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Nanostructured enzymatic biosensor based on fullerene and gold nanoparticles: Preparation, characterization and analytical applications

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ABSTRACT

In this work a novel electrochemical biosensing platform based on the coupling of two different nanostructured materials (gold nanoparticles and fullerenols) displaying interesting electrochemical features, has been developed and characterized. Gold nanoparticles (AuNPs) exhibit attractive electrocatalytic behavior stimulating in the last years, several sensing applications; on the other hand, fullerene and its derivatives are a very promising family of electroactive compounds although they have not yet been fully employed in biosensing. The methodology proposed in this work was finalized to the setup of a laccase biosensor based on a multilayer material consisting in AuNPs, fullerenols and *Trametes versicolor* Laccase (TvL) assembled layer by layer onto a gold (Au) electrode surface.

The influence of different modification step procedures on the electroanalytical performance of biosensors has been evaluated. Cyclic voltammetry, chronoamperometry, surface plasmon resonance (SPR) and scanning tunneling microscopy (STM) were used to characterize the modification of surface and to investigate the bioelectrocatalytic biosensor response. This biosensor showed fast amperometric response to gallic acid, which is usually considered a standard for polyphenols analysis of wines, with a linear range 0.03–0.30 mmol L^{-1} (r^2=0.9998), with a LOD of 0.006 mmol L^{-1} or expressed as polyphenol index 5.0–50 mg L^{-1} and LOD 1.1 mg L^{-1}. A tentative application of the developed nanostructured enzyme-based biosensor was performed evaluating the detection of polyphenols either in buffer solution or in real wine samples.

1. Introduction

It is well established that one of the most important aspects greatly affecting the performances of electron-transfer based biosensors is represented by the characteristics of the immobilization procedure of redox proteins onto the electrode surface. This is particularly important in the development of third generation biosensors (Armstrong et al., 1988; Léger, Bertrand, 2008; Yao and Shiu, 2008), where the most important issue is to obtain an efficient electron transport between redox centers of proteins and electrodes (Willner and Katz, 2000; Zhang et al., 2005) without disrupting the protein native structure. Nonetheless, direct electron transfer (DET) between redox proteins and electrodic surfaces is uncommon as it is often hindered by the inaccessibility of the redox center, which thus limits the development of this type of biosensor (Ghindilis et al., 1997; Zhang and Li, 2004; Shan et al., 2009).

In this context, the use of nanomaterials for the construction of biosensing devices constitutes one of the most promising approaches (Katz and Willner, 2004; Katz et al., 2004). The unique properties of nanomaterials to provide a suitable micro-environment for proteins immobilization, maintaining their bioactivity, and at the same time facilitating electron transfer between their redox center and electrode surfaces, have led to an intensive use of different nanostructured materials for the construction of electrochemical biosensors with enhanced analytical performance (Guo and Wang, 2007). Nanomaterials such as metal nanoparticles (NPs) or carbon nanotubes (CNTs) display unique electronic, optical and catalytic features and in addition, their dimensions are similar to those of several biomolecules. This fosters the integration of nano-objects to biomolecules, thus generating hybrid systems combining the peculiar electronic properties of nanomaterials with the natural recognition and catalytic function of biomolecules (Katz and Willner, 2004; Baron et al., 2007, Antonelli et al., 2009): in particular, they allow to electrically wire redox enzymes with electrodes and to reduce electron transfer distances.

In this respect, most of recent advances in electrochemical biosensors rely on the employment of gold (Au) NPs. Modification

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of electrode surfaces with self-assembled monolayers (SAMs) of thiols provide a simple way to design tailored materials that can be further used as functionalized sites to immobilize AuNPs and enzyme (Gooding and Hibbert, 1999). For example, a recent study has demonstrated a rapid DET between a laccase and AuNPs-modified electrodes, enabling efficient bioelectrocatalytic oxygen reduction (Dagys et al., 2010). Recently, Brondani et al. (2013) proposed a platform for DET, immobilizing a laccase by means of polyethyleneimine (PEI)-coated AuNPs on a glassy carbon electrode. This biosensor was employed for cathecol quantification in spiked water samples. Moreover, AuNPs synthesis (Guo and Wang, 2007) and surface functionalization are simple and easily performed (Ansari and Husain, 2012) and have found various applications (Wang, 2005).

Since the discovery of buckminsterfullerene (C60) in 1985 (Kroto et al., 1985), fullerenes have been widely studied due to their unique structural, electronic, and spectroscopic properties (Dagani, 2002), which may be exploited for their diverse applications in chemistry, biology, and nanoscience (Nakamura and Isobe, 2003; Deng et al., 2004; Chaniotakis, 2007; Gavalas and Chaniotakis, 2009). Fullerenes is a very promising family of electroactive compounds; there have some unique characteristics that make this new type of compound very promising as mediators in amperometric biosensors. An example is represented by a glucose biosensor (Sotiropolou et al., 2003), where a porous carbon rod loaded with fullerene was allowed to adsorb the enzyme. The sensitivity of the resulting biosensor increased with the amount of adsorbed fullerene. These fullerene mediated systems also maintained the good overall analytical characteristics of the unmediated ones, often allowing the resulting electrochemical biosensors to operate at lower potentials thus reducing the interferences from electroactive compounds. Goyal et al. (2007) described an ingenious approach for the fabrication of a promising fullerene (C_{60})-modified electrode for detection of nandrolone in human serum and urine samples.

