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Preparation of active avidin films by a layer-by-layer deposition of poly(vinyl sulfate) and avidin on a solid surface

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Multilayer thin films containing avidin have been prepared by a layer-by-layer deposition of poly(vinyl sulfate) and avidin on the surface of a quartz slide, in which avidin retained its binding activity to biotin and analogues.

Protein-containing thin films are attracting much attention because of their possible applications to electrochemical and optical biosensors. A layer-by-layer deposition technique has been widely used for the preparation of protein-containing multilayer thin films. Lvov and coworkers have reported multilayer thin films containing enzymes and other proteins, which were prepared by an alternate deposition of proteins and oppositely-charged polymers through electrostatic force of attraction.[1–3] Antibodies have also been immobilized in the multilayer thin films composed of dextran sulfate[4] and polystyrenesulfonate.[5] In this context, we have previously reported that avidin-containing thin films can be prepared on the surface of a quartz slide by a layer-by-layer deposition of avidin and biotin-labelled polymers.[6,7] In the avidin-containing films, however, the biological activity of avidin (*i.e.*, strong affinity to biotin) was practically masked because the active sites of avidin were occupied by biotin residues in the biotin-labelled polymers. If the active sites of avidin are available in the film, it would be possible to make the film functional by modifying the film using biotin-tagged functional moieties. We report here the preparation of active avidin film by an alternate deposition of avidin and poly(vinyl sulfate) (PVS) and its binding activity to biotin and analogues.

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}.[8] Since avidin is a basic protein (isoelectric point; *ca.* pH 10) and contains net positive charges at a neutral pH,[8] the multilayer films of avidin may be constructed using avidin and PVS through the electrostatic force of attraction, as illustrated in Fig. 1.

The multilayer films of avidin were constructed on the surface of a quartz slide (5 × 1 × 0.1 cm) and evaluated by UV spectrophotometry. 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-labelled avidin (T-avidin) (Molecular Probe Inc., USA) solution in pure water (0.1 mg ml^{-1}) for 30 min at *ca.* 20 °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.[7] After being rinsed in water for a short time, the T-avidin-modified quartz slide was immersed in an aqueous PVS (molecular weight; 243 000, Nacalai Tesque Co., Japan) solution (3 mg ml^{-1}) for 1 min at *ca.* 20 °C to deposit PVS through electrostatic force of attraction. This treatment would provide a T-avidin–PVS layer 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 monitored.

Fig. 2 shows absorption spectra of the multilayer films prepared on the quartz slide and the change in absorbance at 595 nm as a function of the number of depositions. The spectra

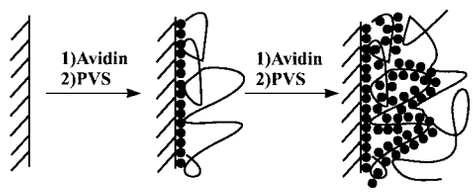

Fig. 1 Schematic representation of layer-by-layer deposition of avidin–PVS multilayers.

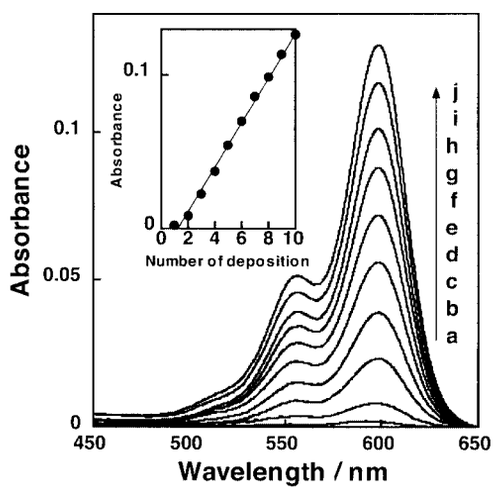

Fig. 2 Absorption spectra of Texas Red avidin–PVS multilayer films as a function of the number of depositions; 1 (a), 2 (b), 3 (c), 4 (d), 5 (e), 6 (f), 7 (g), 8 (h), 9 (i) and 10 layers (j). (Inset) Absorbance of the multilayer films at 595 nm as a function of the number of deposited layers.

exhibited a clear absorption maximum around 595 nm arising from the Texas Red moieties, and the intensity of the spectra was enhanced linearly with the increasing number of depositions. This result suggests that the same amount of T-avidin is immobilized in each deposition to form a layered thin film. The loading of T-avidin on the quartz slide was estimated from the absorbance data, using a molar extinction coefficient of 235 000 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 × 5.5 × 4.0 nm).[8] In other words, for the monomolecular deposition, the absorbance should increase (0.0030 ± 0.0006) per deposition. The slope of the graph in Fig. 2 (inset) is *ca.* 0.013 (or the density of T-avidin is calculated to be *ca.* 2.6×10^{-11} mol cm^{-2}), suggesting that the loading of T-avidin is *ca.* four times higher than that calculated for the monomolecular deposition. The multilayer deposition of T-avidin does not mean a successive and nonspecific adsorption of T-avidin onto the surface or a formation of T-avidin aggregates on the surface. We have ascertained independently that T-avidin cannot be adsorbed nonspecifically onto the surface of a T-avidin monolayer-modified quartz slide. The

