Electrochemical Probing of the Activity of Glucose Oxidase Embedded Sol-Gel Matrices

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Sol-gel chemistry offers new and interesting approaches in the field of biosensors. The fact that such a synthesis is performed at room temperature, allows organic molecules or biomolecules to be incorporated inside the inorganic gel matrix leading to new hybrid materials.[1,2] In recent years, it has become a challenge to build up reproducible and efficient enzyme sensors to monitor biological substrates,[3] such as glucose in water or blood.[4] On the other hand, silica gels and derived glasses are ideal substrates to entrap enzymes because of their easy preparation, chemical stability, and often their transparency. There are so far only a few reports of encapsulation of enzymes in gels or glasses. Recently, a few studies have described the entrapment of glucose oxydase (GOD) in silica gels and xerogels.[5,6] The two leading research groups of research in this new field (Yamanakana et al.[5] and Avnir et al.[1,6]) have employed optical means for the detection of GOD activity and have shown that the activity of the enzyme inside the gel is close to that in water. However optical techniques require transparent medium that cannot always been achieved with sol-gel oxide matrices.[7] Electrochemical detection of enzyme activity can provide an alternative solution.

To specify the level and the conditions of the enzymatic activity inside both colloidal[8] and polymeric[8] silica gels (which are the best understood and more easily available sol gel matrices), as well as to realize the development of electrochemical sensors, we have investigated the activity of GOD directly by the *in situ* electrooxidation of a ferrocene mediator, which immediately transfers its electrons to the enzyme. The current can be a precise measurement of the enzyme activity provided that conditions of total catalysis are reached. The classical mechanism[9,10] of such a system is well-known and can be represented by the following scheme:

$$E + G \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EG \xrightarrow{k_2} EH_2 + GL$$

$$EH_2 + 2Q \xrightarrow{k_3} E + 2P$$

where E and EH_2 are respectively the oxidized and the reduced form of the enzyme (GOD), and Q and P are the oxidized and the reduced forms of the mediator, G represents the substrate, in our case the glucose, GL is the gluconolactone, and EG is the complex formed between the oxidized enzyme and the glucose. The electrochemical current is given by the reoxidation of the reduced form P of the mediator on the electrode, and its increase is a direct quantitative test for the enzyme turnover. In this study, the concentration of the enzyme has been deliberately chosen to be very high, in order to make the catalytic effect as noticeable as possible. The GOD concentrations used are low enough to allow the use of the "stationary state" hypothesis for the GOD. This means that there is no concentration polarization of either the reduced or the oxidized form of the enzyme.

The experimental apparatus (Figure 1) consists of an electrode endcapped with an open pyrex glass hemisphere (according to the scheme so that the gel is mechanically retained without restraining the planar diffusion at the electrode) according to the scheme shown in Figure 1. Therefore the gel is strongly held to the electrode. Three types of gels have been examined:

(1) TMOS gels were made according by a slight modification of a procedure previously described.[11] Typically a homogeneous sol is made within 10 min by vigourously mixing at room temperature 12.4 g of TMOS (Fluka), 4 g of deionized water, and 0.11 g of 0.04 M hydrochloric acid (sol I). Sol I is then cooled to 4 °C immediately after mixing. Separately a solution of GOD (Boeringer Manheim, grade I; sol II; typically 2 mg/mL) in a 0.01 M buffer (pH 8) is prepared. This solution is also cooled to 4 °C. Then in 0.4 mL of sol I is dissolved 1 mg of hydroxymethylferrocene (Aldrich). The resulting solution is quickly mixed (still at 4 °C) with 1.05 mL of sol II to yield the final sol. This final sol is then allowed to fill the cavity of the electrodes with the help of a disposable micropipette. The filled electrodes are maintained at 4 °C for enough time for the sol to gel. The electrodes are then stored at 4 °C for at least 2 h in a 0.1 M aqueous phosphate buffer saturated with (hydroxymethyl)ferrocene, after which most of the methanol resulting from the polymerization of the TMOS has been washed out.[12] The electrodes can then be allowed to rise to room temperature and then transferred to the electrochemical cell. It should be noted that, while a drop of sol is enough to fill the electrode cavity, optical experiments have been performed using 3-mL quartz cells.

