A Layer-by-Layer Deposition of Concanavalin A and Native Glucose Oxidase to Form Multilayer Thin Films for Biosensor Applications

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Glucose oxidase (GOx) can be assembled into multilayer thin films without any chemical modification, by an alternate deposition of Concanavalin A (Con A) and native GOx on the surface of electrode. The Con A/GOx multilayer film-modified platinum electrodes functioned as a glucose biosensor.

The multilayer thin films containing enzymes have been studied extensively for biosensor applications. Willner et al. have prepared enzyme multilayer films on a surface of electrode by treating the electrode with enzyme and cross-linking agents such as dialdehyde and diisothiocyanate.[1] A similar technique was used for the preparation of fiber optic enzyme sensors.[2] On the other hand, Bourdillon et al. have used an immunological technique for the preparation of protein thin films. They deposited antibody and antigen-modified enzyme alternately on an electrode surface to fabricate enzyme biosensors.[3] In this context, we have reported that protein multilayer films can be constructed by a layer-by-layer deposition of avidin and biotin-modified enzymes[4] or Concanavalin A (Con A) and mannose-labeled enzymes,[5] through a strong affinity between avidin and biotin or Con A and mannose, respectively. The enzyme multilayer films prepared by the biological interactions were successfully used to improve the performance characteristics of enzyme sensors. A drawback of these procedures is that, prior to the film preparation, enzymes have to be modified with ligand molecules such as antigen, biotin, and mannose. The procedure for the modification of enzyme and its purification is somewhat time-consuming and, sometimes, the catalytic activity of the enzyme may be impaired by the chemical modification. Recently, we succeeded in overcoming this drawback by using native GOx without any chemical modification. We report here the preparation of multilayer thin films composed of Con A and native GOx on the surface of a platinum (Pt) electrode and their catalytic activity.

It is known that GOx is a glycoprotein and contains intrinsic α-D-mannose residues on the surface of the polypeptide chains.[6] Therefore, it may be possible to construct Con A/GOx multilayer assembly because Con A contains four identical binding sites to α-D-mannose (Figure 1).[7]

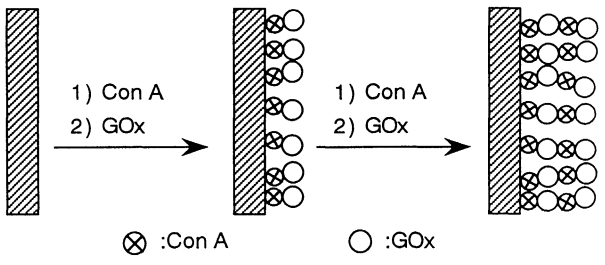

Figure 1. Schematic representation of a layer-by-layer deposition of Con A and GOx.

The formation of a multilayer film was evaluated on the basis of UV spectrophotometry using fluorescein-modified Con A (F-Con A) and GOx (F-GOx). A commercially available F-Con A was used as received (molar extinction coefficient: 486000 M^{-1} cm^{-1} at 495 nm). F-GOx was prepared from GOx (from *Aspergillus niger*) and fluoresceinisothiocyanate according to the reported procedure (molar extinction coefficient: 344000 M^{-1} cm^{-1} at 495 nm).[8] The F-Con A/GOx and Con A/F-GOx multilayer films were prepared by an alternate deposition on a quartz slide. The surface of the quartz slide was first made hydrophobic by the treatment with 10% dichlorodimethylsilane solution in toluene overnight, because Con A is known to be adsorbed on the hydrophobic surface.[5] The silylated quartz slide was immersed in a Con A or F-Con A solution (100 μg ml^{-1} in 0.1 mol dm^{-3} Dulbecco's phosphate buffered saline (D-PBS)) for 30 min at ca. 20 °C. After being rinsed with PBS, the quartz slide was immersed in a F-GOx or GOx solution (100 μg ml^{-1} in 0.1 mol dm^{-3} D-PBS) for 30 min. By this treatment, Con A/F-GOx or F-Con A/GOx double layer would be deposited on both surfaces of the quartz slide. The deposition was repeated 10 times and the absorbance of the quartz slide was monitored at 495 nm after each deposition.

An absorbance of the F-Con A/GOx and Con A/F-GOx multilayer-modified quartz slides was shown in Figure 2 as a function of the number of deposition of the layers. The absorbance increased linearly with increasing the number of deposition for both films, which clearly shows that a constant amount of F-Con A or F-GOx was immobilized upon each deposition to form layer-by-layer structures. In order to check whether or not the specific binding between Con A and mannose residues in GOx is responsible for the formation of the multilayer films, the same procedure was carried out in the presence of $1x10^{-2}$ mol dm^{-3} methyl-α-D-mannopyranose in the Con A solution, in which the binding sites of Con A should be occupied by dissolved methyl-α-D-mannopyranose. As expected the multilayer film did not grow in the presence of methyl-α-D-mannose. This shows clearly that the driving force of the

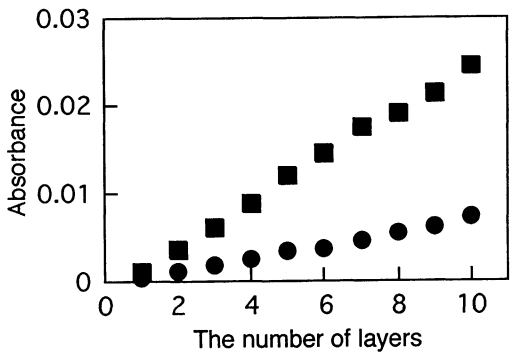

Figure 2. Absorbance of the F-Con A/GOx (■) and Con A/F-GOx (●) multilayer films as a function of the number of deposition.

multilayer formation is not nonspecific adsorption but specific binding between Con A and mannose residues in GOx.

