Assembly of Multicomponent Protein Films by Means of Electrostatic Layer-by-Layer Adsorption

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Abstract: Multilayer films which contain ordered layers of more than one protein species were assembled by means of alternate electrostatic adsorption mostly with positively charged poly(ethylenimine) (PEI) or with negatively charged poly(styrenesulfonate) (PSS). Water-soluble proteins used are cytochrome *c* (Cyt), myoglobin (Mb), lysozyme (Lys), histone f3, hemoglobin (Hb), glucoamylase (GA), and glucose oxidase (GOD). Charged protein layers formed multilayers with linear polymers acting as glue or filler. The assembly was monitored by a quartz crystal microbalance and UV spectroscopy. Linear film growth was observed up to at least 25 molecular layers. The assembly of Mb and Lys, both positively-charged, was realized in alternation with PSS in the form of {PEI/PSS + $(Mb/PSS)_2$ + $(Mb/PSS/Lys/PSS)_4$}. The assembly of oppositely-charged (at pH 6.5) Lys and GOD consists from Lys and GOD layers separated by a polycation/polyanion bilayer: {PEI/PSS/PEI + $(PSS/Lys)_2$ + PSS/PEI + $(GOD/PEI)_6$}. Hb was assembled as "positive" unit at pH 4.5 (in alternation with PSS) and as "negative" unit at pH 9.2 (in alternation with PEI). A multilayer consisting of alternating montmorillonite, PEI, and GOD layers was also assembled. These biomolecular architecture open a way to construct artificially orchestrated protein systems that can carry out complex enzymic reactions.

I. Introduction

Construction of multiprotein systems in predetermined ways is an important research target in biorelated chemistry and in biotechnology. Decisive roles played by spatially organized multiple proteins in the biological systems cannot be overstated. Artificial approaches for multi-protein architecture have been mostly limited to ordered layers based on the Langmuir–Blodgett technique. The pioneering work of Langmuir and Schaefer was followed by an illuminating study of ferritin ordering by Fromherz and, more recently, by ordering of other more complex proteins.[1] Assembly of protein multilayers with specific interlayer interaction at the air–water interface is under intensive study by Ringsdorf and others.[2] In these investigations the protein streptavidin is used, as it possesses four binding sites for its ligand biotin, two on each side, allowing binding of streptavidin to biotinylated layers on both sides of the protein.

Adsorption of charged proteins onto an oppositely charged surface (e.g., mica) has been known for a long time. It is possible to obtain monomolecular protein layers at low protein concentrations and at proper pHs.[3] Incorporation of proteins into the interlayer space of cast multilayer films is another promising method.[4] In this approach positively charged myoglobin and cytochrome *c* were introduced onto negatively charged surfaces of phosphate bilayer membranes in unique spatial orientation.

Examples of protein multicomponent films were recently reported, where antibodies and biotin/streptavidin pairs act as connecting units of different molecular layers.[5a,b] Orientation of bacteriorhodopsin in monolayers,[5c] assembly of glucose oxidase multilayers[5d] through use of bispecific antibody interlayer,[5d] and alternate adsorption of biotinylated polylysine and streptavidin layers[2c] are based on the same principle.

Alternate adsorption of polyanion and polycation has been proposed by Decher and co-workers[6a–e] as a novel preparative technique of molecular multilayers. The principle of multilayer assembly is as follows: a solid substrate with negatively charged surface is immersed in a solution containing cationic polyelectrolyte, and a layer of polycation is adsorbed via electrostatic attraction. Since adsorption is carried out at relatively high concentrations of polyelectrolyte, a number of cationic groups remain exposed to the solution, and thus, the surface charge is effectively reversed. After being rinsed in water, the substrate

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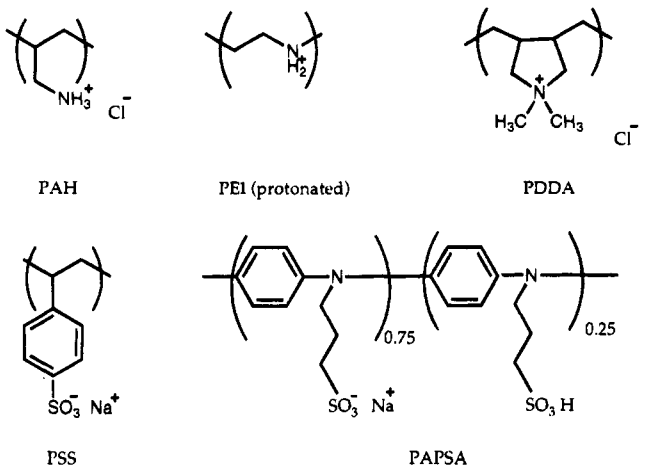
Scheme 1



is immersed in a solution containing anionic polyelectrolyte. A new polymer layer is adsorbed, but now the original surface charge is restored. By repeating these steps, alternating multilayer assembly is obtained. The procedure is related to early pioneering studies by Iler[7a] on sequential deposition of polymer colloids and to more recently study by excessive adsorption of the oppositely-charged polyions onto charged surfaces.[7b]

The protein assembly by the alternate electrostatic adsorption was reported for the combination of negatively-charged poly(styrenesulfonate) and positively-charged myoglobin or lysozyme,[8a] of a cationic bis(pyridinium) salt and glucose isomerase,[8b] and of cytochrome *c* and negatively-charged α-ZrP plates.[8c] The metal−ligand complexation was used as a driving force of the multilayer assembly.[8e]

The idea of applying this assembly procedure for production of films with alternation of two or more polyion components was first realized for linear polymer ions,[9a,b] and spatial separation of neighboring polyion layers with minor interpenetrating was confirmed from neutron reflectivity experiments with deuterated poly(styrenesulfonate).[9a]

We demonstrate in this paper that the alternate adsorption is a highly effective and general procedure for the preparation of multicomponent protein films.

