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Effect of cholesterol content on affinity and stability of factor VIII and annexin V binding to a liposomal bilayer membrane

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ABSTRACT

To investigate the effect of cholesterol composition on the binding of factor VIII (FVIII) and annexin V (AV) to membranes, liposomal membranes with phospholipid bilayers of various compositions of phosphatidylcholine (PC), phosphatidylserine (PS), and cholesterol were constructed. A surface plasmon resonance (SPR) biosensor system was employed to measure the equilibrium and rate constants of the bindings. As expected, PS was found to play a dominant role in the binding of AV; its binding level was directly proportional to the PS composition in a liposome. The binding levels of FVIII and AV to liposome increased with an increase in cholesterol composition in liposome. It seemed to suggest that cholesterol in liposome acts as a 'phospholipid arrangement' factor by inducing the formation of PS-rich microdomains. However, in the absence of PS (20% on a mole basis), cholesterol could not exert the binding enhancement effect, which again confirmed the critical role of PS in the bindings. Stability of the AV binding was significantly improved by the increase in cholesterol content; for AV, the dissociation rate constant was decreased approximately fivefold, from 1.7×10^{-3} s^{-1} in the absence of cholesterol to 3.3×10^{-4} s^{-1} in the presence of only 10% cholesterol. But, for FVIII the binding stability was not so much influenced by the cholesterol addition (up to 50% on a mole basis). In summary, by using liposomes on an SPR system, we were able to demonstrate quantitatively the apparent effects of cholesterol on the binding affinity and stability of the membrane-binding proteins.

1. Introduction

The cell membrane is known to be not only a mechanical barrier of life, but also a mediator for living activity (Boyle, 2005). The membrane works with life materials, including proteins, to activate or inactivate the life pathways. A typical protein that interacts with membrane is protein kinase C, well known in the signal pathway (Newton, 2001). Many researchers have investigated the mechanisms of protein binding to the membrane surface. One of the active research topics in membrane–-protein interactions is the interaction between phosphatidylserine (PS) and membrane-binding proteins. PS is known to correlate with several biological phenomena such as platelet activation (Zwaal and Schroit, 1997) and cellular apoptosis process (Reutelingsperger and van Heerde, 1997; van Engeland et al., 1998). PS is distributed mainly on the inner membrane; however, it is 'translocated' to the outer membrane by internal or external stimuli such as apoptosis or thrombin activation (Dale, 2005). This 'PS flipping' is known to be induced by energy-dependent and unidentified transporters (Stuart et al., 1998). To investigate the mechanism of PS flipping, many researchers have focused on cholesterol effects (Ayala-Sanmartin et al., 2001; Damek-Poprawa et al., 2006; Skrtic and Eanes, 1992). Cholesterol is one of the major components of eukaryotic cell membrane, which is highly distributed in plasma membranes (Lange et al., 1989). The cholesterol composition in the membranes, which may vary up to 50% in some cases (Jedlovszky and Mezei, 2003), provides the physical influences on the membrane properties such as fluidity, permeability, and width (Troup et al., 2004). Recently, many researchers have investigated the role of membrane cholesterol in signal transduction, immune response, cell infection, and cell surface polarity (Batetta and Sanna, 2006; Ramjiawan et al., 1996; Reineri et al., 2007).

Factor VIII (FVIII) is well known to play an important role in blood coagulation cascades. In the blood coagulation cascade, PS should be exposed to the outer membrane for FVIII and FVIIIa to bind to the platelet membrane surface. The translocated PS by thrombin activation, which is related to cholesterol ratio on plasma membranes, was known to provide the binding sites for

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FVIII (Bevers et al., 1983; Heemskerk et al., 1995). Annexin V (AV) is one of the phospholipid binding proteins with high affinity to PS in the presence of calcium ions. AV is used as a powerful tools for detection of PS flipping in apoptosis (Seaton and Dedman, 1998), membrane trafficking, cell signaling, anti-inflammatory, and anti-coagulation processes (Gerke et al., 2005; Reutelingsperger and van Heerde, 1997).

