Chapter 3

Surface Plasmon Resonance for Measuring Interactions of Proteins with Lipids and Lipid Membranes

Aleksandra Šakanovič, Vesna Hodnik, and Gregor Anderluh

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 lipids or 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–lipid and 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, Custom-made chip, Biacore

1 Introduction

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, that is, 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 samples consumption. During the years it became a strong experimental tool that can easily provide qualitative or quantitative data on molecular interactions. Despite the fact that in recent years some new methodologies for measuring molecular interactions were developed, like biolayer interferometry and microscale thermophoresis, SPR still represents a golden standard, especially for evaluating protein–lipid membrane interactions. The technique, however, has certain particularities

that can challenge the inexperienced researcher. The interested reader should therefore also consult some very useful 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, amyloidogenic proteins, etc. [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 [6–8]. Here, we aim to provide the status of SPR in protein–lipid and 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, the 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 sensed 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 on-line 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 the so-called resonance units (RU). There is a linear relationships between the amount of the analyte on the surface of the sensor chip and the increase in the signal, that is, 1 RU equals to approximately 1 pg protein/mm^2 [9]. 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. Due to flexibility and wide
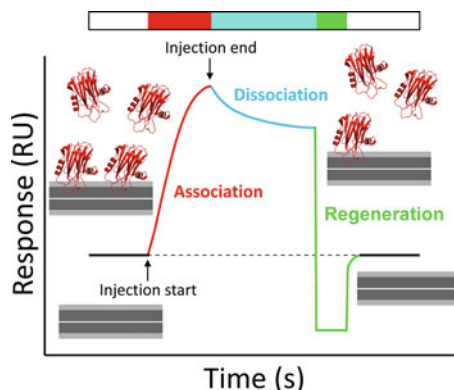
Fig. 1 Sensorgram is a plot that shows response as a function of time. Different phases of an experiment are here labelled with different colors. During the association phase the surface of the sensor chip with immobilized ligand (here a lipid membrane) is in the contact with the analyte (a protein molecule) 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
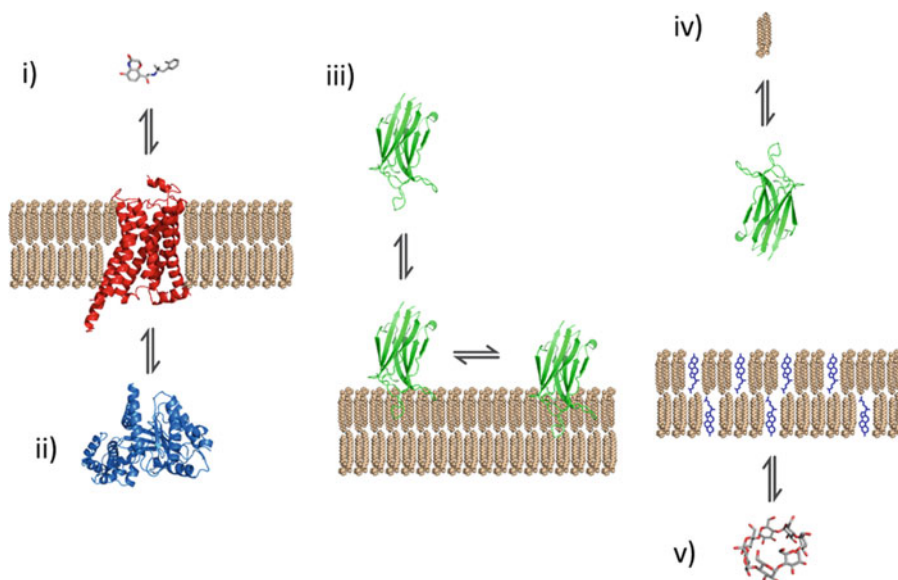
Fig. 2 SPR is a very useful approach for studying various aspects of protein interactions with lipids or lipid membranes. It is possible to capture various ligands on the surface of the sensor chip. Immobilization of membrane proteins allows interactions of small molecules (drugs) with integral proteins (1) or other protein-binding partners (2). The peripheral membrane proteins interactions with lipid membranes can be conveniently studied by immobilizing lipid membrane on the surface of the sensor chip (3). Protein–lipid interactions can be studied by immobilizing proteins as ligands and probing them by injecting various lipid preparations (4). SPR was shown useful also in some other interesting examples of studying lipid membranes structural features. For example the extraction of sterols by methyl-β-cyclodextrin was successfully studied in real time (*see* below) (5)
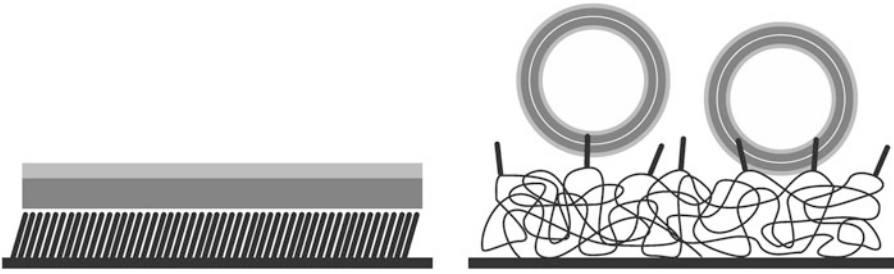
Fig. 3 HPA and L1 sensor chips offered by Biacore (GE Healthcare). Lipid monolayer, also termed hybrid lipid bilayer, is formed after a solution of small unilamellar vesicles is injected across the surface of the HPA sensor chip. Vesicles then fuse to generate a monolayer supported by a hydrophobic alkane layer. L1 chip possesses 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

