Biosensor for luteolin based on silver or gold nanoparticles in ionic liquid and laccase immobilized in chitosan modified with cyanuric chloride

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Novel and effective biosensors based on Ag or Au nanoparticles dispersed in ionic liquid (IL) 1-butyl-3-methylimidazolium hexafluorophosphate (BMI·PF_6) and laccase (Lac) from *Aspergillus oryzae* immobilized in chitosan (Chi) chemically cross-linked with cyanuric chloride (CC) were constructed. This enzyme catalyzes the oxidation of luteolin to the corresponding *o*-quinone, which is electrochemically reduced back to luteolin at 0.35 V *vs.* Ag/AgCl. Square-wave voltammetry was used for the electrochemical determination of luteolin at the Lac-nanoparticles-BMI·PF_6 biosensors. The best performance was obtained with 50:20:15:15% (w/w/w/w) as the graphite powder:Chi-CC:Nujol:Ag-BMI·PF_6 or Au-BMI·PF_6 composition (Lac 0.29 units mL^{-1}) in 0.1 M acetate buffer solution (pH 4.0) with frequency, pulse amplitude and scan increment at 50 Hz, 100 mV, and 5.0 mV, respectively. Under optimized conditions, the cathodic currents increased linearly for the luteolin concentration range of 0.099–5.825 μM with detection limits of 0.054 ± 0.004 μM (Ag-BMI·PF_6) and 0.028 ± 0.002 μM (Au-BMI·PF_6). These biosensors demonstrated high sensitivity, good repeatability and reproducibility, and long-term stability (13% decrease in response over 70 days). The recovery study for luteolin in chamomile tea samples gave values of 91.8–104.8%. The influence of Lac immobilized in Chi-CC and nanoparticles-BMI·PF_6 contributes to the excellent performance of the biosensors.

Introduction

Ionic liquids (ILs) are a class of non-molecular ionic solvents with low melting points (around or below 100 °C) composed of unsymmetrically substituted nitrogen-containing cations, such as imidazolium, with inorganic or organic anions. The large difference between the sizes of a bulky cation and a small anion does not allow packing of the lattice and the ions are disorganized, which results in liquid salts at room temperature.[1,2] Negligible vapor pressure, non-flammability, good thermal, chemical and electrochemical stability, high ionic conductivity and a wide electrochemical window are interesting properties that make ILs promising candidates in several applications: solvents for organic reactions,[3–5] biocatalysis[5,6] and electrochemistry,[7,8] electrolytes in electrochemical devices,[1,9] binders to prepare modified biosensors[10–13] and for the preparation and stabilization of nanomaterials.[14–16]

With the development of nanotechnology, nanoparticles have received substantial attention because they exhibit unique optical, electrical, thermal and catalytic (facilitating electron transfer) properties. Such characteristics, together with an ease of miniaturization of sensing devices to nanoscale dimensions, make nanoparticles suitable for important applications in chemical/biochemical sensing. The modification of electrode surfaces with redox-active metal nanoparticles has led to the development of various electrochemical sensors.[17–22] In this respect, ILs, in particular 1,3-dialkylimidazolium, display a pronounced self-organization structure formed mainly through hydrogen bonds between the cations and anions. This structural organization of ILs can be used to adapt many species, as it provides hydrophobic or hydrophilic regions. The formation and stabilization of transition-metal nanoparticles in imidazolium-based ILs occurs with the re-organization of the hydrogen bond network and generates nanostructures with polar and non-polar regions where the nanoparticles are included.[14–16] The metal nanoparticles dispersed in these fluids are stable and active catalysts and have been applied in the development of high-performance biosensors.[23,24]

Immobilization of an enzyme onto the support (matrix) is a crucial step for the construction of enzymatic biosensors. Encapsulation in membranes, physisorption, entrapment into polymer matrices and cross-linking with bifunctional or multifunctional reagents are immobilization methods that increase the thermostability, operational stability and recovery of enzymes.[12,13,23,25–28] Chitosan (Chi) is an attractive biocompatible, biodegradable and natural biopolymer, with a high percentage of available amino and hydroxyl groups in its chemical structure, which has been widely used as an immobilization matrix.[19–21,23,25–28] In this regard, reactions with substances such as glutaraldehyde, epichlorohydrin, carbodiimide, tripolyphosphate and citrate have been used for the formation of a network between polymer chains of Chi, where the enzymes can

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be covalently linked or entrapped in this modified matrix.[12,23,25–28] Considering the benefits of ILs, metal nanoparticles and Chi, many researchers have integrated them into biosensor fabrication to exploit their synergistic contributions to the improvement of the sensor characteristics, such as stability and sensitivity.[20,21,23] To the best of our knowledge, a laccase (Lac) biosensor with these integrated components has not been previously reported.

Laccase (EC 1.10.3.2, *p*-benzenediol:oxygen oxidoreductase) is a cuproprotein belonging to a small group of enzymes known as 'blue oxidases' produced by higher plants and microorganisms, mainly fungi. This enzyme oxidizes a broad range of aromatic substrates (phenolic compounds preferentially) accompanied by the reduction of dioxygen to water, and its specific affinity for oxygen, as the electron acceptor, is very high.[29] It has potential use in several fields including the pulp and paper (biopulping and biobleaching), textile (degrading dyes and bleaching effluents) and food (bioremediation and beverage processing) industries.[30] Moreover, Lac can be used in the construction of biosensors and then applied in the determination of several phenolic compounds.[10,11,24,28,31,32] Recently, Brondani *et al.*[24] developed a biosensor using Lac in microcapsules and platinum nanoparticles dispersed in 1-butyl-3-methylimidazolium hexafluorophosphate ($BMI{\cdot}PF_6$) IL. The proposed biosensor exhibited high sensitivity, due to the high conductivity of the IL combined with the electron transfer facilitated by the metal nanoparticles. Square-wave voltammetry (SWV) measurements have shown that the biosensor exhibits a linear response for adrenaline concentrations from 0.999 to 213 μM and the detection limit was 0.293 μM.

