Covalent Immobilization of Glucose Oxidase to Poly(*O*-Amino Benzoic Acid) for Application to Glucose Biosensor

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ABSTRACT: A biosensor for glucose utilizing glucose oxidase (GOX) covalently coupled to poly(*o*-amino benzoic acid) (PAB; a carboxy-group-functionalized polyaniline) is described. Amperometric response measurements conducted via unmediated and mediated (with ferrocene carboxylic acid and tetrathiafulvalene) reoxidation of GOX show that glucose can be detected over a wide range of concentrations. An enzyme-conducting polymer-mediator model provides for better charge transport in a biosensor. The optimal response, obtained at pH 5.5 and 300 K, lies in the 1–40 mM range. A kinetic plot yields the value of the apparent Michaelis–Menten constant, K_m^{app}. The operational stability of the PAB-based glucose biosensor was experimentally determined to be about 6 days. © 2000 John Wiley & Sons, Inc. J Appl Polym Sci 78: 662–667, 2000

Key words: poly(*o*-amino benzoic acid); glucose oxidase; amperometric; covalent immobilization

INTRODUCTION

Analytical biosensors combine the high selectivity of biocatalytic reactions with the fast and accurate measurements of electrochemical sensors. In general, a biosensor consists of a biological component in intimate contact with a suitable transducer coupled through immobilization. The response generated as a result of a reaction of a biological component with the analyte is detected by the transducer to give a signal (electrical/optical) that can be used with or without amplification for the estimation of the concentration of the analyte in a given sample.[1] An efficient electrochemical transducer requires a conducting immobilizing matrix to probe the redox reaction at the enzyme active site directly, rather than an electroactive reactant or product as the measuring probe. Conducting polymers are known to be efficient electron-transporting agents for such an application.

Several biosensors have been developed in the last decade with electrochemically polymerized films acting as three-dimensional matrices for the immobilization of enzymes.[2–6] Electronically conducting polymers such as polypyrrole and polyaniline have a number of interesting and advantageous features, such as flexibility of the available chemical structure and compatibility with neutral aqueous media, a prerequisite for enzyme activity. Further, conducting polymers can be doped and undoped electrochemically, a process that is accompanied by significant changes in the conductivity and spectroscopic properties of the film. This property can be utilized for the development of novel enzyme-based devices.[7,8] Several reviews have appeared on different procedures of

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biomolecular immobilization by entrapment in electrogenerated polymers such as polypyrrole, polyaniline, and polyphenol.[9–12]

The immobilization of the biological component (enzyme, microbe, or DNA) enhances its stability in unfavorable pH, temperature, and ionic conditions that otherwise would render it inactive. Depending on the type of component and the characteristics of the matrix (surface area, porosity, hydrophilic nature, etc.), the immobilization of biomolecules (in a desired matrix) can be accomplished in a number of ways, such as physical adsorption, entrapment, and covalent immobilization. However, most of these procedures suffer from a low reproducibility and a poor spatially controlled deposition.

In this study, poly(*o*-amino benzoic acid) was used as a polymer matrix to covalently couple GOX with the carboxy groups of the polymer. Polyaniline and several of its derivatives, such as poly-*o*-phenylene and poly-2-amino aniline, have been widely used for immobilizing different enzymes, including GOX,[13,14] with entrapment. The covalent immobilization of GOX on poly-2-aminoaniline[15] and with glutaraldehyde as a bifunctional agent has also been attempted.[16] Poly(*o*-amino benzoic acid) (PAB), also referred to as polyanthranilic acid, was prepared chemically and covalently coupled to glucose oxidase (GOX) with a carbodiimide reagent. The object of this investigation was to provide proof of the concept of direct coupling of an enzyme to the surface of a polymer with much shorter chains. The novelty lies in the idea of the actual coupling of an enzyme to the polymer rather than it being mixed with a polymer. Because GOX is an important enzyme and has been extensively used for biosensing applications, the idea of covalent coupling will be helpful in giving the enzyme a more natural environment, higher efficiency, and increased stability. Covalent binding also ensures higher macromolecular interaction in comparison with entrapment and has opened newer perspectives to be researched in the field of biosensor construction.

