Amperometric biosensor based on horseradish peroxidase-immobilised magnetic microparticles

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Abstract

Magnetised silica-based microparticles (MMPs) (5 μm) with a high density of nanopores were used for horseradish peroxidase (HRP) immobilization and amperometric biosensor development in batch conditions. The resulting biosensor was applied to study the peroxidation of clozapine (CLZ) which is a dibenzoazepine drug often used in the treatment of neurological disorders. The amperometric response corresponded to the electroreduction of CLZ-oxidized products namely a nitrenium cation and quinoneimine derivative of CLZ. Despite a relatively low amount of immobilized HRP (0.3 μmol/g), clozapine quantification in the micromolar concentration range was achieved by the use of a magnetized solid paraffin carbon paste electrode for microparticles attraction. Diffusion of substrate and products of the enzyme reaction within the nanopores were identified as limiting factors in the biosensor response. This amperometric biosensor configuration has number of interesting advantages such as ease and reproducible microparticle layer renewing, low enzyme consumption, controlled surface immobilization, protective enzyme microenvironment etc.

1. Introduction

The use of magnetic microparticles (microbeads) for the development of sensing systems based on an immobilized biological component is an attractive approach in analytical chemistry [1]. Enzyme, antibody or oligonucleotide-immobilized magnetic beads can be advantageously trapped by magnets and retained close to, or onto an electrode surface [2,3]. The biorecognition event can be detected by electrochemiluminescence [3,4] or electrochemically by monitoring the product of an enzymatic reaction [5–8]. Different electrode configurations can be considered such as screen-printed electrodes [5,6], microelectrodes [7], interdigitated microelectrodes array [8,9] and ISFETs [2]. Most of the applications concern immunosensors (ELISA) since the bioligand-coated magnetic microbeads can be readily confined in close proximity to the electrode surface and in microenvironments allowing for rapid, sensitive and renewed assays [6–8].

Activated beads are commercially available and are generally based on iron oxide microparticles silanized or coated with thin layers of polystyrene and functionalized for subsequent biocomponent immobilization. Silica-based magnetic beads are attracting much recent interest for the immobilization of biological molecules [1,9–14]. Silica particles may be prepared in different shapes and sizes with different degrees of porosity, they are chemically and mechanically stable and are resistant to bacterial attack [9]. The chemistry of silicate materials is well controlled and the substrate can be modified by a variety of reagents. Recently, nano silica-based magnetic particles have been described for biocatalysis and bioseparation applications [1].

The present work is based on our previous observation that highly porous silica-based magnetic microparticles (MMPs) represent an interesting support for the entrapment and immobilization of the enzyme glucose oxidase (GOx) and for their

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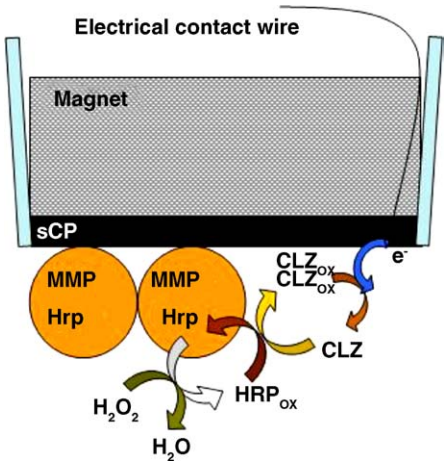

Fig. 1. Schematic drawing of clozapine peroxidation at the horseradish peroxidase-immobilized magnetic microparticles (HRP–MMPs) and subsequent electroreduction at the solid carbon paste electrode (sCPE).

application as a microreactor in flow injection analysis [14]. Here, the enzyme horseradish peroxidase has been immobilized in MMPs and retained onto a magnetized solid carbon paste electrode (sCPE). This original configuration was applied to study the peroxidation of clozapine (CLZ). The latter is an interesting atypical neuroleptic actively metabolized by oxidative processes, thus generating a reactive intermediate (a nitrenium cation) which has been suspected to induce negative side effects [15]. The enzymatic and electrochemical (EC) oxidation patterns of clozapine have been recently compared and the oxidation products identified by liquid chromatography coupled to mass spectrometry (LC–MS) and LC–EC–MS [16,17]. The results confirmed the cyclic voltammetry profile of clozapine oxidation with the formation of two electrochemically reversible species namely, the nitrenium cation and a quinoneimine derivative of CLZ. The presently developed amperometric biosensor is operating by monitoring the peroxidation products of CLZ at the enzyme-immobilized magnetic microparticles (EMMPs) via their electroreduction at the sCPE (Fig. 1).

