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Influence of calcium on glucose biosensor response and on hydrogen peroxide detection

Nathalie Labat-Allietta & Daniel R. Thévenot

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Abstract: Of small species capable of reaching a platinum working electrode from biological samples, calcium cations have been found to inhibit significantly glucose biosensor responses. The sensitivities to glucose of sensors immersed in carbonate buffer saline solutions decreased when 0.5 mM calcium chloride was added. The degree of inhibition was proportional to the glucose response in the absence of calcium (0-17% of the normalized current). Likewise, sensor sensitivities to hydrogen peroxide decreased, in the 5-90% range, in the presence of 0.5 mM calcium.

Bare Pt-Ir wires show a reversible inhibition of hydrogen peroxide sensitivity. This reversible inhibition is directly related to the decrease of hydrogen peroxide oxidation rate at the platinum anode: this has been evidenced, using rotating disk electrodes, by plotting Koutecky-Levich plots. Such inhibition has been found both for free and chelated calcium cations at levels below 1 mM.

Several hypotheses for possible reactions between platinum, hydrogen peroxide and calcium are discussed. © 1997 Elsevier Science Limited. All rights reserved.

Keywords: glucose sensor, calcium, hydrogen peroxide, Koutecky-Levich plots, platinum

INTRODUCTION

Among the numerous applications of biosensors, the development of an implantable sensor for glucose has been one of the most challenging and most studied problems (Wilson et al., 1991). Most glucose biosensors have a reliable in vitro response but show some problems when implanted. They generally demonstrate two effects of this implantation: an important reduction (50-80%) of their sensitivity to glucose; and an important increase (2-4 h) of the time necessary for obtaining a steady-state response, i.e. the run-in time.

As stated by a recent European Survey conducted by Pickup & Thévenot (1993), these modifications have been observed, to differing degrees, by several research groups having realized in vivo evaluations of subcutaneously implantable glucose sensors (Fischer et al., 1987; Pickup et al., 1989; Mascini & Selleri, 1989; Koudelka et al., 1991). Many authors attempted to explain these observations by a modification of surrounding tissue properties (Fischer et al., 1987), the outer membrane fouling by protein adsorption (Shichiri et
al., 1982; Hanning et al., 1986) or even by the inhibition of the biological part of biosensors. But few of them have shown interest in the electrochemical transducer and the detection of the enzyme reaction product, here hydrogen peroxide. In this study, we demonstrate that, in the case of a glucose biosensor, the electrochemical transducer may be incriminated. As do Lobel & Rishpon (1981) and Kerner et al. (1993), we think that modification of H₂O₂ detection could be explained only by the action of small molecules able to reach the platinum electrode. We show here that, among biological ionic constituents, calcium may interfere with the anodic oxidation rate of hydrogen peroxide.

EXPERIMENTAL

Glucose sensor

Glucose sensor and sensing layer fabrication have been described previously (Bindra et al., 1991). The sensing part is situated 3–4 mm from the tip and presents a cylindrical cavity of 1–2 mm in length (Fig. 1). The working cathode is a 0.17 mm diameter Pt-Ir wire (Medwire, Mount Vernon, NY). The sensing cavity is covered with three layers of cellulose acetate (CA), a layer of glucose oxidase (EC 1.1.3.4) (Aspergillus niger, Boehringer, grade II, 230 U/mg) reticulated with glutaraldehyde (GA) and bovine serum albumin (BSA) and, finally, two layers of polyurethane (PU type SG 85A) (Thermedics, Woburn, MA). To prevent glucose sensor responses from acetaminophen interference, supplementary Nation layers are deposited together with CA ones (Zhang et al., 1994). A silver–silver chloride auxiliary reference electrode (100 μm outer diameter; Aldrich) is wrapped around the Teflon-coated Pt–Ir wire. The overall diameter of the sensor is 0.25 mm for the sensing part and 0.45 mm for the reference electrode part.

