Chapter 13

Capture of Intact Liposomes on Biacore Sensor Chips for Protein–Membrane Interaction Studies

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Abstract

Qualitative and quantitative aspects of protein interactions with membranes may be studied by optical sensors. Biacore offers two dedicated chips for working with lipids and membranes: the L1 and HPA sensor chips. The L1 chip is the most frequently used in protein–membrane interaction studies and it allows the capture of intact liposomes. This chapter describes the protocol for immobilization of liposomes on L1 sensor chips and discusses some of the experimental considerations. An alternative approach that utilizes a streptavidin-coated sensor chip (SA sensor chip) is described for cases when it is not possible to use an L1 chip.

Key words: Liposome, sensor chip L1, sensor chip SA, Biacore, protein–membrane interactions.

1. Introduction

Various aspects of protein–membrane interactions may be conveniently studied by optical sensors based on surface plasmon resonance (SPR) (1–4). The number of publications that describe protein–membrane interactions by SPR is steadily increasing since the introduction of commercial machines and dedicated sensor chips that exploit the phenomenon of SPR for detection of bimolecular interactions. Biacore GE has introduced HPA and L1 sensor chips for work with lipids and membranes. The HPA sensor chip is used mainly for the preparation of hybrid lipid bilayers (5). Many researchers use L1 sensor chips for capture of liposomes in order to study protein–membrane interactions (4). Reported applications of this chip include the qualitative description of

N.J. de Mol, M.J.E. Fischer (eds.), *Surface Plasmon Resonance*, Methods in Molecular Biology 627,
DOI 10.1007/978-1-60761-670-2_13,

protein binding to membranes, determination of kinetic constants of protein binding to the membrane, drug–membrane interactions, the kinetics of lipid extraction from liposomes, reconstitution of membrane receptors and functional studies of membrane receptors (4). However, the L1 sensor chip is mostly used to study the kinetics of binding of peripheral membrane proteins (6). Other ways to prepare artificial membrane surfaces on the surface of sensor chips include the preparation of a tethered membrane bilayer on the surface of gold chips, capture of liposomes by the biotin–avidin system, by antibodies and via complementary oligonucleotides (4). Here we describe the procedures for capturing of liposomes on Biacore L1 and streptavidin (SA) chips, as these are the most frequently used.

The properties of the surface generated by the injection of liposomes over a L1 sensor chip were characterized by several researchers (7–10). Some of the reports have indicated that a continuous bilayer is formed on the surface of the chip upon injection of liposomes (8). However, the evidence gathered in the last years indicate that intact liposomes are captured, at least when some of the most common lipids are used for liposome preparation, e.g. DOPC, POPC, SM, DOPG (9, 10). It was, however, noticed that the fusion of liposomes on the surface of L1 chips may be lipid dependent (11). Therefore, we propose to check for the status of liposomes on the surface of L1 sensor chips, if lipids are used of which it is not known which surface they form.

The use of sensor chip L1 offers a number of advantages over other methods of capture, e.g. the capture is easy to perform, the chips are easily regenerated to remove the captured material, the chips may be reused many times. However, lipophilic anchors of L1 sensor chip may interact with some of the substances present in the samples, which can compete with liposomes for binding (12). Consequently, desorption of vesicles from the chip may be observed. Problems may also arise in preparing a proper reference surface, if the protein of interest has a great affinity for lipophilic anchors and complete coverage of the chip is hard to achieve. These and some other considerations are extensively explained in Section 4. If it is not possible to account for these problems, then it is advisable to use other ways to capture the liposomes. As an alternative method the capture of biotin-tagged liposomes on SA chips is described.

2. Materials

2.1. Liposome Preparation

1. Lipid 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) as powder. It should be stored under nitrogen atmosphere at –80°C.