II. Experimental Section

Materials. Cytochrome *c* from horse heart (Cyt *c*, Wako Co., Japan), chicken egg white lysozyme (Lys), calf thymus type YIII-S histone, horse heart myoglobin (Mb), and horse hemoglobin (Hb) (all from Sigma Co.) and glucoamylase *Aspergillus niger* (GA), glucose oxidase *Aspergillus niger* (GOD), and bovine liver catalase (all from Wako Co., Japan) were used as purchased. Aqueous proteins were used at a concentration of 2 mg/mL, except for histone used at a concentration of ca. 0.2 mg/mL because of low solubility. Poly(anilinepropanesulfonic acid) (PAPSA) was synthesized following the work;[10] its structure given in Scheme 1 was confirmed by ^{1}H NMR and by elemental analysis. Sodium poly(styrenesulfonate) (PSS, MW 70 000, Aldrich Co.) at concentration of 3 mg/mL, poly(allylamine) hydrochloride (PAH, MW 50 000−65 000, Aldrich Co.), poly(dimethyldiallylammonium chloride) (PDDA, Aldrich Co.) at a concentration of 2 mg/mL, and branched poly(ethylenimine) (PEI, MW 70 000, Wako Co., Japan) at a concentration of 1.5 mg/mL were dissolved in pure water (Scheme 1).

The pHs of the solutions were adjusted by adding aqueous HCl and are given in Table 1. The standard borate buffer (Wako Co., Japan) was used for Hb solution at pH 9.2. Aqueous dispersion (0.3 mg/mL, pH 6.5) of negatively-charged montmorillonite clay (Kunimine Kogyo Ind. LTD, Japan) was prepared by ion exchange and strong ultrasonic treatment.[11] The ultrapure water obtained by reversed osmosis followed by ion-exchange and filtration (Yamato-WQ500, Millipore, Japan) with the specific resistance better than 18 MΩ cm was used.

Alternate Adsorption. We monitored the assembly process by UV absorption spectroscopy (JASCO, V-570, Japan) and quartz crystal microbalance technique (QCM, USI System, Japan).[12] QCM measurements were operated in two modes. In the stepwise measurement, the resonator was immersed in a polyelectrolyte solution for a given period and dried, and the frequency change was measured. In *in-situ* monitoring of adsorption, only one side of the resonator was in contact with the surface of the solution and the frequency change was recorded continuously. The long-term stability (several hours) of the quartz resonator frequency was within ±2 Hz. All experiments were carried out in an air-conditioned room at a temperature of ca. 22 °C. Resonators used are covered by evaporated silver electrodes (0.16 cm^2) on both faces, and their resonance frequency was 9 MHz (AT-cut). In the *in-situ* measurement, one side of the resonator was in permanent contact with solutions, while the upper electrode was open to air and the upper contact wire was insulated from solution by silicone paint according to the method elaborated by Ebara and Okahata.[12c,d]

In a typical procedure, precursor films were assembled on a silver QCM resonator, on a quartz plate, or on a silicone wafer by repeating four or five alternate adsorptions of PEI and PSS. The outermost layer becomes "negative" or "positive", accordingly. Then the solid surfaces were alternately immersed for 20 min in aqueous solutions of proteins or polyions with intermediate water washing. The process was periodically interrupted for UV spectroscopy and QCM measurements.

For scanning electron microscopy (SEM) observation, a resonator with an assembled film was cut and coated with 20 Å thick Pt by use of an ion-coater (Hitachi E-1030 ion sputter, 10 mA/10 Pa) under Argon atmosphere. Micrographs were obtained with a Hitachi S-900 (Japan) scanning electron microscope (acceleration voltage of 25 kV).

III. Results and Discussion

QCM Frequency−Mass Relation. We can estimate the mass increase due to adsorption from QCM frequency shift by using the Sauerbrey equation.[12a] The following relationship is obtained between adsorbed mass, M (g), and frequency shift, ΔF (Hz), by taking into account characteristics of quartz resonators used:

$$\Delta F = -1.832 \times 10^8 M/A \qquad (1)$$

where A is the surface area of the resonator. This value corresponds to an apparent area of quartz microbalance placed between QCM electrodes: $A = 0.16 \pm 0.01$ cm^2. Then, one finds that 1 Hz change in ΔF corresponds to 0.9 ng. Thus, we can control the mass increase of adsorbed film with high accuracy and reliability.