Sensor calibration

The sensor was dipped into a 37°C thermostatted cell containing 5 ml of stirred 0·027 M carbonate, 0·15 M NaCl, pH 7·4 buffer saline solution (CBS). A 650 mV potential difference was applied between the working and reference electrodes of the sensor, for hydrogen peroxide detection. Amperometry was performed by using a Tacussel (Villeurbanne, France) Model PRGE or PRG-DEL detector. Current–time curves were recorded on a Lineis (Selb, Germany) Model L6514 4 channel strip-chart recorder. After stabilization of the background current (30 min in CBS), standard additions of glucose were performed in order to vary the glucose concentration in the 3–15 mM range.

Calcium dependence of glucose sensor response

All these assays were performed in air saturated CBS, using glucose sensors whose sensitivity to glucose ranged between 1·5 and 30 nA/mM (Table 1).

Sensor responses to glucose or hydrogen peroxide standard additions were determined in two-step experiments (Fig. 2). After stabilization of the background current in CBS, either 3 mM glucose or 20 μM hydrogen peroxide was added and corresponding steady-state responses determined. Then 0·5–2 mM CaCl₂ was added to the
TABLE 1 Analytical parameters of glucose sensors and Pt–Ir anodes used in this study

<table>
<thead>
<tr>
<th>Type of electrode</th>
<th>Analytical parameter</th>
<th>Unit</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose sensors</td>
<td>Sensitivity to glucose</td>
<td>(nA mM⁻¹ mm⁻²)</td>
<td>1–16</td>
</tr>
<tr>
<td></td>
<td>Maximum glucose for 90% linearity</td>
<td>(mM)</td>
<td>3–12</td>
</tr>
<tr>
<td></td>
<td>90% response time to glucose</td>
<td>(s)</td>
<td>60–180</td>
</tr>
<tr>
<td></td>
<td>Sensitivity to H₂O₂</td>
<td>(mA mM⁻¹ mm⁻²)</td>
<td>0.06–0.25</td>
</tr>
<tr>
<td></td>
<td>90% response time to H₂O₂</td>
<td>(s)</td>
<td>30–50</td>
</tr>
<tr>
<td>Bare Pt–Ir wires</td>
<td>Sensitivity to H₂O₂</td>
<td>(mA mM⁻¹ mm⁻²)</td>
<td>2.5–6.7</td>
</tr>
<tr>
<td>Polished rotating Pt disk</td>
<td>Sensitivity to H₂O₂ (extrapolated at infinite r.p.m.)</td>
<td>(mA mM⁻¹ mm⁻²)</td>
<td>3.5–6.2</td>
</tr>
<tr>
<td>Unpolished rotating Pt disk</td>
<td>Sensitivity to H₂O₂ (extrapolated at infinite r.p.m.)</td>
<td>(mA mM⁻¹ mm⁻²)</td>
<td>1.1–2.1</td>
</tr>
</tbody>
</table>

![Graph showing relative glucose sensor responses to successive additions of glucose and calcium.](image_url)

**Fig. 2.** Relative glucose sensor responses to successive additions of either (—) 3 mM glucose or (○) 20 μM H₂O₂ and then 0.5 mM CaCl₂ in CBS solution. Currents have been corrected for background signal I₀ and normalized, i.e. divided by I_max − I₀ in order to show calcium dependence of both glucose and hydrogen peroxide responses.

previous solution and sensor response recorded until a new steady-state response was attained: calcium addition usually yielded a slow but significant decrease of the current. The sensor was rinsed thoroughly in CBS solutions before a further experiment with a different calcium concentration.

**Calcium dependence of bare Pt–Ir wires’ response to H₂O₂**

Pt–Ir wires responses to H₂O₂ standard additions were determined in CBS solutions. After stabilization of the background current, three additions of 2 μM H₂O₂ or one addition of 20 μM H₂O₂ (final concentration) were performed and corresponding steady-state responses determined. Then H₂O₂ responses were determined in CBS solutions containing 0.5–2.5 mM calcium, each calibration curve being obtained at a constant calcium concentration. The sensors were rinsed thoroughly in CBS solutions before a further assay using a different calcium concentration. These responses were compared to the original ones obtained in the absence of calcium. H₂O₂ concentrations were chosen for obtaining response values close to the 5 mM glucose ones for glucose sensors, i.e. in the 2–20 μM range. Pt–Ir wires used for this study present H₂O₂ sensitivities ranging between 2.5 and 6.7 mA mM⁻¹ mm⁻² (Table 1).

