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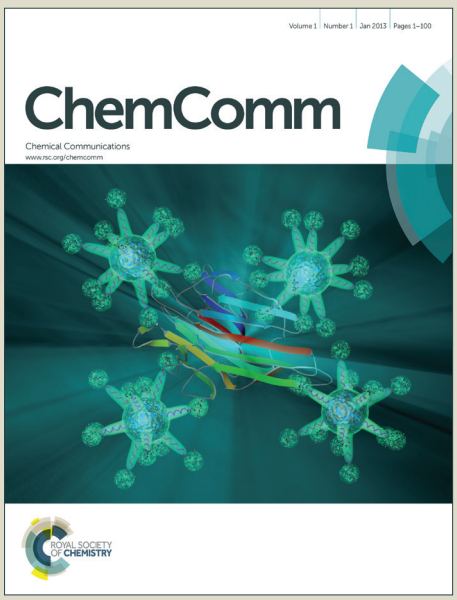

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ARTICLE TYPE

Switching an O_2 sensitive glucose oxidase bioelectrode into an almost insensitive one by cofactor redesign

Emilie Tremey [a,b], Emmanuel Suraniti [a,b], Olivier Courjean [a,b], Sébastien Gounel [a,b], Claire Stines-Chaumeil [a,b], Frédéric Louerat [a,b] and Nicolas Mano *[a,b]

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In the 5-8 mM glucose concentration range, of particular interest for diabetes management, glucose oxidase bioelectrodes are O_2 dependent. Thereby decreasing their efficiencies. By replacing the natural cofactor of glucose oxidase, we succeeded in turning an O_2 sensitive bioelectrode into an almost insensitive.

Glucose oxidase (GOx) is one of the most frequently used enzymes in enzymatic biofuel cells and glucose biosensors [1-4], due in part to its high stability in physiological conditions (neutral pH, 37°C, in presence of NaCl) and its narrow substrate specificity towards glucose. In numerous electrochemical devices, redox mediators (M) are used to electrically connect the GOx active site ($FAD/FADH_2$) to the electrode. These compounds are able to oxidize the reduced form of GOx and shuttle electrons to the electrode surface. (eq 1)

$$GOx\text{-}FADH_2 + (2/x)\ M_{(OX)} \rightarrow GOx\text{-}FAD + (2/x)\ M_{(RED)} + 2\ H^+ \quad (1)$$

(where x = 1 or 2 is the number of electrons collected by the oxidized redox mediator from the GOx)

However, this process is intrinsically limited by the competition with molecular oxygen, which is the natural substrate for the oxidative half-reaction. (eq 2)[5] In addition, O_2 may also interact with the reduced form of the redox mediator if its redox potential is not chosen properly. [6]

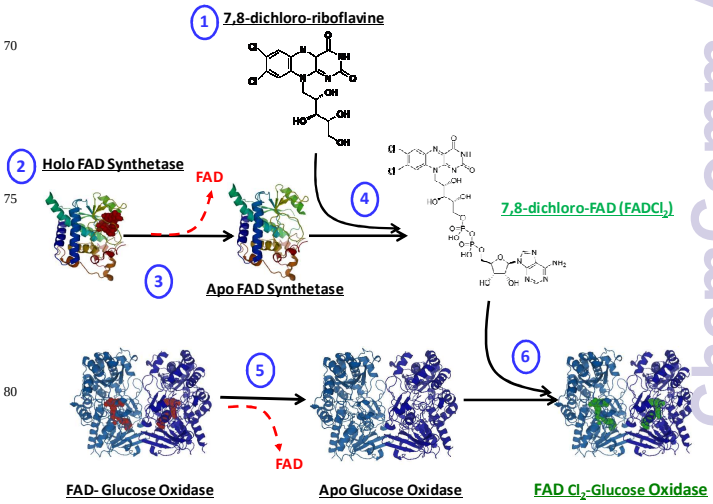
$$GOx\text{-}FADH_2 + O_2 \rightarrow GOx\text{-}FAD + H_2O_2 \quad (2)$$

Minimizing the competitive oxidation by O_2 is a long standing issue of bioelectrochemists and efforts have been essentially directed to the design of new redox mediators or the improvement of enzyme immobilization.[7-11] The use of oxygen-tolerant enzymes such as cellobiose dehydrogenase or FAD-Glucose dehydrogenase is also a promising alternative. [12-14]

In their pioneering work to decipher the electron transfer in GOx from *Aspergillus niger*, Klinman et al [15, 16] showed that the Michaelis–Menten constant, K_M (O_2), was dramatically increased for enzymes containing modified flavins and was attributed to a change in the driving force for outer sphere electron transfer. The rate constants, k_{cat}/K_M (O_2), for native glucose oxidase was $(1.6 \pm 0.2)\times 10^6\ M^{-1}\ s^{-1}$ and $(7.5 \pm 1.6)\times 10^4\ M^{-1}\ s^{-1}$ for a GOx modified with a flavin analogue; 7,8-dichloro-FAD.

