

'The Nano Dog': an *in situ* amperometric biosensor for the detection of vapours from explosive compounds

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5 *Receipt/Acceptance Data* [DO NOT ALTER/DELETE THIS TEXT]

Publication data [DO NOT ALTER/DELETE THIS TEXT]

DOI: 10.1039/b000000x [DO NOT ALTER/DELETE THIS TEXT]

The application of a novel biosensor ensemble enabling the *in situ* detection of trace vapours of explosive compounds is reported. A genetically engineered nitroreductase directly immobilised onto a gold electrode is combined with a novel vapour trap. Vapour samples from a purpose built vapour generator were collected and pre-concentrated using a trap able to concentrate samples 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.

Introduction

The concern about travellers carrying small quantities of hidden explosive material on commercial airlines has grown since the first airline incident attributed to explosives¹ 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 affects 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. Here, we report a key development in bridging this gap in technology by presenting an *in-situ* electrochemical biosensor for the detection of trace vapour levels of explosive compounds.

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 in this work expresses a dimeric NTR, with a subunit molecular mass of 24 kD, which is capable of the reduction, and hence 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.^{8,9}

The 'Nano-Dog'.

The detailed design of the sensor has been previously reported.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 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 cofactor, which associates with the enzyme in a transient manner, and is oxidised. The cofactor then diffuses from the active site and is reductively regenerated at the electrode surface. 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 cofactor 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} We have previously shown that ferrocene dicarboxylic acid (FcDA) can be used as an alternative co-enzyme in the electrochemical studies as it is soluble in water and exhibits well-defined reversible redox

behaviour.^{10,13}

The ensemble achieves its extremely low detection limit by virtue of the immobilisation process. 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 immobilisations.

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.^{14,15} Vapour generators have been used in a range of applications from deodorant sprays to chemical vapour deposition in the coating industry.^{16,17} 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 Cage¹⁸ is used. The 'Nano-Dog' ensemble comprises a sensor and a vapour trap that collects and pre-concentrates vapour samples. Since an aqueous environment is crucial for the enzymatic reactions and for the electrochemical transduction to take place,¹⁹ a vapour trap is employed to transfer the sample from the vapour phase to an aqueous phase. The combination of both novel sensor technology and vapour trapping, allows *in-situ* detection of explosive molecules in the parts per trillion (ppt) range directly from the vapour phase.²⁰

Experimental

Cloning and expression.

The cloning and purification procedure has been previously published.¹⁰ All proteins were expressed from a PCR-generated wild type *nfnB*²¹ gene (Genbank accession number BAA05004, EC 1.5.1.34) from *E.coli* K12 into expression vector pET28a(+).

UV visible spectroscopy.

Activity was assayed with NTR solution ($\approx 1.0 \mu\text{M}^{\text{(Final)}}$, 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 conc.), 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, between 220-500 nm. The reference cuvette contained the same solution, but lacking the NTR enzyme. Assays to determine 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

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cuvette containing solvent with no explosive compound to eliminate any solvent effect.

Vapour generation.

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 consist of an aluminium housing with built-in water circulation, encased within a layer of 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 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 finally directed to a manifold where sampling takes place.

Vapour collection.

Samples of vapour were collected using a novel vapour trap connected to the sensor port of the vapour generator. The trap consisted of a cooled metallic column (kept at 2 °C using a thermostated bath), through which a known volume of gaseous sample was pushed at a fixed rate (30 ml/min).

Due to the low vapour pressure of the nitroaromatic compound, deposition onto the walls of the trap occurs quickly. Following the condensation step, samples are eluted out with two aliquots (2 x 250 μ l) of dimethylsulfoxide (DMSO) and tested 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 ml.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 equation: [DNEB]_{vapour} = ([DNEB]_{DMSO} x vol_{DMSO})/(flow rate x sampling time).

HPLC.

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

Electrochemistry.

All measurements were performed using a battery-powered, handheld 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. Prior to use, the cell was cleaned using a 50:50 mixture of concentrated H₂SO₄:HNO₃ followed by rinsing in deionised water (nominal resistivity > 18 M Ω cm⁻¹ at 25 °C), cleaning in a steam bath, and drying in the oven.

