# INCORPORATION OF ATP SYNTHETASE INTO LONG-TERM STABLE LIPOSOMES OF A POLYMERIZABLE SYNTHETIC SULFOLIPID

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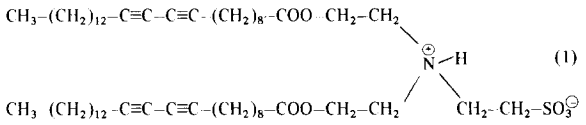
## 1. Introduction

There is increasing interest in elucidating the numerous vectorial processes within a biomembrane mediated by membrane proteins (e.g., oxidative phosphorylation or photophosphorylation, transport phenomena). Studying the properties of these enzymes in their natural environment is a troublesome problem due to the complex structure of biomembranes. Therefore resolution and reconstitution into model membranes is one approach to analyse membrane proteins.

Because of their biomembrane-like bilayer structure spherical liposomes and planar lipid membranes (BLM) are appropriate models for the investigation of proteins embedded in a lipid matrix. One of the problems of reconstituted model systems is their lack of long term stability [1]. The polymerization of synthetica lipids within the bilayer membrane overcomes this problem. This concept of stabilizing model membranes (e.g., liposomes, BLMs) was carried out with lipids bearing diacetylene, butadiene or vinyl moieties as polymerizable groups [2–7]. While synthesis and stability of these polymeric systems have already been carefully investigated, their suitability for the incorporation of membrane proteins is as yet unknown. As an example of an asymmetric membrane-integrated protein we have used the ATP synthetase complex (ATPase, EC 3.6.1.3). This protein appears to have the same general structure in all organisms [8]. It consists of a hydrophobic membrane-integrated part ($F_0$) and a water-soluble ($F_1$) moiety which carries the catalytic centers of the enzyme. The ($F_0$) portion is required for energy transduction from the membrane to ($F_1$) and vice versa [9]. Furthermore, it renders the $F_1$-ATPase moiety sensitive to inhibition by oligomycin and *N,N'*-dicyclohexylcarbodiimide [10].

Here, we describe the incorporation of the ATP synthetase complex from *Rhodospirillum rubrum* into long-term stable polymeric liposomes of the synthetic sulfolipid (1) with the diacetylene group as polymerizable unit.




(1)

The incorporation of the enzyme complex into the monomeric and polymeric liposomes could be demonstrated by agarose gel electrophoresis.

## 2. Materials and methods

*Rhodospirillum rubrum* FR1 cells were grown photoheterotrophically in a 15 l fermenter at 30°C in R8ÄH-medium [11]. Solubilization of the ATP synthetase complex was done as in [12]. The native enzyme complex was solubilized from chromatophores of *Rhodospirillum rubrum* using taurodeoxycholate (TDOC). The enzyme was delipidated by precipitation with PEG and purified by Sepharose CL-4B column chromatography. ATPase activity was measured in 100 mM Tris–HCl (pH 8.0) in the presence of 1 mM ATP at 37°C by determining the liberated ortophosphate [13].

The polymerizable sulfolipid (1) was synthesized according to [6]. The liposomes were obtained by ultrasonication in buffer (10 mM Hepes–KOH (pH 8.0); 0.2 mM EDTA) at 60°C under $N_2$ followed by filtration through millipore filter (8 μm). Monomeric liposomes of (1) were polymerized by UV irradiation (254 nm) at 20°C under $N_2$. Absorption spectra of the polymers were measured on a Perkin Elmer 554

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spectrophotometer. Monomeric and polymeric proteoliposomes were prepared essentially according to [14]. Aliquots of the liposome solution were added to the purified ATP synthetase at ~50:1 lipid:protein (w/w). The mixture was incubated for 10 min at 37°C. Agarose gel electrophoresis was performed using cylindrical gels of 0.5% agarose (Serva, Heidelberg) and 36 mM Tris, 30 mM $NaH_2PO_4$, 10 mM EDTA, at pH 7.5, as electrophoresis buffer. After staining with Coomassie brilliant blue R-250 the gels were scanned at 580 nm with an ISCO UA-5 absorbance monitor. Gels containing lecithin liposomes or monomeric or polymeric liposomes of (1) were scanned at 254 nm in quartz tubes without staining. Electron microscopy was on freeze-fractured preparations of liposomes using a Phillips EM 301. Calorimetric data of liposomes of (1) were obtained on a Perkin Elmer DSC 2B at 10 mg lipid/ml.

