An *in situ* amperometric biosensor for the detection of vapours from explosive compounds†

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An amperometric biosensor based on genetically-modified enzymes was used for the *in situ* detection of trace vapours from a number of explosive compounds. The vapour samples that were generated from a purpose-built vapour generator were collected and pre-concentrated using a trap able to concentrate samples at a rate of 60-fold per minute of sampling. The amperometric biosensor achieved a remarkably low vapour detection limit of 6 parts per trillion from a room temperature sample. The specific activity of the reported enzyme toward a number of explosive compounds was also confirmed using absorbance measurements.

Introduction

The concern about travellers carrying small quantities of hidden explosive material on commercial airlines has grown since the first airline incident attributed to explosives[1] in 1982. In addition to this, the ease of obtaining the information required to make explosives is particularly alarming. Yet, even five years after 9/11 there is still no emerging *in situ* technology in the area of explosives detection and diagnostics.[2,3]

There are two principal obstacles associated with the detection of explosives: firstly, the very low vapour pressures, which vary significantly from a few parts per trillion (ppt) to rarely more than a few parts per million (ppm) by mass,[4,5] which directly affect the amount of explosive available in the air for collection; and secondly, a high requirement for selectivity during detection, essential in order to avoid false positives.[6,7] Furthermore, current methods used to monitor luggage cannot be used for screening passengers due to health reasons.

We have recently reported on the use of an amperometric biosensor based on the orientationally controlled assembly of genetically-modified enzymes.[8] The sensor was tested using solution samples of dinitroethylbenzene (DNEB) and exhibited a reproducible and linear response in the range 100–500 ppt. Here, we report on two further key developments in this work relating to the *in situ* generation and detection of trace vapour levels from a number of explosive compounds.

In the work previously reported and in this work, the surface of an electrode is functionalised with an enzyme to obtain the desired selectivity. The enzyme utilised is a nitroreductase (NTR). The *nfnB* gene used expresses a dimeric NTR, with a subunit molecular mass of 24 kDa which is capable of the reduction, and hence the detection of nitroaromatics; each subunit contains a flavin mononucleotide (FMN), the isoalloxazine ring of which accepts electrons from a soluble co-factor nicotinamide adenine dinucleotide (NADH) and is the redox-active part of NTR.[9,10] Essentially, the function of the enzyme is to provide selectivity by virtue of its biological affinity for explosive compounds during the course of the enzymatic reaction; the explosive compounds are reduced by the enzyme, which in turn is oxidised. The active form of the enzyme, the reduced form, is regenerated using a soluble reduced co-factor, which associates with the enzyme in a transient manner, and is oxidised. The co-factor then diffuses from the active site and is reductively regenerated at the electrode surface (Fig. 1). The charge associated with the reduction step is directly related to the concentration of the nitroaromatic and can therefore be used to quantify it. The natural co-factor used in NTR reactions is NADH. The electrochemical reduction of NAD^+ to NADH, results in two isomers, only one of which may react with the enzyme.[11,12] Therefore, the amount of NADH reduces by half after every cycle.

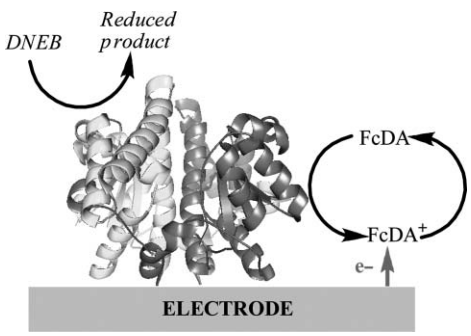

Fig. 1 General model showing the reduction of DNEB by the immobilised enzyme. The model also shows that the enzyme can be regenerated using a co-factor, in this case, ferrocene dicarboxylic acid (FcDA). The oxidised form of FcDA is then reduced at the surface of the electrode.

