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PAPER

Electropolymerized network of polyamidoamine dendron-coated gold nanoparticles as novel nanostructured electrode surface for biosensor construction†

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Polyfuntionalized gold nanoparticles were prepared by using 2-mercaptoethanesulfonic acid, *p*-aminothiophenol and cysteamine core polyamidoamine G-4 dendron as capping ligands. The nanoparticles were electropolymerized on a Au electrode surface through the formation of a bisaniline-cross-linked network. The enzyme tyrosinase was further crosslinked on this nanostructured matrix. The enzyme electrode, poised at −100 mV, was used for the amperometric quantification of cathecol. The biosensor showed a linear response from 50 nM to 10 μM cathecol, with a low detection limit of 20 nM and a sensitivity of 1.94 A M^{-1} cm^2. The electrode retained 96% and 67% of its initial activity after 16 and 30 days of storage at 4 °C under dry conditions.

Introduction

Nowadays, the construction of new electrochemical biosensors with improved bioanalytical performance and robustness is frequently directly linked to the development of novel strategies for tailor-made design of electrode surfaces. Such strategies should provide electrical interfaces with nano- or microsized three-dimensional topology that allow the successful and stable immobilisation of the biomolecules without affecting their biological function, but also favour the fast and efficient occurrence of the electrochemical processes involved in the analytical reaction on such interfaces.[1]

During recent years, nanosized materials have been exhaustively employed in the modification of electrodes for biosensing purposes.[1,2] In general, the development of methodologies for nanostructuring electrode surfaces with such a purpose should consider the following points: (i) the type of surface to be modified, (ii) the nanomaterial to be used, (iii) other compounds/materials that could be also employed, (iv) the physical/chemical method for surface modification, (v) the biomolecule to be immobilized and the method to do that, and (vi) the electrochemical reaction to take place on the functionalized electrode surface.

Strategies for nanostructuring electrode surfaces have been mainly based on the use of "hard" nanomaterials, such as quantum dots,[3] carbon nanotubes,[1,4] graphene,[5] carbon nanofibers,[6] as well as metal[7] and metal oxide nanoparticles.[8] Such approaches include the use of single nanomaterials, mixtures of them, or composites with polymeric materials or low molecular weight compounds.[1,2,4] Since the number of nanomaterials with electroanalytical interest is finite, and the use of most of them in biosensor technology is well documented, the state-of-the-art of nanostructuring electrode surface is mainly addressed to the development of innovative functionalization strategies in which other novel co-materials and modifying ligands are considered.

Recently, the synthesis of polyfunctionalized gold nanoparticles (AuNPs) carrying aniline and boronic acid moieties, which have been employed for the modification of electrode surfaces with a nanostructured conductive matrix through electropolymerization, has been described.[9,10] The presence of boronic acid residues as pedant ligands on such surfaces allowed their successful use for the construction of glycoenzyme-based biosensors,[10] as well as for surface plasmon resonance (SPR) sensors towards molecules having 1,2-vicinal diols.[9]

The possibility of tailor-made design of novel AuNPs with desired and specific properties by manipulation of the capping ligands, as well as the electroconductive character of the resulting nanostructured three dimensional networks, opens a promising field in electroanalytical chemistry for this kind of material. In this regard, we are concerned to evaluate high molecular weight dendritic ligands as capping materials for the preparation of AuNP-based polymeric matrix and its potential application in

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biosensor technology. It should be highlighted that this strategy of surface coverage with electropolymerized tailor-made nanoparticles could be also a valuable tool for the preparation of functional materials for biomedicine, biotechnology, microelectronics and other fields.

Dendrimers and dendrons are nanosized and monodisperse macromolecules with a regular and highly branched three-dimensional architecture. These "soft" nanomaterials have unique properties such as structural homogeneity, high surface reactivity and molecular host capacity.[11] Such properties, resulting from the size, the hydrophilic/hydrophobic character of the internal cavities and the nature of the chemical groups at the surface of these hyperbranched polymers, support their wide application in several fields including the design of novel catalysts, chemical sensors, drug and gene delivery systems, and biosensors.[12,13]

In this work we describe a novel approach for preparing dendron-coated AuNP-based nanostructured electrodes by synthesizing, first, polyfunctionalized nanoparticles with 2-mercaptoethanesulfonic acid, *p*-aminothiophenol and cysteamine core polyamidoamine (PAMAM) G-4 dendron as capping ligands. These polyfunctionalized AuNPs were further electropolymerized on a gold electrode surface through the formation of bisaniline-cross-linked network, yielding a three dimensional matrix on which the enzyme tyrosinase, as a model enzyme, was finally immobilised through covalent crosslinking. The functionalised enzyme electrode was employed for the construction of an amperometric biosensor device towards cathecol as a model target compound.