2. Glass vials with screw caps and Teflon cap-liners (Reacti-Vials, Pierce, USA).
3. Parafilm "M" (Pechiney Plastic Packaging, Menasha, USA).
4. Chloroform (Merck KGaA, Germany).
5. Round-bottom glass flasks.
6. Rotary evaporator.
7. Distilled water of high quality, e.g. MilliQ water.
8. Vesicles buffer: 20 mM Tris–HCl, 140 mM NaCl, pH 7.4. Pass through filters with 0.22 μm pores (Millipore) and store at room temperature.
9. Acid washed glass beads (Sigma-Aldrich, USA).
10. Vortex mixer.
11. Cryogen vials (Pierce).
12. Liquid nitrogen.
13. Lipid extruder (LiposoFast-Basic) and polycarbonate membranes with a pore size of 100 nm (Avestin, Mannheim, Germany).
14. Enzymatic colorimetric test for determination of lipids containing choline headgroups (Wako LabAssay™ Phospholipid; Wako Pure Chemical Industries, Ltd.).

2.2. Liposome Capture on the L1 Sensor Chip

1. Instruments Biacore X or Biacore T100 (Biacore, GE Healthcare).
2. L1 sensor chip (Biacore, GE Healthcare).
3. 50 mL self-standing centrifuge tubes (Corning Life Sciences, the Netherlands).
4. Compressed air spray (Dust Off; Teslanol AG, Germany).
5. 100 mM NaOH. Pass through 0.22 μm pores filter and store at room temperature.
6. Bovine serum albumin (BSA) (Sigma-Aldrich). Prepare 5 mg/mL solution in water, store at –20°C before use. Prepare 0.1 mg/mL solution in vesicles buffer just prior to the injection.

2.3. Liposome Capture on the SA Sensor Chip

1. SA sensor chip (Biacore, GE Healthcare).
2. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(Cap Biotinyl) (biotin-DOPE) (Avanti Polar Lipids) as powder. It should be stored under nitrogen atmosphere at –80°C.
3. 1 M NaCl in 50 mM NaOH.
4. 0.5% SDS (m:v) (Merck KGaA, Germany). Pass through filter with 0.22 μm pores and store at room temperature.

2.4. Regeneration of Sensor Chips

1. Regeneration solution: 50 mM NaOH:isopropanol (Kemika, Zagreb) 2:3 (v:v). Pass through filter with 0.22 μm pores and store at room temperature.
2. 20 mM CHAPS (Sigma-Aldrich, USA).
3. 40 mM *N*-octyl-β-D-glucopyranoside (Sigma-Aldrich, USA).

3. Methods

The capture approaches described below are used with Biacore sensor chips and equipment. All the mentioned procedures, e.g. *Dock*, *Prime*, *Continue*, *Desorb*, are not explained in detail here; the user should refer to application manuals for a particular Biacore instrument (Biacore X or Biacore T100). **Figure 13.1**

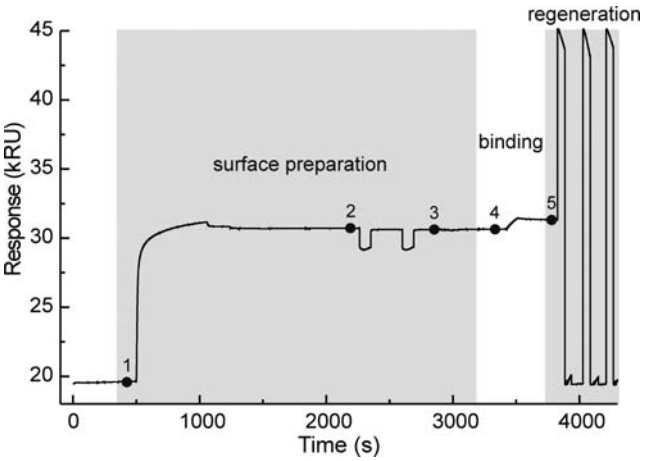

Fig. 13.1. A typical cycle in a protein–membrane interaction experiment. The three parts of the cycle are surface preparation, actual binding experiment and regeneration. Surface preparation (*1–3*) includes deposition of liposomes at a low flow rate of 1–2 μL/min (*1*), conditioning of the surface by two consecutive 1 min injections of 100 mM NaOH at a flow rate of 10 μL/min (*2*) and checking of the surface by binding of 0.1 mg/mL BSA for 1 min at 10 μL/min (*3*). BSA is used to assess the degree of exposure of lipophilic groups on the sensor chip. In this case no BSA is bound, indicating saturation of the surface with liposomes. The protein of interest is injected over the so-prepared surface (*4*). An example of binding of equinatoxin, a pore-forming toxin from sea anemones, is presented. Regeneration (*5*): sometimes the protein binds stably to the lipid membrane and cannot be removed after the binding experiment from the chip by regeneration with some of the commonly used solutions, such as buffers with high salt concentration, low pH or high pH. In that case the liposomes surface cannot be used anymore and should be regenerated by one of the regeneration solutions containing detergents or organic solvents. Usually, three consecutive 1 min injections of 50 mM NaOH:isopropanol 2:3 at a flow rate of 10 μL/min should suffice (*5*).

