FABRICATION, CHARACTERIZATION, AND FUNCTIONALIZATION OF GOLD ELECTRODES WITH POLY(ETHYLENE GLYCOL) FOR BIOSENSING APPLICATIONS

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ABSTRACT - Enzyme-based electrodes are one of the most studied and are applied widely for metabolites detection, like glucose and lactate, byproducts in a variety of diseases, as diabetes, obesity and cancer. The electrodes miniaturization was aided by adaption of semiconductor fabrication processes. But immobilizing distinct biorecognition elements on an electrode array still a challenging process. For this, in our work we developed a multianalyte gold electrode conjugated with Poly(ethylene glycol) (PEG), a widespread polymer used in biomaterials and biotechnology, which provides an excellent matrix for enzymes, and has unique properties, as biocompatible, nontoxic and hydrophilic, creating a robust sensor for biosensing applications. The characterization and functionalization were made by electrochemical assays, using cyclic voltammetry and impedance spectroscopy and the results showed an improvement of the electrode measurements when used the PEG conjugated with ferrocene and enzymes.

1. INTRODUCTION

The field research on biosensor is expanding exponentially; due to nanotechnology which creates new elements and devices through microfabrication process (Sundberg, 2000). These sensors have an increased detection sensitivity, specificity and multiplexing capability on portable devices for use in several areas, e.g. health, safety and environmental evaluations (Xiao et al., 2003; Cai et al., 2006; Bui et al., 2009).

Thus, in biomedical applications, enzyme-based electrodes are an important class of biosensors, being one of the most studied and are applied widely. Where we can detect by electrochemistry the byproducts of enzymatic breakdown of an analyte (Hu et al., 1993; Ohara et al., 1994). For example, enzyme-based sensor can be used to monitoring a variety of diseases such as diabetes, obesity and cancer by detecting energy metabolites like glucose and lactate. (O’Rahilly and Vidal-Puig, 2001; Spiegelman and Filer, 2001; Gatenby and Gillies 2004).

The new generation of biosensors is based on multianalyte detection and miniaturization and this evolution of miniaturization was achieved by adaption of semiconductor fabrication processes for making and packaging of miniature electrodes (Cohen and Kunz, 2000; Revzin et al., 2002; Guenat et al., 2006). However, immobilizing
distinct biorecognition elements (e.g., enzymes) on different members of an electrode array, a requirement for multianalyte detection, remains challenging.

Integration of biorecognition and signal transduction elements has been an important area of biosensors research for several decades. For immobilization of enzyme, distinct approaches are used, such as: chemical cross-linking (Koudelka-Hep, 1997), electrodeposition (Malitesta et al. 1990; Strike et al. 1997), electrostatic interactions (Sirkar et al., 2000; Wang et al., 2006), and membranes or carrier matrices (Yan et al., 2010). Nonetheless, these traditional methodologies can be optimized to depositing specific enzyme types onto a desired member of an electrode array.

An approach most commonly used for creating electrochemical biosensors involves electrodeposition of a conductive hydrogel polymer (Lee et al., 2005; Luo et al., 2005; Chen et al., 2007). Hydrogels are attractive materials in fabricating electrochemical biosensors because it provides an excellent matrix for enzyme encapsulation (Pedrosa et al., 2010).

Several research have used a uniform gel membrane coated on top of the microfabricated electrodes to prevent fouling and enzyme leaching (Mugweru et al., 2007), whereas far fewer reports describe the integration of patterned gel layer with electrodes (Moser et al., 2002). Previous research groups employed acrylamide, hydroxyl ethyl methacrylate (HEMA) or poly vinyl acetate (PVA) for biosensor construction.

Recently, Yan et al. 2010, manufactured a biosensor with poly(ethyleneglycol) (PEG) hydrogel. There is a preference for PEG instead others polymers, because it possess a broad range of properties, making them attractive for biosensor fabrication. PEG hydrogels have been shown to be excellent matrices for entrapment of biomolecules (Rezvin et al., 2001; Koh and Pishko, 2005). These hydrogels are non-fouling and can therefore help eliminate biosensor fouling in complex solutions such as physiological fluids or cell culture media (Drumheller et al., 1995; Quinn et al., 1995). In addition, it can be micropatterned in a manner similar to photolithography and are semiconductors (Rezvin et al., 2003).