Laccases (p-diphenol: oxygen oxidoreductase, EC 1.10.3.2) are copper containing oxidoreductases detected in many plants (Gramss et al., 1999), bacteria (Mayer and Staples, 2002) and secreted by numerous fungi (Luterek et al., 1997). They are able to oxidize many different substrates with the concomitant reduction of oxygen to water (Yaropolov et al., 1995; Solomon et al., 1996; Morozova et al., 2007). Laccases have been applied in paper industry (Crestini and Argyropoulos, 1998), oxidation of organic pollutants (Lante et al., 2000), in food industry (Minussi et al., 2002) and in the development of biosensors (Vianello et al., 2004; Jaroz-Wilkolazka et al., 2004; Shimomura et al., 2011; Shervedani and Amini, 2012) or biofuel cells (Tayas et al., 1999).

In this article a new approach for the development of a nanostructured laccase-based electrochemical biosensor, that exploits the beneficial features of functionalized fullerenols and AuNPs is proposed, in order to obtain a significant improvement of the electroanalytical properties of the device. The choice of AuNPs and fullerenols is due to their remarkable properties such as high surface areas, conductivity, flexibility and reactivity that can be accounted for improving the enzyme biosensor performances.

The biosensor was realized firstly by immobilizing functionalized AuNPs on a gold electrode surface modified with a self-assembled monolayer (SAM) of suitable thiols, bearing chemical moieties that can be further used as functionalization sites to immobilize nanomaterials. Then, the polyhydroxy-fullerene (Full) has been linked onto the AuNPs modified electrode, where the *Trametes versicolor* laccase enzyme (TvL), has been finally immobilized. The ability of this laccase to exert its catalytic activity towards many types of aromatic compounds, has been thoroughly investigated (Yaropolov et al., 1994; Gomes and Rebelo, 2003; Canfora et al., 2008).

The suitability of the modification procedure has been checked by Surface Plasmon Resonance (SPR) while a characterization of the obtained surface was performed by means of Scanning Tunneling Microscopy (STM) experiments. In order to check the efficiency of this strategy, the kinetic and analytical parameters of the resulting nanostructured biosensor with respect to non-nanostructured configuration, have been compared. The electrochemical characterization of the AuNPs-Full-TvL biosensor in both direct and mediated electron transfer phenomena was studied by means of cyclic voltammetry (CV) experiments. Finally, a preliminary application of the developed composite material was performed by employing it in the preparation of a TvL biosensor using a screen printed electrode (SPE) as gold substrate; the resulting biosensor has been proven to be stable enough to be used under flow injection analysis (FIA) conditions for evaluating the polyphenols content in real samples of wines.

2. Experimental

2.1. Chemicals and reagents

Fungal Laccase from *Trametes versicolor* was supplied by Fluka (EC 1.10.3.2, activity: 30.6 U mg^{-1}) and stored at −18 °C. Gallic acid, caffeic acid, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium ferricyanide $K_3[Fe(CN)_6]$, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), 3-mercaptopropionic acid, 11-mercaptoundecanoic acid, polyhydroxy small gap fullerenes, cysteamine, tetrachloroauric (III) acid ($HAuCl_4 \cdot 3H_2O$) and sodium borohydride ($NaBH_4$) were purchased from Sigma-Aldrich (Buchs, Switzerland) and used as received. Solutions of redox mediators were prepared in 0.1 mol L^{-1} acetate buffer, pH 4.5, immediately before use.

Kit for measurement of total polyphenols concentration in wines was purchased from Biogamma Srl (Rome, Italy). The kit contains Folin–Ciocalteau reagent (composed by a mixture of $H_3PW_{12}O_{40}$ and $H_3PMo_{12}O_{40}$), carbonate buffer and a standard solution of gallic acid 3.0 g L^{-1}. Stock solutions of gallic acid were prepared in 0.1 mol L^{-1} Britton–Robinson buffer, pH 5.0 daily. More diluted standard solutions were prepared by suitable dilutions with the same buffer. Other chemicals were all of analytical grade.

High purity deionized water (Resistance: 18.2 MΩ cm at 25 °C; TOC < 10 μg L^{-1}) obtained from Millipore Direct-Q UV_3 (France) has been used to prepare all the solutions. Different wine samples were acquired from a local supermarket in Rome (Italy). The only sample treatment required consisted of an appropriate dilution with a buffer solution before analysis.

2.2. Methods

2.2.1. Synthesis of thiol-funtionalized gold nanoparticles

Synthesis of thiol-derivatised gold nanoparticles (AuNPs-Linker) was obtained by reduction of Au colloid solution in presence of $NaBH_4$ as reducing agent and alkanethiols as the stabilizing ligands as reported in literature (Brust et al., 1994). Au nanoparticles functionalized with 3-mercaptopropionic acid and 11-mercaptoundecanoic acid were prepared by mixing a 10 mL solution containing 197 mg of $HAuCl_4$ in ethanol and 5 mL solution containing 3-mercaptopropionic acid and 11-mercaptoundecanoic acid in methanol (molar ratio 1:2). The two solutions were stirred in the presence of 2.5 mL of glacial acetic acid in an ice bath for 1 h. Subsequently, 7.5 mL of aqueous solution of 1 M $NaBH_4$ was added dropwise, the organic phase changes color from orange to deep brown within a few seconds, associated with the presence of the AuNPs. The solution was stirred for an additional hour in an ice bath and then after further stirring for 14 h at room temperature,

the organic phase was washed with ethanol to remove excess thiol and centrifuged (twice in each solvent) with methanol, ethanol and diethyl ether. This methodology produced spherical functionalized AuNPs with a mean particle size of ca. 4.0–5.0 nm (Willner and Katz, 2000).