Results and discussion

The rationale of the present work is based on the design of novel polyfunctionalized AuNPs, specifically capped with three different thiol derivatives: *p*-aminothiophenol as polymerizable unit, 2-mercaptoethanesulfonic acid as solubilising moiety and cysteamine core PAMAM G-4 dendron as hyperbranched primary amino donor for enzyme immobilization. These nanoparticles were employed for nanostructuring gold electrode surfaces through electropolymerization, which were further used as supports to immobilize tyrosinase for constructing an electrochemical biosensor towards cathecol. A graphical overview of this methodology is shown in Fig. 1.

The strategy used to synthesize the dendron-polyfunctionalized AuNPs was based on the treatment, first, of cystamine core PAMAM G-4 dendrimer with $NaBH_4$ in DMSO to ensure the complete reduction of the disulfide bonds at the dendrimer core, then releasing the thiol-active dendron derivative. This process was performed with the presence of 2-mercaptoethanesulfonic acid and *p*-aminothiophenol in the reaction

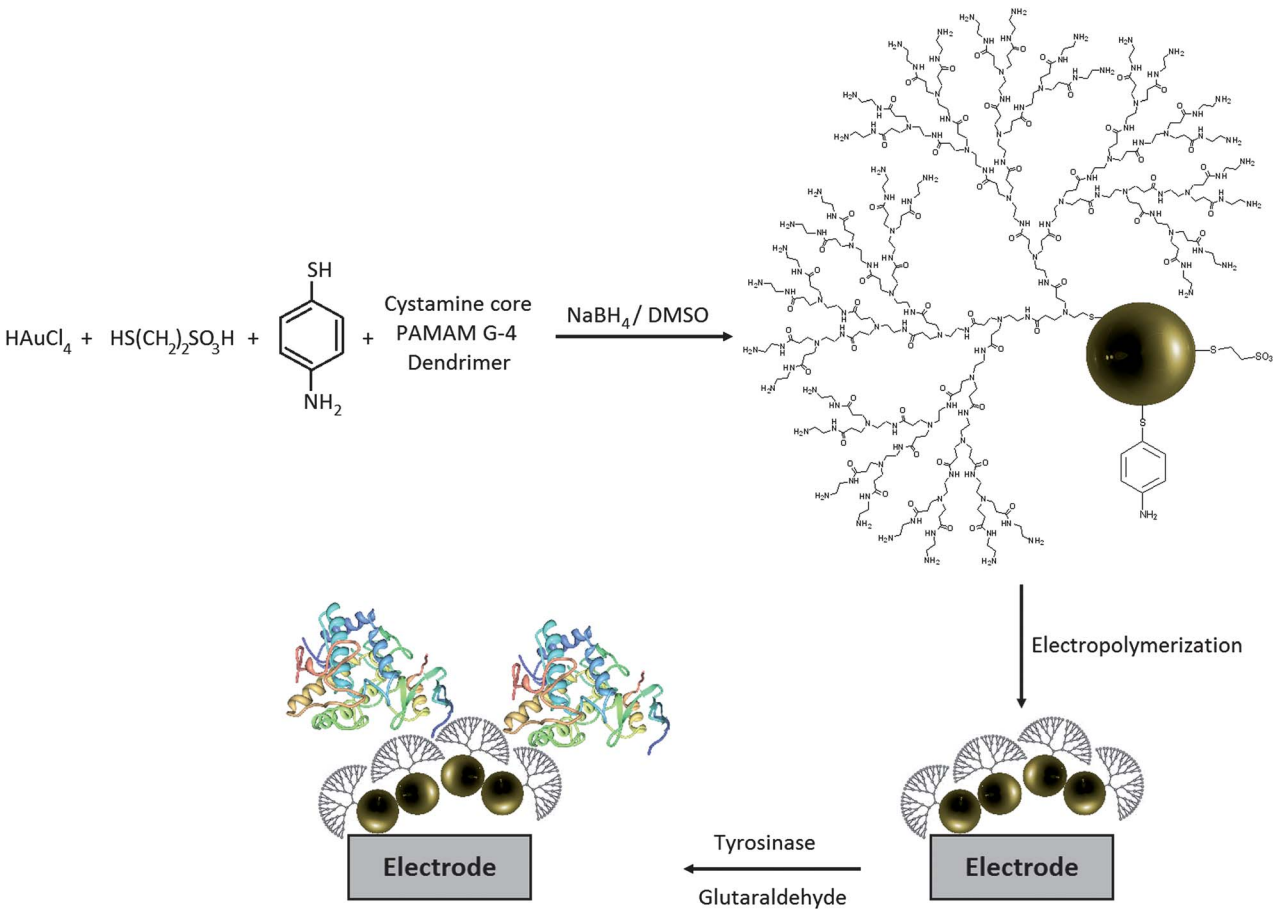

Fig. 1 Scheme displaying the steps involved in the construction of an electrochemical tyrosinase biosensor based on a gold electrode nanostructured with electropolymerized PANAM G-4 dendron-coated AuNPs.

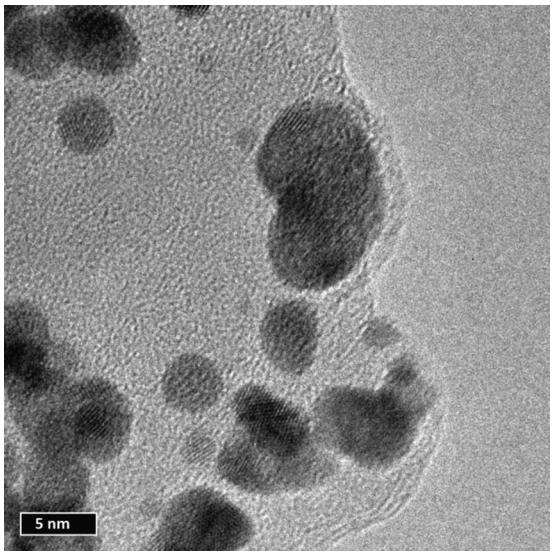