The aim of this study was to elucidate the cholesterol effect on the interaction between PS and PS-binding proteins by using a surface plasmon resonance (SPR) sensor. As the model PS-binding proteins, FVIII and AV were chosen. As artificial membranes, phospholipid bilayer liposomes with various compositions of PC, PS and cholesterol were constructed. Most of the previous studies performed on the cholesterol effect on FVIII/AV binding to PS were based on the biological binding methods such as flow cytometer (Koopman et al., 1994), tryptophan 187 fluorescence (Meers and Mealy, 1993), and a fluorescence-based assay (Heemskerk et al., 1995). We used liposomes with defined chemical compositions, since these biological binding methods could yield different results depending on the binding parameters that may depend on cell conditions.

2. Experimental procedures

2.1. Materials

FVIII was kindly provided by PanGen Biotech, Inc. (Seongnam, Korea) and AV was from the Department of Biochemistry, College of Medicine, Kyungpook National University (Daegu, Korea). Phosphatidylcholine (PC), PS, and cholesterol (Chol) were purchased from Sigma (St. Louis, MO, USA) for liposome preparation. BIACORE 3000 was used as an SPR sensor (Biacore AB, Sweden). L1 chip, which contained a lipophilc polymer layer attached to a dextran layer, was used for liposome immobilization. Human serum albumin (HSA) was purchased from Sigma (USA) to prevent the non-specific binding on L1 chip. All other chemicals used were in a reagent grade.

2.2. Preparation of PC/PS/Chol complex liposome

First, to investigate the dominant role of PS in AV binding *t*, the molar ratio of PS in the liposomes was varied 0, 5, 10, and 15% (see Table 1). Second, to investigate the effect of cholesterol composition on the binding affinity of FVIII and AV, six types of liposomes with various molar ratios of PC/PS/Chol were prepared; PS concentration was fixed at 20% because the FVIII binding was reported to be the highest at this concentration (Saenko et al., 2001), and cholesterol was varied from 0 to 50% (see Table 1). The % PC was calculated from simple deduction; 100% – (% PS + % cholesterol). As PC is known to be the main component of cellular membranes, a 100% PC-containing liposome was used as a control surface. Another control surface, 70% PC/30% Chol, was to observe the cholesterol effect as opposed to the 100% PC-containing membrane.

Table 1
Molar composition ratios used for liposome preparation.

Objective	Component	PC (%)	PS (%)	Chol (%)
Cholesterol effect	a	80	20	0
	b	70	20	10
	c	50	20	30
	d	30	20	50
	e	70	–	30
	f	100	–	–
PS specific binding	g	100	0	–
	h	95	5	–
	i	90	10	–
	j	85	15	–

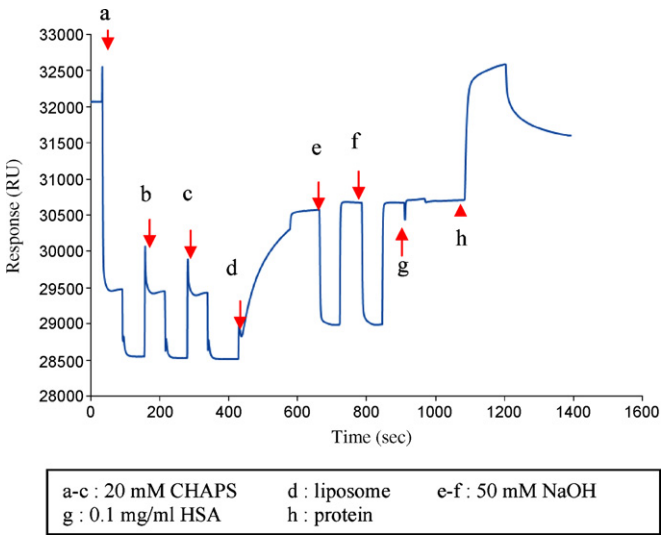

Fig. 1. Typical SPR experimental steps. The injection order was: three times 20 mM CHAPS injections, liposome, two 50 mM NaOH injections, 0.1 mg/ml HSA, and protein. HBS with 5 mM $CaCl_2$ (pH 7.4) was used as a running buffer.

