Full Paper

Development of an Amperometric Enzymatic Biosensor Based on Gold Modified Magnetic Nanoporous Microparticles

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Abstract
Gold modified nanoporous silica based magnetic microparticles have been prepared as support for the immobilization of the enzyme horseradish peroxidase (HRP). The enzyme modified gold microparticles were retained onto the surface of a solid carbon paste electrode with the help of a permanent magnet. The analytical performances of the resulting biosensor were characterized by studying hydroquinone (HQ) and hydrogen peroxide. The former was monitored by the direct electroreduction of the biocatalytically generated quinone. Several experimental parameters influencing the biosensor response were investigated. A linear response to HQ was obtained in the concentration range comprised between 5×10^{-7} and 4.5×10^{-6} M with a detection limit of 4×10^{-7} M. The enzyme electrode provided a linear response to hydrogen peroxide over a concentration range comprised between $5 \times 10^{-7} - 1.3 \times 10^{-4}$ M with a detection limit of 4×10^{-7} M. The inhibition of the biosensor response in the presence of thiols e.g. cysteine, captopril, glutathione and Nacystelyn (NAL) has been pointed out.

Keywords: Amperometric enzymatic biosensor, Magnetic nanoporous microparticles, Enzyme horseradish peroxidase (HRP), Hydroquinone (HQ)

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Dedicated to Professor Petr Zuman on the Occasion of His 80th Birthday

1. Introduction

Magnetic nanoporous microparticles are interesting solid supports for the retention of a high enzyme loading in a protective environment [1]. Different magnetic microbeads are commercially available, they generally consist of iron oxide microparticles silanized or coated with thin layers of polystyrene and functionalized for subsequent biocomponent immobilization [2–7]. Enzyme, antibody or oligonucleotide, immobilized magnetic microparticles can be advantageously trapped by magnets and retained onto an electrode surface [8–12]. Magnetic beads carrying antibodies have been described for the assay of bacteria and viruses [13–15]. Magnetic micro or nanoparticles have been used for the construction of enzyme [12, 16] and DNA [11] immobilized electrodes and immunosensors [17] and for the use as a microreactor in flow injection analysis [18]. The magnetic micro and nanoparticles can also be used for separation and preconcentration steps in analytical chemistry [19]. Due to the attractive properties of magnetic particles they have also been used in radio immunology [20, 21] and cell separation processes [22, 23]. Nanoporous silica based supports are attractive supports for enzyme entrapment and stabilization [24–27]. The biocomponent may be readily retained inside the nanoporous material by adsorption, the strength of the retention depending on the silica and the enzyme net charge [25]. The latter parameter, as well as the pore size relative to the enzyme molecular diameter influence the enzyme diffusion and its loading inside the nanopores [25, 26]. Enzyme aggregation inside the nanopores by chemical reticulation has been shown to minimize enzyme leaking out of the nanopores [27]. Alternatively, the enzymes can be chemically linked to the silica material provided it has been previously functionalized with aminoorganosilane groups [25, 26]. The high chemical reactivity of the functionalized silica material, however, might hinder the free diffusion of the enzyme inside the silica nanopores. In recent years, metal nanoparticles, especially gold nanoparticles have been extensively studied in analytical chemistry for their attractive physicochemical properties and for enzyme immobilization [28]. In the present work, the strategy consisted to create discrete deposits of gold inside the microparticles for subsequent enzyme attachment. The enzyme horseradish peroxidase (molecular radius: 4.6 nm [24]) should readily diffuse inside the porous silica material (pore mean diameter: 34 nm) for subsequent immobilization onto the gold deposits previously functionalized by cysteamine and reacted with glutaraldehyde. The resulting enzyme modified silica based magnetic microparticles were retained onto a

magnetized solid carbon paste electrode (sCPE). The HRP, in the presence of H_2O_2, catalyzed the oxidation of hydroquinone (HQ) to quinone (Q) detected amperometrically by electroreduction at the sCPE. The developed biosensor was characterized with respect to its analytical performances for hydroquinone and hydrogen peroxide assays. Additionally, the inhibition of the biosensor signal was observed in the presence of several thiols, e.g. cysteine, captopril, glutathione and Nacystelyn.