Fig. 2 HRTEM image of dendron-functionalized AuNPs.

medium. Polyfunctionalized AuNPs were then prepared by fast reduction of $HAuCl_4$ with the mixture containing $NaBH_4$ and the capping thiol ligands. Using this procedure, the formation of dendrimer-encapsulated AuNPs was avoided by eliminating the required initial sequestering of Au(III) ions within the dendrimer cavities before reduction.[13,14]

In addition, the presence of thiolated ligands in the reaction medium, including the bulky cysteamine core PAMAM G-4 dendron, ensures the fast coating of the growing Au colloids, then avoiding the formation of dendrimer-encapsulated AuNP adducts.

This fact was confirmed by HRTEM (Fig. 2), revealing that the as-prepared dendron polyfunctionalized-AuNPs showed spherical geometry with an average diameter of 5.7 ± 0.9 nm. The size of the AuNPs was slightly larger than those described for PANAM G-4 dendrimers (about 4.5 nm)[14,15] and was larger than that previously reported for metal nanoparticles encapsulated into dendritic molecules,[16] suggesting that the as-synthesized AuNPs were bigger than the cavities of the cysteamine core PAMAM G-4 dendron.

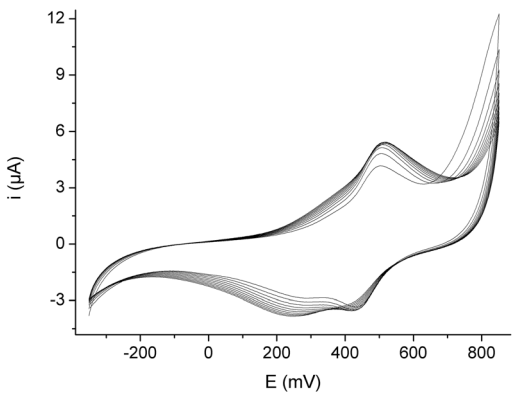

Fig. 3 Cyclic voltammograms for the first electropolymerization process of PANAM G-4 dendron-coated AuNPs on a gold electrode surface, in 0.1 M H_2SO_4. Scan rate: 100 mV s^{-1}.