**Calcium dependence of rotating Pt disk response to H₂O₂**

The rotating disk electrode (RDE) with a 2 mm diameter Pt rod and the motor controller (EDI and Controvit Radiometer-Tacussel, Villeurbanne, France) were associated to a potentiostat (PRGDEL, Radiometer-Tacussel) except for voltamperometry, which was performed on a PRGE model. In some experiments, the RDE was first polished with 5 μm alumina paper (Radiometer-Tacussel) at 600 r.p.m. for 1 min and rinsed with distilled water. Then the RDE was polished again with 0.3 μm diamond paper under the same conditions, followed by a thorough rinsing with deionized water. All these RDE experiments were performed at 25°C using a Ag/AgCl/sat. KCl reference electrode (Radiometer-Tacussel AgCl10) in CBS after stabilization of background current. Two or 20 μM hydrogen peroxide and
0.5 mM calcium chloride spikes were added to the vessel, before scanning the disk rotation rate in the 100–5000 r.p.m. range in six successive steps.

**Chelatant effect on calcium inhibition of rotating Pt disk response to H$_2$O$_2$**

Experiments were performed at 25°C in CBS either on glucose sensors or on polished RDEs at 1250 r.p.m. disk rotation rate. After stabilization of background current, 20 μM hydrogen peroxide spikes were added to the cell and the corresponding steady state determined. Then addition of different chelating agents was performed and the new steady-state response recorded. The chelating solutions tested were: 0.5–1 mM lactic acid, 0.2–0.4 mM β hydroxybutyric acid, 0.03–0.06 mM pyruvic acid, 0.2–0.4 mM α ketoglutaric acid, 0.25–0.75 mM EDTA, 0.25–3 mM citric acid and 0.25–0.75 mM oxalic acid. Same experiments were performed in the presence of 0.5 mM calcium.

**RESULTS AND DISCUSSION**

**Glucose sensor response in the presence of calcium**

Among ionic constituents of biological fluids, i.e. human blood serum or interstitial liquid, which are not taken into account in our PBS and CBS solutions, calcium is clearly the most significant. Its total concentration in blood plasma or extracellular fluid is normally 2.50 mM. Within this amount, a large fraction is bound to proteins (1.0 mM), i.e. albumin (0.7 mM) and globulins (Siggaard-Andersen et al., 1983a). Another fraction, i.e. 0.25 mM, is bound to anions such as bicarbonate, lactate or citrate. The rest, i.e. 1.25 mM, is the so-called ‘ionized calcium’ which itself contains 0.38 mM ‘active calcium’. The ionized calcium level has been assessed as a function of total calcium, total protein and pH (Siggaard-Andersen et al., 1983b).

In order to avoid possible precipitation of calcium carbonate or hydroxide in CBS containing calcium levels that were too high, we performed multi-equilibrium calculations taking into account H$^+$, OH$^-$, Na$^+$, Ca$^{2+}$, Cl$^-$, H$_2$CO$_3$, HCO$_3^-$, CO$_3^{2-}$, CaOH$^+$, CaHCO$_3^+$, CaCO$_3$aq, CaCO$_3$solid, Ca(OH)$_2$aq, Ca(OH)$_2$solid and CO$_2$gas as possible species and their stability constants (Charlot, 1969; Kotrly & Sucha, 1985; Sigg et al., 1992). We used a software, named CINEQ, developed by Jean-Marie Mouchel (CERGRENE, ENPC, Marne la Vallée, France) for multi-equilibrium and kinetics calculation. If no exchange between the solution and the atmosphere is taken into account, i.e. if total carbonate concentration is maintained at 27 mM, solid calcium carbonate is absent, as well as calcium hydroxide, as long as total calcium content is, at pH 7.4, below 0.8 mM. If carbon dioxide is in equilibrium with the atmosphere and partially expelled, which is likely to occur after a few hours stirring CBS in open vessels, then total carbonate concentration decreases below 27 mM and, consequently, maximum level of calcium without solid formation increases, reaching 1 mM. Taking into account these data, we have generally studied glucose sensor operating behaviour in CBS containing 0.5 mM calcium chloride.