## 3. Results

The purified ATP synthetase complex from *Rhodospirillum rubrum* is almost completely inactive. The activity is substantially increased on the addition of amphiphiles and is not restricted to systems with an intact bilayer structure. The stimulation of ATP hydrolysis can be achieved by a variety of amphiphiles (unpublished). These include natural phospholipids, detergents as well as the synthetic sulfolipid (1). For soybean lecithin and (1) the maximal enzyme activity is obtained at a ratio >500 molecules lipid/molecule enzyme. The ATPase activity is stimulated 8-fold using soybean lecithin whereas monomeric (1) causes a 5-fold increase.

The investigation of ATP synthetase in long-term stable spherical model membranes of (1) requires the formation and polymerization of liposomes as shown in fig.1. According to the behavior of a variety of synthetic amphiphiles with two long alkyl chains [15,16] sonication of aqueous suspensions of (1) results in the formation of clear colorless solutions [3]. Electron microscopy (fig.2) proves the formation of monomer liposomes of a defined spherical structure with diameters near 100 nm.

The UV-initiated polymerization of the diacetylene moieties in the hydrophobic region of the bilayer structure occurs with retention of the spherical shape as schematically shown in fig.1 and proven by electron microscopy (fig.2). Extremely stable polymer liposomes with a rigid conjugated polymer backbone are obtained (fig.1) [17]. The polyreaction is documented by an increase in absorbance and a shift to shorter wavelengths in the VIS-absorption spectra (fig.3). One important condition for the polymerization of the diacetylenic units is the existence of a highly ordered state [18] which is realized below the phase transition temperature ($T_t$) in liposomal systems. For monomer liposomes of (1) the transition from solid analogous to liquid analogous state was measured by differential

