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Chemical functionalization of surfaces for building three-dimensional engineered biosensors

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ABSTRACT

This study presents a new approach for developing biosensors based on enzymatic systems with designed three-dimensional structures. Silica glass slides were chemically functionalized at surfaces by reacting with organosilanes, 3-mercaptopropyltriethoxysilane (MPTES), and 3-aminopropyltriethoxysilane (APTES), using sol–gel process at room temperature. The functionalization of the supports was characterized by contact angle measurements and FTIR spectroscopy. The first enzyme layer was covalently immobilized to the support by a bi-functional linker (glutaraldehyde). The second enzyme layer was deposited using the protein conjugation method based on the high affinity "avidin–biotin" interactions. Each enzyme was biotinylated before being added to the nanostructured system and avidin was used as the binder between consecutive enzyme layers. The biochemical response was assayed at all stages to certify that the enzymatic bioactivity was retained throughout the entire layer-by-layer (LBL) process. The model of building 3D-enzymatic systems was evaluated using the enzymatic structure with glucose oxidase (GOx) and horseradish peroxidase (HRP). It was verified that the amino-modified support presented the highest bioactivity response compared to the other chemical functionalities. Moreover, the bienzyme nanostructure demonstrated relevant biochemical activity upon injecting the glucose substrate into the system. Finally, as a proof of concept, the bienzyme systems were assayed using real samples of regular and sugar-free soft drinks where they effectively behaved as structured biosensor for glucose with the built-in 3D hybrid architecture. Based on the results, it can be foreseen the development of promising new nanomaterials for several analytical applications such as monitoring the quality of food and beverages for nutrition purposes.

1. Introduction

Nature has wisely designed biomolecules for performing very specific functions in innumerous living organisms. Each of them is intrinsically related to the others in complex cascade sequences and pathways, frequently with multiple reactions occurring in parallel. One of the most frequent strategies adopted by the researchers is mimicking nature using biomolecules such as proteins and polysaccharides that are selective in their functions and therefore interesting candidates to be associated with different classes of materials [1–6]. Among several alternatives of biomolecules, enzymes have been often chosen as active biosensing molecules due to their specificity, affinity, limit of detection, responsiveness, relative chemical and thermal stability, availability, at reasonable cost compared to other options [7–10]. Enzymes can be usually "temporally" or "permanently" immobilized on insoluble supports. A biomolecule is termed "immobilized" if its mobility has been restricted by chemical or physical means. This relative restraint of mobility may be achieved by several different methods, for instance by covalent bonding or by adsorption to the support. Both methods have positive and negative aspects regarding to their potential use for building sensing systems [11].

Surface functionalization by organic molecules provides several perspectives for the immobilization of enzymes. These surfaces can be effectively used to buildup interesting nano-level architectures. In the last decades, increasing effort has been directed toward potential applications of organosilanes as surface modifiers [12–14]. Furthermore, flexibility with respect to terminal functionalities of the organic molecules allows control of the hydrophobicity or hydrophilicity of surfaces. The attachment is typically mediated by first silylating the surface followed by immobilization of biomolecules of interest [15]. Aminosilanes are attractive for such applications, due in part to significant advances in the understanding of this class of surface modification agents [16,17]. Remarkably, aminosilanes have the advantage of

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catalytic activity by the amine group (nucleophilic) that facilitates formation of siloxane bonds (R—Si—O—Si) with surface silanols (—Si—OH). So, the formation of covalent bonds between an enzyme and an insoluble support is the most frequently used techniques. The strength of binding is very strong and very little leakage of enzyme from the support occurs [18].

Layered construction of enzymes into organized systems has attracted considerable attention in recent years due to its potential application in the areas of bio-electronic and biosensors, etc. There have been a number of approaches for constructing multilayer protein films on the surface of solid matrices, including a layer-by-layer (LBL) deposition of proteins on the surface of electrodes through a coupling reagent and consecutive adsorption of charged biomolecules on a solid surface through attractive electrostatic forces. However, these procedures are complex and commonly not stable enough [19]. As a consequence, research of new biosensors as well as the development of the existent designs has experienced advances in the last years due to the increasing necessity for miniaturized biosensors, particularly for biomedical applications [5].

Since the pioneering work from Clark and Lyons [20] reporting the glucose biosensor, innumerable papers and reviews have been published for the detection of a very large number of analytes [21]. Nevertheless, glucose biosensors are still very important because the metabolism of sugar is crucial for human life and several diseases may be directly or indirectly caused by some unbalance or malfunction of the endocrine system. Patients suffering from diabetes mellitus must monitor and control their blood glucose levels to avoid long-term complications and damage to organs, coma or death. So, the rigid control of blood glucose level by the diabetic sufferer is of paramount importance and biosensing devices comes to play a key role [10,22,23]. However, the glucose level in blood is one way of controlling the diseases but not actually a response for solving the problem and that also can negatively affect the quality of the patient's life. One of the main sources of the sugar intake in the body is associated with the dietary of food and beverages as a consequence of cultural and social habits. That means, the tight control in all sources of food and alternatives can be used as a way for improving the quality of life, reduce the possibility of glycemia shock [24]. Additionally, industrial food products may be unintentionally altered by contamination, by accident caused during the processing, changes in the supplier chain and several other possible factors that may cause severe consequences to highly sensitive people [10,22,23]. Therefore, analysis of compounds or substances could be applied in quality control of beverages and food or to monitor for adulteration or contamination of aliments by means of using rapid accurate biosensor to glucose at the "point-of-care" (PoC).

