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Enzyme Sensors Modified with Avidin/Biotin System-based Protein Multilayers

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Abstract: Enzyme multilayers composed of avidin and biotin-labeled enzymes were prepared on the surface of electrode, through a strong affinity between avidin and biotin (binding constant: ca. 10^{15} M^{-1}). The enzyme multilayers were useful for the improvement of the performance characteristics of enzyme sensors. The output current of the enzyme sensors depended linearly on the number of enzyme layers deposited. Thus, lactate oxidase (LOx) and alcohol oxidase (AlOx) were deposited after being modified with biotin for constructing enzyme sensors sensitive to L-lactate and ethanol respectively. It was also possible to deposit two different kinds of enzymes successively in a single multilayer. The glucose oxidase (GOx) and ascorbate oxidase (AsOx) were built into a multilayer structure on a Platinum electrode. The GOx-AsOx multilayer-modified electrode was useful for the elimination of ascorbic acid interference of the glucose sensor.

Keywords : Enzyme multilayer, Enzyme sensor, Avidin-biotin complexation, Glucose oxidase, Lactate oxidase, Alcohol oxidase, Ascorbate oxidase.

1. Introduction

An avidin-biotin system has been widely used in a variety fields such as affinity chromatography[1], binding assay[2], and immunochemical stains[3]. Avidin is a highly stable glycoprotein (molecular mass:67,000) found in egg-white. It is isolated as a tetramer of identical 128-residue polypeptide chains. The most characteristic feature of avidin is that each subunit contains a binding site to biotin and forms a highly stable complex with biotin or its derivatives.

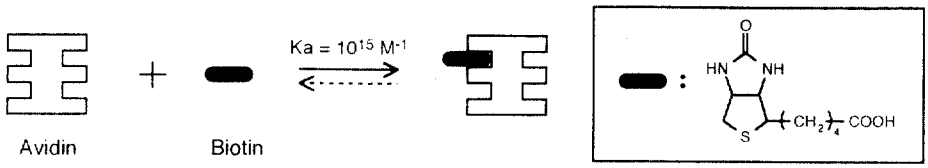

Fig. 1. The complexation between avidin and biotin.

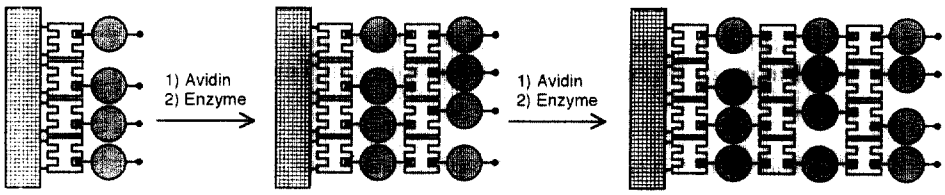

Fig. 2. Stepwise deposition of avidin and biotin-labeled enzymes on a Pt electrode surface.

the association constant being reported to be ca. 10^{15} M^{-1}[4](Fig.1). For this reason, many kinds of biotin- or avidin-labeled reagents, including fluorophores, lectins, DNA and RNA, and enzymes have been developed and are now commercially available.

Based on the strong non-covalent binding between avidin and biotin-labeled enzymes, several authors have reported the preparation of catalytically active enzyme layers on the surface of electrodes[5-8]. We have reported that avidin can be deposited on the electrode surface to form monomolecular or multimolecular layer, by a simple adsorption or electrodeposition[9-14]. A stepwise deposition of avidin monolayer and enzyme monolayer was also possible to prerare a multilayer structure of proteins. Thus, enzyme sensors equipped with a glucose oxidase (GOx) multilayer were developed and the output current of the sensor was controlled by regulating the number of the GOx layers deposited [15-17].

The present paper reports the preparation of enzyme sensors modified with lactate oxidase (LOx) and alcohol oxidase (AlOx) multilayers. The enzyme multilayers composed of GOx and acorbate oxidase (AsOx) monolayers were also prepared to construct the enzyme sensors which are free from an ascorbic acid interference.

2.Experimental

A commercially available avidin (Calzyme Lab.) was used without further purification. The enzymes (GOx, LOx, AlOx, and AsOx) were labeled with biotin using biotinyl-N-ε-aminocaproyl-N-hydroxy-succinimide ester according to the reported procedure[18]. The modified enzymes contain several biotin residues in a single molecule.