2.2.2. Assembling of nanostructured transducer and biosensor preparation

Composite nanostructured material was prepared by step-by-step chemical modification of Au surface either of classical Au microelectrode or screen printed electrode (SPE) for electrochemical experiments or Au substrate for surface plasmon resonance (SPR) or scanning tunneling microscopy (STM) measurement. In the case of classical Au microelectrode, before chemical modification, the electrode surface was mechanically cleaned by polishing with 0.3–0.05 μm alumina slurries, then rinsed with ethanol and deionized water and, finally, sonicated in water for 2 min. The gold surface for SPR and STM measurements were cleaned by immersion for 2 h in piranha solution while the Au SPEs were used as are.

After cleaning, the Au surface was placed into 18 mmol L^{-1} cysteamine (CA) aqueous solution at room temperature in darkness for 4 hours allowing the formation of a self-assembled monolayer (SAM) thereof. Then it was removed from the cysteamine solution, rinsed copiously with water and blow dry with nitrogen gas stream and then the Au-SAM was modified as follows: in a first step, 20 μL of a solution containing AuNPs-linker 2 mg mL^{-1} together with 0.5 mmol L^{-1} EDC and 0.1 mmol L^{-1} NHS, in order to activate the –COOH groups were deposited onto electrode surface; this allows to link the AuNPs to electrode surface via amide bonds. After 30 min the surface was rinsed with water and then 20 μL of polyhydroxy-fullerenes 0.07 mg μL^{-1} with EDC/NHS mixture were deposited onto the modified surface thus forming ester bond and creating the second nanostructured layer. After 30 min it was rinsed again with water and finally, 10 μL of a solution containing 0.076 U μL^{-1} of TvL and EDC/NHS mixture were deposited on the resulting surface to form the enzymatic layer and the electrode was left to dry for about 20 min at room temperature (Scheme 1).

2.2.3. Electrochemical experiments and electrochemical apparatus

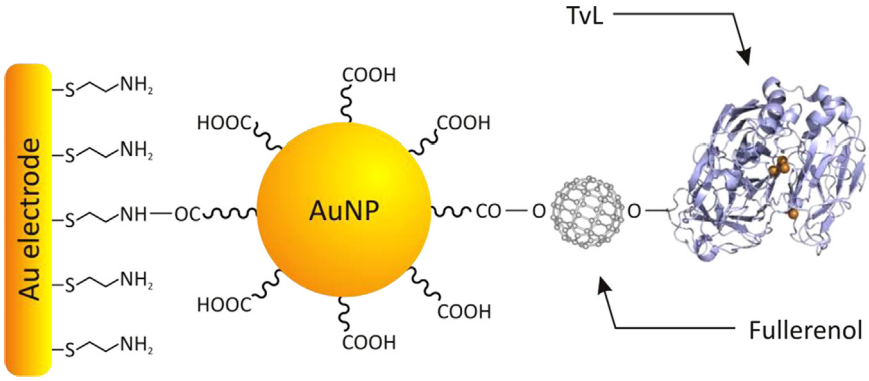
All electrochemical experiments were performed by using a μ-Autolab type III potentiostat from EcoChemie (Utrecht, The Netherlands) controlled by means of the GPES Manager program (EcoChemie). Batch electrochemical experiments were performed in a 5 mL thermostated glass cell (Model 6.1415.150, Metrohm, Switzerland) with a conventional three-electrode configuration constituted by a gold (Au) electrode, with a surface diameter of 3 mm, (Model 6.1204.320, Metrohm, Switzerland) as working electrode, a glassy carbon rod (Model 6.1241.020, Metrohm, Switzerland) as counter electrode and a Ag/AgCl/KCl_{sat} (Model 6.0726.107, Metrohm, Switzerland) as reference electrode (198 mV vs NHE). All the experiments were performed in acetate buffer 0.1 M, pH = 4.5 at 25 °C. Flow experiments, for wine analysis, were carried out using a microliter Flow Cell (DropSens, Oviedo, Spain) and a Gilson Minipuls-3 peristaltic pump; in this case, Screen-Printed Electrodes (SPEs) (Model DPR-220AT, DropSens, Oviedo, Spain), constituted by an Au working electrode with a surface diameter of 4 mm, a graphite counter electrode and an Ag/AgCl/KCl_{sat}, as reference electrode, were used. Measurements were carried out at a fixed potential of −100 mV vs. the internal silver/silver chloride reference electrode with a flow rate of 0.894 mL min^{-1}. The carrier buffer was acetate buffer 0.1 M, pH = 4.5 and aliquots of gallic acid standard solutions at different concentrations in the same buffer were injected (loop volume 250 μL) to obtain the calibration plot. The same procedure has been followed in the analysis of wine samples appropriately diluted.

2.2.4. Spectrophotometric measurements

The spectrophotometric measurements were carried out by using a T60U Spectrophotometer (PG Instruments Ltd., Wibtoft Leicestershire, United Kingdom). Spectrophotometric measurements were carried out according to the assay procedure (Folin and Denis, 1912; Lowental, 1977). This method is specific for the –OH groups of the polyphenolic compounds and the obtained results give the polyphenol index referred as gallic acid concentration (Jaroz-Wilkolazka et al., 2004). All the values reported are the average of at least six measurements.

