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A polyphenol biosensor realized by laser printing technology

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ABSTRACT

An amperometric biosensor sensitive towards phenolic compounds, using the enzyme laccase as biorecognition element, was developed. The enzyme was successfully immobilized in active form onto nonfunctionalized screen printed electrodes by using the laser printing technology. This type of immobilization established efficient electrochemical contact between the enzyme and the electrodes surface. The immobilized laccase was characterized towards catechol in solution, a typical phenolic compound. The biosensor sensitivity was found to be 0.43nA ± 0.04 nA/μM for catechol. This biosensor permits the detection of catechol in aqueous solutions at concentrations in the nanomolar range.

1. Introduction

Polyphenols are plant-derived natural products with various beneficial properties for the human body [1,2]. This class of molecules exhibits an outstanding performance in the prevention of various human-threatening maladies, from cancer to diabetes, and neurodegenerative diseases. Polyphenols include several thousand compounds such as flavonols, catechins, anthocyanidins, and isoflavonoids [3]; and due to their antioxidant activity they have been widely exploited in the market industry in the form of functional foods, dietary supplements, and anti-aging creams [2,4].

The most used analytical methods for the determination phenolic compounds are GC-MS or HPLC [5]. These methods are expensive and involve complex sample pre-treatment procedure. Many biosensors have been developed as an alternative method using the catalytic activity of the enzymes such as tyrosinase or laccase for phenolic compounds determination [6,7].

Laccases are multicopper oxidoreductases, capable of oxidizing several phenolic and non-phenolic compounds, including polycyclic aromatic hydrocarbons, pesticides, organophosphorus, etc. Widely distributed in higher plants, fungi, and microorganisms [8], laccases can be utilized as a biorecognition element (biomediator) for phenolic compounds in the development of biosensors for polyphenols detection [9,10], in particular for catechol detection. Amperometric determination of catechol was realized electrochemically by measuring the variation of current signals in the presence of laccase. Since the oxidation of catechol results in benzoquinone, the latter can be electrochemically reduced and detected in a concentration-dependent manner [11].

A large number of techniques have been developed for the immobilization of enzymes for biosensor applications. Generally, the choice of an appropriate immobilization method is determined by the physical and chemical properties of the surface and the biomediator. The most commonly used immobilization methods in biosensing technology are identified either as chemical or physical processes. Chemical immobilization processes are based on covalent attachment while physical immobilization techniques are mainly based on the following mechanisms: adsorption, entrapment, and cross-linking [12,13]. These methods have several disadvantages such as loss of the biological activity, requirement of surface functionalization, are time consuming, use of laborious reactions, and involvement of toxic chemicals.

In our approach, we use the laser induced forward transfer (LIFT) process as an advanced tool for direct immobilization of the enzyme laccase onto low cost nonfunctionalized screen printed electrodes (SPE). This process has been already used for the deposition of enzymes [14], DNA [15], living cells, [16] and photosynthetic membranes [17]. The high spatial resolution of the LIFT process has led several research groups to employ this technique in order to transfer a wide range of biomaterials for the fabrication of biosensors.

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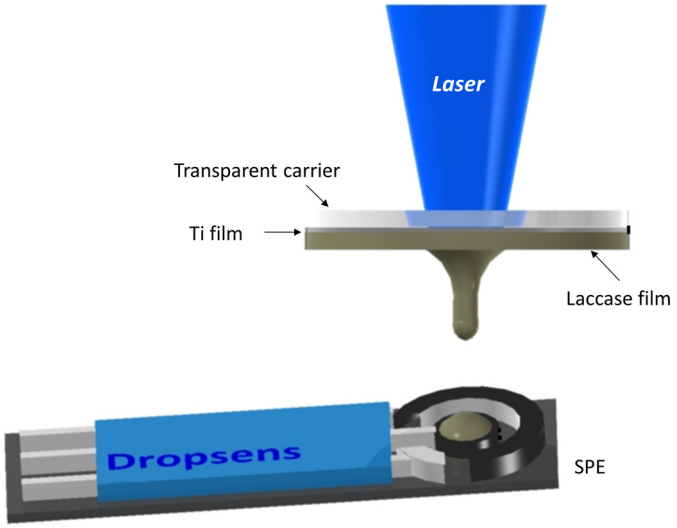

Fig. 1. Schematic illustration of the LIFT process.

This manuscript deals with the use of the LIFT process for the direct laccase immobilization on graphite SPEs for the development of biosensor.