AuNPs were electropolymerized in 0.1 M H_2SO_4 on a thioaniline-modified gold electrode surface. The cyclic voltammograms recorded during the cyclic electropolymerization process are shown in Fig. 3. Repeated potential scanning over the −0.35 V to +0.85 V range resulted in the appearance and continuous growth of anodic and cathodic peaks around 505 mV and 250 mV, respectively. This is the characteristic pattern for the electrochemical formation of dimeric and low molecular weight aniline condensation adducts,[17] clearly indicating the formation

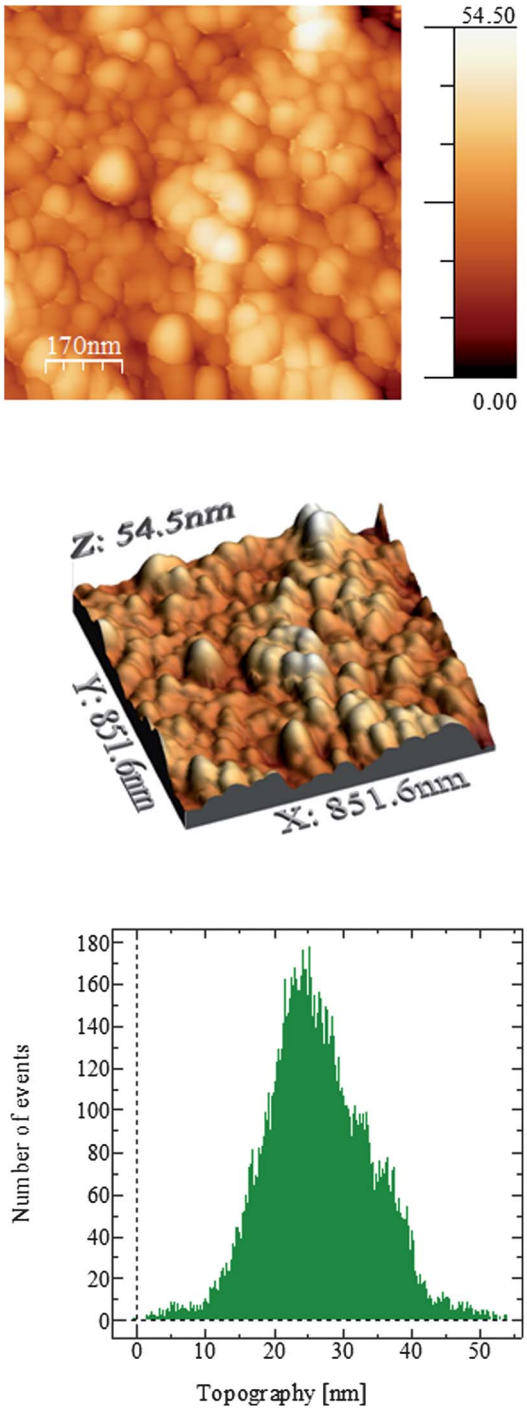

Fig. 4 AFM images and height histogram of the PANAM G-4 dendron-coated AuNP-modified electrode.

of bisaniline-cross-linked network of AuNPs on the electrode surface. In order to prepare highly nanostructured electrodes, and taking into account the unfavourable effect that bulky dendron moieties should cause on the AuNP cross-linking due to steric hindrance, a second electropolymerization process over one hour at a fixed potential of +0.85 V was also carried out.

The topology of the electropolymerized AuNP network on the metal electrode surface was characterised by AFM (Fig. 4). The electrode surface was completely covered by a three-dimensional and dense-packed matrix, showing well defined spherically shaped nanostructures. The average diameter of such electropolymerized nanostructures was significantly higher (110 ± 20 nm) than that estimated for AuNPs by HRTEM. This fact can be justified by the contribution of the capping dendritic moieties to the overall nanostructure size, as well as by the detection of several cross-linked dendron-coated AuNPs as a unique nanostructure by the AFM tip. The roughness average of this nanostructured matrix was estimated to be around 6 nm. This modified surface was characterized by a high content of interstitial holes among the spherically shaped nanostructures, representing 55% of the total area. The Gaussian-type distribution of the nanoparticle-based protuberant structures height showed a mean value of 27.4 nm and a maximum of 54.5 nm for the spherically shaped nanostructures.

