Development of a biosensor assay using membrane proteins reconstituted in liposomes as analyte

Helena Nordström and Olof P. Karlsson
Dept. Biochemistry and Chemistry, Biacore AB, Uppsala, Sweden.

Introduction

Many membrane-associated proteins are not suitable for direct immobilization on sensor surfaces. However, such proteins can be reconstituted in membrane vesicles that are then subsequently used in interaction analysis with an immobilized binding partner. The kinetics of analytes incorporated into liposomes is likely to be affected by the large liposome carrier, thereby deviating from the kinetic behavior of smaller analytes, like globular proteins. Here, the effects of flow rate, liposome size and the number of analytes per liposome were studied using a model system with immobilized antibiotin antibody and biotin-DHPE lipids incorporated into POPC-liposomes.This system, together with another based on the incorporation of EGF receptors into proteoliposomes, were explored using a number of assay formats and were able to establish conditions for and demonstrate the feasibility of this approach to SPR studies of membrane proteins.

Methods

Surface preparation

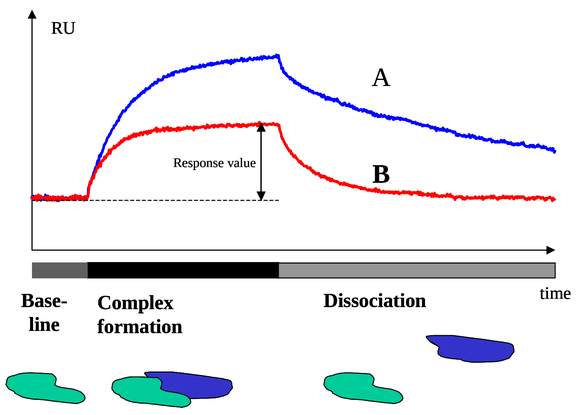
Anti-biotin antibody was immobilized by capture with a rabbit anti-mouse antibody covalently amine coupled to Sensor Chip CM5. This procedure enriched the antibiotin antibody and enabled regeneration of the sensor surface between samples.

Liposome preparation

Various liposome sizes were prepared through different treatment of emulsions of bilayer-forming lipids of desired composition:SUVs <25 nm in diameter were obtained by sonication, until the turbidity disappeared. LUVs were prepared by repeated extrusion of the lipid emulsions through filters of various pore sizes (50, 100 and 200 nm in diameter) [1].

SPR

With surface plasmon resonance (SPR) biosensors it is possible to measure the binding of analytes to immobilized ligands without using labels. Binding curves corresponding to complex formation and the dissociation of analyte from the immobilized ligand are recorded. In this study Biacore 3000 was used.



Abbreviations

DHPE: (N-((6-biotinoyl)amino)hexanoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamin)

EGF: epidermal growth factor, Sigma

LUV: large unilamellar vesicles

OG: (n-Octyl (-D-gluco-pyranoside)

POPC: (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphatidylcholine)

SUV: small unilamellar vesicle

References

[1] Hope, M.J., Bally, M.B., Webb, G. and Cullis, P.R. (1985) Biochimica et Biophysica Acta, vol 812, 55-56

[2] DosRamos, J.G. and Silebi, C.A (1989) AIChE Journal, Vol. 35, No 8, 1351-1364

[3] den Hartigh, J.C., van Bergen en Henegouwen, P.M.P, Boonstra, J., Verkleij, A.J. (1993) Biochimica et Biophysica Acta 1148, 249-256

Basic studies

Influence of liposome size and flow rate

POPC-liposomes of various diameters with 0.5% (mol/mol) biotin-DHPE were injected over immobilized anti-biotin antibody at a flow rate of 20 µl/min (the total lipid concentration was 0.25 mM). The kinetics of the association with the ligand differed for the different liposome sizes. Small liposomes had faster association rates than large ones. The SPR response depends upon the total mass change at the sensor surface. Liposomes with diameters of <25 nm gave a lower maximal response than the larger ones because they are smaller, but probably because they are also fusogenic and less stable.