Luteolin (3′,4′,5,7-tetrahydroxyflavone), a polyphenolic compound available in foods of plant origin, belongs to the flavone subclass of flavonoids, usually occurring as glycosylated forms, and is present in celery, green pepper, parsley, artichoke, perilla leaf and chamomile tea. Studies have shown that this compound has several types of biological activity including anti-inflammatory, anti-bacterial and anti-oxidant, as well as displaying anti-proliferative activity against cancer cells.[33,34] Luteolin has been determined by high-performance liquid chromatography (HPLC),[35–38] column liquid chromatography coupled with mass spectrometry,[39] spectrophotometry and fluorimetry[40] and capillary electrophoresis.[41,42] A reversed-phase HPLC technique has been reported for the determination of luteolin in dog plasma after a single oral administration of 102 mg kg^{-1} of an extract of *Chrysanthemum morifolium* Ramat.[36] The standard curve for luteolin in plasma was linear over the range of 38.5–4350 ng mL^{-1} and the limit of detection was 1.82 ng mL^{-1}. The development of an electrochemical method for luteolin determination is an attractive alternative due to its sensitivity, rapid response, simplicity of operation and cost-effectiveness, as well as the minimal sample pretreatment involved.

In this paper, we used an electroanalytical methodology to combine silver or gold nanoparticles dispersed in IL ($BMI{\cdot}PF_6$) and Lac from *Aspergillus oryzae* immobilized in Chi-CC (chitosan chemically cross-linked with cyanuric chloride) for the fabrication of novel biosensors. The biosensors Ag-$BMI{\cdot}PF_6$ and Au-$BMI{\cdot}PF_6$ were constructed, optimized and used for determination of luteolin in chamomile tea samples. Both the biocompatibility of Chi and Lac and the inherent conductivity of nanoparticles dispersed in IL make this material an excellent biosensing element. The effect of the various experimental conditions on the biosensor response and performance were investigated in detail.

Experimental

Instrumentation and electrochemical measurements

Carbon, hydrogen and nitrogen elemental analyses were performed on a CE Instruments elemental analyzer (model EA 1110 CHNS-O).

Electrochemical measurements, using square-wave voltammetry (SWV), were performed on an Autolab PGSTAT12 potentiostat/galvanostat (Eco Chemie, The Netherlands) connected to data processing software (GPES, software version 4.9.006, Eco Chemie). A conventional three-electrode system was used with a biosensor based on Ag-$BMI{\cdot}PF_6$ or Au-$BMI{\cdot}PF_6$ as the working electrode, a platinum wire as the counter electrode and an Ag/AgCl (3.0 M KCl) reference electrode.

SWV measurements were carried out in an unstirred, non-deaerated acetate buffer solution (0.1 M; pH 4.0) at room temperature (25.0 ± 0.5 °C) and all potentials were measured and reported *vs.* Ag/AgCl (3.0 M KCl). In a typical run, 10 mL of the supporting electrolyte was transferred to a clean, dry cell and the required volume of the luteolin or chamomile tea sample was added by micropipette. The SWV measurements were performed applying a sweep potential between 0.8 and 0.0 V, at a frequency of 10–100 Hz, pulse amplitude of 10–100 mV and scan increment of 0.5–10.0 mV, after successive additions of the analyte. After a stirring period of 60 s to homogenize the solution, square-wave voltammograms were recorded.

Chemicals and solutions

Reagents were of analytical grade and employed without further purification. All solutions were prepared with ultrapure water (18.2 MΩ.cm) obtained from a Milli-Q purification system. An acetate buffer (0.1 M, pH 4.0) solution was used as the supporting electrolyte throughout the experiments. Laccase (Lac) from *A. oryzae* was purchased from Novozymes (Denilite® II BASE) and chitosan (Chi) with a deacetylation degree of 85% from Sigma-Aldrich. The IL 1-butyl-3-methylimidazolium hexafluorophosphate ($BMI{\cdot}PF_6$) and silver and gold nanoparticles dispersed in $BMI{\cdot}PF_6$ (Ag-$BMI{\cdot}PF_6$ and Au-$BMI{\cdot}PF_6$) were synthesized as previously described in the literature.[43–45] Cyanuric chloride (CC) used to modify the Chi was obtained from Fluka and toluene and ethanol from Sigma-Aldrich. Luteolin, rutin, quercetin, apigenin, esculetin, chlorogenic acid, caffeic acid, sinapic acid and 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich. The carbon paste was prepared using graphite powder (Acheson 38, Fisher Scientific) and high purity Nujol purchased from Sigma-Aldrich. A luteolin standard solution (0.2 mM) was prepared daily in ethanol/water solution (40/60%, v/v).

Synthesis of Ag-$BMI{\cdot}PF_6$ and Au-$BMI{\cdot}PF_6$

In a Fischer-Porter bottle containing $BMI{\cdot}PF_6$ (1 mL), a mixture of Ag_2O (25.0 mg, 0.11 mmol) and *n*-butylimidazolium (80 μL, 0.61 mmol)

was stirred at room temperature for 15 min yielding a black dispersion. The system was then heated to 85 °C and hydrogen (4.0 bar) was admitted to the system. After stirring for 2 h a brown 'solution' was obtained. The reactor was evacuated for 1 h at 100 °C to remove excess hydrogen and *n*-butylimidazolium.[44]

In a typical experiment, a solution containing sodium borohydride ($NaBH_4$; 9.25 mg, 250 mmol) dissolved in 0.5 mL of methanol (MeOH) and dispersed in 0.5 mL of BMI·PF_6 was prepared 24 h before use. In a Schlenk tube $HAuCl_4 \cdot 3H_2O$ (10.0 mg, 25 mmol) was dissolved in 0.5 mL of MeOH and dispersed in 0.5 mL of BMI·PF_6. The solution was stirred at room temperature for 15 min yielding in a yellow solution to which 1-methylimidazolium (5.0 μg, 0.06 mmol) was added. After stirring, the solution containing $NaBH_4$ in MeOH and BMI·PF_6 was added to the solution containing $HAuCl_4 \cdot 3H_2O$ in MeOH and BMI·PF_6, and a blue 'solution' was obtained, which was then dried under reduced pressure.[45] The Au nanoparticles were isolated by centrifugation (3500 rpm) with the addition of acetone (5 mL) for 3 min and washed with acetone (3 × 15 mL) and dichloromethane (3 × 15 mL), dried under reduced pressure and isolated.

