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EDGE ARTICLE

Exploration of plasticizer and plastic explosive detection and differentiation with serum albumin cross-reactive arrays†

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Plastic explosives, such as Semtex and C4, are commonly used explosive mixtures. The differentiation and detection of the plasticizers within these mixtures could provide information for anti-terrorism and combat activities. In this study, we demonstrate a strategy of using cross-reactive serum albumin proteins to differentiate and detect the plasticizers found within these explosive mixtures. With our sensing ensemble, comprised of serum albumins, fluorescent indicators and an additive, we successfully classified the five plasticizers found within Semtex and C4 using linear discriminate analysis, and differentiated simulated Semtex and C4 mixtures based on surrogates of the explosive material(s) and the plasticizer composition in these samples. Finally, we have shown the utility of this type of cross-reactive array for real life use in a battlefield setting by examining these mixtures in the presence of soil contamination.

Introduction

Due to the recent increase in terrorism activity and the need for improved homeland security, there is a growing interest in advancing methods for explosive detection.^{1,2} In addition, overseas military endeavours require the need for a facile method to detect and differentiate explosive materials in the context of the battlefield.² There are a plethora of methods used for both bulk and trace explosive detection.^{1,3,4} For trace explosive detection, the primary methods depend upon various forms of chromatography and spectrometry.⁴ The superb canine olfactory senses have also been exploited for such detection, although with some difficulties.^{3,4} In addition, several methods have been reported to utilize colorimetric and fluorescent techniques.³ The number of methods developed for explosive detection is fairly expansive and the vast majority of these methods target the detection of the most common explosive molecules, including: TNT (2,4,6-trinitrotoluene), RDX (1,3,5-trinitro-1,3,5-triazacyclohexane), PETN (pentaerythritol tetranitrate), HMX (1,3,5,7-tetranitro-1,3,5,7-tetrazacyclooctane), EGDN (ethylene glycol dinitrate), AN (ammonium nitrate) and NC (nitrocellulose).^{4–6} Explosive mixtures, however, are far less studied.

The commonly employed and widely known plastic explosive mixtures, Semtex and C4, are known to be relatively easy to use and the components required to prepare these mixtures are readily available, making these explosives popular choices for

terrorist and combat activities.⁷ Although targeting the explosive compounds in these mixtures is logical for detection and differentiation, methods that target the less prevalent compounds in the mixtures, such as plasticizers, may lead to additional information regarding the identity and source of the explosive mixture, specifically because some plasticizers are less accessible in certain countries due to associated health regulations.[‡] Thus, because certain plasticizers are forbidden in specific countries, the presence or lack of these plasticizers may help to identify the geographic origin of these explosive mixtures. Their detection could potentially complement the other well-known explosive detection systems.

Therefore, the primary and fundamental goal was to study whether plastic explosive mixtures can be differentiated by their minor plasticizer composition, to enable the uncovering of further information about the mixtures being tested. In particular, we were interested in developing an assay to detect and differentiate the plasticizers found in the following explosive mixtures: Semtex 1A, Semtex 2P, Semtex H and C4. The Semtex mixtures each contain differing proportions of PETN, RDX, a styrene-butadiene binder, a Sudan dye, plasticizers and in Semtex 1A an antioxidant. C4 mixtures contain RDX, polyisobutylene, motor oil and plasticizers.

Semtex and C4 are termed plastic explosives due to the presence of plasticizers within the explosive mixture, amounting to roughly 10% of the composition.⁷ Plasticizers are usually polyesters of linear or branched aliphatic alcohols and are found in many aspects of our everyday life. They are present in building products, wall papers, medical devices, toys, sealants, rubber materials and paints.⁸ When plasticizers are incorporated into explosives they stabilize the mixture against accidental detonation and preserve the explosive from weathering conditions.⁸

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Three plasticizers are known to exist within Semtex explosive mixtures, (phthalate **P4**, di-*n*-octylphthalate **P5**, and tri-*n*-butyl citrate **P1**) and two plasticizers within C4 explosive mixtures (dioctyl adipate **P3** and dioctyl sebacate **P2**) (Fig. 1).^{9,10} These plasticizers lack functional handles or chemical functional groups to target for detection and differentiation purposes. Additionally, these plasticizers, with the exception of **P4**, are very aliphatic in nature, making them highly hydrophobic molecules. For this reason, we hypothesized that the detection and differentiation of plasticizers and plastic explosives could be achieved by utilizing arrays of cross-reactive serum albumins.