2. Experimental

2.1. Chemicals

Horseradish peroxidase (EC 1.11.1.7 type 11, 240 U/mg), glucose oxidase (EC 1.1.3.4, type VII-S from *Aspergillus niger*), α-D-glucose and clozapine (CLZ; 8-chloro-11-(4-methyl-1-piperazin-yl)-5H-dibenzo[b,e] [1,4] diazepine), were obtained from Sigma (Bornem, Belgium). Super microbead silica gel was purchased from Fuji Silysia Chemical Ltd. (Kasugai, Japan). Graphite powder for the preparation of the solid carbon paste was from Connex (Conmetal, Celle, Belgium). Solid paraffin (solidification point 46–48 °C) was from Merck (Darmstad, Germany). Hydrogen peroxide (30%) was from Vel (Leuven, Belgium). A phosphate buffer (PB) pH 7.4 was prepared by mixing a stock solution of 0.1 M sodium phosphate (Acros Organics (Geel, Belgium)) and stock solution 0.1 M sodium dihydrogen phosphate (Sigma). Glutaraldehyde 25 wt.% (GA) was from Aldrich (Belgium). A stock glucose solution (1.0 M) was left for at least 24 h before being used to allow glucose mutarotation. The solution was stored for a maximum of 2 weeks at 4 °C. Aminopropyltriethoxysilane (APTS) was from Chisso Ltd. (Minimata, Japan). All reagents were of analytical grade and the solutions were prepared with doubly distilled and purified water (Milli-Q quality grade). The electrode material was prepared by thorough blending, in a mortar at 50 °C, the graphite particles and solid paraffin as described earlier [18].

The stock solution of CLZ (1×10^{-2} M) was dissolved in water from CLZ base by addition of a few drops of 2 M HCl and stored in a refrigerator.

2.2. HRP magnetic particles

The enzyme-immobilized magnetic particles were prepared using magnetic silica gels supporting defect spinel-type iron oxide particles inside the pores as described earlier [14]. The microparticles had a nominal pore diameter of 30 nm, a specific area of 101 m^2/g, a pore volume of 1.14 ml/g and mean diameter of 5 μm. Smaller particles (4 μm) were collected by allowing the particles to settle for 4 min in a glass tube and sampling with a micropipette the resulting upper part of the suspension. The MMPs were functionalized with APTS as described previously [14].

2.3. Enzyme immobilization

Two procedures were investigated:

(i) APTS-functionalized silica microparticles (0.5 g) were reacted first with GA (2%) for 2 h and rinsed thoroughly. Then, the HRP solution (2 mg/ml) was reacted with the GA-activated particles in 0.03 M phosphate buffer pH 4.5 for 8 h at 4 °C. The suspension was filtered through a glass filter by vacuum filtration and the retained particles washed with distilled water (5 × 30 ml).

(ii) An amount of 0.5 g MMPs was treated in 20 ml of a 2 wt.% glutaraldehyde solution (in 0.1 M phosphate buffer of pH 7.0) at room temperature for 2 h. The product was washed with PB (pH 7.0, 0.03 M, 5 × 30 ml) then retained on a glass filter and rinsed with water (5 × 30 ml). For the GOx immobilization, a GOx solution (2 mg/ml) was prepared in a 0.03 M PB of pH 7.0. The glutaraldehyde-treated MMPs were reacted with 20 ml GOx solution at 4 °C during 8 h with stirring from time to time. The suspension was first washed with PB (5 × 30 ml) then rinsed with water (5 × 30 ml) on a glass filter. Subsequently the GOx–MMPs were treated in 20 ml diluted glutaraldehyde solution for 8 h at 4 °C with stirring from time to time. The suspension was filtered

over a glass filter and rinsed with 0.03 M PB (5 × 30 ml) then with water (5 × 30 ml). Finally, the GA-activated GOx–MMPs were reacted with 20 ml of HRP solution (4 mg/ml) in 0.03 M PB at 4 °C during 8 h with stirring from time to time. The bienzyme-modified particles (HRP–GOx–MMPs) were first rinsed with phosphate buffer (5 × 30 ml), then rinsed with water (5 × 30 ml) on a glass filter and stored at 4 °C to dry.