2.2.5. SPR experiments

The SPR experiments were performed by an EcoChemie Autolab SPR system (EcoChemie, The Netherlands). It works with a laser diode fixed at a wavelength of 670 nm, using a vibrating mirror to modulate the angle of incidence of the p-polarized light beam on the SPR substrate. The instrument is equipped with a cuvette. The planar gold SPR disks and the gold disks modified with polycarboxylate hydrogel 80-nm thickness (HC80) were purchased from Xantec Bioanalytics (Germany). The gold sensor disks (25 mm in diameter) were mounted on the hemicylindrical lens (with index-matching oil) to form the base of the cuvette. An O-ring (3-mm inner diameter) between the cuvette and the disk prevents leakage. An autosampler (EcoChemie) with controllable aspirating–dispensing–mixing pipette was used to add samples into the cuvette and provide constant mixture by aspiration and dispensing during measurements. This experimental arrangement maintains an homogeneous solution and reproducible



Scheme 1. Au-SAM/AuNPs-Linker/Fullerenols/TvL composite material assembly.

hydrodynamic conditions. The temperature of the cuvette was maintained at 25 ± 1 °C. Data were transmitted to a laptop computer and analyzed by an SPR software 4.1.2 version from EcoChemie.

The planar gold SPR disks were extensively cleaned in a freshly prepared piranha solution (3:1 H_2SO_4 98%:H_2O_2 30%). After 1 h, the disks were thoroughly rinsed with water, dried in a stream of nitrogen gas and immediately incubated into 18 mmol L^{-1} cysteamine (CA) aqueous solution at room temperature in darkness for 4 hours allowing the formation of a self-assembled monolayer (SAM) thereof. Then it was removed, rinsed accurately with water and blow dry with nitrogen gas stream then placed over the glass prism inside the SPR instrument. The modification procedure described in section 2.2.2 was then carried out using the semi-automatic procedure driven by EcoChemie software.

2.2.6. STM measurements

Gold substrates (Arrandee™) with a thickness of 250 nm (± 50 nm) were prepared by evaporation on top of an adhesive chromium layer (2.5 nm) deposited on borosilicate substrates. They were annealed with a butane flame at a temperature of about 1300 °C to obtain re-crystallized terraces and rinsed after annealing. The quality of the annealed gold surface was assessed by STM, which showed atomically flat (111) terraces over hundreds of nanometers. Then, the gold surface was modified accordingly to the procedure described in Section 2.2.2. and was finally rinsed with ultrapure water and blown dry with pure nitrogen. The analysis was restricted to the central region of the sample over an area of about 0.6 cm^2, where a uniform coverage was obtained.

A Picoscan system (Molecular Imaging) equipped with a 10 μm scanner with a final preamplifier sensitivity of 1 nA/V was used for STM measurements. STM tips were prepared by electrochemical etching of Pt/Ir wire (Goodfellow).

3. Results and discussion

3.1. Realization and characterization of composite material

The suitability of the procedure to assemble the nanostructured material was firstly checked by carrying out all the necessary steps along a SPR experiment. As evidenced in Fig. 1, after the activation

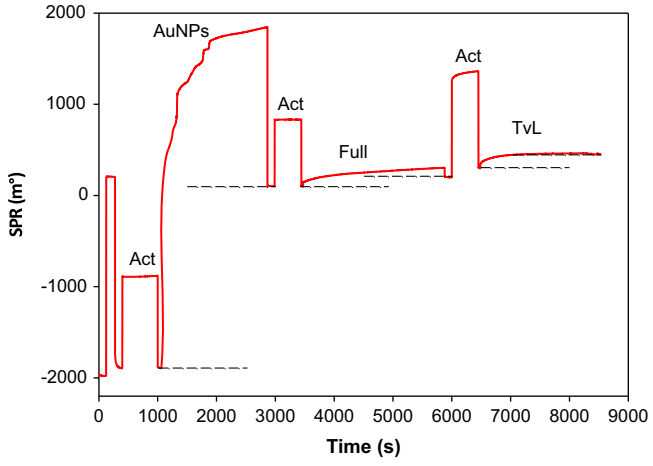


Fig. 1. SPR detection of step-by-step assembly of nanostructured material; (Act): activation of carboxyl groups by EDC/NHS, (AuNPs): gold nanoparticles injection, (Full): fullerenol injection, (TvL): *Trametes versicolor* laccase injection.