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Ferrocene derivatives have been previously used as alternative co-factors because of their solubility in water and their well defined redox behaviour.[8,13]

We have previously shown that ferrocene dicarboxylic acid (FcDA) can be used as an alternative co-factor in the electrochemical studies, and in combination with the genetically-modified NTR provides an ensemble that achieves an extremely low detection limit by virtue of the immobilisation process.[8] The NTRs are genetically modified to incorporate cysteine tags which are positioned to control the orientation of the immobilisation, enabling the enzymatic layer to be self-assembled directly on the gold surface, with no associated diffusion problems that are commonly associated with matrix immobilisation processes.[14]

Experimental

Cloning and expression

The cloning and purification procedure has been previously published.[8] All proteins were expressed from a PCR-generated wild-type *nfnB*[15] gene (Genbank accession number BAA05004, EC 1.5.1.34) from *Escherichia coli* K12 into expression vector pET28a(+).

UV-visible spectroscopy

Activity was assayed with NTR solution (*ca.* 1.0 $\mu M^{(Final)}$, 10 µl) in a 1 ml cuvette with tris-HCl buffer (50 mM, 500 µl, pH 7.1), NADH (1 mM, 100 µl), nitroaromatic compound (various concentrations), and FMN (1 mM, 5 µl), at 25 °C. Spectra were recorded using a Uvikon 943 double beam spectrophotometer at a scan rate of 500 nm min^{-1}, between 220 and 500 nm. The reference cuvette contained the same solution, but lacking the NTR enzyme. Assays to determine the reaction rate were run at 340 nm for 2 min each, against a blank lacking the substrate. The same experiments were also repeated with a blank cuvette containing solvent with no explosive compound to eliminate any solvent effect.

Vapour generation

The evaluation of vapour sensors requires the generation of vapours of an analyte at known concentrations. For this purpose, a vapour generator was employed to produce standardised vapours from substances in the liquid or solid phases. If designed appropriately, the output covers a wide range of concentrations and thus is ideal for low vapour pressure compounds such as nitroaromatics.[16,17] Vapour generators have been used in a range of applications from deodorant sprays to chemical vapour deposition in the coating industry.[18,19] However, generally these generators give no measure of control over the concentration of the vapour produced, which is of particular importance when considering systems to be used in detector development.

Here, a vapour generating system based on a modified design to that reported by Drinkwine and Cage[20] is used (Fig. 2). Since an aqueous environment is crucial for the enzymatic reactions and for the electrochemical transduction to take place,[21] a vapour trap is employed to transfer the sample from the vapour phase to a liquid phase.

The vapour generator consists of a carrier gas source, a purification unit, a cooling/heating unit, a mixing/dilution chamber, a humidity unit, and a manifold of outlet ports (for sampling, sensing, purging, *etc.*). Generation of vapour takes place inside the cooling/heating unit where up to two glass columns are kept at constant temperature and where the analytes are held. The columns are constructed from thin-walled (1 mm), silanised glass tubing packed with glass beads, or glass wool, which are coated with the analyte of interest.

The cooling/heating unit consists of an aluminium housing with built-in water circulation, encased within a layer of