The Ag-BMI·PF_6 and Au-BMI·PF_6 samples thus obtained were prepared for transmission electron microscopy (TEM) and X-ray diffraction (XRD) analyses.

Sample preparation of the Ag and Au nanoparticles for TEM analysis

The analysis of the morphology and the electron diffraction (ED) of the Ag and Au nanoparticles obtained were carried out on a JEOL microscope (JEM-2010) equipped with an energy-dispersive X-ray spectroscopy (EDS) system and a JEOL JEM-1200 EXII electron microscope operating at accelerating voltages of 120 kV. The samples for TEM were prepared by dispersion of the Ag-BMI·PF_6 or Au-BMI·PF_6 nanoparticles at room temperature and then collected on a carbon-coated copper grid. The histograms of the nanoparticle size distribution, assuming spherical shape, were obtained from the measurement of approximately 300 particles and were reproduced in different regions of the Cu grid, found in arbitrarily chosen areas of enlarged micrographs.[46]

Sample preparation of the Ag and Au nanoparticles for X-ray analysis

The phase structures of the Ag-BMI·PF_6 and Au-BMI·PF_6 were characterized by XRD. For the XRD analysis the nanoparticles were isolated as a fine powder and placed in the sample holder. The XRD experiments were carried out on a SIEMENS D500 diffractometer equipped with a curved graphite crystal using Cu Kα R radiation (λ = 1.5406 Å). The diffraction data were collected at room temperature in a Bragg–Brentano θ–2θ geometry. The equipment was operated at 40 kV and 20 mA with a scan range of 20° to 90°. The diffractograms were obtained with a constant step of $\Delta 2\theta = 0.05$. The indexation of Bragg reflections was obtained through a pseudo-Voigt profile fitting using the FULLPROF code.[47]

Obtainment of Chi-CC and measurement of Lac activity

The Chi used as the support for Lac immobilization was chemically modified with CC following steps previously described in the literature.[48]

The enzymatic activity of Lac was determined in triplicate by measurement of the absorbance at 420 nm of the product formed from the reaction between 2.8 mL of 0.5 mM ABTS solution and 0.2 mL of Lac in 0.1 M acetate buffer (pH 5.0) at 25 °C.[28] One unit of Lac activity is defined as the amount of enzyme which oxidizes 1.0 μmol substrate per min.

Biosensor fabrication

Briefly, the biosensors were prepared by hand-mixing 40.0 mg (20% w/w) of Chi-CC containing immobilized Lac (0.29 units mL^{-1}) and 100.0 mg (50% w/w) graphite powder with a mortar and pestle for 20 min to ensure a uniform mixture. To this mixture 30.0 mg (15% w/w) of Nujol and 30.0 mg (15% w/w) of Ag-BMI·PF_6 or Au-BMI·PF_6 were added and then mixed for at least 20 min to produce the final paste. The resulting modified carbon paste was tightly packed into a plastic syringe (1.0 mm internal diameter) and a copper wire was inserted to obtain the external electrical contact. The biosensors without nanoparticles containing Nujol and mixtures of Nujol:BMI·PF_6 and the carbon paste electrode (bare CPE) were prepared in a similar way.

Preparation and analysis of chamomile tea samples

Two chamomile tea samples (*Matricaria recutita* L. – I and II) containing luteolin were acquired from a local drugstore (Florianópolis, Santa Catarina, Brazil) and analyzed using the proposed biosensors. The samples were prepared by extracting 2.0 g of tea with 40 mL of hot water at 50 °C, incubating for 20 min, shaking for 2 min and filtering. An aliquot of 100 μL of the chamomile tea extract was transferred to an electrochemical cell and analyzed by SWV, after successive additions of luteolin standard solution. All measurements were performed in duplicate.

Results and discussion

Preparation and characterization of Ag and Au nanoparticles

The Ag-BMI·PF_6 nanoparticles of 8.9 ± 2.1 nm were prepared by reduction of Ag_2O with stirring, at 85 °C in an oil bath with H_2 (4.0 bar) for 2 h, using a previously described procedure.[44] The Au-BMI·PF_6 nanoparticles of 11.0 ± 1.5 nm were prepared by reduction of $HAuCl_4 \cdot 3H_2O$ dispersed in BMI·PF_6 with $NaBH_4$ also using a previously described procedure, with modifications.[45] These nanoparticles were isolated and characterized by XRD and TEM analysis.

The results showed that the crystalline mean diameters (8.9 ± 2.1 nm and 11.0 ± 1.5 nm) of the Ag (Fig. 1(A)) and Au (Fig. 1(B)) isolated material, respectively, could be estimated from the XRD diffraction pattern by means of the Debye–Scherrer equation calculated from the full-width at half-maximum (*fwhm*) of the (111), (200), (220), (311), and (222) planes.

TEM analysis was performed on samples prepared using Ag-BMI·PF_6 and Au-BMI·PF_6 suspensions that were agitated ultrasonically and then deposited on holey carbon film supported by a copper grid. The grids were placed on filter paper to remove the excess material and allowed to dry out for 2 h under high vacuum. Finally, these TEM samples were examined using a JEM-2010 microscope operating at an accelerating voltage of 120 kV.