Serum albumin is a 66 kDa protein found in plasma that binds hydrophobic molecules.¹¹ One of the primary roles of serum albumin within the body is the binding and transportation of fatty acids.¹¹ A large number of endogenous compounds are known to bind to serum albumin with fairly large binding constants, including long-chain fatty acids ($K_a = 10^6$ – 10^7 M⁻¹),¹² bile acids ($K_a = 10^3$ M⁻¹),¹³ steroids ($K_a = 10^3$ – 10^5 M⁻¹),^{14–16} bilirubin ($K_a = 10^7$ M⁻¹),¹⁷ L-thyroxine ($K_a = 10^6$ M⁻¹),¹⁸ and vitamins D and B-12 ($K_a = 10^4$ – 10^5 M⁻¹ and 10^7 M⁻¹, respectively).^{19,20} In addition, numerous exogenous fluorescent indicators, pharmaceutical drugs and toxins have been found to bind to this protein.¹¹ Specifically, a very recent study has shown that several plasticizers, which pose severe health-related hazards, bind to human serum albumin.²¹ Serum albumins are also known to have significant differences in sequence identity between species. For example, human serum albumin and bovine serum albumin have a 24% difference in amino acid sequence.¹¹ For these reasons, we have previously studied the use of serum albumins as receptors for the detection and differentiation of individual hydrophobic molecules and hydrophobic molecules within complex mixtures. For example, we demonstrated that it is possible to pattern both terpenes and fatty acids, as well as terpenes in perfumes and fatty acids in edible oils.^{22,23} Thus, a second fundamental goal of this work was to further broaden the scope of cross-reactive arrays of serum albumins, ultimately with the goal of showing that the approach could be applied generally to any class of hydrophobic molecules.

Supramolecular chemists have recently employed the use of cross-reactive arrays for sensing purposes, where receptors differentially bind a wide range of analytes.^{24–30} The inspiration for differential sensing arises from the mammalian olfactory system, in which there are a finite number of receptors that differentially bind molecules in the vapour phase.³¹ The differential response results in a pattern stored in the brain which is recalled as a specific odour. Experimentally, to create a pattern from differential sensors we turn to statistical analysis

techniques, such as linear discriminant analysis (LDA). LDA is a multivariate analysis tool that reduces the dimensionality of a data set, with the aim of clustering the data such that maximum differentiation is seen between analyte classes, while minimum differentiation is seen within each analyte class.^{32–34} LDA is a supervised model, meaning that the program is originally told the analyte class for each data point. For this reason, a validation technique called a jack-knife analysis is commonly reported with each LDA plot as a means of testing the predictability of the model.³²

Results and discussion

Cuvette assay with dansyl amide indicator

Our sensing ensemble consists of two serum albumins, human and bovine (HSA and BSA, respectively), fluorescent indicators and a dopant molecule to help increase analyte differentiation. To choose the appropriate fluorescent indicator we screened a variety of fluorophores (6-propionyl-2-dimethylaminonaphthalene, 5(6)-carboxyfluorescein, 1,8-anilinonaphthalene sulfonic acid, dansyl proline, dansyl amide) that bind to serum albumin, and examined whether the addition of plasticizer to a solution of the serum albumin and fluorescent indicator caused a fluorescence signal modulation. Our first successful indicator was dansyl amide, which has previously been studied as an indicator to categorize serum albumin Sudlow Site I binding and is known to have two binding sites on human serum albumin, with binding constants of 1.79×10^5 M⁻¹ and 6.99×10^3 M⁻¹.³⁵ Fig. 2A shows the fluorescence change upon binding of dansyl amide to BSA. Next, Fig. 2B was generated from the data in Fig. 2A and was used to find the concentration of dansyl amide at which approximately 90% saturation occurs, the percentage of indicator at which we have previously found to give an optimal signal for indicator displacement assays.³⁶ We then ran several fluorescence experiments titrating plasticizers into BSA and dansyl amide, for example, see Fig. 2C for the titration of **P1** into a solution of dansyl amide and BSA.