For electrode modification, the EMMP slurry (50 mg) was suspended in 5 ml of 0.1 M PB (pH 7.4). The vial was kept in the refrigerator at 4 °C until needed.

2.4. Determination of amount of immobilized enzyme

Visible spectrophotometry was applied for the determination of HRP immobilized on the GOx–MMPs. Typically, an amount of 0.05 g GA-activated GOx–MMPs was reacted with 2 ml of HRP (4 mg/ml) for 8 h at 4 °C (see above). Then, the suspension was filtered (filter pore diameter 0.2 μm) and the solution was diluted 100 times and absorbance measured. The amount of immobilized HRP was determined by measuring the initial and final concentration of HRP by referring to a calibration curve of HRP realized in 0.03 M phosphate buffer of pH 7 (λ = 402 nm). The amount of HRP retained was expressed with respect to wet or dry MMPs (dried at 100 °C during 2 × 30 min).

Elemental analyses were conducted using a CHN analyzer-type EA 1110 from CE Instrument (Milan, Italy) for the determination of HRP immobilized on the MMP.

2.5. Enzyme-immobilized micromagnetic particles-based electrode

A permanent magnet (i.d. 4 mm × 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 1.5 mm for housing the solid carbon paste layer (Fig. 1). The latter was obtained by manually pressing the solid carbon paste in the hole and the resulting sCPE was smoothed on a clean paper surface. The diameter of the active surface was 2.5 mm. A thin silver wire served as the electrical conductor. Unless otherwise stated, 5 μl of the EMMPs slurry (10 mg/ml in 0.1 M PB of pH 7.4) were spread over the surface of the electrode (in position up side down) allowing the particles to settle and being attracted within a few seconds by the magnet. Subsequently, the EMMP sCPE was inserted in right position in the three-electrode cell along with a Pt wire as the auxiliary electrode and an Ag/AgCl 3 M KCl as reference electrode. The EMMPs were firmly attracted by the magnet allowing appropriate stirring during the amperometric experiments.

2.6. Apparatus and electrochemical measurements

Amperometry was performed in a conventional three-electrode setup with the biosensor working potential polarized at + 0.00 V and the solution (10.0 ml) gently stirred with a magnetic bar during the assays. The potentiostat was a Bruker E 230 LC detector (Bruker, Brussels, Belgium) connected to a Y/t Kipp and Zonen B 111 recorder. All experiments were performed at 25 °C (thermostated conditions) in phosphate buffer of pH 7.4 in the presence of 0.1 mM hydrogen peroxide. The pH of the solutions was controlled by a pH meter (Tacussel Minisis 6000). The working potential was operated at desired potential and the background was allowed to decay to steady state value (for 2 min approximately). Varying amounts of CLZ standard solution were spiked into the stirred electrochemical cell and the current was recorded. Experiments were conducted at several different applied potentials with varying solution pH.

Spectrophotometric experiments were performed with a Pye Unicam PU 8650-Philips.

3. Results and discussion

Experiments were performed in the presence of hydrogen peroxide and in phosphate buffer pH 7.4 i.e. close to physiological conditions where the nitrenium cation was found to be relatively stable ($t_{1/2}$ = 1 min) [19,20]. Amperometric assays at both biosensors were found to be significantly higher using the HRP–GOx–MMPs biosensor (Fig. 2). The response was, however, not linearly related to CLZ in the concentration range studied (5–25 μM).

The amount of HRP immobilized on MMPs was determined by elemental analysis, but the sensitivity of the technique was not high enough to determine such a low amount of immobilized HRP. In order to obtain higher amounts of immobilized HRP, it was attempted to link HRP onto GOx–MMPs. Indeed, the amount of GOx fixed at the

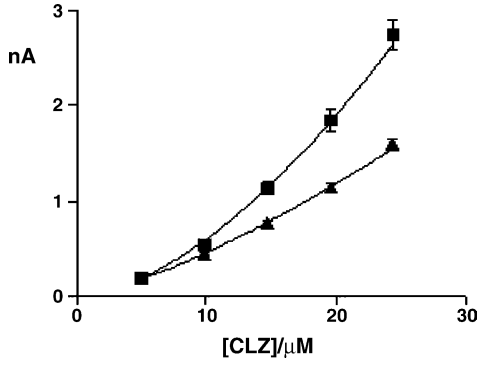

Fig. 2. Amperometric response at the HRP–MMPs (▲) and HRP–GOx–MMPs (■) biosensors. Forty microliters of particles deposited onto the sCPE, 0.1 mM [H_2O_2], 0.1 M PB of pH 7.4. Current measured 1 min after the addition of CLZ, (n = 3).