Enzymes have been extensively explored to develop biosensors with sensitivity, accuracy, fast response, and stability for the determination of glucose by different methods [3]. However, the "ideal" glucose biosensor is yet to be produced because several scientific, technological and industrial challenges must be overcome. In this work, the attention was mostly devoted to developing biosensing systems based on enzymes immobilized to chemically functionalized supports in a sequence of ordered biocatalytic reactions using the detection of glucose as a model but that could be extended and applied in more bioanalytical methods and different substrates. Hence, despite several papers published related to biosensing of glucose, no similar report was found that built 3D-nanostructured bi-enzymatic biosensors with reactions in cascades of GOx and HRP using the LBL method combined with biotin–avidin affinity conjugation and the effect of the alternated architectures were evaluated. Moreover, the biosensor has given a qualitative response to the presence of glucose in commercial beverages with the presence of all possible interferents.

2. Materials and methods

2.1. Materials

Hydrogen peroxide (33%, v/v), ammonium hydroxide (30%) and hydrochloric acid (>96%) were supplied by Vetec, Brazil. Nitric acid (65%), methanol (99.8%) and ethanol (95%) were supplied by Synth, Brazil. 3-Aminopropyl-trimethoxysilane (APTMS, 97%), 3-mercaptopropyl-trimethoxysilane (MPTMS, 95%), 1,5-pentane-dial (glutaraldehyde, GA, 25% aqueous solution), glucose oxidase from *Aspergillus niger* (GOx), glucose (99.5%), horseradish peroxidase (HRP), 3,3′,5,5′-tetramethylbenzidine (TMB), biotin *N*-hydroxysuccinimide ester (NHS-biotin, ≥98% HPLC), avidin from white egg (≥98%), dimethyl sulfoxide (DMSO), Tween-20, sodium phosphate dibasic (>99%), sodium chloride (>99.5%), potassium chloride (>99%), potassium phosphate monobasic (>99%), methanol (>99%), and acetic acid (>99%) were purchased from Sigma–Aldrich, USA. De-ionized (DI) water was obtained from Milli-Q® (Millipore) purification system (resistivity ≥18.2 MΩ cm).

Microscope glass slides (soda-lime glass, chemical composition: $SiO_2 = 75 \pm 5$ wt%; $Na_2O = 15 \pm 2$ wt%; CaO + MgO = 10 ± 2 wt%) and amine modified polystyrene microplates (PS-NH_2, Corning® 96 Well Clear Polystyrene Amine Surface Stripwell™ Microplate, 2×10^{13} reactive sites/cm^2, Product No. 2388) were used as insoluble solid supports.

2.2. Chemical functionalization of insoluble support

2.2.1. Method of surface functionalization

Glass tile surfaces were prepared with two silane coupling agents (Fig. 1a) with different chemical functionality (R_n): thiol (R_1: —SH) (Fig. 1b) and amino (R_2: —NH_2) (Fig. 1c). Glass slides with no chemical modification ("as-supplied") were utilized for enzyme adsorption and compared to the covalently immobilized systems. Prior to silane reaction, glass surfaces were etched by immersion in 20% nitric acid at room temperature (RT = 25 ± 3 °C) for 24 h, followed by rinsing with abundant amount of de-ionized (DI) water. Hydroxylation (–OH) was conducted by soaking glass slides in a 70:30 mixture of DI water:hydrogen peroxide (H_2O_2, 33%, v/v) at 75 ± 5 °C for 45 min. Then, 5 mL of NH_4OH (conc.) was added for each 100 mL of the H_2O:H_2O_2 solution. After cooling, glass slides were copiously rinsed with DI water and dried in methanol. The increase in the hydroxyl groups is usually recommended before performing silane reactions on the surfaces. This process enhances the density of available silanol (Si—OH) sites for silane reaction, improving the efficiency and repeatability of the surface modification process. The effect of this hydrophilic chemical functionalization with silanol groups in the immobilization and activity of enzymes was also evaluated (Fig. 1d).

The surface modification procedure with organosilanes was performed using the sol–gel deposition process from aqueous/alcohol solution (75% distilled water:25% methanol, pH = 4.5 ± 0.2 with acetic acid). The specific organosilane reagent was added to yield a 2% (v/v) final concentration, allowing 10 min for the hydrolysis of alkoxide and silanol formation. Next, the glass slides were immersed in this solution for 30 min and then rinsed with ethanol to remove unbound silane from the glass surface and cured at temperature of 110 ± 10 °C for 30 min for condensation reactions. This procedure is important because it assures that the excess of organosilanes precursors and also unreacted species were fully removed before proceeding to the next step of glutaraldehyde linking.