All the liposomes were prepared at the ambient temperature. Each component (PC, PS, and Chol) was dissolved in chloroform at 10 mM and dispensed into a round bottom flask. Chloroform was added to a final 2 ml volume and completely mixed. After chloroform was removed by evaporation for 2 h, the lipid film coating was hydrated with 2 ml of HBS buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) to prepare 1.4 μM of liposome. The solution was pulse-sonicated for 10 min to detach the coated lipid layer from the flask wall. The hydrated liposome solution was filtered through 0.2 μm pore size polycarbonate filter (Advantec Inc., MFS, Dublin, CA, USA) and stored at 4 °C until use.

2.3. Surface plasmon resonance analysis

To monitor the molecular interactions between liposomes and the proteins, BIACORE 3000 (Biacore AB, Sweden) equipped with a L1 chip was used. The SPR system monitors the whole binding events in real time, from which useful information such as association (recognition) and dissociation (stability) data can be inferred. The PC/PS/Chol complex liposome was captured on a L1 chip surface. Before each liposome injection, the chip surface was cleaned three times by injecting 20 mM CHAPS solution (Sigma, St. Louis, MO, USA) for 1 min at 10 μl/min. The injected liposome volume was adjusted to a similar immobilization level for each type of liposome. To remove unstably bound liposome from the surface, the chip surface was treated twice with 50 mM NaOH for 1 min at 10 μl/min. To block the liposome-unpacked surface, the chip surface was treated with 0.1 mg/ml HSA for 1 min at 10 μl/min (Saenko et al., 2001). For liposome–protein interaction analysis, 125 nM of FVIII and AV diluted in a running buffer (10 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$ at pH 7.4) was injected for 1 min at 5 μl/min. All analyses were performed at 25 °C.

3. Results

3.1. PC/PS/Chol complex immobilization on L1 chip surface

Before liposome immobilization, the L1 chip surface was cleaned three times with 20 mM CHAPS. The first injection showed a different shape from the other two consecutive injections. As observed from the sensorgram in Fig. 1, the first injection showed a steep

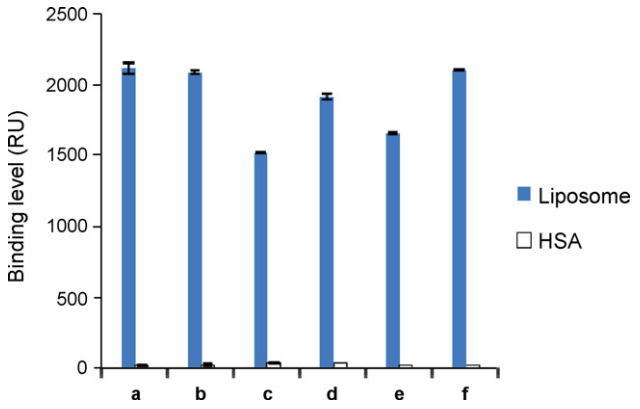

Fig. 2. L1 chip surface blocking. Each liposome surface was treated with 0.1 mg/ml HSA. PC:PS:Chol ratio was: (a) (80:20:0), (b) (70:20:10), (c) (50:20:30), (d) (30:20:50), (e) (70:0:30), and (f) (100:0:0).

downward trend toward a new baseline. Compared to the first injection, the other two CHAPS injections did not show dramatic change in the baseline level. The changing baseline between the second and third CHAPS injections was lower than 50 RU, which was very small compared with the normal liposome immobilization level of greater than 1500 RU (see Fig. 2). Furthermore, liposome immobilization levels were duplicated very reproducibly. The liposome was injected over the L1 chip, and the injected volume was adjusted to reach a range of 1500–2100 RU for each composition of phospholipids. Fig. 2 shows that liposomes containing various compositions of cholesterol yielded the similar immobilization levels, ranging from 1520 to 2160 RU. In other words, liposome binding to the L1 chip appeared independent of either PS or cholesterol content in the liposome. The treatment of 50 mM NaOH after did not cause any significant change in the immobilization level; liposome immobilization yielded a stable baseline and no significant loss during NaOH treatment was observed.