Developing the well plate array

Once we observed that the five plasticizers tested resulted in a unique fluorescent change for each plasticizer, we shifted our cuvette assay to a 96-well plate array, which allowed us to obtain the multiple data repetitions required for LDA. In the 96-well plate, 10% ethanol was added to each well to help solubilize the plasticizer in the solution, while preventing protein denaturation, which occurs at higher ethanol concentrations. We obtained an LDA plot with a jack-knife analysis of 100% from 96-well plate arrays with BSA/dansyl amide and HSA/dansyl amide, analysing all five plasticizers at a concentration of 375 μ M (Fig. 3A). This LDA plot shows **P5** and **P1** on the left of the graph, which correlates well with the most responsive plasticizers clustering on the left side of the graph. Both **P4** and **P2** cluster on the right side of the graph and thus represent the less responsive plasticizers. We hypothesize that **P4** is not very responsive to the sensing ensemble due to its more hydrophilic nature in comparison to the other plasticizers, thus decreasing the driving force of **P4** to bind to the hydrophobic binding sites on serum albumin where the dansyl

Fig. 1 Structures of the five plasticizer analytes, of which **P4**, **P5** and **P1** are found in Semtex and **P3** and **P2** are found in C4.

Fig. 2 A) Addition of BSA (0–400 μM) to dansyl amide (30 μM) in 10 mM phosphate buffer, H_2O , pH 7.00, 0.02% sodium azide, $\lambda_{\text{ex}} = 350 \text{ nm}$. B) Binding isotherm that corresponds to the data in (A) at $\lambda_{\text{em}} = 495 \text{ nm}$. C) Addition of **P1** (tributyl citrate) in EtOH (0–500 μM) to BSA (150 μM), dansyl amide (30 μM) in 10 mM phosphate buffer, H_2O , pH 7.00, 0.02% sodium azide, $\lambda_{\text{ex}} = 350 \text{ nm}$, overall EtOH volume = 0.2%.

indicators reside. **P2**, on the other hand, is the longest and largest plasticizer molecule of the group, and we propose that **P2** may not fit as well into the dansyl indicator serum albumin binding pockets as some of the smaller plasticizers.

Increasing array differentiation with ascorbic acid

Although we were content with our results in Fig. 3A as a first step in discriminating plasticizers with our array, we sought to increase the differentiation in the F2 axis of the LDA plot so that we could obtain a secondary discriminatory axis which carries more weight to enable better differentiation of more complex samples, such as analyte mixtures. To increase differentiation along the F2 axis, we screened a variety of compounds that are known to bind to serum albumin (stearic acid, cholic acid, deoxycholic acid, ascorbic acid) that could be added to the assay to differentially modify the fluorescent signal for each plasticizer. Through our screening, we found that the addition of ascorbic acid (K_a of 10^4 M^{-1} to serum albumin)³⁷ to our assay led to an increase in the F2 axis of the LDA plot from 9% to 14% with a jack-knife analysis of 100% (Fig. 3B). This plot shows a greater

Fig. 3 LDA plots of data collected from 96-well plates with fluorescence parameters of $\lambda_{\text{ex}} 340\text{--}380 \text{ nm}$ and $\lambda_{\text{ex}} 465\text{--}505 \text{ nm}$. The array components consisted of A) bovine and human serum albumins (150 μM), dansyl amide (30 μM), and plasticizers (375 μM) in phosphate buffer with 10% ethanol and B) bovine and human serum albumins (150 μM), dansyl amide (30 μM), plasticizers (375 μM) and ascorbic acid (2250 μM) in phosphate buffer with 10% ethanol.

amount of discrimination along the F2 axis and thus leads to further differentiation of **P1** from the other plasticizers. We see a significant change in the ordering of the plasticizer analytes in the graphs, specifically in the placement of **P2**. The placement of **P2** in the middle of the LDA plot is now indicative of an intermediate response, potentially occurring because the ascorbic acid dopant leads to better binding of this analyte within the serum albumin hydrophobic binding site.

Differentiation of plastic explosive mixtures

Having successfully differentiated the five main plasticizers found within Semtex and C4, we investigated whether our assay could be used to differentiate these plasticizers within their respective explosive mixtures. The compositions of both Semtex and C4 contain a large percentage of explosive material. To ensure our safety during this research, we thought it prudent to find appropriate explosive surrogates for RDX (found in C4 and Semtex) and PETN (found in Semtex) which would mimic the physical and electronic properties of these explosives,

without actually being explosive. As our surrogates, we used 2,4-diamino-1,3,5-triazine as a replacement for RDX and pentaerythritol tetraacetate in place of PETN.³⁸ Next, we found the composition make-ups of three versions of Semtex (Semtex 1A, Semtex H, and Semtex 2P) and the composition make-up of C4. We then made solutions that represented these composition percentages and subsequently took these solutions to dryness and re-dissolved them in the assay solvent system. We either added no plasticizer, **P1**, **P4**, or **P5** for each of the Semtex mimics, and we added no plasticizer, **P2**, or **P3** for the C4 mimics. We consequently made four different samples for each Semtex 1A, Semtex H and Semtex P mixture and three different samples for the C4 mixture. These solutions were then directly used as analytes in the well plate assay consisting of combinations of BSA, HSA, dansyl amide and ascorbic acid. The data from this assay was statistically analysed and an LDA plot was obtained with a jack-knife analysis of 93.3% (Fig. 4A). Fig. 4A shows distinctive separation of the mixtures into their mixture categories, with the C4 and Semtex 1A mixtures located on the

left side of the F1 axis and the Semtex 2P and H mixtures located to the right side.

