Reconstitution of Membrane Proteins into Lipid Monolayer. Two-Step Transfer Technique: From Cell to Liposome, from Liposome to Lipid Monolayer[1]

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A direct reconstitution method, possibly under non-denaturing conditions, was developed to study membrane protein-lipid monolayer at the air/water interface. Proteins were first transferred from intact human erythrocytes or ghosts to liposomes, which spontaneously transformed at the air/water interface. Then, by using the wet-bridge method, a stable protein containing monolayer by means of surface pressure/area diagrams was formed that exhibited erythrocyte acetylcholinesterase activity.

Artificial bioactive surfaces have a great potential with respect to the mimicking of biomembrane processes and the creation of new materials with powerful characteristics.[2] Still, the handling of bioactive materials, embedded in a boundless number of interactions, is as complex as their inherent properties.[3] This is one main reason why the investigation and application of membrane proteins are well behind of that of soluble proteins. If one wants to succeed in reconstitution of membrane proteins, it is crucial to avoid any denaturation or deactivation. It is known for several years that liposomes and biomembranes transform at the air/water interface.[4] However, the systems so far described were either very complex particularly if biomembranes were transformed,[5] or the membrane proteins had to be isolated and solubilized prior to the reconstitution[6] bearing the uncertainty whether their natural activity and/or structure could be successfully preserved.

As previously shown[7] we found evidence that membrane proteins can be efficiently transferred to liposomes containing an artificial boundary lipid, 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine (D_{14}DPC). Membrane proteins keep their native orientation when reconstituted into the liposomal membrane. Furthermore it is believed that D_{14}DPC stabilizes the reconstituted membrane proteins in the lipid bilayer membranes.[7]

Now we developed a method that allows the formation of a stable protein containing lipid monolayer from membrane protein-containing liposomes by direct protein transfer from intact cell membrane (Figure 1).

Membrane proteins from human erythrocyte ghosts and intact bovine erythrocytes were transferred to large unilamellar vesicles containing 60 mol% of D_{14}DPC and 40 mol% dimyristoylphosphatidylcholine (DMPC) as described before.[7] When a suspension of proteoliposomes so obtained was poured into a trough a spontaneous and fast increase in surface pressure was observed. In any case the surface pressure increase continued until the collapse pressure of the corresponding D_{14}DPC/DMPC monolayer spread from an organic solution was reached. It could be also shown by transforming liposomes without proteins reconstituted, that, by increasing the content of D_{14}DPC in the liposomal membrane, the surface pressure increased more rapidly (Figure 2).

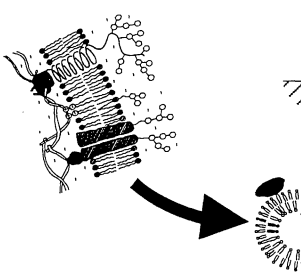

Figure 1. Two-step direct protein transfer from intact cell membrane to liposome and than to lipid monolayer.

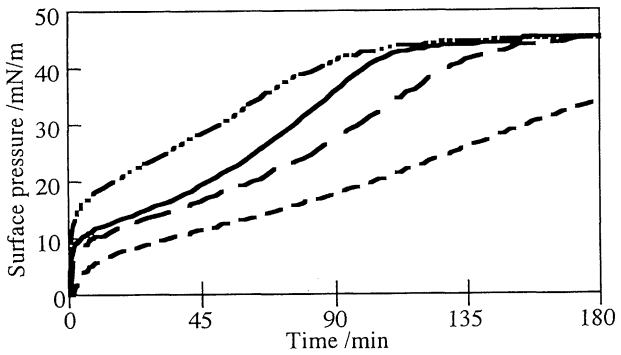

Figure 2. Spontaneous increase in surface pressure of D_{14}DPC/DMPC liposomal suspensions at 37 °C with (— - - — - , protein concentration 40 μg/ml, D_{14}DPC/DMPC molar ratio: 3:2) and without (D_{14}DPC/DMPC molar ratios: - - - - - - 0:1, — — — 3:2, ———— 4:1) proteins reconstituted (lipid concentration 1 mmol).

For separation of the resulting surface active film from the liposomes remaining in the subphase the so called "*wet-bridge*" method was applied.[8] A commercially available miniaturized film balance (USI Systems, Fukuoka, Japan) was equipped with a customized trough made of polytetrafluoroethylene (PTFE), (Figure 3).