In comparison with previous reports dealing with the preparation of electropolymerized matrix of boronic acid-functionalized AuNPs on gold electrodes,[10] AFM studies revealed that dendron-coated AuNPs yield a more homogeneous and less rough surface. This fact could be caused by the presence of the bulky dendritic moieties on the surface of the AuNPs, controlling the rate of electropolymerization by steric hindrance mechanisms to yield a more ordered matrix with soft surface characteristics.

The AuNP-modified electrode was used as support for the covalent immobilization of tyrosinase through glutaraldehyde-mediated crosslinking. Although covalent immobilization methods such as those using carbodiimide coupling could be employed also in general such methods allow lower immobilized protein loadings thus leading to smaller electroanalytical signals, and, therefore we decided to use the crosslinking approach. The optimum enzyme coating conditions, yielding highest electroanalytical response, were established for 100 μg of tyrosinase and 8.3% (v/v) glutaraldehyde through a cross-linking reaction on the PAMAM dendron-polyAuNP modified electrode surface over 2 h at 4 °C in 30 μL of buffer solution of pH 7.0.

Electrochemical impedance spectroscopy was employed to determine the barrier properties of the electropolymerized dendron-coated AuNP network on the electrode surface before and after enzyme immobilization. Fig. 5 shows the Nyquist plots for the electrodes in a 0.1 M KCl solution containing 5 mM $[Fe(CN)_6]^{4-/3-}$ ions. In order to interpret the experimental results, the Randles equivalent circuit was assumed as a model, taking into account the shape of the resulting Nyquist plots.

As can be observed, the Nyquist plot of bare gold electrode was characterized by a small semicircle at high frequencies and a predominant line with the slope close to the unit over the broad range of low frequencies. These facts indicated a predominant diffusion-controlled mechanism for the redox reaction over a broad range of low frequencies, as well as the occurrence of fast electron transfer for the $[Fe(CN)_6]^{4-/3-}$ ions pair on the electrode surface at high values of frequencies. Similar behaviour was observed for the electrode after electropolymerization of PAMAM dendron-coated AuNPs on its surface, but the Nyquist plots revealed that the electron transfer resistance was reduced from 205 Ω to 52 Ω after this modification. This fact can be justified by the synergic contribution of several factors, including: (i) the demonstrated ability of $[Fe(CN)_6]^{4-/3-}$ ions to easily penetrate the surface-confined dendrimers as well as the interstices between them,[15] (ii) the electrostatic attraction of $[Fe(CN)_6]^{4-/3-}$ ions to the positive charged electrode surface due to multiple amino terminal groups in dendron residues, and (iii) the increased electron transfer of $[Fe(CN)_6]^{4-/3-}$ ions to the electrode surface modified with the polyaniline matrix enriched with AuNPs. On the other hand, a noticeable increase in the electron transfer resistance (760 Ω) was observed upon tyrosinase attachment, thus indicating high coverage of the electrode surface by the enzyme molecules.

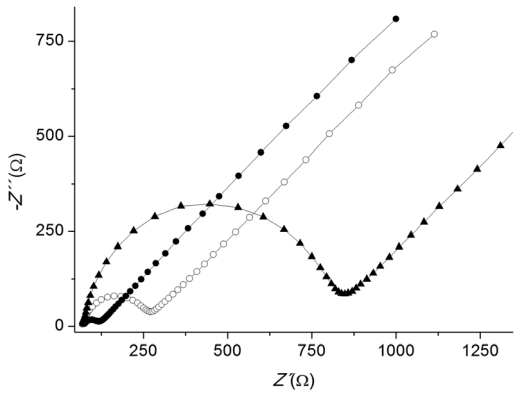

Fig. 5 Impedance plane diagram ($-Z''$ *versus* Z') for the EIS measurements at a bare gold disk electrode (○) and at the electropolymerized PANAM G-4 dendron-coated AuNP-modified electrode before (●) and after tyrosinase immobilization (▲), in 0.1 M KCl solution containing 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1 : 1).

