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Series Title	Methods in Molecular Biology	
Chapter Title	Surface Plasmon Resonance for Measuring Interactions of Proteins with Lipid Membranes	
Chapter SubTitle		
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Copyright Holder	Springer New York	
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Abstract	Surface plasmon resonance (SPR) is an established method for studying molecular interactions in real time. It allows obtaining qualitative and quantitative data on interactions of proteins with lipid membranes. In most of the approaches, a lipid membrane or a membrane-mimetic surface is prepared on the surface of Biacore (GE Healthcare) sensor chips HPA or L1, and the studied protein is then injected across the surface. Here, we provide an overview of SPR in protein–membrane interactions, different approaches described in the literature, and a general protocol for conducting an SPR experiment including lipid membranes, together with some experimental considerations.	
Key words (separated by ',')	Protein–membrane interactions - Lipid membrane - Sensor chip L1 - Sensor chip HPA - Biacore	

Surface Plasmon Resonance for Measuring Interactions of Proteins with Lipid Membranes 2 3

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Abstract 5

Surface plasmon resonance (SPR) is an established method for studying molecular interactions in real time. It allows obtaining qualitative and quantitative data on interactions of proteins with lipid membranes. In most of the approaches, a lipid membrane or a membrane-mimetic surface is prepared on the surface of Biacore (GE Healthcare) sensor chips HPA or L1, and the studied protein is then injected across the surface. Here, we provide an overview of SPR in protein–membrane interactions, different approaches described in the literature, and a general protocol for conducting an SPR experiment including lipid membranes, together with some experimental considerations.

Key words: Protein–membrane interactions, Lipid membrane, Sensor chip L1, Sensor chip HPA, Biacore 13 14

1. Introduction 15

One of the most important approaches to study molecular interactions is the use of optical biosensors that employ the surface plasmon resonance (SPR) phenomenon. This label-free method allows monitoring molecular interactions in real time. The use of commercial biosensors enables facile determination of kinetic parameters of binding. Basically, any molecular interaction can be studied, i.e., protein–protein, protein–DNA, protein–small ligand, virus–antibody, and also protein–lipid membrane interactions. The greatest advantages of the SPR approach are label-free detection, real-time monitoring, and low sample consumption. During the years, it became a strong experimental tool that can easily provide qualitative or quantitative data on molecular interactions. The technique, however, has certain particularities that can challenge the inexperienced researcher. The interested reader should therefore also consult yearly overviews of the rich SPR literature (1, 2) and recent books on SPR (3, 4), to obtain the insights into how

the SPR experiment is properly conducted and the data analyzed and interpreted. SPR was successfully used to study protein–lipid membrane interactions, e.g., membrane binding of proteins that participate in cell signaling, pore-forming proteins and peptides, binding of coagulation factors, enzymes, and amyloidogenic proteins (5). Additionally, some very good reviews were published on the use of SPR in protein–membrane interactions that highlight advantages over other biophysical approaches and are complementary to this review (5–7). Here, we aim to provide the status of SPR in protein–membrane interactions in the last few years by providing examples of qualitative and quantitative data that can be obtained. We will particularly focus on SPR experiments performed on Biacore (GE Healthcare) machines, since they are still the most commonly used.

1.1. SPR Basics

Any biosensor based on SPR is composed of SPR detector, fluidic system that brings interacting molecules together and gold-coated glass slides, so-called »sensor chips«, where interactions occur. A P-polarized laser light is directed through a prism, a medium of high refractive index, to a gold layer on the border of a flow cell with the sample, providing a medium of low refractive index. Laser light is reflected on the ~~sensor chip~~ surface and detected by the detector. At a critical angle of an incident light, the SPR phenomenon occurs and reduces the intensity of the reflected light. Several factors affect the optical properties of the system, the most important for the approach being the refractive index of the medium in the measurement cell. In the Biacore systems, one of the molecular partners is attached to the surface of the sensor chip (in the SPR biosensors terminology named ligand), while the second one (analyte) is injected across the surface of the sensor chip by employing a microfluidic system. Molecular interactions close to the surface of the sensor chip change the refractive index of the solution and consequently the angle at which SPR occurs. This is viewed online as an increase in the signal (Fig. 1). The so-called sensorgram is thus a plot that shows the change of the angle at which SPR occurs against time. The preferred units to describe the rise of the signal are so-called resonance units (RU). There is a linear relationship between the amount of the analyte on the surface of the sensor chip and the increase in the signal, i.e., 1 RU equals to approximately 1 pg protein/mm² (8). Since SPR detects changes of the mass concentration at the ~~sensor chip~~ surface, it is a label-free method, and no additional labeling of ligand or analyte is needed.

