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A Pattern Recognition Based Fluorescence Quenching Assay for the Detection and Identification of Nitrated Explosive Analytes

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Abstract: Herein we report a differential array of micelle-solubilized fluorophores for the detection and identification of small nitrated analytes, such as the explosives TNT, tetryl, RDX and HMX. The quenching ability of the analytes can be used to correlate their analyte identity, wherein the quenching patterns generated from the differential array are used in linear discriminant analysis (LDA). LDA results in a well-clustered two-dimensional plot, and a jack-knife analysis of the data suggests that this system can be used to identify unknown samples of analyte with 96% accuracy and with a detection limit of 19 μM.

Keywords: array sensing · fluorescence · linear discriminant analysis · micelles · nitrated explosives

Introduction

Despite useful roles in construction and demolition, nitrated explosives are most notorious for their use in military capacities and in terrorism campaigns. The presence of nitrated aromatic compounds such as TNT and tetryl, and nitramines such as RDX and HMX, is directly correlated with criminal intent or the presence of ordinances such as unexploded land mines or cluster bombs. As such, nitrated explosives constitute one of the most important molecular targets for the development of highly sensitive and inexpensive molecular sensors.

Numerous analytical methods for explosives detection have been developed[1] including chromatographic methods using UV,[2,3] indirect fluorescence,[4,5] and amperometric detection methods.[6] The use of fluorescence quenching in numerous polymers[7–9] and other solid-state media[10,11] has been extensively investigated. Many of these systems have been well refined and are quite powerful. Additionally, a biomimetic system was recently reported for the detection of dinitrotoluene,[12] and various mass spectrometry methods[13–15] for the detection of nitrated analytes are known.

Fewer studies have probed the non-chromatographic, spectrophotometric detection and differentiation of non-aromatic nitrated explosives. Andrew and Swager recently reported an impressive turn-on system for the fluorescence detection of RDX and PETN that also differentiates the two compounds.[16] Their system relies on the photooxidation of a zinc-coordinated acridine dye to a fluorescent acridinium species in the presence of RDX or PETN, but not TNT. Using this method, RDX and PETN were detectable at a 70 and 130 μM concentration, respectively. This system is one of the few examples of the direct detection of nitrated analytes.

Nitroaromatic and nitramine explosives such as TNT and RDX (see below) generally lack the basic and acidic functionalities that serve as "handles" in the design of selective supramolecular sensors. However, these compounds are known to quench the fluorescence of pyrene as well as other polyaromatic hydrocarbons (PAHs), and this quenching ability differs among compounds.[4] Therefore, by monitoring pyrene fluorescence it is possible to sense the presence of nitrated explosives. To enhance this quenching and hence the sensitivity of a quenching based detection assay, we thought that it would be possible to sequester the pyrene in micelles in order to promote interaction between the micelle-bound pyrene and the hydrophobic explosives. Using polyelectrolyte based micelles, Thayumanavan et al. have recently shown that non-selective electrostatic interactions in combination with covalently[17] or non-covalently[18] bound fluorophores are sufficient to differentiate between a number of biologically relevant proteins. An analogous con-

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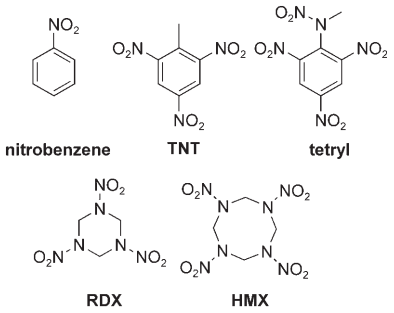
cept is also a well established method for determining the mean aggregation number of micelles,[19] though to our knowledge no one has pursued this idea as part of a sensing array using commercially available surfactants to sense small hydrophobic analytes.