EXPERIMENTAL

Ortho-Amino-benzoic acid (99%), tetrahydrofuran (98%), methanol (99%), *N*-methyl-pyrrolidone (98%), 1-*cyclo*-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate, ferrocene carboxylic acid (FCA), and tetrathiafulvalene (TTF) were procured from Aldrich. GOX (D-glucose:oxygen 1-oxidoreductase; EC 1.1.3.4), Sephadex G-15 gel, and *o*-dianisidine were obtained from Sigma Chemical Co. (USA). Poly(vinyl chloride) (PVC) substrates were procured from Kapoor Plastics (Delhi, India).

PAB was prepared by a modification of the procedure reported by Chen et al.[17] Distilled *o*-amino benzoic acid (100 mL; approximately 50 mM) was mixed with 50 mM ammonium persulfate and 100 mL of 1 N HCl at a pH maintained at 1.0. The polymerization was carried out with constant stirring at 200 rpm in an ice bath, and the temperature was maintained between 0 and 5°C. After 30 min, the dark-green polymer suspension was filtered through a 1-μm-pore-size filter and was thoroughly washed about 10 times with deionized water. The resulting polymer (PAB) was further washed about 10 times with methanol (99%) and acetone to remove any unreacted monomers. The polymer was dried and desiccated for about 24 h and characterized. The conductivity of the polymer pellet (diameter = 1 cm; thickness = 5 mm) was in the insulating region (8.5×10^{-14} S/cm).

The filtrate (1 mL) containing the soluble PAB oligomers (1 mL; a brownish solution) was mixed with 1 mL of 0.15 M 1-*cyclo*-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate in a 0.05 M acetate buffer at pH 4.6 for about 6 h. To this, 12.5 mg of GOX (ca. 1000 units) was added and allowed to react for about 2 h at 4°C.

Solutions containing PAB/GOX, PAB, and carbodiimide were passed through a Sephadex G-15

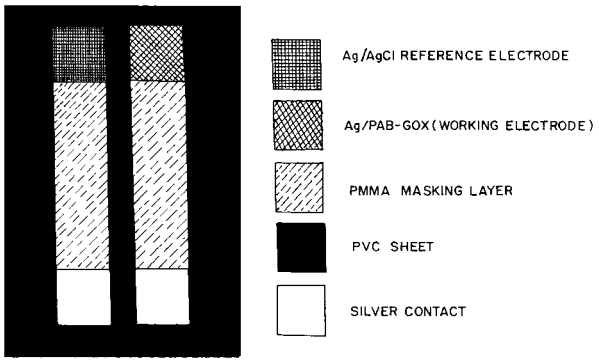


Figure 1 Schematic of the two electrode set-up employed for the amperometric measurements. Each electrode consists of two screen-printed tracks of Ag paste. On one of the silver tracks, the Ag/AgCl reference was printed, and on the other, the PAB/GOX complex was adsorbed. The dimensions of each strip are indicated therein. The working area (4 mm × 2 mm) was covered by a nylon mesh, and the remaining electrode was masked with a PMMA layer.

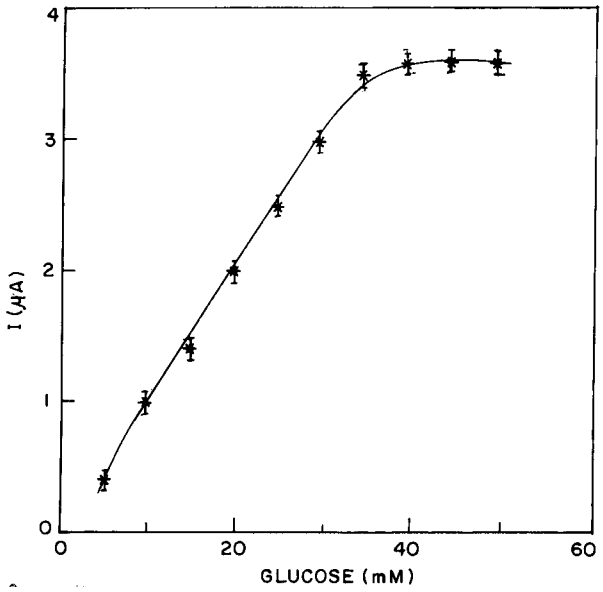


Figure 2 Amperometric response of the PAB/GOX electrode at 0.7 V w.r.t Ag/AgCl for H_2O_2 detection to 40-μL additions of 0–60 mM D-glucose in a 0.1 M phosphate buffer at pH 7.0 and 27°C. Each point on the plot corresponds to the current value after 100 s following the addition of D-glucose. The surface area of the electrode was 8 mm^2.