Inspired by this work, we elaborated a GOx bioelectrode where the native flavin has been replaced by a flavin analogue and studied electrochemically the effect of such substitution on the oxidation of glucose under O_2 et Ar.

As illustrated in Scheme 1, 6 steps are required to obtain GOx where the native flavin has been replaced with 7,8-dichloro-FAD. All experimental details can be found in supporting information and have been adapted from previous reports to simplify the methodology and improve the yield of production.[17-19] Briefly, we first optimized and reported a two-step protocol for the synthesis of 7,8-dichloro-riboflavin with a 2.5-fold increase in yield compared to previous five-step protocol methods (step 1).[20] After overexpression, production and purification of Holo-FAD synthetase (step 2), apo-FAD synthetase is prepared (step 3) and mixed with 7,8-dichloro-riboflavin in presence of an excess of ATP and $MgCl_2$, leading to the formation of 7,8-dichloro-FAD (step 4). Apo-glucose oxidase (step 5) is finally reconstituted with 7,8-dichloro-FAD (step 6) leading to the formation of $FADCl_2$ with a yield of ~ 49%.



Scheme 1. Preparation of $FADCl_2$ - Glucose Oxidase. **1)** synthesis of 7,8-dichloro-riboflavin; **2)** production/purification of FAD-synthetase; **3)** preparation of apo-FAD synthetase; **4)** preparation of 7,8-dichloro-FAD; **5)** preparation of apo-glucose oxidase; **6)** reconstitution of glucose oxidase with 7,8-dichloro-FAD.

Protein	k_{cat} (sec^{-1})	$K_M(FM_{ox})$ (μM)	K_{Mapp} (FMox) (μM)	$k_{cat}/K_M(FM_{ox})$ (μM^{-1} sec^{-1})	$k_{cat}/K_{Mapp}(FM_{ox})$ (μM^{-1} sec^{-1})	K_M(glucose) (mM)	k_{cat}/K_M (Glucose) (mM^{-1} sec^{-1})
WT-GOx (Ar)	822 ± 9	132 ± 6		6.2 ± 0.3		51 ± 7	16 ± 2
WT-GOx (O_2)	689 ± 20		250 ± 27		2.7 ± 0.3	54 ± 11	12 ± 3
$FADCl_2$-GOx (Ar)	1602 ± 32	118 ± 10		13.6 ± 0.2		37 ± 2	43 ± 3
$FADCl_2$-GOx (O_2)	1505 ± 53		126 ± 38		11.9 ± 0.4	35 ± 3	43 ± 5

Table 1. Kinetics parameters (per active site) of native and $FADCl_2$ - Glucose Oxidase with glucose and ferrocinium-methanol as substrates in presence or absence of O_2

Steady state kinetic parameters of the new GOx-$FADCl_2$ (-0.126 V *vs.* NHE) were evaluated with ferrocinium-methanol (FM_{ox}) under Ar and O_2 and compared to the wild type GOx (Table 1). Contrary to native GOx from *P. amagasakiense*, we did not observe any competitive or uncompetitive inhibition of O_2 in presence of FM_{ox} in our experimental conditions.[21] The reason has yet to be determined. As seen in Table 1, whether under Ar or O_2, GOx-$FADCl_2$ is more active than the native GOx: its k_{cat} ~ 2.2 times higher, K_M(glu) ~ 1,5 times lower and $k$cat/K_M(Glu) is 3.5 higher; indicating a more efficient glucose oxidation compared to native GOx. The ratio $(k_{cat}/K_{Mapp}(FMox))O_2/(k_{cat}/K_M(FMox))Ar$ for native GOx is ~ 0.44 and ~ 0.88 for GOx-$FADCl_2$ clearly showing that GOx-$FADCl_2$ is less sensitive to O_2. The better efficiency is also evidenced by the similar values of K_M(FMox) and K_{Mapp}(FMox) for GOx-$FADCl_2$, indicating that the redox mediator is the main substrate for the oxidative half-reaction instead of O_2.