2. Experimental

2.1. Reagents and Materials

Horseradish peroxidase (HRP) (EC 1.11.1.7 type 11, 240 U/mg) and hydroquinone were from Sigma, cysteamine hydrochloride and sodium phosphate dibasic from Fluka, glutaraldehyde 25 wt% from Aldrich and hydrogen peroxide (30%) was from Vel (Leuven, Belgium). Potassium phosphate monobasic was from Janssen Chimica (Belgium). The magnetic silica based microparticles were kindly provided by Fuji Silysia Chemical Ltd. (Kasugai, Japan). Graphite powder was from Connex (Conmetal, Celle, Belgium), and solid paraffin from Merck (Darmstadt). Doubly distilled water was used throughout the experiments. The buffer was 0.1 M phosphate buffer (PB) prepared with KH_2PO_4 and Na_2HPO_4. Cysteine (Merck, Darmstadt), captopril and glutathione (Aldrich) and the lysinate salt of *N*-acetylcysteine or Nacystelyn (SMB-Galephar, Brussels, Belgium) were used without further purification.

Cyclic voltammetry was performed with a PAR Model 175 Universal Programmer. Amperometric experiments were performed using the conventional three electrodes system comprising the enzyme electrode as working electrode , a-Pt wire as auxiliary electrode and a Ag/AgCl, KCl 3 M as reference electrode. Chronoamperometric assays were made with a potentiostat INTRO (Antek, Leyden, The Netherlands) connected to a Y/t Kipp and Zonen B111 recorder.

The pH of the solutions was controlled by a Tacussel Minisis 6000 pH-meter. The spectrophotometric measurements were performed with a Pye Unicam PU 8650-Philips spectrophotometer.

2.2. Biosensor Preparation

2.2.1. Au-Magnetic Microparticles (MMPs/Au)

The silica microparticles used possess defect spinel-type iron oxide nanoparticles inside their pores providing superparamagnetic properties (i.e., do not agglomerate but are attracted by a magnet). They exhibit an important network of nanopores [18] and possess the following characteristics: particle diameter; 5 μm, pores mean diameter; 34 nm, specific area; 101 m^2/g and pore volume; 1.14 mL/g. The deposition of gold on/into the magnetic microparticles was realised as reported in the literature [29]. Briefly, the silica microbeads (30 mg) were first calcinated at 500 °C for one hour. This step permitted to remove any residual water and organic matter. Then, at room temperature, the particles were immersed into a 1×10^{-2} M $AuCl_4^-$ aqueous solution for 20 days allowing diffusion of gold ions into the nanopores. The soaked silica sample was filtered on a sintered glass filter (G4) and thoroughly washed using distilled water and dried at 80 °C for a period not less than 10 hours. This step allowed reduction of the gold adsorbed ions with formation of gold nanowires inside the silica nanopores. The dried soaked samples were annealed at 300 °C in air for about 2 hours and cooled to room temperature. X-ray diffraction (XRD) experiments were attempted but were not sensitive enough to detect gold deposited in/onto the silica particles (the gold loading is likely lower than 0.1% w/w as reported in [29]).

2.2.2. Enzyme Immobilized Gold Based Microparticles (HRP/MMPs/Au)

The Au-modified magnetic micro particles (MMPs/Au) were functionalized by reacting 20 mg MMPs/Au with a 2mM cysteamine solution in ethanol for 17 h at 4 °C. Then the mixture was filtered and the particles washed with water three times. For enzyme coupling to the amino groups of the functionalized MMPs/Au, 10 mL of a 1% glutaraldehyde solution in water was added to the suspension and the solution kept for 2h at 4 °C. After filtration, the suspension was washed three times by phosphate buffer followed by addition of 2 mL of solution HRP in phosphate buffer pH 7 (1 mg/1mL) and the mixture was left for 2h at 4 °C. Excess HRP was removed by washing with phosphate buffer. The obtained enzyme immobilized MMPs/Au were stored in phosphate buffer pH 7 at 4 °C in a refrigerator.

Magnetic particles not modifed with gold (MMPs) were also studied as carrier for enzyme immobilization. The MMPs were immersed in the cysteamine solution as described above and subsequently treated for enzyme insolubilisation as described above for the MMPs/Au.

