Layer-by-layer Architectures of Concanavalin A by means of Electrostatic and Biospecific Interactions

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The surface charge and the sugar receptor site of concanavalin A are used to form layer-by-layer architectures with cationic poly(ethyleneimine) and glycogen, respectively, as evidenced by quartz crystal microbalance and scanning electron microscopy.

Construction of artificially ordered protein systems is an important research target in biotechnology. Immobilization of enzymes has been the subject of intensive attention for many years and a variety of preparative methods have been developed.[1] One of the approaches is based on formation of organized films by the Langmuir–Blodgett technique, as typically illustrated by the pioneering work of Langmuir and Schaefer.[2] Recently a novel technique of ultrathin film assembly was developed by Decher *et al.*[3,4] taking advantage of alternate adsorption of oppositely charged linear polyions. This technique was subsequently extended to include biological macromolecules and inorganic macroions.[5–7] The driving force for the alternate adsorption is not restricted to electrostatic forces. Metal coordination has been used for this purpose by Keller *et al.*[8] and by Watanabe and Regen.[9] In the case of biomolecules, specific interactions can be utilized. Ringsdorf and coworkers[10] used streptavidin as a connector between the neighbouring layers of biotinylated macromolecules. A more sophisticated example is the preparation of a triple layer, streptavidin/concanavalin A/streptavidin, by connecting these components through a biotin-sugar linker.[11] Hong *et al.*[12] combined excessive adsorption of charged species and biospecific interaction between neighbouring layers, and assembled multilayers of streptavidin and biotinylated polylysine.

We found that the strong binding between concanavalin A (Con A) and glucose was directly applicable to the alternate assembly of Con A and glycogen (branched polyglucose). Formation of ordered Con A layers on glycolipid Langmuir monolayers has been studied previously. The glucose containing polymer, dextran, was adsorbed to a Con A monolayer.[13,14] Admixing of glycolipids at a level as low as 1% relative to nonspecific matrix monolayers results in attachment of the Con A layer from water through specific bonding and the presence of only a few glucose molecules in the area of 10 × 10 nm is adequate for anchoring of a Con A monolayer.[14] Con A has an isoelectric point of p*I* 5 and exists as a dimer at pH 5–6.7, as a tetramer at pH 6.8–7.5 and undergoes further aggregation at higher pH.[15] There is one binding centre for its ligand D-glucose in each unit.

Concanavlin A *Canavalia ensiformis* [Con A, M_W 104 000 (tetramer), Wako] was used as purchased, aqueous protein was used at a concentration of *ca.* 1 mg ml^{-1}. Concanavalin A and glycogen (Glyc, M_W 1000 000, Wako) were dissolved in water or Tris buffer containing 0.1 mmol dm^{-3} $MnCl_2$, 0.1 mmol dm^{-3} $CaCl_2$ and 0.12 mol dm^{-3} NaCl. Sodium poly(styrenesulfonate) (PSS, M_W 70000, Aldrich) and branched poly(ethyleneimine) (PEI, M_W 70000, Wako) were dissolved in pure water (Millipore, resistance 18 mΩ) at concentrations of 3 and 1.5 mg ml^{-1}, respectively.

We monitored the assembly process by the quartz crystal microbalance technique (QCM, USI System, Japan). In a typical procedure, a precursor film was assembled on a silver QCM resonator (0.16 cm^2) by repeating three adsorption processes of PEI, PSS and PEI. The outermost layer becomes 'positive'. Then the solid substrate was alternately immersed for 30 min in aqueous solutions of Con A, PEI or glycogen with intermediate water washing. The process was periodically interrupted for QCM measurements. From the Sauerbrey equation, by taking into account characteristics of the quartz resonator used (F_o = 9 MHz), one obtains the following relationship between adsorbed mass ΔM (ng), and frequency shift ΔF (Hz):[16] $\Delta F = -(1.14 \pm 0.1)\Delta M$. For some films scanning electron microscopy (SEM, instrument Hitachi S-900; acceleration voltage, 25 kV) was performed.

Fig. 1 shows results of QCM monitoring of Con A assembly. Initially a precursor film of PEI + $(PSS/PEI)_2$ was prepared. Then five pairs of the Con A/PEI bilayers were assembled onto the cationic PEI layer at pH 7.1 *via* electrostatic interaction between Con A$^-$ and PEI$^+$. An additional Con A$^-$ monolayer was adsorbed electrostatically, and we switched the mode of the assembly from electrostatic to specific ones to perform alternate adsorption of neutral glycogen (in $MnCl_2$, $CaCl_2$ and NaCl) and Con A. The total layer architecture is represented by: {PEI + $(PSS/PEI)_2$ + $(Con\ A/PEI)_5$ + $(Con\ A/Glyc)_9$}. In this second stage, the mode of assembly appears to change with pH of the Con A solution in the dimeric form at pH 5.6, or in the tetrameric form at pH 7.1 (Tris buffer). In all three assembly modes one can see linear frequency shifts (*i.e.* linear mass increases) with adsorption cycles. The QCM frequency shifts for the Con A/Glyc bilayer are 390 ± 30 and 820 ± 10 Hz, respectively. Con A adsorption provides shifts of about 200 and 420 Hz, correspondingly. Electrostatic Con A/PEI assembly shows $-\Delta F$ = 410 ± 20 Hz for bilayer: 360 Hz for Con A and 50 Hz for PEI. The frequency shift corresponding to PEI adsorption is much less than the one for Glyc adsorption, which reflects the difference in their molecular masses.