column (length =15 cm; diameter = 1 cm) with a 0.1 M phosphate buffer at pH 7.0 as the elution buffer at a flow rate of 1 mL/min. Two colored bands were seen on the column. The yellowish band corresponding to unreacted GOX eluted out prior to the brownish-yellow band of the PAB/GOX couple. The latter was collected in 1-mL fractions and was further purified by dialysis against a 0.1 M phosphate buffer at pH 7.0. The preparation was tested for GOX activity prior to use. The GOX bound to PAB was monitored with an *o*-dianisidine procedure.[4]

The screen-printed electrodes consisted of two silver tracks with an active working area of 4 mm $\times$ 2 mm (Fig. 1) printed on a PVC substrate. On one of the tracks, the Ag/AgCl reference was screen-printed, and on the other electrode, the PAB/GOX complex was adsorbed. The area of the electrode exposed to the solution was always kept at 8 mm^2 by masking with a poly(methyl methacrylate) (PMMA) layer. The working area of the screen-printed PAB/GOX electrode was shielded by a nylon membrane.

The amperometric currents at various concentrations of glucose (after the stabilization of the monomers) were measured at a suitable polarization potential with reference to Ag/AgCl with a Keithley electrometer (EC 617). The measurements with mediators (FCA and TTF) were conducted in solution, and no attempts were made to immobilize them. The solutions were degassed with N_2 before each measurement. The thermal measurements were performed on a differential scanning calorimeter (DSC) from PerkinElmer on 5-mg samples of native GOX and PAB/GOX couple at a heating rate of 2°C/min.

RESULTS AND DISCUSSION

While monitoring H_2O_2 during the enzymatic reaction with screen-printed PAB/GOX electrodes, we used a polarization potential of 0.7 V; current response measurements employing FCA and TTF as the mediators were, however, carried out at 0.4 and 0.3 V, respectively. The results of the amperometric response measurements undertaken on the unmediated screen-printed PAB/GOX electrodes as a function of glucose concentration are shown in Figure 2. The measurements constitute an average of three runs, and the current values in each run do not vary by more than 10%. The response is linear up to about 35 mM, beyond which it tends to achieve a steady-state value. A