Visible spectrophotometry was applied for the determination of the amount of immobilized HRP. A quantity of 20 mg of MMPs or MMPs/Au was reacted in 2mL of phosphate buffer in the presence of HRP (1 mg/mL) during 8h at 4°. Then, the suspension was filtered and the solution diluted (100 ×) and the absorbance measured ($\lambda = 402$ nm). The quantity of immobilized HRP was obtained by measuring the initial and final concentration of HRP and referring to a calibration curve of HRP in phosphate buffer 0.1 M [12].

2.2.3. Electrochemical Measurements

A permant magnet (i.d. 4×3 mm) Neody magnet (Nd–Fe–B), purchased from As One, Ltd. (Osaka, Japan) was firmly pressed inside a micropipette tip, Blue F-Gilson 740290 (1 mL volume capacity) leaving a depression at the surface tip of approximately 1 mm for housing the solid carbon paste layer [12]. A thin copper wire was inserted

through the opposite end to establish electrical contact. The carbon paste was prepared by thorough blending in a mortar at 50 °C, the graphite particles and solid paraffin as described previously [30]. Carbon paste was pressed in the electrode hole and the resulting CPE surface was smoothed on a clean paper. Subsequently, 10 μL of the MMPs/Au slurry (1mg/mL in 0.1 M PB of pH 7) were spread over the surface (in position upside down), and the magnetic microparticles firmly attracted within a few seconds by the electrode. Subsequently, the MMP/Au/CPE was inserted in right position in the 3-electrodes cell.

2.2.4. Procedure for Enzyme Inhibition Studies

The HRP/MMPs/Au sCPE was dipped into a 10 mL stirred phosphate buffer solution pH 6.8 in the presence of 0.1 mM H_2O_2 and the initial baseline current was recorded. An appropriate amount of substrate was added to give a final concentration of 4.95 μM HQ and a steady state current was recorded (I_0). Subsequently, solutions of increasing concentrations of the inhibitor were added stepwise and the current value after inhibition (I_1) was measured. The percentage of inhibition (I %) was calculated using the relation [31];

$$(I\ \%) = [I_0 - I_1/I_0\ ] \times 100$$

3. Results and Discussion

3.1. Determination of the Quantity of Immobilized Enzyme

A high enzyme loading was observed with the nanoporous microparticles. The quantity of HRP immobilized on the MMPs/Au and the MMPs were 25 ± 1 mg/g and 29 ± 1 mg/g, respectively. The retention of the enzyme on the non-modified particles was slightly higher than on the particles modified with gold. This difference could likely be explained by the presence of the gold deposits inside the surface of MMPs which occupy and reduce the volume of the pores thus hindering the diffusion of the enzyme inside the MMPs. It is worth to mention that, due to the high density of the pores, most of the enzyme loading occurs inside the nanopores of the microparticles.

3.2. Cyclic voltammetry at the HRP electrode

Figure 1 illustrates cyclic voltammograms at the HRP/MMPs/Au electrode in an unstirred 0.1 M phosphate buffer pH 6.8 solution with 0.1 mM H_2O_2, in the absence (Fig. 1a) and in the presence (Fig. 1b) of hydroquinone. Experiments started at + 0.1 V towards the negative potential direction in order to reduce any species (quinone) enzymatically generated at the electrode surface. Upon addition of hydroquinone a peak appeared at approximately − 0.2 V, attributed to the reduction of quinone generated by the HRP immobilized microparticles.

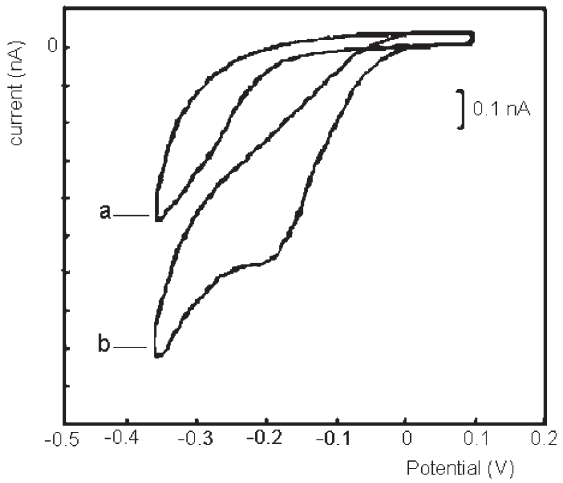


Fig. 1. Cyclic voltammograms at the HRP/ MMP/Au biosensor in PB pH 6.8: 0.1 mM H_2O_2 (a) 0.1 mM H_2O_2 + 20 μM hydroquinone (b). Scan rate 50 mVs^{-1}.