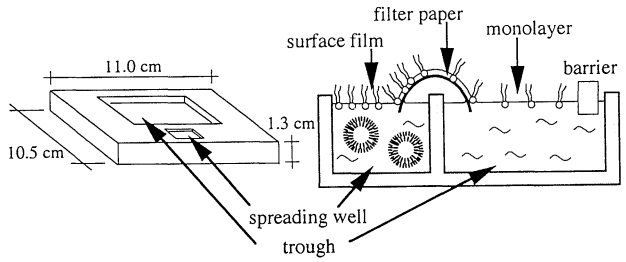

Figure 3. Sketch of trough and "wet-bridge" (top view and cross section).[8]

According to the procedure previously described[8] a corrugated thin strip of wet filter paper was used to bridge between the spreading well and the trough. The whole equipment was placed into a temperature controlled cabinet to keep the temperature constant at 37 °C. The atmosphere inside the box was saturated with water vapor to prevent the filter paper bridge from drying. After

the barrier was moved close to that end of the trough where the filter paper dipped into the subphase, the liposomal suspension (lipid concentration 1~2 mmol) was poured into the spreading well. An increase in the surface pressure of the trough compartment indicated the transformation of liposomes in the spreading well and the expansion of the surface film over the paper bridge to the trough due to the lateral gradient in surface pressure. The barrier was gradually moved so that the surface film gradually expanded at a constant surface pressure onto the surface of the trough. It was necessary to form the surface active film at a constant surface pressure of 10-15 mN/m. It is known that proteins undergo conformational changes under zero or very low surface pressure conditions at the air/water interface.[9] Conditions were usually chosen so that the trough was fully covered with the surface active film within a few minutes. At lower surface pressures (i.e. at 10 mN/m rather than 15 mN/m) the velocity of monolayer formation via the wet-bridge was significantly higher. Also at higher protein concentrations[10] of the liposomal suspension, the transformation process was almost exponentially accelerated (Figure 4A).

Finally the wet-bridge was removed, and the surface active film was allowed to stand for at least 30 min at 15 mN/m. After this procedure, the surface pressure (adjusted to a certain value between 15 and 40 mN/m) kept stable over at least several hours and surface pressure/area isotherms showed an only slightly visible hysteresis. Furthermore, at about 45 mN/m the collapse of the surface layer was observed (Figure 4B). Due to this typical behavior, the surface active film was referred as a monolayer. If the monolayer was further expanded, and the surface pressure dropped to 0 mN/m, a slow time-dependent increase in the surface pressure subsequently occurred. Such behavior has been interpreted regarding to the unfolding of proteins at the air/water interface.[9]

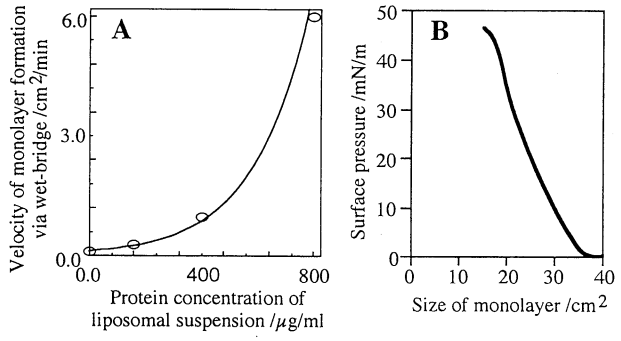

Figure 4. **A**: Velocity of monolayer formation (size of monolayer formed via wet-bridge per time) at 15 mN/m from proteoliposomes as a function of protein concentration of liposomal suspension. **B**: Isotherm of D_{14}DPC/DMPC monolayer with membrane proteins at 37 °C.

To be evident that membrane proteins were certainly transferred from erythrocytes to monolayer via liposome,[10] enzymatic activity of acetylcholinesterase (AchE) transferred was investigated in the monolayer. This enzyme is anchored via a phosphatidylinositol moiety in the membrane of erythrocytes and the active site is not located in the membrane.[11] Therefore, we believe that this enzyme should keep its activity even if transferred into a monolayer at the air/water interface. Acetylthiocholine was injected as substrate into the subphase and thiocholine produced was quantitatively determined via a picomole fluorescence assay.[12] Enzymatic activity was found over at least 3 h (Figure 5). In addition, after cleaning the water surface by aspiration no further increase in enzymatic activity was observed, assuring the reconstitution of enzymatic activity at the interface. Of course we proved that the subphase was not contaminated with liposomes.

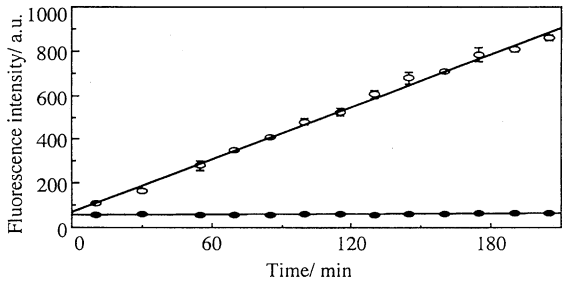

Figure 5. Increase of thiocholine concentration in the subphase due to AChE hydrolysis in the monolayer. Fluorescence intensity corresponds directly to thiocholine concentration. —○— For transformation of proteoliposomes and —●— : For conventional liposome without proteins.

In conclusion, a direct and convenient method was developed that allows the transfer of membrane proteins from intact cell membranes to lipid monolayers via liposomes. In this methodology, D_{14}DPC plays important roles in the protein transfer from cell membrane to liposome and the liposome transformation at the air/water interface.

Based on these results we are currently studying the details about liposomal transformation at the air/water interface and the properties of the resulting surface films especially with regard to reconstituted protein functions.

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References and Notes

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