Enzyme assay using ultra-low volume surface micromachined sensors

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We demonstrate an amperometric enzyme-linked assay within an ultra-low volume (600 pl) micromachined device for the rapid determination of (hypo)xanthine *via* a catalytically generated hydrogen peroxide intermediate, thereby illustrating the potential of this technology in a variety of bioanalytical measurements involving the oxidases.

Techniques used in planar microfabrication have shown considerable promise in analytical biotechnology by enabling miniaturised metallic sensing electrodes to be made routinely, with precise control over their two-dimensional geometry.[1–5] More recently, however, micromachining has provided a complementary technology (to microfabrication), allowing for a greater degree of flexibility in structural design, by defining the geometry of a device in all three dimensions.

In general, the analytical benefits of miniaturisation of such devices become apparent through the scaling laws (which dictate that, for example, within a diffusion limited system, as the dimension of the structure is reduced, so transport of analyte becomes more efficient, with an improvement in both response times and signal to noise).[5,6] As a consequence, within the last two years, the potential for performing biological analyses within micromachined structures has been demonstrated, including examples of novel device configurations, capable of electrophoretic separation of high molecular mass analytes,[6] 'on-chip' PCR[7] and electrophoretic manipulations of cells.[8]

Many of the devices which have been machined have been generated by the annealing of glasses and/or silicon to form three-dimensional channels and chambers.[6–8] However, there are still technical problems in introducing functional sensors or actuators within a micromachined volume. For example, although Clark *et al.*,[9] have embossed very low volume polystyrene vials for electrochemical analysis, the electrodes must be introduced into the device as 'probes' from above. Recently, in order to overcome this problem, we described the use of a photoactive polyimide (Probimide 7020), which can be used in combination with two-dimensional (planar) microfabrication procedures to produce low volume (sub-nl) titre chambers with integrated electrochemical microelectrodes. We have previously characterised these devices by investigating the electrochemistry of the inorganic redox mediator, ferrocene monocarboxylic acid.[10]

The method by which we fabricated this surface micromachined device was adapted from procedures which have previously been described,[10] and involved using photolithography, metal evaporation and lift-off to produce a planar two-electrode array, consisting of gold micro-ring electrodes, adhered on a glass slide using a Ti/Pd underlayer (Ti/Pd/Au 10 : 10 : 100 nm). Importantly, in order to prevent subsequent fouling of the gold electrochemical surface during microchamber processing, the gold was coated with a 10 nm sacrificial layer of NiCr (60% : 40%, also known as 'nichrome'). The volume above the microelectrode (600 pl) was defined by photopolymerisation of the light sensitive polyimide (λ = 436 nm) through an appropriate chrome on quartz mask, followed by exposure to the OCG™ developer. The diameter of the chamber was 200 μm, and the depth of the chamber, as defined by the thickness of the polymer was 20 μm.[10]

After producing the chamber, residual polyimide was removed from the electrochemical surface by wet etching the 'sacrificial' nichrome in 0.6 M acetic acid and 0.37 M ammonium cerium(IV) nitrate, followed by ultrasonication (undercutting the polymer, and thus freeing it from the metal). Fig. 1(*a*) shows the electroanalytical device, coated with the nichrome layer and the contaminating polymer, prior to the wet etching process. Subsequently, Fig. 1(*b*) and (*c*) show the sequential 'cleaning' of the gold by nichrome etching, leaving the electrochemical surface available for further functionalisation (see below).

In order to produce a stable electrochemical surface, platinum was electrodeposited galvanostatically onto both the working and counter gold electrodes from a solution containing 24 mM platinum(IV) chloride and 2.1 mM lead acetate, maintaining a constant current of 1 μA for 1 min.[11,12] Bioelectrochemical measurements were made in a two-electrode configuration, with an outer (larger) micro-ring (inner diameter 120 μm, width 20 μm) acting as the platinum pseudo-reference counter (+270 mV *vs.* Ag/AgCl), and a smaller inner ring electrode (inner diameter 100 μm, width 10 μm) as the (platinum coated) working electrode, see Fig. 1(*a*). The ratio of the respective geometric areas of the counter and the working electrode was > 2 : 1.

