Third Generation Oxygen Sensitive Biosensor Based on Bilirubin Oxidase Modified Nanostructured Electrode

Marcos Pita^{a*}, Cristina Gutierrez-Sanchez^a, Miguel D. Toscano^b, Sergey Shleev^c, Antonio L. De Lacey^{a*}

^a *Instituto de Catalisis y Petroleoquimica, Consejo Superior de Investigaciones Cientificas, C/ Marie Curie 2, L10, 28049 Madrid, Spain. Fax: +34 91 585 4760; Tel: +34 91 585 4813; E-mail: marcospita@icp.csic.es, alopez@icp.csic.es*

^b *Department of Protein Diversity, Novozymes A/S, Krogshøjvej 36, 2880 Bagsværd, Denmark; E-mail: mdto@novozymes.com*

^c *Biomedical Sciences, Faculty of Health and Society, Malmö University, SE-205 06 Malmö, Sweden. Fax: +46 40 665 8100; Tel: +46 40 665 7414; E-mail: sergey.shleev@mah.se*

Keywords

Bilirubin oxidase, biosensor, oxygen, direct electron transfer, gold nanoparticles

Abstract

Gold nanoparticle modified gold disc electrodes have been used as scaffold for the oriented immobilization of bilirubin oxidase. The developed nanostructured bioelectrodes were tested as mediatorless oxygen biosensors in a buffer that mimics both content and composition of human physiological fluids. The resulting biosensor showed a detection limit towards oxygen of 6 μM with a linear range of 6-300 μM , *i.e.* exceeding usual physiological ranges of the bioanalyte in human tissues and fluids.

Introduction

Bioelectrodes that use enzymes as catalysts are promising systems for both *in vivo* biosensing and powering implantable biodevices with biofuel cells that provide electric energy using the naturally existing biochemical substances as fuels and oxidants (Davis and Higson, 2007; Shukla et al. 2004; Katz and Willner, 2003). Electrochemical biosensors can take advantage of the specificity and selectivity of enzymatic processes to be applied in substitution of classic amperometric sensors, which are longer prone to false positive signals or poisoning under physiological conditions. Electrochemical biosensors may be connected to a biofuel cell that

proportionates power to an *in vivo* electric device able to read and transmit the signal from the biosensor.

Bilirubin oxidase (BOx) is a multicopper oxidase (MCO) that oxidizes several natural compounds (*e.g.* bilirubin, tetrapyrroles, polyphenols, aryldiamines) while reducing O₂ directly to H₂O (Solomon et al., 1996). BOx, as other MCOs, performs its catalytic activity by means of four Cu ions split in two interconnected active sites, one to bind and oxidize the organic substrate and another one to bind and reduce O₂ (Mano, 2012). The site for organic matter oxidation includes one Cu ion and known as the T1 site, whereas the O₂ reduction cluster comprises three Cu ions denoted as T2 and T3 sites (Shleev et al. 2005). BOx is able to oxidize a broad range of either natural or artificial electron donors, including electrodes biased with redox potentials lower than that of its Cu T1 site (Solomon et al., 1996; Shleev et al. 2005; Mano, 2012). This later functional property allows BOx to work as a bioelectrocatalyst linked to different electroactive surfaces for developing amperometric biosensors (Göbel et al., 2010; Kannan et al.; 2011). Furthermore, BOx retains significant catalytic activity in solutions with neutral pH and high chloride concentrations, unlike other MCOs, *e.g.* fungal laccases. Therefore, BOx is a suitable enzyme for creation of potentially implantable biosensors and biocathodes of biofuel cells.

BOx has already been matter of bioelectronic development, *e.g.* in 2004 efficient direct electron transfer (DET) based bioelectrocatalytic reduction of O₂ on BOx modified electrodes was demonstrated (Shleev et al., 2004; Tsujimura et al., 2004). DET of BOx on gold electrodes is favored, when the surface is modified with carboxylic groups (Ramirez et al., 2008; Tominaga et al., 2008; Climent et al., 2012) or it is nanostructured (Murata et al., 2009; Salaj-Kosla et al., 2012).

Despite of many publications concerning bioelectrochemical studies MCO modified electrodes in general, and BOx based electrodes in particular, there are only few works reporting the performance of biomodified electrodes in physiological fluids (Kang et al, 2006; Wang et al., 2012; Falk et al., 2012). Moreover, up to our best knowledge, third generation (DET based) O₂ sensitive MCO based sensors have not been designed so far.