However, we need furthermore to consider the surface roughness of the resonator, in order to calculate film thickness from its mass. Scanning electron microscopy of the silver-coated quartz given in Figure 1a suggests that the effective surface area of the silver electrode, and hence, polyion film is approximately 20% larger than that calculated directly from the diameter of the electrode. This estimation comes from repre-

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Table 1. Protein/Polyion Alternate Multilayer Assembly

protein									
	mol wt	isoelect pt	pH used	charge	alternate with	τ (min) (±0.5)	freq change of each step (Hz) protein + polyion (±5%)	thickness of protein + polyion layer (±10%)	protein globule dimens (Å)
cytochrome	12 400	10.1	4.5	+	PSS^-	3	150 + 160	24 + 16	25 × 25 × 37
			4.8		$PAPSA^-$		200 + 50	32 + 8	(ref 3c)
			4.5		PAH^+		does not adsorb		
lysozyme	14 000	11	4	+	PSS^-	4	145 + 115	23 + 19	30 × 30 × 45
			7		$clay^-$		only one step of adsorption		(ref 15b)
			4		PAH^+		does not adsorb		
histone f3	15 300	11	7	+	PSS^-		140 + 260	22 + 42	diameter ≈ 34[a]
myoglobin	17 800	7.0	4	+	PSS^-	3	250 + 194	40 + 31	25 × 35 × 45
			4.6		$PAPSA^-$		240 + 90	39 + 14	(ref 15a)
			6		$clay^-$		only one step of adsorption		
			4		PAH^+		does not adsorb		
hemoglobin	64 000	6.8	4.5	+	PSS^-	4	1100 + 190	175 + 31	50 × 55 × 65
			9.2	−	PEI^+	4	1200 − 60	for bilayer: 182	(ref 15c)
glucoamylase	95 000	4.2	6.8	−	PEI^-		160 + 20	26 + 3	diameter ≈ 63[a]
		4.2	6.8	−	$PDDA^+$		150 + 70	24 + 11	
glucose oxidase	186 000	4.2	6.5	−	PEI^+	5	2150 + 50	344 + 8	diameter ≈ 82[a]
			6.8		$PDDA^+$		for bilayer: 500	for bilayer: 80	
catalase	240 000	5.5	6.5−8	−	PEI^+		does not adsorb		

[a] Protein globule diameters are estimated from molecular weights (in spherical approximation, using 1.3 g/cm^3 protein density).

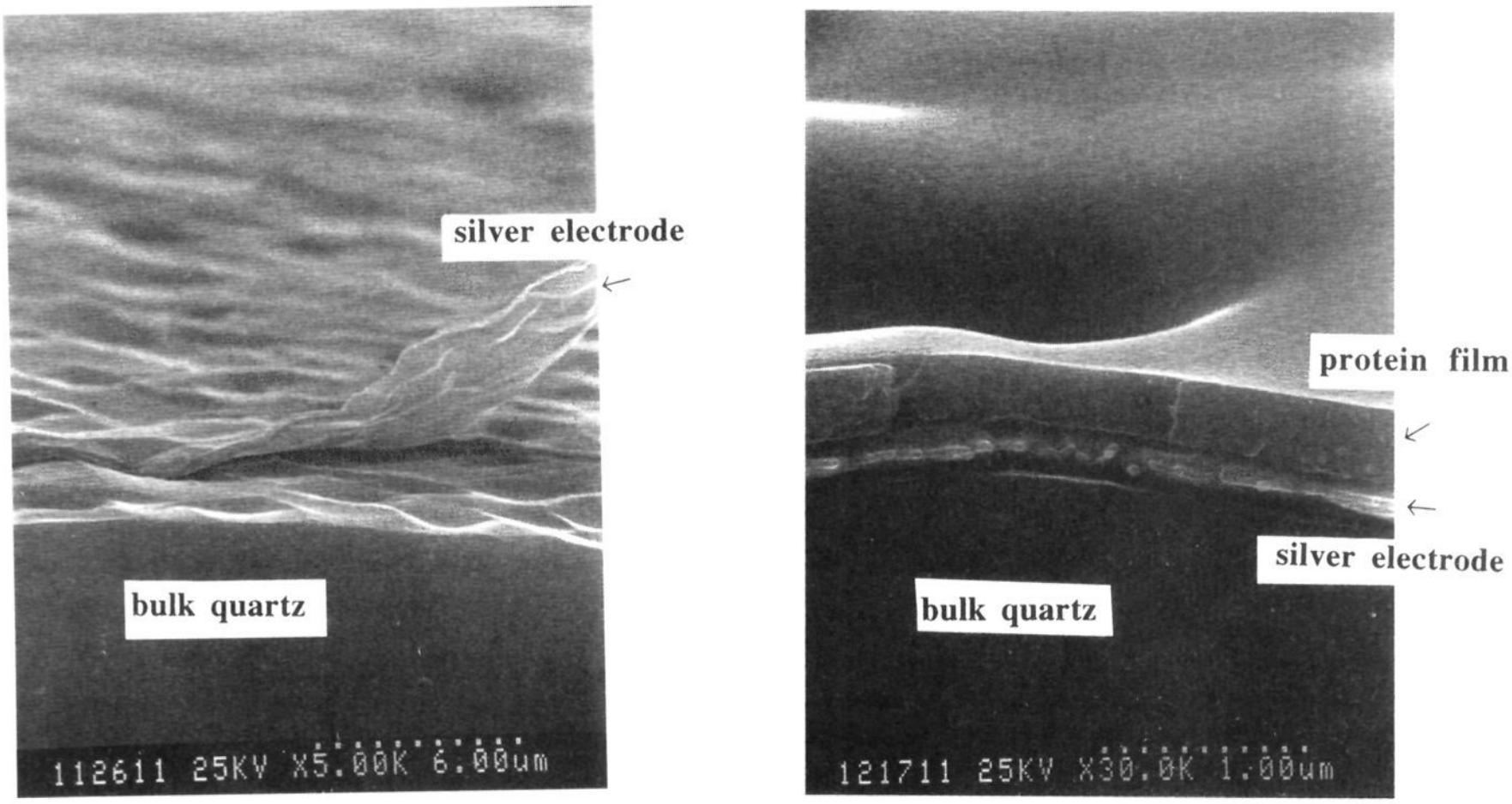

Figure 1. Scanning electron micrographs: (left) silver electrode deposited onto the quartz resonator (part of the silver layer is detached from the quartz); (right) {$(PEI/PSS)_2$ + $(PEI/GOD)_8$ + PEI} film deposited onto the silver-coated resonator.