We considered that by using a micellar solution, we would effectively concentrate the analytes in the hydrophobic portion of the solution (inside the micelle), and thereby provide three advantages to our sensing assay. First, the sensitivity of the assay should be improved because the analyte is more likely to reside in the micelle than in the bulk aqueous solution, and it is therefore more available to quench pyrene than its nominal concentration in the bulk surfactant solution would indicate. Second, while the pyrene is hydrophobic enough to be dissolved almost exclusively in the interior of the micelles,[20] the small, nitrated explosives are present to some degree in the aqueous medium. The differential hydrophobicity of the analytes implies that they will partition to different degrees between the micellar interior and the aqueous medium. This partitioning will affect their ability to quench pyrene, and act as another variable by which the analytes can be differentiated. Finally, pyrene is strongly quenched by molecular oxygen, an attribute that plagues its use in many sensing applications.[21] In the interior of a micelle, however, pyrene is known to be relatively insensitive to O_2,[20] making this micellar sensing assay amenable to routine bench top use without stringent methods to exclude O_2. With these postulates, we set forth to design an array for the detection and differentiation of the explosives.

Results and Discussion

Design criteria: The ability of nitrated compounds to quench the fluorescence of PAHs lessens as one goes from nitroaromatics to nitramines to nitroaliphatics. While the difference in quenching ability between classes is quite large, the differences within a class are subtler. To differentiate closely related species such as TNT and tetryl, or RDX and HMX, we applied our micelle/fluorophore system in an array format. By using solutions of pyrene, pyrene excimer, a pyrene–perylene fluorescence resonance energy transfer (FRET) pair, and a diphenylanthracene (DPA) solution, we created an array of fluorophores dissolved in an aqueous solution of the commercial, nonionic, polysorbate surfactant Tween 80. Different nitrated analytes produce distinct patterns of fluorescence quenching that are diagnostic for the presence of that particular explosive. These quenching patterns can be translated into two-dimensional plots using linear discriminant analysis (LDA). The location of an unknown analyte on these plots reveals its chemical identity. The use of pattern recognition protocols to translate supramolecular sensor output into qualitative and quantitative chemical information has become routine in recent years, with numerous examples from our own group, and from the supramolecular community at large.[22,23]

In designing our sensor array it was important to maintain the simplicity of the system while generating patterns of sufficient complexity to unequivocally identify the analytes. Pyrene was chosen because of its ability to be quenched by nitrated species. DPA is not well quenched by nitrated species, but its high quantum efficiency means that any excitation light intercepted by the UV absorbing nitroaromatics will result in an attenuation of DPA fluorescence. Such absorption is far outmatched by quenching in the case of pyrene. Beyond simple solutions of pyrene or DPA, a higher concentration excimer emitting solution of pyrene was also used as well as a pyrene to perylene fluorescence resonance energy transfer (FRET) system. The logic behind the selection of the last two fluorophore systems is discussed later.

The role of micelles: Before creating the sensor array it was important to confirm the principle that a micellar solution of fluorophore is indeed more sensitive to quenching by the nitrated analytes than an equivalent concentration of fluorophore in an organic solvent. To this end two analogous solutions of pyrene (20 μM) were titrated with TNT (4.4 mM in MeCN). In one case the pyrene was dissolved in MeCN while in the other the pyrene was dissolved in an aqueous 2 mM solution of the nonionic polysorbate surfactant Tween 80. While the absolute fluorescence and hence the absolute change in fluorescence were higher for pyrene in MeCN (Figure 1a), Stern–Volmer data clearly show a higher quenching efficiency in the micellar solution (Figure 1b). The non-linearity of the Stern–Volmer plot in the micellar solution implies that a static quenching complex is forming between TNT and pyrene prior to photoexcitation. An alternate hypothesis is that pyrene is being forced from solution nonlinearly by the incoming TNT, but when pyrene absorbance was monitored by UV/Vis during the titration it was found to be stable. We propose that this complex is the result of pyrene's exclusive residency in the micelles, and the analyte's propensity to gather there as well, creating a high effective concentration of the quencher near the fluorophore.