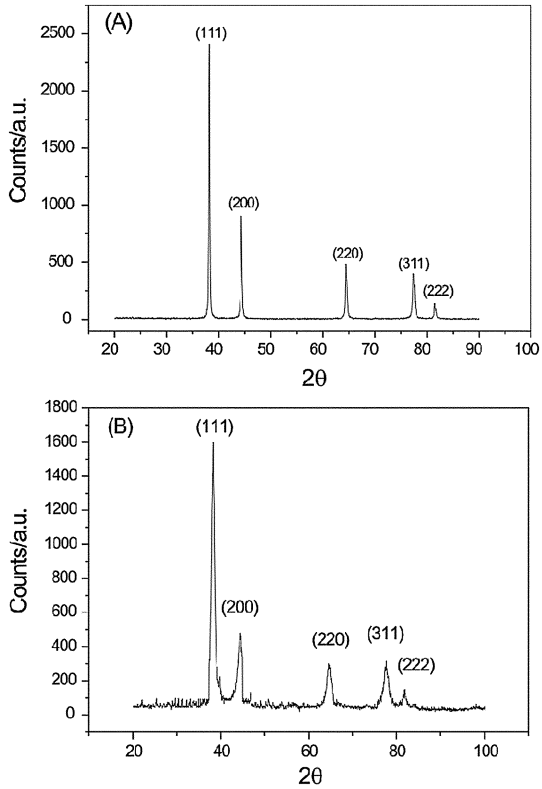


Fig. 1 X-Ray diffraction patterns of (A) Ag-BMI·PF_6 and (B) Au-BMI·PF_6.

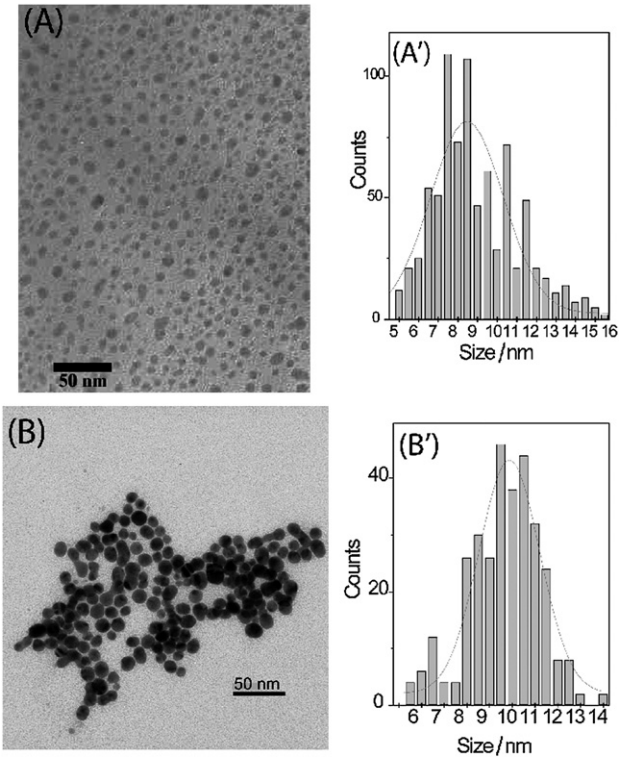


Fig. 2 TEM micrographs (A and B) and histograms (A′ and B′) showing the particle size distribution of Ag-BMI·PF_6 (top) and Au-BMI·PF_6 (bottom).

Bright-field TEM observations were performed under slightly under-focused conditions ($\Delta f \approx -300$ nm), and particle size distributions were determined after the original negative had been digitalized and expanded to 470 pixels cm^{-1} (Fig. 2(A) and 2(B)). The mean diameters were $d_m \approx 8.9 \pm 2.1$ nm for the Ag-BMI·PF_6 and 11.0 ± 1.5 nm for the Au-BMI·PF_6. TEM micrographs at multiple random locations on each sample were obtained. The size distribution histograms were based on the measurement of around 300 particles at multiple random locations on each sample. Fig. 2(A′) and 2(B′) show the particle size distributions obtained, which can be reasonably well fitted by a Gaussian curve.

Chi-CC and Lac immobilization and enzymatic process

The efficiency of enzyme immobilization is determined by the support and enzyme properties. The copolymer Chi is composed of β(1→4)-linked 2-amino-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-glucose, forming a long-chain linear polymer. The presence of amino and hydroxyl groups bonded to the polymer chain makes this biopolymer a promising matrix for enzyme immobilization.[12,23,25–28]

Carbon, hydrogen and nitrogen elemental analysis results for Chi and Chi-CC are in agreement with the percentage values reported by Lopes *et al*.[48] The increase in the amount of nitrogen is an indication of the chemical modification of the precursor biopolymer and CC is a novel alternative cross-linking reagent.[12,23,25–28] Thus, Chi was chemically modified by reaction with an excess of CC followed by Lac immobilization, as shown in Fig. 3. As can be observed, CC can form cross-linking through covalent interaction between its chloride atoms and amino groups (–NH_2) of different units of Chi or, simply, as pendant groups on the Chi polymer backbone (covalent binding between Cl and NH_2). The Lac enzyme is entrapped within the interstitial space of the cross-linked water-insoluble Chi and this system mimics its natural environment which can result in considerable stabilization of the enzyme structure, hence its activity.

The electrochemical behavior of Lac has received increasing attention in recent years. It exhibits electrochemical activity on various electrodes and has been used to construct biosensors for phenolic compounds.[10,11,24,28,31,32] In this study, the Lac-biosensor was employed for the luteolin determination and a schematic representation of the enzymatic reaction on the biosensor surface is given in Fig. 4. Lac catalyzes the oxidation of luteolin to the corresponding *o*-quinone, which is electrochemically reduced back to luteolin at a potential of 0.35 V *vs.* Ag/AgCl, *via* the four-electron reduction of oxygen to water.

