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Short Communication

Cholesterol biosensor based on inkjet-printed Prussian blue nanoparticle-modified screen-printed electrodes

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ABSTRACT

Here we describe the construction and optimization of a cholesterol biosensor based on screen-printed electrodes (SPEs) modified with inkjet-printed Prussian blue nanoparticles (PBNPs). The deposition of PBNPs using inkjet printing led to the highly facile fabrication of sensors with excellent sensitivity and reproducibility for the measurement of H_2O_2. Further integration of the sensor with a microfabricated low volume (4 μL) sample cell allowed the measurement of cholesterol in serum with the addition of cholesterol oxidase. The biosensor exhibited a sensitivity to cholesterol of 2.1 μA/mM cm^2 ($r^2 = 0.97$, $n = 5$) and was linear in the range of 0–15 mM.

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1. Introduction

Despite considerable improvements in medical care over the past 25 years, the greatest cause of death globally is cerebrovascular disease (CVD) [1]. A major modifiable risk factor in CVD is cholesterol. During 2001, out of 16 million estimated CVD deaths, 13 million occurred in low-income and "developing countries" including China and India [2] and 23 million deaths are predicted in 2030 [3].

Currently, assays for cholesterol involve initially collecting a blood sample from a patient and then sending it to a clinical laboratory for analysis: many methods such as fluorescence, surface plasmon resonance and electrochemiluminescence [4–6] have been developed and used in biochemical analysis. However, electrochemical sensors represent a rapid and cost-effective alternative approach to optical methods [7], directly interfaced to patients, especially for those at borderline suffering of hypercholesterolaemia. One of the most frequently used approaches to determine cholesterol concentration is to follow the formation of hydrogen peroxide (H_2O_2) which results from the oxidation of cholesterol by cholesterol oxidase (ChOx) (Eq. (1)). One of the effective electrocatalysts for detecting H_2O_2 is ferric hexacyanoferrate or Prussian blue (PB). It is electrochemically reduced to form Prussian white (PW), which is able to catalyze the reduction of H_2O_2 at low applied potentials [8]. These materials must be coupled to cost-effective techniques which allow the manageable and rational modification of the substrate to be functionalized (i.e. electrochemical processes, solution casting, screen printing [9–11]). However, screen printing presents particular challenges around the rheological properties that inks must possess to make them suitable. An emerging deposition technique that has shown promise is inkjet printing, which has attracted a lot of interest as a manufacturing tool [12]. This stems from its capability in the patterned deposition of picolitre volumes of low-viscosity ink with high accuracy, precision and excellent reproducibility.

Herein, we report on the deposition of PBNPs onto SPEs using inkjet printing as the basis of a cholesterol biosensor in serum. The combination of inkjet printing of the NPs and the screen printing of the electrodes resulted in a simple low cost fabrication process with excellent sensitivity, reproducibility and linear range. The uniqueness of the reported approach is the application of the highly sensitive inkjet-printed PBNP-modified sensor in combination with a simple low volume (4 μL) capillary-filled microfluidic chamber design by means of a simple fabrication procedure.

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2. Experimental

2.1. Chemicals and apparatus

Potassium phosphate monobasic, potassium phosphate dibasic, potassium ferrocyanide, iron(III) chloride, hydrochloric acid, potassium chloride, potassium hydroxide and Triton™ X-100 were obtained from Sigma–Aldrich (USA). Cholesterol oxidase from *Streptomyces* sp. (19.8 U/mg material) was purchased from BBI Solutions. Cyclic voltammetry (CV) was performed using an Autolab PGSTAT-12 potentiostat (Eco Chemie, Netherlands). Amperometric measurements were carried out using a Bio-Logic SP-200 potentiostat (Bio-Logic Science Instruments, France).

2.2. Procedures

Graphite SPEs were manufactured using a 245 DEK (Weymouth, England) screen printing machine as reported previously [10]. PBNPs were synthesized by mixing equimolar amounts of potassium ferrocyanide and iron(III) chloride in the presence of KCl in acidic conditions [13], and SPEs were modified by inkjet printing 20 layers of PBNPs using a Dimatix DMP 2831 piezoelectric printer (Fujifilm Dimatix Inc., USA) as reported previously [14]. SPEs were manually encapsulated using a double sided pressure sensitive adhesive spacer layer (25 μm) and a hydrophilic cyclic olefin polymer lid layer, commercially known as Zeonor, as shown in Fig. 1.