The effect of the different modification processes on the electrochemical characteristics of the modified gold electrode was also studied by cyclic voltammetry, as illustrated in Fig. 6. The cyclic voltammograms of 5 mM $[Fe(CN)_6]^{4-/3-}$ in 0.1 M KCl solution at the bare and AuNP-modified electrodes showed

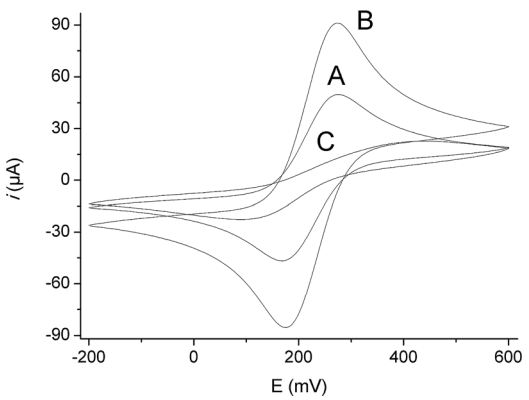

Fig. 6 Cyclic voltammograms recorded at a bare gold disk electrode (A) and at the electropolymerized PANAM G-4 dendron-coated AuNP-modified electrode before (B) and after tyrosinase immobilization (C), in 0.1 M KCl solution containing 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1 : 1).

well-defined typical diffusion-limited patterns, suggesting that the dendron-modified electropolymerized matrix is permeable to the diffusion of the electrochemical probe. Similar behaviour was previously described for gold electrodes coated with PAMAM dendrimers.[15] Electropolymerization of dendron-coated AuNPs increased the electrochemical surface area of the electrode from 3.5 mm^2 to 11.7 mm^2, according to the Randles–Sevcik model, as revealed by the higher peak currents in the voltammogram. This fact can be justified by the electroconductive nature of the bisaniline cross-linked metal nanoparticle-based network. In contrast, a significant decrease in the peak currents and a large separation between the anodic and cathodic peaks was observed upon immobilization of the enzyme on the electrode surface. A significant reduction to 0.98 mm^2 for the electroactive surface area was also noticed, suggesting a high coverage of the electrode surface by the covalent attached tyrosinase molecules, as described above in electrochemical impedance spectroscopy experiments.

The enzyme-modified electrode was further evaluated for the amperometric quantification of cathecol. Optimum conditions for this analytical determination were first established. The applied potential was selected by evaluating the steady-state current measured with the biosensor for 100 nM catechol at different detection potential values (results not shown). The maximum amperometric response was achieved by applying a potential value of −100 mV *versus* Ag/AgCl in buffer solutions of pH 7.0. The measured current corresponded to the electrochemical reduction of the *o*-benzoquinone formed in the enzyme reaction. Fig. 7 shows the typical amperometric behaviour of the enzyme electrode to successive additions of 100 μM cathecol solution under the optimal experimental conditions described above. A control non-nanostructured biosensor, prepared by cross-linking tyrosinase on a *p*-aminothiophenol-coated Au electrode was also tested. Both electrodes showed a fast electroanalytical response, reaching 95% of the steady-state current within 6 seconds. However, the amperometric response of the electrode modified with the dendron-coated AuNP network was 13 times larger than that obtained with the biosensor constructed with no electropolymerized AuNP network. The corresponding calibration curve with the nanostructured bioelectrode exhibited a linear behaviour in the range between 50 nM and 10 μM of cathecol, according to the following equation:

$$i_C\ (\mathrm{mA}) = 19 \cdot \mathrm{c}\ (\mathrm{cathecol/M}) + 2 \times 10^{-6}$$

with a correlation coefficient of 0.999 ($n = 10$).

The range of linear responses obtained was similar to that reported for other tyrosinase biosensors using a glassy carbon electrode coated with Nafion/multi-walled carbon nanotubes/ZnO nanoparticles[18] and screen printed electrodes modified with graphene oxide and AuNPs[19] but shorter than that described for tyrosinase on glassy carbon electrodes coated with polyaniline/ionic liquid/carbon nanofibers.[20]

The biosensor showed a very low detection limit of 20 nM towards cathecol, assuming a signal-to-noise ratio of 3. This detection limit at the nanomolar level was similar to that previously reported for tyrosinase immobilized on glassy carbon electrodes nanostructured with other materials, such as polypyrrole/carbon nanotubes, AuNPs, chitosan/ZnO nanoparticles, Nafion/multi-walled carbon nanotubes/ZnO nanoparticles and chitosan/Fe_3O_4 nanoparticles.[18,21–23] However, a subnanomolar limit of detection has been described for tyrosinase biosensors based on glassy carbon electrodes coated with polyaniline/ionic liquid/carbon nanofibers.[20]

