A Simple and Fast Method for Detecting Glucose in Wines Using a Redox Polymer-Based Amperometric Biosensor

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In our quest for a simple and fast method to detect glucose in wines, a redox polymer (RP)-based amperometric biosensor was developed. The biosensor consists of glucose oxidase and horseradish peroxidase (as recognition tandem), an Os-based RP (as electron transfer mediator), poly(ethylene glycol) diglycidyl ether (as cross-linking agent), and a graphite electrode (as transducer). Each building block of the biosensor was carefully investigated. First, the electrochemical behaviour of the surface immobilized Os-based RP was investigated. Peroxidase- and RP-containing layers were subsequently studied using normal pulse voltammetry (NPV). Finally, glucose oxidase was also added to the sensing layer, and the obtained sensor was calibrated and used to detect glucose in wine. The obtained values agree well with concentrations reported by the producer.

Keywords: amperometric biosensors, glucose detection, os-redox polymer

International competition in a saturated wine market is increasingly quality-driven [1]. As an example, although global wine production volumes have increased and this has led to the problem of over-supply, demand regularly exceeds supply in the market for premium Bordeaux wine [1]. Therefore, one can expect an increasing demand for analytical tools able to conveniently monitor the chemical composition of wine and thus assure its appropriate quality.

Amperometric enzyme-based biosensors are analytical tools making use of the selectivity of enzymes and the sensitivity of electrochemical detection. They are characterized by simplicity, short response times, and low cost [2]. Therefore, they are also very promising for wine analysis. Amperometric enzyme-based biosensors have already been used to detect glycerol [3, 4], glucose [4, 5], polyphenolic compounds [6], lactic acid and malic acid [7] in wine. Some of the most promising were obtained by combining oxidases with peroxidases in order to switch the detection principle from oxidation to reduction and thus gain selectivity and/ or sensitivity, and by using redox hydrogels. Bioelectrochemical approaches, based on glucose oxidase (GOx) working in tandem with horseradish peroxidase (HRP), resulted in some of the most selective and sensitive methods for glucose detection [8-11]. In redox hydrogels the enzymes involved in the detection principle are completed with redox polymers (RPs) as electron transfer mediators. Os-based RPs are especially recommended for such sensors not only by their excellent electron shuttling properties but also by their ability to retain water and thus assure a microenvironment that maintains enzyme activity [5, 12-14]. RPs allow using low applied potentials [14], which are in the optimal potential window for amperometric detection [15].

The aim of the present work was to develop a simple and fast way to detect glucose in wine. A robust GOx and HRP tandem was combined with one of the most efficient electron transfer mediators, namely an Os-based RP, and a simple graphite electrode as transducer. Each building block of the biosensor was carefully investigated.

Experimental part

Reagents and solutions

GOx from *Aspergillus niger* (type VII-s; EC 1.1.3.4., specific activity 184900 U g^{-1} solid) and HRP (type II; EC 1.11.1.7; specific activity 1100 U mg^{-1} solid) were purchased from Sigma-Aldrich (Poole, UK). Poly(ethylene glycol) (400) diglycidyl ether (PEGDGE) was supplied by Polysciences (Warrington, PA, USA). Tobacco peroxidase from *Nicotiana sylvestris* (TOP, specific activity 6170 ± 56 U mg^{-1} solid) was purified, and poly(1-vinylimidazole) complexed with Os (4,4'-dimethylbipyridine)$_2$Cl (PVI_7dmeOs) was synthesized as previously described [14, 16]. D(+) glucose (Merck, Darmstadt, Germany), Na_2HPO4, NaH_2PO_4 x $10H_2O$ and KCl (Sigma, Poole, UK), were used as received. Hydrogen peroxide standard solutions were prepared daily from 30% H_2O_2 solution purchased from Merck (Darmstadt, Germany). The 0.1 M phosphate buffer solution, used as supporting electrolyte, was prepared by mixing the appropriate volumes of Na_2HPO_4 and NaH_2PO_4 solutions. The final ionic strength was fixed at 0.1 M using KCl. If not otherwise indicated, the solutions were prepared in either tridistilled water produced in a Milli-Q system (Millipore, Bedford, MA, USA) or distilled water.

