Alternate deposition of concanavalin A and mannose-labelled enzymes on a solid surface to prepare catalytically active enzyme thin films

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Concanavalin A (Con A) and mannose-labelled enzymes (glucose oxidase and lactate oxidase) have been deposited alternately on a solid surface to prepare Con A–enzyme composite thin films in which enzymes are catalytically active.

A layer-by-layer preparation of enzyme thin films is one of the focal subjects in biomaterial sciences. Willner and co-workers have found that enzyme multilayer films can be constructed on the surface of electrodes by treating the electrode with enzyme and cross-linking agents such as dialdehyde and diisothiocyanate.[1] Bourdillon *et al*. have used antigen–antibody interaction for the preparation of enzyme multilayer-based biosensors.[2] In this context, we have reported that enzyme multilayer films can be prepared by a layer-by-layer deposition of avidin and biotin-labelled enzymes on the surfaces of a quartz slide and a platinum (Pt) electrode, through a strong affinity of avidin and biotin.[3] The enzyme thin films were useful for improving the response characteristics of enzyme biosensors.[1–3] In spite of the successful use of the cross-linkers[1] and biomaterials,[2,3] the strategy for the construction of multilayer films of enzyme is still limited. In the present communication, we report another protocol for the formation of layer-by-layer structure of enzyme films, using a lectin–saccharide interaction. We have used concanavalin A (Con A) (Vector Lab., USA) as a prototype of lectin and either mannose-labelled glucose oxidase (m-GOx) or lactate oxidase (m-LOx). Though the Con A–mannose interaction has been used for designing biofunctional electrodes,[4] the layer-by-layer construction of enzyme films composed of Con A and mannose-labelled enzyme has not been observed.

Con A is a lectin protein (molecular weight; 104 000) found in Jack bean and is known to contain four identical binding sites to α-D-mannose.[5] Therefore, if an enzyme molecule is labelled with several residues of mannose, we can expect that an alternate and repeated deposition of Con A and the mannose-labelled enzyme gives multilayer structure as illustrated schematically in Fig. 1.

Mannose-labelled enzymes were prepared as follows: 1 mg enzyme and 0.5 mg mannopyranosylphenylisothiocyanate (MPITC, Sigma Co., USA) were dissolved in 0.1 M $NaHCO_3$ solution (0.5 ml), and the solution was stirred for 2 h at *ca*. 20 °C. The unreacted MPITC was removed from the reaction mixture by centrifugal filtration.

In order to evaluate the formation of a multilayer film based upon spectrophotometry, fluorescein-modified Con A (F-Con A) (Vector Lab., USA, molar extinction coefficient of F-Con A is 486 000 M^{-1} cm^{-1} at 495 nm) and either m-GOx or m-LOx were deposited alternately on a quartz slide to form the layer-by-layer structure. The quartz slide (5 × 1 × 0.1 cm) was first treated in a 10% dichlorodimethylsilane solution in toluene overnight to make the surface hydrophobic. The silylated quartz slide was immersed in a F-Con A solution [100 μg ml^{-1}, 0.1 M phosphate buffered saline (PBS)] for 60 min at *ca*. 20 °C to deposit the first layer of F-Con A. After being rinsed with the buffer for 10 min, the quartz slide was immersed in a m-GOx or m-LOx solution (100 μg ml^{-1}, 0.1 M PBS) for 60 min and rinsed. This treatment would provide a double layer of F-Con A and enzyme on both surfaces of the quartz slide. The deposition was repeated 10 times and the absorbance of the quartz slide at 495 nm, originating from the fluorescein moiety, was measured after each deposition.

Fig. 2 shows an increase in absorbance of the F-Con A–m-GOx and F-Con A–m-LOx multilayer-modified quartz slides as a function of the number of deposition of the layers. For both cases, absorbance increased in proportion to the number of depositions, suggesting that a constant amount of F-Con A was immobilized upon each deposition to form a multilayer structure on the quartz slide. The adsorption of the first layer of F-Con A on the surface of quartz slide relies probably upon the hydrophobic interaction, while, after the second layer, the strong affinity between Con A and mannose should be responsible for the formation of the enzyme multilayers. In contrast, the absorbance did not increase when unmodified GOx and LOx were used in place of m-GOx and m-LOx, respectively.

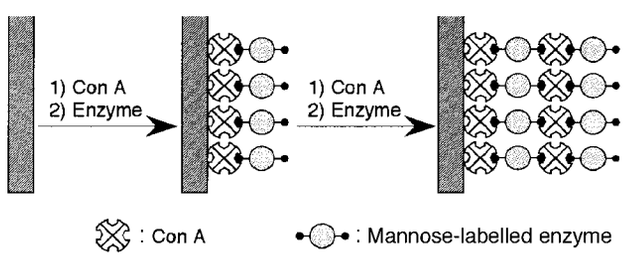

Fig. 1 Schematic representation of layer-by-layer deposition of Con A–enzyme multilayers

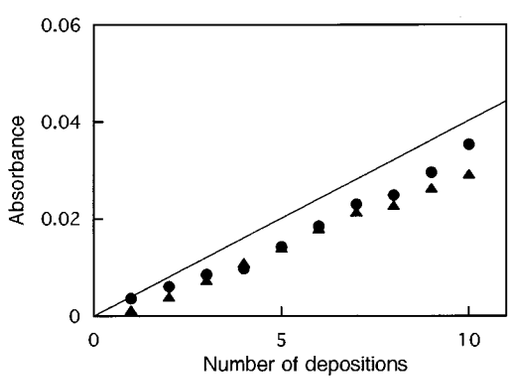

Fig. 2 Absorbance of the multilayer-modified quartz slide at 495 nm as a function of the number of deposition: (●); F-Con A–m-LOx multilayer and (▲); F-Con A–m-GOx multilayer. The straight line shows a calculated value for the formation of monomolecular layers.