of CA SAM, a huge increase (about 3400 m°) of SPR signal relative to AuNPs addition can be observed that remains (about 2500 m°) even after the washing procedure with the buffer solution. This can be explained taking into account not only the nanoparticles dimensions but also the signal amplification due to the changes in the dielectric properties of the matrixes, occurring through the coupling of the localized plasmon of the NPs with the surface plasmon wave (Lyon et al., 1999). On the other hand, the two successive modification steps involving fullerene and laccase, yielded a smaller signal (150–200 m°) if compared to that one of AuNPs but really significant to establish that the considered species are not simply adsorbed onto the surface but they are chemically bound one to the other thus witnessing the efficiency of chemical reactions used for the nanostructure assembly. To assess the influence of different modification steps proposed on the electrochemical properties of the resulting device, cyclic voltammetric (CV) experiments were performed in the presence of different electroactive mediators: $K_3[Fe(CN)_6]$, ABTS, gallic acid and caffeic acid. The electrochemical characterization of the mediators was performed by recording the voltammetric response of 0.5 mmol L^{-1} mediator solutions in 5 mL of acetate buffer 0.1 M, pH 4.5 at scan rate of 5 mV s^{-1}, in oxygen atmosphere. The first considered redox compounds, $K_3[Fe(CN)_6]$ and ABTS, show a quasi-reversible or reversible cyclic voltammogram on the working electrode either before (Au-bare) and after different modification steps (Au-SAM/AuNPs-Linker and Au-SAM/AuNPs-Linker/Fullerenols), respectively (data not shown). Comparing the CVs obtained with the different electrode configurations, a significant improvement of the current response due to the presence of the nanostructured materials can be observed; this can be ascribable to a large increase in surface area, resulting in an increase in the surface-roughness factor (ρ). Table SM1 (see Supplementary Materials) reported roughness values for each proposed configuration, calculated as the ratio of the electrochemical surface area to the geometric surface area. The electroactive surface area of the electrodes was calculated by cyclic voltammetry in 1.1 mmol L^{-1} $K_3[Fe(CN)_6]$ solution containing 0.1 mol L^{-1} KCl at different scan rates, according to the Randles–Sevcik equation (Bard and Faulkner, 2000). The high roughness values of the modified electrodes should promote a more effective immobilization of the protein, when used as electrochemical transducers in biosensors assembly.

The electron transfer rate constant (k°) values for ferricyanide reported in Table SM1 were calculated from CVs data by applying the method described by Kochi (Klinger and Kochi, 1981) considering (i) $n=1$ the number of electrons transferred, (ii) $D=7.6\times10^{-6}$ cm^2 s^{-1} the diffusion coefficient for ferricyanide and (iii) $\alpha=0.5$ the dimensionless parameter known as electron transfer coefficient for the reversible reaction; it appears evident the increase in electrochemical performances achieved once the nanostructures are used to modify the electrodic surface. Moreover, the higher efficiency of electron transfer becomes evident once the electrochemical response obtained in presence of not fully reversible redox mediators, is considered: Figure SM1 (see Supplementary Materials) shows a comparison of the CVs of 0.5 mmol L^{-1} caffeic acid solution in acetate buffer 0.1 mol L^{-1}, pH 4.5, at scan rate of 5 mV s^{-1}. It is known that the electrochemical behavior of caffeic acid is consistent with an Electrochemical-Chemical mechanism (EC mechanism), involving a two electron transfer followed by a first order chemical reaction (Trabelsi et al., 2004). As a consequence of this secondary process, the electroactive substance produced in the oxidation of caffeic acid is removed from the electrode surface and therefore the associated cathodic peak disappears being the chemical reaction rate faster than the electron transfer kinetic. Comparing the current responses obtained for the different electrode

configurations, it can be observed that in the presence of AuNPs and fullerenols, the CVs tended to be more reversible, suggesting that the nanomaterials improve the electron-transfer kinetic of the process. In particular, the increase of system reversibility can be evaluated from both the peak currents intensity ratio (i_c/i_a) and peaks separation (ΔE_p): for (i) Au-SAM, (ii) Au-SAM/AuNPs-Linker and (iii) Au-SAM/AuNPs-Linker/Fullerenols, the i_c/i_a corresponding values are 0.33, 0.45 and 0.70, while ΔE_p values are 110, 100 and 85 mV, respectively.

3.2. Development of TvL-biosensor

Once established that the assembled nanocomposite material (Au-AuNPs-Fullerenols) increases the electrochemical performances either regarding the electroactive surface and the electron transfer kinetics, the resulting modified electrode was employed as transducer of an electrochemical biosensor based on laccase; to this end *Trametes versicolor* Laccase (TvL) enzyme was chemically bound to electrode surface after each one of all three steps of modification, namely on (i) Au-SAM, (ii) on Au-SAM/AuNPs-Linker and (iii) Au-SAM/AuNPs-Linker/Fullerenols as described in the Experimental section.

The TvL modified Au-SAM/AuNPs-Linker/Fullerenols surface displays direct electrocatalytic behavior in the absence of redox mediators (see Fig. 2) (Shleev et al., 2005). The voltammograms at different scan rate in anaerobic buffer at 25 °C (starting from low scan rate 0.1 V s^{-1} up to 1.0 V s^{-1}) show well definite, chemically reversible reduction–oxidation peak pair with a reduction potential of 130 mV vs Ag/AgCl that remains constant with scan rate and with an intensity ratio close to unit even at high scan rate. With the increase of scan rate, the redox peak currents also increased gradually. The linear dependence of the voltammetric peak currents vs the potential scan rate (reported in the inset of Fig. 2 only for Au-SAM/AuNPs-Linker/Fullerenols/TvL as an example) confirms the immobilization of the redox protein and indicates that the redox process was not controlled by diffusion but it was a typical of surface-controlled process, as expected for immobilized systems.

