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Laccase biosensor based on screen-printed electrode modified with thionine–carbon black nanocomposite, for Bisphenol A detection

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ABSTRACT

The relevance of Bisphenol A (BPA) in human health is well-known. For this reason we designed and developed a biosensor based on a bionanocomposite (laccase–thionine–carbon black)-modified screen-printed electrode. Thionine, a commercially available dye, was used as electrochemical mediator coupled with a nanostructured carbon black. By means of cyclic voltammetry, the interaction of thionine adsorbed on modified screen printed electrode with laccase/BPA reaction products has been studied. In addition, the immobilization of laccase by physical adsorption on the surface of thionine–carbon black modified screen printed electrodes was investigated. The response of the biosensor has been optimized in terms of enzyme loading, pH and applied potential reaching a linear concentration range of 0.5–50 μM, a sensitivity of 5.0 ± 0.1 nA/μM and a limit-of-detection (LOD) of 0.2 μM. The developed biosensor has been also challenged in tomato juice samples contained in metallic cans where release of BPA due to the epoxy resin coating can be assumed. A satisfactory recovery value comprised between 92% and 120% was obtained.

1. Introduction

Bisphenol A [BPA, 2,2-(4,4-dihydroxydiphenyl) propane; CAS Registry No. 80-05-7] is produced by combining acetone and phenol and is an important organic chemical owing to its wide use as intermediate in the manufacture of polycarbonate plastics, epoxy resins, and flame retardants [1]. Its main use is as coating of metallic cans, powder paints, dental fillings, and antioxidant in plastics. It has also been used as an inert ingredient in pesticides, antioxidants and polyvinyl chloride stabilizer [2]. Recently, BPA has received considerable attention due to its endocrine disrupting activity and possible toxic impact on environment [3–5]. BPA levels in the μg/L range have been found in biological, food and water samples [6,7]. The presence of BPA is still a "hot topic": the European Food Safety Authority (EFSA) is still investigating on new risk assessment of BPA and, very recently, in its newsletter, invited "*. . .Member States, research institutions, academia, food business operators, packaging business operators and other stakeholders to submit data on BPA, in particular its occurrence in food and beverages, migration from food contact materials, occurrence in food contacts materials. Deadline: 31 July 2012*". The above findings suggest that it is necessary to determine the BPA presence also in trace amounts.

Traditional methods for BPA detection include chromatographic techniques coupled with mass spectrometry, capillary electrophoresis and solid phase microextraction, methods that are time consuming, cannot be performed on-site and require sample pre-treatment [8]. Electrochemical sensors can provide rapid and on-site BPA detection. For this purpose many researchers have developed electrochemical sensors using tyrosinase as biological element and different immobilization procedures [9–13]. Recently, the use of different tyrosinase-functionalised nanoparticle systems has shown interesting results [14].

All the developed electrochemical sensors for BPA use the enzyme tyrosinase, differently from the approach used for other phenol compounds for which other enzymes, such as peroxidase [15] and laccase [16], have been successfully employed. In particular the use of laccase combined with a properly chosen redox-active compound could represent a potential good candidate for designing and fabricating novel BPA sensors.

Laccases (E.C. 1.10.3.2) are dimeric or tetrameric glycoproteins, containing four copper atoms per monomer distributed in three redox sites: one in each T1 and T2 sites, and two in T3 site. It is assumed that the catalysis firstly involves T1 Cu reduction by the substrate, followed by internal electron transfer from T1 Cu to T2 and T3 Cu and, finally, dioxygen reduction at T2 and T3 sites

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[17]. Laccases catalyze the oxidation of ortho- and para-diphenols, aminophenols, aryl diamines, polyphenols, polyamines, lignin as well as some inorganic ions, coupled to the reduction of molecular dioxygen to water [18,19]. In the case of BPA not all the reaction products have been characterized. For instance, an interesting study was performed by Fukuda et al. [20], in which they demonstrated that the BPA was metabolized by laccase in two kinds of compounds: one kind composed by high molecular weight compounds and another kind composed by low molecular weight compounds, one of which was identified as 4-isopropenylphenol by means of gas chromatography–mass spectrophotometry. The 4-isopropenylphenol as oxidative degradation product has been identified using chromatography mass–spectrometry also in other works reported in literature [21,22].

It has been also reported that some redox-active compounds, known as "mediators", allow enlarging the range of compounds that can be targeted for oxidation by laccase [23]. The mediator interacts with the enzyme or with the reaction product. In some cases, typically the enzyme firstly oxidizes the mediator, which diffuses away from the enzyme active site, and sequentially oxidizes a compound that may be or not to be substrate of the enzyme. The mediator then returns to its original form and can subsequently be used to accomplish the conversion of more target species. The employment of mediators makes the use of laccase more attractive because it increases the number of pollutants that can be targeted [24]. When mediators are involved in laccase-assisted processes, the electron transfer from the mediator to the enzyme is followed by electron donation from the target molecule to oxidized mediator, which gives rise to the regeneration of the mediator [25–27]. In other cases, instead, the mediator reacts directly with the reaction products, as it occurs for tyrosinase interacting with phenols [10].

