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# P-glycoprotein inserted in planar lipid bilayers formed by liposomes opened on amorphous carbon and Langmuir–Blodgett monolayer

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**Abstract**

The insertion of proteins into planar lipid layers is of outstanding interest as the resulting films are suitable for the investigation of protein structure and aggregation in a lipid environment and/or the development of biotechnological applications as biosensors. In this study, purified P-glycoprotein (P-gp), a membrane drug pump, was incorporated in model membranes deposited on solid supports according to the method by Puu and Gustafson, Biochim. Biophys. Acta 1327 (1997) 149-161. The models were formed by a double lipid layer obtained by opening P-gp-containing liposomes onto two hydrophobic supports: amorphous carbon films and Langmuir–Blodgett (L–B) lipid monolayers, which were then observed by transmission electron microscopy and atomic force microscopy, respectively. Before the opening of liposomes, the P-gp structure and functionality were verified by circular dichroism spectroscopy and enzymatic assay. Our micrographs showed that liposomes containing P-gp fuse to the substrates more easily than plain liposomes, which keep their rounded shape. This suggests that the protein plays an essential role in the fusion of liposomes. To localize P-gp, the immunogold labeling of two externally exposed protein epitopes was carried out. Both imaging techniques confirmed that P-gp was successfully incorporated in the model membranes and that the two epitopes preserved the reactivity with specific mAbs, after sample preparation. Model membranes obtained on L–B monolayer incorporated few molecules with respect to those incorporated in the model membrane deposited onto amorphous carbon, probably because of the different mechanism of proteoliposome opening. Finally, all particles appeared as isolated units, suggesting that P-gp molecules were present as monomers. 

*Keywords:* P-glycoprotein; Lipid bilayer; Circular dichroism; Atomic force microscopy; Transmission electron microscopy

## 1. Introduction

P-glycoprotein (P-gp), a protein encoded by the human MDR1 gene, belongs to the superfamily of

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ABC (ATP-binding cassette) transporters. This protein is embedded in the lipid bilayers and acts as a nanoscale pump, utilizing the energy of ATP hydrolysis to translocate molecules across cellular membranes. P-gp evolved to protect cells from xenobiotic molecules and it is normally found in cells of the digestive tract, the kidney, the liver and in endothelial cells lining capillaries in the brain [1]. In tumor cells P-gp provides cross-resistance to a variety of chemotherapeutic drugs (multidrug resistance, MDR) and its overexpression is responsible for the failure of the chemotherapeutic treatment of several tumors [2].

P-gp structure and function are far from being completely elucidated. P-gp is generally described as constituted by α-helices and β-structures [3]. However, the actual folding of P-gp in a lipid bilayer is still a matter of debate. Experimental data have so far generally supported a model describing the protein as a toroidal ring of 12 α-helices, deployed in two arcs of six helices each (6+6 helix model), forming a single aqueous pore in the bilayer [4,5]. However, Jones and George [6] have recently proposed a different interpretation of the bulk of experimental data: an alternative model in which the protein forms two transmembrane pores constituted by β-barrels.

To elucidate the structure–function link, it is of crucial importance to understand the physiological and functional aggregation state of the protein. For P-gp, data suggest that the minimum functional unit should be a monomer, but do not exclude the possibility that these monomers might form dimers or larger oligomers under certain physiological conditions, and that oligomerization might alter the structural and functional properties of the constituting monomers [5,7].

Immunogold labeling, if performed with well-characterized monoclonal antibodies recognizing defined protein epitopes, would detect the single monomeric unit in the cellular plasma membrane. Transmission electron microscopy (TEM) localizes, with high resolution, gold particles up to 5 nm diameter, but admits only thin samples, and the information that it furnishes is limited to a bi-dimensional projection. In studies performed by scanning electron microscopy (SEM) on cells expressing P-gp, previously labeled with immunogold complexes, the protein epitopes were detected on the cellular membrane surface [8]. However, conventional SEM has a low spatial resolution limit (about 5–10 nm) and does not yield a fine detailed three-dimensional (3D) analysis by which the presence of P-gp dimers or oligomers can be assessed. In addition, sample preparation for SEM could introduce artifacts that modify the native structure of P-gp.

In order to overcome these limitations we used atomic force microscopy (AFM) to study the structure of P-gp in a model phospholipid double-layer membrane. AFM is a very attractive tool for biological investigations because it is suitable to observe the 3D shape of a whole cell as well as its substructures, e.g. proteins and DNA, without having to resort to impairing treatments like fixation, which can completely alter the original structure. Moreover, lateral high-resolution images can be attained (up to few nanometers) and useful 3D topological maps of the sample can be reconstructed. However, if molecular resolution has to be attained, AFM needs very flat and homogeneous samples where only molecular discontinuities occur.