With each Semtex mixture class, we note that the clusters with no plasticizer (*i.e.*, just the explosive mixture background) and the clusters with **P4** are located closest to each other. This is consistent with the earlier results that show **P4** having little impact on the indicator signal modification of the sensing ensemble, again due to the lack of hydrophobic driving force for the binding of **P4** by serum albumin. There is no clear trend with plasticizer order for each explosive mixture or we see most prominently that the sensing ensemble is best able to distinguish the large differences between explosive mixture categories, with small amounts of differentiation seen according to the plasticizer composition within the mixtures. Thus, our assay best discriminates explosive mixtures by plasticizer composition when making comparisons within the same category, but is still capable of providing significant differentiation by plasticizer identity when explosive mixture categories are considered. In this study, the concentration of plasticizers within these mixtures is approximately 7–80 μM . We should note, however, that the array could potentially be optimized to obtain lower detection limits, by adding more discriminating variables to the assay.

Optimizing the array by adding dansyl cadaverine indicator

Although the LDA plot in Fig. 4A shows clear discrimination of the explosive mixtures into their corresponding mixture category (*i.e.*, C4, Semtex 1A, Semtex 2P, and Semtex H mixture category), there is considerable overlap between the explosive mixtures within each category, where the mixtures only differ by which plasticizer (if any) is added. To further aid in discrimination, we screened several more indicators for the assay (anthracene carboxylic acid, *p*-nitrobenzofurazan-fatty acid, *p*-nitrobenzofurazan-monoacylglycerol, dansyl cadaverine). Our search led us to the indicator dansyl cadaverine. Interestingly, this indicator has the same dansyl core that exists in dansyl amide, however, it possesses a positive charge at neutral pH conditions, giving it differing properties from the dansyl amide indicator. After first studying the binding of dansyl cadaverine to serum albumin, we introduced dansyl cadaverine to our array. The addition of dansyl cadaverine to the array resulted in a third axis (F3), which carried enough discriminatory weight to allow us to obtain a 3D plot with a jack-knife analysis of 98.9%. The 3D plot shown in Fig. 4B adds a layer of topography, which accounts for further plasticizer differentiation within each explosive mixture class. In particular, we see improved discrimination within the C4 and Semtex 2P mixtures. Additionally, the loading plot shown in Fig. 5 uncovers the significant receptor performance of the BSA–dansyl cadaverine, BSA–dansyl cadaverine–ascorbic acid, and HSA–dansyl cadaverine–ascorbic acid receptors, accounting for the increased discriminatory power of the array. Furthermore, if individual LDA plots are run on each explosive mixture category (Fig. 6), we find very distinct discrimination between the plasticizers in the mixtures. Thus, the addition of dansyl cadaverine to the sensing ensemble improves the ability of the array to differentiate plasticizers contained within the explosive mixture classes.

Fig. 4 LDA plots of data collected from 96-well plates with fluorescence parameters of $\lambda_{\text{ex}} = 340\text{--}380\text{ nm}$ and $\lambda_{\text{em}} = 465\text{--}505\text{ nm}$. The array components consisted of A) bovine and human serum albumins (150 μM), dansyl amide (30 μM), explosive mixtures (C4 600 μM , Semtex 100 μM) and ascorbic acid (2250 μM) in phosphate buffer with 10% ethanol and B) bovine and human serum albumins (150 μM), dansyl amide (30 μM), dansyl cadaverine (30 μM), explosive mixtures (C4 600 μM , Semtex 100 μM) and ascorbic acid (2250 μM) in phosphate buffer.

Fig. 5 A loading plot corresponding to the LDA in Fig. 4B. The variables including dansyl cadaverine are highlighted in green to show the improved discriminatory power of the array with dansyl cadaverine.

