Layer-by-layer deposition of avidin and biotin-labeled antibody on a solid surface to prepare a multilayer array of antibody

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Multilayer thin films containing an antibody have been prepared successfully by depositing avidin and biotin-labeled antibody alternately and repeatedly on the surface of a quartz slide, in which the antibody retains its binding activity.

Enzyme-containing thin films have been studied extensively because of their possible applications to biosensors and other bioelectronic devices. Recently, much attention has been devoted to the development of spatially-ordered enzyme thin films in which the geometry of the enzyme molecules is designed at the molecular level.[1–7] Willner and Riklin reported a multilayer array of enzymes in which monomolecular layers of enzymes are arranged in a layer-by-layer configuration, through covalent bonding.[1] Chen *et al.* also prepared a molecular assembly of enzyme multilayers through covalent bonding for the construction of optical biosensors.[2] On the other hand, non-covalent interactions such as electrostatic forces of attraction[3] and protein–ligand binding[4] were also employed for the preparation of enzyme multilayer films. 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 or lectin and sugar-labelled enzymes, through the strong biological interactions.[5,6] These enzyme films have been used for improving the response characteristics of enzyme biosensors. In contrast to the successful use of enzyme multilayer films, few reports have appeared on spatially-ordered thin films of antibodies. Though the antibody-containing thin films have been prepared by alternate deposition of polymer and antibody on a solid surface through electrostatic and hydrophobic forces of attraction, no attempt has been made to regulate the arrangement of the antibodies at the molecular level in thin films.[7,8] We report here the preparation of a spatially-ordered multilayer array of antibody, based on the strong affinity between avidin and a biotin-labelled antibody.

Avidin is a glycoprotein (molecular weight; 68 000) found in egg white and is known to contain four identical binding sites to biotin. The binding constant between avidin and biotin is reported to be *ca.* 10^{15} M^{-1}.[9] Therefore, in analogy with avidin–biotin system-based enzyme films, the multilayer films of antibody may be constructed using avidin and biotin-labelled antibody, as illustrated in Fig. 1.

The multilayer films of antibody were prepared on the surface of a quartz slide (5 × 1 × 0.1 cm) and evaluated by spectrophotometry. Two different procedures were employed for the preparation of antibody thin films. The first procedure relied upon the alternate deposition of avidin and biotin-labelled antibody (Method A, Fig. 1). Before use, the quartz slide was treated in a 10% dichlorodimethylsilane solution in toluene overnight to make the surface hydrophobic. The silylated quartz slide was immersed in a Texas Red-tagged avidin (T-avidin) (Molecular Probes Inc., USA) solution [20 μg ml^{-1}, Dulbecco's phosphate buffered saline (D-PBS)] overnight at *ca.* 4 °C to deposit the first layer of T-avidin. It has been reported that avidin forms a monomolecular layer on the surface of a hydrophobic quartz slide.[10] After being rinsed in D-PBS for 3 min, the T-avidin-modified quartz slide was immersed in an antibody [biotin-labelled anti-fluorescein isothiocyanate (B-anti-FITC), Sigma Co., USA] solution (10 μg ml^{-1}, D-PBS) for 30 min at *ca.* 4 °C to deposit B-anti-FITC through avidin–biotin interactions. This treatment would provide a double layer of T-avidin and B-anti-FITC on both surfaces of the quartz slide. The deposition was repeated to prepare the multilayer films composed of the desired number of layers. After each deposition, the absorbance of the quartz slide at 595 nm, originating from the Texas Red moiety, was measured. Another type of antibody film was prepared by depositing T-avidin, B-anti-FITC, and antigen (FITC-glycine) successively (Method B). For this purpose, the procedure for Method A was slightly modified; after each deposition of B-anti-FITC, the multilayer film-modified quartz slide was immersed in an FITC-glycine solution (10^{-4} M in D-PBS) for 1 h at *ca.* 4 °C to bind FITC-glycine through an immunological interaction. In Method B, the absorbance of the quartz slide at 495 and 595 nm was measured simultaneously to monitor the deposition of FITC-glycine and T-avidin, respectively.