The surface of the sensor chip is composed of chemical groups that allow the capture of the ligand. The study of protein–lipid interaction can be performed by attaching a protein to the surface of the sensor chip and lipid vesicles injected over the protein (9). However, more researchers use an approach in which a lipid membrane or membrane-mimetic surface is formed on the surface of the

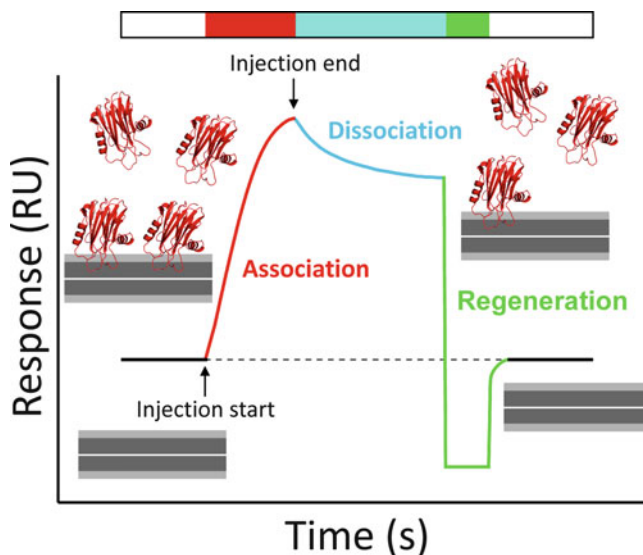


Fig. 1. Sensorgram is a plot that shows SPR response as a function of time. Different phases of an experiment are here labeled with different colors. During the association phase, the surface of the sensor chip with immobilized ligand (here a lipid membrane) is in the contact with the analyte (a protein molecule) dissolved in the running buffer. In the dissociation phase, the sensor chip is flushed with the running buffer only. Regeneration is used to remove the remaining analyte from the ligand surface, so that the new cycle of binding analysis can be performed.

sensor chip and protein then injected across such a surface. Over 79
 the years, many different approaches on how to prepare a lipid 80
 membrane on the surface of the ~~sensor chip~~ were described and 81
 reviewed (5, 10). However, the most used sensor chips for 82
 membrane-related work offered by Biacore are HPA and L1 sensor 83
 chips (Fig. 2). HPA sensor chip harbors a layer of alkanes on the 84
 gold surface. When small unilamellar liposomes are injected across 85
 such a surface, they attach and fuse to generate a hybrid bilayer 86
 membranes (11). An L1 sensor chip possesses lipophilic alkane 87
 groups on the dextran matrix, which efficiently captures intact 88
 liposomes (Fig. 2). Since capturing of intact liposomes is possible, 89
 this sensor chip is the most preferred of all. The use of L1 sensor 90
 chip is described below in a more detail. Captured liposomes 91
 were characterized in a more detail, and these papers should be 92
 also consulted for more information (12, 13). The experiment is 93
 typically performed in several steps: surface preparation, binding 94
 experiment, and regeneration of the ~~sensor chip~~ surface (Fig. 3). 95
 Both sensor chips can be regenerated easily by injecting a detergent 96
 solution, and thus, it is possible to use them many times. 97

Other ways to attach intact liposomes are by using liposomes 98
 that possess traces of biotinylated lipids and sensor chip with immo- 99
 bilized streptavidin (SA sensor chip), liposomes that contain trace 100
 amounts of lipopolysaccharide and sensor chip with immobilized 101

