Chapter 4

Application of a Nanostructured Enzymatic Biosensor Based on Fullerene and Gold Nanoparticles to Polyphenol Detection

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Abstract

Electrochemical biosensors provide an attractive means of analyzing the content of a biological sample due to the direct conversion of a biological event to an electronic signal. The signal transduction and the general performance of electrochemical biosensors are often determined by the surface architectures that connect the sensing element to the biological sample at the nanometer scale. The most common surface modification techniques, the various electrochemical transduction mechanisms, and the choice of the recognition receptor molecules all influence the ultimate sensitivity of the sensor. We show herein a novel electrochemical biosensing platform based on the coupling of two different nanostructured materials (gold nanoparticles and fullerenols) displaying interesting electrochemical features. The use of these nanomaterials improved the electrochemical performance of the proposed biosensor.

An application of the nanostructured enzyme-based biosensor has been developed for evaluating the detection of polyphenols either in buffer solution or in real wine samples.

Key words Enzymatic biosensor, Nanomaterials, Direct electron transfer, Laccase, Polyphenols

1 Introduction

According to the International Union of Pure and Applied Chemistry (IUPAC), a biosensor (Fig. 1) is defined as "a self-contained integrated device that is capable of providing specific quantitative or semiquantitative analytical information using a biological recognition element which is in direct spatial contact with a transduction element" [1].

An important class of biosensors is represented by the electrochemical biosensors, in which the physicochemical transducer is an electrochemical sensor. They are based on the electrochemical species consumed and/or generated during a biological and chemical interaction of biological active substance with substrate.

Ben Prickril and Avraham Rasooly (eds.), *Biosensors and Biodetection: Methods and Protocols, Volume 2: Electrochemical, Bioelectronic, Piezoelectric, Cellular and Molecular Biosensors*, Methods in Molecular Biology, vol. 1572,
DOI 10.1007/978-1-4939-6911-1_4,

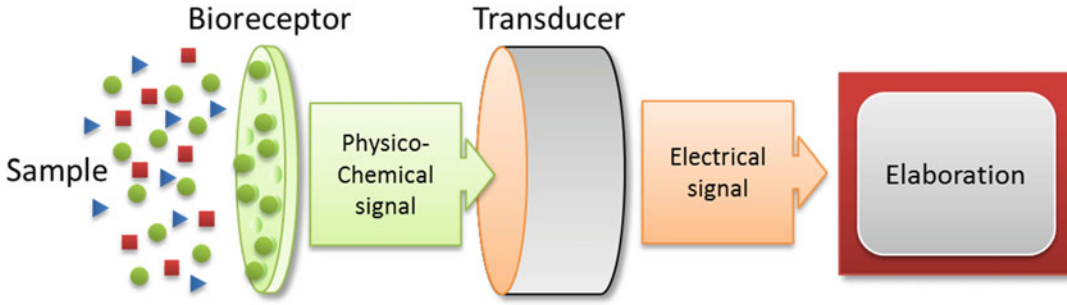

Fig. 1 Scheme of a biosensor

In such a process, an electrochemical detector measures the electrochemical signal produced by the interaction. Thus the differences among the three types of electrochemical biosensors (conductometric, potentiometric, and amperometric detectors) include their techniques of measuring biochemical changes in solution [2, 3].

The most widely used biosensors are the amperometric/enzymatic ones, which are based on the use of enzymes that catalyze specific redox reactions. These biosensors, according to the pathway followed by electrons in the functioning scheme, can be classified into first, second, and third generation biosensors.

Based on different generation instruments, a short description is reported:

First generation biosensors: Even if many complex detection schemes can be found in biosensor designs, the simplest approach to a biosensor is the direct detection of either the increase in concentration of an enzymatically generated product or the decreasing concentration of a substrate of the redox enzyme. Additionally, a natural redox mediator that is participating in the enzymatic reaction can be monitored. In all three cases it is necessary that the compound monitored is electrochemically active [4–8].