Biosensor preparation

Graphite electrodes (Ringsdorff-Werke GmbH, Bonn-Bad, Germany, type RW001, 3.05 mm diameter) were polished on a wet fine emery paper (Tufbak, Durite P1200, Allar, Sterling Heights, MI) before modification. Peroxidase-modified graphite electrodes (denoted G/RP-TOP) were prepared as described elsewhere [17]. Amperometric biosensors for glucose detection (denoted G/RP-HRP-GOx) were developed according to a previously described method [4]. Shortly, appropriate volumes from stock solutions of RP, peroxidase, GOx, and cross-linker were mixed. A small droplet of the obtained mixture was carefully placed on the electrode surface using a micropipette. The modified electrodes were allowed to

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cure at room temperature until the droplet dried out, the cross-linking occured, and the redox hydrogel film was formed. All bioelectrodes were stored at + 4 ^{0}C until tested.

Electrochemical measurements

Normal pulse voltammetry (NPV) experiments were done using a BAS CV-50W potentiostat (from Bioanalytical Systems, West Lafayette, IN, USA), while cyclic voltammetry measurements were carried out using an AutoLab PGSTAT 10 computer controlled voltammetric analyzer (from Echochemie, Utrecht, Netherlands). All electrochemical measurements were performed in a conventional single-compartment three-electrode cell, using the modified electrode as working electrode, a Ag/AgCl, $KCl_{0.1M}$ as reference electrode (from either Bioanalytical Systems, USA or Radiometer, France), and a platinum plate as counter electrode. Amperometric measurements were done using a rotating disc electrode (Radiometer, France).

Results and discussions

As already mentioned, all biosensor building blocks were carefully investigated, first separately and then together.

First, the electrochemical behavior of the surface-immobilized RP was studied. Cyclic voltammograms, recorded at G/RP modified electrodes (fig. 1) present a well-defined pair of peaks, having the formal potential (estimated as the average of the cathodic and anodic peak potentials) around ~0.14 V *vs.* Ag/AgCl, $KCl_{0.1M}$. The redox polymer exhibits a quasi-reversible behavior as proved by the peak separation (~65.4 mV) and the peak current ratio [$I_{p,a}/I_{p,c}$~1.05 (50 mV/s)]. The width at half-way height values are higher than the theoretical one (90.5 mV) indicating the presence of lateral repulsive interactions between the adsorbed molecules [18].

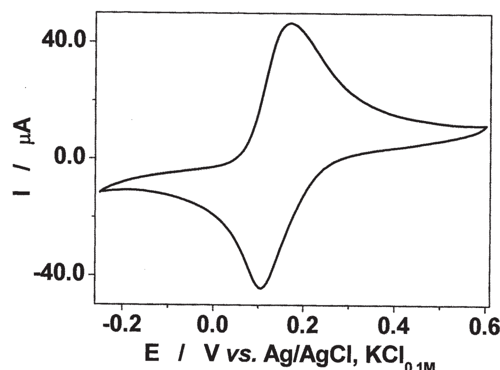

Fig.1. Cyclic voltammogram recorded at G/RP electrode. Experimental conditions: supporting electrolyte, 0.1 M phosphate buffer containing 0.1 M KCl (pH 7.0); starting potential, -0.25 V vs. Ag/AgCl, $KCl_{0.1\,M}$; scan rate, 50 mV s^{-1}

In the next step, peroxidase was added to the RP-modified electrodes. Two major changes were made compared to most of the papers reporting on similar systems. First, TOP was chosen instead of the classically used HRP since this enzyme was previously shown to exhibit a wider optimum stability *p*H range, higher stability to inactivation with hydrogen peroxide, and higher efficiency in direct electron-transfer processes than HRP [19]. Moreover, upon immobilization together with the Os-based RP on graphite electrodes, TOP exhibited sensitivity and stability comparable to those of horseradish peroxidase and a wider linearity range [17]. Secondly instead of the usual cyclic voltammetry, normal pulse voltammetry was used to investigate the electrochemical behaviour of the system. NVP is a sampled-current voltammetry method that uses a series of potential pulses of increasing amplitude. The current is measured near the end of each pulse and thus it is to a great extent free of the charging current. Figure 2 shows typical normal pulse voltammograms, recorded at G/RP-TOP electrodes, for various sampling times (τ, ms). As expected, the NPVs are similar to those observed for $(IrCl_6)^{3-/2-}$ incorporated in poly(4-vinylpyridine) film [20].