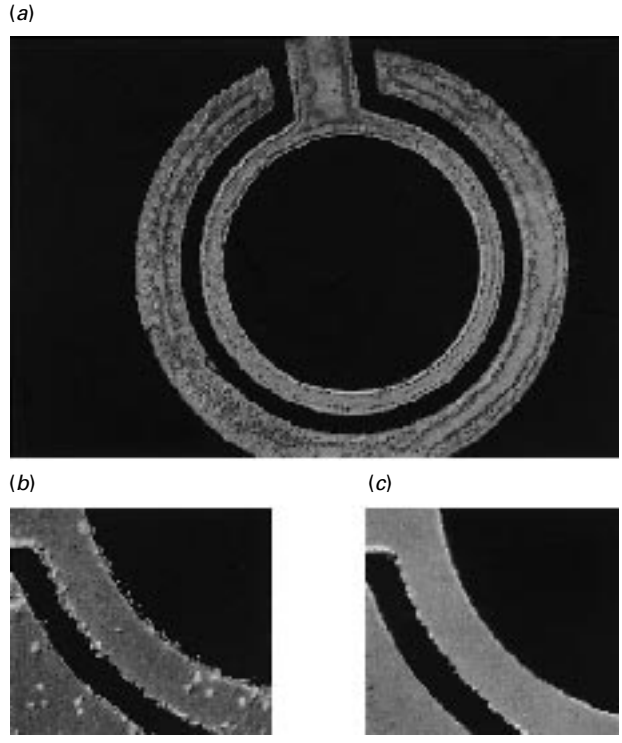

Fig. 1 A method for producing polyimide free electrodes, within the μ-electrochemical chamber: (*a*, above) shows the nichrome layer (with contaminating polymer); (*b*, below, left) and (*c*, below, right) show the sequential etching of the 'sacrificial' nichrome to remove the polymer residue. The opaque polymer surrounding the microelectrode is the photocured polyimide, which defines the three dimensional structure.

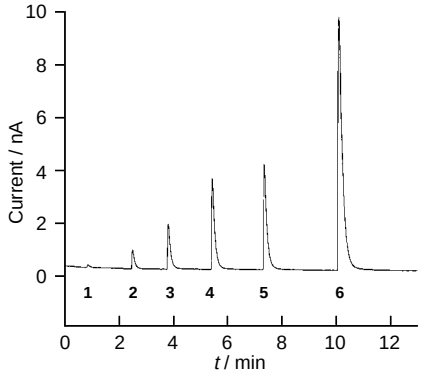

Fig. 2 *i–t* responses for successive additions of 8 pl buffer (1) and subsequently 1 pl (2), 8 pl (3), 30 pl (4), 66 pl (5) and 220 pl (7) of hydrogen peroxide to 600 pl μ-electroanalytical chamber containing a platinised micro-ring working electrode (geometric area = 3.3×10^3 μm²), poised at a potential equivalent to +420 mV *vs.* Ag/AgCl

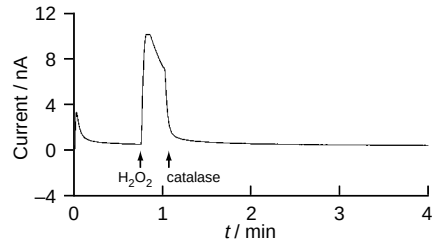

Fig. 3 Corroborative evidence that the responses in Fig. 2 are due to the electro-oxidation of hydrogen peroxide, shown by the addition of 2.5 nmol of catalase (equivalent to 25 units of enzyme), thereby acting as an effective catalytic scavenger. Responses were measured at a potential equivalent to +420 mV *vs.* Ag/AgCl.