However, even with several works on microfabrication of enzyme-biosensors and the extensively using of PEG polymers for entrapment of functional enzymes (Koh and Pishko, 2005; Yan et al. 2009), there is a lack of research on the improvement of the functionalization of such sensors, and for this, in this article we verified how the inclusion of ferrocene (redox molecules) could enhance the electron transfer through the hydrogel and calculated the optimum ratio of ferrocene (FC), polymer, and enzyme to avoid destabilizing the sensor and achieve a better electrochemical signal.

The strategy for integrating redox molecules into hydrogels onto miniature electrodes described in this paper should be broadly applicable in construction of robust and sensitive enzyme-based biosensors.

2. MATERIAL AND METHODS

2.1 Materials

Poly (ethylene glycol) diacrylate (PEG-DA, MW 575), 2-hydroxy-2-methyl-propiophenone (photoinitiator), 99.9% toluene, (EC 1.1.3.4, type II-S from Aspergillus niger (18 000 U g⁻¹ solid), Ferrocene, D-(+)-glucose and 3-(trichlorosilyl)propyl methacrylate (silanization agent) were purchased from Sigma (St Louis, MO). Phosphate buffer (PBS) 0.10 M was used as an electrolyte for all electrochemistry experiments. Water used for preparation
of aqueous solutions came from a Millipore Direct-Q water purification system (resistivity, 18 MΩ cm$^{-2}$).

D-(-)+Glucose solutions were allowed to muta-rotate overnight at room temperature before use. Stock solutions were prepared in bidistilled water or PBS solution and stored in the dark at 4 °C.

2.2 Equipment

The voltammetric and spectroscopy impedance experiments were performed using a µAutolabType III/response analyzer (Metrohm Autolab) with a frequency range of 100 kHz–100 mHz and signal amplitude of 5 mV. The experiments were conducted in a three electrode system containing a counter electrode wire of titanium coated with platinum, a saturated Ag/AgCl (3 M) reference electrode and enzyme-modified working electrode. All electrochemical measurements were performed at room temperature.

2.3 Fabrication of Au electrode arrays

The electrode array layout was designed in AutoCAD and converted into plastic transparencies by DGM design (Curitiba, PR). The design of the Au electrode arrays is shown in Figure 1A. The fabrication of gold electrode arrays, were made at Brazilian Nanotechnology National Laboratory (LNNano/CNPENM, Campinas/SP), we sputter-coated standard (75 mm × 25 mm) glass slides with 15 nm Cr adhesion layer and 100 nm Au layer. The electrodes were fabricated using traditional photoresist lithography and wet etching processes. The etching of Au/chrome layers resulted in an array of eight working microelectrodes patterned on a glass slide. Each Au electrode was 300 μm in diameter with 15 μm wide leads and 1 mm × 1 mm square contact pad (see Figure 1A for layout of electrodes).

2.4 Functionalization of electrodes substrates

For improving hydrogel adhesion on electrode substrate, the electrodes were modified with 3-(trichlorosilyl)propyl methacrylate. Glass surfaces were silanized as the following standard protocol (Yan et al., 2011). Briefly, glass slides were cleaned in “piranha” solution
consisting of 3:1 ratio of H₂SO₄ and H₂O₂ for 30 minutes, washed in mili-Q water and subsequently immersed in 2mM toluene solution of 3-(trichlorosilyl)propyl methacrylate (TPM) for 1 h to obtain a self-assembled monolayer of silane on glass regions then the substrates were washed in toluene to remove the excess of silane agent and finally were placed in an oven for 3h at 100°C to crosslink the silane layer.

2.5 Integration of Au Electrodes with Enzyme-Carrying Hydrogel Microstructures

When preparing enzyme electrodes, to testing the influence of different concentrations of FC, we had used five FC concentrations (2.5 mg/mL, 5 mg/mL, 10 mg/mL, 15 mg/mL and 20 mg/mL). GOX was dissolved in PBS buffer (pH 6.0) to reach the concentration of 20 mg/mL and glutaraldehyde was added to the enzyme solution at 2% v/v to improve enzyme retention and function of the biosensor.

The prepolymer solution was prepared by adding 2% (v/v) of photoinitiator (2-hydroxy-2-methyl-propiophenone) and FC to PEG-diacylate (DA) (MW 575). Enzyme and prepolymer solution were combined by adding 0.1 mL of the enzyme solution to 0.4 mL of PEG-DA/ferrocene. The mixture was stirred for 4 h at 4 °C to ensure the homogeneous dispersion of the enzyme molecules.