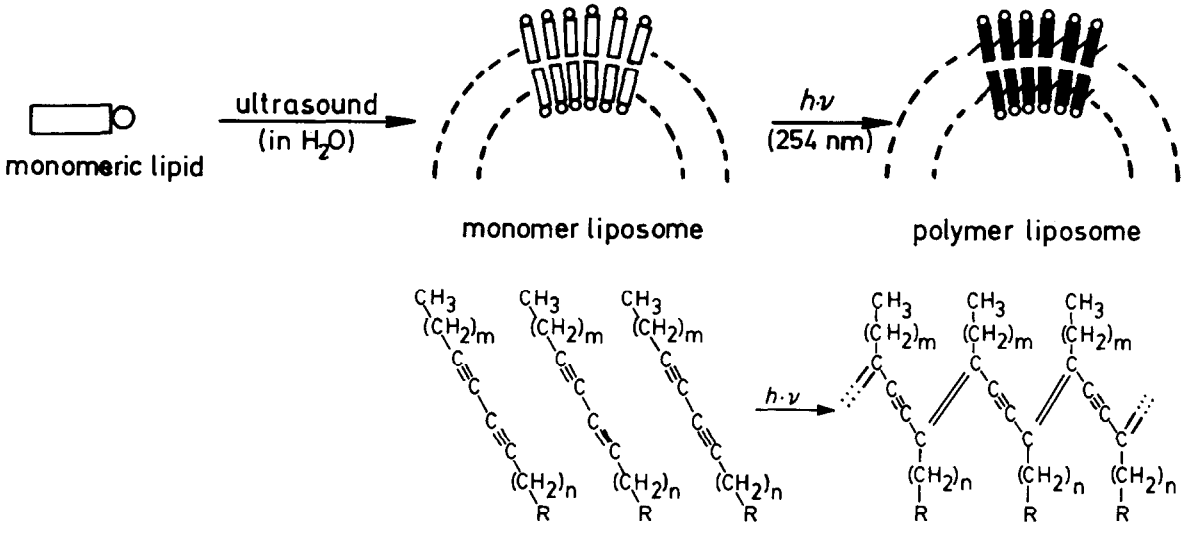
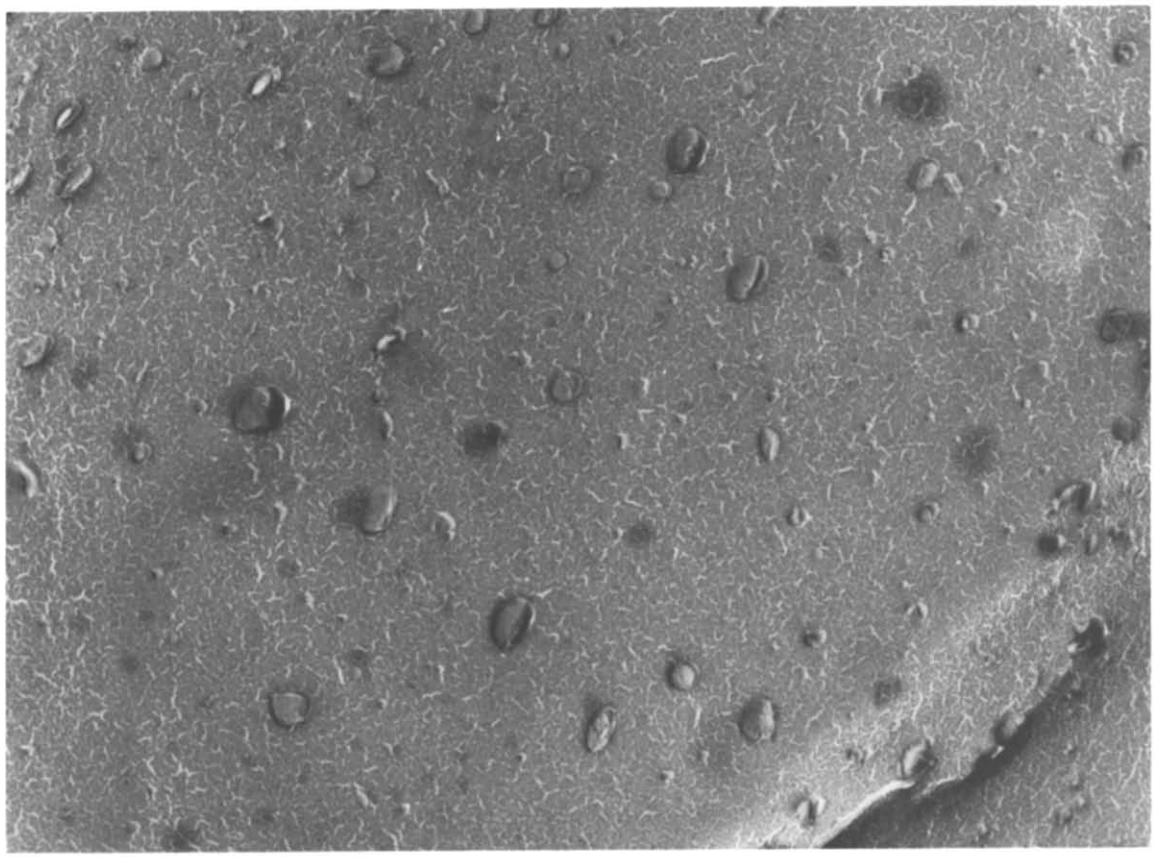



Fig.1. Formation of monomeric liposomes of (1) and UV-initiated polymerization of the diacetylenes in the bilayer membrane.

