Available online at www.sciencedirect.com
ScienceDirect
Biosensors and Bioelectronics 23 (2007) 355–361
BIOSENSORS & BIOELECTRONICS
www.elsevier.com/locate/bios

Improved sensitivity and stability of amperometric enzyme microbiosensors by covalent attachment to gold electrodes

Jean-Francois Masson [a], Christine Kranz [a], Karl S. Booksh [b], Boris Mizaikoff [a,*]

[a] *School of Chemistry and Biochemistry, Georgia Institute of Technology, 770 State St., Atlanta, GA 30332-0400, United States*
[b] *Department of Chemistry and Biochemistry, Arizona State University, Mail Code 1604, Tempe, AZ 85287-1604, United States*

Received 2 January 2007; received in revised form 25 April 2007; accepted 25 April 2007
Available online 29 April 2007

Abstract

Covalent attachment of glucose oxidase to a pre-activated 16-mercaptohexadecanoic acid at a gold ultramicroelectrode surface improves sensitivity, stability, and reproducibility of enzyme-based amperometric microbiosensors. Self-assembled monolayers of the *N*-hydroxysuccinimide ester of 16-mercaptohexandecanoic acid (NHS-MHA) at gold electrodes enable spontaneous covalent linking of glucose oxidase to the gold surface of ultramicroelectrodes. By self-assembling NHS-MHA for 30 min, approximately 93% of the electrode surface is covered, thereby maximizing both the number of attachment sites for glucose oxidase, and sufficient diffusion of hydrogen peroxide to the gold electrode. The glucose oxidase reaction with NHS-MHA was optimized at pH of 6.5, and at a temperature of 43 °C, resulting in a surface concentration of $6.8 \pm 0.6 \times 10^{11}$ enzyme molecules cm^{-2}. Thus obtained amperometric microbiosensors were calibrated in the range of 1–10 mM providing excellent correlation with the theoretical prediction of the microbiosensor response. The reported sensitivity of these microbiosensors documents an improvement by one order of magnitude compared to other approaches for covalent enzyme attachment. This is attributed to the NHS-MHA layer spacing the enzymatic recognition interface further from the electrode surface, thereby minimizing quenching of the enzyme activity.

Keywords: Microbiosensor; Amperometric microbiosensor; Glucose microbiosensor; Covalent enzyme attachment; Surface plasmon resonance; Quartz crystal microbalance; Glucose monitoring; Diabetes; Microbiosensor response modeling

1. Introduction

Miniaturization of electrodes has been of great interest to the field of scanning electrochemical microscopy (SECM), ultimately leading to the integration of micro-and nanoelectrodes into atomic force microscopy (AFM) cantilevers (Macpherson and Unwin, 2000; Kranz et al., 2001; Macpherson and Unwin, 2001; Macpherson et al., 2002a,b; Kueng et al., 2003a,b; Abbou et al., 2004; Hirata et al., 2004; Kranz et al., 2004). This combined AFM-SECM approach enables simultaneous measurements of the sample topography and of the electrochemical surface properties. Decreased electrode dimensions yield improved lateral resolution during SECM measurements for the laterally resolved determination of localized concentrations of biologically active molecules, or for imaging concentration profiles of these molecules above live tissues or cells (Kueng et al., 2003a; Kranz et al., 2004). Many of these molecules cannot be directly detected at an unmodified electrode surface, and frequently the complex biological matrix and the low concentration levels require specific and sensitive detection schemes, as provided by amperometric biosensors. However, miniaturization of electrodes gives rise to new challenges for the immobilization of biorecognition interfaces related to the decreased electrode dimensions. When immobilizing enzymes or other analyte-specific receptors at micro- or nanoelectrodes, the reproducibility of non-covalent immobilization processes and the stability of the resulting microbiosensors decrease along with the dimensions of the electrode. Membranes

* Corresponding author. Tel.: +1 404 894 4030; fax: +1 404 385 6447.
E-mail address: boris.mizaikoff@chemistry.gatech.edu (B. Mizaikoff).

0956-5663/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2007.04.013

are subject to delamination, and deposition techniques based on surface adsorption frequently show limited reproducibility. Furthermore, as the signal-to-noise ratio achievable with miniaturized amperometric electrodes decreases with decreasing electrode dimensions, sufficient sensitivity of miniaturized devices relies on the efficiency associated with the biosensing interface. Hence, the development of advanced molecular immobilization strategies improving reproducibility, stability and sensitivity of miniaturized biosensors is primordial to their more widespread application in electrochemical sensing and imaging techniques.