We have now a typical frequency shift of *ca.* 200 Hz for the Con A assembly at a lower pH, and of 360–420 Hz for the other two modes at higher pH assembly. These shifts correspond to an

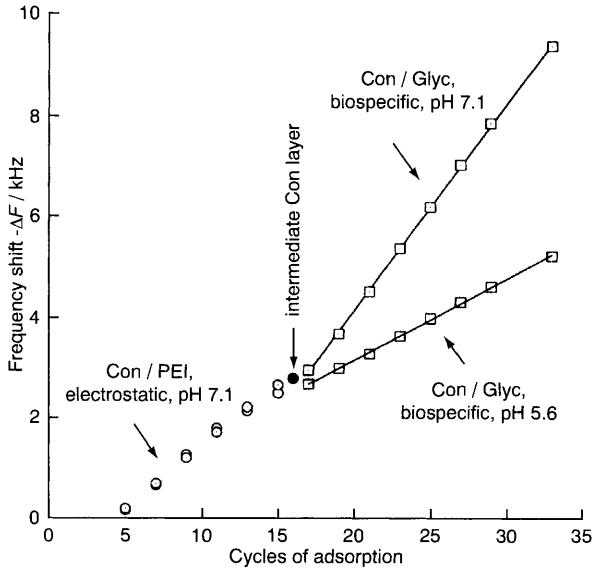

Fig. 1 QCM frequency shifts ($-\Delta F$) and cycles of alternate adsorption: {$(PEI/PSS)_2$ + PEI + $(Con\ A/PEI)_5$ + Con A + Glyc + $(Con\ A/Glyc)_8$} at pH 7.1 and pH 5.6. At steps 30–33 intermediate drying was not carried out.

adsorption of *ca.* 87 and 170 ± 20 ng on either side of the QCM resonator, respectively. Ebara and Okahata[14] found a 100 ng mass increase on a similar QCM device for maximal binding of Con A monolayer to a preformed glycogen monolayer at pH 7.4. The discrepancy may be attributed to the difference in QCM techniques used: their measurements were performed *in situ* in water, but we registered a mass increase of dried films. The surfaces to which Con binds are different in these two approaches: ordered glycolipid monolayer or glycogen layer with statistically distributed sugar-ending.

The thickness of Con A layers is estimated from mass increase as 4.1 and 8.1 nm at low and at high pH, respectively, by using a protein density of *ca.* 1.3 g cm^{-3} and a film area of 0.16 cm^2. These thicknesses are consistent with the monolayer formation from Con A in dimer or tetramer forms, since the dimensions of the Con A subunit are $3.9 \times 4.0 \times 4.2$ nm^3. The adsorption process at pH 7.1 appears more stable than that at pH 5.6, as the frequency change is more reproducible under the former conditions. This probably reflects better interlayer anchoring due to the presence of four binding sites in the tetrameric Con A.

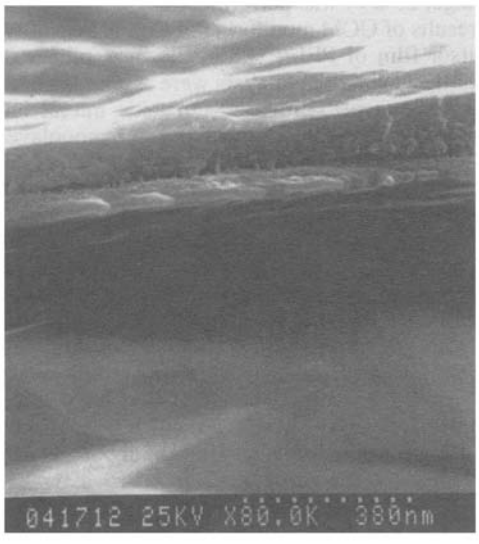

Fig. 2 SEM micrograph of $\{(PEI/PSS)_2 + PEI + (Con\ A/PEI)_5 + (Con\ A/Glyc)_9\}$ film. A resonator with the assembled film was cut and coated with 2 nm thick Pt by use of an ion-coater (Hitachi E-1030 ion sputter, 10 mA/10 Pa) under an argon atmosphere.