MMP was quite high (325 μmol/g) [14] and this structure was inferred to be a good matrix for further HRP immobilization.

The quantity of HRP immobilized onto GOx–MMPs, determined by spectrophotometry, was found to be equal to 0.3 μmol/g for the dried HRP–GOx–MMPs and approximately 0.1 μmol/g for the wet HRP–GOx–MMPs slurry. This is very low compared to the amount of GOx immobilized onto the MMPs but is in the same order of magnitude than HRP physisorbed onto mesoporous silica particles [10] and onto silica nanoparticles [1]. The origin of the relatively low amount of immobilised HRP may be related to some diffusion constraints inside the pores of the particles and also likely to a poor chemical efficiency of HRP linking via Schiff base reaction of amino groups to free pending groups of GA. HRP has an extensive glycosylation pattern on its surface and other immobilisation procedures should be studied in future work [20].

All further experiments were performed using the HRP–GOx–MMPs. It was checked that the GOx–MMPs biosensor, in the presence of glucose (to liberate hydrogen peroxide), gave no response on CLZ addition.

The response of the biosensor was studied at different applied potentials in the range −0.2 and +0.2 V. At potentials lower than 0 V, no signal enhancement was obtained compared to 0 V, but the noise increased. By operating at positive potentials, the signal was lower with no response detected above +0.25 V.

The amperometric response studied as a function of amount of deposited EMMPs (5, 10, 20, 30, 40 and 50 μl) showed that the highest signal was obtained with 40 μl of deposited volume of MMPs suspension. The slope of the calibration curve for 40 μl was twice the slope for 5 μl deposited MMPs. Higher volumes gave lower signals. This is likely to be related to diffusion limitation of CLZ towards the electrode created by the microparticles layer thickness. Further experiments, however, were performed with 5 μl deposit since it provided better reproducibility of results.

Amperometric responses at the HRP–GOx–MMPs biosensor were compared in the presence of hydrogen peroxide and in the presence of glucose. As reported in Fig. 3, a similar trend was observed in the presence of both

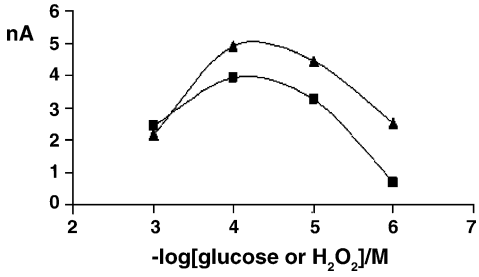

Fig. 3. Amperometric response at the HRP–GOx–MMPs biosensor as a function of hydrogen peroxide (▲) and glucose (■) concentrations ($n = 2$). Deposit of 5 μl particles, 1.10^{-4} M CLZ, 0.1 M PB of pH 7.4. Signal measured 30 s after addition of clozapine.

co-substrates with slightly higher signals in the presence of hydrogen peroxide. Interestingly, at high co-substrate concentration (above 1.10^{-4} M), a signal decrease was obtained which is attributed to some HRP inactivation [21]. When the co-substrate concentration was lower with respect to clozapine, a signal decrease was observed due to a lower amount of activated HRP.

Responses as a function of clozapine concentration, in the presence of 0.5 mM glucose, were studied by renewing the HRP–GOx–MMPs (the latter are readily removed by flushing a water burst over the biosensor surface with a wash bottle) after each calibration curve. A typical Michaelis–Menten plot was obtained (Fig. 4A) which, when converted to an Eadie–Hofstee plot (Fig. 4B), shows a concave trend towards the origin thus suggesting diffusion limitation of the CLZ oxidation products inside the nanopores and/or chemical instability of the CLZ oxidation products (especially in relation with the nitrenium cation). A linear trend, not passing through the origin, was found between 3×10^{-5} and 2×10^{-4} M ($R^2 = 0.9999$, R.S.D. of slope = 4.7%, $n = 5$).

