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Laccase–MWCNT–chitosan biosensor—A new tool for total polyphenolic content evaluation from *in vitro* cultivated plants

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ABSTRACT

Laccase from *Trametes versicolor*, an enzyme with broad substrate specificity for the phenolic substrates was employed as a biorecognition element in order to develop a biosensor for total content evaluation of phenolic secondary metabolites from two "*in vitro*" cultivated plants: *Salvia officinalis* and *Mentha piperita*. The enzyme immobilization was carried out by entrapment into the nanocomposite film during electrodeposition process from multiwall carbon nanotubes (MWCNT)–chitosan (CS) solution containing 25 U/mL laccase. Optimum conditions for MWCNT–CS film deposition (2 mg MWCNT/mL chitosan 1% solution prepared in 1% acetic acid) on gold support using a −1.5 V *vs.* Ag/AgCl controlled potential V for 5 min were established taking into consideration the layer capacity value. FTIR studies were performed to obtain information about the secondary structure of enzyme entrapped into the MWCNT–CS nanocomposite film. Calibration of the laccase biosensor was performed on four phenolic acids (caffeic acid, chlorogenic acid, gallic acids and rosmarinic acid) as substrates at −0.2 V *vs.* Ag/AgCl reference electrode. The developed biosensor was sensitive to micromolar concentration of the tested polyphenols. The performance characteristics of the biosensor for rosmarinic acid were: limit of detection 2.33×10^{-7} mol L^{-1}, response linear range 9.1×10^{-7}–1.21×10^{-5} mol L^{-1} and sensitivity 846 μA/mmol. The obtained values of the Km^{app} for all tested substrates proved that nanocomposite film provides a proper environment for enzyme immobilization, preserving enzyme catalytic specificity. The functionality of the developed biosensor was tested to evaluate the total polyphenolic content from real samples (*S. officinalis* and *M. piperita* extracts), results being expressed in equivalent rosmarinic acid.

1. Introduction

Polyphenols, one of the most important classes of natural antioxidants, widely occurring in fruit, vegetables and medicinal plants have been found to have a protective role against many chronic human diseases associated with oxidative stress, like cancer or cardiovascular disease [1,2]. They have also been employed as markers in taxonomic studies and food quality control [3]. Polyphenols are divided into three large groups: phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), flavonoids (anthocyanidins, flavonols, flavonones, flavones, isoflavones and chalcones) and tannins (hydrolyzable tannins and condensed tannins) [4]. Due to the importance of this class of compounds, many analytical strategies to evaluate the total polyphenolic content from plant extract and to establish phenolic profile have been reported in the literature. Liquid chromatography (LC) is the most used technique for polyphenolic compounds separation and quantification. LC with different types of detectors have been employed, among which are the photodiode array detector (DAD) [5,6], mass spectrometer (MS) [7,8], fluorescence detector [6] or electrochemical detector [4]. Gas Chromatography (GC) coupled to Flame Ionization or Mass Spectrometer detectors are techniques also used for separation, identification and quantification of volatile phenolic compounds or non-volatile compounds readily derivatized [9,10]. However these techniques do not easily allow continuous monitoring in real samples. Moreover, there are expensive, time-consuming, need skilled operators, and sometimes require preconcentration and extraction steps that increase the risk of sample loss.

Another strategy to assess total polyphenolic content is the Folin-Ciocalteu (F-C) method [11,12]. However this method requires mandatory steps and conditions to obtain reliable results: (1) proper volume ratio of alkali and F-C reagent; (2) optimal reaction time and temperature for color development; (3) monitoring of the absorbance at 765 nm; (4) use of gallic acid as the reference standard phenol; (5) correction for interfering substances [13].

Considering all these tedious steps of analysis, the development of a simplest procedure capable to provide reliable data with respect to total polyphenolic content determination appear as necessary and useful. Different enzymatic biosensors with redox

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enzymes have been proposed as an alternative device for total polyphenolic content (TPC) assessment. The main advantage of using redox enzymes in amperometric biosensor developing is the value of the potential applied to monitor reduction or oxidation of the species at the electrode surface, that generally occur in −0.2 to 0 V range, potential frame that allows to reach a minimum of possible electrochemical interferences [19].

Various electrochemical biosensors based on immobilized tyrosinase [14,15], laccase [16,17], peroxidase [18,19], or laccase-tyrosinase [20,21] have been developed for the TPC evaluation in wine, beer, tea, olive oil, and vegetable extract. The mechanism of polyphenols detection using these types of biosensors is based on monitoring the reduction current of quinones resulted as consequence of polyphenols enzymatic oxidation [22].

Immobilization of enzymes on the electrode surface is considered as one of the critical steps that dictate the effectiveness of the enzymatic biosensor, different approaches being developed in order to preserve enzyme specificity and to retain their native structure. Enzyme immobilization on electrode surfaces by physical adsorption, covalent linking of laccase to the surfaces of carbon materials, gold or platinum via bi-functional reagents or enzyme incorporation into a polymer matrix has been described [23–28].