Evidence of a similar assembly phenomenon is found in the pyrene surfactant solution itself. According to published values, the concentration of micelles in a 2 mM Tween 80 solution is 34 μM[24] indicating a ratio of 0.59 pyrene molecules per micelle. Indeed, at this concentration some pyrene exci-

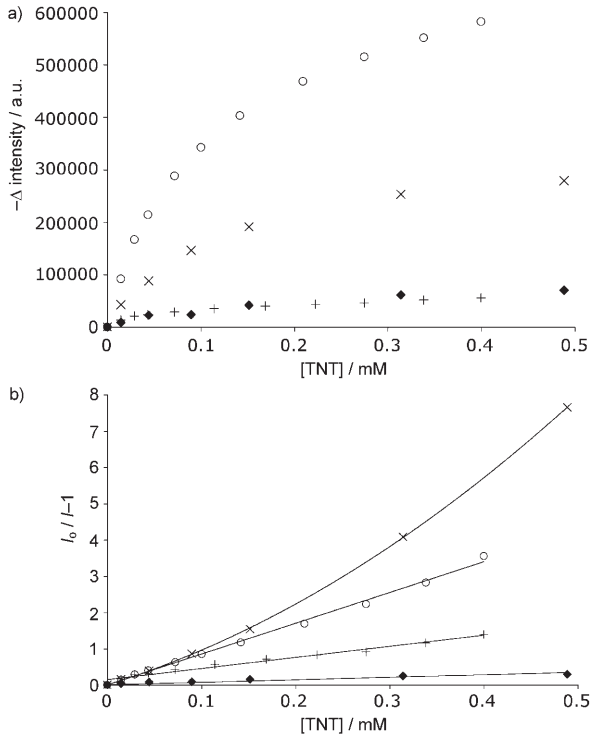


Figure 1. a) Quenching of pyrene monomer emission (λ_{max} 372 nm). ×: TNT into pyrene (20 μM) in Tween 80 (2 mM, aq.); ○: TNT into pyrene (20 μM) in MeCN (anaerobic); +: TNT into pyrene (20 μM) in MeCN (aerobic); ◆: MeCN into pyrene (20 μM) in Tween 80 (2 mM, aq.), as a control. Note the inversion of sign along the *y* axis. a.u. = arbitrary units. b) Stern–Volmer plots of the titration data shown in Figure 1a. I_o/I = original intensity over intensity after addition of quencher.

mer emission is already apparent, and at higher pyrene concentrations in the 2 mM Tween 80 solution, pyrene monomer emission continues to give way to excimer emission (Figure 2), a phenomenon not seen in MeCN until pyrene concentration reaches ≈1 mM.[25] The low concentration excimer formation is further evidence of the small habitable

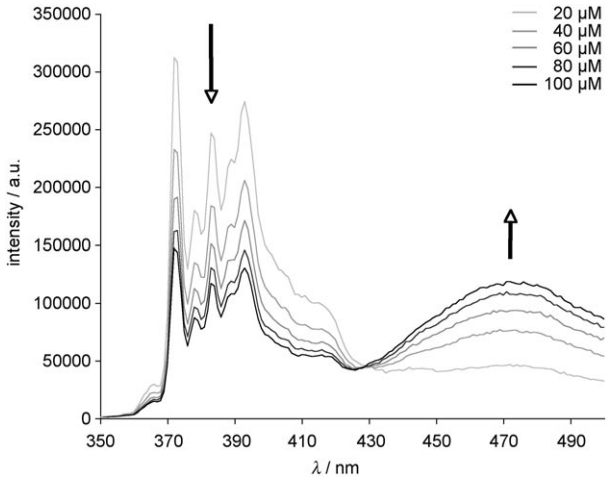


Figure 2. Pyrene excimer formation in Tween 80 (2 mM, aq.). Excitation λ_{max} increases slightly with pyrene concentration from λ_{max} (20 μM)/336 nm to λ_{max} (100 μM)/342 nm.

volume for hydrophobes in the micellar solution. It should be pointed out that while pyrene resides in the hydrophobic interior of the micelle, smaller compounds such as TNT and RDX are expected to exist primarily at the micelle–water interface, migrating inward only as their concentration increases.[20] Despite the different micellar solvation modes, the pyrene and nitrated explosives are proximal and quenching is promoted in the micellar solutions as evidenced by the Stern–Volmer plot.