variety of surface chemistries available for the immobilization of the ligand, a lot of different approaches can be designed in order to provide useful functional insights into protein–lipid or protein––membrane interactions (Fig. 2).
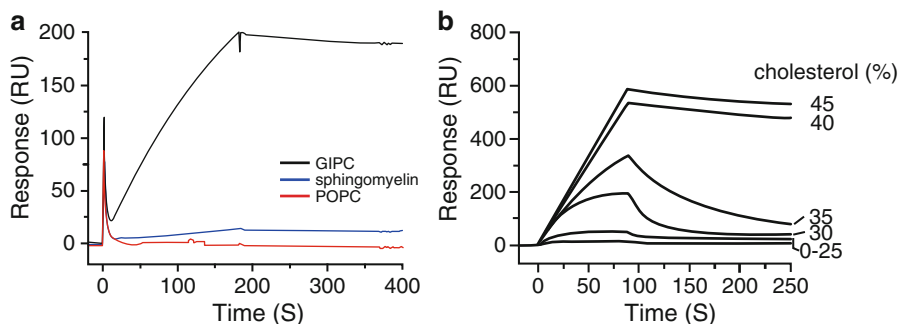
The study of protein–lipid interaction can be performed by attaching a protein to the surface of the sensor chip and lipids [10–12] (Fig. 2) or vesicles injected over the protein surface [13, 14]. However, more researchers use an approach in which a lipid membrane or membrane-mimetic surface is formed on the surface of the sensor chip and protein then injected across such a surface. Over the years many different approaches on how to prepare a lipid membrane on the surface of the sensor chip were described and reviewed [5, 15]. However, the most used sensor chips for membrane-related work offered by Biacore are HPA and L1 sensor chips (Fig. 3). HPA sensor chip harbors a layer of alkanes on the gold surface. When small unilamellar liposomes are injected across such a surface, they attach and fuse to generate a hybrid bilayer membrane [16]. An L1 sensor chip possesses lipophilic alkane groups on the dextran matrix, which efficiently captures intact liposomes (Fig. 3). Since capturing of intact liposomes is possible, this sensor chip is the most preferred of all. The use of L1 sensor chip is described below in a more detail. Captured liposomes were characterized in a more detail and these papers should be also consulted for more information [17, 18]. The experiment is typically performed in several steps: surface preparation, binding experiment and regeneration of the sensor chip surface (Fig. 4). Both sensor chips can be regenerated easily by injecting a detergent solution and thus it is possible to use them many times.