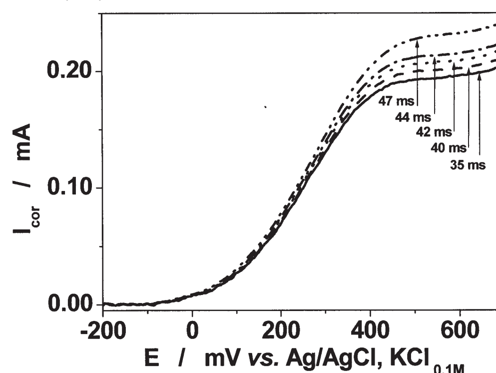

Fig. 2. NPVs recorded at G/RP-TOP electrodes. Experimental conditions: supporting electrolyte, 0.1 M phosphate buffer (pH 7.0); initial potential, -200mV *vs.* Ag/AgCl, $KCl_{0.1\,M}$; pulse width 50 ms; pulse period, 500 ms; scan rate, 5 mV s^{-1}. I_{cor} stands for base line corrected current values

The current-potential relationship for normal pulse voltammograms recorded for a simple electrode oxidation process is given by [20]:

$$E = E^r_{1/2} - \frac{RT}{\alpha nF}\ln\left\{\frac{4}{3^{1/2}}\frac{k_s^0\tau^{1/2}}{D^{1/2}}\right\} - \frac{RT}{\alpha nF}\ln\left\{\frac{I}{I_{\lim}}\left[\frac{1.75+\left(\frac{I}{I_{\lim}}\right)^2\left(1+\exp\left[-\frac{nF}{RT}\left(E-E^r_{1/2}\right)\right]^2\right)}{1-\left(\frac{I}{I_{\lim}}\right)\left[1+\exp\left[-\frac{nF}{RT}\left(E-E^r_{1/2}\right)\right]\right]}\right]^{1/2}\right\} \quad (1)$$

where:

E is the electrode potential (V);

$E_r^{1/2}$ - reversible half-wave potential (V);

α - anodic transfer coefficient;

k_s^o - standard rate constant (s^{-1});

τ - sampling time (s);

R - gas constant (J mol^{-1} K^{-1});

T - absolute temperature (K);

F - Faraday's constant (C mol^{-1});

n - the number of electrons involved in the redox process;

D - the diffusion coefficient (cm^2 s^{-1}) expressed as $(D_{app,\,anodic})^{\alpha}(D_{app,\,cathodic})^{1-\alpha}$;

I - the current intensity (A);

I_{lim} - limiting diffusion current (A) expressed by equation (2).

From the slope of the Cottrell dependence (fig. 3):

$$I_{\lim} = nFAc^0\left(\frac{D_{app}}{\pi\tau}\right)^{1/2} \quad (2)$$

where:

n = 1, A = 0.0707 cm^2 and c^0 = 1.92 mol cm^{-3} (c^0 = ΓA/V, where c^0 is the superficial concentration of redox species;

Γ - under peak area for the oxidation process recorded at G/RP-TOP electrodes;

A - electrode area;

V stands for the volume of the polymer film deposited on the electrode surface, supposing its density as 1g cm^{-3}),

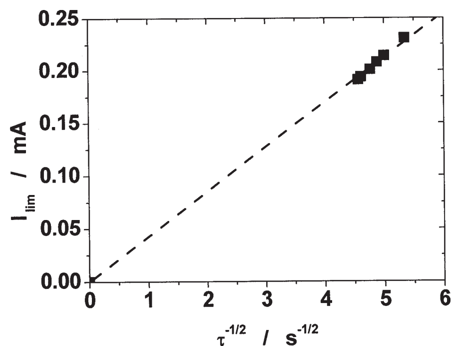

Fig. 3. Plots of limiting current, I_{lim}, *vs.* inverted square root of sampling time, $\tau^{-1/2}$, for the oxidation of Os^{2+} incorporated in the RP at G/RP-TOP electrode. Experimental conditions: as in figure 2

a rough estimation of the electron diffusion coefficient (D_{app}) value was done.

Taking

$$Y = \ln\left\{ \frac{I}{I_{\lim}} \left[\frac{1.75 + \left(\frac{I}{I_{\lim}}\right)^2 \left(1 + \exp\left[-\frac{nF}{RT}\left(E - E_{1/2}^r\right)\right]^2\right)}{1 - \left(\frac{I}{I_{\lim}}\right)\left[1 + \exp\left[-\frac{nF}{RT}\left(E - E_{1/2}^r\right)\right]\right]} \right]^{1/2} \right\} \quad (3)$$

equation (1) becomes:

$$Y = \frac{\alpha nF}{RT}\left(E - E_{1/2}^r\right) + \ln\left\{\frac{4}{3^{1/2}} \frac{k_s^0 \tau^{1/2}}{D^{1/2}}\right\} \quad (4)$$