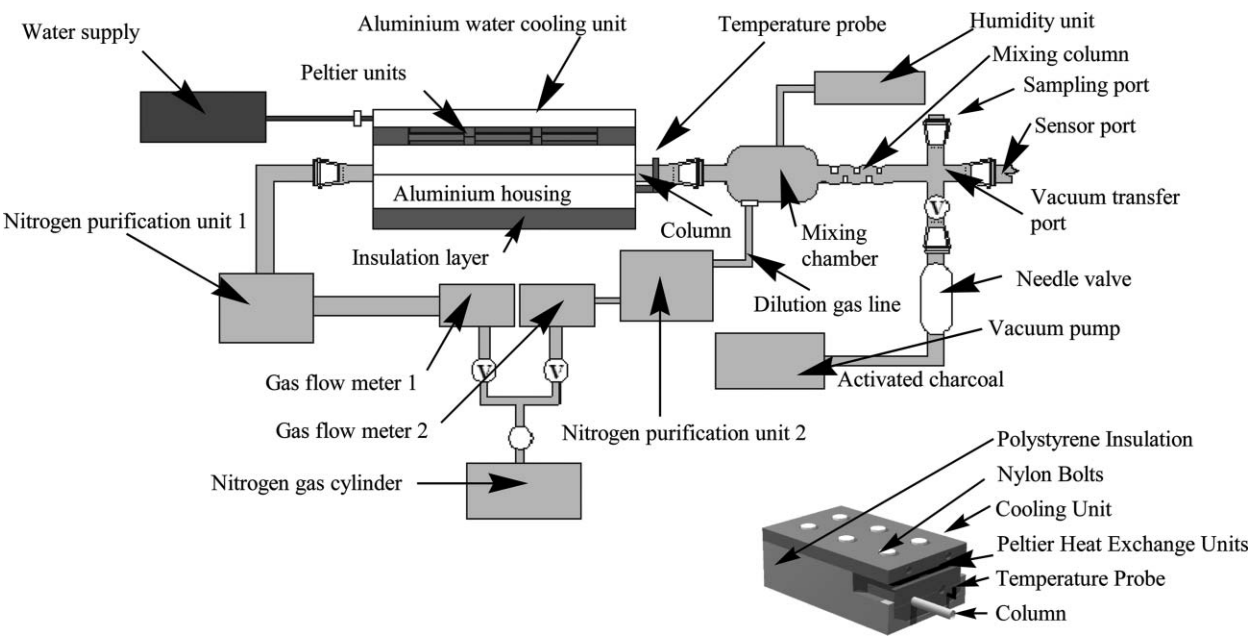

Fig. 2 Schematic diagram of the vapour generator. (Insert) Diagram of heating/cooling unit with one column in place.

insulation (polystyrene) to prevent temperature drifting. A set of three thermoelectric units govern heat transfer to or from the aluminium housing upon application of a current regulated by a control unit allowing temperature control within ±0.1 °C.[22]

The concentration of the vapour is regulated by the amount of compound held in the columns (using glass beads or glass wool), the flow rate of the carrier gas and the temperature of the columns. If dilution is employed, then the flow rate through the dilution line must also be taken into account.

The vapour stream is formed as the dry/purified carrier gas flows through the columns where the analyte is held, and carries it through to the mixing chamber. Here, if desired, dilution takes place before the stream is homogenised as it flows through a turbulence-inducing column and is finally directed to a manifold where sampling takes place.

Vapour collection

Samples of vapour were collected using a vapour trap connected to the sensor port of the vapour generator. The trap consisted of a cooled metallic tube (kept at 2 °C using a thermostated bath), through which a known volume of gaseous sample was passed at a fixed rate (30 cm^3 min^{-1}).

Owing to the low vapour pressure of the nitroaromatic compounds, deposition onto the walls of the trap occurs quickly. Following the condensation step, samples are eluted out with two aliquots (2 × 250 µl) of dimethyl sulfoxide (DMSO) and measured at the bioelectrochemical sensing chamber. The ratio of flow rate to volume of eluted solvent determines the pre-concentration factor during vapour collection. The concentration of the vapour was increased 60-fold per minute of collection (*e.g.* 30 cm^3 min^{-1}/0.5 ml = 60 min^{-1}). Dinitroethylbenzene (DNEB) was utilised as the example analyte. The concentration of DNEB in the vapour phase ($[DNEB]_{vapour}$) was determined from the concentration of DNEB in the liquid phase ($[DNEB]_{DMSO}$) using the equation:

$$[\mathrm{DNEB}]_{\mathrm{vapour}} = \frac{\left([\mathrm{DNEB}]_{\mathrm{DMSO}} \times \mathrm{vol}_{\mathrm{DMSO}}\right)}{\left(\text{flow rate} \times \text{sampling time}\right)}.$$

The collection efficiency was tested using two vapour traps that were connected in series to the sensor port output of the vapour generator. Each trap comprised a copper tube (inner diameter 1 mm, outer diameter 1.8 mm and 230 mm length). The dimensions of the vapour trap resulted from trials of several columns of varied lengths, inner diameter, and volume. It was found that thin columns (inner diameter 0.6 mm) were efficient at collecting nitroaromatics from vapour streams but were prone to blockage. The use of wide columns prevented blockage but collection of the nitroaromatic from vapour streams was not quantitative. Therefore, a flow-through trap must be of sufficient inner diameter to allow a continuous flow of gas but thin enough to quantitatively collect the analyte from the vapour stream flowing through it.