3. Results and discussion

3.1. PBNPs-SPE analytical performance toward H_2O_2 detection

Recently [14], we demonstrated the excellent performance of inkjet-printed PBNPs-SPE with regard to the reduction of H_2O_2 at 0 V vs. Ag/AgCl, with a sensitivity of 762 μA/mM cm^2, limit of detection of 0.2 μM, linear range of 1–4500 μM with a relative standard deviation of <5%. This excellent analytical performance indicated the opportunity to use it as the platform of a cholesterol biosensor employing H_2O_2 as the measurand, as reported by the following equation:

$$\text{Cholesterol} + O_2 \xrightarrow{\text{ChOx}} \text{Cholest} - 4 - \text{en} - 3 - \text{one} + H_2O_2 \quad (1)$$

3.2. Optimization of cholesterol measurement parameters

Sequential optimization of a number of experimental parameters for the measurement of cholesterol using the PBNP-SPEs was performed using amperometry at 0 V vs. Ag/AgCl, with current measurement taken at 200 s. Parameters such as enzyme concentration, pH, surfactant concentration and reaction time were optimized ($n = 5$). A concentration of 5 mM cholesterol was chosen for all the optimization steps to obtain the best experimental conditions (Fig. 2). The optimum pH was selected within the range of 6.5–8.0 as shown in Fig. 2a. Maximum assay sensitivity was achieved at pH 7.0 and it was selected in all further experiments. This behavior relates to the optimum activity of both the ChOx enzyme as reported in other work [15], as well as the PB electrocatalysis which decreases with increasing pH due to a possible Fe—CN bond breaking by OH^- ions [16]. To ensure cholesterol solubilization across the physiological range, Triton X-100 was added to aid the solubilization of cholesterol (a lipid) by forming micellar structures, from which its optimal concentration was investigated. As shown in Fig. 2b, the current increased with increasing surfactant concentration up to 10% (v/v). Subsequent studies were carried out at this concentration of surfactant, as excessive foaming occurs at higher concentrations. The concentration of ChOx enzyme employed in the assay was studied in the range from 1 to 10 mg/mL (0.08–0.8 U). As illustrated in Fig. 2c, the response increased up to 5 mg/mL and reached a plateau above this concentration; this value was chosen for the rest of work. Finally, the enzymatic reaction time was evaluated (Fig. 2d). Currents increased linearly from 1 to 4 min, but did not increase further at 8 min. As a consequence a 4 min reaction time was chosen as the best compromise in terms of sensitivity and time required for the cholesterol biosensor. Having optimized all the analytical parameters, cholesterol was then measured with serum as the matrix using the same procedures.

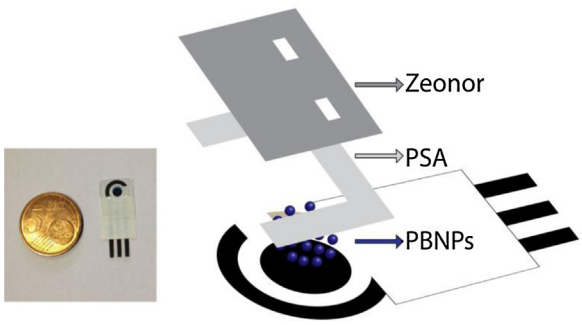

Fig. 1. Components and configuration of for the inkjet-printed PBNP-modified screen-printed electrode. PBNPs: Prussian blue nanoparticles; PSA: double sided pressure sensitive adhesive spacer layer (25 μm); Zeonor: hydrophilic cyclic olefin polymer lid layer.

3.3. Interference study

To estimate the selectivity of the cholesterol biosensor, common potential serum electroactive interferents, including ascorbic acid (0.1 mM), uric acid (0.5 mM) and acetaminophen (0.06 mM), as well as cholesterol were introduced to the biosensor and measured in the manner already described, and the results obtained are shown in Fig. 3. The concentrations tested were those found at their maximum physiological or therapeutic levels in serum, as reported in the literature [17–19]. As shown in Fig. 3, the amperometric responses of the interferents were comparable to the background current at 200 s, demonstrating that these interferents had no significant effect on the response of the sensor. This suggests that this PBNP-based SPE is acceptable for the selective determination of cholesterol in serum in the presence of these electroactive species.

3.4. Measurement of cholesterol in serum

In this work, the cholesterol concentration in serum was measured in the extended range up to 15 mM. Fig. 4a displays the amperometric curves, at 0 V (vs. Ag/AgCl), which show an initial decay of reduction current of H_2O_2 and a quasi-steady state response at approximately 200 s. The response of the biosensor to cholesterol was linear from 0 to 15 mM (0–580 mg/dL) ($n = 5$) as shown in Fig. 4b. The biosensor yielded a sensitivity of 2.1 μA/mM cm^2 ($s = 0.2$ μA/mM cm^2, $r^2 = 0.97$, $n = 5$) after normalizing the slope of the calibration plot in Fig. 4 by the electrode surface area. The limit of detection calculated as LOD = $3\sigma_b$ ($n = 5$)/slope was found to be 0.2 mM (8.5 mg/dL). The inter-electrode repeatability which was evaluated at 5 mM (approx. 190 mg dL^{-1}) cholesterol was 8% ($n = 5$). In addition, intra-electrode repeatability of 6.3% was obtained by measuring cholesterol with the same sensor ($n = 5$) by removing the lid, washing the SPE with distilled water, allowing water to evaporate and re-encapsulating the SPE. The performance of the developed biosensor compares very favorably with alternative assay systems, with regards to its sensitivity, linear range and sample volume requirements. For example, EnzyChrom™ is a colorimetric kit that detects serum cholesterol up to a concentration

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