Electrochemical characteristics of the luteolin biosensors based on nanoparticles dispersed in BMI·PF_6

The electrochemical behavior of luteolin in relation to the biosensors based on Nujol, BMI·PF_6, Ag-BMI·PF_6, and Au-BMI·PF_6, and to the bare carbon paste electrode (CPE), was investigated by SWV in the potential range of 0.8 to 0.0 V *vs.* Ag/AgCl. Fig. 5 shows the voltammograms obtained using the (a) bare CPE, (b) Nujol-biosensor (free Lac), (c) Nujol-biosensor, (d) Nujol/BMI·PF_6-biosensor, (e) Nujol/Ag-BMI·PF_6-biosensor and (f) Nujol/Au-BMI·PF_6-biosensor (c, d, e and f – immobilized Lac)

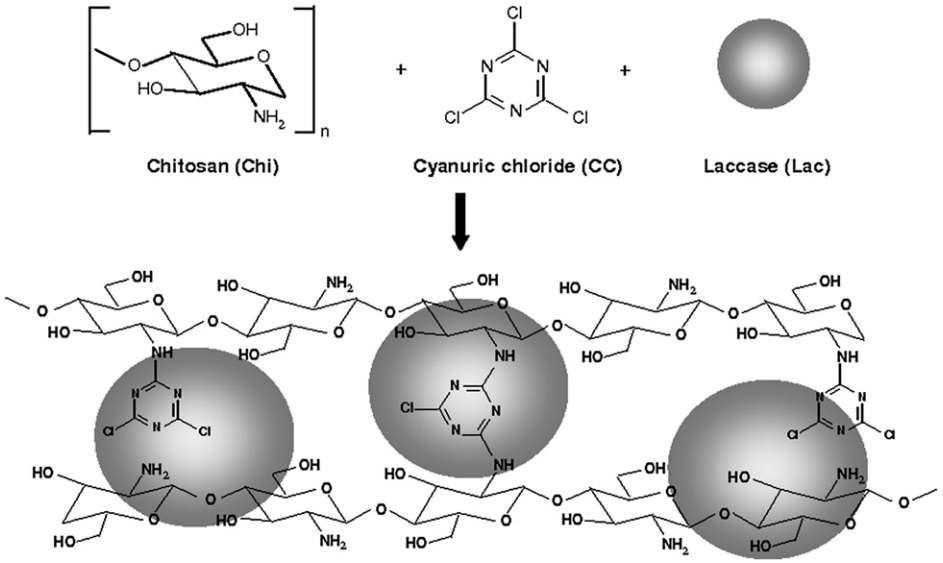


Fig. 3 Reaction between Chi, CC and Lac enzyme: proposed immobilization of Lac in chitosan chemically modified with cyanuric chloride (Chi-CC).

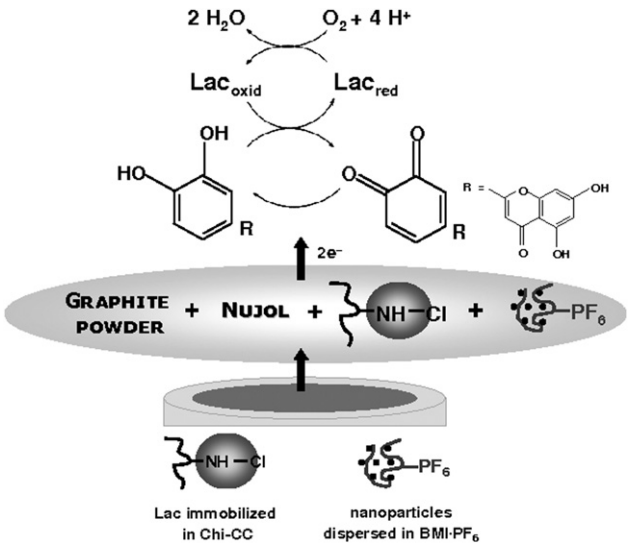


Fig. 4 Schematic representation of the Lac-catalyzed oxidation of luteolin with its subsequent electrochemical reduction on the biosensor surface and components utilized for fabrication of the biosensor.

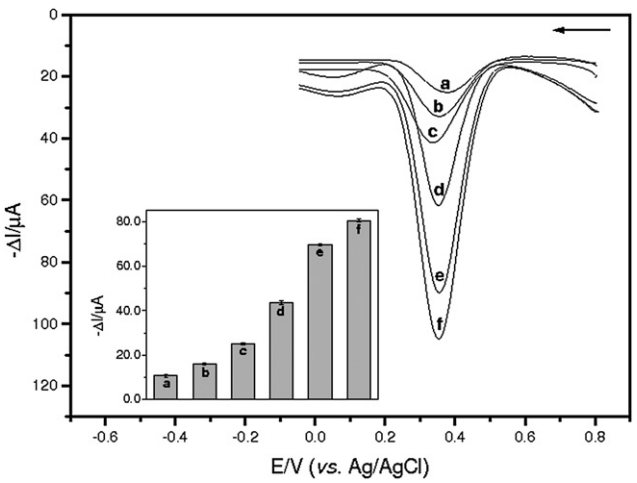


Fig. 5 Square-wave voltammograms obtained using (a) bare CPE, (b) Nujol-biosensor (free Lac), (c) Nujol-biosensor, (d) Nujol/BMI·PF_6-biosensor, (e) Nujol/Ag-BMI·PF_6-biosensor and (f) Nujol/Au-BMI·PF_6-biosensor (c, d, e and f – immobilized Lac) in 4.798 μM luteolin in 0.1 M acetate buffer solution (pH 4.0) at frequency 50 Hz, pulse amplitude 100 mV and scan increment 5.0 mV. Inset: cathodic peak current values for bare CPE and different biosensors.

in 4.798 μM luteolin in 0.1 M acetate buffer solution (pH 4.0), under optimized parameters. A peak reduction of *o*-quinone to luteolin was used as the analytical response for luteolin determination and the cathodic peak current values are shown in the inset of Fig. 5. The response of the bare CPE (a) is increased with the addition of free Lac (b) and this behavior is attributed to the catalytic properties of the enzyme. However, the higher response shown for the Nujol-biosensor (curve c) confirms that the enzyme was efficiently immobilized on Chi-CC, resulting in a more sensitive electrode. The incorporation of BMI·PF_6 as a binder for the construction of biosensor (d) improved the wave of *o*-quinone reduction to luteolin at a potential of 0.35 V, due to its hydrophobicity and high ionic conductivity.