The temperature of the traps was kept between 1 and 2 °C to induce the deposition of the nitroaromatic, but was kept above 0 °C to avoid the formation of ice. If the collection efficiency is high, then the majority of the analyte should be retained in the first trap with a minimal amount escaping into the second. A high vapour concentration regime was used in the test (DNEB column set at 30 °C, carrier gas flow 30 cm^3 min^{-1}) with long collection times (5, 10 and 20 min). The contents of the traps were then analysed using HPLC. The data showed that trap 1 retained 98.5% after 5 min of sampling, 99.5% after 10 min and 99.8% after 20 min, implying an excellent efficiency for vapour collection.

HPLC

HPLC analysis of vapour samples collected in DMSO was performed using an Agilent 1100 series HPLC system, with degasser, quaternary pump, thermostated column oven, autosampler and diode array, and with Agilent Chemstation control and data processing software. The instrument was operated with a Waters Sherisorb ODS2 4.6 × 250 mm Analytical column. The mobile phase was methanol and water 50 : 50 and elution of DNEB occurred at 8 min.

Electrochemistry

All measurements were performed using a battery-powered, hand-held potentiostat and PC (Palm Instruments BV). The analysis was carried out with a three-electrode cell, using a saturated calomel reference electrode and a platinum counter electrode.

The working electrode was an enzymatically-modified gold-coated glass slide. A seal was made between the working electrode and the electrolyte solution with O-rings defining a geometric area of 0.6 cm^2. The solution in the cell contained potassium phosphate buffer pH 7.1 (20 ml, 0.1 M), mixed with the co-factor FcDA (2 mM). Additions of the substrate were made by pipetting through the top of the cell. All the chemicals used were of an analytical grade. Prior to the enzyme layer formation, the gold(111)-coated glass slides (Winkler GmbH, Germany) were flame-annealed in a Bunsen burner until they attained red heat several times. After cooling in air for a short time, the slide was quenched in water. This produces a flat gold surface with strong Au(111) characteristics.[23] The gold surface was modified by immersing the slide in a potassium phosphate buffer solution containing the enzyme, for a period of 12 h at 5 °C. After assembly, the modified electrode was rinsed with copious amounts of the buffer solution.

Results and discussion

UV-visible spectroscopic analysis

The enzymatic activity was first monitored in solution through the oxidation of NADH by measuring the change in its absorbance peak at 340 nm;[24] the explosive compounds and NTR did not significantly overlap the 340 nm region specific to NADH.

The initial studies on the activity of NfnB-cys_{12} have been published elsewhere.[8] Here, the comparison for the activities for different explosive compounds, Fig. 3, reveals the high degree of selectivity towards a broad range of explosives. Benzene has also been included to illustrate the selectivity of the enzyme towards the target analytes. In order to make such a comparison the concentration of all the explosives was kept identical (10 µM) so as to avoid any alterations in the comparison of the rates