Modified electrodes at their optima enzyme/redox polymer/cross-linker ratio were prepared as previously reported.[22] The redox polymer is a PVP –[Os(1,1'-dimethyl-2,2'biimidazole)$_2$-2-[6-methylpyrid-yl]imidazole)]$^{2+/3+}$ with a redox potential of -30mV and insensitive to O_2.[23] The ratio was identical for both enzymes and each electrode consisted of 35 wt% enzyme, 55 wt% redox polymer and 10 wt% of PEGDGE for a total dry loading of 200 μg.cm^{-2}.

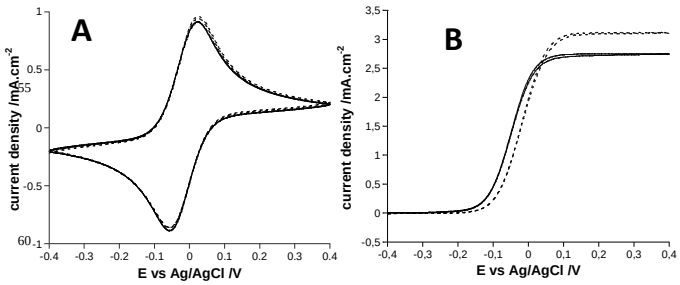


Fig 1. Cyclic voltammograms of modified electrodes with native GOx (thin line) or $FADCl_2$-GOx (dotted line) in absence (A) or presence (B) of 100 mM glucose. 35 wt% enzyme, 55 wt% redox polymer and 10 wt% of PEGDGE for a total dry loading of 200 μg.cm^{-2}. 20 mM phosphate buffer, 140 mM NaCl, pH 7, 37 °C, 1000 rpm and 100 (A) or 5(B) mV.s^{-1}.

Fig 1A shows the resulting cyclic voltammograms under Ar for the native GOx (black line) and GOxFAD-Cl_2 (dotted line) obtained at 100 mV.s^{-1} in a 20 mM sodium phosphate buffer pH 7.2+140 mM NaCl. Those CVs are characteristics of a polymer bound osmium complex with an apparent redox potential of - 30 mV *vs.* Ag/AgCl. Peak heights, peak separation between the oxidation and reduction and the width of the peaks at half-height are identical for both electrodes. It suggests a similar structure/morphology of both hydrogels and a similar apparent diffusion coefficient of electrons.[22] The electrocatalytic oxidation of 100 mM glucose under Ar obtained at 5 mV.s^{-1} is seen in Figure 1B. A current density of 2.5 mA.cm^{-2} is reached for the native GOx (black line) and 3.1 mA.cm^{-2} for the $FADCl_2$ electrode (dotted line). The onset for glucose oxidation is slightly shifted toward more anodic potential because of the difference between the redox potential of both enzymes. It also explains the difference in current densities which are related to some extent to the difference between the redox potential of both enzymes and the redox polymer, as already observed for other systems.[23, 24] To maximize the effect of O_2 at low glucose concentration (<10 mM), we performed our experiments under 1 atm O_2, forced convection and used an hydrogel film of intermediate thickness.[25] Because reproducibility is a key issue in those experiments, we tested two different protocols of measure. In the first one, calibration curves were first recorded under Ar at +0.3 V *vs.* Ag/AgCl with at least two electrodes, which were then washed with buffer and tested under O_2. This protocol was also repeated with other electrodes in the reverse order. In the second protocol, each experiment started in a buffer saturated with Ar and after each addition of glucose the solution was bubbled with O_2 until the current reached a baseline. It was then bubbled again with Ar before the next addition of glucose. This last protocol gave the best results in terms of reproducibility (±10 % *vs.* ±25%) and was therefore used. One example is illustrated in Fig 2 in presence of 0.5 mM glucose (Fig 2A) and 5 mM glucose (Fig 2B) for electrodes modified as in Figure 1.

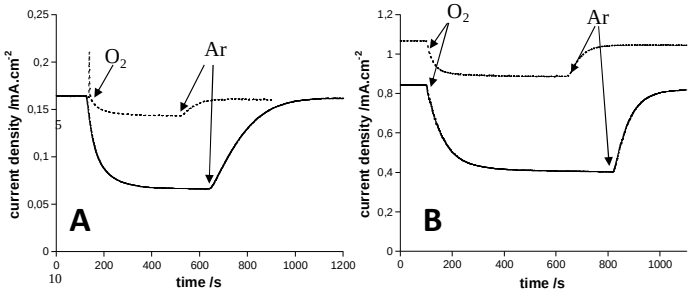


Fig 2. Chronoamperogramms at +0.3 V *vs.* Ag/AgCl of modified electrodes with native GOx (thin line) or $FADCl_2$-GOx (dotted line) in presence of 0.5 mM glucose (A) or 5 mM glucose (B). The arrows show the beginning of the corresponding gas bubbling. Other conditions as in Fig 1