Other ways to attach intact liposomes are by using liposomes that possess traces of biotinylated lipids and sensor chip with immobilized streptavidin (SA sensor chip); liposomes that contain trace amounts of lipopolysaccharide and sensor chip with immobilized LPS-specific antibody; or by using DNA-derivatized liposomes that
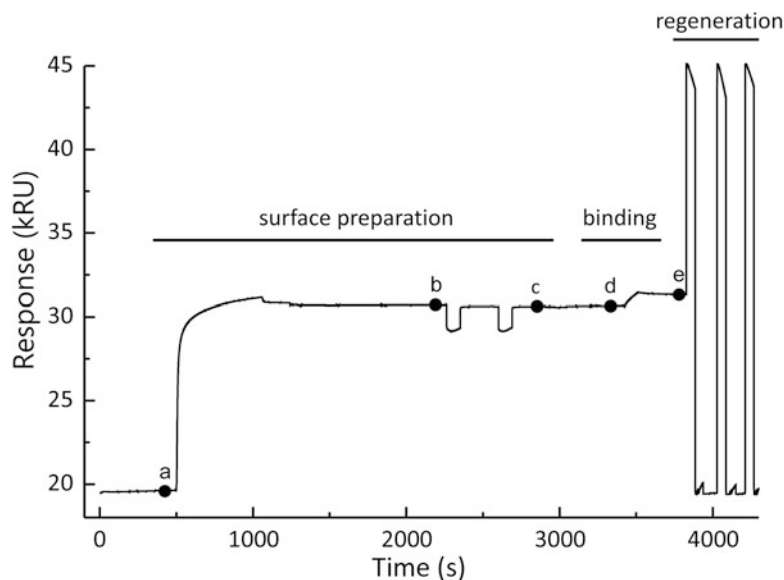
Fig. 4 A single cycle in a protein–membrane interaction typically consists of surface preparation, binding experiment and regeneration of the sensor chip. In the first step liposomes are injected (a) over an L1 sensor chip surface at a low flow rate (typically 2 μL/min) and then conditioned with several injections of 100 mM NaOH (b) to remove the loosely bound liposomes. A single injection of bovine serum albumin (BSA) 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 since there was no BSA binding. 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 the 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 β-ᴅ-glucopyranoside). Sometimes protein can be removed from the surface of liposomes by a brief injection of high-salt solution (0.5–2 M NaCl), low pH (10 mM glycine pH 2–3), or high pH (10–200 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

allows hybridization to DNA tethers attached on a gold chip (for overview see Beseničar Podlesnik et al. [5, 15]). 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 [10, 19].

Fig. 5 Qualitative assessment of protein–lipid specificity. (**a**) Protein–lipid interactions can be studied by immobilizing proteins to the surface of the sensor chip and the binding of lipids can then be evaluated. In the presented case binding of plant sphingolipids by Nep1-like protein was shown to be extensive, while control lipids sphingomyelin and phosphatidylcholine did not bind [12]. (**b**) A qualitative study of protein–lipid membrane interaction is shown here for binding of a protein toxin listeriolysin O at 40 nM concentration to phosphatidylcholine liposomes containing different amounts of cholesterol. 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 Lenarčič et al. [12] and Bavdek et al. [19] with permission