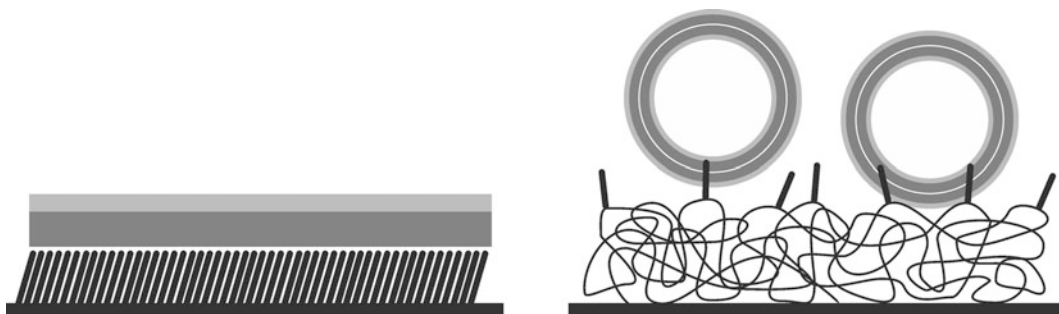


Fig. 2. HPA and L1 sensor chips offered by Biacore (GE Healthcare). Lipid monolayer, also termed hybrid lipid bilayer, is formed after a solution of small unilamellar vesicles is injected across the surface of the HPA sensor chip. Vesicles then fuse to generate a monolayer supported by a hydrophobic alkane layer. L1 chip possess long aliphatic anchors that efficiently capture intact liposomes. This is the preferred approach for studies of integral membrane proteins and proteins that need both monolayers for efficient membrane binding.

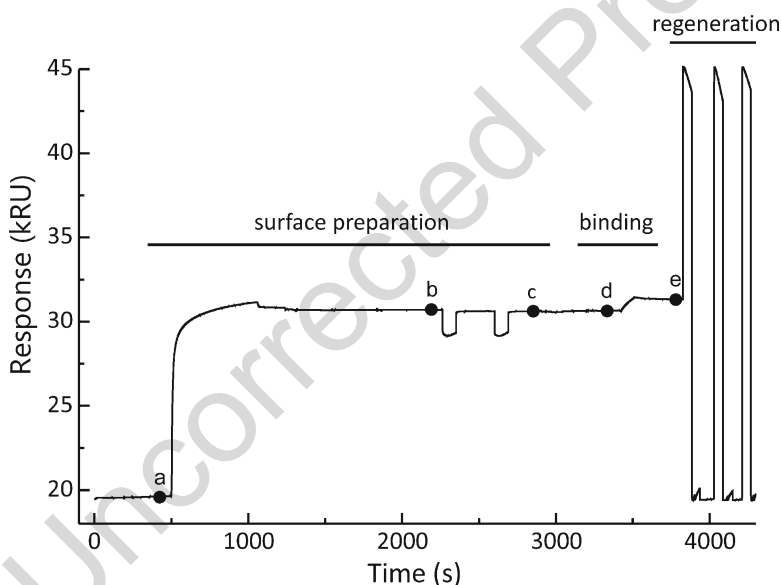


Fig. 3. A single cycle in a protein-membrane interaction consists of surface preparation, binding experiment, and regeneration of the sensor chip. In the first step, liposomes are injected (**a**) over an L1 sensor chip surface at a low flow rate (typically $2 \mu\text{l}/\text{min}$) and then conditioned with several injections of 100 mM NaOH (**b**) to remove the loosely bound liposomes. A single injection of bovine serum albumin is then used (**c**) to assess the degree of sensor chip exposure. It is possible to immobilize enough liposomes so that the whole surface of the chip is covered and no lipophilic anchors are exposed, as shown here. In the binding step, the protein of interest is injected across the so-prepared surface (**d**). Regeneration is the last step (**e**) and is used to remove the liposomes with the bound protein, and so cleaned chip is ready for another measurement cycle. The best regeneration solution is mixture of isopropanol and 50 mM NaOH (2:3, vol:vol) or detergent solution (40 mM octyl glucoside). Sometimes, protein can be removed from the surface of liposomes by a brief injection of high-salt solution ($0.5\text{--}2 \text{ M NaCl}$), low-pH (10 mM glycine pH 2–3), or high-pH ($10\text{--}200 \text{ mM NaOH}$) solution. In such case, many injections of different protein concentration can be performed on a single liposome surface, and sensor chip is regenerated at the end of the experiment.

LPS-specific antibody, or by using DNA-derivatized liposomes that allows hybridization to DNA tethers attached on a gold chip (for overview, see Beseničar Podlesnik et al.(5, 10)). The liposomes used for the interaction studies can be composed of a single synthetic lipid or mixtures of several lipids. Lipid extracts from whole cells, plasma membrane, or some other cell compartment were also employed for the preparation of liposomes. L1 sensor chip also allows capturing of various cellular membrane preparations, such as red blood cell ghosts (14, 15).

1.2. SPR in Protein–Membrane Interactions

In general, SPR gives qualitative and quantitative data of molecular interactions. The most straightforward experiment in protein–membrane interaction is determination of lipid specificity or influence of some other factor on the binding of protein to the lipid membrane, i.e., pH, buffer composition, and salt concentration (Fig. 4) (14). Such qualitative experiments are easy to perform and can be done fast, since it is easy to change lipid composition of the liposomes attached on the sensor chip or change the buffer composition.