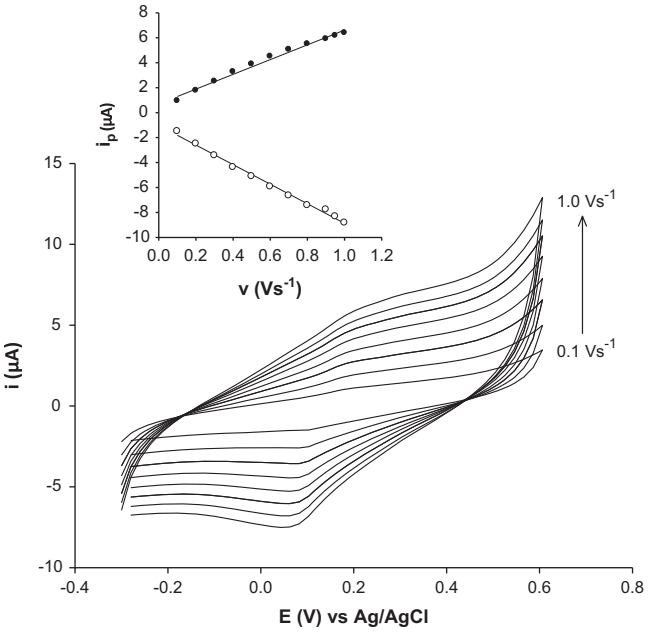


Fig. 2. CVs of TvL immobilized on Au-SAM-AuNPs-Linker/Fullerenols electrode, at different scan rate, from 0.1 Vs^{-1} to 1.0 Vs^{-1} in the potential range of of −0.3÷0.6 V vs Ag/AgCl. The experiments were performed under anaerobic conditions in 0.1 mol L^{-1}, pH 4.5 acetate buffer, at 25 °C. Inset: variation of anodic (black dots) and cathodic (white dots) peak intensities (i_p) as a function of scan rate (v).

Fig. SM2 (see Supplementary Materials) shows the linear relationship between the peak potential (E_p) and the natural logarithm of scan rate (ln v) for Au-SAM/AuNPs-Linker/Fullerenols/TvL biosensor, in order to calculate the value of apparent heterogeneous electron transfer rate constant (k_s), the following Laviron equation (Laviron, 1979) was used:

$$\log k_s = \alpha \log(1-\alpha) + (1-\alpha)\log \alpha - \log \frac{RT}{nFv} - \frac{\alpha(1-\alpha)nF\Delta E_p}{2.3RT} \quad (1)$$

where α is the electron transfer coefficient, n is the number of electron, ΔE_p is the separation of the redox peaks, v is the scan rate, R, T and F have their usual meanings (R=8.314 J mol^{-1} K^{-1}, T=298 K, F=96,493 C mol^{-1}). Obtained results for all three steps of modification are summarized in Table 1 where the formal potential ($E^{0'}$) of TvL, the electron transfer rate constant (k_s) and the surface concentration of immobilized electroactive protein (Γ^*) are compared.

The k_s values for nanostructures-modified electrodes were higher than those obtained for TvL immobilized on Au-SAM electrode; this can be explained taking into account that it is well documented in literature (Chen et al., 2009; Feng et al., 2006) how the nanostructured-modified electrodes can greatly enhance electron transfer rate of immobilized proteins due to the increase of roughness of the electrodic surface (see roughness increase in Table SM1). Particularly, the presence of nanostructured material increases the protein loading due to high surface-active area available for protein binding, improve the reversibility thereof and provide an ideal microenvironment to retain protein activity.

The surface concentration of immobilized electroactive protein (Γ^*) was estimated from the integration of reduction peak currents and using Faraday's law according to the following equation:

$$\Gamma^* = Q/nFA \quad (2)$$

where Q is the charge, n the electron transfer number, F the Faraday constant and A is the electroactive area of the working electrode. The Γ^* values for modified electrode were markedly higher than that obtained for Au-SAM electrode surface, indicating that nanostructured materials provide a very large active area for enzyme immobilization, as expected.

In the case of the third step of modification (Au-SAM/AuNPs-Linker/Fullerenols), a microscopic characterization of the transducer surface before and after modification with TvL has been carried out by scanning tunneling microscopy: in Fig. 3(a) it can be observed as before the enzyme immobilization, several nanoparticles of 15 nm width are detected. This size increase can be explained considering that each AuNP (diameter 5 nm) is modified both sides with fullerenol tethered by an eleven methylenic chain of mercaptoundecanoic acid. After modification with TvL (Fig. 3 (b)) a huge increase of particles size is detected (about 35 nm) that could be attributed to the establishment of a hybrid system more conductive than that found on the surface before linking the protein.

Table 1
Electrochemical parameters of *Trametes versicolor* biosensors employing different electrochemical transducers; formal potential ($E^{0'}$), diffusionless electron transfer rate constant (k_s) determined by Laviron method and amount of immobilized electroactive protein (Γ^*).

Transducer	$E^{0'}$ (mV) vs Ag/AgCl	k_s (s^{-1})	Γ^* (mol cm^{-2})
Au-bare	170	0.4	6.6×10^{-11}
Au-SAM/AuNPs-Linker	175	0.5	1.2×10^{-10}
Au-SAM/AuNPs-Linker/Full	186	0.9	3.0×10^{-10}