sentation of a rough silver surface as a distribution of small sections with average 20° tilting. Then, thickness (d) of adsorbed film on both sides of the electrode is estimated by taking into account the film density as follows:

$$d\,(\text{Å}) \approx -0.16\Delta F\,(\text{Hz}) \qquad (2)$$

The density was assumed to be 1.2 ± 0.1 g/cm^3 for polyion films and 1.3 ± 0.1 g/cm^3 for proteins.[13] Thus, for example, the thickness of the Lys/PSS composite bilayer was calculated to be 41 ± 5 Å (Table 1). Because of uncertainties in the film area and density, the calculated thickness is reliable to ±10%. The validity of this estimation is supported by X-ray reflectivity measurement and SEM observation. X-ray reflectivity measurements[14] of one and two Lys/PSS bilayers adsorbed onto the $(PEI/PSS)_3$ precursor film gave the bilayer thickness of 40 ± 5 Å. This value agrees with that calculated from QCM data within the experimental error.

Figure 1b shows a SEM of GOD/PEI film on Ag-coated resonator: the bulk quartz, silver electrode, and protein film are clearly seen. The thin bright layer under the thicker protein film is the silver electrode layer which has been deposited onto quartz. The PEI/GOD layer has a constant thickness of 3000 ± 200 Å and is molecularly smooth. The roughness of this film surface (with the outermost PEI layer) is estimated to be no more than 50 Å. QCM frequency shift for the {$(PEI/PSS)_2$

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(14) X-ray reflectivity measurements and following film thickness calculations for the Lys/PSS bilayer adsorbed onto a polyion precursor film was performed by Dr. R. L. Kayushina at the Institute of Crystallography, Moscow 117333, Russia.

+ (PEI/GOD)$_8$ + PEI} composite film was 17 500 Hz. Therefore, 1 Hz corresponds to 0.17 Å. This is consistent with eq 2. ΔF for each GOD/PEI layer was 2100 Hz in this experiment.

Generality of Protein Assembly. We have shown in our previous paper[8a] that the alternate assembly can be used for combinations of positively charged Mb or Lys and polyanion. It is important to confirm that this approach is generally applicable to charged protein molecules, in order to develop proper multiprotein architecture. Table 1 summarizes alternate adsorption of eight water-soluble proteins in combination with oppositely charged polyions. The pHs of the protein solutions were set apart from the isoelectric point so that proteins are sufficiently charged under the experimental conditions. All the proteins except for catalase undergo the alternate adsorption with organic polyions for unlimited numbers of cycles. However, negatively-charged montmorillonite underwent only one step of adsorption with positively charged lysozyme (Lys) or myoglobin (Mb). The alternate adsorption naturally does not proceed between proteins and polyions of identical charges. The mass increment at each step is reflected in the frequency shift and is quite reproducible.

It is noteworthy that the frequency change for the protein adsorption step of proteins correlates approximately with their molecular weights. Protein layer thicknesses as estimated from ΔF (Table 1) and molecular dimensions of proteins obtained from crystallographic data show good correspondence, suggesting a relatively uniform monolayer formation. Of course, such comparison is of only qualitative character because we know nothing about orientation, packing density, and hydration of proteins in a layer. All these parameters may have significant influence on the thickness of the protein monolayer. Unlike other proteins, the layer thickness for Hb was much larger as compared with its globule dimension. This may be caused by formation of Hb aggregates, but still its adsorption process was saturated and the layer thickness at every step of growth was constant. We may point out one interesting feature for GOD films: alternate assembly with polycation PDDA gave a step of growth for the GOD/PDDA bilayer as $-\Delta F = 500$ Hz. This value corresponds to the layer thickness of ca. 80 Å. However, alternate assembly with polycation PEI gave the growth step for the bilayer of ca. 2200 Hz (thickness, ca. 352 Å). This suggests aggregation of the protein during assembly.

Recently, QCM monitoring of specific binding of concanavalin A (molecular weight 104 000) to a water–surface monolayer of synthetic glycolipids was reported by Ebara and Okahata.[16] Binding of concanavaline A as monolayer onto one side of a resonator resulted in the frequency change ranging from 66 to 200 Hz in dependence on binding conditions (i.e., deposition on both sides of a resonator would be 132–400 Hz). These values are in the same range as those given in Table 1.

Let us analyze in more detail assembly of the glucose oxidase (GOD)/PEI film. As shown in Figure 2, the first two steps are not linear, but the subsequent steps provide linear mass increases up to at least 21 molecular layers. The frequency increment of each cycle, $-\Delta F = 2200 \pm 100$ Hz, is composed of 2150 Hz for GOD and 50 Hz for PEI. A linear increase of UV absorbance was also observed with the number of deposited GOD layers. The adsorption kinetics of this process are given in Figure 3. GOD adsorption produces a large frequency shift