In order to visualize possible deposits of CaCO$_3$ precipitate on the electrode surface, microscopic observations were realized. Three Pt–Ir wires were observed under optical microscopy (magnification 100) in CBS, spiked with 0.5–2.5 mM calcium (final concentration). After 90 min of immersion of these wires into the solution, there was no visible CaCO$_3$ precipitate for 0.5 mM total calcium concentration. On the contrary, crystals of a few micrometres length were observed after 90 min immersion in a 2.5 mM calcium CBS solution: an electrode covering apparently lower than 10% was then evaluated.

Thus, both calculations and observations demonstrate that, within 27 mM CBS at pH 7.4, no calcium carbonate precipitates below at least 0.8 mM total calcium concentration.

After initial stabilization of background current in CBS and calibration with 3 mM glucose or 0.02 mM H$_2$O$_2$, 0.5 mM calcium was added to the solution. An 18 and 30% decrease of the current was observed, respectively, for glucose and H$_2$O$_2$, reaching a new steady state after 15–30 min ($t_{90\%}$) (Fig. 2). These sensors recovered their initial sensitivity after 15–30 min washing with calcium-free CBS solutions. Such calcium inhibition of sensor responses to glucose and hydrogen peroxide was observed both for freshly prepared sensors and for those used in calcium-containing solutions and then rinsed in CBS solutions.
When comparing calcium effect on several sensors, a linear correlation was observed between the residual glucose sensitivity and the initial sensor glucose sensitivity in the absence of calcium ($r^2 = 0.84, n = 11$) (Fig. 3(A)). Thus, the less sensitive sensors (1–3 nA mM$^{-1}$ mm$^{-2}$) were almost unaffected by the presence of 0.5 mM Ca, whereas more sensitive ones presented a 5–17% inhibition in the presence of 0.5 mM Ca. Although responses to hydrogen peroxide are much less reproducible than glucose ones, since Pt anodes of such sensors cannot be polished mechanically, a linear correlation was also observed for H$_2$O$_2$ sensor response ($r^2 = 0.67, n = 9$) (Fig. 3(B)).

Calcium concentration effect on glucose and H$_2$O$_2$ response was evaluated in the 0–5–2 mM calcium range by plotting glucose and H$_2$O$_2$ calibration curves in CBS solutions spiked with calcium: a linear correlation was observed between the relative sensitivity and the calcium concentration in CBS (Fig. 4). This batch of sensors being in the lower sensitivity range of those described in Fig. 3, they present a 0.5 mM Ca inhibition for glucose and H$_2$O$_2$ in the 2–5 and 7–17% ranges, respectively.

Although the effect of calcium on glucose and H$_2$O$_2$ responses presents some qualitative differences, Figs. 2–4 seem to indicate that the calcium inhibition mechanism should be common both to glucose and to hydrogen peroxide responses and increase when sensors have higher sensitivity to both analytes. When considering all possible rate-limiting steps, glucose response depends upon:
1. oxygen transport through the PU outer layer;
2. glucose transport through the PU outer layer;
3. glucose oxidation catalysed by glucose oxidase;
4. hydrogen peroxide transport through...
CA, BSA-GA and Nafion inner layers; and (5) anodic detection of hydrogen peroxide.

Hydrogen peroxide response is controlled only by hydrogen peroxide transport through inner and outer layers and its anodic oxidation on Pt. Thus, any effect of calcium on oxygen transport and glucose oxidase activity has to be ruled out. Similarly, it seems very unlikely that calcium would act in the same manner on glucose and hydrogen peroxide transport through the outer PU layer: such a mechanism may indeed be different, i.e. micro porosity and homogenous permeability. Comparison of glucose and hydrogen peroxide responses before and after PU layer deposition has shown very different permeabilities for these two species (Sternberg et al., 1988): whereas sensitivity to glucose is decreased by a factor of about 30 when PU is deposited, sensitivity to hydrogen peroxide is only slightly reduced. Thus, it appears that calcium is probably inducing an inhibition of the common step to glucose and hydrogen peroxide responses, namely anodic detection of the latter.