1.2 SPR in Protein–Membrane Interactions

In general, SPR give 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 (pH, buffer composition, salt concentration, etc.) (Fig. 5) [10, 12, 19]. Such qualitative experiments are easy to perform and can be done fast, since it is easy to change the solution containing lipids that are injected across the protein-immobilized surface (Fig. 5a), change the liposomes surface attached on the sensor chip (Fig. 5b), or make some other variations in the system, that is, change in 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 sensorgrams by numerical integration analysis (Fig. 6) [6, 20]. 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
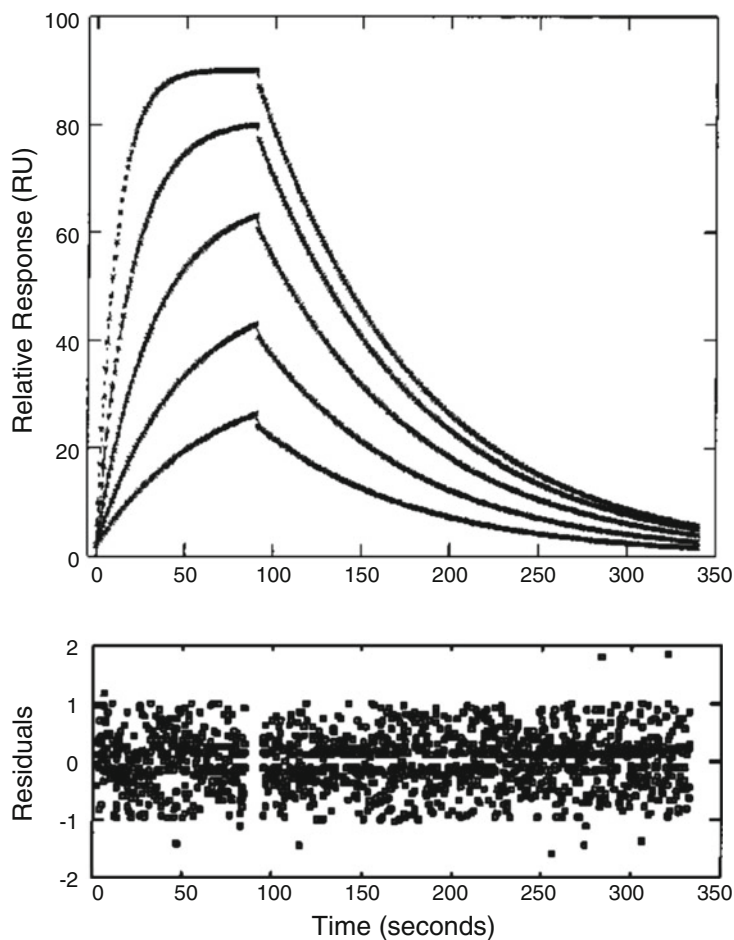
Fig. 6 Binding analysis of *Naja naja atra* phospholipase A_2 to lipid vesicles composed of 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine. Different concentrations of the protein (0.1, 0.2, 0.4, 0.8, and 1.6 μM from bottom to top) were injected over the vesicles surface and association and dissociation was monitored as shown in the upper panel. The data fit well to the 1:1 Langmuir model (*see* **Notes 11** and **12**), as is indicated by low and random residual scatter. The fit is presented with solid lines. Reproduced from Stahelin and Cho [21] with permission

a range of protein concentrations by fitting the data to a Langmuir adsorption isotherm (Fig. 7) [22].

In addition, SPR can provide some further insights into the mechanisms of protein action on membranes, as highlighted with some elegant examples of the rich SPR 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. 8a) [23]. Molecular interaction of ternary complexes on membranes can also be easily studied. Sometimes molecular
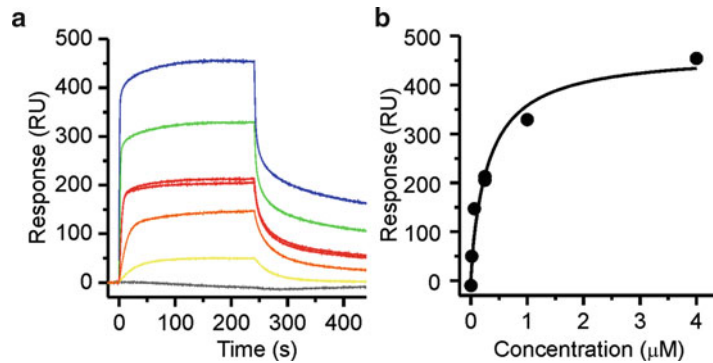
Fig. 7 Equilibrium binding analysis allows determination of equilibrium affinity constant. (**a**) Pacsin was injected at varying concentrations (2.6 nM (gray), 15.6 nM (yellow), 62.5 nM (orange), 250 nM (red, in duplicate), 1 μM (green), 4 μM (blue); from bottom to top) over the liposome surface. Here long association times were used in order to reach equilibrium responses. (**b**) A binding isotherm was then generated from equilibrium responses (R_{eq}) versus the concentration of the protein. A solid line represent 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 Kostan et al. [22] with permission