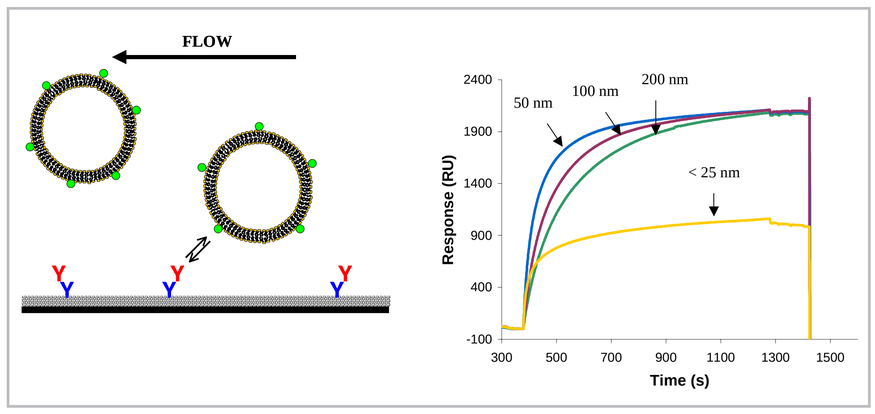


Figure 1: Influence of liposome size

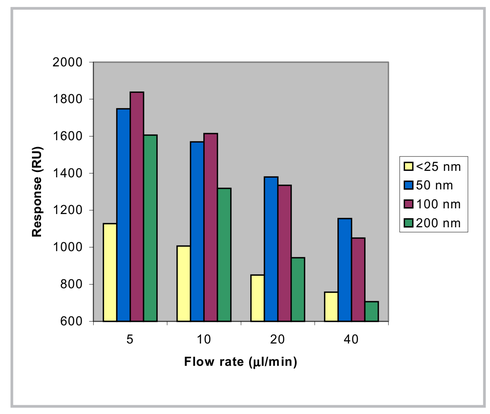


Figure 2: Influence of flow rate

The four differently sized liposomes were injected for 6 min at various flow rates. The flow rate that gave highest response was 5 µl/min. For all sizes an increase in flow rate gave a decrease in response. Large liposomes showed, however, a more pronounced decrease in response than small liposomes. A possible explanation for this is that at high lamellar flow rate, large particles tend to assemble in the middle of the flow cell [2], counteracting the diffusion to the sensor surface. Considering this, 5 µl/min appears to be an appropriate flow rate.

Direct binding assay

By including small amounts of biotin-DHPE in liposomes with a diameter of 100 nm, the minimum number of binding sites required on every liposome for ligand binding was tested. Based on a liposome structure containing about 81 000 lipids/liposome with 0.0012 % (mol/mol) biotin-DHPE, the liposomes were calculated to harbor only one single biotin per vesicle. Even at this low degree of biotin incorporation, significant response levels could be obtained. The liposome analyte gave a small response on the reference surface (rabbit anti-mouse antibody) and for that reason no reference subtraction was performed. The figure demonstrates an overlay plot of experimental data (colored curves) and data calculated with the 1:1 Langmuir binding model with drifting baseline (black curves).

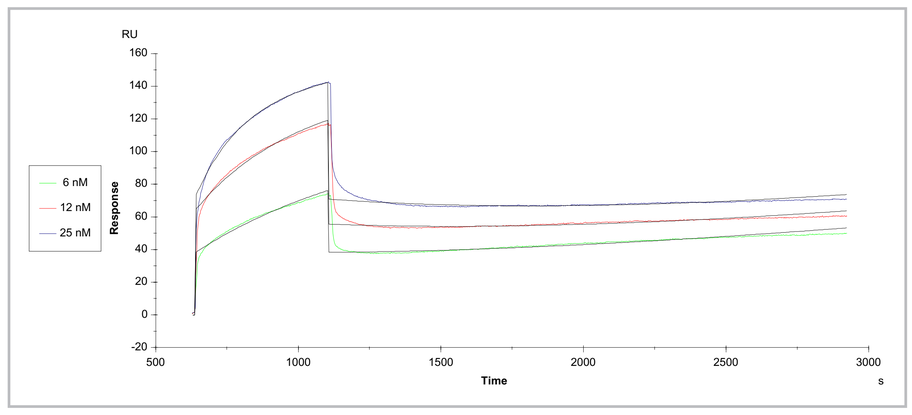


Figure 3: Direct binding assay

Kinetic Parameters	1:1 Langmuir binding with drifting baseline
k_a (1/Ms)	2.2e+5
k_d (1/s)	6.0e-4
K_A (1/M)	3.7e+8
K_D (M)	2.7e-9