As can be seen, a greater response to the luteolin solution was obtained using the biosensors containing nanoparticles dispersed in BMI·PF_6 (e and f). A major advantage in terms of the preparation of the biosensors lies in the ability of nanoparticles to provide a suitable microenvironment for Lac, permitting direct electron transfer between the immobilized Lac and the biosensor surface. This behavior is in agreement with that obtained for the biosensor containing Ir-BMI·PF_6 and Pt-BMI·PF_6.[23,24] The use of nanoparticles dispersed in IL for the construction of electrochemical biosensors is of great interest, since it leads to enhanced analytical performance with respect to other biosensor designs. In addition, the Au-BMI·PF_6-biosensor displayed a higher cathodic peak current for the reduction of *o*-quinone to luteolin when compared with the Ag-BMI·PF_6-biosensor (a response 13.7 ± 0.1% greater than that obtained using the biosensor based

on silver nanoparticles). This finding can be attributed to a synergistic effect between Au and BMI·PF_6 on the stabilization and dispersion of the nanoparticles in the solution (7 days)[45] and with MeOH addition in the synthesis (14 days), when compared with Ag which undergoes dispersion in BMI·PF_6 (3 days).[44]

Optimization conditions of the biosensors and experimental parameters

Several experimental conditions were evaluated to optimize the biosensor performance: Nujol:IL (BMI·PF_6, Ag-BMI·PF_6 or Au-BMI·PF_6) composition, Lac concentration (0.22–0.94 units mL^{-1}), supporting electrolyte and pH, along with the frequency (10–100 Hz), pulse amplitude (10–100 mV) and scan increment (0.5–10.0 mV) used in the SWV.

Initially, the effect of the Nujol:IL composition (100:0; 75:25; 50:50; 25:75 and 0:100% (w/w)) on the biosensor response was investigated by SWV. The addition of up to 50% IL (BMI·PF_6, Ag-BMI·PF_6 or Au-BMI·PF_6) improved the electroanalytical response of the biosensor to a 4.798 μM luteolin solution. The performance of the biosensor decreased at higher percentages of IL (data not shown). These results are in agreement with those obtained in previous studies.[10,11,24] Thus, the Nujol:IL ratio of 50:50% w/w was used for construction of the biosensors.

In order to determine the most suitable Lac concentration, the effect of different enzyme unit values (0.22–0.94 units mL^{-1}) on the biosensor response was investigated. The analytical signal (cathodic peak current) for a 4.798 μM luteolin solution increased with a laccase concentration up to 0.29 units mL^{-1}. Consequently, this enzyme concentration was selected in the construction of the biosensors.

The effect of the different supporting electrolytes and pH, that is, 0.1 M phosphate buffer solution (pH 6.0–8.0), 0.1 M acetate buffer solution (pH 3.0–5.0) and 0.1 M KCl, on the biosensor response to a 4.798 μM luteolin solution was investigated. The best voltammetric responses were obtained in acetate buffer solution at pH 4.0.

Finally, the instrumental parameters of the SWV were studied to obtain the best performance of the biosensors. Frequency, pulse amplitude and scan increment were then evaluated in the ranges of 10–100 Hz, 10–100 mV and 0.5–10.0 mV, respectively. The best analytical signals obtained for the biosensors were at frequency 50 Hz, pulse amplitude 100 mV and scan increment 5.0 mV. These experimental conditions were selected for subsequent experiments. Table 1 summarizes the range over which each variable was investigated and the optimal value found.

Table 1 Optimization of the experimental conditions for the biosensors[a]

Parameters investigated	Range studied	Optimal value
Nujol:IL (%, w/w)	100:0–0:100	50:50
Laccase (units mL^{-1})	0.22–0.94	0.29
Electrolyte support/pH	Phosphate buffer/6.0–8.0 Acetate buffer/3.0–5.0 KCl	Acetate buffer/4.0
Frequency (Hz)	10–100	50
Pulse amplitude (mV)	10–100	100
Scan increment (mV)	0.5–10.0	5.0

[a] Biosensors investigated: BMI·PF_6, Ag-BMI·PF_6 and Au-BMI·PF_6.

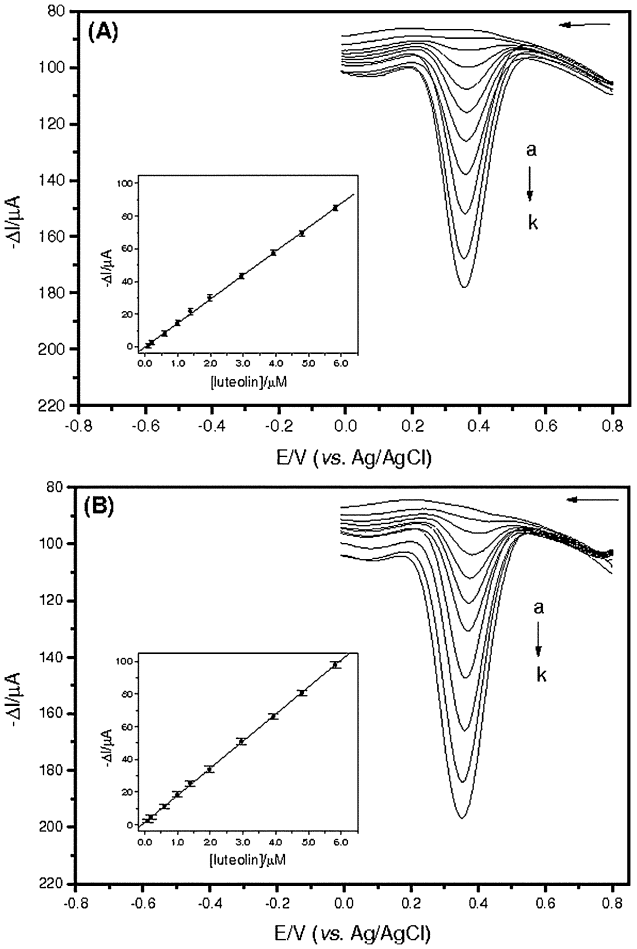


Fig. 6 Square-wave voltammograms obtained using (A) Ag-BMI·PF_6 and (B) Au-BMI·PF_6 biosensors for (a) blank in acetate buffer solution (0.1 M; pH 4.0) and luteolin solutions at the following concentrations: (b) 0.099; (c) 0.199; (d) 0.598; (e) 0.995; (f) 1.390; (g) 1.980; (h) 2.955; (i) 3.921; (j) 4.798 and (k) 5.825 μM at frequency 50 Hz, pulse amplitude 100 mV and scan increment 5.0 mV. Inset: calibration curves of luteolin.