A typical procedure for the preparation of enzyme multilayer is as follows; a platinum (Pt) disk electrode (3 mm diameter) was polished with alumina powder and sonicated in distilled water. The Pt electrode was immersed in an avidin solution (100 μg·ml), a phosphate buffered-saline, and a biotin-labeled enzyme solution (100 μg·ml) successively for 20-30 min each. By this treatment, the Pt electrode can be modified with a double-layer composed of avidin monolayer and biotin-labeled enzyme monolayer. The same procedure was repeated in order to deposit the protein multilayers (Fig. 2). The electrochemical measurements of the enzyme multilayer-modified electrodes were carried out in a

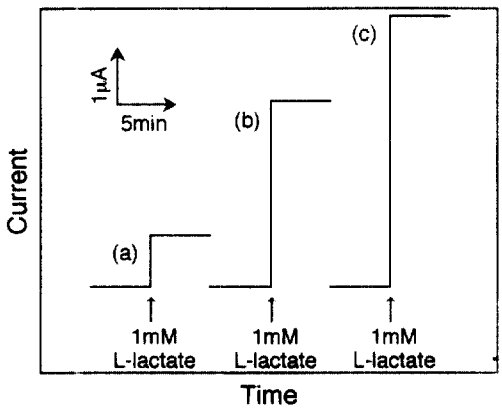

Fig. 3. Typical responses of the avidin-LOx multilayer-modified lactate sensors to 1 mM L-lactate. LOx layers: monolayer (a), 5 layers (b), and 10 layers (c).

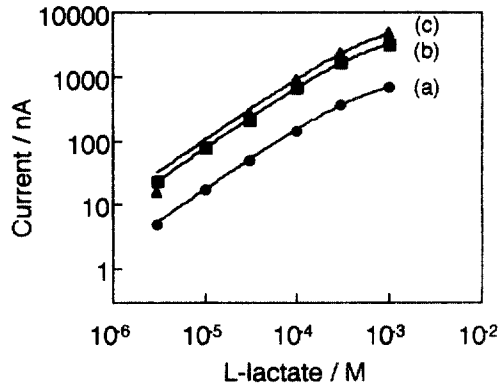

Fig. 4. The calibration graphs of the avidin-LOx multilayer-modified sensors. LOx layers: monolayer (a), 5 layers (b), and 10 layers (c).

phosphate buffer pH 7.4) at 23 °C, using a Pt counter electrode and a Ag/AgCl reference electrode. The electrode potential was set at 0.6 V.

3. Results and Discussion

Figure 3 shows a typical response of the lactate sensors modified with a monolayer, 5-layer and 10-layer LOx. The electrochemical response of the lactate sensors originates from the fact that LOx catalyzes the oxidation reaction of L-lactate, in the presence of oxygen, to produce pyruvate and hydrogenperoxide (H_2O_2) (Eq. 1), the latter of which can be oxidized at the Pt electrode surface at 0.6 V vs Ag/AgCl. The output current (Δi) of the lactate

$$\text{L-Lactate} + O_2 \xrightarrow{\text{LOx}} \text{Pyruvate} + H_2O_2 \qquad (1)$$

sensors increased with increasing the LOx layers, suggesting that the LOx was accumulated successively on the electrode surface through avidin-biotin complexation and that the biotin-labeled LOx is still catalytically active in the multilayer structure. On the contrary, when native LOx bearing no biotin residue was used in place of biotin-labeled LOx, Δi was small and did not increase even after several treatments with avidin and LOx. This shows clearly that the repeated deposition of avidin and biotin-labeled LOx relies on the specific binding between avidin and biotin.

Another interesting feature of the lactate sensors is that the response time was very fast (ca. 10 sec) and remained virtually unchanged, irrespective of the number of LOx layers. These results suggest that the enzyme layers are so thin that they do not influence significantly the mass transfer of analyte and reaction products of the enzymatic reaction. In this case, the response time of the sensors would be determined by the rate of the enzymatic reaction.

Figure 4 illustrates calibration graphs of lactate sensors loaded with a monolayer, 5 layers and 10 layers of biotin-labeled LOx. The monolayer-modified sensor gave a useful calibration to L-lactate concentration ranging from 1×10^{-3} to 1×10^{-5} M, below which the output current was too small (less than 10 nA) for practical use. The lower detection limit was extended to 0.5×10^{-6} M by the deposition of 10 LOx layers.