The liposome-immobilized chip surface was blocked with 0.1 mg/ml of HSA by a 1 min injection. This step was performed for two reasons; to block the unoccupied space and to prevent non-specific binding. As shown in Fig. 2, HSA binding ranged only from 9 to 33 RU. It indicated that non-specific binding of hydrophilic proteins such as HSA would be very low due to the lipophilicity of the L1 chip surface. Fig. 3 showed that the presence of cholesterol generally increased the ratio of HSA binding to liposome binding. It

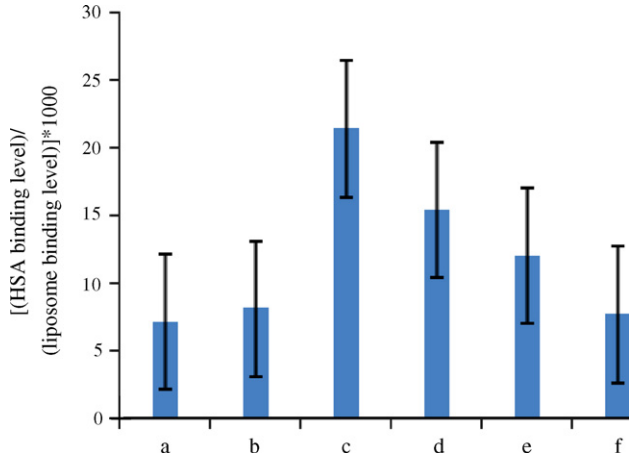

Fig. 3. Normalization of HSA binding to each liposome surface. HSA binding was divided by liposome binding and multiplied by 1000. PC:PS:Chol ratio was: (a) (80:20:0), (b) (70:20:10), (c) (50:20:30), (d) (30:20:50), (e) (70:0:30), and (f) (100:0:0).

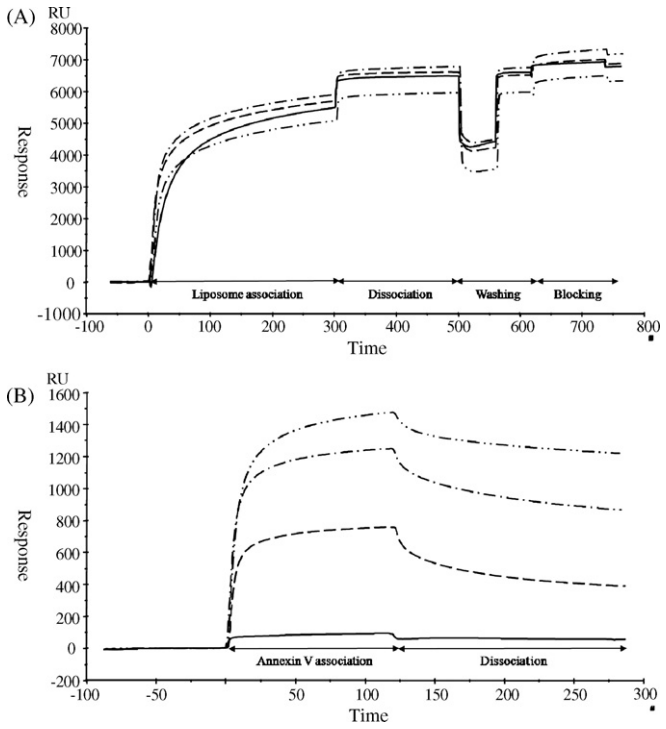

Fig. 4. Sensorgrams of annexin V binding to PS measured by SPR. (A) Immobilization of several molar ratios of PS/PC liposome. (B) Annexin V binding on PS/PC liposome surface, solid line: 0% PS/100 PC, long dash line: 5% PS/95% PC, dash-dot-dash line: 10% PS/90% PC, and dash-dot-dot-dash: 15% PS/85% PC.

was the highest on the liposome surface containing 30% Chol, i.e., PC:PS:Chol (50:20:30). This is in agreement with the other report (Meierhofer et al., 2009), in which HSA seemed to bind specifically to cholesterol in lipid vesicles but at a negligible level compared to those of FVIII and AV. A similar observation was reported by another group (Kim et al., 2001).

