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A Kinetic Study of Concanavalin A Binding to Glycolipid Monolayers by Using a Quartz-Crystal Microbalance

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Abstract: Specific binding and dissociation processes of concanavalin A (Con A) to a mixed monolayer containing synthetic glycolipids at the air−water interface were observed from frequency changes of a quartz-crystal microbalance (QCM) that was attached horizontally on the monolayer from the air phase. Binding constants (k_1), dissociation rate constants (k_{-1}), and binding amount (Δm) could be obtained from time courses of the frequency decrease (mass increase) of the QCM. Con A showed the specific binding to $2C_{18}$-mal lipids having a glucopyranosyl head group but not $2C_{18}$-lac lipids having a galactopyranosyl head group in the matrix of phosphoethanolamine lipids ($2C_{18}$PE). The maximum binding amount (Δm_{max}) was independent of the mole fraction of the $2C_{18}$-mal, but on the contrary, binding (k_1), dissociation rate (k_{-1}), and association constants ($K_a = k_1/k_{-1}$) were affected largely by the content of the $2C_{18}$-mal in the monolayer.

Introduction

Specific protein binding to a receptor at the cell membrane surface is common to various cell functions such as immune cell recognition and response. A monolayer lipid film at the air−water interface has been demonstrated to be useful in cell surface modeling. Studies in binding behavior of proteins from solution with a lipid monolayer have been reported by using various *in situ* techniques: surface tension measurements,[1,2] fluorescent-labeling techniques,[3−5] radio-labeling techniques,[6] fluorescence reflection methods,[7] surface plasmon resonance,[8,9] and surface force measurements.[10] These methods have a future potential in observing protein bindings; however, these methodologies require large and expensive equipment for *in situ* measurements or have some associated difficulties in obtaining quantitatively the amount of protein adsorbed and the time courses of both binding and dissociation processes.

In this paper, we report a new, easy, and *in situ* technique for detecting the specific binding of Con A from a subphase to a mixed monolayer of synthetic glycolipids ($2C_{18}$-mal or $2C_{18}$-lac) in the phosphoethanolamine matrix ($2C_{18}$PE) at the air−water interface, on which a QCM was horizontally attached from the air phase (see Figure 1). QCMs are known to provide very sensitive mass measurings because their resonance frequency decreases upon the increase of a mass on the QCM in the nanogram level.[11−14,22] Binding amount (Δm), binding and dissociation rate constants (k_1 and k_{-1}), and association constants

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(1) Watanabe, N.; Shirakawa, T.; Iwahashi, M.; Seimiya, T. *Colloid Polym. Sci.* **1988**, *266*, 254.
(2) Ivanova, M.; Panaiotov, I. *Colloid Surf.* **1986**, *17*, 159.
(3) Blankenburg, R.; Meller, P.; Ringsdorf, H.; Salesse, C. *Biochemistry* **1989**, *28*, 8214.
(4) Heckl, W. H.; Thompson, M.; Möhwald, H. *Langmuir* **1989**, *5*, 390.
(5) Heyn, S.-P.; Egger, M.; Graub, H. E. *J. Phys. Chem.* **1990**, *94*, 5073.
(6) Brash, J. L.; Uniyal, S.; Pusineri, C.; Schmitt, A. *J. Colloid Interface Sci.* **1983**, *95*, 28.
(7) (a) Tanimoto, S.; Kitano, H. *Langmuir* **1993**, *9*, 1315. (b) Zao, S.; Reichert, W. M. *Langmuir* **1992**, *8*, 2785.
(8) Kooyman, R. P. H.; Bruijn, H. E.; Eenink, R. G.; Greve, J. *J. Mol. Struct.* **1990**, *218*, 345.
(9) Häussling, L.; Ringsdorf, H.; Schmitt, F.-J.; Knoll, W. *Langmuir* **1991**, *7*, 1837.
(10) Leckband, D. E.; Israelachivili, J. N.; Schmitt, F.-J.; Knoll, W. *Science* **1992**, *255*, 1419.