Besides enhanced quenching, a second advantage of micellar solutions of pyrene is the protection of pyrene from quenching by adventitious oxygen. When TNT is added to pyrene in MeCN in the presence of oxygen (Figure 1b), the Stern–Volmer plot indicates a markedly weaker sensitivity to the presence of the nitroaromatic analyte. Figure 3 illustrates the rapidity with which a rigorously anaerobic solution of pyrene in MeCN can become quenched by atmospheric oxygen. Differential O_2 exposure across a 96-well plate during assay preparation would skew quenching profiles for the nitrated analytes, thus making pattern recognition impossible.

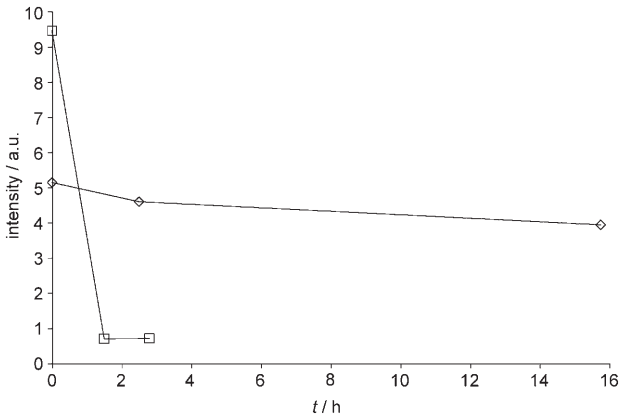


Figure 3. Oxygen quenching of pyrene (20 μM) over time (emission λ_{max} 372 nm) in ◇: Tween 80 (2 mM, aq.) and □: MeCN.

Pyrene response: Figure 4a shows differential quenching of pyrene by a series of nitrated compounds. Nitroaromatics are distinctly better quenchers of pyrene fluorescence than the nitramine compounds, and it is possible to differentiate singly, triply and quadruply nitrated aromatics (nitrobenzene, TNT and tetryl) at the ≈0.1 mM point of the titration. RDX and HMX, however, are hardly distinguishable from each other even late in the titration data. In fact, at the micromolar concentrations at which an effective explosives sensor must operate, all of the analytes show highly similar signals. Therefore, the fluorescence modulation of a pyrene–micelle solution alone is insufficient to reliably sense and differentiate the explosive compounds at low concentrations. However, Stern–Volmer plots (Figure 4b) reveal the fundamental differences in the quenching behavior of these nitrated species. Hence, we postulated that a series of subtly dif-

ferential sensors would eventually result in a distinct pattern that is diagnostic for single analytes even at low concentrations. We thus set out to develop a series of fluorophore solutions that would amplify and elaborate the subtle differences between minimally diverse nitrated analytes.