In the present work we have studied the performance of BOx electrodes as O₂ biosensors, when immersed in a serum-mimicking buffer. BOx was covalently immobilized on Au nanoparticle (AuNPs) modified Au electrodes (Murata et al., 2009) functionalized *via* reduction of a diazonium compound and subsequent thiol self-assembled monolayer (SAM) (Pita et al., 2011). The goal was to optimize the DET between the immobilized enzyme and the electrode surface in order to obtain a sensitive third generation amperometric biosensor well operating in both simple buffers and complex physiological fluids.

Materials and Methods

Enzyme

A highly purified preparation of *Myrothecium verrucaria* BOx (MvBOx), expressed recombinantly in *Aspergillus oryzae*, has been kindly provided by Novozymes A/S (Denmark).

Chemicals

All reagents were purchased on ACS purity grade. 6-amino-2-naftoic acid (NA), NaNO₂, 3-mercaptopropionic acid (MPA), HAuCl₄, sodium citrate, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), 30% H₂O₂, 2-morpholinoethanesulfonate (MES) hydrate 99.5%, were purchased from Sigma-Aldrich (USA). 98% H₂SO₄ was purchased from Panreac (Spain). Water Milli-Q (18.2 MΩ·cm) was used for all the experiments.

Gold nanoparticle synthesis

AuNPs were prepared following a procedure based on citrate reduction. (Frens, 1973) Briefly, 125 mL of a sodium citrate solution 38.8 mM was heated to boiling temperature, afterwards 12.5 mL of 1 mM HAuCl₄ solution was added. The reaction was allowed for 15 min and then to cool down to RT.

Modification of gold electrodes with AuNPs and further covalent attachment of BOx

Gold disc electrodes (Metrohm, 2 mm diameter, 99,999% purity) were cleaned as reported previously (Pita et al., 2011). The method used for the modification of the Au electrodes with AuNPs is based on that reported by Murata *et al.*, 2009. Firstly, AuNPs were concentrated 100-fold by centrifugation. A single 15 µL drop of the concentrated AuNPs dispersion was deposited on the surface of the electrodes and let to dry. Secondly, the modified electrode was taken into an electrochemical cell containing 0.1 M H₂SO₄ and 20 CVs were run from 0 to 1.5 V at 100 mV·s⁻¹ scan rate. The AuNPs modified electrode was immersed in an electrochemical cell filled with an ice-cooled solution, prepared immediately before its use by mixing 2 mL of 20 mM NA in acetonitrile and 2 mL of 2 mM NaNO₂ in 2 M HCl aqueous solution. A reductive CV from 0.6 V to -0.6 V at 200 mV·s⁻¹ was recorded. Thirdly, the electrodes were immersed into a 1 mM MPA aqueous solution during 20 hours. Finally, the electrodes were rinsed with water, dried with a gentle N₂ gas flow and immersed in a 20 µL solution containing 0.83 mg/mL BOx in 10 mM MES buffer pH 6 during 1 h. Afterwards, the electrode was taken into a different MES buffer solution containing 16 mM EDC and 32 mM NHS freshly prepared and let to incubate for 2 h. The electrode was then rinsed with 100 mM phosphate buffer, pH 7.4.

Buffers

Two buffers were used in our studies, *viz.* 100 mM phosphate buffer, pH 7.4 and a complex serum-like buffer, pH 7.4. The human serum-like buffer was prepared by the addition of 150 mM NaCl, 18 mM NaHCO₃, 5.55 mM glucose, 4.3 mM urea, 0.4 mM fructose, 0.4 mM uric acid, 0.055 mM ascorbic acid, 0.1 mM L-cysteine and 0.87 mM MgSO₄ into 100 mM phosphate buffer and adjustment of pH to 7.4. Contrary to the bare phosphate buffer this solution mimics quite well both the content and composition of human serum (Jung, 2008).

Electrochemical measurements

Experiments were performed with an Autolab PGSTAT30 analyzer controlled by GPES 4.9 software from Eco Chemie (The Netherlands). The experiments were run in a three-electrode glass cell using a BAS Ag/AgCl/3 M KCl reference electrode (204 mV vs. NHE) and a platinum wire counter electrode. All redox potentials mentioned in the text and figures are given vs. the Ag/AgCl reference electrode.

UV-Vis spectroscopy

The measurements were performed with an UV-2401PC spectrophotometer from Shimadzu (Japan) under thermostatic conditions using disposable polycarbonate cuvettes. 100 μ L of the AuNPs dispersion were diluted up to 1 mL and taken to a polycarbonate cuvette for UV-Vis characterization. A spectrum ranging from 400 to 600 nm was recorded.