3.2. A dominant role of PS in AV binding to liposome

Liposomes with a different composition of PS (0–15% molar ratio) were prepared and immobilized on the L1 chip surface, and AV bindings were monitored by SPR. The immobilized liposomes were in the range of 6000–6800 RU (Fig. 4(A)). The AV binding level increased proportionally with an increase in PS composition (0, 5, 10, 15%) (Table 2, Fig. 4(B)). HBS containing 5 mM $CaCl_2$ was used as a running and dilution buffer, and the calcium concentration was kept constant during the entire experiment. This is known to be a sufficiently high concentration for FVIII and AV to bind to exposed PS (Stuart et al., 1998). This result showed that an increase in PS concentration in a liposome membrane considerably enhanced the binding level. In addition, it was important to note that the bound AV on the PS surface was hardly dissociated.

3.3. Cholesterol effect on FVIII and AV binding to liposome

To check the cholesterol effect on FVIII and AV binding to PS-containing liposome, each type of liposome described in Section 2 was immobilized independently on L1 chip surface. Fig. 5(A) shows that the AV binding level to PC:PS:Chol liposome was ranked from high to low as: (30:20:50), (50:20:30), (80:20:0), (70:20:10), (70:0:30), (100:0:0). To analyze the relative binding of AV to each liposome surface, the protein binding level was 'normalized' by dividing the mass of the bound AV by the mass of immobilized liposome. Fig. 5(B) shows that the normalized binding level was

Table 2
Binding levels of annexin V to liposome complexes with various PS compositions as analyzed by SPR.

Composition of lipids complex	PC (mg/ml)	PS (mg/ml)	Immobilized on chip (RU)	Annexin V binding (RU)	Ratio (AV/LIP%)
0% PS/100% PC	7.28	0.000	6597	61	0.92
5% PS/95% PC	6.92	0.039	6475	575	8.87
10% PS/90% PC	6.55	0.079	6737	1092	16.2
15% PS/85% PC	6.19	0.118	5979	1369	22.9

96, 73, 64, 56 for (30:20:50), (50:20:30), (80:20:0), (70:20:10), respectively. On the other hand, the binding levels to the surfaces without PS (e and f), i.e., PC only (100:0:0) and PC:PS:Chol (70:0:30) surfaces, were only 11 and 16, respectively. Those were approximately five- to ninefold lower than the PS-containing surfaces (a–d). It was clear that PS played a critical role in the AV binding (Fig. 5(B)). In addition, the relative binding level of AV increased with respect to cholesterol composition (10–50%). The FVIII binding produced an interaction ranking slightly different from that of AV. Fig. 6(B) showed that the normalized values were 30, 28, 20, 17, 3.0, and 2.4 for (50:20:30), (30:20:50), (70:20:10), (80:20:0), (70:0:30), and (100:0:0), respectively. Again, by comparing the PS-containing liposomes with those without PS, we could confirm that PS was the dominant factor in the FVIII binding and cholesterol would also enhance the binding but only in the presence of PS.

3.4. Change in binding stability

The dissociation rate constant, k_d, was calculated with BIA evaluation software 4.1 (Biacore AB, Uppsala, Sweden). Fig. 7 indicated that, for AV, cholesterol had profound effect on the