3.3. Influence of experimental parameters

The pH effect of the buffer solution on the biosensor amperometric response was investigated over the pH range 6 – 8 in PB solution containing 0.1 mM H_2O_2 and 2 μM hydroquinone. The reduction current increased substantially from pH 6 to 6.8 and then decreased (Fig. 2). At pH 6.8, the enzyme electrode exhibited a maximum of sensitivity. This pH was close to the optimum pH between 6 and 7 reported for the soluble peroxidase [32] and was in agreement with the results obtained at a HRP immobilized nanogold modified biosensor for hydroquinone assays [33].

The effect of the applied potential (E_{app}) on the amperometric signal was studied in the potential range − 280 to − 220 mV. The highest signal was obtained at − 250 mV (figure not shown) and this potential was selected for further experiments. The amperometric response was studied as a

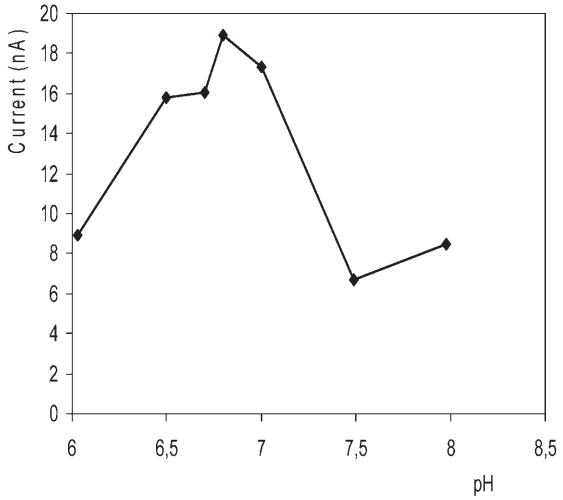


Fig. 2. Amperometric response as a function of pH. HRP/MMPs/Au biosensor, 2 μM HQ, 0.1 M PB solution, 0.1 mM H_2O_2 , $E_{app} = -250$ mV vs. Ag/AgCl, 3 M KCl.

function of H_2O_2 in the concentration range $3 \times 10^{-5} - 3 \times 10^{-4}$ M. The response increased by raising the concentration of H_2O_2 till 1×10^{-4} M then remained constant. The latter concentration was selected for subsequent experiments.

3.4. Amperometric Detection of Hydroquinone

A typical chronoamperometric response at the HRP biosensor for successive additions of different volumes of hydroquinone (HQ) under the selected experimental conditions (pH 6.8, 0.1 mM H_2O_2, $E_{app} = -250$ mV vs. Ag/AgCl, 3 M KCl) is illustrated in Figure 3. A rapid and stable response to HQ was achieved; 95% of the steady state current was observed in less than 9 seconds.

The hydroquinone biosensor exhibited a typical Michaelis Menten behavior (Fig. 4) with a linear trend in the concentration range comprised between 5.0×10^{-7} and 4.5×10^{-6} M ($I = 8.44\ C + 1.298$), slope units in nA/μM, correlation coefficient = 0.9918) with a detection limit ($S/N = 3$) of 4×10^{-7} M . The apparent Michaelis–Menten constant K^{app}_{m} , determined from the slope and intercept of the Lineweaver–Burk equation, was found to be 15 μM.

3.5. Repeatability, Reproducibility and Lifetime of the Biosensor

The repeatability of the response was studied in the concentration range $5 \times 10^{-7} - 4.5 \times 10^{-6}$ using the biosensor without renewing the microparticles. The relative standard deviation (RSD) of the slope at the HRP/MMP/Au was 3.0% ($n = 3$) and only 45.2% at the HRP/MMPs ($n = 3$). The poor results with the latter was likely due to a progressive leaching of the enzyme into the analyzed solution despite its agglomerated state.