However, the most important advantage of SPR over other biophysical approaches is the ability to determine the apparent rate and affinity constants from sensorgrams. This is particularly important when the differences between different conditions, e.g., different variants of studied protein, are small. Typically, such experiments are performed to get an insight into the magnitude of the effects particular amino acid side chain of the protein has on membrane association or dissociation. Here, one needs to perform binding experiment with several different concentrations of the protein, and then binding constants can be determined directly from the

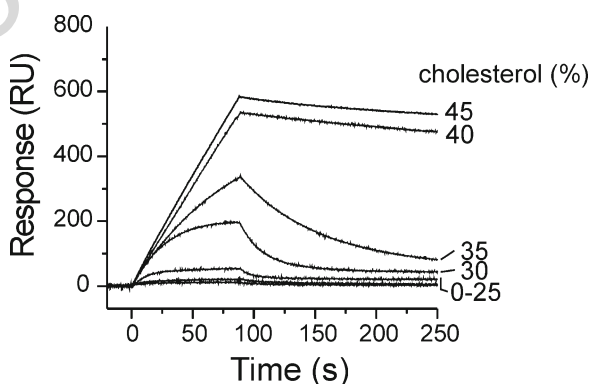


Fig. 4. Qualitative assessment of protein lipid specificity. Here, binding of a protein toxin listeriolysin O at 40 nM concentration to phosphatidylcholine liposomes containing different amounts of cholesterol was monitored. Listeriolysin O activity is dependent on cholesterol content in the membranes. Cholesterol enables initial interaction of the toxin with the membrane, which is evident from the experiment. The amount of cholesterol was 0, 10, 20, 25, 30, 35, 40, and 45% (mol) (from bottom to top) (Adapted from Bavdek et al. (14) with permission).

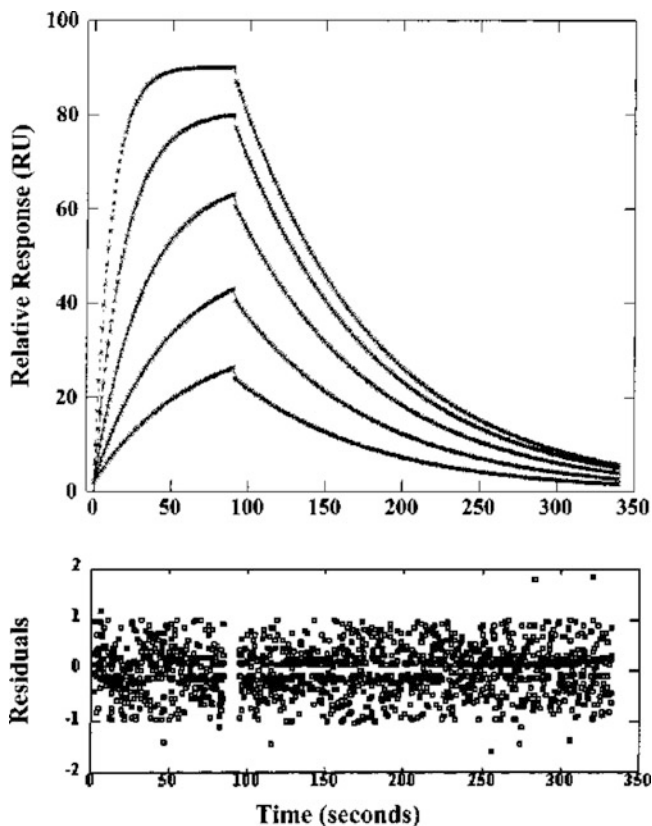


Fig. 5. Binding analysis of *Naja naja atra* phospholipase A₂ to lipid vesicles composed of 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine. Different concentrations of the protein (0.1, 0.2, 0.4, 0.8, and 1.6 μM from *bottom to top*) were injected over the vesicle surface, and association and dissociation were monitored as shown in the *upper panel*. The data fit well to the 1:1 Langmuir model (see Note 10), as is indicated by low and random residual scatter. The fit is presented with solid lines (Reproduced from Stahelin and Cho (17) with permission).

sensorgrams by numerical integration analysis (Fig. 5) (6, 16). This is conveniently done by Biacore evaluation software or some other dedicated programs, employing the appropriate binding model. The equilibrium affinity constants can also be directly determined from the equilibrium binding responses over a range of protein concentrations by fitting the data to a Langmuir adsorption isotherm (Fig. 6) (17).