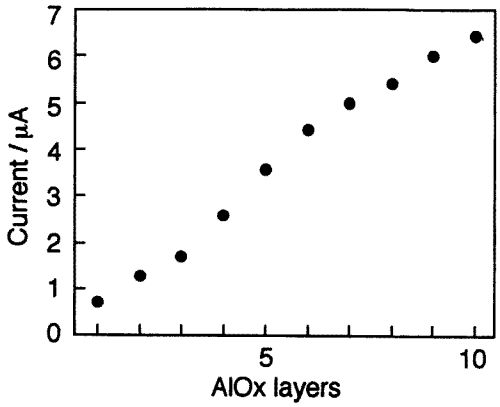

Fig. 5. Effect of successive deposition of biotin-labeled AlOx layers on the output current of alcohol sensor.
Ethanol concentration: 10 mM.

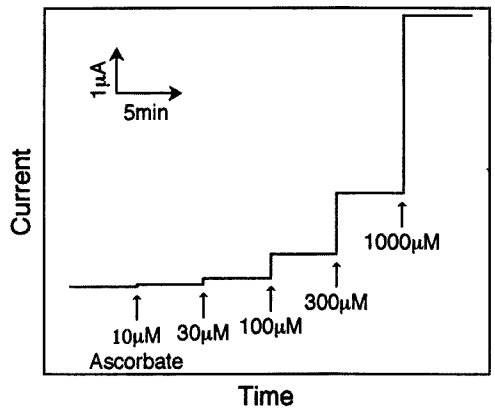

Fig. 6. The response of 10-layer GOx-modified glucose sensor to ascorbic acid.

Another example of the enzyme multilayer-modified sensor is alcohol sensors, which were prepared by depositing avidin AlOx multilayers on a Pt electrode. AlOx catalyzes the oxidation reaction of ethanol to acetoaldehyde (Eq. 2). The resultant

$$CH_3CH_2OH + O_2 \xrightarrow{AlOx} CH_3CHO + H_2O_2 \qquad (2)$$

H_2O_2 can be oxidized electrochemically on the Pt surface. Figure 5 plots the output current of the alcohol sensors to 10 mM ethanol as a function of the number of AlOx layers on the electrode. A clear dependence of the Δi value on the number of AlOx layers was observed. The AlOx multilayer sensor can be used for the determination of μM and mM level of ethanol in solution. The long-term stability of the alcohol sensor was examined for one month. The Δi value to 10 mM ethanol was recorded once a day, and the sensor was stored in the working buffer at 4 °C when not in use. More than 90% response of the original Δi value was maintained after one month.

Thus, it is clearly demonstrated that the alternate and repeated deposition of avidin and biotin-labeled LOx and AlOx can give catalytically active enzyme multilayers and that the total activity of the enzyme multilayers depends directly on the number of enzyme layers.

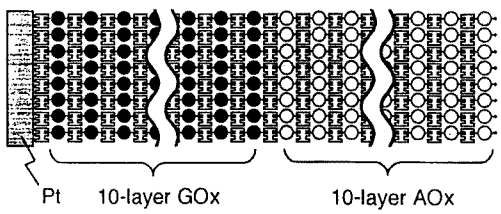

Fig. 7. A schematic representation of an idealized multilayer structure composed of GOx and AsOx on the Pt electrode.

One of the drawbacks of the electrochemical enzyme sensors is the interference arising from the direct oxidation of easily oxidizable substances such as ascorbic acid and uric acid in biological fluid. In order to eliminate the interference, the surface of the enzyme sensors are often covered with an appropriate permselective membrane. For example, the negatively charged Nafion and Eastman AQ membranes have been used to eliminate interferences from the anionic ascorbic acid and uric acid[19,20]. Cellulose

membranes are also used to minimize the interference, due to the size exclusion properties[21]. An alternative strategy for the elimination of interference is to use an outer enzyme layer in which the interfering substances can be decomposed enzymatically. Thus, AsOx has been used to eliminate the ascorbic acid interference[21].(Eq. 3).

$$\text{Ascorbic acid} + 1/2\,O_2 \xrightarrow{\text{AsOx}} \text{Dehydroascorbic acid} + H_2O \quad (3)$$

These results have prompted us to prepare enzyme multilayers composed of GOx and AsOx layers, for the development of glucose sensors which are free from ascorbic acid interference.