In the presence of 10 μM of glucose as the co-substrate, a linear trend was obtained between 5 and 25 μM of clozapine ($R^2 = 0.9973$, R.S.D. = 3%, $n = 5$). The repeatability was determined in this calibration range by using the same biosensor and rinsing in PBS during 5 min after each calibration curve. A substantial increase was obtained between the first and the following calibration curves. The R.S.D. of the slope

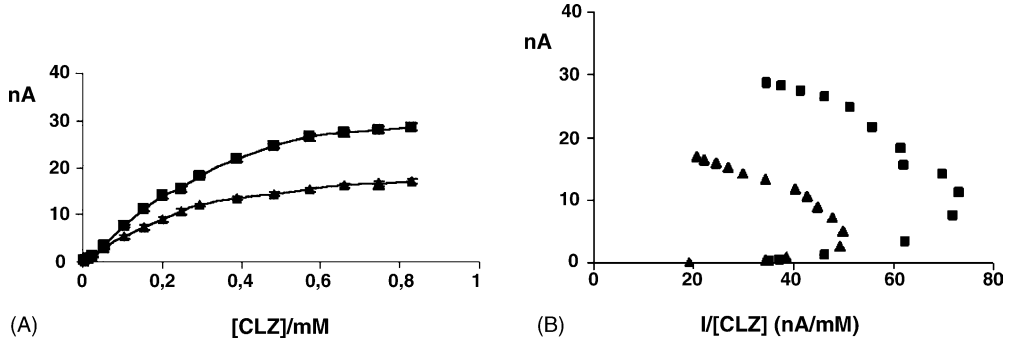

Fig. 4. Amperometric response as a function of clozapine concentration ($n = 3$). HRP–MMP in the presence of 0.1 mM hydrogen peroxide (■) and in the presence of 0.1 mM glucose (▲) (A) and Eadie–Hosfstee plot (B). Five microliters of particles deposited onto sCPE, 0.1 M PB of pH 7.4. Current measured 30 s after addition of CLZ.

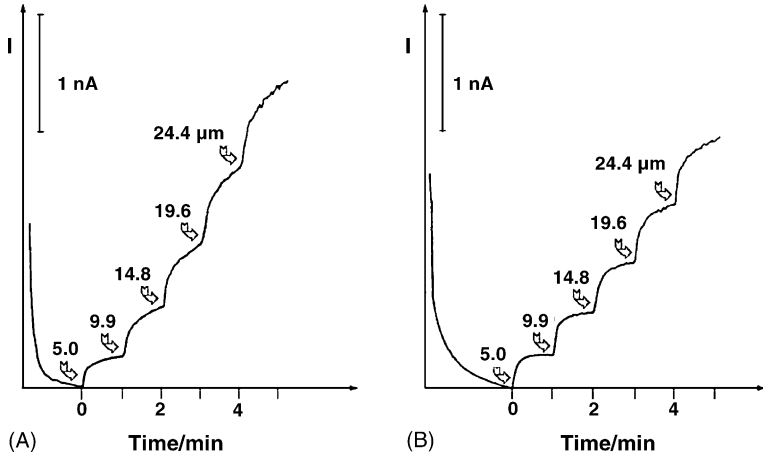

Fig. 5. Typical amperometric response at the HRP–GOx–MMPs biosensor using the method of standard addition. Five microliters of deposited microparticles (diameter particles = 5 μm) (A) and 10 μl of deposited microparticles (diameter of particles = 4 μm) (B). 0.1 M PB of pH 7.4.

for five calibration curves (not counting the first one) was 8%.

No significant influence of ionic strength on biosensor response was found by addition of NaCl in the concentration range 0.01–0.2 M. The biosensor response was also studied as a function of pH in the pH range 3–7.4. A maximum response was obtained at pH 5.5, which is a compromise between HRP activity and clozapine oxidation products formation and stability.

Fig. 5 illustrates a typical amperometric response as a function of increasing CLZ concentrations. As shown in Fig. 5A, a steady state current plateau was rapidly obtained after the first addition of clozapine but further addition showed a continuous plateau current raise. The former was a result of CLZ reacting with HRP immobilized at the outer surface of MMP and the subsequent trend may likely be attributed to progressive diffusion of increasing amounts of CLZ oxidation products out of the HRP–GOx–MMP pores. Experiments performed with smaller particle diameter (4 μm) showed improved steady state current plateaus attributed to a shorter diffusion path length for the molecules to diffuse out of the pores (Fig. 5B).