Thus, given the intrinsic complexity of biomembranes, it seemed more appropriate to approach the study by using simplified model membranes such as lipid bilayers, where all experimental parameters can be accurately monitored. The visualization of P-gp monomers and of dimers and oligomers, if any, could be improved by incorporating the protein in a lipid bilayer. Puu and Gustafson [9] developed a method by which stable lipid bilayers can be prepared by transferring liposomes to a planar support, constituted by a Langmuir–Blodgett (L–B) lipid monolayer [10] or amorphous carbon. They performed a systematic investigation to determine factors of importance involved in the kinetics and stability of lipid bilayer formation. In particular, they studied the influence of lipid composition of liposomes and monolayers, and the influence of $Ca^{2+}$ concentration on the fusion process. They evaluated bilayer stability after storage and transfer between different media by crossing the air–water interface. By their method, proteins with retained activities can be successfully introduced in the bilayer. The same authors refer that lipid bilayers can also be created by directly opening liposomes onto hydrophobic supports such as amorphous carbon, suggest-

ing that two different mechanisms of the planar bilayer formation operate.

In this paper we compare results obtained by TEM on model membrane with P-gp prepared by direct deposition of proteoliposomes onto amorphous carbon support, with those achieved by AFM on a model membrane prepared by opening proteoliposomes onto an L–B lipid monolayer deposited onto mica.

The aims of this study were to look inside the biochemical and structural features of P-gp reconstituted in liposomes, to obtain information about the mechanisms of bilayer formation on two different hydrophobic surfaces, in the presence and absence of P-gp, to find out whether the insertion of P-gp in the model membrane affects its conformational features, at least in the surroundings of two external epitopes and, finally, to elucidate P-gp aggregation state.

## 2. Materials and methods

### 2.1. Cell culture

The MDR variant of human leukemic lymphoblastic cell line (CEM VBL100) was grown in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1% L-glutamine and 1% penicillin and streptomycin at 37.0°C in a 5% $CO_2$ humidified atmosphere in air. CEM VBL100 cells were grown in complete medium containing 10 ng/ml vinblastine sulfate (Velbe, Lilly, Fegersheim, France).

### 2.2. mAbs reagents

mAbs MM4.17 [11] and MRK-16 (Kamiya, Thousand Oaks, CA, USA), that recognize two distinct human-specific epitopes of extracellular domains of MDR-1-P-gp, were used in this study. MM4.17 is an $IgG_{2ak}$ monoclonal immunoglobulin reacting with a continuous-linear epitope on the apical part of the fourth loop of P-gp. MRK-16 is an $IgG_{2ak}$ monoclonal immunoglobulin reacting with a conformational epitope distributed on the first and fourth loop of P-gp [12].

### 2.3. P-gp purification, liposome preparation and functionality tests

P-gp was extracted from a plasma membrane fraction separated by means of a sucrose density gradient as described by Dong et al. [13]. Briefly, the membranes were dissolved with 2% sodium dodecyl sulfate (SDS) and ultracentrifuged for 30 min at $100\,000 \times g$ at 20.0°C. The supernatant was diluted to 1% SDS with an equal volume of 10 mM phosphate buffer, pH 7.0, containing 1 mM dithiothreitol (DTT). P-gp was purified by hydrophobic interaction chromatography on a hydroxyapatite high-performance liquid chromatography (HPLC) column equilibrated with 50 mM phosphate buffer (pH 7.0), 1% SDS and 1 mM DTT. After binding of the P-gp extract and extensive washing, the protein was eluted with a linear gradient of phosphate ($0 \rightarrow 0.5$ M) at 28.0°C. The chromatographic fractions from the TSK column were analyzed by electrophoresis and immunoblot. SDS–polyacrylamide gel electrophoresis (PAGE) was performed on slab gels by the method of Laemmli [14] using 10% acrylamide. After electrophoresis, proteins separated by SDS–PAGE were transferred to nylon membranes (Immobilon P from Millipore), and stained with a mouse monoclonal anti-P-gp C219 (Signet Laboratories Inc., working dilution 1:100) at 37°C for 60 min. Bound antibodies were detected with alkaline phosphatase-conjugated secondary anti-mouse immunoglobulin (Sigma, working dilution 1:1000). The purification grade of P-gp was determined by HPLC analysis on reverse-phase C8 (Vydac) columns.

Reconstitution of purified P-gp into liposomes was carried out following the method by Rigaud et al. [15]. 25 mg of L-α-phosphatidylcholine (PC) and 2.5 mg of L-α-phosphatidic acid (AP) were dissolved in chloroform and dried under a stream of nitrogen. The lipid film was dissolved in 20 mM Tris–HCl, pH 7.4, 75 mM NaCl, 1 mM DTT and 0.5 mM EDTA buffer and sonicated on ice until the mixture became transparent. A 100 μl aliquot of liposome preparation was sequentially supplemented with 610 μl of buffer and 40 μl of 10% dodecyl maltoside and, after 15 min, 250 μl of 0.2 mg/ml purified P-gp under constant stirring at room temperature. To remove the detergent, the solution was dialyzed overnight in Tris–HCl buffer.

The functional properties of liposome-reconstituted P-gp were tested by a fluorimetric assay of its ATPase activity, as described by Gonzalo et al. [16]. This assay follows NADH fluorescence decay as a function of ADP produced by ATPase activity of P-gp, which triggers a coupled enzymatic cascade involving pyruvate kinase and lactate dehydrogenase in the presence of phosphoenolpyruvate and NADH. Cyclosporin A (CsA) has been used to verify the P-gp ATPase function, since it is known to inhibit P-gp both at the basal and stimulated activities [17].