According to equation (4) the Y = f(E) plots for different values of τ, and a diffusion controlled electrochemical process, should correspond to parallel straight lines. As other systems of metal complexes in films [20], our redox hydrogel also follows quite well equation 4 (fig. 4). Taking D_{app} = 3.3 x 10^{-5} cm^2 s^{-1}, the slope gives the transfer

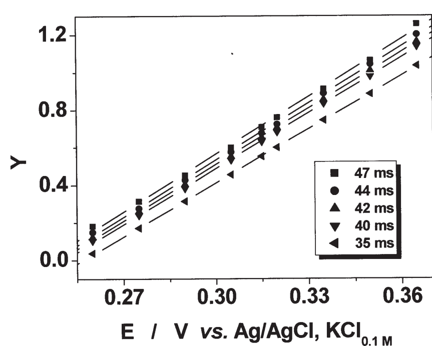

Fig. 4. Y vs. E plots (see equation 4) for the NPVs depicted in figure 2. Experimental conditions: as in figure 2

coefficient (α = 0.256), while the intercept gives the standard rate constant (k_s^o = 0.34 s^{-1}).

In order to sense glucose, in the last step of our biosensor development, GOx was also added to the sensing layer. As it can be seen from figure 5, the presence of bulky insulating enzyme molecules changed a little bit the electrochemistry

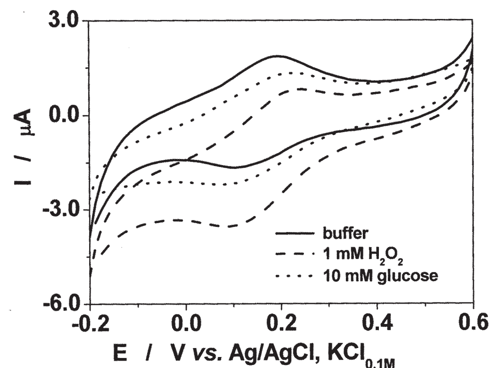

Fig. 5. Electrocatalytic activity of G/RP-HRP-GOx electrode for amperometric detection of H_2O_2 and glucose. Experimental conditions: supporting electrolyte, 0.1 M phosphate buffer containing 0.1 M KCl (pH 7.0); starting potential, -0.2 V vs. Ag/AgCl, $KCl_{0.1\ M}$; scan rate, 50 mV s^{-1}.

displayed by the RP. The formal potential shifted to a more positive value (~ 0.15 V *vs.* Ag/AgCl, $KCl_{0.1M}$). The slightly higher peak potential separation (~ 79 mV) and the peak current ratio [$I_{p,a}/I_{p,c}$ ~1.7 (50 mV/s)] are both indicating that the reversibility of the redox process was negatively affected. Judging from the peak currents, the surface concentration of the Os redox centers significantly decreased.

In the presence of H_2O_2 or glucose, the cathodic peak current, $|I_{cp}|$, increases, while the anodic peak current, I_{ap}, decreases, indicating a bioelectrocatalytic effect. The electrocatalytic efficiency, calculated as the ratio $\frac{I_{cp,[substrate]\neq 0} - I_{cp,[substrate]}}{I_{cp,[substrate=0]}}$ = 0 was found 1.13 for H_2O_2 and 0.33 for glucose. After proving the existence of a bioelectrocatalytic effect, amperometric measurements were also performed using the rotating disk electrode. From the I *vs.* time recordings in presence of increasing concentrations of glucose, calibration curves were plotted both for glucose (fig. 6) and H_2O_2 (results not shown) and

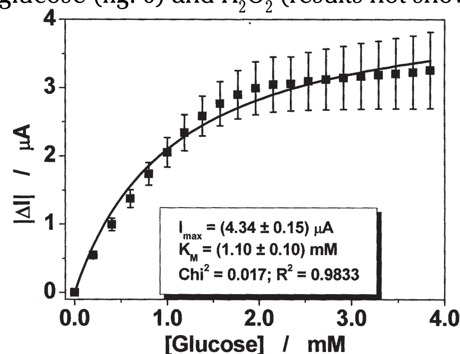

Fig. 6. Calibration curve for glucose recorded at a G/RP-HRP-GOx electrode. Experimental conditions: supporting electrolyte, 0.1 M phosphate buffer containing 0.1 M KCl (*p*H 7.0); applied potential, -50 mV vs. Ag/AgCl, $KCl_{0.1\ M}$; rotating speed, 1500 rpm. The error bars represent the difference between two successive measurements performed with the same electrode