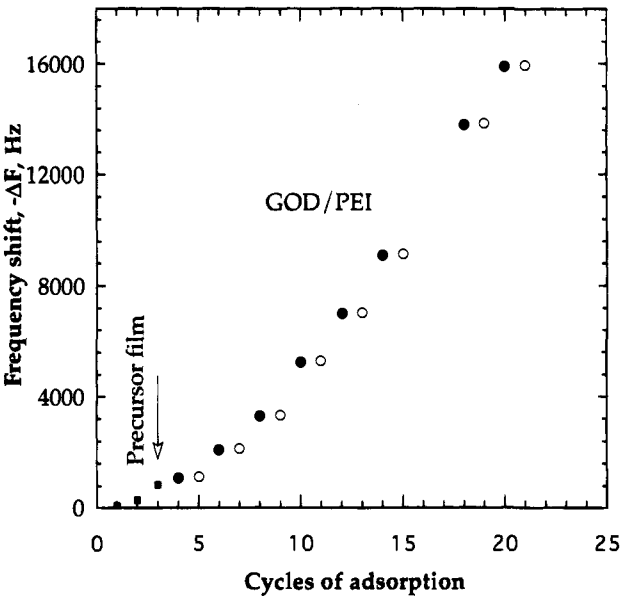

Figure 2. Frequency shift ($-\Delta F$) due to cycles of alternate GOD/PEI adsorption: (●) GOD adsorption steps and (○) PEI adsorption steps. Steps 16–18 were continued without intermediate drying, but the linear mass increase still apparent.

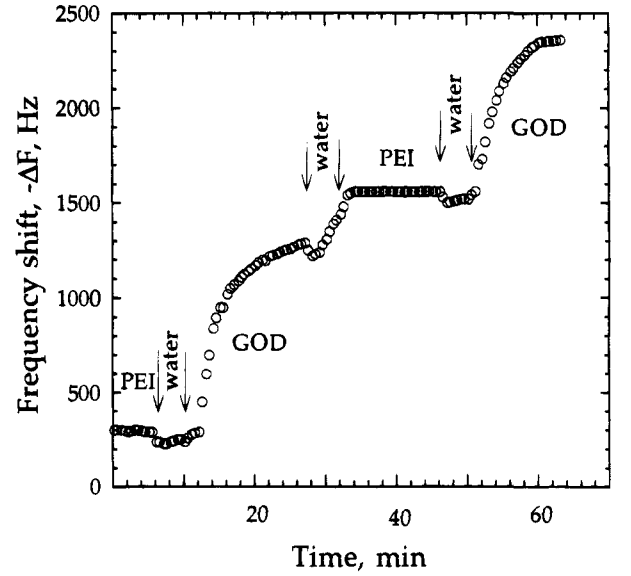

Figure 3. *In-situ* monitoring of the frequency change (ΔF) during GOD/PEI assembly without interrupting the drying process. Time intervals of intermediate water washing and GOD or PEI adsorption are marked.

(≈1000 Hz), whereas PEI adsorption gives only a small frequency change (≈30 Hz) because of the small molecular weight of the monomer unit. One can see that GOD adsorption reaches saturation in ca. 15 min. The first-order rate constant is obtainable by curve fitting as a characteristic time of adsorption, τ, of 5 min. The rate constants were determined for other proteins from the *in-situ* experiments and are included in Table 1. They are 3–5 min in all these cases, indicating facile saturation on the surface at $t \approx 3\tau$.

UV monitoring of the assembly process was useful for heme-containing proteins. Figure 4 shows the UV spectra for a Mb/PSS film at consecutive steps of the assembly. The absorbance at the Soret band increased linearly with the number of Mb layers in the film.

The assembly process was more stable when it was not interrupted for UV spectroscopy of the terminal protein layer.

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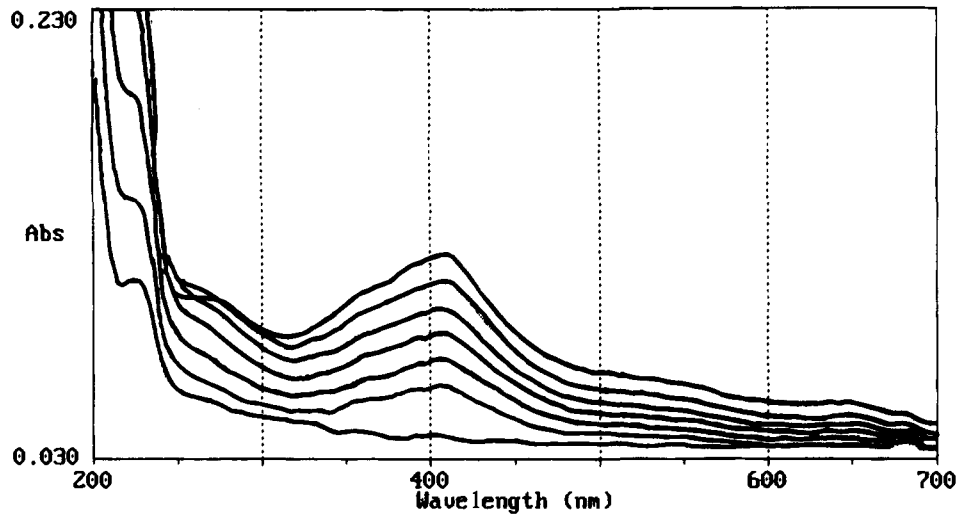

Figure 4. UV absorption change with Mb/PSS assembly. The lowest curve corresponds to the precursor film. The following curves, from bottom to top, correspond to adsorption of 2, 4, 6, 8, 10, and 12 alternate Mb/PSS layers.