H$_2$O$_2$ responses of bare Pt-Ir wires

In order to detect the effect of calcium on hydrogen peroxide detection, bare Pt-Ir wires were prepared. The H$_2$O$_2$ response of 11 bare Pt-Ir wires was evaluated in CBS in the presence of calcium within the range 0.5-2.5 mM: a 30-50% decrease of the sensitivity was observed as compared to the original value $I_i$ in calcium-free CBS (Fig. 5(A)). A positive correlation between the sensitivity decrease and the calcium concentrations in CBS was observed but calcium was found to yield a non-proportional inhibition of hydrogen peroxide detection and, apparently, to reach a maximum value for high calcium concentrations. In order to evaluate such maximum inhibition, inverse plots were drawn (Fig. 5(B)), giving, by linear regression, the following equation:

$$\frac{I_i}{I(1/\%)} = 0.019 - 0.0027/[Ca_{ion}] \text{ (mM}^{-1})$$

($n = 53, r^2 = 0.41$)

Thus, a maximum decrease of hydrogen peroxide sensitivity on bare Pt-Ir wires was evaluated as 47%. The half-maximum inhibition was obtained for about 0.3 mM calcium in CBS: in such solution, calcium concentration was clearly below values necessary for carbonate or calcium hydroxide deposition. These sensors recovered their initial sensitivity after washing with calcium-free CBS solutions. The degree of inhibition of hydrogen peroxide responses of such bare Pt-Ir wires, in the presence of 0.5 mM Ca, i.e. 28 ± 8% (Fig. 5(A)), was in the same range as for glucose sensors, i.e. 50 ± 30% (Fig. 3(B)) or 12 ± 5% (Fig. 4). Apparently the inner and outer layers covering the Pt-Ir surface do not affect the calcium inhibition mechanism.

H$_2$O$_2$ response of rotating Pt disk

In order to differentiate any deposit invisible with optical microscopy from a reversible inhibition of the anodic oxidation of H$_2$O$_2$, we have performed
experimental measurements of kinetic oxidation current $I_k$ and of the kinetic oxidation constant $k_f$ on a RDE.

All measurements were realized at a constant applied potential of 650 mV against a Ag/AgCl reference electrode. Voltamperometric studies have shown that this potential is indeed appropriate for H$_2$O$_2$ oxidation on rotating Pt electrodes: under our experimental conditions, i.e. 2 mV/min, this plateau potential was found to be independent of the rotation speed (64–5000 r.p.m.) and not significantly modified by an increase of the hydrogen peroxide concentration in the 2–20 μM range, or by the presence of 0.5 mM calcium (Fig. 6). Finally, 650 mV against Ag/AgCl was found, under all our experimental conditions, to be situated on the plateau of the H$_2$O$_2$ anodic wave.

The general equation (1) for a slow electrode reaction contains both kinetic and mass transfer components (Bard & Faulkner, 1980):

$$I = nF_Ak_fC^*/(1 + k_f/m)$$  (1)

where $k_f$ is the heterogeneous electron transfer rate constant and $m$ is the mass transport coefficient. These two terms can be separated using reverse plots:

$$1/I = 1/I_k + 1/I_{le}$$  (2)

where $I_k$ is the kinetic current, i.e. the current at infinite rotation rate in the absence of any mass transfer limitation, and $I_{le}$ is the current under limiting current conditions. When using a RDE, $I_{le}$ is defined by the Levich equation:

$$I_{le} = 0.620nFAD_0^{2/3}ω^{1/2}ν^{-1/6}C^*$$  (3)

where $A$ is the electrode active area, $D_0$ the analyte diffusion coefficient, $ν$ the cinematic viscosity, $ω$ the disk electrode rotation speed and $C^*$ the analyte bulk concentration. As kinetic current $I_k$ in Eq. (2) is proportional to $k_f$:

$$I_k = nF_Ak_fC^*$$  (4)

Eq. (2) becomes:

$$1/I = 1/(nF_Ak_fC^*) + 1/(0.620nFAD_0^{2/3}ω^{1/2}ν^{-1/6}C^*)$$  (5)

Thus, $ω^{1/2}C^*$ is a constant only when $I_k$ or $k_f$ are large, i.e. when electrode reaction is rapid. When this is not the case a plot of $I$ versus $ω^{1/2}$ (Levich plot) will be curved and reach a maximum $I_k$. A plot of $1/I$ versus $1/ω^{1/2}$ (Koutecky–Levich, KL, plot) should be linear and extrapolated at infinite rotation rate to yield $1/I_k$. Furthermore, the slope of a KL plot, i.e. $1/(0-620nFAD_0^{2/3}ν^{-1/6}C^*)$, is directly related to the reciprocal of the electrode area $A$ and should be modified by its variation, for example by crystal deposits.