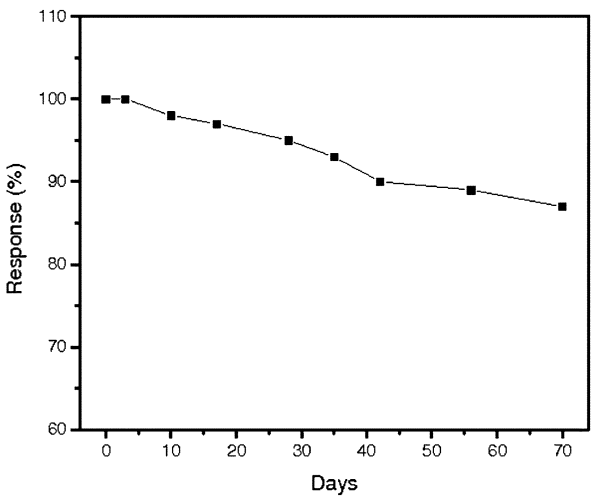
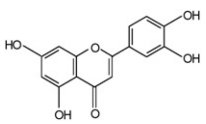
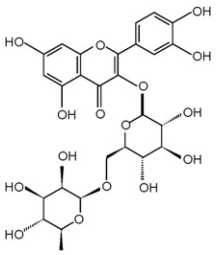
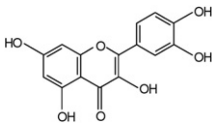
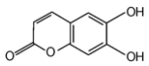
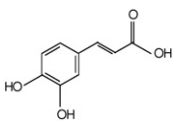
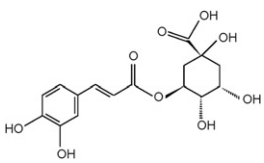
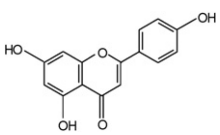
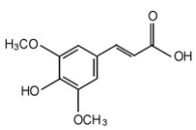


Fig. 7 Study on stability of the Au-BMI·PF_6 biosensor using 4.798 μM luteolin in 0.1 M acetate buffer solution (pH 4.0).

Table 2 Relative response of the Au-BMI·PF_6 biosensor to different phenolic compounds and the tolerated concentration of possible interferents on luteolin determination

Phenolic compound	Structure	E_{pc} (V)	Relative response[a] (%)	Tolerated concentration[a] (μM)
Luteolin		+0.35	100	—
Rutin[b]		+0.31	24.7 ± 0.3	9.6 ± 0.1
Quercetin[b]		+0.33	19.3 ± 0.4	15.4 ± 0.3
Esculetin[b]		+0.36	14.2 ± 0.2	20.7 ± 0.3
Caffeic acid[b]		+0.25	9.6 ± 0.1	39.8 ± 0.2
Chlorogenic acid[b]		+0.22	4.9 ± 0.1	64.5 ± 0.3
Apigenin[b]		NR[c]	NR[c]	NI[c]
Sinapic acid[b]		NR[c]	NR[c]	NI[c]

[a] $n = 3$. [b] Phenolic compounds as possible interferents. [c] NR = no response was observed; NI = no interfering phenolic compounds.

Analytical curve for the electrochemical analysis of luteolin

Under the optimal working conditions the analytical curves were obtained with the biosensors in the potential range of 0.8 to 0.0 V *vs.* Ag/AgCl. The electrochemical reduction to luteolin was obtained at a potential of 0.35 V for both biosensors. Fig. 6 shows the square-wave voltammograms and analytical curve, constructed from cathodic current peak *vs.* luteolin concentration, obtained employing the proposed biosensors: (A) Ag-BMI·PF_6 and (B) Au-BMI·PF_6. As can be observed, the cathodic current increased with luteolin concentration and the corresponding linear ranges and regression equations obtained using these biosensors were:

(A) 0.099–5.825 μM ($-\Delta I = 0.257(\pm0.029) - 14.662(\pm0.103)$ [luteolin]; $R^2 = 0.9998$)

(B) 0.099–5.825 μM ($-\Delta I = -0.738(\pm0.017) - 16.550(\pm0.085)$ [luteolin]; $R^2 = 0.9999$)

where $-\Delta I$ is the cathodic current peak (μA), and [luteolin] is the luteolin concentration (μM). The detection limits (three times the standard deviation of the intercept/slope) using the Ag-BMI·PF_6 and Au-BMI·PF_6 biosensors were found to be 0.054 ± 0.004 and 0.028 ± 0.002 μM, respectively. The sensitivity of these biosensors is better than that of other biosensors described in the

literature based on gold, silver, iridium and platinum nanoparticles.[17,19,20,23,24] In particular, the Lac biosensor based on Pt-BMI·PF_6 developed by Brondani *et al.*[24] showed a higher detection limit (lower sensitivity) for adrenaline when compared with these biosensors for luteolin determination.