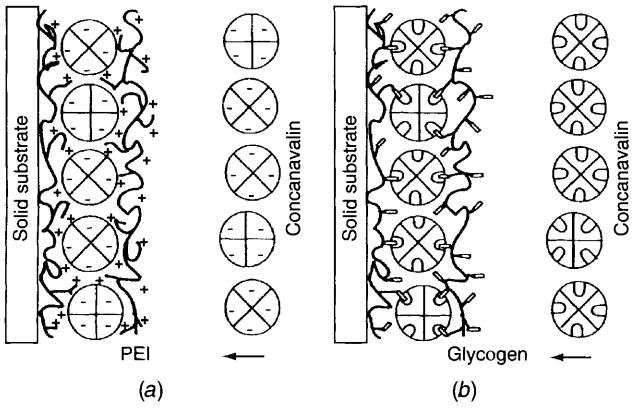

Fig. 3 Two types of the alternate adsorption technique used in the Con A architecture: (*a*) assembly *via* electrostatic attraction of tetrameric Con A, (*b*) assembly *via* specific interaction in the case of tetrameric Con A

A scanning electron micrograph of a Con A/Glyc film which contains 14 protein layers is shown in Fig. 2. The assembled film follows the contours of the silver electrode in the QCM resonator with a thickness of 150 ± 10 nm. This corresponds to the *ca.* 10 nm thickness of one protein/polymer bilayer and is close to the calculated thickness from QCM data. One can see that the silver electrode is detached from the underlying quartz probably during cutting.

The specific Con A–Glyc interaction is the driving force in the second stage of the assembly. This is established by the following results: (*a*) a new Con A layer is not adsorbed onto the terminal Con A layer; (*b*) only a minor amount of Glyc is adsorbed onto the terminal Glyc layer; (*c*) proteins of the same charge as Con A (glucose oxidase and catalase) are not adsorbed onto the terminal glyc layer; (*d*) alternate assembly of Con A with non-charged poly(vinyl alcohol) (instead of glycogen) does not proceed.

We have demonstrated in the present work that the multilayer assembly of Con A was achieved by two modes of interaction. They are schematically shown in Fig. 3 for the case of tetrameric Con A. In the neutral pH region Con A molecules exist as tetramer species and are assembled as polyions in combination with polycations, or as a biospecific receptor in combination with glycogen. The potent multireceptor sites are effectively used for alternate assembly with a polymeric specific guest (glycogen in this instance) in the neutral and weakly acidic regions. The observed multiple modes of the alternate assembly are advantageous for constructing more elaborate protein architectures.

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References

1 V. N. Hasirci, *Immobilization of bioactive species*, in *Biomaterials, European Materials Research Society Monographs 3*, ed. D. Muster, Elsevier, Amsterdam, 1993.
2 I. Langmuir and V. Schaefer, *J. Am. Chem. Soc.*, 1938, **60**, 1351; see also *Thin Solid Films*, 1994, **244**, special issue, *Proceedings of 6 Langmuir–Blodgett Conference*.
3 G. Decher and J.-D. Hong, *Ber. Bunsenges. Phys. Chem.*, 1991, **95**, 1434.
4 Y. Lvov, G. Decher and H. Möhwald, *Langmuir*, 1993, **9**, 481.
5 Y. Lvov, K. Ariga and T. Kunitake, *Chem. Lett.*, 1994, 2323.
6 Y. Lvov, K. Ariga, I. Ichinose and T. Kunitake, *J. Am. Chem. Soc.*, 1995, **117**, 6117.
7 W. Kong, L. Wang, M. Gao, H. Zhou, H. Zhang, W. Li and J. Shen, *J. Chem. Soc., Chem. Commun.*, 1994, 1297.
8 S. Keller, H.-N. Kim and T. Mallouk, *J. Am. Chem. Soc.*, 1994, **116**, 8817.
9 S. Watanabe and S. Regen, *J. Am. Chem. Soc.*, 1994, **116**, 8855.
10 M. Ahlers, R. Blankenburg, D. Grainger, P. Meller, H. Ringsdorf and C. Salesse, *Thin Solid Films*, 1989, **180**, 93.
11 W. Müller, H. Ringsdorf, E. Rump, G. Wildburg, X. Zhang, L. Angelmaier, W. Knoll, M. Liley and J. Spinke, *Science*, 1993, **262**, 1706.
12 J.-D. Hong, K. Lowack, J. Schmitt and G. Decher, *Progr. Colloid Polym. Sci.*, 1993, **93**, 98.
13 H. Haas and H. Möhwald, *Thin Solid Films*, 1989, **180**, 101.
14 Y. Ebara and Y. Okahata, *J. Am. Chem. Soc.*, 1994, **116**, 11209.
15 A. Kalb and A. Lustig, *Biochim. Biophys. Acta*, 1968, **168**, 366.
16 Y. Okahata, K. Ariga and K. Tanaka, in *Organic Thin Films and Surfaces: Directions for Nineties*, ed. A. Ulman, Academic Press, 1995, p. 317.