a



b

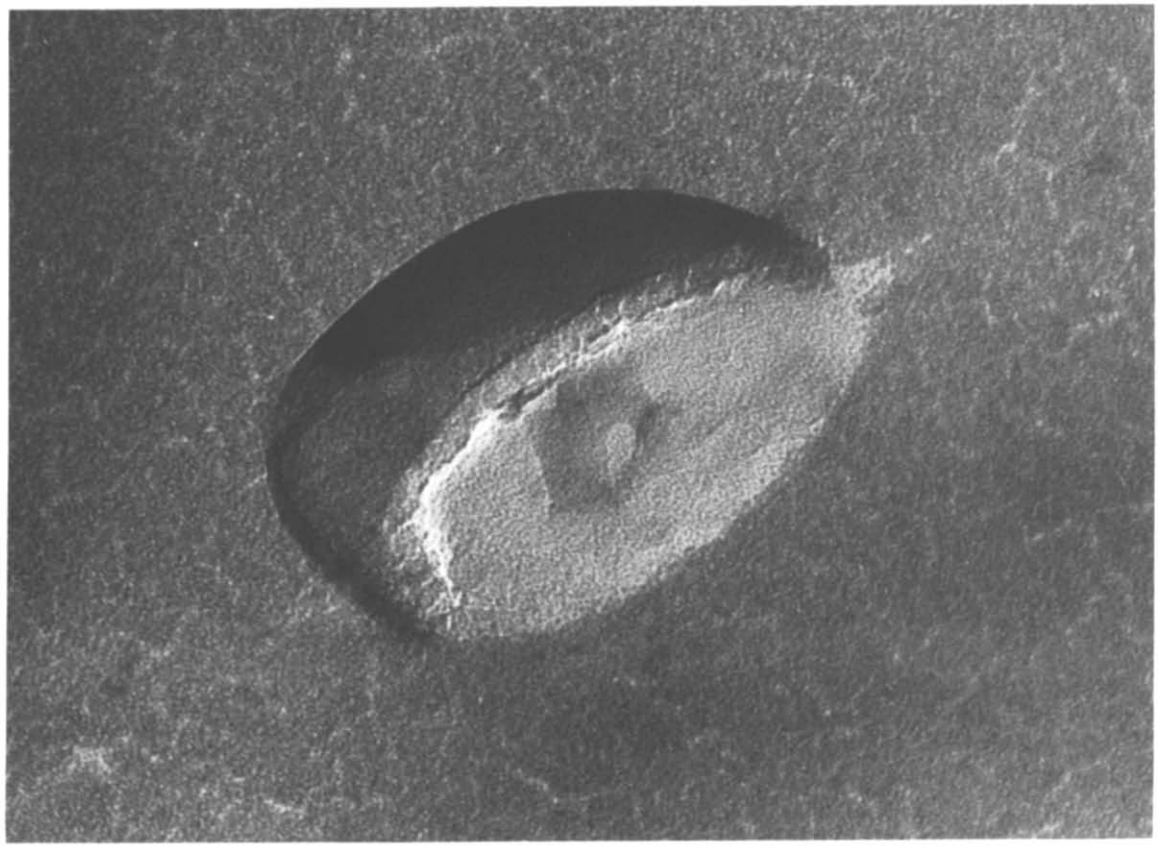


Fig.2. Freeze fracture electron microscopy of: (a) monomeric liposomes of 1 (× 54 000); (b) polymerized liposome of 1 (× 387 000).

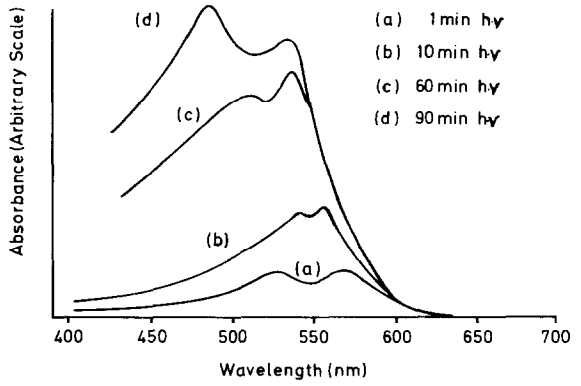



Fig.3. VIS-absorption spectra of polymer liposomes of (1).

scanning calorimetry (DSC). Fig.4 illustrates the phase transition behaviour of (1) as a function of the polymerization time. The pure monomer liposomes show a $T_t$ of 53°C. Only below this temperature they are in a highly ordered, crystalline state and can thus be polymerized. During the polymerization a decrease in the heat of phase transition indicates a restricted mobility of the polymerized hydrocarbon core. Moreover, the phase transition finally disappears after complete polymerization of the monomer.

The incorporation of the ATP synthetase into monomeric and polymeric liposomes of (1) was achieved by simple incubation. For comparative studies the enzyme was also incorporated into liposomes of soybean lecithin.

There is a remarkable difference of ATPase activity depending on the method of proteoliposome preparation (fig.5).