Second generation biosensors: In order to produce biosensors which operate at moderate redox potentials the use of artificial redox mediators was introduced for the "second generation" biosensors [9–11]. The redox mediator can be used to read out the analyte concentration within a sample. The employed redox enzyme for the analyte of interest is able to donate or accept electrons to or from an electrochemically active redox mediator. It is important that the redox potential of this mediator is in tune with the cofactor(s) of the enzyme. Preferably, the redox mediator is highly specific for the selected electron transfer (ET) pathway between the biological recognition element and the electrode surface. The difference in potential between the different cofactors and the introduced artificial redox mediator should not be less than

ΔE ~50 mV in order to provide a reasonable driving force of the reaction. As free-diffusing mediators, a large variety of compounds such as ferrocene derivatives, organic dyes, ferricyanide, ruthenium complexes, and osmium complexes have been utilized [12].

Third generation biosensors: A different approach to realize biosensor architectures is the immobilization of a redox enzyme on the electrode surface in such a manner that direct electron transfer (DET) could be possible between the active side of the enzyme and the transducer [13, 14]. Thus, free-diffusing redox mediators are not necessary for these types of biosensors [15–17]. Biosensor designs based on DET have been studied thoroughly [13, 18–24].

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 electrochemical biosensors where the most important issue is to obtain an efficient electron transport between redox centers of proteins and electrodes without disrupting the protein native structure. Nonetheless, 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 [18, 25, 26].

The introduction of different nanomaterials, such as gold nanoparticles and fullerene, in the development of electrochemical biosensor, due to their unique properties provides a suitable microenvironment for proteins immobilization, maintaining their bioactivity, and at the same time facilitating electron transfer between their redox center and electrode surfaces [27].

Laccases (EC 1.10.3.2) are cuproproteins belonging to the group of blue oxidase enzymes [28, 29]. They are widely distributed in fungi [30], higher plants [31], and in some bacteria [32]. Laccase is a polyphenol oxidase catalyzing the oxidation of several inorganic substances as phenol, associated to the reduction of oxygen to water. Laccases have four neighboring copper atoms distributed among different binding sites and classified into three types: copper types 1, 2, and 3. Copper type 1 is involved in electron capture and transfer, copper type 2 activates molecular oxygen, while copper type 3 is responsible for oxygen uptake. Mediator oxidation using laccase is one-electron reaction, which generates a free radical [33] (Fig. 2).

Here, we introduce a preliminary application of the developed *Trametes versicolor* Laccase (TvL) biosensor using a screen printed electrode (SPE) as gold substrate; the resulting biosensor is utilized under flow injection analysis (FIA) conditions for the determination of polyphenols content in real samples of wines and our results are compared to the Folin–Ciocalteau method as spectrophotometric reference method. Also we carry out a microscopic