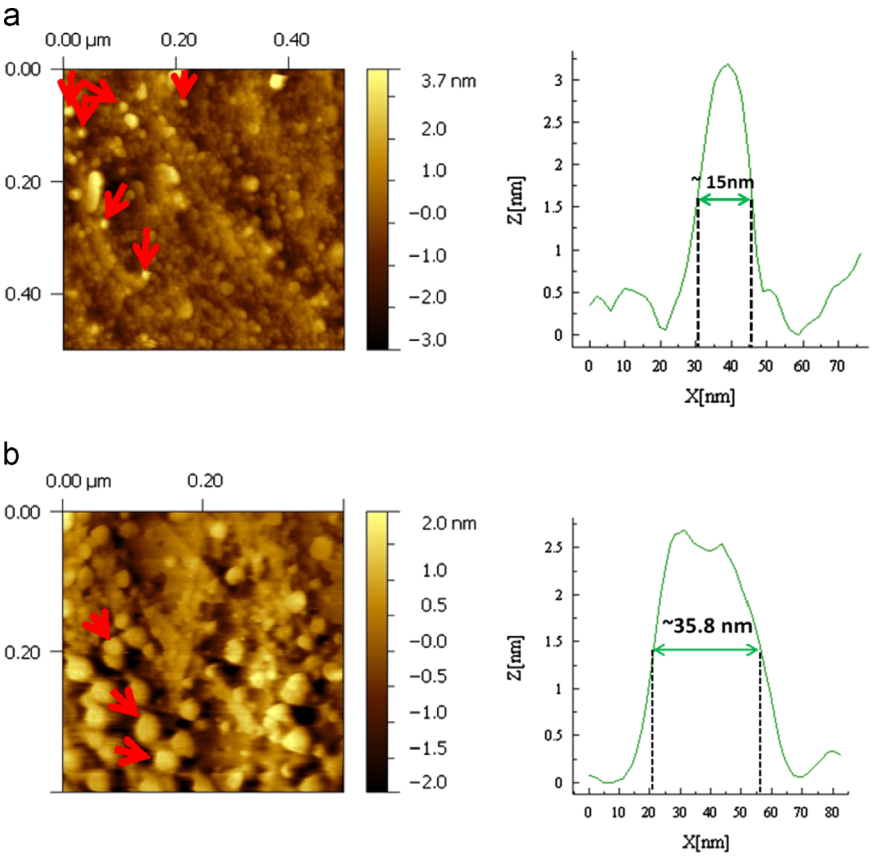


Fig. 3. STM images obtained for Au-SAM/AuNPs-Linker/Fullerenols before (a) and after (b) modification with TvL; on the right, the cross section profiles referring to the objects indicated by the arrows.

In order to characterize the electrocatalytic activity of TvL once immobilized onto electrodic material, slow-scan (scan rate of 5 mV s^{-1}) voltammograms were recorded in 0.5 mmol L^{-1} mediator solutions, pH 4.5 in the absence and in the presence of TvL immobilized on the electrode surface, in oxygen atmosphere; in particular, the obtained results for gallic acid were reported as examples in Figure SM3 in Supplementary Materials. In this case it must be taken into account that the CV of gallic acid is characteristic of an electrochemical irreversible reaction: the CV shows a typical sigmoidal catalytic curve characterized by the disappearance of the anodic process, due to the catalytic oxidation of the mediator, and a significant enlargement of the cathodic process, related to the reduction of the oxidized form of the mediator generated by the enzymatic reaction (Léger et al., 2003), thus suggesting that the immobilized laccase on any electrodic surface, retains its catalytic activity in the presence of substrate, also displaying an increase of bioelectrochemical performances. In addition, in the presence of nanostructured materials on the electrode, a significant increase of the catalytic current with respect to that one registered on Au-SAM, was also observed (Borisov et al., 2000), suggesting an enhancement of the electrochemical performances of the biosensor according to the different procedures of electrode modification with nanostructured materials, as already observed. These results agree with the observed improvement of the electron transfer rate between the modified electrode and immobilized enzymes, fostered by the considered nanomaterials; such an increased rate accelerates the regeneration of the enzyme and enhances the activity of the enzyme, that resulted in an increase of redox currents obtained by the biosensor.

In view of the possible application of the proposed nanocomposite material as electrochemical transducers for biosensors development, a TvL-biosensor was prepared by employing the described material on an Au-SPE as substrate and a full electroanalytical characterization of the system has been carried out. Hence, kinetic parameters using gallic acid as substrate were calculated by monitoring the variation of catalytic steady-state current at increasing mediator concentration at an appropriate applied potential (−100 mV) operating under flow-injection analysis. In Figure SM4(a) (see Supplementary Materials) the amperometric detection of gallic acid on Au-SAM/AuNPs-Linker/Fullerenols/TvL biosensor is showed. The current-concentration dependence (Figure SM4(b) in Supplementary Materials) was modeled by using Michaelis-Menten nonlinear fitting (Lindgren et al., 1999), thus allowing the calculation of the main kinetic parameters; the obtained results are shown in Table 2.

Table 2
Kinetic and analytical parameters of *Trametes versicolor* biosensors employing different electrochemical transducers obtained by FIA amperometry in 0.1 mol L^{-1} acetate buffer, pH 4.5 at a fixed potential of −100 mV using gallic acid as substrate.