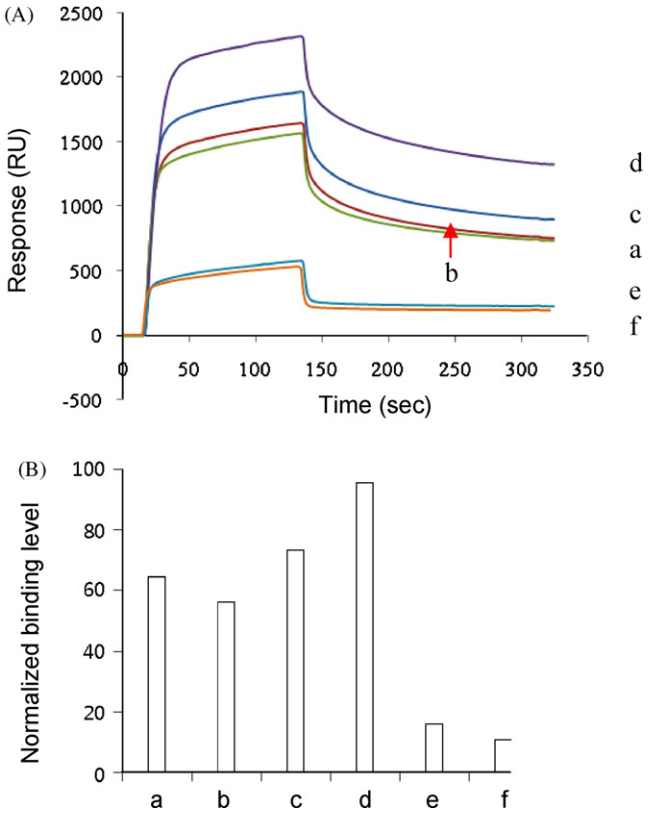

Fig. 5. Typical binding sensorgram of: (A) the binding of 125 nM AV and (B) normalized binding of AV. The binding of each protein was normalized against each liposome immobilization level and multiplied by 100. PC:PS:Chol ratio was: (a) (80:20:0), (b) (70:20:10), (c) (50:20:30), (d) (30:20:50), (e) (70:0:30), and (f) (100:0:0).

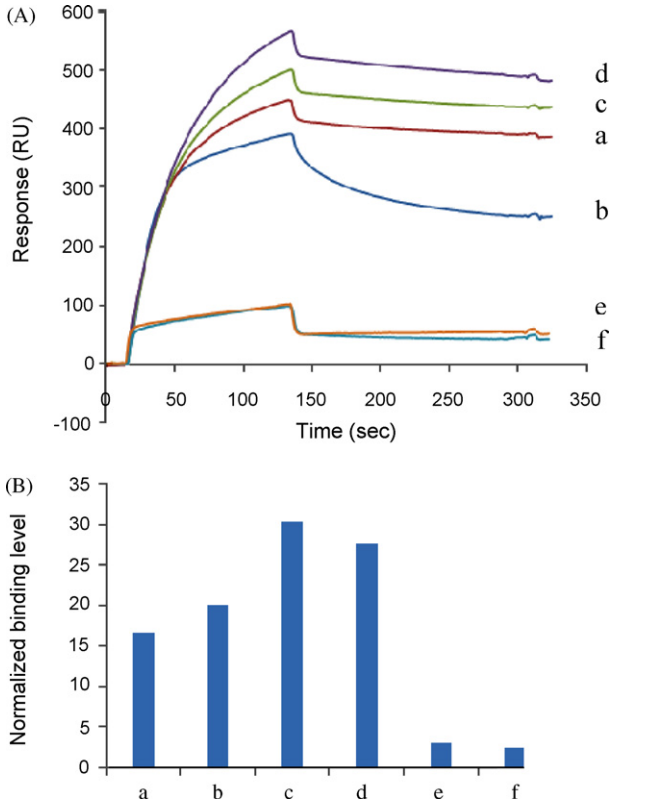

Fig. 6. Typical binding sensorgram of: (A) the binding 125 nM FVIII and (B) normalized binding FVIII. The binding of each protein was normalized against each liposome immobilization level and multiplied by 100. PC:PS:Chol ratio was: (a) (80:20:0), (b) (70:20:10), (c) (50:20:30), (d) (30:20:50), (e) (70:0:30), and (f) (100:0:0).

k_d values; between (80:20:0) and (70:20:10), $1.69 \times 10^{-3}\ s^{-1}$ and $3.24 \times 10^{-4}\ s^{-1}$, respectively, which was nearly fivefold difference. It strongly suggested that AV binding could be stabilized by the presence of membrane cholesterol. There was no significant stability change of AV binding between (70:20:10), (50:20:30), and