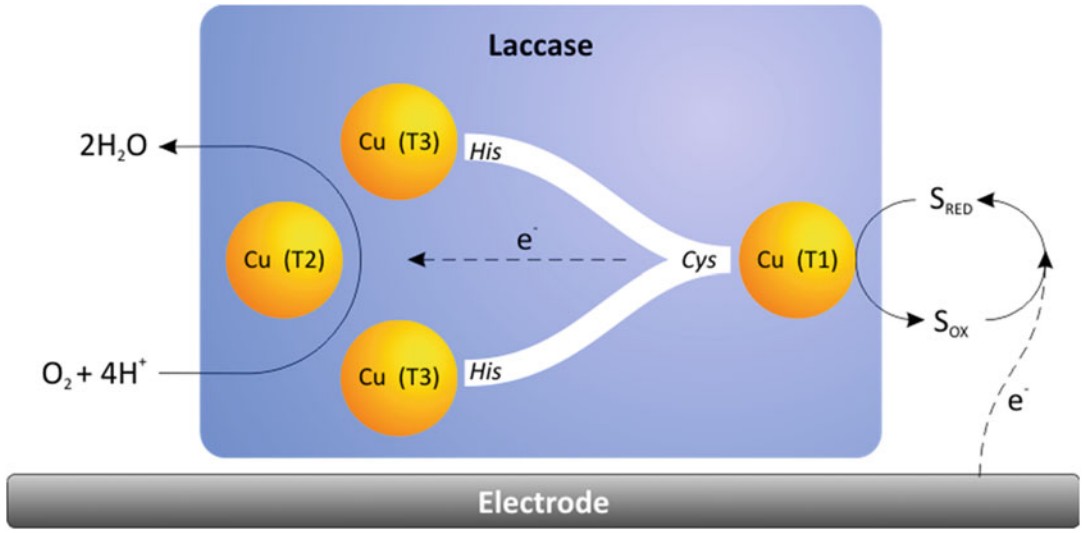

Fig. 2 Electron transfer mechanism of the substrates' (S_{ox}/S_{red}) reaction on the laccase modified electrode

characterization of the transducer surface before and after modification with TvL by scanning tunneling microscopy (STM) and show the DET of our proposed modified biosensor in absence of redox mediators.

The system can be used for repetitive measurements and as far as the 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, has been observed.

2 Materials

2.1 Electrochemical Apparatus (See Note 1)

Batch electrochemical experiments are performed using:

1. 5 mL thermostated glass cell (model 6.1415.150, Metrohm, Switzerland).
2. Gold (Au) electrode, with a surface diameter of 3 mm, as working electrode (model 6.1204.320, Metrohm, Switzerland); a glassy carbon rod as counter electrode (model 6.1241.020, Metrohm, Switzerland) and a Ag/AgCl/KClsat as reference electrode (198 mV vs NHE) (model 6.0726.107, Metrohm, Switzerland).

Flow experiments are carried out using:

3. Microliter Flow Cell (Dropsens, Spain) (Fig. 3).
4. Flow injection valve (Dropsens, Spain) (Fig. 4), loop volume 250 μL.
5. Peristaltic Minipuls-3 peristaltic pump (Gilson, Inc., USA).