Drying of the protein upper layer and the subsequent UV spectroscopy often led to deterioration of the assembly in the next step. In contrast, this problem was not experienced when the outermost polyion layer covered the protein layer. Drying of films on the resonator did not cause growth deterioration, and the resulting protein films were water insoluble, i.e. an assembly process is irreversible.

The assembled proteins are in most cases not denaturated. Thus UV spectral patterns of the Mb and Hb films are similar to those of the same proteins in solutions, including the location of the Soret band at 409 nm. In addition, for Mb/PSS and Cyt/PSS films, electrochemical study performed on the protein films attached to gold electrodes[17a] indicates that the redox activity (Fe(III) ↔ Fe(II)) of the heme in protein/polyanion films is close to that in solution. The enzymatic activity of GOD in the film was confirmed by oxidation of glucose which was followed by oxidation of iodonitrotetrazolium violet (INT) in the presence of phenazine methosulfate (PMS) as a mediator.[17b,c] Quartz slides with assembled films of {PEI/PSS/PEI + $(GOD/PEI)_1$} and {PEI/PSS/PEI + $(GOD/PEI)_2$} were immersed in an INT/PMS solution. A purple color of iodonitroformazane was observed on the film within 30 min after the film was immersed in glucose solution, and the absorbance at 500−800 nm was proportional to the number of GOD layers.

Multicomponent Protein Films. An initial attempt to obtain a multilayer was conducted by the alternate adsorption applied directly to proteins of opposite charges. At pH 6.5, lysozyme (Lys) is positively charged and GOD is negatively charged. Thus a precursor film possessing the PEI/PSS/PEI + $(PSS/Lys)_2$ composition was prepared with the outermost Lys layer. In the subsequent attempt GOD molecules were not adsorbed in spite of the fact that these proteins were oppositely charged at pH 6.5. In order to amend this failure, we built an intermediate PSS/PEI layer above the Lys layer, and then the GOD monolayer was formed successfully onto the terminal PEI layer. The growth mode was thus switched from Lys^+/PSS^- to GOD^-/PEI^+ by intercalation of the PSS/PEI bilayer. The final layer structure of the superlattice is expressed as {PEI/PSS/PEI + $(PSS/Lys)_2$ + PSS/PEI + $(GOD/PEI)_6$}.

In a second approach, a hemoglobin film was prepared. Since hemoglobin (Hb) possesses an isoelectric point of 6.8,[13b] it is positively charged at pH 4.5 and negatively charged at pH 9.2. Thus the Hb layer can be alternated either with polycation or polyanion by using these pH conditions. Figure 5 shows QCM monitoring of the assembly. The first four steps correspond to a precursor film of $(PEI/PSS)_2$, and the following steps are Hb/PSS assembly at pH 4.5, to give $(Hb/PSS)_5$. One can see that beginning from the second protein layer the film growth is linear with an increment of $-\Delta F = 1290$ Hz: 1100 ± 50 Hz for Hb adsorption and 190 ± 5 Hz for PSS adsorption. At the fourteenth step we changed the assembly mode and polycation PEI was adsorbed. Then assembly mode was switched to PEI/Hb (pH 9.2). Stable assembly was realized only after two (nos. 15 and 16) intermediate steps. Successful assembly of negatively charged Hb with PEI was repeated at steps 17−26. The growth cycle corresponds to $-\Delta F = 1140$ Hz: 1200 Hz for Hb adsorption and −60 Hz for PEI. Apparently PEI peels off some of outermost proteins and recharges the surface. The Soret band of Hb assembled at pH 4.5 was very broad, although absorbance was still proportional to the number of adsorption cycles. After switching to the second mode, the UV data also indicated removal of the upper two Hb layers assembled at pH 4.5 probably by replacement with more competitive PEI

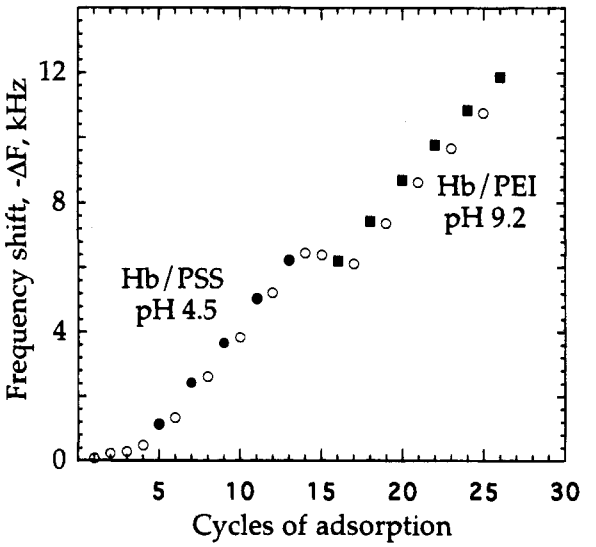

Figure 5. Frequency shift with cycles of alternate Hb/polyion adsorption $\{(PEI/PSS)_2 + (Hb_{pH4.5}/PSS)_5 + (Hb_{pH9.2}/PEI)_6\}$: ●, Hb adsorption at pH 4.5; ■, Hb adsorption at pH 9.2; ○, adsorption of PSS or PEI.