Table 1 Binding constants (K) and maximum loadings (Γ_m) of HABA to the avidin–PVS films

No. of depositions, (Avidin–PVS)$_n$/ mol cm^{-2}	pH 4.0		pH 7.4	
	K/ 10^5 M^{-1}	$\Gamma_m/10^{-10}$ mol cm^{-2}	$K/10^5$ M^{-1}	$\Gamma_m/10^{-10}$ mol cm^{-2}
5	1.1 ± 0.1	7.9 ± 0.4	0.50 ± 0.08	8.8 ± 0.7
10	1.0 ± 0.3	23 ± 1	0.54 ± 0.09	20 ± 2

results may be rationalized by taking into account the molecular geometry of PVS. A significant part of the polymer chains of the adsorbed PVS protrudes from the surface to form loops as schematically shown in Fig. 1, resulting in enhanced surface density of negative charges for T-avidin binding. We observed previously a similar behaviour for avidin thin films prepared using biotin-labelled polyamines.[7] Thus, the alternate deposition of T-avidin and PVS gave layered thin films which contain four times as many avidin molecules in each layer as for the monomolecular deposition.

It is interesting to elucidate the binding activity of avidin in the multilayer films. For this purpose, the avidin multilayer films were prepared using native avidin and PVS. The avidin–PVS film-coated quartz slide was exposed to a 2-(4′-hydroxyphenylazo)benzoic acid (HABA) solution, and the absorbance at 500 nm was measured to estimate the binding of HABA to avidin. It is known that avidin binds HABA as an analogue of biotin to induce a new absorption band of HABA at 500 nm.[9] When the multilayer film containing *ca*. 2.5×10^{-10} mol cm^{-2} avidin was immersed in 1×10^{-5} M HABA solution (phosphate buffer, pH 7.4), the absorbance of the film at 500 nm was increased and reached a steady-state value in *ca*. 15 min, showing HABA was bound to avidin in the multilayer film. The absorption band disappeared rapidly upon addition of 1×10^{-5} M biotin into the solution, suggesting that the bound HABA was displaced by biotin since the binding constant between biotin and avidin (*ca*. 10^{15} M^{-1}) is much higher than that for HABA–avidin complexation (*ca*. 1.7×10^5 M^{-1}). These results clearly show that the avidin–PVS film retains its binding activity to biotin and its analogue HABA.

The effects of pH and thickness of the film on the binding properties of the avidin–PVS film were evaluated using HABA. The apparent binding constant of HABA to the film was estimated based on the Langmuir adsorption isotherm [eqn. (1)],

$$\Gamma = \Gamma_m C/(K^{-1} + C) \quad (1)$$

where Γ denotes the amount of HABA adsorbed at the concentration C, Γ_m is the maximum loading of HABA in the film, and K represents the apparent binding constant of HABA to the film. Table 1 summarizes the K and Γ_m values for the avidin–PVS films at pH 4.0 and 7.4. Almost the same K values were obtained for the five-layer and ten-layer films. In other words, the effect of thickness of the film on the HABA binding is negligibly small, which in turn shows that all avidin molecules in the film are involved equally in the binding of HABA. On the other hand, the K values depended upon the pH of the solution; slightly lower K values were observed at pH 7.4 than at pH 4.0. This probably originates from the facts that the avidin–PVS films contain net negative charges due to the sulfate residues in PVS chains and that HABA dissociates into carboxylate anion at pH 7.4. Thus, the binding constant was lowered by the electrostatic repulsion between the negative charges in HABA and PVS chains. The binding constant of HABA to avidin is reported to be 1.7×10^5 M^{-1} in solution.[9] Therefore, the K values observed for the avidin–PVS films are slightly lower than the value in solution. This is reasonable because, in the multilayer films, access of HABA toward avidin may be hindered to some extent due to the network chains of PVS in which avidin is entrapped. It is calculated that, from the Γ_m values, almost all the binding sites of avidin can be available in the film for the binding of HABA at both pH 4.0 and 7.4.

In conclusion, multilayer thin films containing biologically active avidin were prepared on the surface of a quartz slide by the alternate deposition of avidin and PVS. The loading of avidin in each layer of the multilayer film is *ca*. four times higher than that calculated for the monomolecular deposition. The binding ability of avidin in the multilayer film is nearly comparable to that in solution. The avidin–PVS thin films may be useful for preparing many different types of functional thin films, by modifying with biotin-tagged functional molecules. It is a merit of the avidin–PVS multilayer film to be able to accommodate a larger amount of functional molecules than in a monolayer film.

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