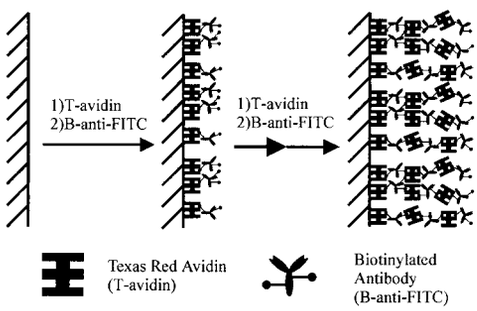

Fig. 1 Schematic representation of layer-by-layer deposition of avidin–antibody multilayers.

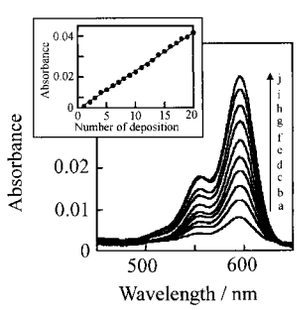

Fig. 2 Absorption spectra of Texas Red avidin–anti-FITC multilayer films as a function of the number of deposited layers: 2 (a), 4 (b), 6 (c), 8 (d), 10 (e), 12 (f), 14 (g), 16 (h), 18 (i) and 20 layers (j). (Inset) Absorbance of the multilayer films at 595 nm as a function of the number of deposited layers.

Fig. 2 shows typical absorption spectra of the multilayer thin films prepared on the quartz slide by Method A and the change in absorbance at 595 nm as a function of the number of depositions. The spectra exhibited a clear absorption maximum around 595 nm arising from the Texas Red residues, and the intensity of the spectra was enhanced linearly with the increasing number of layers, suggesting the formation of a layer-by-

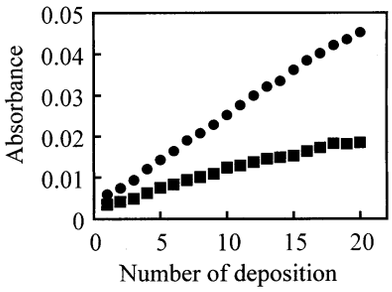

Fig. 3 Absorbance of Texas Red avidin–anti-FITC-FITC-glycine multilayer films at 595 (●) and 495 nm (■) as a function of the number of deposition.

layer configuration of T-avidin in the film as illustrated in Fig. 1. In contrast, the absorbance did not increase when biotin-free antibody was used instead of B-anti-FITC, confirming the avidin–biotin interaction as the driving force of the multilayer formation. The loading of T-avidin onto the quartz slide was estimated from the absorbance data, using a molar extinction coefficient of 206 000 M^{-1} cm^{-1} at 595 nm for T-avidin. Assuming that T-avidin forms a closely packed monomolecular layer on the quartz slide, the density of T-avidin on the surface is calculated to be $(6.3 \pm 1.3) \times 10^{-12}$ mol cm^{-2}, depending on the orientation of the T-avidin molecule (the molecular dimensions of avidin are reported to be $6.0 \times 5.5 \times 4.0$ nm).[9] In other words, for the monomolecular deposition, the absorbance should increase (0.0026 ± 0.0004) per deposition. The slope of the graph in Fig. 2 (inset) is *ca.* 0.0022 (or the density of T-avidin is calculated to be *ca.* 5.0×10^{-12} mol cm^{-2}), suggesting the formation of a nearly monomolecular layer of T-avidin in the multilayer film.