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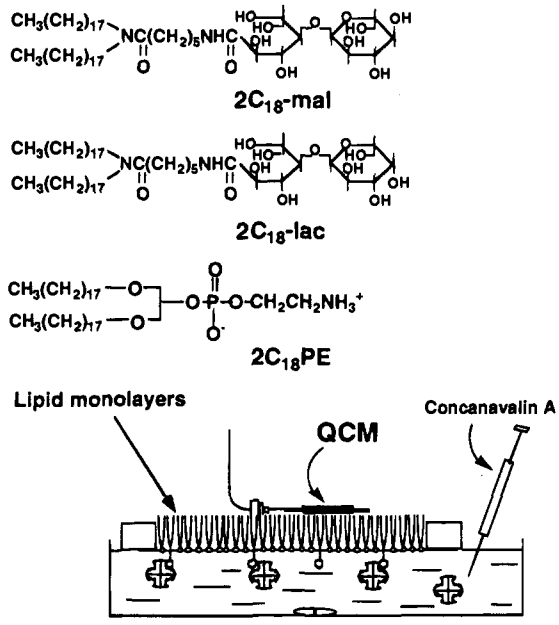


Figure 1. Chemical structures of monolayer-forming lipids and a schematic illustration of the experimental setup.

(K_a) could be determined quantitatively from the time courses of the frequency decrease (mass increase) of the QCM on the monolayer, responding to the addition of proteins into a subphase.

Recently, QCMs have become popular tools for detection of bioactive compounds such as odorous and bitter substances,[11] protein adsorption,[12,13] immunoassay,[14-17] DNA hybridization,[18-20] enzyme reactions,[21,22] and cell growth.[23]

Experimental Section

Materials. Preparations of synthetic phosphoethanolamine lipid ($2C_{18}$PE) were reported elsewhere.[24,25] Structures and purities of the prepared lipids were confirmed by TLC with a flame ionization detector (TLC-FID, Iatroscan MK-5, Iatron Co., Ltd., Tokyo), NMR and IR spectroscopies, and elemental analyses (C, H, and N, within ±0.2% errors).

A synthetic glycolipid, $2C_{18}$-mal, containing α-D-glucopyranosyl-D-gluconamide as a hydrophilic head group was prepared as follows. ω-Bromo(*N,N*-dioctadecyl)hexanamide was prepared from *N,N*-dioctadecylamine and ω-bromohexanoyl chloride in chloroform in the presence of triethylamine: yield 32 g (76%). ω-Amino(*N,N*-dioctadecyl)hexanamide was prepared by the Gabriel synthesis from the bromo compound and potassium phthalimide and then converted to the final product by treating with hydrazine in ethanol and recrystallizing twice with ethyl acetate: yield 2.5 g (13%); TLC-FID (8:2 chloroform/ethanol) showed one peak (R_f = 0.4). Maltose [*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose] was oxidized to maltonolactone in methanol in the presence of iodide.[26,27] Maltonolactone was allowed to react with ω-amino(*N,N*-dioctadecyl)hexanamide in refluxing methanol for 20 h and recrystallized from ethyl acetate/dichloroethane (3:1): yield 3.8 g (54%); white powder; mp 155–157 °C; TLC-FID (8:2:0.1 chloroform/methanol/NH_3) showed a single peak (R_f = 0.2).

A synthetic glycolipid, $2C_{18}$-lac, containing β-D-galactopyranosyl-D-gluconamide as a hydrophilic head group was prepared from the dialkylamino compound and the oxidized lactose [*O*-β-D-galactopyranosyl-(1→4)-D-glucopyranose] in the same way.[26,27] The obtained pale yellow powder was purified through a silica gel column [dichloromethane/ethyl acetate (3:1)]: yield 0.7 g (14%); mp 155–157 °C; TLC-FID (8:2:0.1 chloroform/methanol/NH_3) showed a single peak (R_f = 0.4)