interaction between different proteins occurs only after one of the binding partners is first associated with the membrane. Membrane binding may cause conformational change that exposes the binding site for the other partner [24, 25]. Some proteins can extract lipids from membranes and SPR was successfully used to study the kinetics of removal of particular lipid component. Here the decrease of the signal during the protein injection is indicative of lipid removal from liposomes (Fig. 8b). Some nice examples include extraction of lipids by saposin [26], ceramide by a CERT protein [27] and also cholesterol by methyl-β-cyclodextrin [28]. Finally, kinetics of pore formation by human perforin was followed by SPR (Fig. 8c) [29]. Perforin is a pore-forming protein from the immune system. Its pore-forming ability was studied by liposomes filled with fluorescent probe calcein. Such vesicles can be attached to the surface of L1 sensor chip without compromising the integrity of the liposomes. In fact, this is a useful control for proteins that bind only to the outer vesicle leaflet, since in this case liposomes are not ruptured or damaged during the binding process, since no fluorescence can be detected during the binding process [21]. However, in the case of perforin the SPR signal dropped considerably during the association phase (Fig. 8c). Additional controls were done to show that this decrease of the signal is due to the released calcein, that is, the eluted solution was strongly fluorescent [29]. Finally, the use of both L1 and HPA sensor chips for a particular protein–membrane interaction can provide information about the depth of the protein
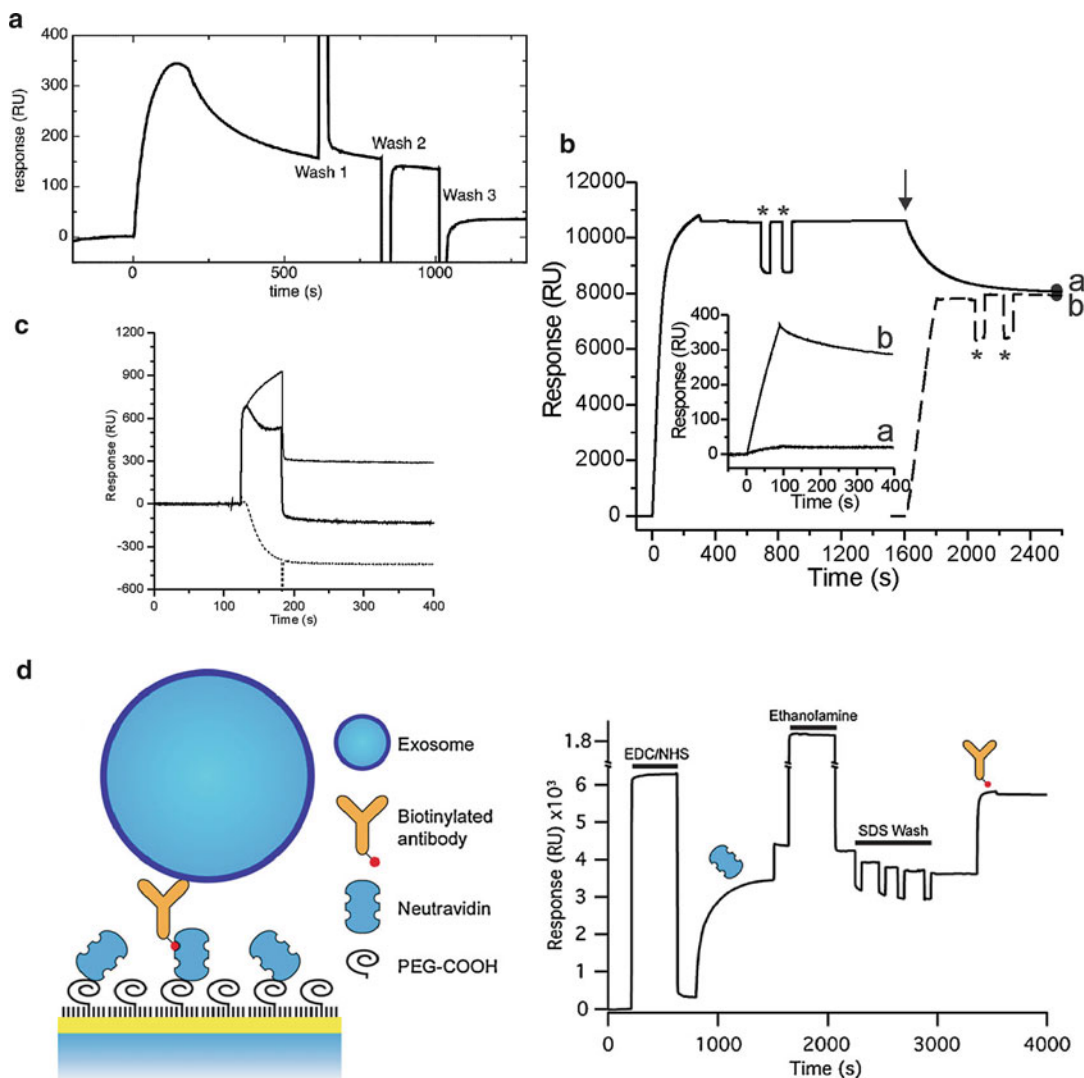
Fig. 8 Some additional examples from the literature on the certain aspects of protein–membrane interactions. (**a**) The stability of protein–membrane complexes may be assessed on line by washing the surfaces with buffers commonly used to disrupt protein–membrane interactions. TorA–GFP fusion was bound to the liposome and subsequently washed with 300 mM NaCl (wash 1), 100 mM Na_2CO_3 (wash 2), and 100 mM NaOH (wash 3) [23]. (**b**) 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 was surface that contains approximately the same amount of original liposomes (dashed trace (**b**) and trace (**b**) of the inset) [28]. (**c**) Interaction of pore-forming protein perforin with the liposomes [29]. 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 vesicles. The difference between the responses (dashed curve) shows the kinetics of the probe release from the liposome. (**d**) SPR can be used for analysis of exosomes. The gold chip was modified using a mixture of short and long PEGs to which NeutrAvidin was bound, followed by functionalization with biotinylated antibodies (left panel). Typical sensorgram of the surface functionalization is shown (right panel). Adapted from refs. 23, 28, 29, 39 with permission