Transmission Electron Microscopy

200 KV JEOL 2100 transmission electron microscope equipped with an Oxford Instruments EDX analyzer (UK) was used for the analyses. Free software ImageJ 1.46R from NIH (USA) was used for nanoparticle statistic purposes. The sample was prepared by adding a drop of diluted AuNPs dispersion on a carbon-coated copper grid 200 Mesh and let it dry.

Results and Discussion

Three-dimensional Au electrodes with a high surface were designed by deposition of AuNPs. AuNPs were synthesized as described above, and they were characterized by transmission electron microscopy (TEM) and UV-Vis spectroscopy (Figure 1). Analysis of the TEM images with ImageJ gave an AuNPs average size of 10.9 ± 2.3 nm by counting 240 nanoparticles (Figure 1C). Besides, the AuNPs average size and concentration was determined from their Localized Surface Plasmon Resonance (LSPR) measured by UV-Vis spectroscopy (Haiss et al., 2007). The UV-Vis spectrum of a 10-fold diluted AuNPs dispersion showed a resonance-absorption band centered at wavelength $\lambda_{\text{SPR}} = 519\text{nm}$ with an absorbance of $A_{\text{SPR}} = 0.220$ (Figure 1D). Another relevant parameter for the AuNPs characterization was $A_{450} = 0.141$. According to Haiss' numerical method, the synthesized nanoparticles were *ca.* 11 nm in diameter and dispersed at a concentration of approximately 17 nM. Therefore, the AuNPs size obtained by TEM and UV-Vis is coherent.

A clean Au disc electrode was modified with the AuNPs dispersion to increase the surface area of the gold electrodes while maintaining their geometric area, and to favor DET with the immobilized enzyme. By consecutive AuNPs deposition cycles it is possible to linearly increase the electrode area (Murata et al., 2009), which is of interest for developing a highly sensitive BOx based O₂ biosensor. The surface enhancement of the Au electrode after one deposition of AuNPs was estimated by integrating the Au reduction electrochemical wave in 0.1 M H₂SO₄ (Figure S1). The non-modified gold electrode had an electroactive surface of 0.068 cm², whereas the deposition of AuNPs allowed an increase of *ca.* 30-fold, reaching of about 2 cm² area. The enhancement was considered sufficient for developing a sensitive

biosensor, allowing for a higher bioelectrocatalytic signal while not increasing too much the background current. Besides, the area increase of the AuNPs-modified Au electrode can also be tuned by the number of gold oxidation-reduction CVs run under 0.1 M H₂SO₄ solution after the AuNPs deposition (Figure S2). The area obtained for Au reduction slowly decreased during the process, suggesting that the oxidation/reduction cycles of closely placed AuNPs alloys their aggregation.

The AuNPs modified electrode was modified with a mixed monolayer designed to favor the DET. Modification of a graphite electrode with 6-naftocarboxylic groups has been proven to facilitate DET of at BOx at graphite electrodes (dos Santos et al., 2010). By a similar procedure, we have made react 6-amino-2-naftoic acid inside the electrochemical cell with NaNO₂ and HCl to synthesize the diazonium salt derivative. A fast cyclic voltammogram was run to reduce the diazonium salt and form a NA layer *via* Au-C bonds (Laurentius et al, 2011; Pita et al., 2011). This modification yields an incomplete coverage of the surface, needing for an additional thiol SAM to protect the enzymes from denaturizing at direct gold contact (Pita et al., 2011). MPA has proven to support DET process between gold and BOx (Ramirez et al., 2008; Tominaga et al., 2008; Climent et al., 2011), so it was chosen as blotting thiol to build a SAM on the Au surface by immersion of the NA-AuNPs electrode in a 1 mM MPA solution overnight.

