8$ cells/mL. For each bacterial species, nine measurements were conducted: i.e., the three amounts of bacterial suspensions were added to each dye concentration.

The absorption spectra were recorded using a UV/Vis spectrophotometer (Varian Cary 50 Bio), at a wavelength range between 350 and 750nm. All measurements were taken in 1cm plastic cuvettes. Absorption measurements were collected of all dye-bacteria solutions and blank (no bacteria) dye solutions.

The excitation and emission spectra, as well as the emission-enhancement kinetics, were recorded with a fluorescence spectrophotometer (Fluorolog 3-22). For the emission spectra and the kinetics measurements, the excitation wavelength was set near the absorption maximum of the dye, $\lambda_{ex} = 420$nm. Measurements of fluorescence intensity at the spectral maximum were collected over ten-minute periods for all samples. Control experiments with samples containing pure buffer, only THIA in buffer, and only bacteria in buffer were also conducted, allowing us to establish the baseline fluorescence (autofluorescence) of the Tris buffer solution and of the bacteria solutions in absence of dye. About 50 seconds into the ten-minute measurement period, 3, 30, or 300µL of bacterial suspension was added. In order to eliminate fluctuations in fluorescence intensity due to diffusion of the bacteria through the dye solution, the samples were continuously stirred during the measurements. The samples were also kept at about 37°C in order to prolong the viability of the bacteria. Emission, excitation, and absorption spectra of the dye-bacteria solutions were taken before and after the kinetics measurements. The kinetic measurements were repeated twice (with one-to-two month separations between the measurements).

From the fluorescence and absorption data, the quantum yield, $\Phi$, of THIA for different solvents (glycerol, methanol, 70% methanol, isopropanol, and water) was estimated:

$$\Phi = \Phi_0 \frac{S}{A} \frac{1 - 10^{-A}}{n^2}$$

where $S$ is the areas under the fluorescence spectra; $A$ is the absorption at the excitation wavelength; and $n$ is the medium index of refraction. The subscript “0” indicates the quantities for the fluorescence standard, coumarin 151 in ethanol, $\Phi_0 = 0.49$.

Quantification and analysis of the spectroscopic and quantum yield data was performed using IGOR and Microsoft Excel software.

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References