Transducer	I_{max} (µA)	K_M^{app} (mM)	I_{max}/K_M^{app} (µA/mM)
Au-bare	4.55	0.84	5.42
Au-SAM/AuNPs-Linker	5.21	0.75	6.94
Au-SAM/AuNPs-Linker/Full	6.00	0.66	9.09

By comparing the calculated K_M^{app} for the different laccase-modified electrodes, we observed that K_M^{app} decreases after successive addition of AuNPs and Fullerenol suggesting an increase of the enzymatic affinity for the substrate. This behavior can be explained taking into account that the modification of electrode surfaces with nanostructured materials provides a favorable microenvironment for the protein similar to that of the redox

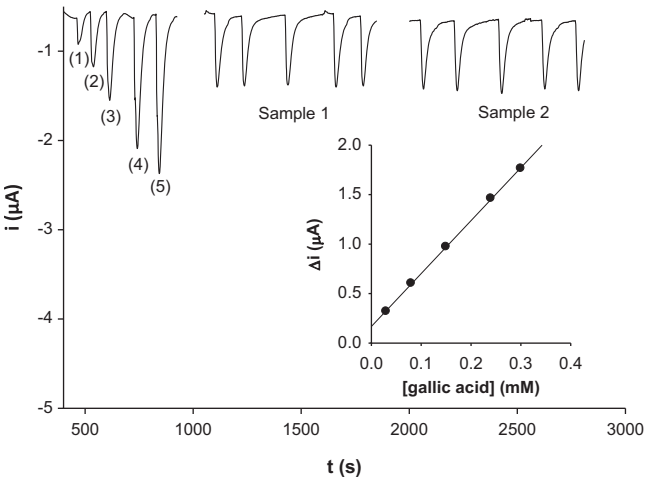


Fig. 4. Chronoamperometric detection of gallic acid in real samples operating at -100 mV vs Ag/AgCl under FIA conditions using a SPE-Au-SAM/AuNPs-Linker/Fullerenols/TvL biosensor; experimental response for different concentrations of gallic acid (0.03 mmol L^{-1} (1), 0.08 mmol L^{-1} (2), 0.15 mmol L^{-1} (3), 0.24 mmol L^{-1} (4), 0.30 mmol L^{-1} (5)), for five injections of white wine sample diluted 10 times (Sample 1) and five injections of red wine sample diluted 100 times (Sample 2). Inset: calibration plot for gallic acid.

Table 3
Comparison of polyphenols index obtained for two wines with SPE-Au-SAM/AuNPs-Linker/Fullerenols/TvL biosensor by FIA amperometry in 0.1 mol L^{-1} acetate buffer, pH 4.5 at a fixed potential of − 100 mV and the values obtained using the Folin–Ciocalteau reference method.

Wine sample	Folin–Ciocalteau (mg L^{-1})	TvL biosensor (mg L^{-1})	Recovery (%)
White wine	271 ± 3	273 ± 7	101%
Red wine	2327 ± 38	2443 ± 72	103%

proteins in native systems and gives the protein molecules an adequate mobility in order to correctly orientate its redox centers, thus to achieving a better electron transfer with the electrode surface (De Groot et al., 2007; Frasconi et al., 2009). At the same time, the conservation of a native-like structure for the enzyme and the increase of electroactive surface gained by using a nanostructured transducer, could explain also the observed increase of I_{max}/K_m which can be considered proportional to efficiency of enzymatic turnover (see Table 2). Hence, the improvement of performances results from both increased surface area and increased catalytic activity.

3.3. Application of TvL-biosensor to polyphenols index analysis

The possibility of applying the proposed laccase SPE-based biosensor for measurements in real samples was checked by determining the polyphenol content in wines samples, by operating in FIA conditions (Di Fusco et al., 2010).

In Fig. 4 the amperometric behavior obtained after the addition of gallic acid, usually as standard for polyphenols in real samples, white wine (Sample 1), red wine (Sample 2) and in the inset the corresponding calibration plot constructed by using the considered TvL biosensor, are reported.

In Table SM2 (see Supplementary Materials) the main analytical parameters obtained operating in FIA conditions are reported, indicating the possibility to detect polyphenols in the range from 0.03 to 0.3 mmol L^{-1} (about 5.0 mg L^{-1} to 50 mg L^{-1}) of gallic acid with a good repeatability of the measurement (RSD=2.2%) and a detection limit as low as 1.1 mg L^{-1}mg L^{-1}. As far as the system stability is concerned, a reduction to 87% of the initial value of the sensitivity after 120 days from preparation and of about 53% after 240 days, was observed.

The obtained results for two different commercial Italian wines have been compared to those obtained by the Folin–Ciocalteau as reference method. As it can be seen in Table 3 the values of polyphenols index obtained with TvL-biosensor are slightly higher than those obtained with the Folin–Ciocalteau method (namely, 0.74% for the white wine and 4.98% for the red one). Nevertheless, a Student-based t-test gives that at 95% probability there are no significant differences between the two methods and thus the obtained results with the biosensor method for the real samples of commercial wines are reliable and in good agreement with those obtained with the conventional reference method.

4. Conclusion

This paper describes the realization of nanostructured based modified electrodes by using AuNPs and fullerenols derivative, the resulting device has been characterized in the surface characteristics as well as in the electrochemical properties. The modification of the electrode resulted in an enhancement of the electrochemical properties. Furthermore the modified electrode has been employed, as transducer, in the development of a laccase (TvL) based biosensor, the characterization of this biosensor put in evidence good stability and reproducibility and the increase the amount of electroactive protein on the electrode and enhanced the electron transfer between the redox center of the protein and the electrode surface, resulting in an enhancement of the bioelectrochemical performances of the device. The laccase biosensor has also been used in the analysis of wine samples showing interesting electroanalytical properties.

These results suggest the possibility to consider the modification procedure of electrode by means of the integration of gold nanoparticles and fullerenols an interesting new platform for biosensor design and other biological applications.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.12.028.

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