The MPA/NA-AuNPs Au electrode was used as support for the covalent immobilization of BOx *via* formation of amide bonds between the carboxylic groups at the Au surface and the lysine residues of the enzyme. The nanostructured electrodes modified with BOx were firstly tested by cyclic voltammetry in 100 mM phosphate buffer pH 7.4 (Figure 2A). It should be noted that the immobilized BOx required electrochemical activation before offering an appropriate electrocatalytic signal, so a CV between 0.7 and 0 V was performed prior to the analysis. Similar electrocatalytic behavior has already been reported very recently for BOx adsorbed on graphite electrodes and has been attributed to the reductive activation at low potential of the enzyme in the “alternative resting” form (Kjaergaard et al, 2012). Afterwards, the two CVs shown in Figure 2A were recorded. The first one was measured in O₂ saturated solution and second one in N₂-saturated solution. A clear bioelectrocatalytic process for O₂ reduction is observed starting already at 0.52 V, which is near to the redox potential of the T1 site of MvBOx (Christenson et al., 2006). There is a fast increase of the current value until 0.35 V, where the current increase slows down but does not reach the theoretical plateau for an electrocatalytic process. The residual slope of the bioelectrocatalytic current at high overpotentials is typical for BOx-based biocathodes, when O₂ transport is not a rate-limiting step of bioelectrocatalysis. It indicates that a dispersion of interfacial electron transfer rates is affecting on the bioelectrocatalytic process, even if a preferential orientation of the immobilized BOx is favored (dos Santos et al., 2011). The catalytic current obtained after saturating the cell atmosphere and solution with N₂ is almost negligible, as expected for a system with a very low O₂ concentration. After testing that the electrode was active, it was taken into a cell filled with the serum-like buffer to evaluate its electrochemical response (Figure 2B). At first sight the CVs obtained show several differences with the ones recorded in phosphate buffer. The presence of redox active interferences such as uric and ascorbic acids cause a high oxidative

current above 0.35 V that diminishes the BOx biocatalytic reductive current measured at the higher potentials, in other words, depolarization of biocathodes in human physiological fluids occurs (Falk et al., 2012). The resulting CVs also show that the O₂ reduction current measured in serum-like buffer for values lower than 0.35 V is hampered approximately 50%, when compared to the ones obtained using the phosphate buffer. The decrease in the current at low oxidative potentials can be also attributed to the high concentration of chloride and other compounds in the medium, which may cause a reversible and partial inhibition of BOx (Murao and Tanaka, 1982; Wang et al., 2012).

The potential to characterize the O₂ biosensor amperometrically was selected to be 0.1 V to minimize the interference of uric and ascorbic acids (Figure 3). The electrochemical cell with the MvBOx-NA/MPA-AuNPs electrode and the serum-like buffer was saturated with N₂ and a chronoamperometry was run while N₂ was flowed above the solution (Figure 3, inset). The signal was stabilized during 20 min, and then aliquots of O₂ saturated serum-like buffer were added gradually, recording the volume added and the current value obtained for each addition. At t = 120 min the solution was bubbled again with N₂ and the background current was reached again. The concentration of O₂ was correlated with the current values, obtaining a calibration curve that adjusted to a straight line for values below 300 μM, which is the approximately the concentration of O₂ in an air-equilibrated solution (ca. 250 μM at 25 °C (Truesdale and Downing, 1954)). The fitted linear equation for this range, which shows an r factor of -0.997, is the following:

$$i (\mu A) = -0.054 - 0.87 [O_2] (mM) \quad (1)$$

The reproducibility of 3 replicate calibrations was within a 17% relative standard deviation and the detection limit determined for O₂ was 6 μM.

Conclusions

The bioelectronic system developed by modifying a Au electrode with AuNPs and further functionalized by *in situ* diazotation of NA and a thiol SAM of MPA for the covalent attachment of BOx was able to function as O₂ biosensor in a solution mimicking the composition and content of human serum. A linear response was measured in the broad range, starting from natural concentration of O₂ in living beings of about 6 μM and ending with 300 μM, *i.e.* exceeding the maximal O₂ concentration in solutions saturated with air.

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Figures

Figure 1. AuNPs characterization. (A) Low magnification TEM picture. (B) High amplification TEM picture. (C) Particle size counts corresponding to the picture shown in (A). (D) UV-Vis spectra of a 10-fold diluted AuNPs dispersion in water.

Figure 2. CVs of a *MvBOx*-MPA/NA-AuNPs modified Au electrode under N_2 -saturated atmosphere (a) or O_2 saturated atmosphere (b): (A) in 100 mM phosphate buffer, pH 7.4; (B) in serum-like buffer, pH 7.4. Inset in (B) corresponds to the (b)-(a) subtraction of the CVs forward scans. Scan rate was $10 \text{ mV} \cdot \text{s}^{-1}$.

Figure 3. Calibration curve of the current response of a BOx-MPA/NA-AuNPs modified Au towards different O₂ concentrations in solution. Inset: chronoamperometry at +0.1 V upon consecutive additions of O₂ on MvBOx-MPA/NA-AuNPs modified Au electrode (a) and unmodified electrode (b). At 120 min the cell solution was flushed with N₂.