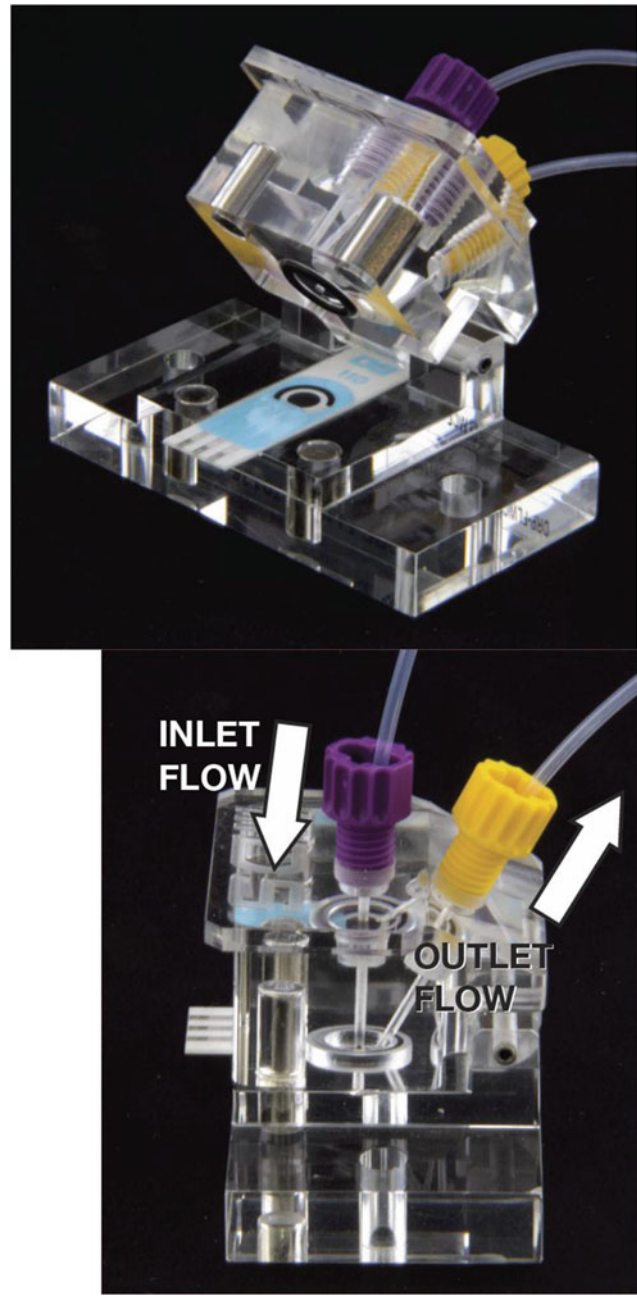

Fig. 3 Microliter flow cell (DropSens ref. FLWCL) is a methacrylate wall-jet Flow-Cell for FIA

6. Screen-printed electrodes (SPEs) constituted by an Au working electrode with a surface diameter of 4 mm (model DPR-220AT, Dropsens, Spain), a gold electrode as counter electrode, and silver as reference electrode are used (Fig. 5).

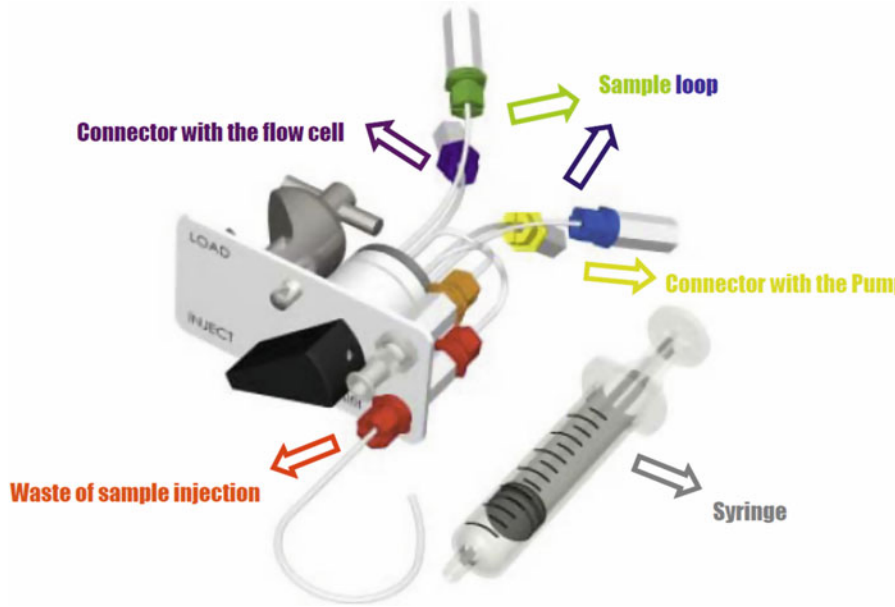

Fig. 4 Flow injection valve (DropSens DRP-Valve)

Fig. 5 Au screen-printed electrode (DropSens ref. DRP-220)

2.2 Potentiostat (See Note 2)

All electrochemical measures are performed by using a μ-Autolab type III potentiostat from EcoChemie (Utrecht, The Netherlands) controlled by means of the GPES Manager program (EcoChemie).

2.3 Spectrophotometric Measurements (See Note 3)

The spectrophotometric measurements are carried out by using a T60 U Spectrophotometer (PG Instruments Ltd., Wibtoft Leicestershire, UK).

2.4 Working Solutions (See Note 4)

1. Methanol (99.8%).
2. Ethanol (96%).
3. 3-mercaptopropionic acid and 11-mercaptoundecanoic acid in methanol (molar ratio1:2).