In addition, SPR can provide some further insights into the mechanisms of protein action on membranes, as highlighted with some elegant examples of the recent literature. SPR allows assessing the strength of the protein interaction with the membranes. It is easy to perform the screening of conditions that desorb the protein from the surface of the lipid membrane after the binding (Fig. 7a) (19). Molecular interaction of ternary complexes on membranes can also be easily studied. Sometimes, molecular interaction between

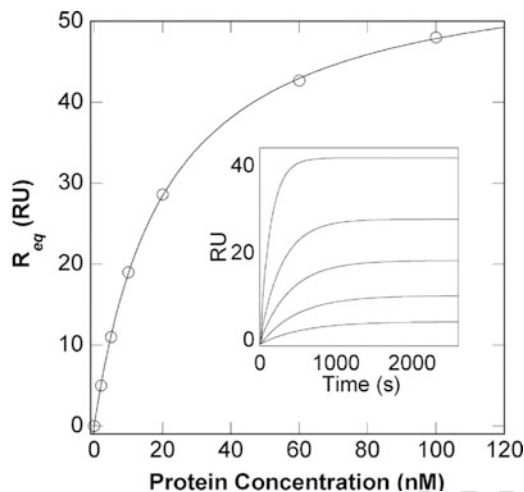


Fig. 6. Equilibrium binding analysis allows determination of equilibrium affinity constant. The PLD1 PX domain was injected at varying concentrations (2, 5, 10, 20, and 60 nM from *bottom* to *top*; inset) over the liposome surface. Here, long association times were used in order to reach equilibrium responses. A binding isotherm was then generated from equilibrium responses (R_{eq}) versus the concentration of the protein. A solid line represents the fit of the data by using the following equation: $R_{eq} = R_{max}/(1 + K_D/C)$, where R_{max} is the maximal response, K_D is equilibrium dissociation constant, and C is the concentration of the protein (Reproduced from Stahelin et al. (18) with permission).

different proteins occurs only after one of the binding partners is 145
 first associated with the membrane. Membrane binding may cause 146
 conformational change that exposes the binding site for the other 147
 partner (Fig. 7b) (20, 21). Some proteins can extract lipids from 148
 membranes, and SPR was successfully used to study the kinetics of 149
 removal of particular lipid component. Here, the decrease of the 150
 signal during the protein injection is indicative of lipid removal from 151
 liposomes (Fig. 7c). Some nice examples include extraction of lipids 152
 by saposin (22), ceramide by a CERT protein (23), and also cholest- 153
 erol by methyl- β -cyclodextrin (24). Finally, kinetics of pore forma- 154
 tion by human perforin was followed by SPR (Fig. 7d) (25). 155
 Perforin is a pore-forming protein from the immune system. Its 156
 pore-forming ability was studied by liposomes filled with fluorescent 157
 probe calcein. Such vesicles can be attached to the surface of LI 158
 sensor chip without compromising the integrity of the liposomes. 159
 In fact, this is a useful control for proteins that bind only to the outer 160
 vesicle leaflet, since in this case liposomes are not ruptured or 161
 damaged during the binding process, since no fluorescence can be 162
 detected during the binding process (17). However, in the case of 163
 perforin, the SPR signal dropped considerably during the associa- 164
 tion phase (Fig. 7d). Additional controls were done to show that 165
 this decrease of the signal is due to the released calcein, i.e., the 166
 eluted solution was strongly fluorescent (25). Finally, the use of 167
 both LI and HPA sensor chips for a particular protein-membrane 168