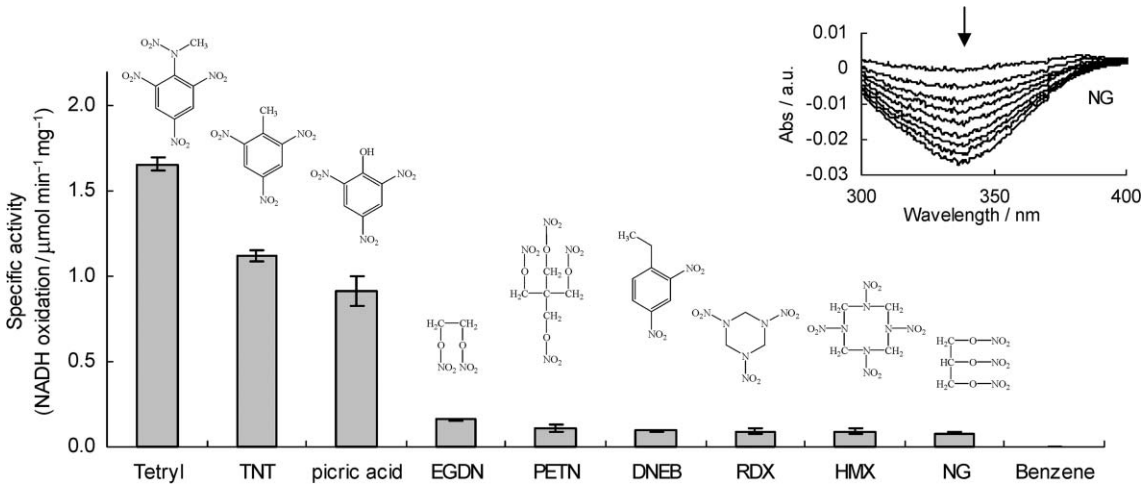

Fig. 3 Comparisons of the activity of NfnB-cys$_{12}$ against various analytes. The insert shows the UV-vis data resulting from the reaction of NfnB-cys$_{12}$ against NG, where each spectrum was recorded every minute (1–9 min). The error bars were calculated from the average of three sets of results obtained from three separate NTR expressions.

with changes in the Michaelis constant values. It is important to point out that despite what looks like a relatively low level of reactivity with compounds such as nitroglycerine (NG), the spectral response is measurable and is only dwarfed in the figure by the very large response from compounds such as TNT and Tetryl (Fig. 3, insert).

Surprisingly, the NTR exhibited a response similar in magnitude to cyclotetramethylene tetranitramine (high melting explosive, HMX), towards triacetone triperoxide (TATP) and hexamethylene triperoxide diamine (HMTD). We are currently trying to optimise this response.

The effect of temperature on the concentration of vapours

Vapours were generated using a constant stream of N_2 (30 cm^3 min^{-1}) over glass wool coated with 100 mg of DNEB. The output of the vapour generator was examined by varying the column temperature between 0 and 20 °C and, as expected, an increase in concentration with temperature was observed. The vapour concentration was calculated by recording the volume of gas passed through the trap and the mass of analyte collected. The mass of analyte collected was determined by HPLC analysis of the solutions resulting from elution of the trap with 500 µl DMSO against standard solutions of DNEB in DMSO. As expected, temperature is an important factor in controlling the concentration of vapours; indeed, it was found that for the same flow rate at temperatures between 0 and 20 °C the vapour concentration varied between 4 and 32 ppt (w/v) (Fig. 4). This variation is crucial when considering a design for an integrated system comprising sampling and sensing components.

Amperometric detection of the explosive compounds

The enzymatic activity of the immobilised enzyme was examined electrochemically by monitoring the current at a fixed potential of 100 mV *vs.* SCE. The potential was chosen so that any ferrocenium dicarboxylic acid produced during the regeneration of the active form of the enzyme is reduced back to ferrocene

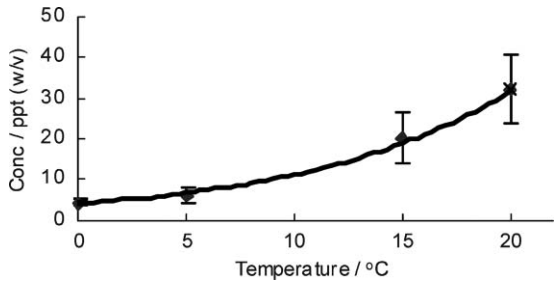

Fig. 4 Graph showing the influence of temperature on the concentration of DNEB in the vapour phase.

dicarboxylic acid. The current provides a direct measure of the amount of analyte present.

A solution sample containing DNEB (50 ppt) was obtained from the generation of a 6 ppt vapour which was collected for 5 min at room temperature.