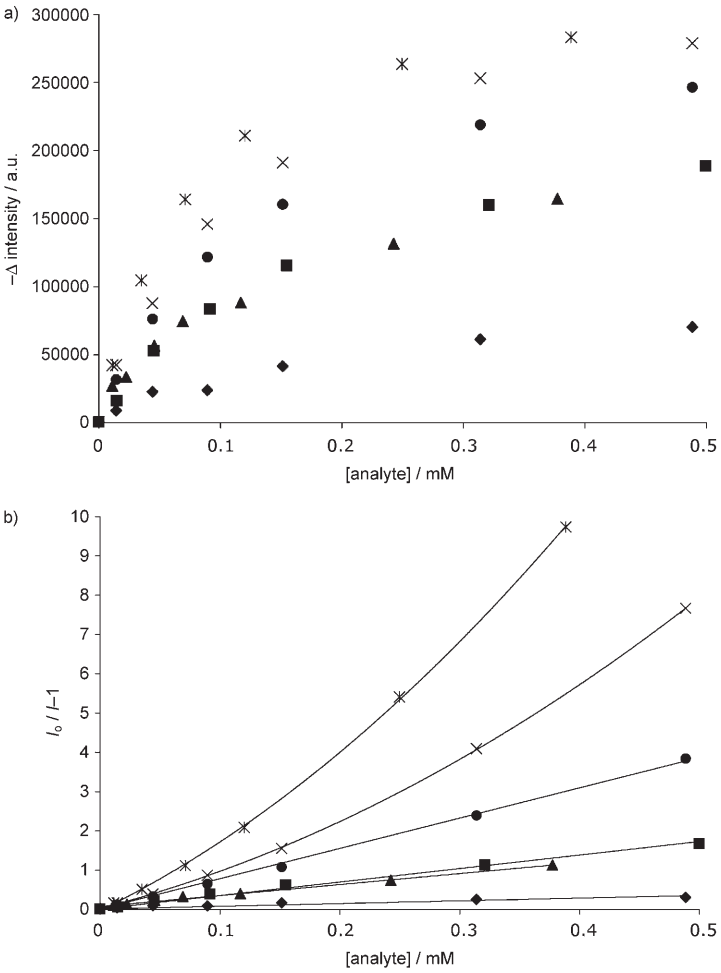


Figure 4. a) Quenching of pyrene (20 μM) in Tween 80 (2 mM, aq.) by nitrated analytes (emission λ_{max} 372 nm). *: Tetryl; ×: TNT; ●: nitrobenzene; ■: RDX; ▲: HMX; ◆: MeCN, as a control. Note the inversion of sign along the *y* axis. b) Stern–Volmer plots of the titration data shown in Figure 4a.

Pyrene excimer response: According to Focsaneanu and Scaiano, pyrene monomer emission and pyrene excimer emission are quenched in different ratios by different nitrated species in MeCN.[25] We confirmed that this is also the case in Tween 80 micelle solution. RDX and TNT show diverging ratios of monomer and excimer emission as their concentrations increase (Figure 5). Such ratiometric quenching is detectable in the 96-well plate reader by reading emissions through two separate filters. The sample (fluorophore solution in the presence of analyte) is excited by light passed through a 340/11 nm bandwidth filter, then emission readings are taken from a 380/20 nm filter and a 460/40 nm filter, bandwidths corresponding roughly to pyrene monomer and excimer emissions (Figure 6). Along with an analyte's ability to quench pyrene monomer fluorescence as a function of concentration, the array also includes the diagnostic ratio of monomer and excimer emission. Clearly the addition of this data will lead to further separation of TNT and RDX.

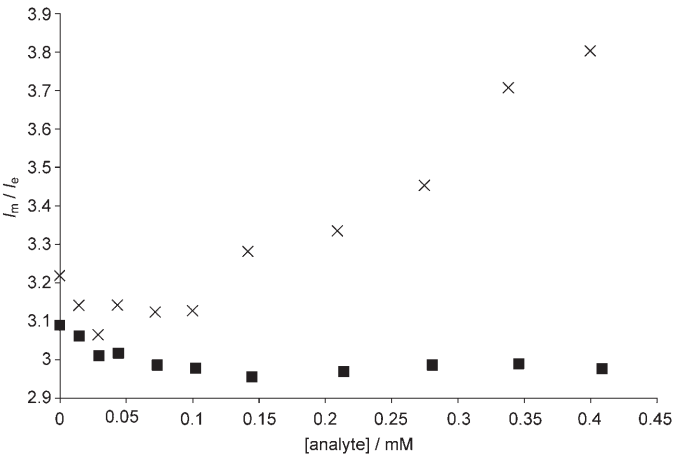


Figure 5. Ratio of monomer (emission λ_{max} 372 nm) over excimer (emission λ_{max} 470 nm) intensity for pyrene (20 μM) in Tween 80 (2 mM, aq.) as a function of ×: TNT and ■: RDX concentration.