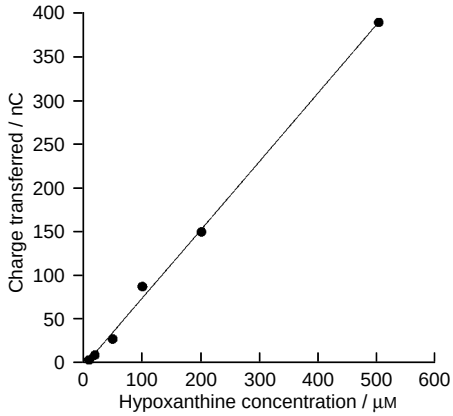

Fig. 4 Calibration curve for hypoxanthine in the concentration range between 10 μM (60 fmol) and 500 μM (3 pmol), (r = 0.99). Substrate additions were made to stock solutions of Ringers buffer containing 1.75×10^{-5} units of XOD in a volume of 600 pl.

In the first instance, hydrogen peroxide was ultramicropipetted into the μ-electroanalytical chamber through a layer of mineral oil (to prevent solvent evaporation),[10] and *i–t* measurements were made using a BAS CV 37 potentiostat (Bioanalytical Systems, UK) at a potential equivalent to +420 mV *vs.* Ag/AgCl.[11,12] Responses to successive additions of hydrogen peroxide are shown in Fig. 2, which are very fast, owing to the short electrode diffusion lengths. Measurements were integrated, as the total oxidation charge for hydrogen peroxide, giving a linear calibration curve over a range of analyte concentrations between 8.3 μM (5 fmol) and 1.83 mM (1.10 pmol), *i.e.* $y = 0.1\,x + 6.1$ nC (r = 0.99, n = 3). That the observed signals were generated by the electro-oxidation of hydrogen peroxide was corroborated by the addition of catalase during one such experiment (Fig. 3), which scavenged the analyte efficiently, as expected.

In order to further demonstrate the potential application of this device to bioanalytical measurements, a series of experiments involving the measurement of hypoxanthine were performed, using the oxidase enzyme, xanthine oxidase (XOD). The quantification of hypoxanthine is of interest in biomedicine, not least as it is produced during purine catabolism, and therefore has relevance in assessing the nucleotide pool in tissue. In the XOD catalysed reaction, under study here, two hydrogen peroxide equivalents are generated as a consequence of the oxidation of hypoxanthine, Eqn. (1) and (2), and these are subsequently measured through their electrochemical oxidation at the working electrode, according to Eqn. (3).

$$\text{Hypoxanthine} \xrightarrow[\text{XOD, pH 7.4}]{} \text{Xanthine} + H_2O_2 \quad (1)$$

$$\text{Xanthine} \xrightarrow[\text{XOD, pH 7.4}]{} \text{Uric acid} + H_2O_2 \quad (2)$$

$$H_2O_2 \xrightarrow[+420\text{ mV } \textit{vs.}\text{ Ag/AgCl}]{} O_2 + 2H^+ + 2e^- \quad (3)$$

The calibration curve for the electrochemical measurement of hypoxanthine is shown in Fig. 4. Measurements are the integral of the total (*i–t*) response, as before, after the addition of substrate within the range 10 μM (60 fmol) to 500 μM (3 pmol), $y = 0.79\,x - 5.04$ nC (n = 1, r = 0.99).

Although in both cases (for the generalised measurement of H_2O_2 and for the specific measurement of hypoxanthine) the responses do not correspond exactly to theoretical values, calculated on the assumption that all analyte is consumed, the results do demonstrate the general principle of bio-electrochemical detection within a confined ultra-low volume.

At present, routine *in situ* single cell measurements are limited to determinations of ions and ATP. The technique that we have developed has potential implications for the rapid, low volume detection of a wide range of new analytes, *e.g.* for determinations from single cells using oxidase enzymes to catalytically generate an electroactive coproduct, including glucose (*cf.* glucose oxidase) or lactate (*cf.* lactate oxidase).

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Notes and References

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