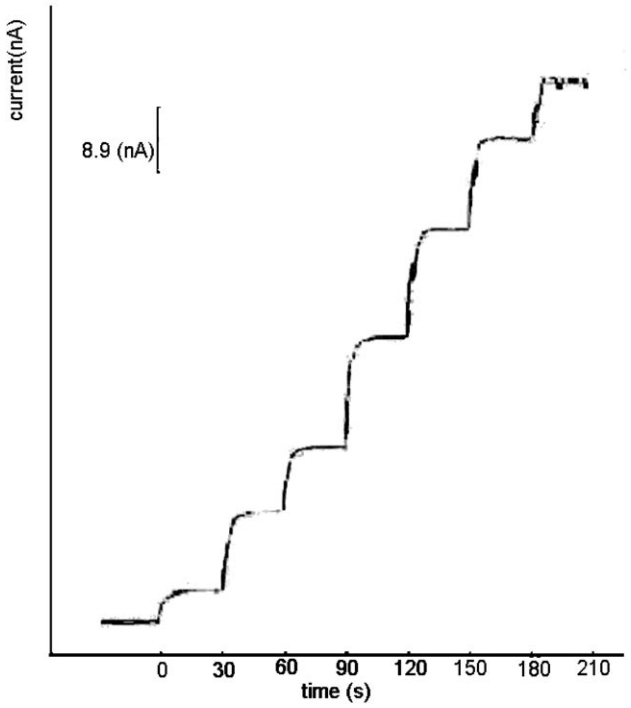


Fig. 3. Current–time response curve at the HRP/MMPs/Au biosensor for increasing HQ concentrations in 0.1 M PB (0.49–16.99 μM), 0.1 mM H_2O_2, pH 6.8 , $E_{app} = -250$ mV vs. Ag/Ag/Cl, KCl 3 M.

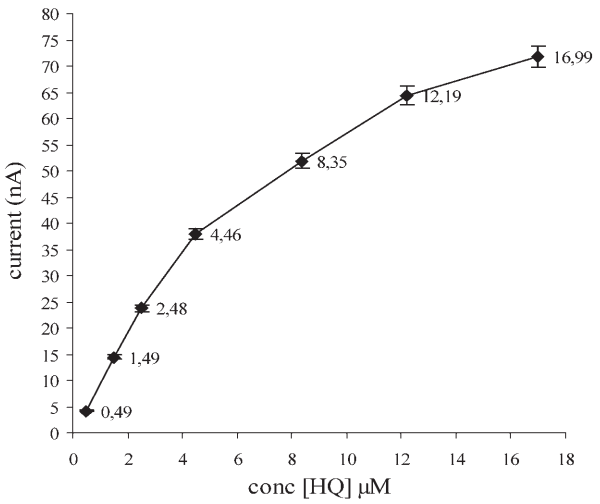


Fig. 4. Calibration curve at the HRP/MMPs/Au biosensor for HQ in PB pH 6.8, 0.1 mM H_2O_2 , $E_{app} = -250$ mV vs. Ag/AgCl , 3 M KCl ($n = 3$). Numbers at data points represent final concentrations of added HQ.

The reproducibility of the response was studied with four newly prepared HRP/MMPs deposits and renewing the particles between each calibration curve by flushing with a water jet. A relative standard deviation of the slope equal to 7.1% was obtained with the HRP/MMP/Au compared to the 5.8% RSD obtained with the biosensor prepared with the HRP/MMPs.

The stability of the HRP/MMPs/Au and the HRP/MMPs was studied comparatively by performing measurements, 2 to 3 times per week, in the hydroquinone linear concentration range. The particles were stored in 0.1 M phosphate buffer (pH 7) at 4 °C during all the time period of the stability study (two months). The results reported in Figure 5 show the trend of the amperometric signal for both biosensors for a hydroquinone concentration of 4.46 μM. The response recorded after two days of immobilization of the enzyme is regarded as the starting experiment. It is noted that the HRP/MMPs/Au exhibited a good stability as inferred from the biosensor response which retained 91% of its activity after one month and 80% after two months of storage. On the other hand, the particles not modified by gold (HRP/MMPs) exhibited a substantially lower response (despite a higher enzyme loading) and markedly less repeatable signals ($n = 3$), with a drastic reduction of the response after 45 days of storage. These results clearly point out the relatively poor retention, with leaking out into the PB solution, of the insolubilized enzyme in the MMP compared to the MMP/Au and the advantages provided by the gold deposit for enzyme retention and improved analytical characteristics.