presents an overview of a typical experiment performed to study protein–membrane interactions. It is composed of three parts: preparation of the surface, binding experiment and regeneration of the surface. The protocols below cover the first and last steps, which have a general character. How an actual binding experiment is performed depends on the protein of interest.

3.1. Liposome Preparation

1. Equilibrate lipids, e.g. DOPC, at room temperature (approximately 22°C). Weigh a few milligrams into the small glass vials with screw cap and Teflon cap-liner. Add chloroform to reach a final 100 mg/mL concentration. Screw tightly and additionally wrap the cap with parafilm. Store at –20°C before use.
2. Pipette approximately 5 mg of DOPC dissolved in chloroform to a round-bottom glass flask. Prepare a thin lipid film by a rotary evaporator. Dry under vacuum for at least 3 h to remove all traces of chloroform. Add 1 mL of vesicles buffer to the lipid film, together with small amount of glass beads. Vortex vigorously for 1 min or until the entire lipid is removed from the walls. Remove the lipid suspension from the glass flask, put in a cryogen vial and freeze–thaw six times in liquid nitrogen. The resulting multi-lamellar vesicles are converted to large unilamellar vesicles (LUVs) by extrusion through 100 nm polycarbonate membranes (*see* **Note 1**) (13). Usually, 30 passages through the membrane should suffice. Generated LUVs have a well-defined and narrow distribution of size with average of approximately 100 nm.
3. Determine the concentration of lipids by an enzymatic colorimetric test according to the instructions of the producer (*see* **Note 2**). Store vesicles at 4°C immediately after preparation and use within 2 days.

3.2. Liposome Capture on the L1 Sensor Chip

1. Equilibrate an L1 sensor chip at room temperature (approximately 22°C) for 30 min. If you are using a new chip, open the protective pouch by scissors. If you are using a previously used chip, take the polystyrene support with the mounted chip out from the 50 mL centrifuge tube in which it was stored. If the chip was fully immersed in the buffer, gently wipe it with paper tissue. Do not touch the glass from either side. Blow away the remaining buffer with compressed air (*see* **Note 3**). Put the chip in protective sheath.
2. Degas the vesicles buffer, if you work with Biacore X apparatus. Stop the *Continue* mode of the instrument. Set the temperature of the instrument to 25°C. Insert the sensor chip with the *Dock* procedure. Perform the *Prime* procedure with vesicles buffer for at least two times. Start new sensorgram. Set the flow rate to 10 μL/min over all flow cells.

3. Clean the sensor chip with three 1 min injections of regeneration solution at the same flow rate (*see* **Section 3.4** below). Wait till the baseline stabilizes (*see* **Note 4**).
4. Change the flow rate to 1 or 2 μL/min. Inject the liposome solution at a final 1 mM lipid concentration for 10 min over all flow cells (*see* **Notes 5** and **6**). Repeat injection, if the level of the immobilized lipids is not enough (*see* **Note 7**).
5. Change the flow rate to 150 μL/min for at least 2 min. Change the flow rate to 10 μL/min. Inject twice 100 mM NaOH for 1 min. Wait till the baseline stabilizes. Inject 0.1 mg/mL BSA for 1 min (*see* **Note 8**).
6. Perform your binding experiment (*see* **Note 9**).