The sensitivity of the biosensor was determined as 1.94 A M^{-1} cm^{-2}, considering the electroactive area of the enzyme-modified electrode. In addition, the apparent kinetics constants K_M and I_{max} showed values of 21.9 μM and 505 nA, respectively. This value of the apparent Michaelis–Menten constant ranks among the lower values reported for tyrosinase-based amperometric biosensors,[18,19,22] suggesting that the affinity of the enzyme for cathecol was not greatly affected by immobilization on the PAMAM dendron-coated AuNP matrix.

The electroanalytical response of the electrodes was highly reproducible, showing a relative standard deviation value of 3.1% by measuring ten independent signals corresponding to 100 nM cathecol and using the same electrode. On the other hand, the electrode-to-electrode reproducibility was evaluated from the response of 10 equivalently prepared biosensors towards 100 nM cathecol, yielding a relative standard deviation value of 8.8%.

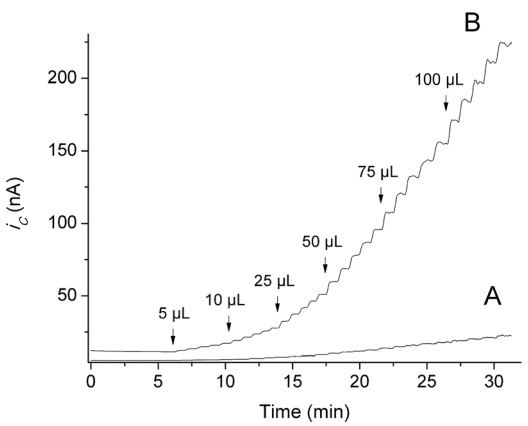

Fig. 7 Amperometric response of the tyrosinase-functionalized *p*-aminothiophenol-coated non-nanostructured gold electrode (A) and electropolymerized PANAM G-4 dendron-coated AuNP-modified electrode (B) to successive additions of 100 μM cathecol solution to 10 mL of 0.1 M sodium phosphate buffer, pH 7.0. $E_{app.} = -100$ mV.

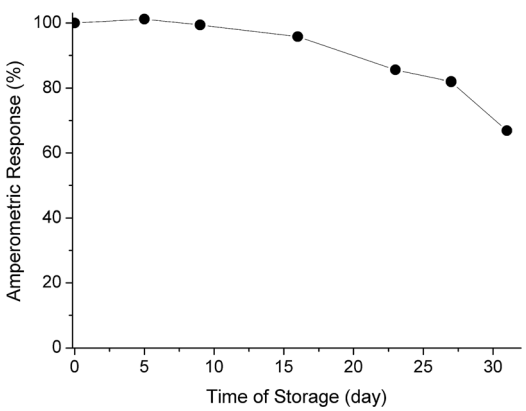

Fig. 8 Effect of the storage time at 4 °C of the tyrosinase biosensor on the relative bioelectrocatalytic activity towards 100 nM cathecol determination.

The long-term storage stability of the biosensor at 4 °C in dry conditions was determined by monitoring its analytical response towards 100 nM cathecol with intermittent usage. The AuNP-modified enzyme electrode retained about 96% and 67% of its initial bioelectroanalytical activity after 15 and 30 days of storage, respectively (Fig. 8). This stability can be ascribed to the glutaraldehyde-mediated multipoint attachment of the enzyme to the primary amino groups at the surface of the PAMAM hyperbranched structures capping the AuNP-based electropolymerized network. Such multipoint covalent cross-linking tends to preserve the catalytic activity of the enzyme by fixing the three dimensional active enzyme structure and by avoiding the release of tyrosinase from the electrode surface.

Experimental

Reagents and apparatus

Tyrosinase (Tyr, 5370 U mg^{-1}), $HAuCl_4$, $NaBH_4$, 2-mercaptoethanesulfonic acid, *p*-aminothiophenol and cystamine core PAMAM G-4 dendrimer were purchased from Sigma (USA). All other chemicals were of analytical grade.