Following injection of the analyte, Fig. 5, an increase in current caused by the disturbance of the sample being introduced to the cell is quickly followed by a sharp current drop of around 1 nA. This drop in current was not apparent in

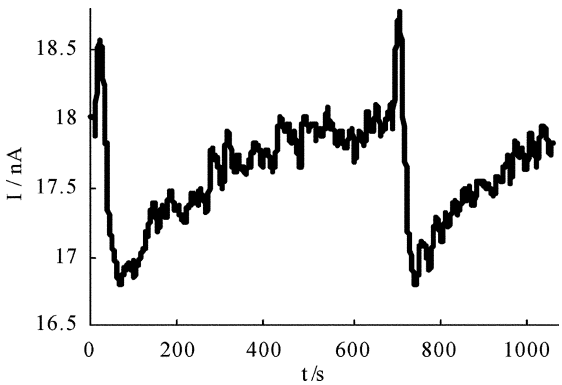

Fig. 5 Current transients obtained at a fixed potential of 100 mV after the addition DNEB [50 ppt (w/v)] at 0 and 700 s. All measurements were performed in phosphate buffer (pH 7.1, 0.1 M) and co-factor FcDA (2 mM) with an NfnB-cys$_{12}$-modified Au(111) electrode.

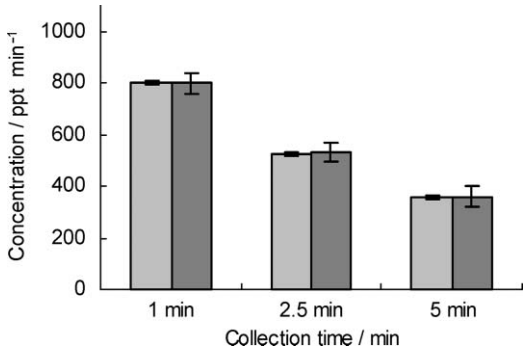

Fig. 6 Plot showing the concentration of DNEB vapours normalised against time as a function of sampling time. The data shown are for 13 ppt (1 min), 9 ppt (2.5 min) and 6 ppt (5 min). Key: [DNEB] measured by HPLC; [DNEB] measured using the biosensor and multiplied by the dilution factor. The errors were calculated from the standard deviation resulting from three sets of data.

the absence of the enzyme, FcDA, or the nitroaromatic. This drop is caused by the reduction at the electrode surface of the ferrocenium dicarboxylic acid that had been produced by the immobilised NTR. The current drop was followed by recovery to the steady state baseline, thus enabling successive measurements to be recorded. Injection of another sample was followed by a reproducible response in the current drop. This confirms that the sensor exhibits reproducible behaviour and, equally important, the potential for 're-usability'.

For practical use, the sensor needs to exhibit a response from various concentrations of the explosive materials in the vapour phase. To assess this, DNEB vapour streams of different concentrations were generated and samples were collected for different periods (13 ppt for 1 min, 9 ppt for 2.5 min, and 6 ppt for 5 min). The collected samples were then eluted as described in the experimental section. Detection of DNEB was carried out using both HPLC and the electrochemical biosensor. The concentration of DNEB in samples immediately after elution (HPLC) and as detected by the biosensor are shown together for direct comparison (Fig. 6). For the sake of clarity, the calculated concentrations were normalised against time. For practical purposes, the electrolyte volume in the electrochemical cell was 20 ml; therefore, the concentration of DNEB in the eluted samples was diluted 666-fold (30 µl aliquot of DNEB DMSO in 20 ml of electrolyte), and this has been taken into account in Fig. 6.

The data show striking similarities between the concentrations calculated using the amperometric biosensor and those calculated from HPLC and indeed confirm the viability of the biosensor as a tool for detecting explosives.

Conclusions

The utilisation of genetically-engineered enzymes used in conjunction with a vapour trap has enabled the detection of vapours from explosive compounds in the low ppt range. This successful demonstration of the amperometric biosensor for the detection of nitroaromatics can also be adapted for other analytes.

The main hurdle in its adaptation is the correct positioning of the cysteine amino acids within different protein structures.

A prototype hand-held unit (NanoDog), comprising a sampling system and the detector in a miniaturised form, is currently under development.

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