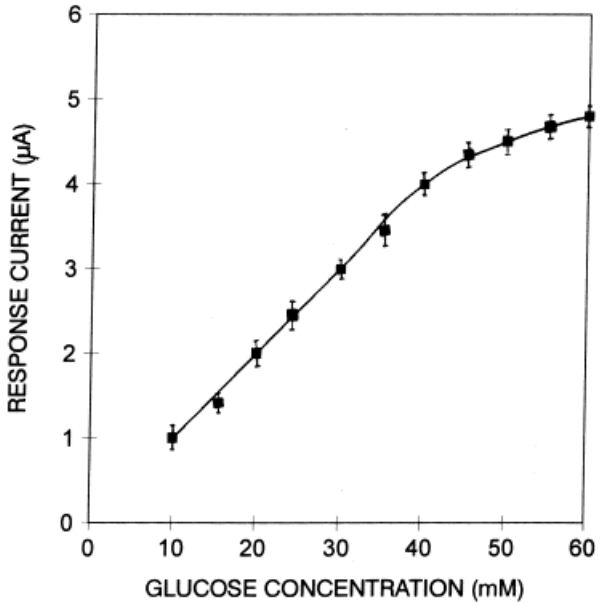
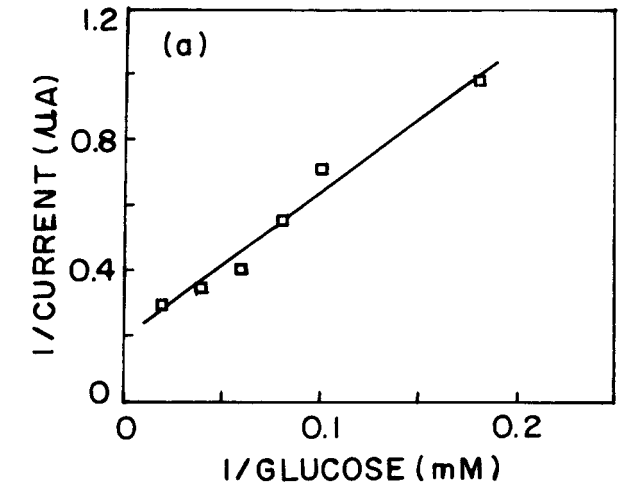
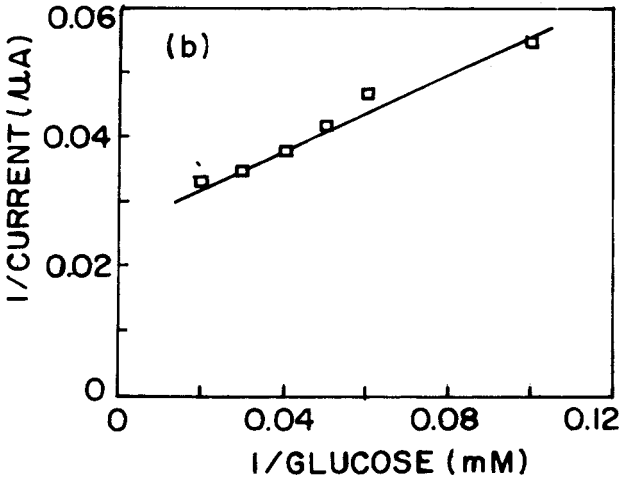
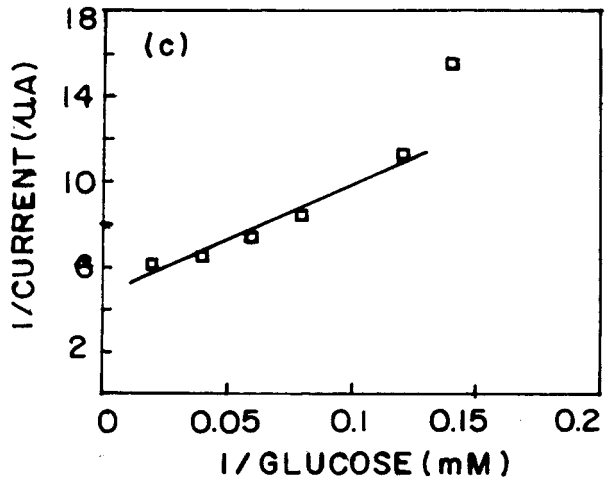


Figure 3 Amperometric response of the GOX (physically adsorbed) electrode at 0.7 V w.r.t Ag/AgCl for H_2O_2 detection to 40-μL additions of 0–60 mM D-glucose in a 0.1 M phosphate buffer at pH 7.0 and 27°C. Each point on the plot corresponds to the current value after 100 s following the addition of D-glucose. The surface area of the electrode was 8 mm^2.

linear regression analysis yields a correlation coefficient of 0.998. The response time is 60 s. The results are comparable to those obtained with GOD physically adsorbed on screen-printed electrodes under similar reaction conditions (Fig. 3).







Lineweaver–Burke plots (double reciprocal plots) were analyzed for the unmediated and mediated reactions at the PAB/GOX couple. The plots (Fig. 4) indicate that the systems follow classical Michaelis–Menten-type kinetics. The $I_{max}(\mu A)$ intercept, the maximum current due to the uninhibited reaction, for the PAB/GOX/FCA system increases in comparison with the unmediated PAB/GOX/O_2 system. The K_m^{app} value simultaneously decreases. This is due to favorable reaction kinetics and mediation in the presence of FCA compared with dioxygen. The larger K_m^{app} value of PAB/GOX could perhaps be attributed to the increased hydrophobic environment[18] around GOX and the restricted conformational freedom or, probably, the elevated diffusion barrier at the polymer/solution interface. The I_{max} value for the PAB/GOX/TTF system, however, is lower than that of the PAB/GOX/O_2 system despite the fact that TTF is known to be a good mediator.[19,20] This anomaly is not yet understood.