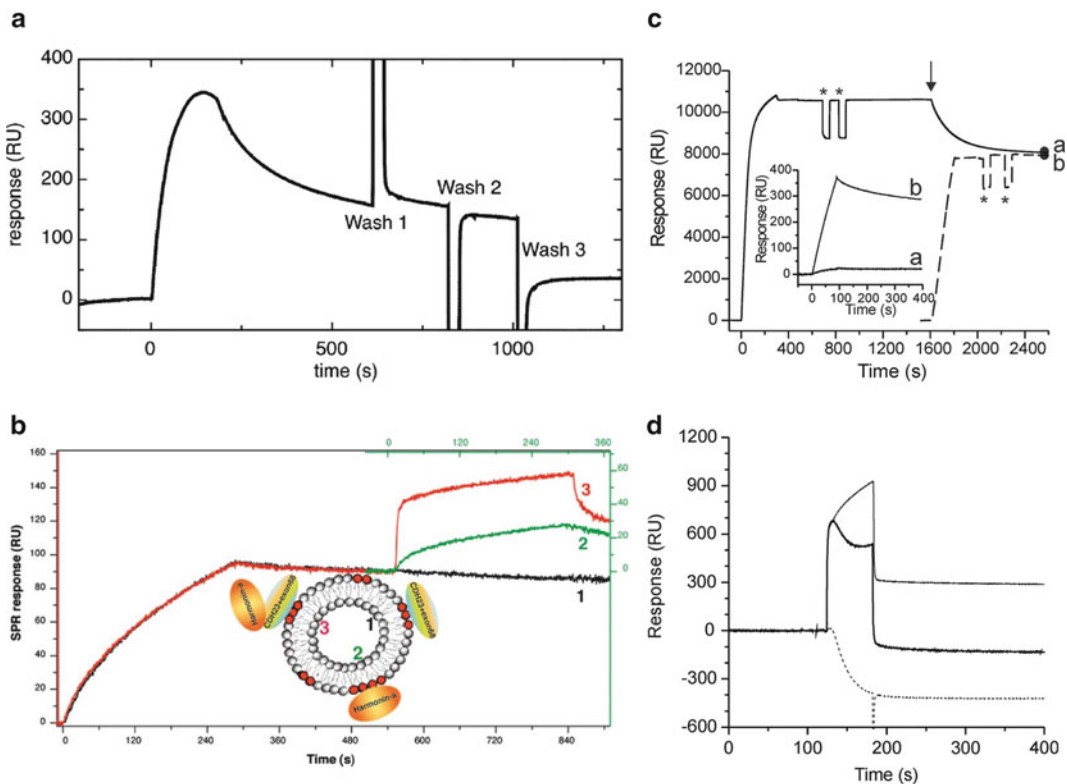


Fig. 7. Some additional examples from the literature on the certain aspects of protein–membrane interactions. **(a)** The stability of protein–membrane complexes may be assessed on-line by washing the surfaces with buffers commonly used to disrupt protein–membrane interactions. TorA–GFP fusion was bound to the liposome and subsequently washed with 300 mM NaCl (wash 1), 100 mM Na₂CO₃ (wash 2), and 100 mM NaOH (wash 3) (19). **(b)** Assessment of molecular complexes attached to the lipid vesicle. In such assay, binding of one of the partners to the lipid membrane is checked in the absence or presence of the other partner (20). **(c)** Selective extraction of cholesterol from membranes by using methyl- β -cyclodextrin. The decrease of the signal is observed after injection of methyl- β -cyclodextrin across the sensor chip surface covered by the liposomes composed of phosphatidylcholine and cholesterol at 40 mol%. Two injections of 100 mM NaOH are denoted by asterisks. The arrow denotes the injection of methyl- β -cyclodextrin. The surface after depletion was tested for the binding of cholesterol-dependent cytolysin streptolysin, which was negligible (*inset*). The control surface that contains approximately the same amount of original liposomes (trace b and dashed trace of the inset) (24). **(d)** Interaction of pore-forming protein perforin with the liposomes (25). The interaction with liposomes (*thin curve*) is compared to the liposomes filled with the fluorescent probe calcein (*thick curve*). In the latter case, the signal decreases in the association phase, which is indicative of the pore formation in the membrane of the liposome and release of calcein from the vesicle. The difference between the responses (*dashed curve*) shows the kinetics of the probe release from the liposome (Adapted from refs. (19, 20, 24, 25) with permission).

interaction can provide information about the depth of the protein 169
insertion. If protein attaches only superficially to the lipid mem- 170
brane, then kinetic constants of the binding should not differ in 171
both systems, as indeed observed in the case of coagulation factor 172
VII (26). However, if protein needs both monolayers for insertion, 173
then weaker binding is observed in the case of HPA chip (27). 174

All these examples show the capability and versatility of the SPR 175
approach in studying protein interactions with membranes. Other 176
examples of using SPR and various membrane preparations include 177

the reconstitution of receptors and assessment of their functionality (28), attachment and characterization of membrane systems prepared from cellular membranes, i.e., nanosomes with functional proteins (29), and transport of solutes across biological membranes by membrane protein (30). Some new approaches to prepare model membranes, such as nanosized bilayer disks, were also reported recently (31). We will next describe the most commonly used approach to study protein–membrane interactions by employing L1 and HPA sensor chips (the following protocol describes the binding experiment as presented on Fig. 3). Some variations of this protocol, other different approaches on preparing membrane surfaces for protein interactions studies, and some additional experimental considerations may be found in some recent reviews (32, 33).