In order to estimate the loading of B-anti-FITC in each layer of the multilayer film, FITC-glycine (*i.e.*, an antigen for B-anti-FITC) was deposited simultaneously in the T-avidin–B-anti-FITC multilayer film through the antigen–antibody interactions (Method B), and the absorbance of the films arising from FITC-glycine was monitored at 495 nm after each deposition. Fig. 3 shows an increase in absorbance at 495 and 595 nm of the multilayer films prepared by Method B as a function of the number of deposited layers. The absorbance increased at both wavelengths in proportion to the number of depositions. These results show that a constant amount of FITC-glycine is immobilized upon each deposition in the multilayer film, which, in turn, strongly suggests that a constant amount of antibody (B-anti-FITC) exists in each layer of the multilayer film. Thus, we can envisage the layer-by-layer structure of the films depicted in Fig. 1. It is possible to estimate the loading of B-anti-FITC in each layer on the basis of the assumption that all the binding sites in B-anti-FITC are occupied by FITC-glycine in the film. Assuming that B-anti-FITC forms a monomolecular layer in close packing upon each deposition and using the molecular dimensions of the B-anti-FITC antibody (*ca.* $6.7 \times 7.3 \times 9.5$ nm), the occupied area of the antibody in each layer is calculated to be 59 ± 10 nm^2, depending upon the orientation of the antibody. If all antibody molecules are active in the multilayer film to bind antigen, two FITC-glycine molecules should be bound to a single B-anti-FITC molecule because antibodies usually contain two binding sites for its antigen. From these considerations and using the molar extinction coefficient of FITC-glycine (*ca.* 90 000 M^{-1} cm^{-1}), the absorbance arising from FITC-glycine which is bound to the monomolecular layer of antibody is calculated to be *ca.* 0.0010 per deposition. The experimental data in Fig. 3 for the deposition of FITC-glycine show that the slope of the plot is in fair agreement with the calculated value, suggesting that the B-anti-FITC antibody also forms a monomolecular layer in the multilayer films. Thus, it is concluded that both T-avidin and B-anti-FITC form a nearly monomolecular layer in the layer-by-layer structure of the multilayer films. To the best of our knowledge, this is the first example of antibody multilayer films prepared by a stepwise deposition of monomolecular layers.

It is interesting to elucidate the binding activity of the antibody in the multilayer films. For this purpose, the antibody multilayer films prepared by Method A were exposed to an FITC-glycine solution, and the absorbance at 495 nm was measured before and after the treatment. When the multilayer film composed of 20 layers of antibody was immersed in a 10^{-4} M FITC-glycine solution for 1 h, the absorbance of the film at 495 nm was enhanced by *ca.* 0.0038. This result shows that FITC-glycine was bound to *ca.* 4 layers of the 20 layers of B-anti-FITC and the remaining 16 layers did not accommodate the antigen. By the treatment of the FITC-glycine-bound multilayer film in the 0.01 M HCl solution for 30 min, *ca.* 75% of the FITC-glycine was removed from the multilayer film. Conceivably only the outermost 3–4 layers in the multilayer film participate in the binding of the FITC-glycine. In other words, FITC-glycine may be unable to penetrate deep into the multilayer films probably due to a compact arrangement of avidin and antibody in the multilayer film. This view is supported by the fact that almost the same results were obtained for 10-layer antibody films; the absorbance increased by 0.0038–0.0046 upon binding of FITC-glycine and 70–80% of the absorbance was decreased by treatment with HCl solution. These results clearly show that only the outermost 3–4 layers are involved in the binding of the antibody, irrespective of the total number of layers in the multilayer film. Essentially the same results were obtained using the multilayer film prepared by Method B, in which whole layers of antibody are intrinsically attached with FITC-glycine. When the 10- and 20-layer films were exposed to HCl solution, 2–3 layers of FITC-glycine were desorbed from the multilayer films. The remaining FITC-glycine could not be removed probably because the diffusion of the antigen was suppressed in the multilayer films.

In conclusion, a spatially-ordered multilayer array of antibody was prepared on the surface of a quartz slide by the alternate deposition of avidin and biotin-labelled antibody. The antibody multilayers are characterized by a layer-by-layer structure composed of a monomolecular layer of avidin and antibody. The antibody molecules in the outermost 3–4 layers of the multilayer films can participate in the binding of the antigen.

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