4. Cysteamine in aqueous solution.
5. Polyhydroxy small gap fullerenes.
6. Tetrachloroauric (III) acid ($HAuCl_4 \cdot 3H_2O$).
7. Sodiumborohydride ($NaBH_4$).
8. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide(EDC) and *N*-hydroxysuccinimide (NHS).
9. Fungal laccase from *Trametes versicolor* (Fluka) (EC 1.10.3.2, activity: 30.6 U/mg, stored at −18 °C) is dissolved in 0.1 mol/L acetate buffer, pH 4.5.
10. Stock solutions of gallic acid are prepared in 0.1 mol/L Britton–Robinson buffer, pH 5.0 daily. More diluted standard solutions are prepared by suitable dilutions with the same buffer.
11. 10.0.1 mol/L acetate buffer, pH 4.5.
12. Britton–Robinson buffer (BR buffer) (a mixture of 0.015 M H_3PO_4 (85%) + 0.015 M H_3BO_3 + 0.015 M CH_3COOH, that has been titrated to the desired pH with 0.2 M NaOH).
13. High purity deionized water (Resistance:18.2 MΩ cm at 25 °C; TOC < 10 μg/L) obtained from MilliporeDirect-Q UV3 (France) has been used to prepare all the solutions.
14. Different Italian commercial wine samples are acquired from a local supermarket. The only sample treatment required consisted of an appropriate dilution with a buffer solution and deaerating action by passing a N_2 stream before analysis.
15. Kit for measurement of total polyphenols concentration in wines is purchased from Biogamma Srl (Roma, 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.

3 Methods

3.1 Synthesis of Thiol-Functionalized Gold Nanoparticles (Diameter ca. 4–5 nm)

1. Mix 10 mL solution containing 197 mg of $HAuCl_4$ in ethanol to 5 mL solution containing 3-mercaptopropionic acid and 11-mercaptoundecanoic acid in methanol (molar ratio 1:2), then add 2.5 mL of glacial acetic acid in an ice bath for 1 h stirring.
2. Add dropwise 7.5 mL of aqueous solution of 1 M $NaBH_4$ (solution color changes from orange to deep brown within few seconds). Stir for another 1 h in an ice bath and stir for 14 h at room temperature.
3. Wash the organic phase with 25 mL ethanol to remove excess thiol using a separatory funnel and then the solution (ca. 3–4 mL) is transferred into a centrifuge tube for centrifuging (twice in each solvent) with methanol, ethanol, and diethyl ether.

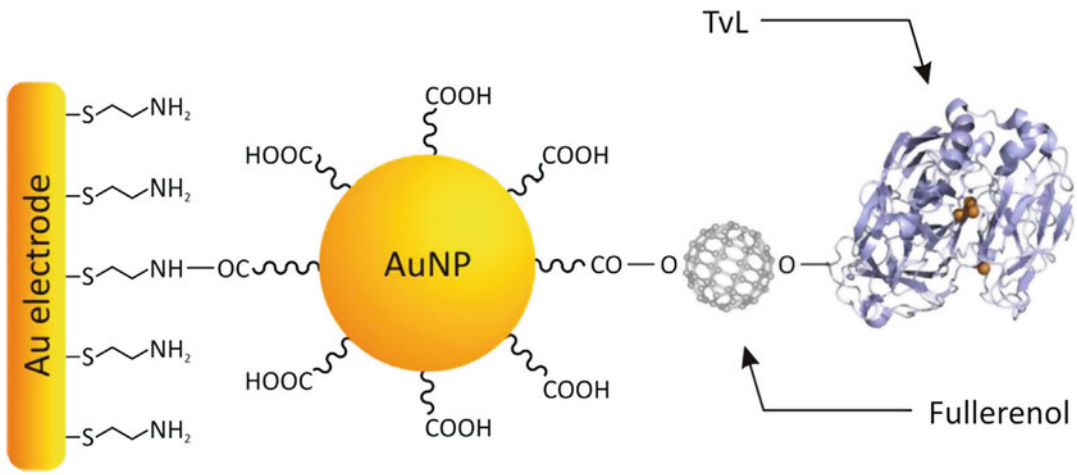

Fig. 6 Assembling of biosensor

3.2 Cleaning Electrode Surface

1. Chemical-polish mechanically surface of Au electrode with 0.3–0.05 μm aqueous alumina slurries. Begin from 0.3 μm crystal size and move on to 0.05 μm. Use a circular motion on the clean polishing cloth.
2. Rinse with ethanol and deionized water, after each cleaning step.
3. Sonicate in water for 2 min, after each cleaning step.
4. Finally rinse with deionized water.

