Sensors and Actuators B 231 (2016) 837–846

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

Colorimetic biosensing dispositive based on reagentless hybrid biocomposite: Application to hydrogen peroxide determination

J. Pla-Tolós, Y. Moliner-Martinez, C. Molins-Legua *, P. Campins-Falcó *

Department of Analytical Chemistry, Faculty of Chemistry, University of Valencia, Doctor Moliner 50, Burjassot,Valencia, Spain

ARTICLE INFO

Article history:
Received 14 October 2015
Received in revised form 23 February 2016
Accepted 20 March 2016
Available online 22 March 2016

Keywords:
Colorimetric biosensor
3,3′,5,5′-Tetramethylbenzidine
Horseradish peroxidase
Sol-gel entrapment
Enzyme adsorption
Hydrogen peroxide

ABSTRACT

An efficient approach to enhance the performance of colorimetric biosensors has been developed. The biosensor is based on the co-immobilization of the reagent 3,3′,5,5′-teramethylbencidine (TMB) and the enzyme horseradish peroxidase (HRP) in a PDMS-TEOS-SiO_2NPs support. The HRP, in presence of H_2O_2, catalyzes the oxidation of TMB, producing a blue color. The generated biosensor, doped with the substrate (TMB) and the enzyme (HRP) (entrapped or adsorbed), has been used to determine H_2O_2 in real samples. Firstly, the immobilization of TMB and HRP in the composite has been studied in order to find the best suitable configuration. The kinetic parameters V_{max} (maximum reaction rate) and K_m (substrate affinity) of the different assayed systems have been determined and compared. Secondly, the analytical properties of the H_2O_2 method have been obtained. From the analytical point of view, no significant differences were observed between the adsorption and entrapment immobilization procedures used for the HRP. This method is simple, inexpensive, highly sensitive and selective for the determination of H_2O_2, with detection limits of 1.3 μM and a good linearity over the range 4.2–72 μM. The LOD can be improved to 0.4 μM by acidifying the solution with sulphuric acid. Precision was also satisfactory (relative standard deviation, RSD < 10%). Results were compared to those obtained by the conventional derivatization method in solution. The developed biosensor is a reagent-release support which significantly simplifies the analytical measurements, because it avoids the need to prepare derivatization reagents and sample-handling, and it allows in situ measurements.

1. Introduction

Enzymes have the ability to catalyze reactions with a very high degree of substrate specificity and selectivity [1]. Classical enzymatic assays are performed in solution; the reagents are prepared and added in the appropriate proportion. In some cases, these preparations are complex and time-consuming. Nowadays, besides the traditional analytical properties, other factors such as simplicity, cost, toxicity or times are required. For this reason, since Clark and Lyons coined the word "biosensor" in 1962, the scene changed, giving rise to a new field in the miniaturized techniques [2].

Immobilization of the enzyme is a key factor to develop efficient biosensing devices with a good operational and storage stability, high selectivity, short response time and high reproducibility [3,4]. For this aim, several immobilization techniques have been applied, including physical adsorption, covalent binding and entrapment into the material [1,4–7]. Each of these techniques has its own advantages and drawbacks:

Physical adsorption onto solid supports represents the easiest method of physical immobilization. Herein, the enzymes are attached to the matrix through hydrogen bonding or Van der Waals forces. This method may suffer from problems such as enzyme leakage from the matrix when the interactions are relatively weak [4].

Covalent attachment ensures the highest strength of the bonding between the support and the enzyme, minimizing leakage issues. However, the binding reaction has to be performed under conditions which do not affect the active site of the enzyme.

The entrapment method consists of the physical occlusion of an enzyme in the lattice of a polymeric matrix [4,8].

Another critical step in the development of the biosensor is the selection of the support to perform the immobilization. The matrix has to provide a biocompatible and inert environment and it should not interfere with the native structure of the protein [9]. Polydimethylsyloxane is a good choice because it offers an appropriate way to fix biomolecules because of its porosity, optical transparent matrix, low temperature curing and inherent versatility [5,10,11].

* Corresponding authors.
E-mail address: cmolins@uv.es (C. Molins-Legua).

http://dx.doi.org/10.1016/j.snb.2016.03.094
0925-4005/

In addition, the combination of polydimethylsiloxane with crosslinkers such as triethoxymethylsilane (MTEOS) or tetraethyl orthosilicate (TEOS) allows the regulation of the polymerization process, controlling the pore size distribution and improving the resistance of the films to cracking. Consequently, the entrapped enzymes are accessible to react with external species due to the presence of pores [8]. Wang et al. [12] have reported that the incorporation of copolymers into silica based glasses improves the activity of entrapped enzymes, and the storage stability.

One interesting aspect which has to be commented is that, as far as we know, enzymes are immobilized while the reagent is added into the solution, or immobilized separately [13]. Immobilization of all reagents would be a comfortable way to avoid the solution limitations and could achieve a totally "solid-state" scheme, making easy the miniaturized configuration [14].

There are many examples of biosensors capable of determining H_2O_2 in the literature [14–26]. This compound is present in a large variety of products and has a great importance in pharmaceutical, clinical, chemical, industrial, and environmental analyses [19,24,25,27,28]. Some of the proposed sensors are based on the immobilization of the enzyme horseradish peroxidase in a modified electrode [15,19,22,29]. However, these sensors need instrumentation to perform the analysis and the results cannot be analyzed by naked eye. Other publications come up with sensors based on colorimetric methods, but, as it has been comment above, only the enzyme is immobilized while the substrate is added to the solution separately [26,30,31].