Injection	Lipid concentration (mM)	Biotin-DHPE concentration (nM)
1	0.5	6
2	1.0	12
3	2.0	25

Surface competition assay

POPC-liposomes with 0.2% (mol/mol) biotin-DHPE (3 nM total concentration) were mixed with various concentrations of biotin-jeffamine and subsequently injected over immobilized anti-biotin antibody. The interaction between the liposomes and the anti-biotin was inhibited, indicating specific competitive binding. The IC_{50} value was 60 nM.

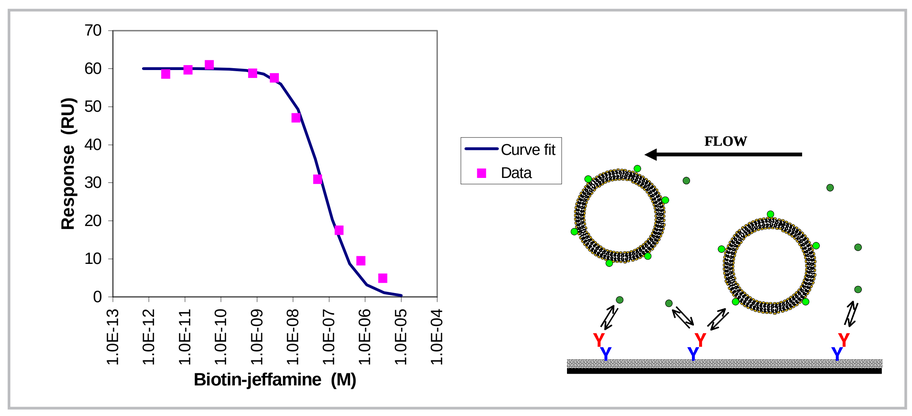


Figure 4: Surface competition assay

Solution inhibition assay

Immobilization of EGF

EGF was covalently immobilized to the dextran hydrogel on Sensor Chip CM5 through amine coupling via one of its primary amine groups.

Proteoliposome preparation

The EGF-receptor was reconstituted in lipid membrane vesicles by slow removal of the detergent OG from a mixture of POPC, OG and EGF-receptor. This was accomplished by dialysis.

In a competition assay with reconstituted receptor and soluble EGF at varying concentrations an IC_{50} value of 3 nM was obtained. This appears reasonable in comparison to literature data of K_D = 1.8 nM [3].

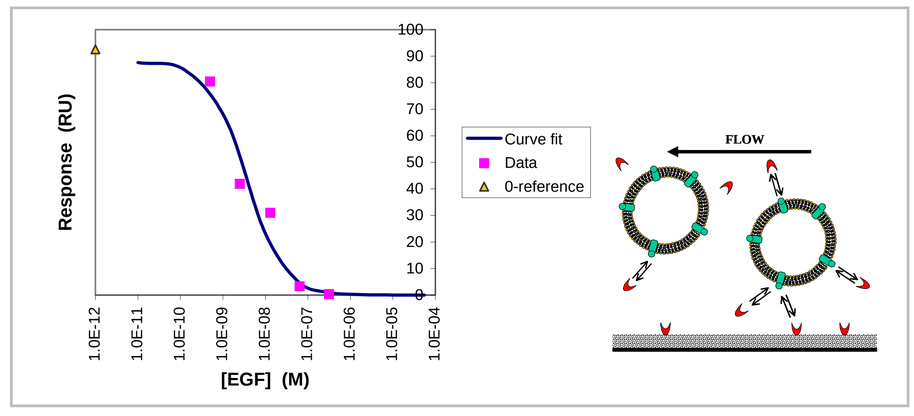


Figure 5: Solution inhibition assay

Conclusions

- Liposomes with diameters between 50 - 100 nm are preferable.
- Use a low flow rate - 5 µl/min.
- Incorporation of one analyte molecule per liposome is sufficient to generate readable signals for kinetic and affinity analysis.
- These studies demonstrate that using reconstituted proteoliposome analytes provides a useful approach for the study of membrane protein interactions using Biacore's SPR technology.

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