The good analytical performance for the proposed biosensors, such as good linearity, better correlation coefficient and low limit of detection, can be attributed to the efficient immobilization and stabilization of Lac in Chi-CC and to the high conductivity of nanoparticles dispersed in the IL BMI·PF_6, for the fast electron transfer between luteolin and the biosensor surface. A detection limit (three times the standard deviation of the intercept/slope) of 0.102 μM was obtained using the BMI·PF_6 biosensor (analytical curve not shown), confirming the catalytic contribution of the metal nanoparticles in the electron transfer.

Repeatability, reproducibility and stability of the biosensors

The repeatability of the same biosensors used in the measurements was obtained by recording the response to a 4.798 μM luteolin solution in acetate buffer solution (0.1 M; pH 4.0) in five successive experiments. The relative standard deviations (RSDs) were calculated as 2.05% and 1.32% for the Ag-BMI·PF_6 and Au-BMI·PF_6 biosensors, respectively. Additionally, to evaluate biosensor-to-biosensor reproducibility, three biosensors of each type were independently prepared using the same conditions and revealed an acceptable reproducibility with low RSD values of 3.12% (Ag-BMI·PF_6) and 2.77% (Au-BMI·PF_6).

The stability and lifetime of the constructed biosensors were examined by intermittently measuring the current response to a luteolin standard solution over a 70-day storage period. The biosensors were stored at room temperature in a dry place when not in use. Three days later no change was observed and ten days later the biosensors still retained a 98% response. The catalytic current maintained 87% of its initial response over 70 days (both biosensors based on Ag or Au nanoparticles demonstrated the same response for the stability). As can be seen in Fig. 7, the Au-BMI·PF_6 biosensor showed an excellent operational stability, confirming that Lac was immobilized and stabilized in a favorable microenvironment.

Study of selectivity, interference, recovery and luteolin determination

In order to investigate the selectivity of the proposed biosensors to different phenolic compounds (luteolin, rutin, quercetin, apigenin, esculetin, chlorogenic acid, caffeic acid and sinapic acid), a comparative study was carried out. The responses were obtained from solutions containing 4.798 μM of the substrates in acetate buffer solution. Table 2 shows the potential E_{pc} (cathodic peak current) and relative response (%). The Au-BMI·PF_6 biosensor sensitivity decreased in the following order: luteolin, rutin, quercetin, esculetin, caffeic acid and chlorogenic acid. No response was observed for apigenin and sinapic acid (the same behavior was observed for the Ag-BMI·PF_6 biosensor). Thus, luteolin was selected for the optimization of the biosensors and application to chamomile tea samples.

The effect of possible interferents on the determination of luteolin in chamomile tea (phenolic compounds mentioned above) was investigated by using solutions containing 4.798 μM of the luteolin and adding various concentrations of an interferent. The current response was calculated by reading the current of the Au-BMI·PF_6 biosensor in the solution containing luteolin and the interferent and comparing it with the current from the biosensor in the same solution containing luteolin. The tolerated concentration of each interferent for the detection of luteolin was taken as the measured signal variation ± 3%. The results obtained are listed in Table 2 (similar responses were obtained for the Ag-BMI·PF_6 biosensor). None of these substances interfered with the proposed procedure, that is, the biosensors were able to determine the amount of luteolin in the presence of these phenolic compounds and can therefore be considered selective. The selectivity and interference studies were performed in triplicate, and according to the Student's *t*-test the results lay within the 95% confidence level.

Table 3 Study of recovery of luteolin (mg L^{-1}), as the standard solution, from chamomile tea determined using the proposed biosensors

		Found[a]		Recovery[a]	
Samples	Added	(A)[b]	(B)[b]	(A)[b]	(B)[b]
I	0.85	0.78 ± 0.1	0.81 ± 0.1	91.8	95.3
	1.12	1.17 ± 0.2	1.16 ± 0.2	104.5	103.6
	1.67	1.69 ± 0.1	1.66 ± 0.1	101.2	99.4
II	0.85	0.82 ± 0.2	0.79 ± 0.1	96.5	92.9
	1.12	1.10 ± 0.1	1.15 ± 0.1	98.2	102.7
	1.67	1.75 ± 0.2	1.69 ± 0.2	104.8	101.2

[a] $n = 3$; Found: detected value of luteolin (mg L^{-1}), in the standard solution, for the biosensors; Recovery: [(found value/added value) × 100%]. [b] (A) = Ag-BMI·PF_6-biosensor; (B) = Au-BMI·PF_6-biosensor.

Analytical recovery measurements were obtained by adding different amounts of luteolin (0.85, 1.12 and 1.67 mg L^{-1}) to two chamomile tea samples (I and II). The percentage recovery values were calculated by comparing the concentration obtained from the samples with and without the addition of known concentrations of the luteolin standard solution. Recoveries of 91.8–104.8% of luteolin from chamomile tea samples were obtained using the Ag-BMI·PF_6 and 92.9–103.6% using the Au-BMI·PF_6 biosensors, as shown in Table 3. It can be clearly observed that the recovery results obtained suggest an absence of matrix effects in these determinations. Also, to demonstrate the accuracy of the biosensors, the samples of chamomile tea were used in the quantitative determination of luteolin applying the standard additions method ($n = 3$). The luteolin contents in the chamomile tea infusion were 8.58 mg L^{-1} (I) and 4.97 mg L^{-1} (II) using the Ag-BMI·PF_6 biosensor and 9.15 mg L^{-1} (I) and 5.75 mg L^{-1} (II) using the Au-BMI·PF_6 biosensor. The results obtained using the proposed biosensors for different chamomile teas were in agreement and it can thus be concluded that the biosensors are suitable for this application.

Conclusions

In this study, novel biosensors based on Lac immobilized in chitosan chemically modified with cyanuric chloride (Chi-CC), comprised of silver and gold nanoparticles dispersed in BMI·PF_6, were used for the electrochemical determination of

luteolin. The combination of excellent immobilization of Lac, high conductivity of BMI·PF_6 and the advantages of the metal nanoparticles in the electron transfer, enhance the sensitivity of the biosensors significantly. The results showed that the method was simple and offered sufficient sensitivity for the determination of luteolin in chamomile tea samples with good precision and accuracy.

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