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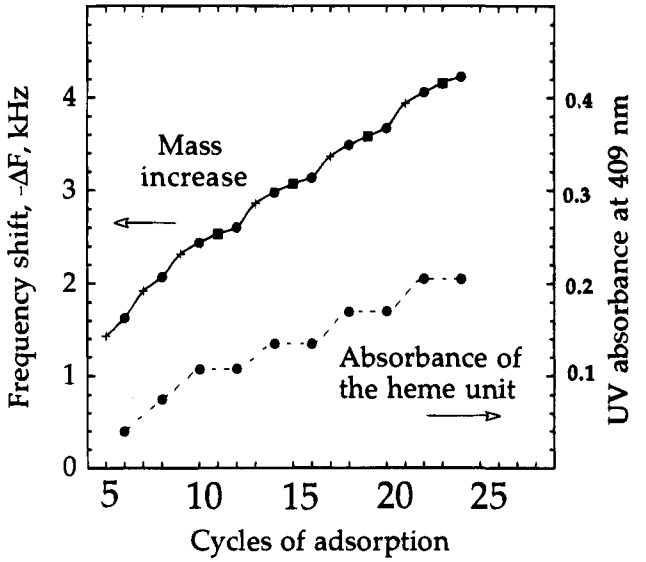

Figure 6. Assembly process of a two-protein multilayer as observed by resonator frequency shifts ($-\Delta F$) and by UV absorption (the total cycle is given by $\{(PEI/PSS)_2 + (Mb/PSS)_2 + (Mb/PSS/Lys/PSS)_4\}$: +, Mb adsorption; ■, Lys adsorption; ●, PSS adsorption.

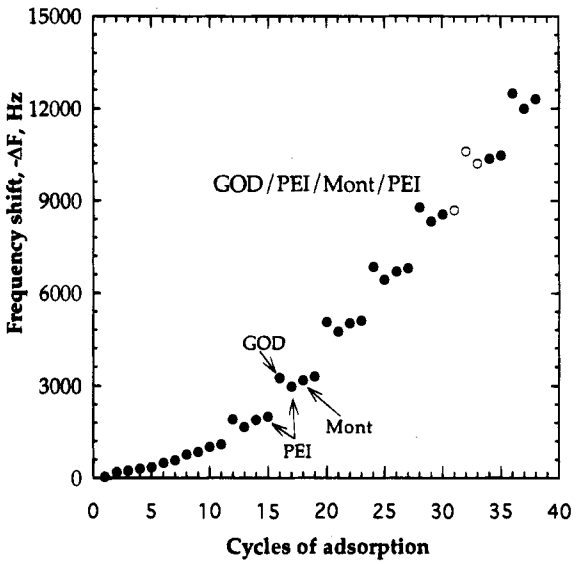

Figure 7. Assembly process of GOD/PEI/montmorillonite/PEI multilayer monitored by resonator frequency shifts ($-\Delta F$). The total cycle is given by $\{(PEI/PSS)_2 + (PEI/Mont)_2 + PEI + (GOD/PEI/Mont/PEI)_7\}$. At steps 31−33, intermediate drying was not carried out and empty circles just symbolize the probable positions of appropriate points.

polycation, but the subsequent alternate assembly proceeded as expected. The corresponding Soret band was typical of native Hb, and the absorbance increment was linear. Thus, it appears that Hb molecules assembled at pH 4.5 are denatured (though still keeping their globular shape), but those assembled at pH 9.2 retain their native structure.

A third example is a combination of identically charged proteins. Figure 6 give results of QCM monitoring of adsorption involving positively charged myoglobin (Mb) and Lys. The four-layer (Mb/PSS/Lys/PSS) unit was assembled onto a $(PEI/PSS)_2 + (Mb/PSS)_2$ precursor film. This unit assembly is represented by the frequency increase of $-\Delta F = 550 \pm 10$ Hz: 250 Hz for Mb, 130 Hz for PSS, 90 Hz for Lys, and 80 Hz for PSS. Different increments are found for the two PSS layers because the underlying proteins are different. The four layer cycle was repeatable: the four layer unit of Mb/PSS/Lys/PSS looks like repeating arcs in the frequency shift. We can estimate from frequency shifts the thickness of each layer as 40 + 21 + 15 + 13 Å. In the example of Figure 6, 24 molecular layers were deposited, with the total film thickness of ca. 500 Å. The absorbance of the Soret band at 409 nm increases only at the assembly step corresponding to Mb layer deposition. In contrast, the absorbance at 225 nm (phenyl band) increased at every step of deposition, i.e. at the Mb, PSS, Lys, and PSS steps.

In addition, a film containing GOD and glucoamylase (GA), both negatively charged at pH 6.8, was assembled in the following architecture: $\{(PEI/PSS)_2 + (PDDA/GOD)_2 + (PDDA/GOD/PDDA/GA)_5\}$. The frequency shift for the four-layer unit was 800 ± 50 Hz. This value is consistent with frequency shifts for separate two-component adsorptions measured for GOD/PDDA and GA/PDDA films (Table 1).

The fourth type of an architecture is a protein/polycation/ceramic film where we demonstrate the way to assemble protein monolayers in alternation with molecularly-thin (10 Å) montmorillonite clay sheets. At first we attempted to assemble negatively-charged montmorillonite and positively charged Mb or Lys by alternate adsorption, but only one layer of charged proteins was adsorbed and no further film growth was observed, as summarized in Table 1. The reason for this is not clear. Denaturation may occur for proteins adsorbed onto solid charged substrates, but only minor conformational changes were found for Mb and Lys.[8a,3d] This situation was remedied by using interlayer polyion "glue". We chose PEI as polycation to keep together neighboring layers of negatively-charged montmorillonite and GOD. Figure 7 describes QCM monitoring of the assembly process. The first 11 cycles represent formation of a $\{(PEI/PSS)_2 + (PEI/Mont)_3 + PEI\}$ precursor film. The tetrad unit of (GOD/PEI/Mont/PEI) was then assembled. One can follow the assembly by individual steps: at step 12 the frequency shift corresponds to adsorption of a thicker GOD layer; some of the GOD molecules are peeled out during the following immersion in PEI solution (step 13), montmorillonite sheets are absorbed stable at step 14, and PEI adsorption of step 15 completes the formation of superlattice unit. The identical mass increase is repeated from the second cycle on as follows: $-\Delta F = 1950 \pm 40$ Hz; 2000 Hz for GOD and −400 Hz for PEI (which peels off some of the underlying GOD molecules); 250 Hz for Mont and 100 Hz for PEI. This cycle was also repeatable when the characteristic GOD absorption was monitored.