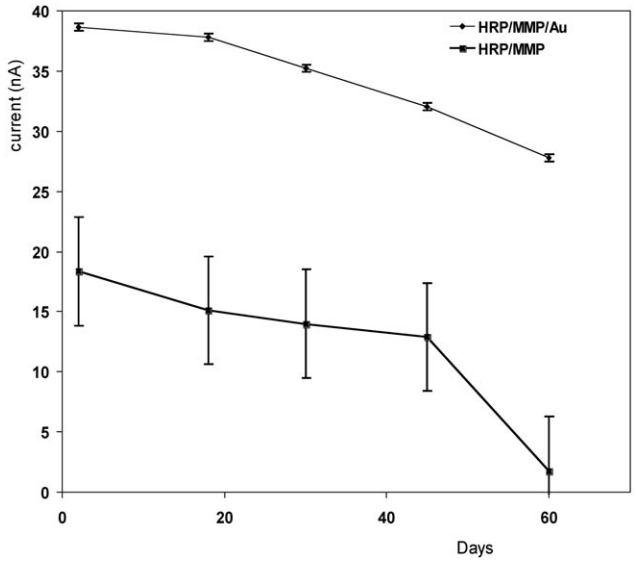


Fig. 5. Biosensor response as a function of enzyme modified microparticles storage time. Comparison between gold modified (HRP/MMR/Au) and nonmodified (HRP/MMPs) microparticles. Hydroquinone 4.46 μM, all experimental conditions are identical as in Figure 4.

3.6. Inhibition Studies

The effect of some thiol compounds on the biosensor response was studied by chronoamperometry. It is well known that thiol containing compounds such as cysteine, captopril, glutathione and nacystelyn etc. readily react with quinones leading to the production of a chemically reduced adduct (hydroquinone-thiol) as shown in Scheme 1 [34 – 37]. Recently, cysteine and glutathione were studied as inhibitors of quinone during its generation at an immobilized HRP on controlled pore glass in a flow injection step up, a glassy carbon electrode served to monitor the reduction of the quinone and its consumption in the presence of the studied thiols [36]. Yet the hydroquinone-thiol conjugate can be reoxidized, (i) electrochemically [37], or (ii) in the presence of an excess of quinone or (iii) enzymatically by HRP, giving quinone-thiol adducts [34]. The latter are also electroreduced at the presently applied potential [34, 37].

Taking into account these literature data it was expected that a possible signal enhancement (due to biocatalytical regeneration of electroactive quinone-thiols) would likely be observed at the biosensor in the presence of a thiol in solution. Interestingly, stepwise additions of a thiol derivative to a constant concentration of HQ caused rather a substantial decrease of the biosensor response. Repetitive experiments with a new HQ solution realized with the same biosensor permitted to point ou that the inhibition was reversible (i.e. complete restauration of the HQ signal). A typical chronoamperometric response curve is illustrated in Figure 6 in the case of cysteine hydrochloride additions.

Given the reversible pattern of the signal inhibition, the current decrease could be explained by the fact that less quinone derivatives reach the electrode surface because (i) HRP generated quinone was reduced homogeneously in solution by the added thiol following Scheme 1, (ii) the hydroquinone-thiol conjugate reoxidation by HRP was limited due to enzymatic steric problems and kinetic limitations and (iii) the HRP regenerated quinone-thiol adducts had diffusional restrictions to freely diffuse out of the microparticles.

This trend allowed the sensitive determination of the thiol compounds by plotting the percentage of inhibition as a function of thiol concentration. Typical calibration curves are shown in Figure 7. A linear range domain was obtained in the concentration range $9.89 \times 10^{-7} - 9.80 \times 10^{-6}$ M for all the thiol compounds studied. The limit of detection were 2.6×10^{-7}, 7.7×10^{-7}, 1.3×10^{-7} and 3.9×10^{-7} M for cysteine (cyst), captopril (cap), glutathione (GSH) and Nacystelyn, respectively. The concentration of the thiol compounds corresponding to 50% inhibition were 7.9, 15.3, 6.2 and 10.4 μM for cysteine, captopril, glutathione and Nacystelyn, respectively.