3.3 Assembling (Fig. 6) and DET of the TvL Biosensor (Fig. 8)

1. Immerse Au electrode into 18 mmol/L cysteamine aqueous solution at room temperature in darkness for 4 h (*see* **Note 5**).
2. Rinse with water (*see* **Note 6**) and dry with nitrogen gas.
3. Immerse the Au electrode modified by SAM with 20 μL of a solution containing AuNPs-linker 2 mg/mL together with 0.5 mmol/L EDC and 0.1 mmol/L NHS for 30 min (*see* **Note 7**).
4. Rinse with water and then deposit 20 μL of polyhydroxy-fullerenes 0.07 mg/μL with EDC/NHS mixture onto the modified surface for 30 min.
5. Rinse with water and deposit 10 μL of a solution containing 0.076 U/μL of TvL and EDC/NHS mixture onto the resulting surface and dry for about 20 min at room temperature.
6. Carry out a microscopic characterization of the transducer surface before (Fig. 7a) and after modification (Fig. 7b) with TvL by scanning tunneling microscopy (STM).
7. Measure cyclic voltammetries (CVs) of the TvL biosensor at different scan rates, from 0.1 to 1.0 V/s, in the potential range −0.3 ÷ 0.6 V vs Ag/AgCl, in absence of O_2, in in 0.1 mol/L acetate buffer, pH 4.5, at 25 °C (Fig. 8).