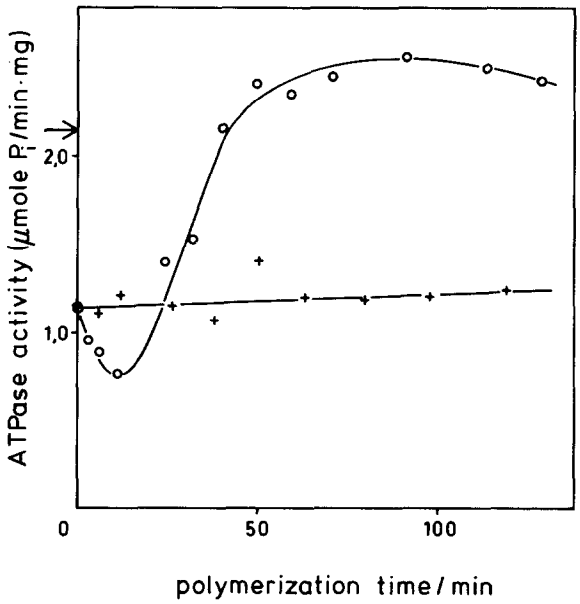
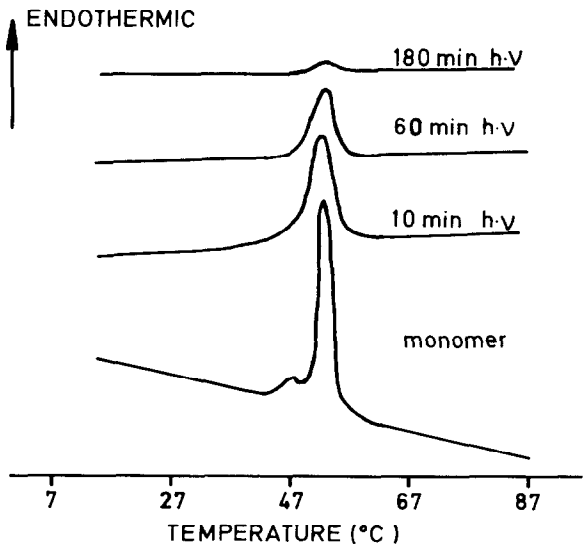



Fig.5. ATPase activity of ATP synthetase incorporated into liposomes: (-○-) incubation of the enzyme with polymerized liposomes (method A); (- + -) incubation of the enzyme with monomeric liposomes of 1 followed by polymerization (method B); (→) activity of ATP synthetase in soybean lecithin liposomes.



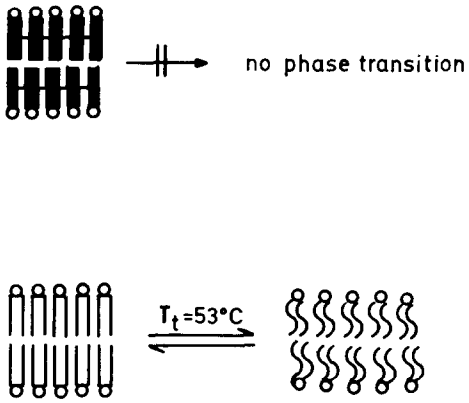




Fig.4. Measurement of phase transition of liposomes of (1) by DSC (Perkin Elmer 2 B, $c$ = 10 mg/ml).

*Method A:* The ATP synthetase incorporated into partially polymerized liposomes shows a significant dependence of its hydrolytic activity on the polymerization time of the momomer liposomes. A minimum of activity after 10 min polymerization time is followed by a strong increase by prolonged irradiation of the monomer liposomes. This minimum of enzyme stimulation corresponds with a red shift of absorption in the VIS-spectra of the polymers (fig.3). The color shift of polydiacetylenes occurring in bulk [19] as well as in solution [20], points to changes in the conformation of the polymer.

*Method B:* Incubation of the enzyme with monomeric liposomes of (1) followed by the polymerization results in a slight increase in ATPase activity (fig.5) as a function of the polymerization time. In contrast to method A no drastic increase in activity is observed. In order to verify that the ATPase activity is not decreased by UV irradiation (254 nm, $E = 0.09$ $J/m^2 \cdot s$) the enzyme was also irradiated in buffer solution and after incorporation into liposomes from soybean lecithin. While ATPase activity was slightly decreased in buffer (50 mM Tris–HCl (pH = 8.0), 5 mM TDOC, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 20% glycerol) it was completely retained if the enzyme was embedded in soybean lecithin liposomes. Further evidence that the ATP synthetase remains intact after the UV-initiated polymerization of the sulfolipid (1) in liposomes is given by the inhibition experiments with *N,N'*-dicyclohexylcarbodiimide and oligomycin. The ATPase activity is inhibited to the same extent in soybean lecithin liposomes as well as in monomeric and polymeric liposomes. Final evidence for the incorporation of active ATP synthetase requires the investigation of energy-linked reactions in proteoliposomes. This is currently under investigation. A preliminary hint for the incorporation of the enzyme is given by agarose gel electrophoresis. This method has also been a useful tool in analyzing monomers and dimers of bacteriophage capsides with diameters of ~60 nm [21]. Thus sonicated liposomes and proteoliposomes (30–100 nm diam.) are expected to migrate into low concentration agarose gels. Electrophoresis was performed as in section 2.