At low glucose concentration, both modified electrodes display the same current density. When O_2 was bubbled, the current density of the electrode modified with the native GOx decreased by 60 % (thin line) but only by 13% for the modified GOx (dotted line). At 5 mM (Fig 2B), the decrease was respectively 52 % and 18% for the native and modified GOx. Resulting calibration curves for native GOx (triangles) and GOx-$FADCl_2$ (circles), under Ar (black symbol) and O_2 (empty symbols), are plotted in Fig 3. For both modified electrodes, the current density increased with the glucose concentration up to 50 mM and displays a typical shape for GOx-based electrodes. [25] Fig 3B shows the normalized data of Fig 3A.

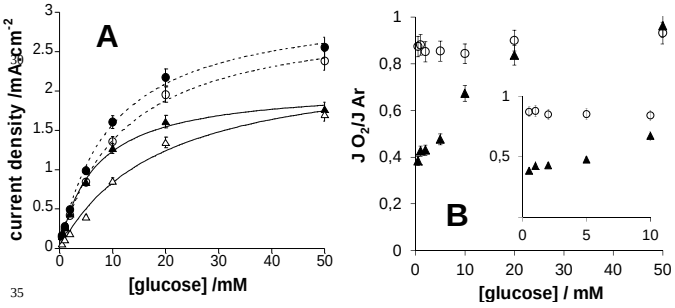


Fig 3. A) Calibration curves of modified electrodes made with native GOx (triangles) or $FADCl_2$-GOx (circles) under O_2 (empty symbols) and Ar (full symbols). Measured at +0.3 V *vs.* Ag/AgCl. B) Dependence of the normalized current density on the glucose concentration for electrodes modified with native GOx and polymer I (triangles) and $FADCl_2$-GOx and polymer I (circles). Inset: Zoom in the 0-10 mm glucose concentration range. Other conditions as in Fig 1

For the lowest glucose concentration (<1 mM), the effect of O_2 is maximal for both electrodes. All the electrons generated by the oxidation of glucose are captured by O_2 either directly from the GOx or from the reduced osmium complex (when an O_2-sensitive mediator is used).[6, 25] It explains the same current observed for both electrodes. For higher glucose concentration (>20 mM), most of the O_2 is consumed in the outer layers of the hydrogel and its impact is therefore limited on the current density. As a result, JO_2/JAr is identical for both electrodes (Figure 3B). In the 0.5-20 mM glucose concentration range, the current density for the GOx-$FADCl_2$ modified electrode (circles) is higher because the enzyme is more active and less sensitive to O_2. Most of the electrons generated during the oxidation of glucose are transferred to the redox mediator and not to O_2. It is also evidenced by the values of the apparent Michaelis–Menten constants (K'_M) which are almost identical under O_2 or Ar, $(K'_M)_{Ar}$ (9.8 mM) and $(K'_M)_{O2}$ (11.8 mM). For the native GOx (triangles), however, K'_M increases upon increasing the partial pressure of O_2 from $(K'_M)_{Ar}$ (6.71 mM) to $(K'_M)_{O2}$ (18.8 mM); because O_2 competes with the redox mediator for electrons. A greater glucose concentration is then required to reach half of the maximal catalytic current. This trend is in agreement with the data of table 1.

Conclusions

For the first time, we report the use of unnatural GOx from *Aspergillus niger* for the electrochemical detection of glucose. By replacing the native FAD by an unnatural flavin, 7,8-dichloro-FAD, we succeeded in switching an O_2 sensitive electrode into an almost insensitive. The novel GOx is not only more active than the native GOx but also less sensitive to O_2. In the 5-8 mM glucose concentration range, of particular interest for diabetes management, an electrode made with a native GOx loses 60 % of its current under O_2, while only 15% with the new electrode.

Large quantities of apo GOx lacking FAD can now be isolated and reconstituted with unnatural flavins with high yields. This strategy may offer a promising alternative to more classical methods such as directed mutations and may be extended to other electrochemical enzymatic systems.

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Notes and references

[a] *CNRS, CRPP, UPR 8641, Pessac F-33600, France E-mail: mano@crpp-bordeaux.cnrs.fr*
[b] *Univ. Bordeaux, CRPP, UPR 8641, Pessac F-33600, France*

† Electronic Supplementary Information (ESI) available: production and purification of the differents enzymes. See DOI: 10.1039/b000000x/

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