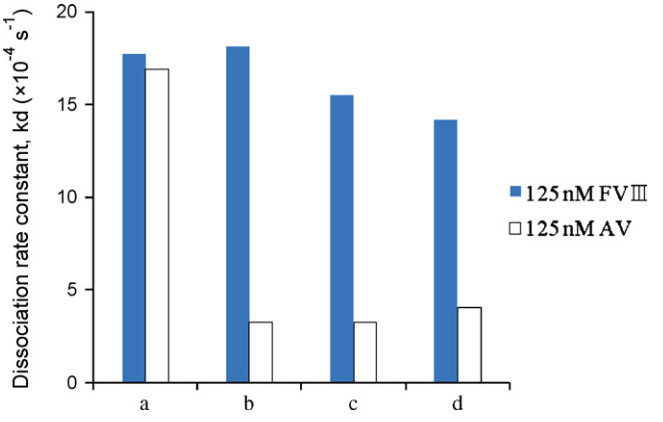

Fig. 7. Change of dissociation rate constant with respect to cholesterol composition. PC:PS:Chol ratio was: (a) (80:20:0), (b) (70:20:10), (c) (50:20:30), and (d) (30:20:50).

(30:20:50). For FVIII, as can be seen in Fig. 7, the binding was relatively less influenced by cholesterol concentration. The k_d values obtained for FVIII were comparable with a previous report (Saenko et al., 1999).

4. Discussion

In this study, an SPR sensor was used to investigate the cholesterol effect on the interaction between phospholipid binding proteins and liposomal lipid bilayer, using FVIII and AV as the model proteins. Liposomes of varying compositions of PC, PS, and cholesterol were prepared to quantitatively evaluate the critical role of PS and augmentative effect of cholesterol in the FVIII/AV binding (Table 1).

Liposomes were immobilized on L1 chip surface which is composed of a lipophilic polymer layer attached to a dextran layer coated on a chip surface. To prevent non-specific binding on the free moiety, HSA was injected to the liposome-immobilized surface. The binding level of HSA increased with an increase in cholesterol molar ratio in liposome. It was the highest for the 50% PC/20% PS/30% Chol surface (Fig. 3), corroborating the results on the cholesterol concentration effect reported by another group (Kim et al., 2001). The binding could be mediated by the specific interactions between HSA and cholesterol (Meierhofer et al., 2009). However, the binding level of HSA to liposome surface was negligible (<35 RU) compared to those of FVIII and AV (ranging from several hundreds to thousands RU).

From Fig. 4(A) it was clearly seen that PS was a dominant force for AV binding. Also, comparing the data from the liposomes containing PS with that from those without PS (see Figs. 5(B) and 6(B)), we could see that PS was necessary for FVIII/AV to bind to the liposome surface. This result would explain why annexin II failed to bind to PC/Chol liposome surface (Harder et al., 1997). In addition, the binding level of FVIII/AV to PS could be increased by raising the cholesterol composition in PC/PS/Chol liposome (Figs. 5(B) and 6(B)). Similar results were observed by another group (Ayala-Sanmartin et al., 2001). The reason for cholesterol to enhance the FVIII/AV binding to PS-containing liposome could be attributed to one of the cholesterol characteristics; cholesterol has higher affinity towards PC than PS (Huster et al., 1998), which might induce the formation of PS-rich microdomains (Ayala-Sanmartin et al., 2001; Lopez-Revuelta et al., 2007). Physically, this observation was in line with the previous report that specific binding between cholesterol and HSA would result in the increased membrane permeability (Meierhofer et al., 2009). It could also provide a potential reason why cholesterol-dependent lipid assembly regulated the activity of the PS translocators involved in its externalization (Damek-Poprawa et al., 2006; Sanchez-Yague et al., 1987).

Fig. 7 showed the cholesterol effect on the binding stability of FVIII and AV was different. In the case of AV, binding to liposome became dramatically stable at the presence of cholesterol (even 10%). In contrast, the binding stability of FVIII was not improved by the cholesterol composition. Therefore, it could be concluded that cholesterol would enhance the binding of both FVIII and AV to PS, but influence the binding stability differently.

The work herein has provided a label-free method to quantitatively investigate the binding behavior of a protein to membranes. Through the simple steps, the membrane properties could be precisely modified to meet the experimental goals. SPR biosensor assay provided detailed information on real-time stability change of a protein, which might provide valuable information on the relationship between compositional structure and biological function. The combination of artificial liposome membranes and the SPR biosensor assays can be very useful tools to determine the causes of the disorder of peripheral proteins.

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