Covalent attachment of glucose oxidase (GOD) to self-assembled monolayers (SAMs) is an attractive strategy toward improved stability of enzymatic recognition layers at gold electrodes. SAMs are solidly tethered to the gold surface via a mostly covalent Au–S bond (Evans and Ulman, 1990). The enzyme molecules are covalently attached via an amide bond formed between free amine group of the enzyme and the carboxylic group at the ω-position of the SAM (Gooding et al., 2000; Gooding et al., 2001). Several groups reported microbiosensors for glucose with significantly improved fabrication reproducibility by covalently attaching GOD to 3-mercaptopropionic acid (MPA) (Gooding et al., 2000, 2001; Campuzano et al., 2002). However, microbiosensors based on MPA-tethered GOD also yielded less sensitivity compared to sensors based on conventional polymer entrapment. Attaching enzymes to a SAM-modified gold surface generates a two-dimensional scaffold with a maximum surface concentration of $1.6–2.3 \times 10^{12}$ enzyme molecules cm^{-2} for GOD. Improvement of the surface coverage of glucose oxidase was demonstrated using gold nanoparticles located at the surface of a gold electrode (Zhang et al., 2005a,b). Although locating enzymes in close proximity of the electrode decreases the electron transfer distance and facilitates the diffusion of the electroactive specie to the electrode for electrochemical biosensors, tethering GOD with a C_3-linker may lead to quenching of the enzyme activity by the gold electrode. Similar effects were previously observed for antibodies immobilized at gold surfaces of surface plasmon resonance (SPR) chips (Masson et al., 2006), and for photochromes located near gold surfaces (Chance et al., 1978; Yang et al., 2000). For example, by locating antibodies at an increased distance to the gold surface of the SPR chip, an increase in sensitivity was observed. The sensing principle is different for SPR biosensors compared to electrochemical biosensors. However, SPR theoretically should be more sensitive for biorecognition element located closer to the gold surface. Thus a similar quenching of the biorecognition element can be observed with electrochemical biosensors. Moreover, pre-activating 16-mercaptohexadecanoic acid (MHA) with *N*-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide in a dioxane solution yields nearly 100% of NHS-MHA, hence nearly 100% coverage of NHS-MHA at the gold surface (Masson et al., 2006). Typically, reaction yields of 30–40% are observed for reacting SAMs with carboxylic acid functionality while immobilized on gold surfaces utilizing carbodiimide chemistry (Johnsson et al., 1991). Thus, by pre-activating the SAM with NHS combined with an increased length of the spacer yields enhanced sensitivity in SPR sensing with antibodies as molecular recognition elements (Masson et al., 2004a; Battaglia et al., 2005).

This study demonstrates the utility of NHS-MHA to covalently attach enzyme monolayers to gold ultramicroelectrodes. The experimental factors affecting the formation of a dense and functional monolayer of enzymes are investigated in detail along with theoretical limitations and advantages of covalently attaching enzymes via NHS-MHA to the surface of microelectrodes.

2. Experimental

2.1. GOD enzyme layer using NHS-MHA self-assembled monolayer

The preparation of the enzyme layer is similar to the attachment of antibodies for surface plasmon resonance microbiosensors (Masson et al., 2004a; Battaglia et al., 2005). NHS-MHA was synthesized according to the previously published procedure (Battaglia et al., 2005). The bare gold surface of the 25 μm-diameter ultramicroelectrode (supporting information for preparation) was exposed to a 0.005 M NHS-MHA solution in tetrahydrofuran establishing a self-assembled monolayer (SAM). As described in more detail in the following section, the optimal self-assembly time of NHS-MHA at a gold surface was determined to be 30 min at room temperature. The formed SAM was immediately rinsed with ethanol. Then, the surface was rinsed with water for 10 min. Amine coupling between GOD and this activated surface was thereafter initiated with a 10 mg/mL GOD solution in 100 mM phosphate buffer. Phosphate buffers with variation in pH and reaction temperature were investigated to optimize covalent attachment of GOD to the activated gold surface. Non-specifically bound enzymes and the unreacted sites at the monolayer were deactivated by rinsing the ultramicroelectrodes with an aqueous solution of 1 M ethanolamine at a pH of 8.5 for 10 min. The microbiosensors were then stored at 0 °C in 100 mM phosphate buffer at a pH of 7.4 for at least 2 h prior to use.

2.2. Flow injection analysis system

The calibration and optimization of the glucose microbiosensor were performed using flow injection analysis (FIA). Glucose detection experiments were performed at room temperature. The peristaltic pump of a FIA system (FIAlab, FIAlab Instruments, Bellevue, WA) was utilized in combination with an 8-way valve to deliver glucose solutions at different concentrations to the microbiosensor. An electrochemical flow cell was fabricated using a 5-way manifold comprising an inlet and outlet for the solutions, a port for an Ag/AgCl reference electrode, a port for a 1 mm diameter Pt counter electrode, and a port for the amperometric glucose microbiosensor. A flow rate of 8 $\mu L\,s^{-1}$ was selected resulting in a pulse rate of 0.8 Hz with the utilized system. Therefore, in order to minimize the effects of the pulse generated by the peristaltic pump, a pulse damping module was