2. Materials and methods

Graphite SPEs (DRP110) were purchased from DropSens (Oviedo, Spain). Laccase from *Trametes versicolor* (E.C.1.10.3.2) was purchased from Sigma–Aldrich (St. Louis, MO USA), claimed activity of at least 0.5 U/mg. All reagents were purchased from Sigma–Aldrich (St. Louis, MO USA) and were of analytical grade.

2.1. Laser printing setup

The laser printing setup is comprised of a pulsed Nd:YAG (neodymium–doped yttrium aluminum garnet) laser (266 nm wavelength, 10 ns pulse duration) and a high power imaging micromachining system [18]. A donor substrate consists of one inch quartz and 40 nm titanium layer which acts as the dynamic release layer for the laser-assisted process. The liquid film to be deposited is coated to the donor substrate using a microblade and forms a uniform liquid film of 10 μm in thickness.

This method relies on the displacement of the material to be deposited from a donor substrate to an acceptor substrate. Irradiation of the donor substrate results in the rapid heating of the Ti film and the explosive boiling of the adjacent liquid, thus producing a high vapor cavity, which expands and drives through the remaining film. The acceptor substrate is placed parallel to the donor substrate at 300 μm, so that following irradiation, the biomaterial is printed onto the former (Fig. 1).

2.2. Enzyme preparation

Laccase solutions were prepared by dissolving a suitable amount of lyophilized enzyme with known enzymatic activity (40 U), in phosphate buffered saline (PBS) solution pH 4.5. Graphite SPEs DRP110 were used for these experiments, purchased by DropSens, which consist of a counter, a reference, and a working electrode (4 mm diameter). The working electrode was printed with the enzyme (86 mU) in PBS via LIFT technique and afterwards, 50 μL PBS were added for amperometric measurements.

2.3. Biosensor set-up

Tests with the immobilized laccase on the SPEs were performed by using the potentiostat AMPBIO-SPE (Biosensor s.r.l., Rome, Italy; www.biosensor.it). The SPE, with the immobilized enzyme, was polarized at −30 mV relative to an Ag/AgCl reference electrode.

Amperometric detection of the electric current was carried out by the redox reactions taking place in the surface of the working electrode which correspond to the electrochemical oxidation of the catechol and consequently the electrochemical reduction of the generated benzoquinone. Amperometric measurements were carried out at room temperature.

3. Results and discussion

3.1. Laser printing

The direct immobilization of the biomaterials relies on the high velocities that the liquid droplets are being transferred by means of laser printing. The liquid film impacts with high pressure at the rough substrate, resulting in a physical absorption of the biomaterials to the substrate. In this endeavor, the LIFT technique was used for both the deposition and the immobilization of the laccase enzyme onto the surface of commercial graphite SPEs. Two-dimensional patterns of continuous enzyme layers were printed on the electrodes by adjusting the droplet separation distance and size. The transfer was carried out in such a way that each droplet was deposited by a single pulse and a 16×16 array with pixel distance 80 μm resulted to a continuous laccase enzyme film on the electrode. According to the optimum laser transfer conditions, the energy fluence was 400 mJ/cm^2 and the spot size was 75 μm. The physical absorption of the enzyme in the rough substrate was studied by contact angle (CA) measurements. There is a wetting variation in relation to the laser printing energy that can lead to the direct immobilization of the biomaterials on a rough substrate [19] as can be seen in Fig. 2.

As the laser fluence was increased from 125 to 300 mJ/cm^2, there was a gradual transition from a partial wetting state to a complete wetting state. This is a result of the extremely high impact pressure (1–10 MPa) produced by the high velocity transfer of the biomaterial. The reference partial wetting state obtained by the pipetting method is shown for comparison. The volume of the printed laccase enzyme was calculated through Eq. (1) 1.68 ± 1.61 nL and therefore the concentration of the immobilized laccase enzyme on the graphite SPE was 0.086 ± 0.008 U.

$$V = \frac{\pi h}{6}(3a^2 + h^2) \quad (1)$$

where, a is the radius and h is the height of the printed droplet.

3.2. Biosensor response

In order to evaluate the biosensor performance, tests were conducted on different SPEs with immobilised laccase in the presence of catechol at various concentrations. After a stabilization period the biosensor showed a cathodic current when catechol was added to PBS buffer. Before each test, the SPE was rinsed twice with 50 μL PBS so as to remove any nonphysically absorbed enzyme layers to the graphite SPE. The biosensor showed high signal to noise ratio and in the presence of catechol solution, a reduction current was generated. The reproducibility of the biosensor was good, showing a relative standard deviation (RSD) value of 11.7% and a detection limit of 150 nM. Concerning the repeatability, the biosensor has been used up to four times for the detection of 300 nM catechol solution without a decrease at the amperometric signal response. A dose-response curve was obtained in the presence of several