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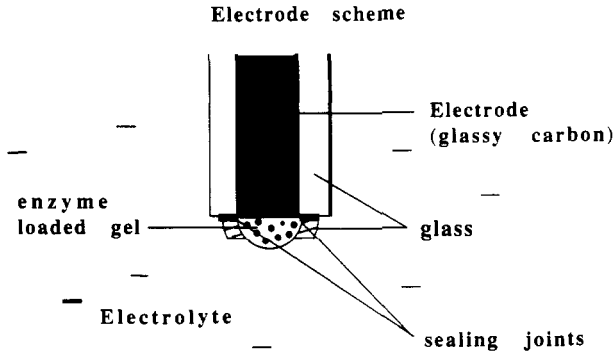

Figure 1. Electrode scheme.

(2) Gels were prepared from silicon tetraacetate, according to the following procedure: 1.3 g of $Si(OAc)_4$ was added to a minimum quantity of acetone (about 1 cm^3). To this solution were added successively 2 cm^3 of pure water (yields a clear solution) and 3 cm^3 of 1 M aqueous NaOH. The resulting clear sol has a pH of 5. GOD (5–10 mg) is then added, and the sol is saturated with ferrocene methanol ($C = 2 \times 10^{-4}$ M) before being introduced into the electrode cavity.

(3) Gels from colloidal silica: Commercial monosized silica sols (with diameters 40, 140, and 1000 Å in diameter) stabilized at a basic pH (NYACOL) are neutralized to pH 6–7 with an adequate amount of perchloric acid;[8] then the desired amount of GOD (a few milligrams is added to 1 mL of this sol. The electrode cavities are filled with the resulting sol and covered to avoid evaporation. The gelation time depends upon the size of the silica sol but is between 30 min and a few hours. The electrodes are then equilibrated in a buffer solution saturated with ferrocene methanol before use. The electrodes are handled as previously.

Freshly prepared electrodes and aged electrodes stored inside a buffer solution exhibit the same electrochemical activity. However the drying of the gel causes a shrinkage that bypass the electrical contact and stops the electrochemical detection.

Our experiments show that the GOD was active in several types of gels, as evidenced by the electrochemical response of the electrodes. The typical cyclic voltammetry curve of a GOD loaded TMOS gel is shown in Figure 2. Without glucose added, we observe the classical response of the ferrocene included in the gel, similar to what could be observed in solution. When 0.1 M glucose is added to the electrolyte, after ca. 20 min, the cyclic voltammetry curves change as an effect of the catalytic process occurring inside the gel. At a high scan rate, the electrochemical reduction of the included ferrocene, occurring at the backward sweep is faster than the transfer to the enzyme and the curves are unchanged. At slow scan rate, the enzymatic kinetics are predominant and the curve displays the behavior of a polarogram with the limiting current depending only upon the reduction rate of the ferricinium, that is the enzymatic turnover. Varying the glucose concentration between 0.2 and 1 M leads only to qualitative (that is, not linearly correlated) differences in the electrochemical response of the device. Thus, the developpement of sensors for low glucose concentrations probably would require an improvement of the system.

With colloidal silica gels, the maximum catalytic effect is obtained with medium size silica (the diameter of

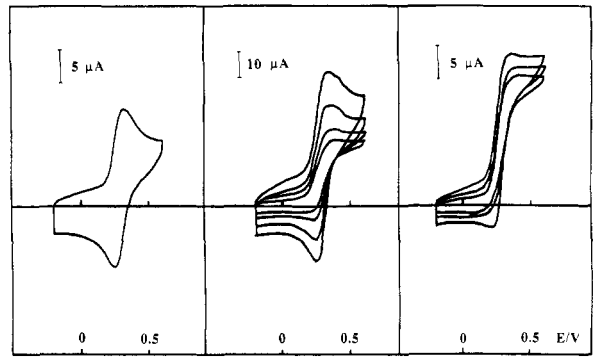

Figure 2. Catalytic activity of GOD embedded in a polymeric silica gel obtained from TMOS; the mediator was ferrocene methanol at a concentration of 4×10^{-3} mol/L and the GOD concentration was 2.5 g/L (about 1.6×10^{-5} M). The aqueous electrolyte was saturated with ferrocene methanol to avoid leaching of it from the gel. (a) Cyclic voltammogram of the gel before the addition of glucose. (b) and (c) Cyclic voltammograms with 0.1 M glucose added; for curves (b) scan rates were respectively 2, 1, 0.5, and 0.2 V/s and for curves (c) 0.1, 0.05, and 0.02 V/s, respectively, from top to bottom curve in both cases.