Several organic and inorganic compounds have been reported as effective mediators for the above purposes. The laccase reduction of dioxygen to water in the presence of different redox mediators or nanomaterials [28] has been studied in other electrochemical applications [29–34].

It has been also demonstrated that the use of redox mediators coupled with carbon nanomaterials can further improve the electrochemical performances of the developed sensor. For example carbon nanotubes were used together with ferrocenedicarboxylic acid [35], azure dye [36], thionine [37]. Among the carbon nanostructured materials such as carbon nanotubes and graphene, it was recently demonstrated the useful use of carbon black (CB). CB is an industrially manufactured colloidal material that consists of approximately spherical carbon primary particles with diameter comprised from 15 to 100 nm, which typically forms fused aggregates with sizes below 1000 nm. CB was demonstrated: (i) to have electrocatalytic properties towards many compounds such as ascorbic acid, dopamine, NADH, benzoquinone, epinephrine, cysteine, thiocholine, hydrogen peroxide [38–43], and (ii) that it can be used as the basis for construction of a tyrosinase biosensor for catechol detection [44]. In addition, as highlighted in our papers [41–43], this material allows the production of a stable dispersion using a cheap carbon nanostructured material, as recently confirmed by Compton's group [45].

In this paper we describe a disposable BPA biosensor based on laccase. To our knowledge, this is the first report in literature concerning biosensor for BPA detection based on laccase coupled with disposable sensor. By means of cyclic voltammetry (CV), the effect of thionine as a mediator has been investigated when the immobilized laccase interacts with BPA. In order to increase the analytical performance and the easiness of the proposed biosensor, a screen printed electrode (SPE) modified by using a nanocomposite formed by CB and thionine has been used. Also in this case, to our knowledge this is the first time that the CB was used coupled with a redox mediator in developing an electrochemical sensor.

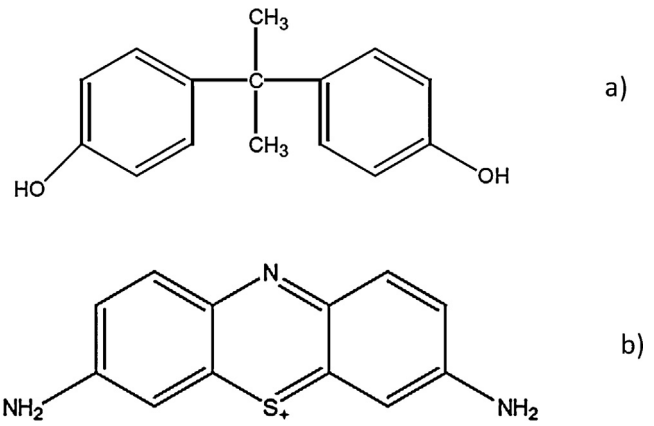

Fig. 1. Chemical structure of BPA (a) and of thionine (b).

The biosensor response was examined in terms of immobilized enzyme units, applied electrical potential and pH. Finally the optimized biosensor has been used for the amperometric determination of BPA in aqueous buffer solutions and in peeled tomatoes stored in metallic cans.

2. Materials and methods

2.1. Materials

Laccase (20 Units/mg) from *Trametes Versicolor*, Bisphenol A (see chemical structure in Fig. 1a), Thionine acetate (see chemical structure in Fig. 1b), Nafion® and all the other chemicals were purchased from Sigma (Sigma–Aldrich, Milan, Italy) and used without further purification. Carbon Black N220 (CB) was obtained from Cabot Corporation (Ravenna, Italy). Screen-Printed Electrodes G-Sensor (SPEs) was obtained from Ecobioservice and Research (Firenze, Italy).

The working electrode was made of graphite and its diameter was 0.3 cm.

2.2. Methods

The enzymatic working electrode was prepared by modifying the surface of a screen printed electrode with 6 μL of a dispersion of CB 1 mg/mL in acetonitrile [41]. After solvent evaporation, 5 μL of thionine 0.4 mM aqueous solution were added and then the electrode was put in an oven at 60 °C for about 15 h [13]. After this treatment, the electrode was further modified by adding 5 μL of laccase solution at different concentrations, in order to obtain enzymatic unit values ranging from 0.59 U to 6.52 U. After drying, the electrodes were covered with 3 μL of a neutralized aqueous solution of Nafion® 2.2% (v/v) and left to dry at room temperature for 30 min in order to avoid rapid enzyme leaking. The whole scheme is reported in Fig. 2a.

The BPA/laccase reaction products were separately obtained by the following procedure: 500 μL of 0.05 M citrate buffer at pH 4.5 were added to 100 μL of 1 mM BPA and 100 μL of laccase (40 mg/mL) both in the same buffer and allowed to react at 37 °C for 1 h to obtain total conversion of BPA in product.

To obtain a thionine–CB-modified SPE, two steps were carried out: (1) 6 μL of a dispersion of CB 1 mg/mL in acetonitrile were placed on SPE as described in our previous work [41]; (2) thionine was made to adsorb on the modified SPE as previously described.

All the measurements were carried out using SPEs connected to a PalmSens instrument (Palm Instruments, the Netherlands) coupled to a PC and were performed at room temperature (25.0 ± 0.5 °C). The potentials were referred to the internal Ag