Array reproducibility

We investigated the experimental reproducibility of this sensing assay by fully replicating the experiment in its entirety (see supporting information†). We found that the *x*-axis in the LDA plot was inverted for the reproduced results with respect to the original results (which has no physical meaning) and there was slightly less prominent clustering (jack-knife analysis of 95.6%). However, the data was similar to the original data and, thus, we feel confident that the assay reproducibly discriminates plastic explosive mixtures.

Differentiation of contaminated plastic explosive mixtures

Finally, as a first step toward accessing the utility of this sensing ensemble to discriminate explosive mixtures and the plasticizers within explosive mixtures in a real-life setting, we developed a protocol to analyse the mixtures in the presence of soil contaminates. To prepare the soil-contaminated samples, we took the previously made explosive mimics and mixed them with soil by adding each explosive mixture to a petri dish with 20 mL of ethanol and 10 g of soil. We then filtered the soil from the solution, took the solution down to dryness and re-dissolved the remaining residue in our solvent system. These 15 contaminated samples were then analysed by directly introducing them into an array consisting of BSA, HSA, dansyl amide, dansyl cadaverine and ascorbic acid. The ensuing LDA plot (Fig. 7), with a jack-knife analysis of 96.5%, shows the dirt-exposed samples in new locations within the plot. These contaminated samples (in blue dashed boxes) were always clustered to the left of their corresponding non-contaminated clusters (in red dashed boxes). The new location of these clusters is likely due to a combination of factors. First, there is a concentration change in the main responsive components in these mixtures – a consequence of how the contaminated *versus* non-contaminated samples were prepared – and second, there could be a change in the responsiveness of these components when in the presence of unknown contaminates found in soil. Although these dirty samples do carry a larger amount of noise (*i.e.* less tightly clustered replicates), it is important to note that our ensemble of receptors, indicators and an additive can still

Fig. 6 LDA plots obtained for the data from the following sensing ensemble: bovine and human serum albumins (150 μ M), dansyl amide (30 μ M), dansyl cadaverine (30 μ M) and ascorbic acid (2250 μ M) in phosphate buffer with 10% ethanol. A) Discrimination of C4 mixtures (600 μ M), 100% jack-knife analysis. B) Discrimination of Semtex 1A mixtures (100 μ M), 100% jack-knife analysis. C) Discrimination of Semtex H mixtures (100 μ M), 95.6% jack-knife analysis. D) Discrimination of Semtex 2P mixtures (100 μ M), 100% jack-knife analysis.

Fig. 7 LDA plot of data collected from 96-well plates with fluorescence parameters of λ_{ex} 340–380 nm and λ_{em} 465–505 nm. The array components consisted of bovine and human serum albumins (150 μM), dansyl amide (30 μM), dansyl cadaverine (30 μM), explosive mixtures (C4 600 μM , Semtex 100 μM), contaminated explosive mixtures (C4 600 μM , Semtex 100 μM) and ascorbic acid (2250 μM) in phosphate buffer with 10% ethanol.

discriminate small differences in composition, even in the presence of soil, which could otherwise potentially destroy the performance of our assay.

Conclusions

In conclusion, this study shows that differential arrays of serum albumins, an additive and indicators can differentiate plasticizers, even when they are the minor components in explosive mixtures. These results, coupled with our previous studies on terpenes in perfume and fatty acids in edible oils, supports the generality of this sensing approach. Thus, the method has the promise to classify other small differences in complex mixtures based upon the content and structure of their hydrophobic components.

Looking to the future of this particular sensing system, the ability to differentiate plastic explosive mixtures according to their explosive mixture class and plasticizer composition has the possibility to uncover the identity of the explosives, as well as its geographic origin. Furthermore, the sensing array was shown to successfully respond in the presence of soil contaminants, although with a shift in the patterns from pure samples. Admittedly, other contaminants would also likely lead to a shift in the patterns, yet with a retention of the differentiating power of the array. We are currently exploring the robustness of the 96-well plates as potential kits for field use.

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Notes and references

- ‡ Ref. 8 details the health regulations associated with certain plasticizers in the USA. Other regulations presumably exist in other countries.
- § To make our Semtex and C4 mixtures, we found compositions of these explosive mixtures which were widely available on the internet to the public and therefore are most likely to be used by potential terrorists. We did, however, check these compositions with more reliable sources (see ref. 9 and 10) to ensure that the compositions we used were within a reasonable limit. The websites used were: Wikipedia Semtex <http://en.wikipedia.org/wiki/Semtex> (accessed Sept 8, 2010) and Wikipedia C-4 (explosive) http://en.wikipedia.org/wiki/C-4_explosive (accessed Sept 8, 2010).
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