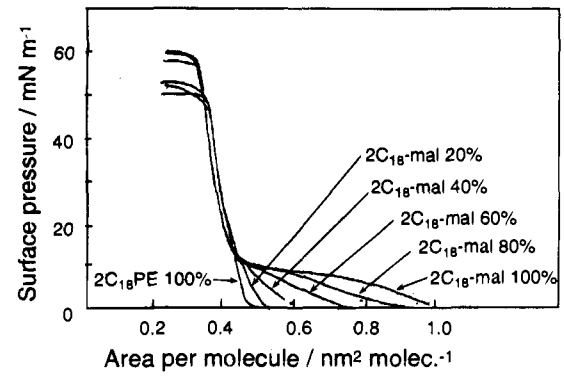


Figure 2. π–*A* isotherms of the mixed monolayers of $2C_{18}$-mal and $2C_{18}$PE (20 °C, pH 7.4, NaCl 150 mM, Ca^{2+} and Mn^{2+} 0.1 mM).

Measurements. A chloroform solution of a synthetic glycolipid, $2C_{18}$-mal or $2C_{18}$-lac, and a synthetic phospholipid, $2C_{18}$PE, was spread on an aqueous solution (pH 7.4, NaCl 150 mM, Ca^{2+} and Mn^{2+} 0.1 mM) in a Teflon-coated trough with a microcomputer-controlled Teflon barrier (San-Esu Keisoku Co., Fukuoka).[28,29] The stable mixed monolayer formation was confirmed from surface pressure (π)–area (*A*) isotherms at 20 °C in various compositions of the $2C_{18}$-mal glycolipids and $2C_{18}$PE, and their π–*A* curves are shown in Figure 2. Similar π–*A* curves were also obtained in the case of the mixed monolayer of $2C_{18}$-lac and $2C_{18}$PE (data not shown). The plots between mean molecular areas and the mole fraction of the glycolipid in the mixed monolayer showed good linear correlation at given surface pressures of 10, 20, 30, and 40 mN m^{-1}. The collapse pressures of the monolayer obtained from π–*A* isotherms changed depending on the glycolipid mole fraction. These findings indicate that glycolipids, $2C_{18}$-mal or $2C_{18}$-lac, mixed *homogeneously* with $2C_{18}$PE matrices in the mixed monolayer.

The QCM employed is a commercially available 9-MHz, AT-cut quartz (diameter 9 mm) on both sides of which Au electrodes were deposited (area 16 mm^2) obtained from Kyushu Dentsu Co., Ltd., Tokyo. The one side of the QCM was covered with a rubber case to avoid the contact with the ionic buffer solution. The bare Au electrode side of the QCM plate was attached horizontally to the mixed monolayer at a surface pressure of 30 mN m^{-1}, and the frequency change of the QCM responding to the addition of Con A from aqueous solution was followed with time. The subphase was stirred gently in order to avoid the effect of the diffusion process of Con A, and the stirring did not affect the stability of the monolayer (see Figure 1). The QCM was connected to a handmade oscillator designed to drive the quartz at its resonance frequency in aqueous solution or at the air–water interface.[11,18,21,24] The frequency changes were followed by a universal frequency counter (Iwatsu Co., Tokyo, model SC 7201) attached to