If no detergents are present in the gel the pure delipidated ATP synthetase complex migrates as a double band indicating the formation of oligomeric enzyme complexes. The incubation with increasing amounts of monomer or polymer liposomes of (1) results in the formation of a single but broadened band with a higher electrophoretic mobility. At a ratio of ~2 molecules enzyme/liposome a single new band is evident. The mobility of this band is identical with that of pure monomeric or polymeric liposomes of (1). In comparative experiments the same results were obtained with proteoliposomes made of ATP synthetase and soybean lecithin, whereas under the same conditions two hydrophilic proteins (BSA, ferritin) show no interaction with liposomes (not shown).

## 4. Discussion

The pure isolated ATP synthetase complex from *Rhodospirillum rubrum* shows no enzymic activity due to the delipidation during preparation. The reactivation of enzymatic activity by the addition of natural phospholipids has been reported [12]. Here, we describe for the first time the same reactivation with the synthetic sulfolipid containing polymerizable diacetylene units in the hydrophobic chains.

Both natural phospholipids as well as the synthetic polymerizable sulfolipid have amphiphilic properties and are thus able to form sufficient hydrophobic structures required for the stabilization of the ($F_0$) moiety of the ATP synthetase.

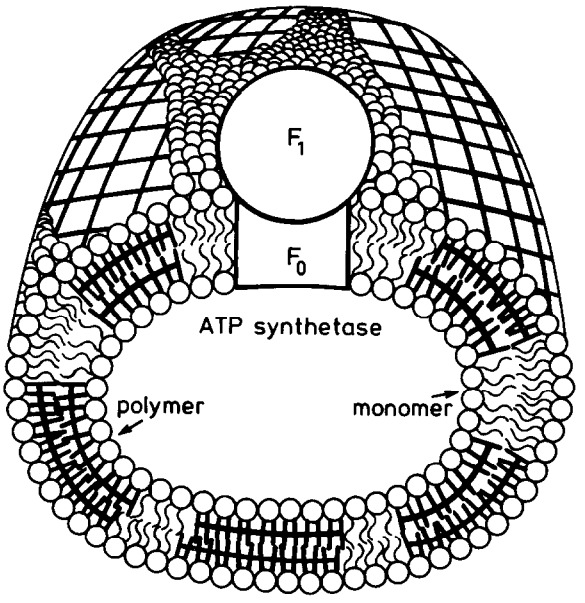



Fig.6. Schematical representation of ATP synthetase incorporated into a partially polymerized liposome.

Here the incorporation of an active membrane protein into polymerized long term stable liposomes is demonstrated. The polymerization within the bilayer membrane influences the enzymatic behavior of the ATP synthetase. This is documented by the fact that the polymeric proteoliposomes show an unexpected 2-fold increase of activity compared with the monomeric liposomes. This result can be explained by a structural change in the bilayer organization during the polyreaction.

DSC data indicate residual 'monomeric domains' in the partially polymerized liposomes. Thus one can assume that incorporation of the enzyme takes place in the region of the monomeric lipids, stabilized by the polymer matrix as schematically shown in fig.6.

Due to the high phase transition of the sulfolipid one would expect a decreased enzyme activity [22]. But as illustrated by fig.5, even below the phase transition temperature of the monomeric lipid (1) the enzymatic activity is retained.

The incorporation of the ATP synthetase into the bilayer membrane of liposomes by simple incubation is demonstrated for the first time by agarose gel electrophoresis. This method may be a generally applicable and rapid test for the incorporation of membrane proteins into sonicated liposomes.

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