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Biosensing at disk microelectrode arrays. Inter-electrode functionalisation allows formatting into miniaturised sensing platforms of enhanced sensitivity

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ABSTRACT

Biosensor performance depends on the effective functionalisation of a transducer with suitable biorecognition elements. During functionalisation, surface blocking steps are normally carried out to avoid later binding of undesirable molecules and thus guarantee biosensor specificity. However, these blocking steps may be deleterious in electrochemical systems where transduction ultimately relies on electron transfer between the electrode and a redox species in solution. This work presents a novel approach to develop improved amperometric biosensing platforms using microfabricated disk microelectrode arrays, based on the functionalisation of the inert surface surrounding the active microdisks. These devices more than doubled assay sensitivity compared to conventional biosensors produced using the same arrays. This approach benefits from three advantages: the functionalisation of a broader surface, the possibility to activate the microelectrodes immediately before detection, and access to enhanced rates of mass transport to microelectrodes that improve device sensitivity. To demonstrate this, we first studied the electrochemical behaviour of tetramethylbenzidine (TMB) at gold disk microelectrode arrays, and then used TMB as the redox mediator for the amperometric biosensing of HRP/H_2O_2. Down to 0.54 pM H_2O_2 or as little as 25 pM HRP were detected within 5 s of enzyme activity in just 10 μl of enzyme substrate solution. We postulate that microelectrode arrays may be used to develop novel electrochemical biosensing platforms that are faster and more sensitive than conventional biosensors.

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1. Introduction

Affinity-based biosensors often need blocking of the transducer surface with inert materials to minimise non-specific signals, because biosensor specificity is only guaranteed in the absence of non-specific adsorption of unwanted components. Bovine serum albumin, BSA, is the protein most widely used for this purpose, and it works by adsorbing on the gaps left among the biorecognition elements used to functionalise the transducer. However, blocking agents may also exert adverse effects in electrochemical systems where signal transduction ultimately relies on electron transfer steps.

Microelectrode arrays are powerful tools in electroanalysis as they allow access to mass transport rates comparable to microelectrodes and current levels similar to macroelectrodes (Ordeig et al., 2006, 2007). The microelectrode arrays used in this work consist of a large number of individual microdisks that are wired in parallel, patterned in a regular lattice over an inert surface and separated from their closest neighbours by a distance several times their diameter. This means that only a small fraction of the whole transducer surface area is at work. In this work we also exploit the electrochemically inert surface of microelectrode arrays to develop immunosensors that are more sensitive than previously reported electrochemical systems. We functionalised the inert part of the arrays and kept the microelectrodes electrochemically active. Electrode passivation could be avoided by electrochemical activation after the various steps in a typical immunoassay. Since the activation only affects the microelectrodes, biomolecules such as enzymes, antibodies or DNA probes could be safely attached to the surface among them.

Here we prove this concept using a well-known electrochemical system based on the enzyme horseradish peroxidase, HRP, and 3,3′,5,5′-tetramethylbenzidine, TMB^0, as the redox mediator. HRP catalyses the oxidation of numerous reducing substrates by hydrogen peroxide (H_2O_2) following the general reaction summarized in Fig. 1a. A variety of HRP substrates have been described, including aromatic phenols, phenolic acids, indoles, amines and sulfonates, which regenerate the enzyme active centre, oxidised as a result of the reaction with H_2O_2 (Regalado et al., 2004; Veitch, 2004). Using reversible redox substrates allows for enhanced electrochemical detection, as the mediator shuttles electrons between the enzyme active site and the electrode. The rate of mediator reduction can then serve to estimate HRP and/or H_2O_2 concentration. Compared with other widely used enzymes like alkaline phosphatase, HRP is