(11) (a) Okahata, Y.; Ebato, H. *Trends Anal. Chem.* **1992**, *11*, 344. (b) Okahata, Y.; En-na, G.; Ebato, H. *Anal. Chem.* **1990**, *62*, 1431.
(12) Ebara, Y.; Okahata, Y. *Langmuir* **1993**, *9*, 574.
(13) Tamiya, E.; Suzuki, M.; Karube, I. *Anal. Chim. Acta* **1989**, *217*, 321.
(14) Thompson, M.; Arthur, C. L.; Dhaliwal, G. K. *Anal. Chem.* **1986**, *58*, 1206.
(15) Muramatsu, H.; Dicks, J. M.; Tamiya, E.; Karube, I. *Anal. Chem.* **1987**, *59*, 2760.
(16) Ebersole, R. C.; Ward, M. D. *J. Am. Chem. Soc.* **1988**, *110*, 8623.
(17) Ebato, H.; Gentry, C. A.; Herron, J. N.; Mueller, W.; Okahata, Y.; Ringsdorf, H.; Suci, P. A. *Anal. Chem.* **1994**, *66*, 1683.
(18) Okahata, Y.; Matsunobu, Y.; Ijiro, K.; Mukai, M.; Murakami, A.; Makino, K. *J. Am. Chem. Soc.* **1992**, *114*, 8299.
(19) Ebersole, R. C.; Miller, J. A.; Moran, J. R.; Ward, M. D. *J. Am. Chem. Soc.* **1990**, *112*, 3239.
(20) Yamaguchi, S.; Shimomura, T.; Tatsuma, T.; Oyama, N. *Anal. Chem.* **1993**, *65*, 1925.
(21) Okahata, Y.; Ebara, Y. *J. Chem. Soc., Chem. Commun.* **1992**, 116.
(22) Guilbault, G. G. *Anal. Chem.* **1983**, *55*, 1682.
(23) Ebersole, R. C.; Foss, R. P.; Ward, M. D. *Biotechnology* **1991**, *9*, 450.
(24) Okahata, Y.; Ebato, H. *J. Chem. Soc., Perkin Trans. 2* **1991**, 475.
(25) Seki, T.; Okahata, Y. *J. Microencapsulation* **1985**, *2*, 13.
(26) Okahata, Y.; Nakamura, G.; Noguchi, H. *J. Chem. Soc., Perkin Trans. 2* **1987**, 1317.
(27) Kobayashi, K.; Sumitomo, H.; Ina, Y. *Polymer J.* **1983**, *15*, 667.
(28) Okahata, Y.; Kimura, K.; Ariga, K. *J. Am. Chem. Soc.* **1989**, *111*, 9190.
(29) Ariga, K.; Okahata, Y. *J. Am. Chem. Soc.* **1989**, *111*, 5618.

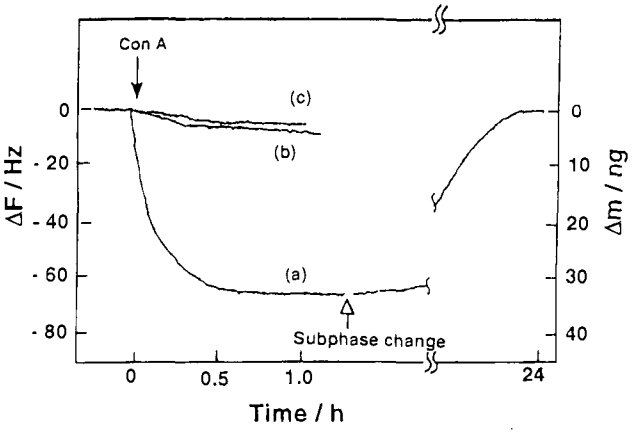


Figure 3. Typical time courses of frequency changes (ΔF) of a QCM on the mixed monolayers of (a) $2C_{18}$-mal:$2C_{18}$PE = 40:60 and (b) $2C_{18}$-lac:$2C_{18}$PE = 40:60 and the homo-monolayer of (c) $2C_{18}$PE at a surface pressure of 30 mN m^{-1}, responding to the addition of Con A (10 ppm, 0.1 μM, M_w = 104 000) from an aqueous solution (20 °C, pH 7.4, NaCl 150 mM, Ca^{2+} and Mn^{2+} 0.1 mM) at the closed arrow. The subphase was changed to a new buffer solution at the open arrow.

the microcomputer system (NEC Co., Tokyo, model PC 9801). Calibration showed that a frequency decrease of 1 Hz corresponded to a mass increase of 0.5 ng on the QCM electrode in an aqueous solution,[18,21,24] according to a Sauerbrey's equation.[30]

$$-\Delta F = (2.1 \pm 0.1)\Delta m \quad (1)$$

When the 9-MHz QCM was attached horizontally to the water subphase without monolayers, the frequency decreased 4350 ± 10 Hz compared with that in air, which was consistent with the theoretical frequency change due to the high viscosity and density of water compared with that in air.[12,30,31] When the QCM was attached horizontally to the lipid monolayer on the water subphase, the frequency deceased 4400 ± 10 Hz, which is the total of the effect due to the mass of the lipid monolayer under the electrode ($-\Delta F$ = 50 Hz) and the effect of the water medium ($-\Delta F$ = 4350 ± 10 Hz). The frequency decrease due to the mass of the monolayer was consistent with the theoretical value calculated from the molecular area (0.4 nm^2 at 30 mN m^{-1} from π–A isotherms) and the electrode area of the QCM (16 mm^2). These results suggest that the QCM plate is maintained intact on the monolayer and is sensitive to the mass under the monolayer. The resonance frequency when the QCM was attached to the monolayer was defined as the standard (zero position).[12] The stability or the drift of the QCM frequency on the subphase was ±2 Hz for 12 h at 20–30 °C.