The HRP/MMPs/Au sCPE was also used for determination of H_2O_2 using hydroquinone (0.1 mM) as redox mediator. The biosensor provided a linear response to hydrogen peroxide over an extended concentration range, $5 \times 10^{-7} - 1.3 \times 10^{-4}$ M, with a sensitivity of 0.84 A mM^{-1} and a limit of detection of 3.6×10^{-7} M. The repeatability and the reproducibility of the biosensor for H_2O_2 gave a

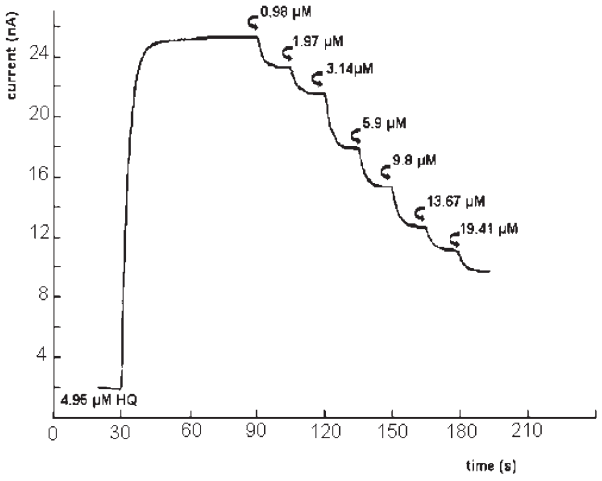
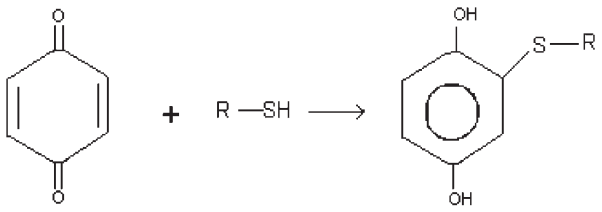


Fig. 6. Response of the biosensor to 4.95 μM HQ (left wing) and stepwise addition of cysteine hydrochloride aliquots (right wing).0.1 M PB pH 6.8, 0.1 mM H_2O_2 , $E_{app} = -250$ mV vs. Ag/AgCl, 3 M KCl . Numbers in figure represent final concentration of added cysteine.



Scheme 1.

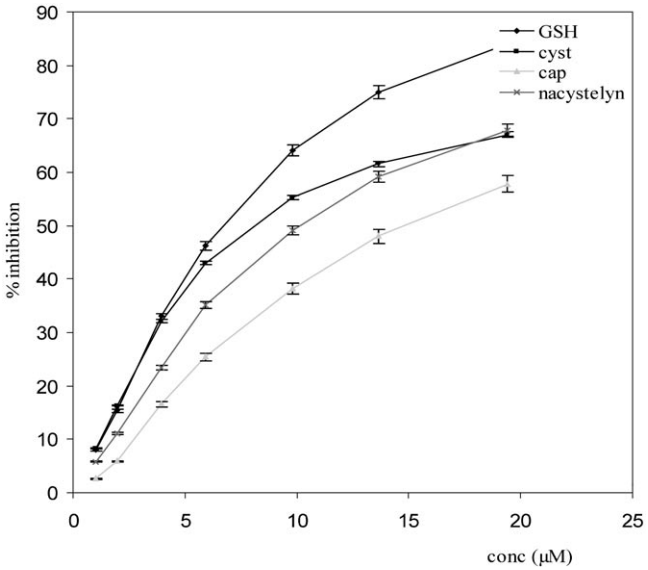


Fig. 7. Inhibition calibration curves for thiol containing compounds. HRP/MMPs/Au biosensor, 0.1 mM PB pH 6.8, 4.95 μM HQ, 0.1 mM H_2O_2, $E_{app} = -250$ mV vs Ag/AgCl, 3 M KCl.

RSD for the slope equal to 7.5% and 1%, respectively ($n = 3$).

4. Conclusions

Nanoporous superparamagnetic silica based microparticles can be advantageously modified by gold deposits for subsequent functionalization and enzyme immobilization. The enzymatic microbeads may be trapped by a "magnetized" electrode for amperometric assays. Such biosensor configuration offers interesting characteristics such as a high enzyme loading in a protective environment. The enzyme immobilized microbeads may be readily characterized and renewed and the electrode surface recycled or regenerated. Hydrogen peroxide and hydroquinone can be determined in a broad concentration range with high sensitivity allowing a broad number of analytical applications to be performed.

5. Acknowledgement

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