Table 1
BIOELECTROANALYTICAL PARAMETERS FOR GLUCOSE AND H_2O_2 AT G/RP-HRP-GOx ELECTRODE. EXPERIMENTAL CONDITIONS: AS IN FIGURE 6

Analyte	I_{max} (µA)	K_M (mM)	Sensitivity* (µA mM^{-1})	Linear range (mM)	Detection limit** (µM)	R^2/N
Glucose	4.34 ± 0.15	1.07 ± 0.10	4.06 ± 0.52	Up to 1	7	0.9833/21
H_2O_2	0.34 ± 0.01	0.35 ± 0.02	0.97 ± 0.08	Up to 0.5	54.5	0.9966/15

* calculated as the I_{max}/K_M ratio;
** estimated for signal/noise ratio equal to 3.
R - correlation coefficient;
N – number of experimental points.

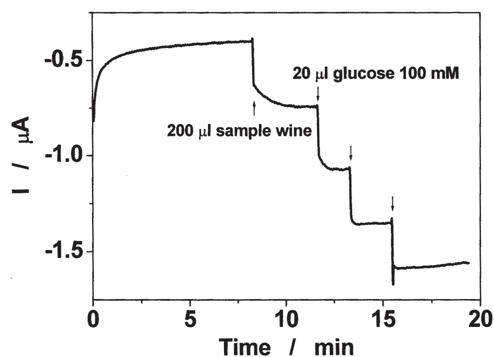

Fig. 7. Amperometric response recorded at a G/RP-HRP-GOx biosensor following injection of a wine sample (1:10 v/v dilution in phosphate buffer) and three injections of glucose standard solution. Experimental conditions: buffer volume, 10 ml; glucose standard solution, 100 mM; other conditions, as in figure 6.

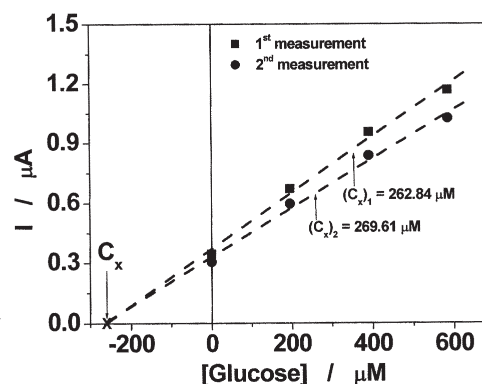

Fig. 8. Standard addition plot obtained with a G/RP-HRP-GOx biosensor. Experimental conditions: as in figure 6

the bioelectroanalytical parameters were calculated (table 1).

As a proof of concept, the glucose content of a wine sample was measured with the developed biosensor. The method of standard addition was used in order to overcome possible matrix effects. As shown in figure 7, after injecting a 10 times diluted wine sample, three injections from a glucose standard solution were also performed. Figure 8 graphically summarizes the obtained results by using the standard addition method. For the wine taken into study (Tamâioasă, from Cotnari vineyard, a white sweet wine) an average value of 136 mM glucose was found with our biosensor from two successive measurements (1 and 2) performed with the same electrode. This value is in good agreement with that indicated by the producer.

Conclusions

An amperometric biosensor for the fast and simple detection of glucose in wine was developed. The building blocks of the biosensor were first individually taken into study. The osmium polymer used as electron transfer mediator is characterized by a quasi-reversible redox process and a formal standard potential of ~0.14 V *vs.* Ag/AgCl, $KCl_{0.1 M}$. When peroxidase was added to the system, the electron transfer reaction was characterized by an apparent electron diffusion coefficient of 3.3 x 10^{-5} cm^2 s^{-1}, a transfer coefficient of 0.256 and a standard rate constant of 0.34 s^{-1} (as determined by NPV). Moreover, the G/RP-TOP sensor presented a strong electrocatalytical activity towards H_2O_2 reduction at -50 mV vs. Ag/AgCl, $KCl_{0.1 M}$. In order to sense glucose, in the last step of biosensor development, GOx was also added to the sensor. The bioelectrochemical parameters found for the G/RP-HRP-GOx biosensor (sensitivity, 4.06 ± 0.52 μA mM^{-1}; linear range, up to 1 mM and detection limit, 7 μM) were in good agreement with the results found in the literature. Preliminary experiments proved that the biosensor can be used for glucose detection in wine.

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