Results and Discussion

Figure 3 shows typical time courses of frequency changes (ΔF) of the QCM on the monolayer responding to the addition of Con A (10 ppm, 0.1 μM, M_w = 104 000) into the subphase. When the mixed monolayer of $2C_{18}$-mal:$2C_{18}$PE = 40:60 in mole percent was employed, the frequency of the QCM decreased (mass increased) gradually responding to the addition of Con A and saturated at $-\Delta F$ = 66 ± 5 Hz (Δm = 31 ± 5 ng according to eq 1) within 1 h (curve a). When the subphase was changed to a new buffer solution at the open arrow in the figure, the frequency very slowly reverted to the original value (mass decrease), which indicates fast binding and very slow dissociation of Con A from the monolayer surface. When the QCM was moved to an excess amount of glucose solution (70 mM) at the open arrow, the dissociation rate was largely increased due to the exchange reaction between the glycolipid monolayer and excess glucose in the subphase. When the QCM plate was moved upward to the air phase at the open arrow and dried in air, the binding amount of Con A was calculated to be Δm = 35 ± 10 ng from the frequency change in the air phase before and after the deposition. This value was consistent with the binding amount (Δm = 31 ± 5 ng) obtained directly from the time course of the frequency decrease in the subphase.

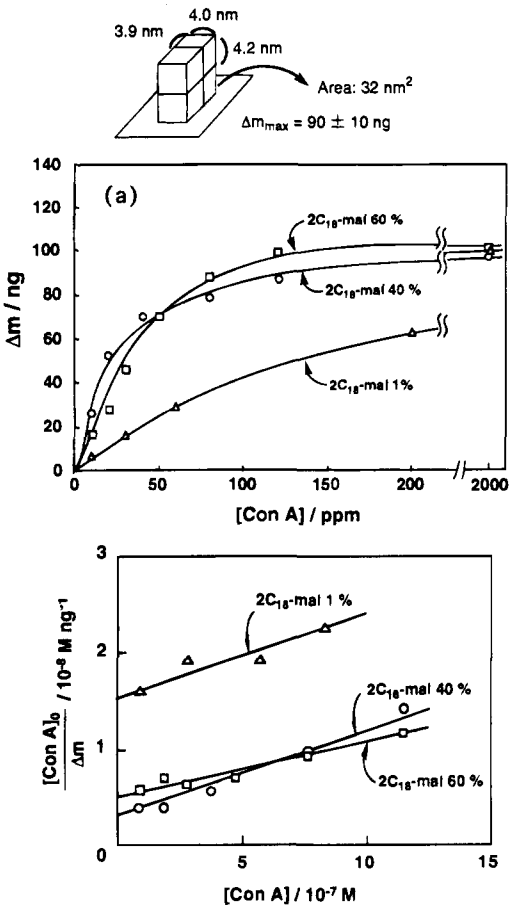


Figure 4. (a) Saturation binding behavior of Con A to the mixed monolayer of $2C_{18}$-mal (1, 40, and 60 mol %) and $2C_{18}$PE depending on the concentration of Con A in the subphase (20 °C, pH 7.4, NaCl 150 mM, Ca^{2+} and Mn^{2+} 0.1 mM, at a surface pressure of 30 mN m^{-1}). (b) Linear correlation between $[\text{Con A}]_0/\Delta m$ and $[\text{Con A}]_0$ according to eq 2.

When the mixed monolayer of $2C_{18}$PE and $2C_{18}$-lac having a galactopyranosyl head group or the $2C_{18}$PE phospholipid homo-monolayer was employed, the binding of Con A was hardly observed, as shown in curves b and c in Figure 3. Con A is known to have four binding sites for α-D-glucopyranosyl, but not β-D-galactopyranosyl, in glycolipids and polysaccharides on the cell surface.[32–34] Therefore, curve a in Figure 3 indicates the selective binding of Con A to a glucopyranosyl head group of $2C_{18}$-mal in the mixed monolayer.