Table 1 Catalytic activity of the enzyme multilayer films as a function of the number of enzyme layers

No. of enzyme layers, n	Enzyme multilayer			
	$(\text{Con A–m-GOx})_n$		$(\text{Con A–m-LOx})_n$	
	I_{max}/μA	K_m^{app}/mM	I_{max}/μA	K_m^{app}/mM
1	3.0	26	0.2	0.36
5	22	19	3.1	0.42
10	37	13	8.0	0.50

This is further support of the Con A–mannose interaction as the driving force for the multilayer formation. The loading of F-Con A in each layer was estimated using the following approximations regarding the shape and size of Con A. Although Con A is known to have a 222 symmetry of tetramer composed of $3.9 \times 4.0 \times 4.2$ nm^3 polypeptides,[5] the shape of Con A would induce complexity into the calculation of surface area occupied by a single Con A molecule in the close packing formation. For this reason, in this study, Con A was simply approximated to be spherical with a density of 1.3 g cm^{-3}.[6] Assuming that F-Con A forms a monomolecular layer in close packing on the surface of the quartz slide upon each deposition and using a molar extinction coefficient of 486 000 M^{-1} cm^{-1} for F-Con A at 495 nm, the slope of the plot is calculated to be *ca*. 0.0040 per deposition. The slopes of the plots in Fig 2 are *ca*. 0.0031 for the F-Con A–m-GOx multilayer and *ca*. 0.0036 for the F-Con A–m-LOx multilayer, showing that the coverage of F-Con A is *ca*. 78% (F-Con A–m-GOx) and *ca*. 90% (F-Con A–m-LOx) of the monomolecular layer. Thus, the F-Con A layer was found to be in nearly a monomolecular layer in both enzyme multilayers.

The enzyme multilayer films were prepared on the surface of a Pt electrode and the catalytic activities of the enzymes were assessed by measuring the oxidation current which originates from the electrochemical oxidation of hydrogen peroxide (H_2O_2) produced in the enzymatic reactions (1) and (2). Both

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

$$\text{Lactate} + O_2 \xrightarrow{\text{LOx}} \text{Pyruvate} + H_2O_2 \quad (2)$$

m-GOx and m-LOx exhibited catalytic activity in the oxidation reactions (1) and (2). Table 1 summarizes the catalytic activities of the enzymes in the 1-, 5- and 10-layer films. The maximum current (I_{max}) and apparent Michaelis constant (K_m^{app}) were obtained using the Eadie–Hofstee form of the Michaelis–Menten equation (3),[3,7] where I is the steady-state current,

$$I = I_{max} - K_m^{app}(I/C) \quad (3)$$

I_{max} is the maximum current under stationary substrate conditions, K_m^{app} denotes the apparent Michaelis constant and C is the concentration of substrate. For both enzymes, I_{max} values increased significantly with increasing number of enzyme layers. This is reasonable because I_{max} should reflect total enzyme activity (or enzyme loading) on the electrode surface. These data also suggest the formation of the layer-by-layer structure of enzyme multilayers. On the other hand, K_m^{app} values depended slightly upon the number of the enzyme layer. The K_m^{app} values for Con A–m-GOx multilayer films are comparable to those reported for other enzyme films prepared on the electrode surface.[3,8] For Con A–m-LOx films, the K_m^{app} values are almost consistent with the reported values for the LOx multilayer films prepared based upon an avidin–biotin system[3] and for native LOx.[9] Note that the K_m^{app} value for Con A–m-GOx films decreased with increasing number of enzyme layers while, for the Con A–m-LOx films, the higher K_m^{app} values were observed for the thicker films. This discrepancy may arise from the fact that lactate is negatively charged in the sample solution (pH 6.8) though glucose is an electrically neutral substrate. The distribution of glucose and lactate into the multilayer film and their diffusion inside the film may be modified differently with increasing film thickness.

We checked whether or not the binding between Con A and mannose-labelled enzymes in the multilayer is displaced by free mannose dissolved in solution. The enzyme multilayer-modified electrodes were immersed in a 5 mM mannose solution for 20 min, and the electrochemical response of the electrodes to glucose or lactate was measured after the treatment. No deterioration in the response was observed, confirming that the multilayer assemblies are stable against free mannose in solution. This is probably due to the fact that Con A and enzymes are cross-linked tightly with each other into a three-dimensional network structure. The stability of the enzyme activity was also evaluated by monitoring the response of the multilayer-modified electrodes once a day; almost no deterioration in response of Con A–m-GOx multilayer-modified electrode was observed after two weeks. The activity of Con A–m-LOx multilayer was less stable; about a half of the original activity remained after two weeks.

In summary, we have demonstrated that the layer-by-layer structure of Con A–m-GOx and Con A–m-LOx multilayer films can be prepared on the solid surface in which the catalytic activity of the enzymes is retained.

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