2. Materials

2.1. Preparation of Lipid Vesicles

1. Lipid stocks in organic solvents (Avanti Polar Lipids, USA). 193
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2. Acid-washed glass beads (Sigma-Aldrich, USA). 195
3. Cryogen vials (Pierce, USA). 196
4. Vesicle buffer: 20 mM Tris–HCl, 140 mM NaCl, 1 mM EDTA, pH 7.4 in ultrapure water. Pass through cellulose acetate filters with 0.22 μm pores (Sartorius, Germany) and store at room temperature. 197
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2.2. Immobilization of Vesicles on the Surface of L1 and HPA Sensor Chips

1. Solutions for conditioning and regeneration of the L1 sensor chip: isopropanol:50 mM NaOH 2:3 (vol:vol), 100 mM NaOH, 0.1 mg/ml bovine serum albumin (BSA; Sigma-Aldrich, USA). 40 mM octyl glucoside is used instead of isopropanol:50 mM NaOH 2:3 (vol:vol) when regenerating HPA sensor chip. 201
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2. L1 or HPA sensor chips, T100 optical biosensor (Biacore, GE Healthcare, Sweden). 207
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2.3. Binding Experiment

1. Stock solution of protein in the vesicle buffer. Usually micromolar concentrations of proteins should be enough. 209
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3. Methods

3.1. Preparation of Lipid Vesicles

1. Add 5 mg of desired lipids dissolved in the appropriate organic solvent to a round-bottom flask and dry under vacuum using the rotary evaporator for at least 3 h (see Note 1). 213
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2. Add 1 ml of vesicle buffer and one-third of teaspoon of glass beads. Agitate vigorously on vortex approximately 1 min or 217
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	until all lipids are removed from the walls of the flask.	219
	The temperature of the vesicle buffer should be above the	220
	gel–liquid crystal transition temperature.	221
	3. Transfer the suspension of large multilamellar vesicles to the	222
	cryogen vial and freeze it by using liquid nitrogen. Repeat	223
	the freeze–thaw cycles 6 times.	224
	4. Use extruder equipped with polycarbonate filters of the defined	225
	size (Avestin, Germany) to obtain large unilamellar vesicles.	226
	Pass the suspension through filters at temperature that is	227
	above the gel–liquid crystal transition temperature until trans-	228
	lucent solution is obtained. Store the vesicles at 4°C and use	229
	them within 2 days. Do not freeze (see Note 2).	230
3.2. Immobilization		
of Vesicles on		
the Surface of L1		
Sensor Chips		
	1. Equilibrate sensor chip at room temperature, dock it into the	231
	apparatus, and prime the system twice with the vesicle buffer.	232
		233
	2. Set the flow rate to 10 µl/min and precondition the surface	234
	with two 1-min injections of isopropanol:50 mM NaOH 2:3	235
	(see Note 3).	236
	3. Prepare 200 µl of 1 mM lipids. Use slow flow rate (2 µl/min)	237
	and long injection time (10 min) to immobilize the vesicles in	238
	the desired flow cells (see Notes 4 and 5).	239
	4. Increase the flow rate to 100 µl/min for few minutes to rinse	240
	the loosely bound vesicles from the surface.	241
	5. Stabilize the lipid surface with two 1-min injections of 100 mM	242
	NaOH at 10 µl/min. To cover the unbound area on the chip,	243
	inject 0.1 mg/ml BSA for 1 min. Allow the surface to stabilize	244
	(baseline drift should be lower than 1 RU/min) before	245
	performing the analysis.	246
3.3. Immobilization of		
Vesicles on the Surface		
of HPA Sensor Chips		
	1. Clean the instrument with desorb and sanitize procedures. Run	247
	the ultrapure water with low flow rate over the surface over-	248
	night to remove all traces of detergent (see Note 6).	249
	2. Equilibrate sensor chip at room temperature, dock it into the	250
	apparatus, and prime the system twice with the vesicle buffer.	251
	3. Set the flow rate to 10 µl/min and precondition the surface	252
	with 5-min injection of 40 mM octyl glucoside.	253
	4. Prepare 200 µl of 1 mM lipids. Use slow flow rate (2 µl/min)	254
	and long injection time (30 min–3 h) to immobilize the vesicles	255
	in the desired flow cells (see Notes 4 and 5).	256
	5. Continue with the procedures 4 and 5 in paragraph 5.2.	257 AU1
3.4. Binding Experiment		
	1. Set flow rate to 10 µl/min. Inject the protein at appropriate	258
	concentration for several minutes and follow the dissociation	259
	for several minutes to half an hour (see Notes 7 and 8).	260