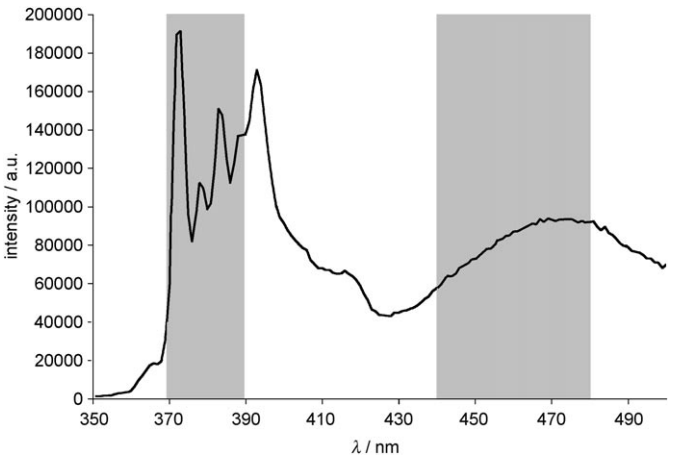


Figure 6. Pyrene (60 μM) in Tween 80 (2 mM). Illustration of the emission filter bandwidths of the fluorescence intensity measurement in the 96-well assay plate reader.

Pyrene–perylene FRET pair: We were inspired by the ratiometric quenching of pyrene monomer and excimer emission, and thereby lead to explore an analogous system of dual emission using a pyrene–perylene FRET pair. When pyrene and perylene are co-dissolved in a micellar solution, excitation of pyrene results in some relaxation via emission of pyrene monomer along with some FRET to excite perylene which then relaxes *via* its own emission (Figure 7, topmost spectrum). Titration studies of this system revealed that while the ratio of pyrene and perylene emission does change

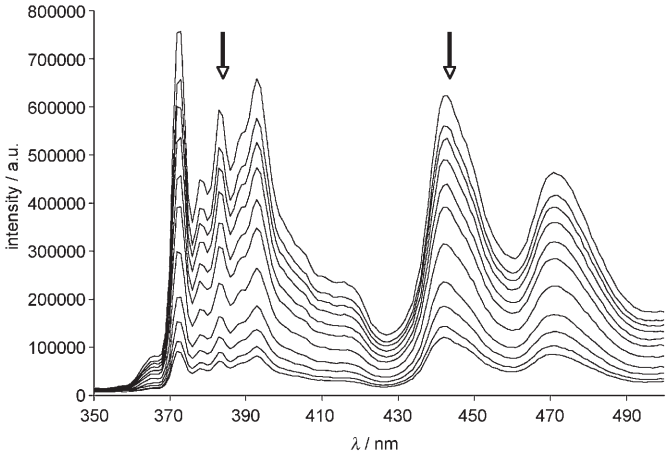


Figure 7. Emission spectra of pyrene (20 μM) and perylene (20 μM) in Tween 80 (2 mM, aq.) (excitation λ_{max} 336 nm). Pyrene monomer and perylene FRET emissions are quenched as the concentration of TNT increases from 0 mM to 0.4 mM.

as the two signals are quenched by a nitrated analyte, this change in ratio was the same for RDX and HMX. Although the FRET system did not display ratiometric quenching, it does represent an efficient transformation of data from the ultraviolet to the visible region because of the high perylene emission within the 460/40 nm bandwidth, and therefore opens the possibility for future naked eye detection systems. The FRET system was therefore included in the 96-well array sensor.