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 cm². 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

Ultraviolet visible spectroscopy 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-cys₁₂ have been published elsewhere.¹⁰ Here, the comparison for the activities for different explosive compounds, fig 1, reveals the high degree of selectivity within a broad range of explosives. In order to make such a comparison the concentration of all the explosives were kept

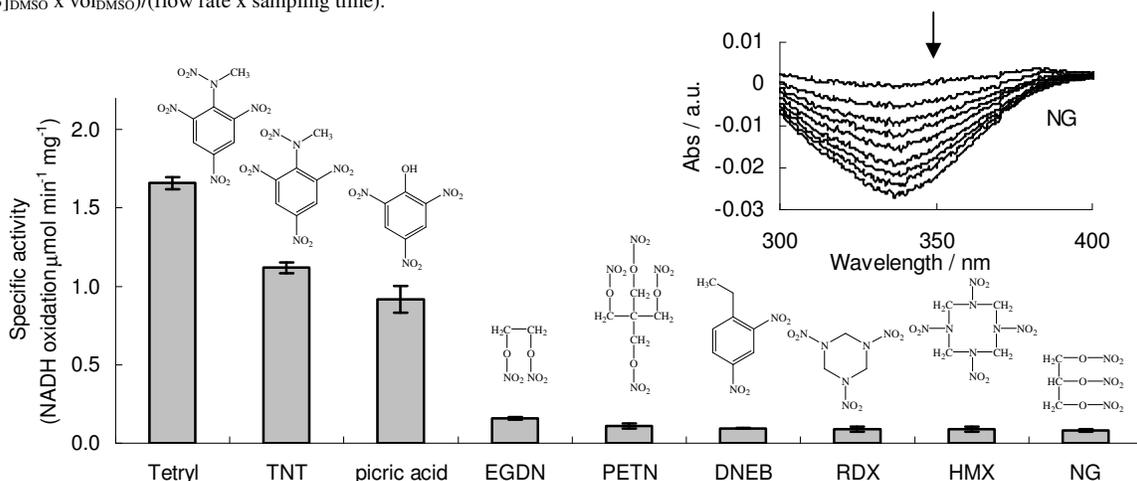


Figure 1 Comparisons of the activity of NfnB-cys₁₂ against various analytes. The insert is the UV-vis data resulting from the reaction of NfnB-cys₁₂ against NG, each spectrum corresponds to one min. The error bars were calculated from the average of three sets of results obtained from three separate NTR expressions.

identical (10 μM) so as to avoid any alterations in the comparison of the rates 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 1 insert).

Surprisingly, the NTR exhibited a response similar in magnitude to HMX, towards triacetone triperoxide (TATP) and hexamethylenetriperoxidodiamine (HMTD). We are currently trying to optimise this response.

Vapour generation.

Vapours were generated using a constant stream of N_2 (30 ml/l) over glass wool coated with 102 milligram of DNEB. The output of the vapour generator was accessed by varying the column temperature between 0 $^\circ\text{C}$ and 20 $^\circ\text{C}$ and the expected increase in concentration with temperature was observed. Vapour concentration was calculated by recording the volume of gas pushed 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 $^\circ\text{C}$ and 20 $^\circ\text{C}$ vapour concentration varied between 4 and 32 ppt (w/v).

Vapour collection.

The design of the vapour trap resulted from trials of several columns of varied lengths, inner diameter, and volume. It was found that thin columns (0.6 mm, 100 μl) 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 whilst thin enough to quantitatively collect the analyte from the vapour stream flowing through it.

The efficiency of the trap system was assessed by connecting two traps in series whilst monitoring the amount of analyte collected at each trap after set intervals of sampling. The setup developed towards achieving 100 % collection on the first trap. Long collection periods were implemented to increase the likelihood of losses from the first trap during collection. A 230 μl copper column (ID 1 mm, OD 1.8 mm, 230 mm length) was found to retain 99.8 % of the total mass after 20 minutes of collection from a 70 ppb DNEB vapour. This column was found to be appropriate and used for the duration of the tests. The column temperature was set between 1-2 $^\circ\text{C}$ in order to induce fast nitroaromatic deposition, but was kept above 0 $^\circ\text{C}$ to avoid formation of ice and minimise condensation of water.