3.3. Liposome Capture on the SA Sensor Chip

1. The liposome preparation is in principle the same as described in **Section 3.1**, only a small amount of the biotinylated lipid should be included in order to allow capture by the SA sensor chip. Add 0.1% of biotin-DOPE (by mole) to DOPC dissolved in chloroform. The rest of the liposome preparation is the same as described in **Section 3.1**.
2. Equilibrate the sensor chip at room temperature and prepare the instrument as described in **Section 3.2**. Set the flow rate to 10 μL/min over both flow cells.
3. Condition the chip with three consecutive 1 min injections of 1 M NaCl in 50 mM NaOH.
4. Change the flow rate to 1 or 2 μL/min. Inject diluted liposome solution at a final 1 mM lipid concentration for 10 min over both flow cells. Repeat injection, if the level of the immobilized lipids is not enough.
5. Change the flow rate to 150 μL/min for 2–5 min. Change the flow rate to 10 μL/min.
6. Perform your binding experiment.
7. Set flow rate to 10 μL/min. Regenerate with consecutive 1 min injections of 0.5% SDS until the response level drops to the value at the beginning of the experiment (*see* **Note 10**).
8. Finish the experiment as explained in point 2 of **Section 3.4**.

3.4. Regeneration of Sensor Chips

1. This procedure cleans the surface of L1 sensor chip. One should use it prior to injection of liposomes, when chip is docked in the instrument to start with the new experiment or after the binding experiment for the preparation of fresh liposome-covered surface. Set flow rate to 10 μL/min. Regenerate with three 1 min injections of regeneration solution at the same flow rate (*see* **Notes 11** and **12**).

2. Stop the sensorgram and remove the chip from the machine with the *Undock* procedure. Take the chip from the protective sheath and store it in the 50 mL centrifuge tube at 4°C (*see* **Note 13)**. Insert the Biacore Maintenance chip, perform the *Prime* procedure two times with water and perform the *Desorb* procedure according to the instructions (*see* **Note 14**). Leave the instrument in the *Continue* mode.

4. Notes

1. Liposomes of composition or size different from the one specified here may be used with the L1 sensor chip. Small unilamellar vesicles may be prepared by sonication of multilamellar vesicles (14). It is also possible to use L1 sensor chips to deposit cell membrane preparations, e.g. red blood cell ghosts, plasma membrane remnants, synaptosomes (15).
2. The concentration of the phospholipids can be measured also by other methods, e.g. according to Bartlett (16).
3. Prevent contaminating either sides of the glass slide of the chip by dust particles. Keep the sensor chip support fully inserted into the sheath at all times.
4. It is very important to use solutions and equipment as clean as possible. Liposomes represent very hydrophobic surfaces into which hydrophobic impurities from the buffer will partition. Use only water of high purity, i.e. MilliQ water, and thoroughly cleaned and washed glassware. Extra care should be taken that no traces of detergents, used for example in washing procedures, are present. As a guide, a baseline drift of 0.3 response units (RU) per min is acceptable (17).
5. Approximately 10,000 RU should be deposited under these conditions. The amount of captured liposomes depends on the concentration of lipids injected over the sensor chip (9).
6. The amount of the liposomes deposited on the sensor chip depends on the assay developed by the user. It is advisable to perform a test for the binding of protein to lipophilic groups on the sensor chip, i.e. in the absence of liposomes. If binding of the studied protein is negligible, then an

empty flow cell, i.e. flow cell 1, can be used as a reference to account for changes in the refractive index due to bulk effects. But if binding of protein to lipophilic groups of the sensor chip is significant, then other ways of referencing should be used. In such cases the user should aim to cover completely the sensor chip with liposomes.

7. Liposomes that contain negatively charged lipids, such as phosphatidylglycerol or phosphatidylserine, contain a net excess of negative charge. It will, therefore, not be possible to fully cover the sensor chip due to electrostatic repulsion between the vesicles (9). The binding may be improved by including a higher concentration of NaCl in the running buffer. Avoid using buffers with a low salt content.
8. BSA is used as an indicator that gives information about the extent of coverage of the chip with liposomes. At full coverage of the sensor chip with liposomes no binding of BSA should be observed (7, 9). If the surface is not fully covered by liposomes, BSA will bind to exposed lipophilic groups on the sensor chip, since it has great affinity for alkyl chains. In this way BSA will prevent non-specific binding of the protein to the lipophilic groups on the chip. However, for detailed kinetic analysis this may not be the most optimal approach, as BSA dissociates slowly from the lipophilic groups and one needs to account for that in the kinetic models used for the description of the binding.
9. It is not explained here how to perform the actual binding experiment, as this largely depends on the nature of the protein and the information you wish to obtain. Many binding cycles may be performed on surfaces prepared in this way, if the protein binds to lipid membranes reversibly or if it is possible to remove the protein from the surface of the liposomes with regeneration solutions that do not contain detergents or organic solvents, which may damage or disintegrate the liposomes. The removal of the protein may be accomplished with high-concentration salt solution (2 M NaCl), low pH (glycine pH 2–3) or high pH (100 mM NaOH). Sometimes proteins will bind very strongly and it will be impossible to remove them from the surface of liposomes by these solutions. In this case, the sensor chip surface should be regenerated by a solution containing detergents or organic solvents and a new liposome-covered surface should be prepared for each cycle (*see* **Fig. 13.1**).