Dendron-coated gold nanoparticles were characterized by high resolution transmission electron microscopy (HRTEM) using a JEOL JEM-4000 EX microscope. The morphology of the nanostructured gold surface was studied by atomic force microscopy (AFM) with a Nanotec Cervantes SPM microscope.

Cyclic voltammetry and electrochemical impedance spectroscopy experiments were performed using a FRA2 μAutolab Type III potentiostat/galvanostat and the data were acquired using GPES Ver. 4.9 and Frequency Response Analyser softwares, respectively (Metrohm Autolab B.V., The Netherlands). Amperometric measurements were performed with a dual-channel ultrasensitive Inbea potentiostat (Inbea Biosensores S.L., Spain). A conventional three-electrode system was employed in all electrochemical studies. The working electrode was a gold disk (CHI Instruments, UK, 2.0 mm diameter) modified with the electropolymerized AuNP network and the immobilized enzyme. An Ag/AgCl/KCl (3 M) and a Pt wire were used as reference and counter electrodes, respectively. Bioelectrode measurements were carried out at 25 °C in 0.1 M sodium phosphate buffer, pH 7.0 (working volume 10 mL). The solutions were stirred at 300 rpm with a magnetic bar during amperometric measurements. For analytical purposes, 100 μM cathecol solutions in 50 mM sodium phosphate buffer, pH 7.0, were freshly prepared.

Synthesis of the dendron-coated AuNPs

To prepare the dendron-coated AuNPs, 150 mg of sodium borohydride, 18 mg of 2-mercaptoethanesulfonic acid, 250 mg of cystamine core PAMAM dendrimer G-4 and 5 mg of *p*-aminothiophenol were dissolved in 25 mL of de-aerated dimethyl sulfoxide (DMSO) and stirred for 30 min. Another solution was prepared by dissolving 99 mg of $HAuCl_4$ in 50 mL of de-aerated DMSO and it was rapidly added to the first reducing mixture. The reaction mixture turned deep brown immediately, but the reaction was allowed to proceed for 24 h under N_2 atmosphere. Then, the functionalized AuNPs were precipitated by adding 50 mL CH_3CN, collected by centrifugation, and washed with 50 mL CH_3CN : DMSO (1 : 1, v/v), 50 mL ethanol and 50 mL diethyl ether. The nanoparticles were finally isolated by centrifugation and dried under N_2. The nanoparticles were characterized by FT-IR and HRTEM.

Preparation of the electropolymerized AuNP-modified enzyme electrode

A clean gold disk electrode was first dipped into a 10 mM ethanolic solution of *p*-aminothiophenol in order to modify the metal surface with a monolayer of the aniline derivative. After 2 h of incubation, the electrode was washed several times with ethanol and double distilled water, and further dipped into a freshly prepared 2 mg mL^{-1} solution of PAMAM dendron-AuNPs in 0.1 M H_2SO_4. Electropolymerization was performed by applying 10 potential cycles between −0.35 V and +0.85 V *vs.* Ag/AgCl at a scan rate of 100 mV s^{-1}, followed by application of a constant potential of 0.85 V for 1 h. The electrode coated with the electropolymerized PAMAM dendron-AuNP network was exhaustively washed with 0.1 M H_2SO_4 and double distilled water before enzyme immobilization.

To functionalize the electrode with tyrosinase, a mixture of 20 μL of 5 mg mL^{-1} enzyme solution in 50 mM sodium phosphate buffer, pH 7.0, and 10 μL of 25% (v/v) glutaraldehyde was dropped on the electrode surface and kept at 4 °C for 2 h in a wet atmosphere. The enzyme-modified electrode was then exhaustively washed with cool 50 mM sodium phosphate buffer, pH 7.0, and finally kept at 4 °C in dry conditions until used.

Conclusions

A novel nanostructured electrode surface, based on the modification of gold substrates with a polymeric matrix of PAMAM G-4 dendron-coated AuNPs, was developed and used as support for the covalent immobilization of tyrosinase. The nanostructured enzyme electrode was evaluated for biosensing cathecol, showing excellent analytical performance regarding detection limit, sensitivity and stability. Considering the experimental results here presented, we suggest that the covalent immobilization of enzymes on metal electrodes coated with the electropolymerized network of dendron-modified metal nanoparticles constitutes an excellent strategy to construct robust and reliable amperometric biosensors.

Acknowledgements

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