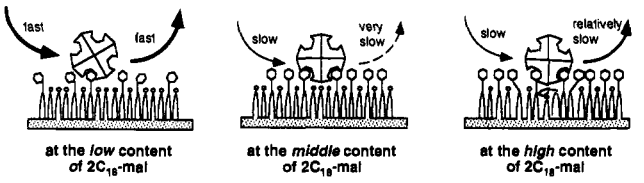
When the concentration of Con A was increased in the subphase, the amount binding to the $2C_{18}$-mal/$2C_{18}$PE mixed monolayer *vs* the concentration in the subphase showed typical saturation curves at the given mole fractions of $2C_{18}$-mal (see Figure 4a). Linear correlations between $[\text{Con A}]_0/\Delta m$ and [Con A] according to eq 2 are shown in Figure 4b. Association constants (K_a) of Con A to the $2C_{18}$-mal were calculated from

(30) Sauerbrey, G. *Z. Phys.* **1959**, *155*, 206.
(31) (a) Kanazawa, K. K.; Gordon, J. G. *Anal. Chim. Acta* **1985**, *175*, 99. (b) Schmacher, R.; Borges, G.; Kanazawa, K. K. *Surf. Sci.* **1985**, *163*, L621.
(32) Kawaguchi, T.; Matsumoto, I.; Osawa, T. *Biochemistry* **1974**, *13*, 3169.
(33) Novogradsky, A.; Biniaminov, M.; Ramot, B.; Katchalski, E. *Blood* **1972**, *40*, 311.
(34) Becker, J. W.; Reeke, G. W., Jr.; Edelman, G. M. *Nature* **1976**, *259*, 406.

Table 1. Binding (k_1) and Dissociation Rate Constants (k_{-1}) and Association Constants (K_a) of Con A with the Mixed Monolayer of $2C_{18}$-mal and $2C_{18}$PE

monolayer content ($2C_{18}$-mal:$2C_{18}$PE)	k_1 (M^{-1} s^{-1})	k_{-1} (10^{-6} s^{-1})	$K_a = k_1/k_{-1}$ (10^6 M^{-1})	K_a[a] (10^6 M^{-1})
1:99				0.6
5:95	1800	1400	1.3	
20:80	420	170	2.5	
40:60	190	61	3.1	2.8
60:40	250	210	1.2	1.6

[a] Obtained from eq 2.



the slopes and intercepts of Figure 4b, and the results are summarized in Table 1.

$$\frac{[\text{Con A}]_0}{\Delta m} = \frac{[\text{Con A}]_0}{\Delta m_{max}} + \frac{1}{\Delta m_{max} K_a} \quad (2)$$

The maximum binding amounts (Δm_{max}) at various $2C_{18}$-mal mole fractions were constant and were calculated to be 100 ± 10 ng (*ca.* 1 pmol). Con A has been observed by X-ray crystallography to contain the 222 symmetry of a tetramer of 3.9 x 4.0 × 4.2 nm^3 peptides, each of which has one binding site.[34] Since it is difficult to bind four binding sites of Con A with the planar lipid membrane at the same time, Con A is expected to bind using two binding sites as shown in the illustration in Figure 4. When Con A binds to the $2C_{18}$-mal mixed monolayer by using two binding sites, Δm_{max} is calculated to be 90 ± 10 ng as a Langmuir adsorption, which is consistent with Δm_{max} = 100 ± 10 ng obtained from eq 2.

When a small amount of dextran (1 mM as a glucose unit) was injected into the subphase at the maximum binding of Con A to the glycolipid monolayer, a frequency decrease (mass increase) was observed due to the binding of glucose units in dextran chains to Con A under the monolayer. The addition of dextran was confirmed not to affect the viscosity or density of the subphase in these conditions. These results also suggest that Con A binds to the glycolipid monolayer using two binding sites and has other binding sites for dextran.