One can estimate the loading (or surface coverage) of F-GOx and F-Con A in each layer of the multilayer film on the basis of its UV absorption. Using molar extinction coefficients of 344000 M^{-1} cm^{-1} for F-GOx and 486000 M^{-1} cm^{-1} for F-Con A and assuming that, upon each deposition, F-GOx and F-Con A form close-packed monomolecular layers, the loading of F-GOx and F-Con A is calculated to be 2.6×10^{-12} and 4.3×10^{-12} mol cm^{-2}, respectively (for simplicity, F-GOx and F-Con A were approximated to be spherical in shape with a density of 1.3 g cm^{-3}).[6,9] The experimental data in Figure 2 show that the loading of F-GOx and F-Con A is ca. 1.1×10^{-12} and 2.7×10^{-12} mol cm^{-2}, respectively. Consequently, the surface coverage in the films is ca. 42% for F-GOx and ca. 63% for F-Con A. These results may be reasonable in view of the fact that the actual molecular shape of the proteins is much more complicated than the spherical model employed here. The surface coverage is slightly lower than that of the GOx/Con A multilayer film prepared using mannose-labeled GOx,[5] probably due to a lack of mannose residues.

The Con A/GOx multilayer films were prepared on the surface of Pt electrode using unmodified Con A and GOx to assess the catalytic activity. The response of the Con A/GOx multilayer-modified Pt electrode to β-D-glucose was evaluated by measuring the current at +0.6 V *vs* Ag/AgCl originating from the electro-oxidation of hydrogen peroxide produced in the enzymatic reaction (Eq. 1). Figure 3 plots the output current of the

$$\text{Glucose} + O_2 \xrightarrow{\text{GOx}} \text{Gluconolactone} + H_2O_2 \quad (1)$$

modified electrodes to 1×10^{-3} and 5×10^{-3} mol dm^{-3} β-D-glucose as a function of the number of Con A/GOx layers. The response current depended linearly on the number of layers, showing a constant amount of GOx was immobilized upon each deposition. Thus, the electrochemical and UV absorption results clearly show that the catalytically active thin films composed of GOx multilayers can be prepared using native GOx and Con A.

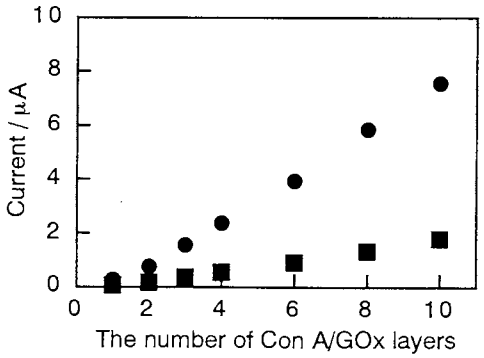

Figure 3. The output current of the Con A/GOx multilayer film-modified electrodes to 1×10^{-3} (■) and 5×10^{-3} mol dm^{-3} (●) β-D-glucose solutions as a function of the number of the layers.

The response characteristics of the Con A/GOx multilayer-modified electrode as glucose sensor were evaluated in the presence of β-D-glucose. The Con A/GOx modified glucose sensors showed a rapid response to β-D-glucose, the response time being 10-15 sec even for the sensor modified with 10 Con A/GOx layers. The rapid response of the sensors can be ascribed to the thin nature of the Con A/GOx films. The thickness of the Con A/GOx unit layer is calculated to be 10-15 nm on the basis of their molecular dimensions.[6,7] The glucose sensors exhibited satisfactory calibration graphs over the concentration range of 1×10^{-5} - 3×10^{-2} mol dm^{-3} β-D-glucose, which covers the normal and diabetic blood level of β-D-glucose. The apparent Michaelis constant (K_m^{app}) for the Con A/GOx multilayer films were 1.0×10^{-2}, 2.3×10^{-2}, and 1.7×10^{-2} mol dm^{-3} for the monolayer, 5-layer, and 10-layer Con A/GOx films. In order to check the long-term stability of the sensor, the 10-layer Con A/GOx-modified sensor was used once a day for two months and stored in the phosphate buffer at 4 °C when not in use. About 50% and 40% responses of the original value were observed after one and two months, respectively. The K_m^{app} values and stability of the sensors are nearly comparable to those reported for the GOx multilayer films prepared using mannose- or biotin-labeled GOx.[4,5]

We have found in a separate experiment that the catalytic activity of GOx in solution was decreased down to ca. 70% of the original activity when GOx was modified covalently with mannose according to the reported procedure.[5] Therefore, the present technique is useful to circumvent this problem.

In summary, Con A/GOx multilayer films can be prepared using native GOx without any chemical modification and the resulting GOx multilayer films are useful for the sensitive layer of glucose biosensors. The present procedure may be applicable to other glycoproteins such as glucoamylase and peroxidase.

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