In the next step, PEG prepolymer solution containing enzyme molecules and redox species was photopolymerized on top of the Au electrodes in a process similar to photolithography (see Figure 1B). Briefly, PEG-based prepolymer solution was spin-coated at 800 rpm for 4 s onto glass slides containing Au electrode patterns. A photomask was made with an electrode pattern and then exposed to unfiltered UV light at 70 mW/cm² for 10 s to convert liquid prepolymer into cross-linked hydrogel. The surfaces were developed in mili-Q water to remove unpolymerized PEG precursor solution. Enzyme carrying hydrogel microstructures were made larger than Au electrodes, 600 and 300 μm diameter for hydrogel and Au features, respectively. This was done to ensure effective anchoring of the polymer structures to silanized glass substrate. To help visualize deposition of the polymer microstructures on adjacent electrodes, we utilized an optical microscope coupled with a digital camera (Figure 2).

2.6 Electrochemical Characterization and Functionalization of Enzyme Electrodes

Electrodes were tested in a custom-made, Plexiglas electrochemical cell with a volume of ~1 mL. PBS (pH 6.0) Two electrochemistry techniques, cyclic voltammetry and spectroscopy impedance, were used to characterize the sensor response to glucose and lactate. D-Glucose solutions with various concentration were made (0.02mM, 0.05 mM, 0.1mM and 0.2 mM) were stored overnight at room temperature to allow equilibration of the α and β-forms. To characterize redox properties of ferrocene-containing hydrogel, we used cyclic voltammetry with scan rates ranging from 20 to 250 mV/s. When performing impedance spectroscopy experiments, we used a µAutolab Type III/FRA2 with a frequency range of 0.01 – 5000 hz and signal amplitude of 5 mV, we also poised the enzyme-containing working electrode at 0.3 V (vs Ag/AgCl) (anodic peak potential of immobilized ferrocene) and
exposed it to different aliquots of glucose, all impedance spectra were fitted to the equivalent electrical circuits using Nova Autolab software (Metrohm).

3. RESULTS AND DISCUSSIONS

3.1 Surface Modification and Fabrication of Au electrodes

This paper describes the use of enzyme-carrying hydrogels for development of electrochemical biosensor with enhanced both sensibility and sensitivity, thus we tried to take advantage of PEG properties, functionalized as a non-fouling surface and as matrix for enzyme entrapment.

The micropatterned electrode surfaces were enclosed inside an electrochemistry cell with an approximately 1 mL volume. The experimental design allowed us to use small amounts of PEG, FC and enzymes, in the vicinity of gold electrodes.

The device consisted of an array of 2 x 4 electrodes with 300 μm diameter connected to contact pads via 10 μm wide leads. An overview of one electrode arrays is shown in Figure 2A. Following the fabrication of Au electrodes, substrates were modified with methacrylate silane agent to secure covalent attachment of PEG hydrogel to glass (Figure 2B). The silanization did not influence the electrochemical experiments as we see the results bellow.

![](image1.png)

Figure 2 - (A) Portion of eight-electrode array. (B) PEG-ferrocene enzyme microstructures polymerized around the Au electrode. (C) Cyclic voltametric characterization of different ferrocene ratios.
3.2 Enhancement of Charge Transport in Ferrocene-PEG Hydrogels

We hypothesized that inclusion of ferrocene will improve the conductivity of PEG hydrogels. To test this hypothesis we used different ratios of ferrocene in the fabrication of PEG-Hydrogels, in order to find an optimum ratio of ferrocene and polymer. As seen in Figure 2C the cyclic voltammetry showed that the use of FC increased the catodic and anodic peak by improvement of electrolytes no PEG. The optimal concentration of FC found was 2.5mg/mL rather than high concentrations like the usual concentration of 10 mg/mL encountered in several papers (Yan et al. 2010; Yan et al. 2011,). This was primarily attributable to the microenvironment of the electrode cell; nanofabrication allows the use of lower amounts of the compounds with an enhanced electrochemical signal response. Despite the high concentrations of FC virtually predicts higher levels of current, using them, the PEG-prepolymer solution became more unstable, compromising the cross-link with enzymes. Therefore, we used the 2.5mg/mL concentration in the subsequent experiments with enzymes.

![Figure 3- Schematic of the enzyme reaction at the PEG/Au electrode site](image)

3.4 Fabrication, characterization and enzyme detection of enzyme-carrying hydrogel on Au Electrodes

The enzyme immobilization into the PEG hydrogel was micropatterned on the substrate in a process similar to traditional photoresist lithography (29, 35 artigo verde). This methodology involves the same spin-coating, alignment, UV exposure and development, briefly we used a mask with the same design of the Au electrode, but with 600 µm of diameter instead 300 µm (figure 3.).