Amperometric detection.

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 is reduced back to ferrocene dicarboxylic acid. DNEB was injected into the cell after the system had attained equilibrium.

Following injection of the analyte, fig 2, 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 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. The drop in current is comparable between the two samples. The magnitude of the change in current was directly related to the concentration of the analyte, signifying tremendous promise towards the development of the biosensor.¹⁰ The solution sample of 50 ppt was obtained from the generation of a 6 ppt vapour which was sampled for 5 minutes at room temperature.

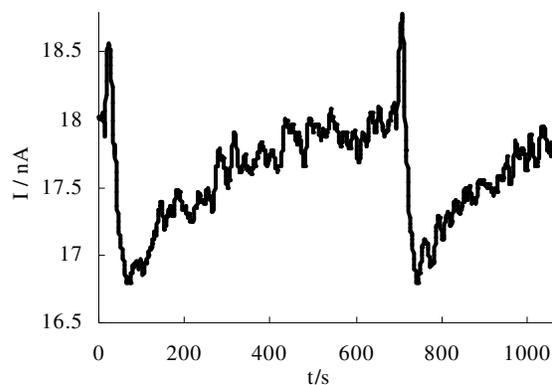


Figure 2 Typical consecutive current transients at a fixed potential of 100 mV obtained after the addition DNEB (50 ppt (mass)) at 0 and 700s after the addition of DNEB (50 ppt (mass)) into the solution. All measurements were performed in phosphate buffer (pH 7.1, 0.1 M) and co-factor FcDA (2 mM) with a FcDA-cys₁₂ modified Au(111) electrode.

Sensor evaluation.

To assess the practical use of the 'Nano-Dog' during real-time sensing, a vapour stream of 9.3 ppt was generated from which samples were collected for 1, 2.5 and 5 minutes and eluted as described in the experimental section. Detection of DNEB was done using both HPLC and the electrochemical biosensor. The concentration of DNEB in samples immediately after elution (HPLC) and as detected by the 'Nano-Dog' biosensor are shown together for direct comparison (fig 3). For practical purposes, the electrolyte volume in the 'Nano-Dog' 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 figure 3.

Conclusions

The utilisation of this method of immobilisation used in conjunction with the vapour trap has enabled detection limits in the low ppt range, thus making the overall process highly applicable for the detection of explosive vapours. Our successful demonstration of the amperometric biosensor for the detection of nitroaromatics using a genetically modified nitroreductase can be adapted for other analytes. The main hurdle in its adaptation is the correct positioning of the cysteine amino acids within different protein structures.

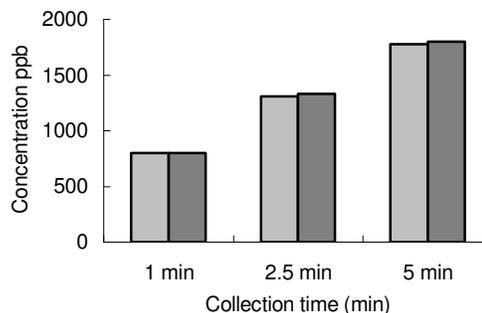


Figure 3 DNEB concentrations in DMSO samples as collected after elution from the vapour trap column (HPLC) and as detected by the 'Nano-Dog' sensor calculated from the charge under the transient. (Vapour generator temp. = 5 $^\circ\text{C}$; vapour conc. 9 ppt).

Key: \square [DNEB] as measured by HPLC.

\blacksquare Nano-Dog concentration at the interface multiplied by the dilution.

Acknowledgments

CG would like to acknowledge the financial support from Trwyn Ltd.
285 (UK) and the Welsh Assembly Government for their funding in support of the patent and proof of concept.

Interest declaration

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