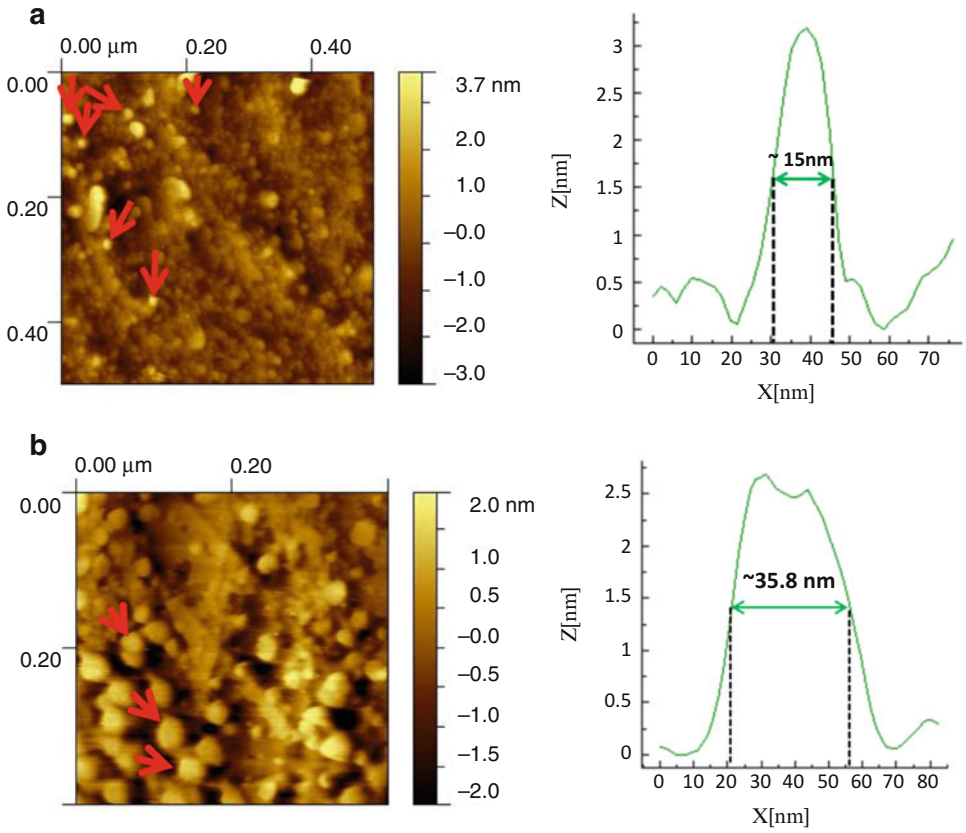

Fig. 7 STM images obtained for Au-SAM/AuNPs-Linker/Fullerenols before (**a**) and after (**b**) modification with TvL; on the right, the cross section profiles referring to the objects indicated by the arrows

3.4 Determination of Calibration Plot of Gallic Acid

1. Modify Au-SPE surface with the same procedure described in Subheading 3.3.
2. Au-SPE is placed into the flow cell.
3. A fixed potential of −100 mV vs internal silver/silver chloride reference electrode is applied.
4. For stabilization of baseline in the flow cell, BR buffer is circulated by a peristaltic pump with a flow rate of 0.894 mL/min.
5. In this system, different standard solutions of gallic acid are flowed into the flow cell. Between the different solution injections, the flow cell is washed with acetate buffer until stabilization of baseline (Fig. 9) (*see* **Note 8**).

3.5 Determination of Polyphenols in Wines by a Microliter Flow Cell

1. Modified Au-SPE is placed into the flow cell.
2. A fixed potential of −100 mV vs internal silver/silver chloride reference electrode is applied.

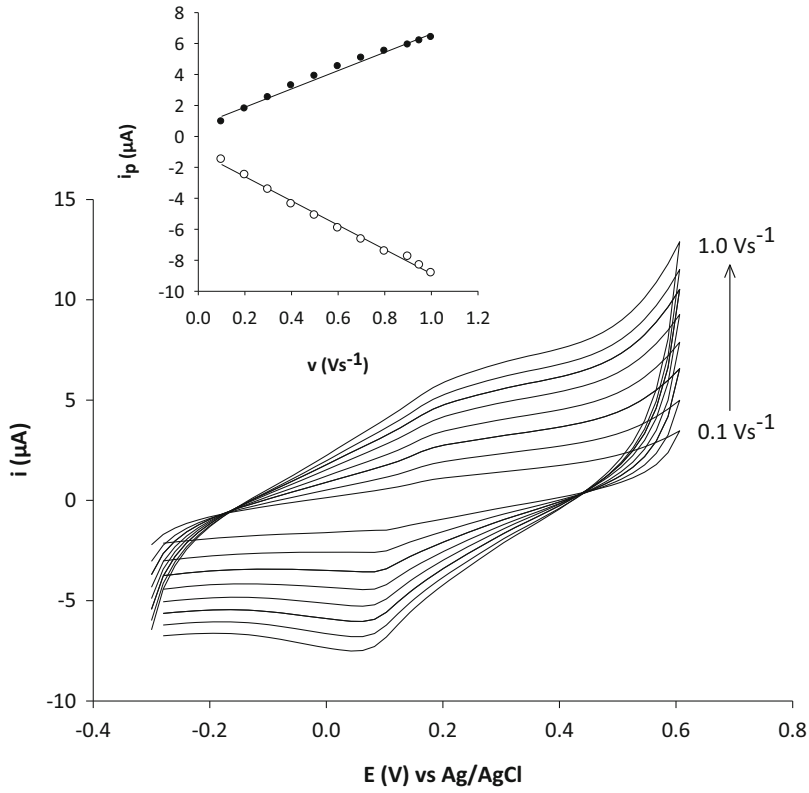

Fig. 8 CVs of TvL biosensor at different scan rates (0.1 ÷ 1.0 V/s). The experiments were performed in anaerobic conditions in 0.1 mol/L, pH 4.5 acetate buffer, at 25 °C. *Inset*: variation of anodic (*black dots*) and cathodic (*white dots*) peak intensities (i_p) as a function of scan rate (*v*)