Figuer 6 shows a typical response of the glucose sensor modified with 10-layer GOx membranes to ascorbic acid. The sensor exhibited a high response to ascorbic acid at 0.6 V vs Ag/AgCl (in other words, a significant interference to glucose determination);the oxidation current for ascorbic acid being higher than that for the same concentration of glucose. Thus, the physiological level of ascorbic acid in blood (ca. 0.1 mM) would cause a significant overlapping in the output current of the sensor in glucose determination. It should be noted here that a bare Pt electrode gave a several times higher oxidation current than the 10-layer GOx-modified electrode to the same concentration of ascorbic acid. This means that the diffusion of ascorbic acid from solution to the surface of Pt electrode is suppressed to a considerable extent by the 10-layer avidin/GOx multilayer. However, it is clear the further elimination of ascorbic acid interference is still needed.

We have prepared enzyme multilayers composed of 10 GOx layers plus outer 10 layers of AsOx on the Pt electrode (Fig. 7), by a stepwise deposition of avidin and biotin-labeled GOx and AsOx. In this configuration of GOx and AsOx layers, one can expect that ascorbic acid is oxidized to the electrochemically inactive form (dehydroascorbic acid) in the outer AsOx layer and the concentration of ascorbic acid at the Pt surface can be lowered depending on the catalytic activity of the AsOx layer.

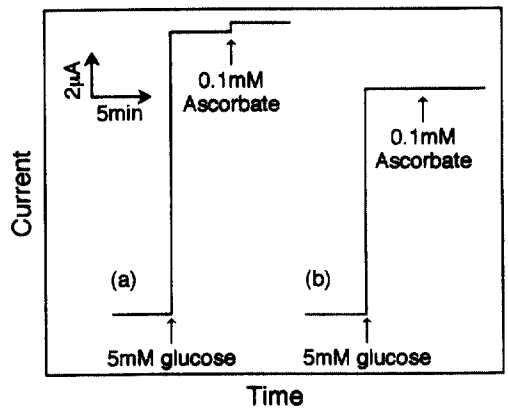

Fig. 8. Effect of ascorbic acid on the glucose determination by glucose sensors with and without AsOx outer layers.
(a) 10-layer GOx modified sensor and (b) (10-layer GOx plus 10-layer AsOx)-modified sensor.

On the other hand, the outer AsOx layers may have no significant influence on the diffusion of glucose into the GOx layers. Thus, we can eliminate the ascorbic acid interference based on the GOx/AsOx multilayer-modified sensors. Figure 8 depicts the response of the 10-layer GOx-modified glucose sensors with and without the outer AsOx layers. The influence of the physiological level of ascorbic acid (0.1 mM) on the output current of the sensors to 5 mM glucose was recorded. The glucose sensor without AsOx layers showed ca. 12 μA of output current in the 5 mM glucose solution and the further addition of 0.1 mM ascorbic acid induced 0.4 μA of interference (Fig. 8-a). On the contrary, as shown in Fig. 8-b, the ascorbic acid interference was eliminated completely in the case of the GOx/AsOx-modified sensor. These results show that the outer AsOx layers catalyzed the oxidation reaction of ascorbic acid to reduce the concentration of ascorbic acid at the Pt electrode surface.

Conclusions

We have demonstrated that enzyme multilayers can be prepared on an electrode surface using avidin and

biotin labeled enzymes, by a stepwise deposition. The deposition was performed simply by immersing the electrode in the avidin and biotin-labeled enzyme solutions, alternately. The avidin and enzyme layers are connected with each other through a strong non-covalent binding between avidin and biotin. The enzyme loading or the number of enzyme layers can be controlled by regulating the deposition number, which enabled a stepwise and precise control of the size of the output current of the enzyme sensors.

It is also possible to construct bienzyme multilayers composed of monolayers of GOx and AsOx. The GOx AsOx multilayer-modified glucose sensor is useful for the elimination of ascorbic acid interference, because the interfering ascorbic acid can be oxidized in the AsOx layers to the electrochemically inactive dehydroascorbic acid. Thus, it is possible to determine the blood level of glucose without ascorbic acid interference.

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