Levich plots, i.e. $(I - I_0)$ versus $ω^{1/2}$, were found not to be linear, except at low rotation rate. They show a horizontal asymptote for highest rotation rates. This demonstrates the limiting role played by electrochemical kinetics during the establishment of the steady-state response of the glucose sensor.

As for experiments realized on Pt-Ir wires, addition of 0.5 mM calcium chloride yielded a significant decrease of H$_2$O$_2$ kinetic oxidation current. KL plots, i.e. $1/(I - I_0)$ versus $ω^{-1/2}$ where, for each rotation rate, steady-state current $I$ was corrected from its background value $I_0$ in the absence of hydrogen peroxide, were found to be linear with identical slopes to those observed in the absence of calcium (Fig. 7 and Table 2). This demonstrates that the Pt disk active area $A$ is not significantly modified in the presence of 0.5 mM calcium. Furthermore, both kinetic oxidation current $I_k$ at infinite rotation speed and kinetic oxidation constant $k_f$ decrease reversibly by ca 30–45% when 0.5 mM calcium is added to CBS. These results were observed on both unpolished and polished platinum disk and within 2 or 20 μM hydrogen peroxide solutions: the main effects of electrode polishing were the increase of $k_f$ and the increase of the inhibition effect of 0.5 mM Ca, which reached 30–35 and
Chelant effect on calcium inhibition

In biological fluids, calcium is present both under ionized and complexed forms, so it seems interesting to study the effect of different chelating agents on the calcium inhibition of H$_2$O$_2$ response. Under physiological conditions, organic acids, i.e. pyruvate, lactate, α-ketoglutarate and β-hydroxybutyrate, show small affinity to calcium and have no effect on the inhibition of H$_2$O$_2$ oxidation by this cation.

Chelating agents which present higher affinity to calcium, i.e. oxalic and citric acids and EDTA, have also been studied. They have been chosen for the calcium dissociation constant values of CaC$_2$O$_4$, CaHCit$^-$ and CaEDTA$^{2-}$, i.e. 10$^{-3.0}$, 10$^{-4.8}$ and 10$^{-7.8}$, respectively (apparent constants at pH 7.4). In the absence of calcium, 0-25 and 0-5 mM oxalic and citric acid yielded only a small modification of H$_2$O$_2$ response at a polished rotating Pt disk. On the other hand, EDTA plays an important promoting role on H$_2$O$_2$ oxidation: 0-25 mM EDTA addition gives a 65% increase of 20 μM H$_2$O$_2$ response (Fig. 8). In the presence of 0-5 mM calcium, 0-25-0-75 mM oxalic and citric acids still have no effect on H$_2$O$_2$ response and do not permit one to regain the original sensitivity to H$_2$O$_2$. On the contrary, addition of EDTA enables one to outmatch the original sensitivity obtained in the absence of calcium.

No direct oxidation of EDTA was observed within the 0.2-1.0 mM range, using 50 mV/s cyclic voltammetry in CBS.

KL plots, i.e. $1/(I - I_0)$ versus $1/ω^{1/2}$ on a polished rotating Pt disk in the presence of 1 mM EDTA, confirm the promoting effect of this chelating species (Fig. 9). Extrapolation to infinite rotation rate shows that, in the absence as well

35-45% on unpolished and polished Pt RDE, respectively. Such inhibition of hydrogen peroxide oxidation on a Pt RDE, by the addition of 0.5 mM Ca$^{2+}$, is very similar, both in amplitude and reversibility, to what was observed on bare Pt-Ir wires (Fig. 5(A)). On the other hand, organic layers, such as those covering the Pt-Ir surface in these glucose sensors, do not appear to decrease the amplitude of the 0.5 mM Ca$^{2+}$ inhibition, which is in the 10-90% range (Fig. 3(B)). Finally, the complexity of the H$_2$O$_2$ oxidation mechanism on Pt is further evidenced by the H$_2$O$_2$ concentration dependence of $k_f$: the measured kinetic constant ranges 1.85 and 3.3 × 10$^{-3}$ cm/s in 2 and 20 μM H$_2$O$_2$, respectively (Table 2).