Chitosan is a biocompatible polysaccharide which exhibits good film forming capacity, good adhesion to various supports, high mechanical strengths and high water permeability. The presence of the reactive amino and hydroxyl functional groups allows chitosan to be easily modified by covalent functionalization, making the resulting composites more stable and with tuned properties. Chitosan has been widely used as matrix for enzyme immobilization through ionic or covalent crosslinking, dip-coating or the electrodeposition processes [27–30]. Electrochemical deposition of chitosan has been reported as an effective method for enzyme immobilization and formation of chitosan film with controllable thickness. During electrodeposition other substances such as redox mediators [31], gold nanoparticles [32,33], or carbon nanotubes [27–30] can be incorporated into the chitosan film, leading to biocomposites with enhanced electrical conductivity.

In present work, laccase from *Trametes versicolor*, an enzyme with broad substrate specificity for the phenolic substrates [24] was employed as a biorecognition element in order to develop a biosensor for total content evaluation of polyphenolic secondary metabolites from two "*in vitro*" cultivated plants: *Salvia officinalis* and *Mentha piperita*. The enzyme immobilization was achieved by entrapment into the nanocomposite film during the electrodeposition process from multiwall carbon nanotubes (MWCNT)–chitosan (CS) solution.

From our knowledge, there are no data in literature reporting entrapment of laccase into the CS–MWCNT layer through the one step electrodeposition process. Fourier Transform Infrared Spectroscopy (FTIR) studies performed to obtain information about the secondary structure of laccase entrapped into the CS–MWCNT nanocomposite layer provided the proof of potential preservation of enzyme conformational specificity.

2. Experimental

2.1. Reagents and solutions

Gold foil (Au) (99.9% purity, 0.25 mm thickness), chitosan (CS) from crab shells (medium molecular weight, 85% deacetylated), acetic acid, laccase (Lacc) from *T. versicolor* (25 U/mg solid), $K_3[Fe(CN)_6]$ 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) caffeic acid, rosmarinic acid, chlorogenic acid and gallic acid were purchased from Sigma–Aldrich. Multiwall carbon nanotubes (MWCNT) (97% purity, 10–30 nm diameter, length > 5 μm) were provided by Nanothinx SA, Greece and they were used as received.

Stock solutions of the phenolic compounds (10^{-3} mol L^{-1}) were prepared daily by dissolving appropriate amount in either buffer solution or in ethanol depending on the phenolic compounds solubility. Diluted solutions were prepared in McIlvaine buffer (mixture of 0.1 M citric acid and 0.2 M disodium phosphate) at working pH (pH 4.5) which is also used as supporting electrolyte. All reagents used in this study were of analytical grade and all solutions were prepared from a Millipore Milli Q system.

The plant extract samples were provided by University of Pisa, Department of Pharmacological Sciences, Pisa, Italy and Institute of Soil Science and Plant Cultivation Pulawi Poland, partners in the FP6 Nutrasnack project.

2.2. Equipment

Electrochemical measurements were performed on Autolab System PGSTAT 302N, at room temperature. Experiments were carried out using a three electrode cell operating with an Ag/AgCl (3 M KCl) as reference electrode, a platinum wire as counter electrode and gold sheets (Au), gold/chitosan (Au/CS), gold/chitosan–multiwall carbon nanotubes (Au/CS–MWCNT), gold/Laccase–chitosan–multiwall carbon nanotubes (Au/Lacc–CS–MWCNT) as working electrodes. Cyclic voltammetry was employed to assess the electrochemical behavior of the modified electrodes. Performance characteristics of the Au/Lacc–CS–MWCNT were evaluated using chronoamperometry.

Scanning electron microscopy images of chitosan (CS) film and CS–MWCNT nanocomposites film were recorded using the TESCAN VEGA II LMU Scanning Electron Microscope at an accelerating potential of 5 kV and working distance of 3.5 mm.

Spectra of free laccase and immobilized laccase were recorded on a Bruker Tensor 27 Fourier Transform Infrared spectrometer using KBr pellets technique, or Variable angle reflectance (VAR-FTIR) technique, angle 45°, under dry air at 25 °C. Each FTIR spectrum represents the average of 64 scans at 4 cm^{-1} resolution. Brucker's OPUS 6 software was used to record and process samples spectra.

2.3. Preparation of the modified electrodes

Before each modification gold foil used as working electrode was polished with 0.05 μm α-Al_2O_3 nanopowder. After 5 min of sonication in double distilled water, the electrodes were immersed in freshly prepared Piranha solution (30% H_2O_2:concentrated H_2SO_4 3:1, v:v). After 15 min of Piranha treatment electrodes were submitted to ultrasounds once more in double distilled water for 5 min.

A 1% CS solution was prepared by dissolving CS in 1% acetic acid solution with magnetic stirring for about 4 h and used for preparation of solution undergoing electrodeposition.

MWCNT were dispersed in the CS solution by ultrasounds. Before the deposition, suspension was sonicated 6 h, until a homogenous dispersion of MWCNT was achieved. Non-covalent wrapping of the tubular surface with chitosan chain during sonication led to a stable and homogenous dispersion of MWCNT in aqueous solution. After that Laccase was also homogenously dispersed in the chitosan solution due to their electrostatic interaction with chitosan chain. It has been widely described in the literature that the mechanism of chitosan deposition at negative potentials is based on their charge and solubility pH dependence [30,34,35]. At slightly acidic pH (pH < 6.00) chitosan is protonated and soluble. When the pH nears a negative electrode surface it raises above 6.3 (pK_a of CS), due to the proton consumption, the amino group becomes deprotonated, leading to an insoluble hydrogel network onto the electrode surface.