Diphenylanthracene response: By far the simplest of the fluorophore systems we used is that containing diphenylanthracene. Attenuation of the DPA signal upon addition of TNT is nearly linear (not shown), and is therefore due to simple absorption of excitation light by TNT in the UV. This conclusion is further supported by the observation that the non-chromophores RDX and HMX show a negligible suppression of DPA fluorescence. While one may argue that it would be much easier to measure TNT's absorbance directly, the sensitivity of fluorescence measurements is inherently higher than that of absorbance. Additionally, as mentioned previously in the case of perylene, transformation of ultraviolet absorbance information into the visible spectrum (emission λ_{max} (DPA)/411 nm) holds potential for future naked eye detection systems.

The sensor array: The sensor array consists of a series of 2 mM Tween 80 solutions with varying fluorophores that all undergo fluorescence attenuation when exposed to nitrated analytes. Those solutions are: a low concentration pyrene solution (20 μM) exhibiting predominately monomer fluorescence, a more concentrated pyrene solution (60 μM) with marked excimer fluorescence, a FRET displaying pyrene (20 μM) and perylene (20 μM) solution, and a DPA (20 μM) solution whose fluorescence attenuation is linked to the absorption of incident light by the nitroaromatics. These four solutions were treated with nitrated analytes and their fluorescence emissions observed over the two bandwidths discussed previously. In the case of the DPA solution only one bandwidth was useful and so a total of seven variables were submitted to LDA. Using this method, 48 samples were correctly classified as belonging to one of six classes at a final analyte concentration of 19 μM. Cross-validation, or jackknife analysis, was 96% accurate in predicting the identity of initially omitted observations. Figure 8 shows analyte clustering and the 95% confidence ellipse for each grouping.

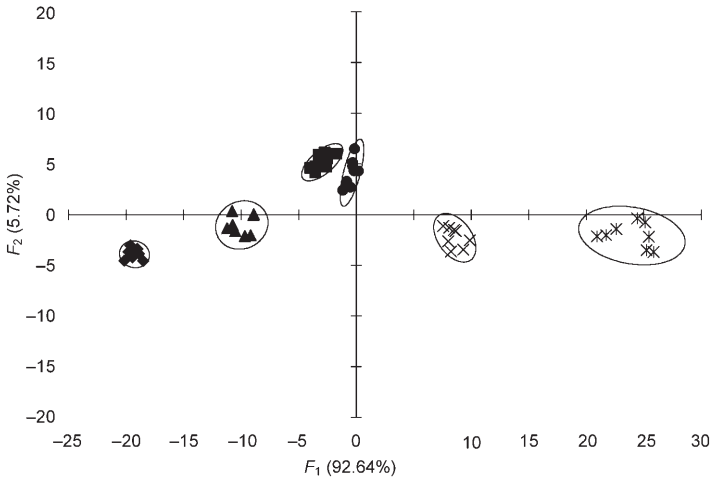


Figure 8. LDA plot of 96-well plate assay. Analytes are at 19 μM concentration. *: Tetryl; ×: nitrobenzene; ●: RDX; ▲: HMX: ◆: MeCN.

Similar 96-well assays with analyte concentrations of 1.9 μM were markedly less successful at sensing and differentiating the nitrated compounds, and therefore 19 μM was considered to be the limit of detection.

Conclusion

We have presented a powerful and relatively inexpensive sensor design for nitrated organic explosives. The well known ability of these analytes to quench pyrene fluorescence was parlayed into a series of similar systems including ratiometric sensing using the pyrene excimer, a pyrene–perylene FRET pair, and a simple DPA signal attenuation due to UV absorption by aromatic analytes. Combining these fluorophore solutions in an array, and examining the fluorescence over two bandwidths resulted in a "fingerprint" for each analyte that allowed it to be classified according to its molecular identity using LDA. The sensor detects these explosive compounds with good sensitivity (19 μM), and also differentiates between highly similar structures such as RDX and HMX. The modular nature of this array means that it is expandable as alternative surfactants and fluorophores are considered for this application, and we are pursuing this line of research to enhance the scope and sensitivity of this method.