PEG was used because is a biocompatible polymer with nonfouling properties and it is broadly used in tissue engineering and biosensor development (Charnley et al., 2009).

Insulating properties make PEG hydrogel an excellent bioelectrode packaging material and one advantage of our mask design was the possibility to polymerize the PEG around the Au electrodes without overlapping, creating thin films and avoiding an accumulation of layer over the electrode, which could undermine the diffusion of electrons in the electrochemical measurements.

After fabricating enzyme-carrying hydrogel/Au Electrodes we characterized the response of these biosensors to the analyte of interest: glucose as seen in Figure 3, the enzymatic reaction releases gluconolactone and hydrogen peroxide, and finally the electrons are generated into the electrochemical cell, improving the electric current.

Cyclic voltammetry (CV) and impedance spectroscopy were used to analyze the biosensor response to glucose. The activity of the redox species within the hydrogel was
evaluated by cyclic voltammetry a 50 mV/s scan rate in PBS and glucose solutions. As shown in Figure 4A, the anodic and cathodic peaks at 300 and 100 mV, respectively, this is consistent with redox behavior of ferrocene (Ju et al., 1997).

Importantly, Figure 4A also shows the change in CV with an increased of current as function of glucose solutions with a major increment at higher concentrations of glucose. Concomitantly to the increase in current, an important result was the proximity of catodic and anodic peaks at 0.2mM glucose concentration, this suggests fast and reversible redox processes occurring in the PEG hydrogelmodified electrodes (Pedrosa et al., 2010; Yan et al, 2010).

The amplitude of anodic and cathodic peaks has changed significantly when testing the electrodes with glucose solutions, pointing to retention of the redox species in the gel, suggesting electrocatalytic oxidation of the substrate (Figure 4B). This suggests fast and reversible redox processes occurring in the PEG hydrogel modified electrodes resulting in a reliable functional electrode.

To corroborate CV results, enzyme-carrying hydrogel/Au electrodes were analyzed by impedance spectroscopy (EIS) (Figure 4C). EIS is an optimal methodology for evaluate the interfacial properties of modified electrodes (Pardo-Yissar et al., 2001; Feng et al., 2008). The semicircle diameters of EIS spectra are related to electron-transfer resistance where the diameter of the semicircle represents charge-transfer resistance ($R_{ct}$) at the electrode surface, which means that smaller semicircles had an enhanced electrons transfer.

EIS measurements were performed in the presence of a redox molecule FeMeOH (dissolved in 0.1 M KCl). Figure 4C compares the electrochemical impedance spectra (Nyquist plots, $Z''$ vs. $Z'$) for hydrogels with glucose variations. As seen from these data, the
diameter of the semicircle or charge transfer resistance $R_{ct}$ decreased as the glucose concentration was increased, suggesting an enzymatic reaction that facilitated the electron transfer through the hydrogel. Our result indicates that incorporating ferrocene to the PEG hydrogels, enhances electron transfer and increases conductivity of the hydrogel.

4. CONCLUSIONS

This paper describes an optimized biomaterial microfabrication technique. PEG hydrogel photolithography with ferrocene and glucose oxidase, as an enhanced strategy for development of electrochemical enzymatic sensor. Similar Photolithograph process was used to functionalize Au electrode arrays with enzyme-carrying hydrogel microstructures. The biosensors were characterized electrochemically and the inclusion of ferrocene was found to enhance conductivity of PEG hydrogels but the optimal ratio was found at lower concentration of ferrocene (2.5 mg/mL). The cross-link of GOX molecules in PEG hydrogel microstructures allowed constructing biosensors with an improved current and lower detection limit of 0.02 mM.

The microfabricated biosensors were highly reproducible and stable. Inclusion of PEG into the vicinity of the Au electrode hydrogel without covering it up reduced the diffusion layer and improved the electron transfer between polymer and the electrode. In addition to providing an excellent matrix for enzyme entrapment, PEG hydrogels are nonfouling and have been used extensively by several researchers for micropatterning of living cells (Lee et al., 2009; Zhu et al., 2009). The present paper provides an optimization for fabricating miniature enzyme-based electrodes using PEG hydrogel as a biocompatible, nonfouling, and cell-friendly polymer. The next effort will be aimed at the use of PEG microstructures as biosensor for monitoring analytes in cell cultures or complex biological fluids.

5. REFERENCES