3. For stabilization of baseline in the flow cell, BR buffer is circulated by a peristaltic pump with a flow rate of 0.894 mL/min.
4. For stabilization of baseline in the flow cell, Britton–Robinson solution is circulated by a peristaltic pump with a flow rate of 0.894 mL/min.
5. A white wine sample diluted ten times is flowed into the flow cell by flow-injection valve for three injections (red wine sample is diluted 100 times) (Fig. 9) (*see* **Note 9**).
6. Polyphenols index is obtained by calibration plot of gallic acid and multiplied by the dilution factor.
7. The obtained result for two different commercial wines have been compared to those obtained by the Folin–Ciocalteau as reference method. The recovery of white wine sample is 101% and of red wine is 103% (Table 1).

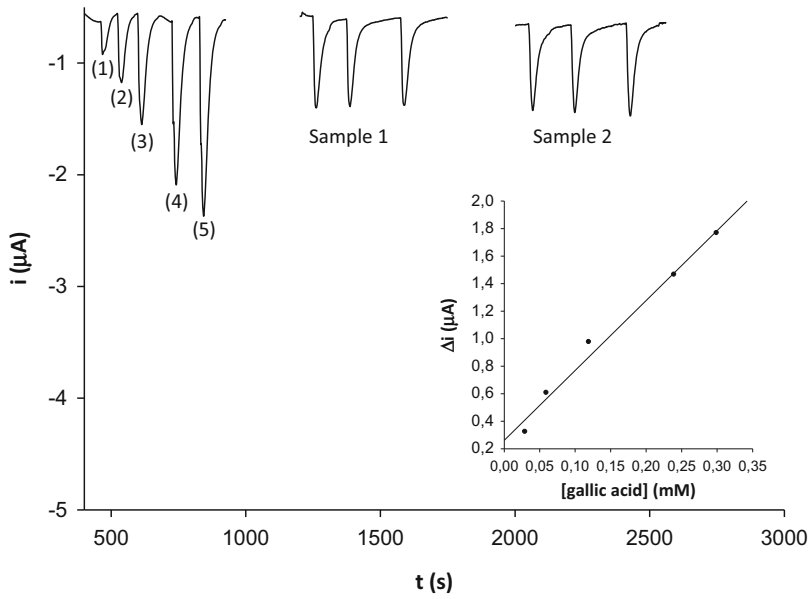

Fig. 9 Flow injection analysis (I (μA) vs t (s)) of gallic acid and analysis of two wine samples. In particular, is reported the experimental response for different gallic acid concentrations: (1) 0.03 mmol/L, (2) 0.08 mmol/L, (3) 0.15 mmol/L, (4) 0.24 mmol/L, (5) 0.30 mmol/L. Sample 1 is white wine diluited ten times and Sample 2 is red wine diluited 100 times. In the *inset* ir reported the calibration plot for gallic acid (I (μA) vs [gallic acid] (mM))

Table 1
Comparison of polyphenols index obtained for two wines with Au-AuNPsLinker-Fullerenols-TvL biosensor by FIA amperometry in 0.1 mol/L 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)	TvL biosensor (mg/L)	Recovery (%)
White wine	271 ± 3	273 ± 7	101%
Red wine	2327 ± 38	2443 ± 72	103%

4 Notes

1. The apparatus and the electrodes are also available from other commercial sources.
2. The potentiostat is available from other suppliers.
3. The spectrophotometer is available from other suppliers.
4. Numerous competitive reagents are available from different commercial sources.
5. Although a SAM of alkanethiol on gold is formed within a few minutes, we usually choose to leave gold substrates in a solution of alkanethiol for approximately 4 h.

6. The rinsing step is important to eliminate physically adsorbed cysteamine.
7. EDC and NHS are dissolved in deionized water.
8. It is important to wait the stabilization of the baseline to carry out with the experimental measurements.
9. Wine samples are diluited in 0.1 mol/L Britton–Robinson buffer, pH 5.0.

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