The results of the amperometric response measurements carried out on the PAB/GOX/O_2 system at 20–50°C are shown in Figure 5. I_{max} increases (Fig. 5) and then decreases, indicating that the measurements can be made up to about 40°C. Beyond 40°C, it appears that the enzyme loses considerable activity and I_{max} decreases. Figure 6(a) shows the results of DSC studies conducted on PAB at 20–100°C at 2°C/min, indicating an endothermic peak at about 70°C. Figure 6(b) shows the endotherm observed for the PAB/GOX couple at ~40 and ~70°C. The 40°C peak corresponds to the denaturation temperature of GOX, which remains the same in solution and when bound to PAB. This behavior indicates that GOX retains its physicochemical properties even after being bound to PAB.

The PAB/GOX couple was stored between 5 and 10°C in a 0.1 M phosphate buffer at pH 7.0 for

Figure 4 Lineweaver–Burke plots (double reciprocal plots) obtained from the amperometric response current measurements versus the glucose concentration (a) for the PAB/GOX electrode at 0.7 V w.r.t Ag/AgCl to 40-μL additions of 0–20 mM D-glucose, (b) in the presence of 2 mM FCA measured at 0.4 V w.r.t Ag/AgCl for FCA (red) to 40-μL additions of 0–70 mM D-glucose, and (c) in the presence of 2 mM TTF measured at 0.2 V w.r.t Ag/AgCl for TTF (red) in a 0.1 M phosphate buffer at pH 7.0 and 27°C. Other conditions were the same as those in Fig. 2.

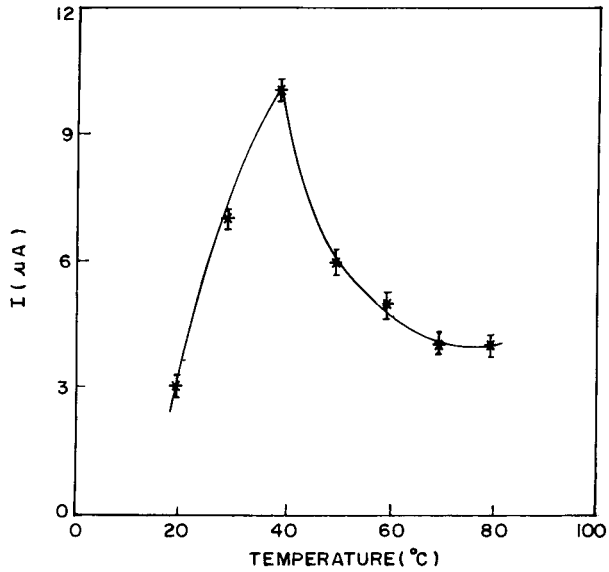


Figure 5 Amperometric response to 10 mM D-glucose additions to PAB/GOX preparations heated to temperatures between 35 and 70°C in a 0.1 M phosphate buffer at pH 7.0.

a period of about 6 days to test the storage stability of a poly-*ortho*-amino benzoic acid-based glucose biosensor. As shown in Figure 7(a), GOX bound to PAB retains about 60% of its initial activity over a period of 6 days. The change in the amperometric current [Fig. 7(b)] for a given unmediated screen-printed PAB/GOX electrode over the 6-day period is negligible.

CONCLUSIONS

It has been demonstrated that PAB-coupled GOX functions as a stable glucose biosensor. It should be interesting to investigate the effect of increasing the loading of GOX onto PAB on the stability and sensitivity of the PAB/GOX biosensor. Detailed experiments concerning the role of interferents in the amperometric current of PAB/GOX electrode biosensors are currently in progress.

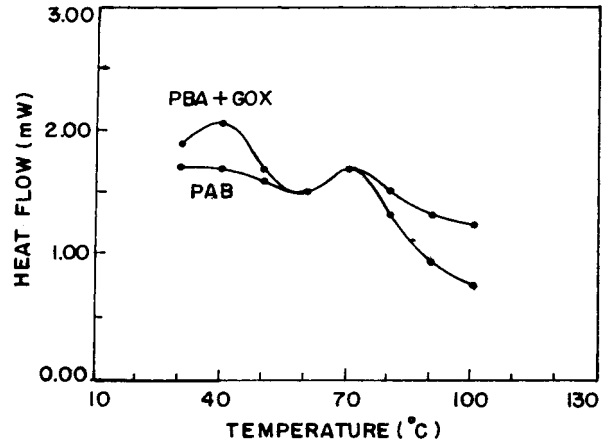


Figure 6 Thermogram recorded for 10 mg of (a) PAB and (b) the PAB/GOX couple at a heating rate of 2°C/min between 30 and 100°C.