10. The treatment with SDS will remove lipids with bound protein, but not biotinylated lipid. As a consequence less and less liposomes can be immobilized and finally the chip cannot be used any more.
11. Regeneration of the sensor chip L1 can be performed by detergents or organic solvents. This will remove any liposomes or other hydrophobic substances attached to lipophilic groups of the sensor chip. We have found that the most efficient regeneration is achieved by the regeneration solution specified in this protocol (50 mM NaOH:isopropanol 2:3); however, if used extensively it may damage the microfluidic cell of the instrument. We found equally effective solutions of 50 mM HCl:isopropanol 2:3 or 40 mM *N*-octyl-β-D-glucopyranoside in water. Some researchers also use 0.5% SDS or 20 mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) in water for regeneration, but we found them less effective (*see* **Fig. 13.2**).

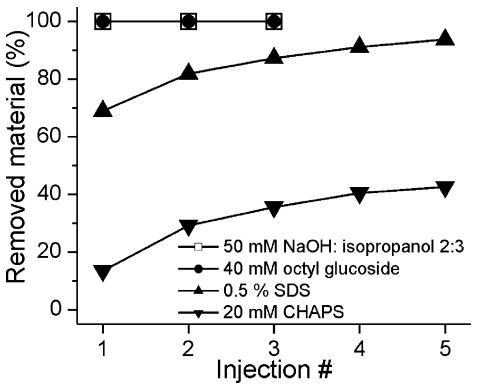

Fig. 13.2. Efficiency of some of the most commonly used regeneration solutions in removing DOPC liposomes from an L1 sensor chip. DOPC liposomes were immobilized to approximately 10,000 RU to the surface of an L1 sensor chip. Various regeneration solutions, as specified in the figure, were then injected for 1 min to desorb liposomes from the surface of the chip. The signal of remaining material on the surface of the sensor chip was determined after each injection and percentage of removed material calculated.

12. Regeneration solutions described in **Note 11** will remove mostly the lipid material from the surface of the sensor chip. If the response level after regeneration does not drop to the initial value this implies that the proteins are irreversibly attached to the lipophilic groups on the chip. Additional treatment with Pronase may further remove the

bound material. Take the sensor chip support from the protective sheath and place it in the 50 mL centrifuge tube filled with 1 mg/mL Pronase (Protease, type XIV from *Streptomyces griseus*; P5147, Sigma) in 20 mM NaH_2PO_4, 150 mM NaCl, pH 7.2 (18). Leave for 1 h at room temperature. Take the support from the tube, rinse it well with distilled water and remove excess water as explained in **Section 3.2**. Put the support with the sensor chip in the protective sheath, place it in the instrument and check the response level.

13. L1 and SA sensor chips may be stored in 50 mL centrifuge tubes for longer periods. The sensor chip support is removed from the protective sheath and placed in a 50 mL centrifuge tube filled with running buffer. This method of storage is equally effective if only 5 mL of the running buffer is put at the bottom of the tube (*see* **Fig. 13.3**).
14. It is important to perform the *Desorb* procedure regularly, when working with hydrophobic lipids and proteins. We perform it at the end of each day of experimentation.

Fig. 13.3. Long-term storage of Biacore sensor chips. Chips may be stored for longer periods of time (months) at 4°C in 50 mL centrifuge tubes as shown. Tubes may be filled to the top with the preferred buffer (*right*). However, it is also possible to store chips in tubes filled with only a few millilitres of the buffer (*left*), which will provide a moist atmosphere.

Acknowledgments

We would like to thank Slovenian Research Agency for support and Mojca Podlesnik Beseničar for critically reading the manuscript.

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