### Table 2: Effects of 0-5 mM calcium addition to CBS on hydrogen peroxide kinetic parameters, as determined on KL plots similar to Fig. 6: mean obtained for three to six experiments, each using six rotation rate values in the 100-5000 r.p.m. range

<table>
<thead>
<tr>
<th>RDE</th>
<th>H$_2$O$_2$ (μM)</th>
<th>EDTA (mM)</th>
<th>$I/A$ (nA/mm$^2$)</th>
<th>$k_f$ (10$^{-3}$ cm/s)</th>
<th>KL slope (nA cm$^{-1}$ rad$^{1/2}$ s$^{1/2}$)</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polished</td>
<td>2</td>
<td>0</td>
<td>7.0</td>
<td>4.3</td>
<td>1.85</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0</td>
<td>124</td>
<td>69</td>
<td>3.3</td>
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<tr>
<td></td>
<td>20</td>
<td>1</td>
<td>229</td>
<td>205</td>
<td>6</td>
<td>5.4</td>
</tr>
<tr>
<td>Not polished</td>
<td>2</td>
<td>0</td>
<td>3.0</td>
<td>2.1</td>
<td>0.80</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0</td>
<td>33</td>
<td>21</td>
<td>0.85</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Fig. 7. Koutecky-Levich curves in the 64-5000 r.p.m. range on polished Pt RDE: 20 μM H$_2$O$_2$ oxidation at 650 mV/AgCl in CBS at 25°C containing either (○) 0 or (●) 0.5 mM calcium. Each steady-state current $I$ was corrected from its background values $I_0$ at each rotation rate.

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Fig. 8. ( ■) EDTA, ( ●) citrate and ( ×) oxalate concentration effect on polished Pt disk steady-state response (I_{lim} - I_0) to 20 μM H_2O_2 in CBS solution containing either (——) 0 or (-----) 0.5 mM calcium, at 650 mV/AgCl and 1250 r.p.m. rotation rate.

![Graph](image)

Fig. 9. Koutecky-Levich curves in the 64–5000 r.p.m. range on polished Pt RDE: 20 μM H_2O_2 oxidation at 650 mV/AgCl in CBS at 25°C containing either (□) 0 or (■) 1 mM EDTA and (——) 0 or 0.5 mM (-----) calcium chloride. Each steady-state current I was corrected from its background values I_0 at each rotation rate.

![Graph](image)

CONCLUSION

Within small species capable of reaching glucose biosensor platinum working electrode from biological samples, we have shown that calcium inhibits significantly glucose responses. Calcium (0.5 mM) addition inhibits relatively slowly (t_{50%} = 15 to 30 min) biosensor responses to both glucose and hydrogen peroxide (0–45 and 30–90%, respectively). Such inhibition is reversible, i.e. disappears after 15–30 min washing in CBS solutions, and appears to be correlated to sensor sensitivities. It is limited to 0–7 and 10–20% for glucose and hydrogen peroxide, respectively, when sensors are prepared and selected for a limited sensitivity, as it is often necessary for in vivo implantation. Calcium acts similarly on both enzymatic-electrochemical (glucose) and electrochemical (hydrogen peroxide) responses. Taking into account the very different PU permeabilities to glucose and hydrogen peroxide, it seems that calcium is interfering with the anodic oxidation of hydrogen peroxide. Results obtained on bare Pt-Ir wires indicate that the interaction with calcium is reversible and presents an amplitude within the range observed on glucose sensors. On the contrary, this inhibition is not proportional to the total calcium concentration: it reaches a 47% maximum value for calcium concentration higher than 3 mM in CBS, i.e. in over-saturated calcium solutions, in which the Pt surface is likely to be partially covered by carbonate crystals. Experiments performed on rotating platinum disks clearly show that under-saturated calcium solutions decrease the H_2O_2 kinetic oxidation rate but do not affect the active electrode area. Multi-equilibrium calculations, optical microscopy observations and KL plots on rotating platinum disks indeed rule out the occurrence of calcium carbonate deposits in 0.5 mM calcium CBS solutions at pH 7.4.