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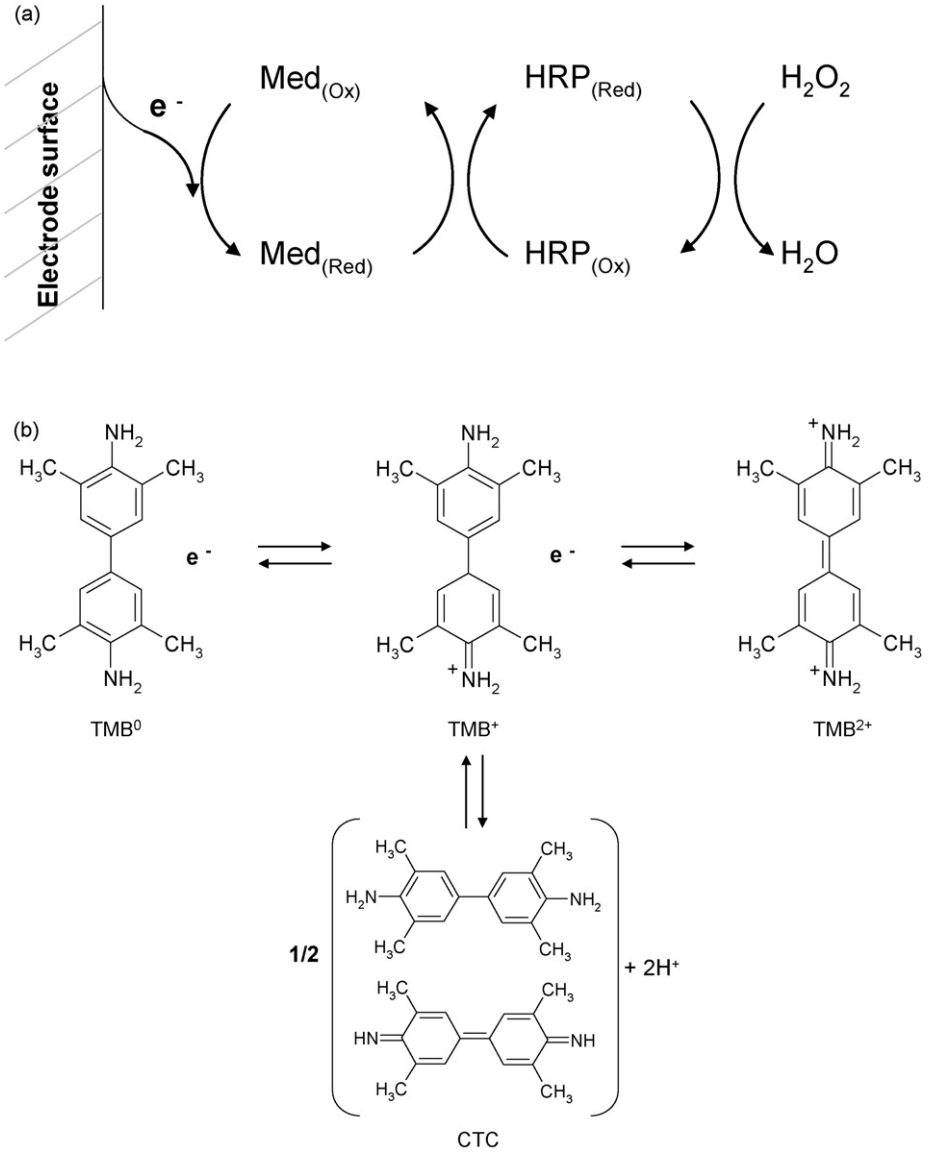

Fig. 1. (a) Reduction of H_2O_2 by a redox mediator catalysed by HRP. (b) TMB^0 electro-oxidation in aqueous solution at neutral pH involves two consecutive one-electron steps. The radical intermediate, TMB^+, coexist in solution with a blue charge-transfer complex, CTC, attributed to the reversible complexation of TMB^0 and TMB^{2+}.

less costly, smaller, more stable and has a high turnover rate that allows the generation of strong signals in a relatively short time. The availability of a variety of substrates has encouraged the use of HRP in the preparation of enzyme-labelled biocomponents, especially in immunohistochemistry and immunoassays (Kricka, 1994; Regalado et al., 2004).

TMB^0 is one of the most widely used chromogenic substrates for HRP-based detection systems and commercial ELISA test kits because it is less toxic and provides higher assay sensitivity and faster reactions than other HRP substrates such as *O*-phenylenediamine (OPD) and 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). TMB^0 shows in aqueous solution at pH 4.0–7.0 near fully reversible electro-oxidation that involves two consecutive one-electron steps. These are attributed to the formation of a radical intermediate, TMB^+, and a yellow two-electron oxidised product, TMB^{2+}. As shown in Fig. 1b, TMB^+ is present in solution in equilibrium with a charge-transfer complex, CTC (Volpe et al., 1998; Liu et al., 2008). This CTC has been attributed to the reversible complexation of the reduced TMB^0 (a neutral diamine that behaves as the electron donor) and TMB^{2+} (a diimine that behaves as the CTC electron acceptor) (Awano et al., 1990; Misono et al., 1997; Liu et al., 2008). The CTC, which is blue, is also electroactive and presents a single reversible voltammetric peak (Josephy et al., 1982; Liu et al., 2008). Under strong acidic conditions, however, the voltammetry of TMB^0 shows a single two-electron oxidation/reduction wave, because low pHs favour the formation of TMB^{2+} (Misono et al., 1997; Crew et al., 2007; Liu et al., 2008).

The electrochemical monitoring of HRP activity using H_2O_2 and TMB has been successfully applied to ELISA and Enzyme-linked Aptamer Assay (ELAA) detection. Reports exist for chronoamperometry, cyclic voltammetry, differential pulse voltammetry, and square wave voltammetry, often coupled to flow injection analysis (FIA) and nearly always based on macroelectrodes. In these examples, the electrochemical assay format usually shows wider assay dynamic ranges, lower detection limits, and shorter incubation times than the colorimetric counterparts (He et al., 1997, 2003; Draisci et al., 2001; Pyun et al., 2001; Valentini et al., 2003; Baldrich et al., 2005; Fanjul-Bolado et al., 2005; Liu et al., 2005, 2006; Crew et al., 2007). While HRP is one of the most widely used enzymes in biosensing, the use of TMB as the substrate is restricted to a few reports relying on amperometric detection using

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