From π–A isotherm experiments, the $2C_{18}$-mal was found to have a molecular area similar to that of the $2C_{18}$PE (0.4 nm^2) and to be mixed homogeneously with the $2C_{18}$PE matrix membrane. Therefore, the $2C_{18}$-mal content in mole percent in the mixed membrane reflects the surface density of the glycolipid in the membrane. The binding area of Con A (32 nm^2) is very large relative to that of the $2C_{18}$-mal lipid molecule (0.4 nm^2), and *ca.* 80 lipid molecules are calculated to be covered under one Con A binding. This means that the existence of less than 1 mol % of the $2C_{18}$-mal lipid is enough to bind Con A as a monolayer from the subphase. Therefore, the Δm_{max} value was independent of the $2C_{18}$-mal content in the monolayer in the range of 1–60 mol %.

The binding between a receptor ($2C_{18}$-mal) and a legend (Con A) is described by eq 3.

$$[2C_{18}\text{-mal}] + [\text{Con A}] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [2C_{18}\text{-mal/Con A}] \quad (3)$$

The amount of the $2C_{18}$-mal/Con A complex, formed at time t after injection, is given by eq 4.

$$[2C_{18}\text{-mal/Con A}]_t = [2C_{18}\text{-mal/Con A}]_\infty (1 - e^{-\frac{1}{\tau}t}) \quad (4)$$

where $[2C_{18}\text{-mal/Con A}]_t = \Delta m_t$, $[2C_{18}\text{-mal/Con A}]_\infty = \Delta m_{max}$, and $\frac{1}{\tau} = k_1[\text{Con A}] + k_{-1}$.

The binding (k_1) and dissociation rate constants (k_{-1}) were obtained from the time courses of the binding process (curve a in Figure 3) by using eq 4 at four different Con A concentrations (1.0×10^{-7}, 4.0×10^{-7}, 7.5×10^{-7}, and 11.5×10^{-7} M). k_1 and k_{-1} and the association constant ($K_a = k_1/k_{-1}$) are summarized in Table 1, at different contents of $2C_{18}$-mal in the $2C_{18}$-PE matrix membrane. K_a values obtained from the curve fitting method of eq 4 showed good consistency with those obtained from the equilibrium method of eq 2. The k_1, k_{-1}, and K_a values depended on the glycolipid content in the monolayer. Both k_1 and k_{-1} values decreased largely and then increased slightly with increasing the glycolipid content. As a result, association constants (K_a) showed a maximum at 40 mol % content of $2C_{18}$-mal in the matrix. The mixed monolayer of $2C_{18}$-mal/$2C_{18}$PE has been confirmed from π–A isotherms to form a homogeneously mixed monolayer (see Figure 2). These effects of glycolipid contents on the binding kinetics are explained as follows (see schematic illustrations below Table 1).

At a low content (*ca.* 5 mol %) of the glycolipid in the monolayer, the binding rate (k_1) is large due to the low steric hindrance between proteins and the glucopyranosyl unit on the membrane and the dissociation rate (k_{-1}) is also large due to the binding with one site. As a consequence, the K_a value becomes small. Around a content of 40 mol % glycolipids, the k_1 value slightly decreases and the k_{-1} value largely decreases due to the strong binding of Con A using two binding sites and K_a showed the maximum value of 3.1 × 10^6 M^{-1}. At a high content of $2C_{18}$-mal, the k_{-1} value is relatively increased due to the steric hindrance between the two binding sites of Con A and the tightly packed glucopyranosyl head groups in the monolayer; therefore, the K_a value decreases again.

The interaction between Con A and the glucopyranosyl units has been studied by using turbidity changes due to the aggregation of erythrocyte or lymphocyte cells and by using radioisotope-labeled Con A.[32,33] The apparent binding constants have been obtained to be *ca.* (1–5) x 10^6 M^{-1}, and our results [K_a = (1–3) x 10^6 M^{-1}] were well consistent with these values.

In conclusion, the method in which a QCM is attached horizontally to the lipid monolayer at the air–water interface will become a new, useful technique for detecting quantitatively time courses of specific binding and dissociation of proteins with a receptor lipid monolayer.