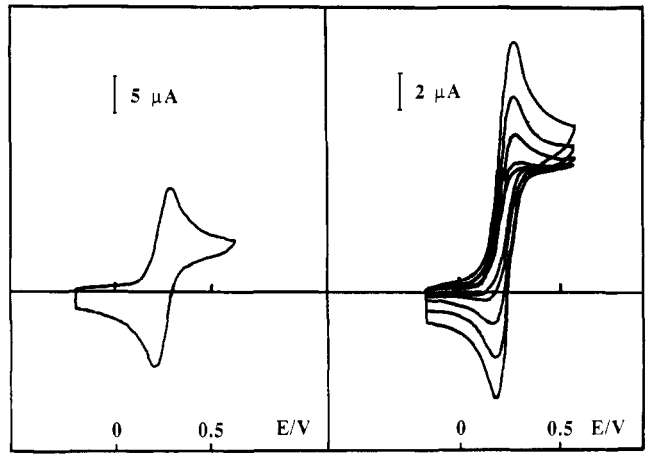

Figure 3. Catalytic activity of GOD embedded in a colloidal silica gel; the silica content was 30% (after neutralization) and the average diameter of the particles was 140 Å. The mediator was ferrocenemethyl trimethyl ammonium chloride at 4×10^{-3} mol/L. GOD concentration was 1.2 g/L (about 0.8×10^{-5} M). For curves (b) the scan rates were respectively 1, 0.5, 0.2, and 0.1 V/s from top to bottom; catalysis was total at scan rates lower than 0.1 V/s.

colloidal silica particles is 140 Å) (Figure 3). With small size silica (particles diameter = 30–40 Å), the catalytic effect is very weak, as a probable consequence of the steric compression of the enzyme. With larger silica (particles diameter = 1000 Å), the size of the channels is bigger than 300 Å and therefore leaching of the enzyme probably slowly occurs which diminishes the catalytic efficiency.

With gel made from silicon tetraacetate, no enzymatic activity has been recorded. Although there is no straightforward explanation for this phenomenon, we suppose that the high quantity of acetate ions provided in the gel by the polymerization reaction generates a high ionic strength which is deleterious to the enzymatic activity. Further experiments to establish this point are currently in progress.

For both polymeric gels obtained via hydrolysis and condensation of TMOS and colloidal gels enzymatic activity has been quantified using the recent theoretical treatment proposed by Moiroux and Savéant.[12] We have been able to estimate the amount of active enzyme in the

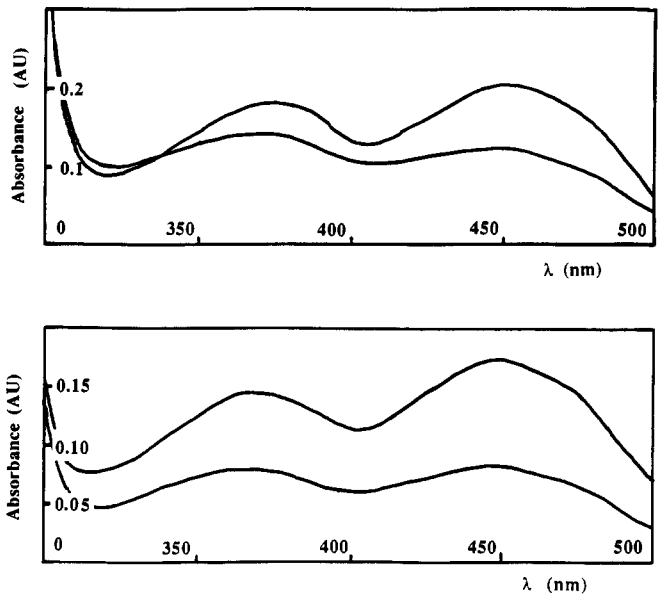

Figure 4. UV spectra of glucose oxidase. (a) GOD solution (0.7 g/L) in distilled water, before (upper curve) and after (lower curve) quenching with glucose. (b) Same feature in a fresh TMOS sol made according to the described procedure; the GOD concentration was 1.6 g/L.