insertion. If protein attaches only superficially to the lipid membrane, then kinetic constants of the binding should not differ in both systems, as indeed observed in the case of coagulation factor VII [30]. However, if protein needs both monolayers for insertion than weaker binding is observed in the case of HPA chip [31]. In recent years the SPR was used also to determine the presence of exosomes (Fig. 8d).

All these examples show the capability and versatility of the SPR approach in studying protein interactions with membranes. Other examples of using SPR and various membrane preparations include the reconstitution of receptors and assessment of their functionality [32], attachment and characterization of membrane systems prepared from cellular membranes, that is, nanosomes with functional proteins [33], and transport of solutes across biological membranes by membrane protein [34]. Some new approaches to prepare model membranes, such as nanosized bilayer disks, were also reported lately [35]. Recently several reports of detecting extracellular vesicles with SPR were published. CM5 sensor chips with covalently bound anti-ICAM-1 antibodies [36] or custom-made sensor chips [37–40] have been utilized. The SPR can also be used to determine the active concentration of the analytes without the use of any standard. In the Calibration Free Concentration Analysis (CFCA) the active concentration of protein can be determined using protein diffusion coefficient and observed changes in binding rates at different flow rates under diffusion-limited conditions [41]. The assay was additionally successfully implemented in determining the concentration of exosomes [40].

The binding analysis can be done in a multicycle format with separate cycles for different protein concentrations including the regeneration step or in a single-cycle mode with no regeneration step between different analyte concentrations [42, 43]. The latter is especially appreciated when the analyte binds tightly with the lipids on chip since in these cases the regeneration can be tricky. Single cycle analysis also reduces the time needed to complete the experiment. We will 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. 4). The protocol of lipid binding to immobilized protein will also be described. Some variations of these protocols, other different approaches on preparing membrane surfaces for protein interactions studies and some additional experimental considerations may be found in some other recent reviews [44, 45].

2 Materials

2.1 Preparation of Lipid Vesicles

1. Lipid stocks in organic solvents (Avanti Polar Lipids, USA).
2. Acid washed glass beads (Sigma Aldrich, USA).
3. Cryogen vials (Pierce, USA).
4. Vesicles 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.