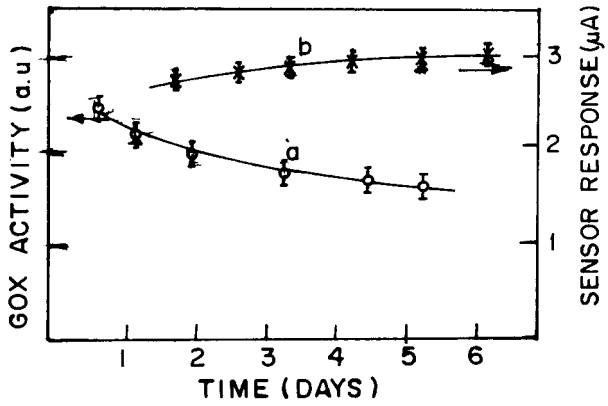


Figure 7 Stability of the PAB/GOX preparation: (a) the activity of GOX in the PAB/GOX couple measured chemically every 24 h with the *o*-dianisidine assay as a function of storage time between 0 and 6 days in a 0.1 M phosphate buffer at pH 7.0 and 27°C and (b) the amperometric response to 40-μL additions of 10 mM D-glucose measured every 24 h in a 0.1 M phosphate buffer at pH 7.0.

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REFERENCES

1. Griffiths, D.; Hall, G. Trends Biotechnol 1993, 11, 122.
2. Hoa, D. T.; Kumar, T. N. S.; Punekar, N. S.; Srinivasa, R. S.; Lal, R.; Contractor, A. Q. Anal Chem 1992, 64, 2645.
3. Bartlett, P. N.; Cooper, J. M. J Electroanal Chem 1993, 363, 1.
4. Ramanathan, K.; Annapoorni, S.; Malhotra, B. D. Sens Actuators B 1994, 21, 165.
5. Verghese, M. M.; Ramanathan, K.; Ashraf, S. M.; Malhotra, B. D. J Appl Polym Sci 1998, 70, 1447.
6. Cosnier, S.; Senillou, A.; Gratzel, M.; Comte, P.; Vlachopoulos, N.; Renault, N. J.; Martlett, C. J Electroanal Chem 1999, 469, 176.
7. Bartlett, P. N.; Birkin, P. R. Synth Metals 1993, 61, 15.

8. Schumann, W. Mikrochim Acta 1995, 121, 1.
9. Contractor, A. Q.; Sureshkumar, T. N.; Narayanan, R.; Sukeerthi, S.; Lal, R.; Srinivas, R. S. Electrochim Acta 1994, 39, 1321.
10. Trojanowicz, M.; Krawczynski vel Krawczyk, T. Sens Actuators B 1995, 28, 191.
11. Cosnier, S. Biosens Bioelectron 1999, 14, 443.
12. Wallace, G. G.; Smyth, M.; Zhao, H. Trends Anal Chem 1999, 18, 245.
13. Cooper, J. C.; Hall, E. A. H. Electroanalysis 1993, 5, 385.
14. Karyakin, A. A.; Lukachova, L. V.; Karyakina, E. E.; Orlov, A. V.; Karpachova, G. P. Anal Commun 1999, 36(4), 153.
15. Losada, J.; Armada, M. P. G. Electroanalysis 1997, 9, 1416.
16. Parente, A. H.; Marques, E. T. A.; Azevedo, W. M.; Diniz, F. B.; Melo, E. H. M.; Felho, J. L. L. Appl Biochem Biotechnol 1992, 37, 267.
17. Chen, H. S. O.; Ng, S. C.; Seow, S. H.; Sim, W. S.; Hor, T. S. R. Therm Anal 1993, 39, 177.
18. Scheller, F.; Schubert, F. In Techniques & Instrumentation in Analytical Chemistry; Elsevier: Amsterdam, 1992; Vol. 11, p 53.
19. Gunasingham, H.; Tan, C. H. Analyst 1990, 115, 35.
20. Mulchandani, A.; Bassi, A. S. Biosens Bioelectron 1996, 11, 271.