Experimental Section

Materials and methods: Pyrene (98%), perylene (99+%), nitrobenzene (99%), and Tween 80 were obtained from Acros Organics and used without further purification. Diphenylanthracene (98%) was obtained from Sigma-Aldrich and used without further purification. DMSO was obtained from Fisher Scientific and used without further purification, while MeCN (Optima grade) was obtained from Fisher Scientific and degassed prior to use via bubbling N_2 for 1 h. TNT (4.40 mM), tetryl (3.48 mM), RDX (4.50 mM) and HMX (3.38 mM) were all obtained as 1000 $\mu g mL^{-1}$ MeCN solutions in sealed glass vials from Ultra Scientific. Water was distilled, deionized and filtered prior to use.

Single-cuvette fluorescence measurements were made using a Photon Technology International QuantaMaster spectrofluorimeter. 96-well plate fluorescence intensity data was gathered using a Biotek Synergy 2 Multi-detection Microplate Reader.

Fluorescence titrations: Fluorophores were dissolved in DMSO near their solubility limits (pyrene, 200 mM; perylene, 20 mM DPA, 10 mM) and added to 2 mM Tween 80 in water to affect the desired final concentration of fluorophore.

A sample titration is described. A cuvette was prepared with 3 mL of an aqueous solution of pyrene (20 μM) in Tween 80 (2 mM). This solution was excited with 336 nm light (pyrene excitation wavelength varied with solvent but emission maxima were consistently observed at 372 nm) and an emission spectrum recorded. An aliquot of TNT (4.4 mM) in MeCN was added, and after thorough mixing the emission spectrum at 336 nm excitation was again recorded. This was repeated until 0.3 mL of the MeCN solution had been added. The fluorescence at the emission maximum (372 nm) for each spectrum was then plotted against the corresponding TNT concentration.

96-well plate experiments: Two 96-well assay plates (Costar, #3632) having 8 rows and 12 columns were used to develop the plot seen in Figure 8. Each of the four Tween 80/fluorophore solutions detailed earlier was added to four contiguous rows of a plate resulting in two plates each of which contained two types of fluorophore solution. The wells of a plate were filled to 300 μL with three 100 μL aliquots of fluorophore solution using a Biotek Precision Microplate Pipetting System.

The five nitrated analyte solutions and the MeCN blank were added to the columns of the plate so that each analyte resided in two of the 12 columns. In this way, eight samples of each analyte existed for each of the fluorophore solutions. The nitrated analyte solutions were made by adding 3 μmol of analyte dissolved in MeCN (1000 $\mu g mL^{-1}$; neat MeCN in the case of the blank) to a 10 mL volumetric flask, and then adding MeCN to standardize the MeCN volume in each solution at 1 mL. The volumetric flask was then filled to 10 mL with an aqueous 2 mM Tween 80 solution for an analyte concentration of 0.3 mM. Using the microplate pipetting system, the explosive analyte solutions were added in a single 20 μL aliquot to the wells for a final in-well analyte concentration of 19 μM.

The 96-well assay plate was then submitted to measurements of fluorescence intensity. The fluorophore solutions were excited using a tungsten light source with a 340/11 bandwidth filter. Two readings of the emission radiation were taken: 1) the emission radiation was passed through a 380/20 bandwidth filter and read from a top 50% optics position with a sensitivity of 45, and 2) the emission radiation was passed through a 460/40 bandwidth filter and read from a top 400 nm optics position with a sensitivity of 45. In the case of the DPA solution only the 460/40 nm filter was used.

Data processing: Data processing was done using XLSTAT (version 2007.6). Fluorescence intensity data was transformed by standardization using unbiased standard deviation ($n-1$). The transformed data was then processed using linear discriminant analysis (LDA) to produce Figure 8. Cross-validation was performed using the leave-one-out method commonly known as the jack-knife method.

Acknowledgements

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