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 β-D-glucopyranoside is used instead of isopropanol–50 mM NaOH 2:3 (vol:vol) when regenerating HPA sensor chip.
2. L1, HPA, or custom-made sensor chips, T100 optical biosensor (Biacore, GE Healthcare, Sweden).

2.3 Binding Experiment on L1 and HPA Sensor Chips

1. Stock solution of protein in the vesicles buffer. Usually micromolar concentrations of proteins should suffice.

2.4 Immobilization of Proteins on the Surface of CM5 Sensor Chip

1. Running buffer: 10 mM Hepes, 140 mM NaCl, pH 7.4.
2. Solutions for immobilizing proteins: *N*-Hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 1 M ethanolamine hydrochloride-NaOH pH 8, 10 mM sodium acetate with pH 0.5–1 unit below protein isoelectric point.
3. CM5 sensor chips, T100 optical biosensor (Biacore, GE Healthcare, Sweden).

2.5 Binding Experiment on CM5 Sensor Chips

1. Stock solution of lipids in solvent mixture. Dilute lipids just prior the experiment in the running buffer.
2. 0.05% SDS for regeneration of the surface.

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**).
2. Add 1 mL of vesicles buffer and one third of teaspoon of glass beads. Agitate vigorously on vortex approximately 1 min or until all lipids are removed from the walls of the flask. The

temperature of the vesicles buffer should be above the gel–liquid crystal transition temperature.

3. Transfer the suspension of large multilamellar vesicles to the cryogen vial and freeze it by using liquid nitrogen. Repeat the freeze-thaw cycles six times.

4. Use extruder equipped with polycarbonate filters of the defined size (Avestin, Germany) to obtain large unilamellar vesicles. Pass the suspension through filters at temperature that is above the gel–liquid crystal transition temperature until translucent solution is obtained. Store the vesicles at 4 °C and use them within 2 days. Do not freeze (*see* **Note 2**).

3.2 Immobilization of Vesicles on the Surface of L1 Sensor Chips

1. Equilibrate sensor chip at room temperature, dock it into the apparatus and prime the system twice with the vesicles buffer.

2. Set the flow rate to 10 μL/min and precondition the surface with two 1-min injections of isopropanol–50 mM NaOH 2:3 (*see* **Note 3**).

3. Prepare 200 μL of 1 mM lipids. Use slow flow rate (2 μL/min) and long injection time (10 min) to immobilize the vesicles in the desired flow-cells (*see* **Notes 4** and **5**).

4. Increase the flow rate to 100 μL/min for few minutes to rinse the loosely bound vesicles from the surface.

5. Stabilize the lipid surface with two 1-min injections of 100 mM NaOH at 10 μL/min. To cover the unbound area on the chip inject 0.1 mg/mL BSA for 1 min. Allow the surface to stabilize (baseline drift should be lower than 1 RU/min) before performing the analysis.

3.3 Immobilization of Vesicles on the Surface of HPA Sensor Chips

1. Clean the instrument with Desorb and Sanitize procedures. Run the ultrapure water with low flow rate over the surface overnight to remove all traces of detergent (*see* **Note 6**).

2. Equilibrate sensor chip at room temperature, dock it into the apparatus and prime the system twice with the vesicles buffer.

3. Set the flow rate to 10 μL/min and precondition the surface with 5-min injection of 40 mM octyl β-D-glucopyranoside.

4. Prepare 200 μL of 1 mM lipids. Use slow flow rate (2 μL/min) and long injection time (30 min to 3 h) to immobilize the vesicles in the desired flow-cells (*see* **Notes 4** and **5**).

5. Continue with the **steps 4** and **5** in Subheading 3.2.

3.4 Binding Experiment on L1 and HPA Sensor Chips

1. Set flow rate to 10 μL/min. Inject the protein at appropriate concentration for several minutes and follow the dissociation for several minutes to half an hour (*see* **Notes 7** and **8**).

2. Check the reference flow cell for the nonspecific binding since proteins can bind to the sensor surface. If the nonspecific

binding is higher than 10% of the response obtained on the lipid membrane different strategies can be used to minimize nonspecific adsorption (*see* **Note 9**).