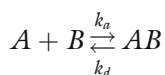
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2. Regenerate surface with brief injection (1–2 min) of one of the following solutions: 0.5–2 mM NaCl or up to 200 mM NaOH (see Note 9). If protein cannot be removed from the surface of liposomes, proceed to point 4. 261
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3. Repeat the binding of protein by injecting different concentration. 265
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4. Regenerate the surface of the sensor chip by three 1-min injections of isopropanol:50 mM NaOH 2:3 (Fig. 3). 267
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5. Fit the obtained sensorgrams using the evaluation program and the appropriate binding model (see Notes 10 and 11). 269
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4. Notes

1. L1 sensor chip allows capture of liposomes of different composition or size. It is also possible to deposit membrane preparations from cells, such as red blood cell ghosts, plasma membrane remnants, and cellular organelles (5). 271
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2. Small unilamellar vesicles prepared by sonication may also be effectively used. 278
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3. It is important to clean the surface of the sensor chip before the deposition of the liposomes. This is conveniently done by regeneration solutions. Apart from isopropanol:50 mM NaOH 2:3, also some detergent solutions may be used, i.e., 0.5% SDS or 40 mM octyl glucoside. 280
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4. The maximum immobilization level depends on the lipids used. It is higher (11,000–12,000 RU) for the noncharged lipids, such as phosphatidylcholine, and lower (up to 8,000 RU) for negatively charged phospholipids, such as phosphatidylglycerol or phosphatidylserine (13). 285
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5. In general, L1 sensor chip allows capture of intact liposomes (12, 13, 34), although some reports indicate that vesicles may fuse to form the bilayer (7, 35). 290
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6. The surface of HPA chip is composed of long alkanethiol chains that form hydrophobic layer which is very sticky for various hydrophobic molecules. Extra care should be taken when preparing solutions. Be sure that no traces of detergents are present in buffers. 293
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7. The concentrations that should be used in the analysis cover the range from the lowest, where there is hardly any binding seen, to the highest concentration, reaching the saturation. In other words, concentrations used should be $0.1 \times K_D$ – $10 \times K_D$. Use at least five different concentrations to cover this range, do at least one repetition, and include the buffer injection. 298
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8. The association time should be optimized for each interaction separately. For equilibrium analysis, the sensorgrams should reach the equilibrium level during the injection. The duration of dissociation phase is thus not crucial, since the equilibrium response levels are used for the evaluation of the interaction.
9. The level of lipids on the surface should remain the same during the whole experiment. If the analyte could not be effectively removed from the lipid vesicles (see Fig. 7a for the procedure that is used to determine the most effective way in how to remove the analyte from the membrane), then liposomes with bound analytes should be removed with three 1-min injections of isopropanol:NaOH 2:3, and the lipids should be applied for each concentration of protein separately.
10. Special care should be taken to perform experiments at conditions where interaction is not affected by mass transport effect, rebinding of the analyte during the dissociation phase, etc. (16). The evaluation programs allow data to be fitted to several models. The appropriate model should be carefully chosen, possibly also by the use of some supportive data from other experiments.
11. The simple 1:1 interaction model (also termed Langmuir model) implies that molecules bind without other interactions. The dynamic equilibrium is given by



where A represents the analyte and B is the ligand. k_a and k_d are association and dissociation rate constants, respectively. The association and dissociation rate constants thus determine the formation and breakdown of the complex at the surface of the sensor chip. The net rate equation is expressed as

$$\frac{d[AB]}{dt} = k_a \cdot [A] \cdot [B] - k_d \cdot [AB]$$

In SPR experiments, the response, R , scales linearly with the complex concentration, $[AB]$, so the rate equation is expressed as

$$\frac{dR}{dt} = k_a \cdot C \cdot (R_{\max} - R) - k_d \cdot R$$

where C is the concentration of the analyte and R_{\max} is the response signal at the saturation. This equation is used to fit the data, as presented on Fig. 5, to obtain k_a , k_d , and R_{\max} . The equilibrium dissociation constant, K_D , is expressed by the rate constants:

$$K_D = \frac{k_d}{k_a}$$

339 **Acknowledgments**

340 We would like to thank to the Slovenian Research Agency for the
 341 continuing support of the Infrastructural Centre for Molecular
 342 Interaction Analysis at the Department of Biology, Biotechnical
 343 Faculty, University of Ljubljana, Slovenia.

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Author Queries

Chapter No.: 2	214093_1_En
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Query Refs.	Details Required	Author's response
AU1	Please provide the appropriate Subheading and step for "procedures 4 and 5 in paragraph 5.2".	
AU2	Please clarify whether "point 4" can be changed as "Step 4" per MIMB series style.	

Uncorrected Proof