Calcium complexation by different chelating agents, such as oxalate and citrate, indicates that, even when complexed, calcium inhibits electrochemical H_2O_2 oxidation. However, this inhibiting effect may be partly or completely compensated by a promoting effect of EDTA.

Several hypotheses for possible reactions as in the presence of 0.5 mM calcium, addition of 1 mM EDTA increases by a factor of approximately 2 the H_2O_2 oxidation rate k_f (Table 2).

The comparison between the magnitude of the 1 mM EDTA-promoting effect and 0.5 mM Ca^{2+} or the CaL^n-inhibiting effect on H_2O_2 oxidation shows we have two antagonist mechanisms: one decreasing H_2O_2 kinetic oxidation constant k_f by Ca^{2+} or CaL^n when L is oxalate or citrate and another one increasing k_f in the presence of EDTA.
between platinum, hydrogen peroxide and calcium can be proposed:

(1) A catalytic dismutation of $\text{H}_2\text{O}_2$, thermodynamically unstable, is unlikely. Measurements of $\text{H}_2\text{O}_2$ kinetic oxidation constant on Pt rotating disks are reproducible within several hours: this rules out $\text{H}_2\text{O}_2$ decrease even at low concentration levels such as 2 $\mu$M.

(2) Lowry et al. (1994) described a homogenous reaction between ascorbic acid and hydrogen peroxide catalysed by metallic ions traces, thus confirming the hypotheses of Hand & Greisen (1942), Timberlake (1960) and De Chatelet et al. (1972). In our experiments such homogenous chemical reaction of hydrogen peroxide could be considered only if this reaction was fast and not complete. Furthermore, we have observed the calcium inhibition of hydrogen peroxide response even in the absence of ascorbate.

(3) A heterogeneous chemical reaction between hydrogen peroxide and a solid at the platinum surface may occur. Electrochemical oxidation of hydrogen peroxide has been studied by Zhang & Wilson (1993) on similar Pt disk and Pt-Ir wires, within phosphate buffer saline solution either at pH equal to 7.4 or fixed in the 4–13 range by additions of HCl or NaOH. It has been found that the oxidation rate constant $k_r$ of 0.1–3 mM hydrogen peroxide solutions is in the 5–7$\times$10$^{-6}$ cm/s range, much lower than the 2–3$\times$10$^{-3}$ cm/s values observed in this work (Table 2). The rate-limiting step has been estimated by Zhang & Wilson (1993) to be either an electron-transfer reaction or preceding chemical reaction that would occur between hydrogen peroxide and the Pt(PtO) surface. At the +650 mV/Ag–AgCl applied potential and at pH 7.4, the electrode is partially covered by platinum oxides such as PtO, PtO$_2$ and Pt(OH)$_2$. The amount of such species on the Pt surface may depend upon experimental conditions and influence $k_r$.

(4) Pacault (1958) describes the formation of calcium peroxides between calcium hydroxide and $\text{H}_2\text{O}_2$ but no kinetic or thermodynamic data are given on such a reaction.

In conclusion, if there is a chemical reaction of $\text{H}_2\text{O}_2$ with calcium it should be relatively slow, i.e. reach an equilibrium within 15–30 min, and be not total even in the presence of a large excess of calcium compared to hydrogen peroxide, i.e. 3 mM versus 2 $\mu$M.

This work demonstrates the importance of biosensor detection, especially the electrochemical one, in the understanding and control of biosensor inhibition mechanisms. This work does not prove that free or complexed calcium is the only factor responsible for biosensor inhibition in biological fluids. In fact, the amplitude of the observed inhibition of glucose response in the presence of 0.5 mM Ca, i.e. 0–7%, is significantly lower than the inhibition observed when similar sensors are implanted subcutaneously or placed in blood serum, i.e. 50–80%. It nevertheless shows that calcium is likely to contribute to such in vivo inhibition: as the metal surface state and covering layers may modify such a calcium effect, we recommend assessing its value for each type of glucose sensor developed for either in vivo or in vitro measurements if the latter are performed in calcium-containing samples.

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