3. 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 10**). If protein cannot be removed from the surface of liposomes proceed to **step 5**.

4. Repeat the binding of protein by injecting different concentration.

5. Regenerate the surface of the sensor chip by three 1-min injections of isopropanol–50 mM NaOH 2:3 (Fig. 4).

6. Fit the obtained sensorgrams using the evaluation program and the appropriate binding model (*see* **Notes 11** and **12**).

3.5 Immobilization of Proteins on the Surface of CM5 Sensor Chips

1. Equilibrate sensor chip at room temperature, dock it into the apparatus and prime the system twice with the running buffer (*see* **Note 13**).

2. Set the flow rate to 10 μL/min and inject EDC/NHS 1/1 through both flow-cells for 7 min. Prepare 50 μg/mL of protein in a 10 mM sodium acetate with appropriate pH (*see* **Note 14**). Inject it over second flow-cell for 5 min. Block both flow cells with 7-min injection of 1 M ethanolamine.

3.6 Binding Experiment on CM5 Sensor Chips

1. Set flow rate to 10 μL/min. Inject lipids diluted in running buffer for few minutes and follow the dissociation for several minutes.

2. Regenerate surface with brief injection (5 s) of 0.05% SDS (*see* **Note 15**).

3. Repeat the binding of lipid by injecting different concentration or use different lipid. Regenerate the surface between each lipid injection.

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, cellular organelles, etc. [5].

2. Small unilamellar vesicles prepared by sonication may also be effectively used.

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 β-D-glucopyranoside).

4. The maximum immobilization level depends on the lipids used. For example, for L1 sensor chip it is higher (11,000–12,000 RU) for the noncharged lipids, such as phosphatidylcholine, and lower (up to 8000 RU) for negatively charged phospholipids, such as phosphatidylglycerol or phosphatidylserine [18].

5. In general L1 sensor chip allows capture of intact liposomes [17, 18, 46], although some reports indicate that vesicles may fuse to form the bilayer [7, 47].

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.

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.

8. The association time should be optimized for each interaction separately. For equilibrium analysis the sensorgrams should reach the equilibrium level during the injection of each protein concentration. The duration of dissociation phase is thus not crucial, since the equilibrium response levels are used for the evaluation of the interaction.

9. The first step to minimize the nonspecific binding of analytes is injecting the 1–3 min pulse of 0.1 mg/mL bovine serum albumin over both flow cells. Another approach is using different chips, like streptavidin sensor chip with adding small percentage (0.1%) of biotinylated lipids during preparation of the vesicles. Also custom-made chips can be prepared soaking bare gold chip into the solution of alkanethiols or some other coupling molecules [48].

10. 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. 8a for the procedure that is used to determine the most effective way in how to remove the analyte from the membrane) than 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.

11. 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. [20]. 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 independent experiments.

12. The simple 1:1 interaction model (also termed Langmuir model) implies that molecules bind without other interactions. The dynamic equilibrium is given by:

$$A + B \underset{k_d}{\overset{k_a}{\rightleftarrows}} AB$$
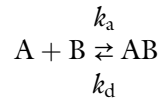
where A represents the analyte and B is the ligand. k_a and k_d are association and dissociation rate constants, respectively. The association and dissociation rate constants thus determine the formation and breakdown of the complex at the surface of the sensor chip. The net rate equation is expressed 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:

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

13. Since the coupling of the protein is achieved by its amine groups, completely avoid buffers that contain amine groups (like Tris).

14. The sensor surface is at most conditions negatively charged. In order to obtain sufficient preconcentration at the surface of the chip, the protein should be diluted just prior the injection in a 10 mM sodium acetate with pH that is 0.5–1 point below the isoelectric point of the protein.

15. Proteins can denaturate if too harsh pulses of detergent are being used. Very short injections (5 s at 30 μL/min) of low detergent concentration should be used as a starting point. If the lipids remain bound to the protein, longer time or slightly increased detergent concentration should be utilized. The optimization of the regeneration conditions is crucial in all SPR binding experiments.

Acknowledgments

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