The aim of this paper is the development of a biosensor based on the integration of all the reagents in a solid support, in order to obtain a biocomposite with the enzyme and the substrate co-immobilized in the same composite. As a model biosensor, the assayed enzyme was horseradish peroxidase (HRP), which catalyzes the oxidation of a wide variety of substrates in presence of H_2O_2 [32,33]. In this case, 3,3′,5,5′-tetramethylbenzidine (TMB) was chosen as a chromogenic peroxidase substrate [32,34,35], which can be easily quantified by colorimetry or visual inspection. In this work, substrate (TMB) and/or enzyme (HRP) have been successfully immobilized together into the same sol-gel matrix of PDMS-TEOS-SiO_2NPs. The films have been used to develop reliable sensors for hydrogen peroxide, which give rise to a blue color that can be measured spectrophotometrically or simply by naked eye. This work improves the toxicity, portability and rapidness of the typically used TMB/HRP catalytic method. This new approach can be a promising tool to determine analytes which requires the use of biomolecules and organic reagents as substrate.

2. Materials and methods

2.1. Instruments

UV–vis measurements were recorded by a HP-8453 UV–vis spectrophotometer from Hewlett Packard (USA) furnished with 1 cm path length quartz microcell. Absorption spectra were registered from 190 to 900 nm. For preparing the composite, ultrasonic bath (300 W) from Sonitech and magnetic stirrer (45 W) from Stuart Scientific was used. Plastic well-plates were used as template to fabricate the sensor (Sharlau, Spain).

2.2. Reagents and solutions

Nanopure water obtained using Nanopure II system (Barnstead, USA) was used for preparation and dilution of all solutions. Sylgard® 184 silicon elastomer base and Sylgard® 184 silicon elastomer curing agent were purchased from Dow Corning (USA). Silicon dioxide was provided from Sigma-Aldrich (China). Tetraethyl orthosilicate (TEOS) and 3,3′,5,5′-tetramethylbenzidine (TMB) were obtained from Sigma-Aldrich (Germany). Hydrogen peroxide 30% was acquired from Merk (Germany). Horseradish peroxidase was obtained from Sigma (USA). Sodium acetate was obtained from Panreac (Spain). Acetic acid was purchased from Sharlau (Spain).

2.3. Preparation of the PDMS-TEOS-SiO_2NPs-TMB composites

PDMS-TEOS-SiO_2NPs-TMB composites were prepared by mixing 8 mg of 3,3′,5,5′-tetramethylbenzidine and 5 mg of silicon dioxide in 2 g of TEOS, under ultrasonic conditions during 10 min. Then, 2 g of base were added to the mixture, followed by vigorous stirring for 15 min. After that, the curing agent was added to the dispersion at a weight ratio of 1:10 to the base and stirred 15 min. The individual films were formed weighting 0.2 g of the blend in a well polystyrene plate (d = 1 cm). Finally they were cured at 30 °C during 12 h. The preparation is based on the patent developed by Campins-Falcó et al. [36]

2.4. Preparation of the PDMS-TEOS-SiO_2NPs-TMB-HRP biocomposites

Two varieties of PDMS-TEOS-SiO_2NPs-TMB-HRP composites were tested: (i) the HRP was entrapped inside the matrix; (ii) the enzyme was adsorbed on the surface.

PDMS-TMB-HRP (entrapped) biosensors were developed by mixing 8 mg of 3,3′,5,5′-tetramethylbenzidine, 5 mg of silicon dioxide and 20 μl of a dispersion of HRP in TEOS (1 mg ml^{-1}) in 2 g of TEOS, under ultrasonic conditions during 10 min. Then, 2 g of base were added to the suspension, followed by vigorous stirring for 15 min. After that, curing agent was added to the dispersion at a weight ratio of 1:10 to the base and stirred for other 15 min. The individual PDMS composites were obtained weighting 0.2 g of the blend in a well polystyrene plate (d = 1 cm). Finally they were cured at 30 °C during 12 h.

PDMS-TEOS-SiO_2NPs-TMB-HRP (adsorbed) composites were synthesized by mixing 8 mg of 3,3′,5,5′-tetramethylbenzidine and 5 mg of silicon dioxide in 2 g of TEOS. The blend was sonicated during 10 min and 2 g of base were added to the suspension, followed by vigorous stirring for 15 min. Curing agent was added to the dispersion at a weight ratio of 1:10 to the base and stirred for other 15 min. The individual PDMS composites were formed weighting 0.2 g of the blend in a well polystyrene plate (d = 1 cm) and cured at 30 °C during 12 h. Then, composites were placed above 200 μl of a solution 0.03 mg ml^{-1} of HRP in carbonate buffer (pH 9, 0.05 M) and left to react for 1 h (each side) at room temperature. Lastly, the composites were rinsed with phosphate buffer at pH 7.

The dimensions of the films were 1 cm diameter and 0.6 mm thickness.

2.5. H_2O_2 measurement procedures

Two hydrogen peroxide measurements were carried out: firstly, when all the reagents were placed in the solution (solution derivatization); secondly, by using the different configurations studied with the composite.

2.5.1. Solution derivatization

The enzyme HRP catalyzes the TMB oxidation by hydrogen peroxide, producing the apparition of a blue color with a maximum at 653 nm which corresponds to the one electron oxidation of TMB. The blue color intensity increased with reaction time. To perform this reaction, 1 ml of buffer NaAc/HAc (0.1 M pH 5), 20 μl of TMB (3.5 mg ml^{-1}), 20 μl of HRP (1 μg ml^{-1}) and the hydrogen peroxide,