Polyion Shape and Film Formation. We demonstrated in this article the assembly pattern of different types of mono- and multiprotein films with precise control of their architectures. The protein multilayers were constructed from two proteins with opposite charges, different proteins of the same charge, and one protein that is charged positive or negative at different pHs, and by alternation of protein, polycation, and clay layers. The kinetics of the adsorption process could be delineated by the QCM technique. The *in-situ* measurement is indispensable for establishing proper experimental conditions. The saturation and peel-off processes cannot be readily identified by other methods. Therefore, the multilayer formation is quite general. Schematic illustrations of such films are given in Figure 8. The physical separation of the individual layers in the films is assumed on the basis of the regular, stepwise mass increase during the assembly. However, the layer separation between proteins and polyions may not be definite, as illustrated in Figure 8. The linear polyions would rather cover the protein surface and bridge protein molecules, so that the protein molecules are stabilized. Additionally, in montmorillonite containing films, some ran-

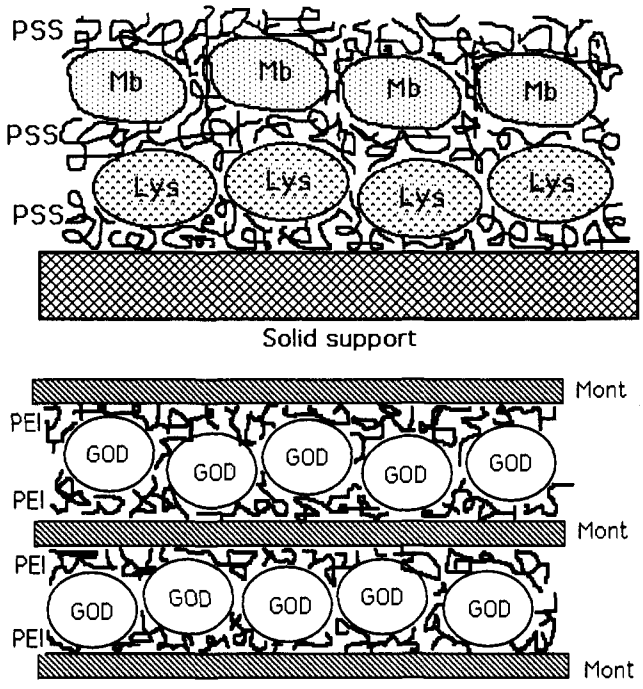

Figure 8. Schematic simplified illustrations of protein films: (top) Mb/PSS/Lys/PSS multilayer and (bottom) GOD/PEI/montmorillonite/PEI multilayer.

domly distributed defects due to occasional overlapping of clay sheets were visible on scanning electron micrographs.

The assembly process is straightforward, since it depends basically on electrostatic attraction. This appears to be essentially valid with linear polyions. However, the situation would be different for macroions with specific three-dimensional shapes and charge distributions. We failed to prepare a GOD/Lys composite layer and alternate assembly of negatively-charged montmorillonite and positively-charged Mb or Lys. Direct assembly of oppositely-charged protein molecules was difficult, probably because electrostatic attraction cannot be maximized with globular proteins. The density and location of charged units on the protein surface should be crucial in this respect. The "patched" nature of the protein surface charge is known to favor polyion anchoring.[18a] This problem also applies to the planar montmorillonite ion. On the other hand, flexible linear polyions can produce optimized electrostatic attraction. They may penetrate in between proteins molecules and act as electrostatic glue.

Deposition of charged proteins onto the oppositely charged polyion layer may be accompanied by polyion-induced aggregation. It was reported that up to tens of serum albumin molecules underwent PDDA-induced aggregation in solution.[18b] Certainly, some of linear polyions have "open" arms extended from a film surface to a bulk solution, and during the contact with incoming proteins, they may induce protein aggregation. As a consequence, more than one layer of protein molecules may be adsorbed in one step. We see this situation for the case of GOD/PEI assembly, where the step of film growth can be larger than protein monolayer thickness (Table 1).

Finally, the surface structure of solid support can affect the stability of the assembly. Among the proteins employed in this study, the assembly processes of Hb, GOD, and Mb were more stable than those of Cyt *c* and Lys. Some failures happen at their assembly onto quartz slides: the alternate Lys/PSS adsorption on slides sometimes showed a sudden stop after 4−5 cycles, and only one cycle proceeded satisfactorily for the Cyt *c*/PSS adsorption. Interestingly, multilayer assembly onto the QCM resonator was performed successfully for all of these proteins.

The protein multicomponent films are extremely interesting as novel biologically-active materials. We can arrange given protein layers according to specific biological activity. Sequential enzyme reactions and vectorial transfer of electrons and energy become feasible targets by preparation of anisotropic protein layers and precise control of distances of active layers.

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