The stability of the HRP–GOx–MMPs was of interest since no loss in signal was obtained after 1 month storage in 0.1 M PB of pH 7.4 at 4 °C and the slurry was stable for periods longer than 5 months.

4. Conclusions

Magnetic silica-based microparticles represent an interesting means for biocomponent (enzyme, antibody etc.) entrapment and immobilization and for subsequent attraction onto magnetized electrodes. The particles allow for a variety of surface reactions to be performed for biocomponent attachment. The amount of biological material immobilized can be determined with accuracy and high signals can be obtained thanks to the close proximity of the MMPs and the magnetized electrochemical transducer. The MMPs may be readily renewed and the stability of the enzymes entrapped in the nanopores appears to be high thanks to the protective silica-based environment. Batch and flow conditions may be considered for analytical application. The immobilization procedure, however, needs to be judiciously selected and carefully controlled for efficient enzyme immobilization. The relatively large size of the highly porous microparticles create some substrate and product diffusion limitations inside the dense nanopore-sized network.

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References

[1] H.-H. Yang, S.-Q. Zhang, X.-L. Chen, Z.-X. Zhuang, J.-G. Xu, X.-R. Wang, Anal. Chem. 76 (2004) 1316 (references cited therein).
[2] S. Solé, S. Alegret, F. Cespedes, E. Fàbregas, Anal. Chem. 70 (1998) 1462.
[3] J.T. Soini, M.E. Waris, P.E. Hänninen, J. Pharm. Biomed. Appl. 34 (2004) 753.
[4] A.J. Bard, G.M. Whitesides, US Patent 5,238,808, 1993.
[5] A.R. Varlan, J. Suls, P. Jacobs, W. Sansen, Biosens. Bioelectron. 10 (1995) XV.
[6] M. Dequaire, C. Degrand, B. Limoges, Anal. Chem. 71 (6) (1999) 2571–2577.
[7] J.H. Thomas, S.K. Kim, P.J. Hesketh, H.B. Halsall, W.R. Heineman, Anal. Biochem. 328 (2004) 113–122.
[8] S.K. Kim, P.J. Hesketh, C. Li, J.H. Thomas, H.B. Halsall, W.R. Heineman, Biosens. Bioelectron. 20 (2004) 886.
[9] J. Deere, E. Magner, J.G. Wall, B.K. Hodnett, Chem. Commun. (2001) 465.

[10] J. Deere, E. Magner, J.W. Wall, B.K. Hodnett, Catal. Lett. 85 (2003) 19.
[11] J. Deere, E. Magner, J.G. Wall, B.K. Hodnett, Biotechnol. Prog. 19 (2003) 1238.
[12] X. Liu, Z. Ma, J. Xing, H. Liu, J. Magn. Magn. Mater. 270 (2004) 1.
[13] Y. Sun, L. Duan, Z. Guo, Y. DuanMu, M. Ma, L. Xu, Y. Zhang, N. Gu, J. Magn. Magn. Mater. 285 (2005) 65.
[14] A. Nomura, S. Shin, O. Oulad Mehdi, J.-M. Kauffmann, Anal. Chem. 76 (2004) 5498.
[15] Z.C. Liu, J.P. Uetrecht, J. Pharmacol. Exp. Ther. 275 (1995) 1476.
[16] B. Blankert, O. Dominguez, W. El Ayyas, J. Arcos, J.-M. Kauffmann, Anal. Lett. 37 (2004) 917.
[17] S.M. van Leeuwen, B. Blankert, J.-M. Kauffmann, U. Karst, Anal. Bioanal. Chem., 2005, in press.
[18] C. Petit, A. Gonzales-Cortes, J.-M. Kauffmann, Talanta 42 (1995) 1783.
[19] J.L. Maggs, D. Williams, M. Pirmohamed, B.K. Park, J. Pharmacol. Exp. Ther. 275 (1995) 1463.
[20] A.M. Azvedo, V. Vojinovic, J.M.S. Cabral, T.D. Gibson, L.P. Fonseca, J. Mol. Catal B: Enzyme 28 (2004) 121.
[21] M. Dequaire, B. Limoges, J. Moiroux, J.-M. Savéant, J. Am. Chem. Soc. 124 (2002) 240.