freshly made sol by spectroscopy. Figure 4 represents the spectra recorded at 4 °C from a freshly made polymeric sol, before and after quenching of the enzyme with an excess of glucose (in this case the solvated oxygen is the mediator). The FAD site (the flavine redox center) of the active enzymes is then reduced while the inactive FAD remains oxidized. The percentage of active enzyme can be extracted from the comparison between the curves and therefore this percentage is about the same in the gel than in the sol (the gelation time is 5–10 mn and at 4 °C the enzyme is not deactivated). Figure 4 shows that while about 90% of the commercial GOD is active in water, this proportion falls to about 70–80% in the fresh polymeric silica sol (probably as an effect of the methanol added).

Knowing the exact amount of active GOD and the mediator concentration in the gel, it becomes therefore possible to compare the values of the catalytic current with the theoretical predictions of Moiroux and Savéant: $I_p/I_p^\circ = (\lambda^{1/2}/0.446)\{2/\sigma[1 - (1/\sigma)\ln(1-\sigma)]\}^{1/2}$ where $\lambda = 2k_3C_E^\circ(RT/Fv)$ and $\sigma = (k_3C_P^\circ/k_2)[(1 + (k_{-1} + k_2)/k_1C_G^\circ]$

(12) (a) The diffusion coefficient of methanol in water is about 10^{-5} cm²/s. It has also been shown[12b] that small molecules diffuse almost freely in fresh gels, or gels aged but maintained in the presence of solvents. This means that the average distance effected by a methanol molecule during 1 h is $(3600 \times 10^{-5}\pi)^{1/2}$, i.e., 0.3 cm. Since our electrode is never larger than 1 mm, this means that within a few hours the largest part of the methanol has gone and within a few hours there are only traces left. (b) Audebert, P.; Hapiot, P.; Griesmar, P.; Sanchez, C. *J. Mater. Chem.* **1992**, *2*, 1293.

Table I. Catalytic Effects of GOD Inside Silica Gels

nature of the gel	GOD concn, mg/mL	ferrocene concn, mol/L	I_p/I_p° theor	I_p/I_p° measd
TMOS gel	2.5	4×10^{-3}	2.6	1.8 (at 0.1 V/s)
colloidal silica	1.2	4×10^{-3}	1.9	1.8 (at 0.02 V/s)
silicium acetate	1–2	$(2–5) \times 10^{-3}$		no catalysis

are kinetic dimensionless parameters representative of the catalysis mechanism, and I_p/I_p° represents the ratio of the currents respectively with and without catalysis. C_E°, C_P°, and C_G° are respectively the total concentrations of GOD, ferrocene, glucose, and k_i are kinetic constants with their usual meanings as presented earlier.[13]

The theoretical and experimental values are listed in Table I. A very good agreement with theory is obtained for both experimental cases (colloidal and polymeric gels). Using electrochemical probes, these results show that entrapped enzymes in different sol–gel matrices behave as free enzymes dissolved in an aqueous medium.[14] Such results are in complete agreement with conclusions previously reported[5,6] on the basis of optical measurements.

For polymeric silica gels, the fit appears slightly less accurate than for colloidal silica gels probably because the value of k_3 is slightly overestimated for the former. However values of k_3 between 10^7 and 5×10^6 mol/s (around pH 7) are typical values for GOD in aqueous solutions.

Small differences in k_3 can be observed when the access of the mediator to the enzyme is slightly hindered by the polymeric network. Differences affecting the k_2 value are also possible but have much less influence on the kinetics.

Conclusion

We have shown using electrochemistry of a mediator that the enzymatic activity of glucose oxidase was retained in two types of silica gels. Quantitative results show that a very good agreement with theroetical predictions is obtained, especially in colloidal silica gels where organic solvents are avoided and only water comes into contact with the enzyme. Further work is in progress to explore the details of the GOD kinetics in the gels as well as to attempt to fabricate efficient gel based electrochemical sensors. Moreover the electrochemical probing of GOD activity does not require transparent matrices, consequently this work can be extended to a variety of other transition metal oxide based sol-gel matrices.

Acknowledgment. The authors wish to express their warm thanks to Professor J. Moiroux (Laboratoire d'Electrochimie Moléculaire Universite Paris 7) for many fruitful discussions and advice.

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