### 2.4. Circular dichroism (CD) on liposomes

CD spectra of liposomes with and without P-gp were recorded on a Jasco J-710 spectropolarimeter (Jasco Corporation, Tokio, Japan). Spectra were recorded in the far-UV region (200–260 nm) using 1 mm path-length QS cell. Resolution and sensitivity were 0.2 nm and 50 mdeg, respectively. In order to maximize signal-to-noise ratio, each spectrum was averaged on four different scans.

### 2.5. Lipid bilayer onto mica

Air-stabilized lipid bilayers were prepared following the procedure proposed by Puu and Gustafson [9]. This method involves the fusion of PC–AP liposomes with dipalmitoylphosphatidylcholine (DPPC) L–B monolayer deposited onto mica. DPPC monolayers were prepared under controlled physical conditions at the air–water interface following the Langmuir technique [10]. Surface pressure measurements as function of area per molecule (isotherm curve) reflect both the structures of the molecules in the monolayer and the interactions between them. A dedicated apparatus, the Langmuir trough, consists of a rectangular Teflon trough filled with the aqueous subphase, and two sliding barriers that are used to sweep the surface. With the aid of an organic solvent the lipid is spread between the barriers; when the solvent evaporates, the lipids remain at the air–water interface. Following Wilhelmy's method [10] the barriers are made to approach and surface pressure can be easily measured, using a roughened platinum plate, with a precision of 1 mN/m. We used a commercial apparatus (Minitrough, KSV, Helsinki, Finland) enclosed in a Plexiglas box and controlled by a computer. Lipid was dissolved in chloroform (1 mg/ml) and appropriate portions of the solution (about 24 μl) were spread with a microsyringe over the aqueous solution. All experiments were carried out on a subphase of deionized water, thermostated at 25.0°C. A symmetric, non-linear compression was achieved by making the two barriers approach at a constant rate of 10 mm/min. Monolayers were deposited onto a mica substrate, keeping surface pressure constant at 35 mN/m, which corresponds to the liquid condensed state occurring in the cell membrane. Before spreading the Langmuir film, a substrate of freshly cleaved mica has been immersed in the aqueous subphase; when the monolayer was formed, the substrate was raised at a constant rate of 0.1 mm/min.

According to the method proposed by Puu and Gustafson [9], we prepared lipid bilayers on the mica substrates by fusion of liposomes with the L–B DPPC monolayer after an incubation of about 24 h, in the presence of 20 mM $CaCl_2$. Samples were then withdrawn from the liposome suspension, washed with distilled water and dried under a weak nitrogen stream for about 24 h. This procedure was repeated three times, using liposomes with and without P-gp, and we obtained three series of lipid bilayers.

### 2.6. Lipid bilayer onto amorphous carbon and immunolabeling of P-gp

Liposomes, with and without P-gp, were deposited onto thin carbon film-coated grids for TEM observation and air-dried. The labeling of P-gp was performed by the immunonegative stain technique. The grids were incubated for 30 min at room temperature with mAbs MM4.17 or MRK-16 diluted to a final concentration of 10 μg/ml in phosphate buffer (0.15 M NaCl, 0.05 M $NaH_2PO_4$, 0.05 M $Na_2HPO_4$, pH 7.2) containing 1.0% bovine serum albumin (Sigma, St. Louis, MO, USA). After washing with phosphate buffer, the grids were incubated with goat anti-mouse Ig-gold conjugate (Sigma; average diameter of gold particles 5 nm), diluted 1/10 in phosphate buffer for 30 min at room temperature. Negative controls were obtained by incubating samples with mouse $IgG_{2a}$ or with immunoconjugate alone. After washing with phosphate buffer, grids were negatively stained with

2.0% aqueous phosphotungstic acid for 2 min. Samples were examined with a Philips 208 transmission electron microscope (FEI Company, Eindhoven, The Netherlands).

For AFM measurements, the labeling of P-gp incorporated in lipid bilayers was performed on samples obtained by the fusion process of liposomes, with or without P-gp, with L–B DPPC monolayers prepared as described above. The immunolabeling reaction was performed as above, but using 10 nm gold particles.

### 2.7. AFM

AFM measurements were performed with a commercial atomic force microscope (Bioprobe, Thermomicroscopes, Sunnyvale, CA, USA) equipped with a 100 μm scanner and I-shaped silicon cantilevers (Nanosensors, Germany) with a pyramidal tip (radius $< 10$ nm) and a force constant of 2.8 N/m (typical values). To minimize the mechanical vibration of the system, the microscope was placed on an anti-vibration table and the samples (about $6 \times 10$ mm) were glued onto a brass disc. Preliminary tests were made in order to define the most appropriate operation mode and tip. The samples were imaged in contact mode in air. With this technique we obtained a good resolution and observed that the sample structures were firm enough. In fact, multiple scanning of the same region did not sweep the sample structures away. Each sample was observed in different regions always starting from a scan size of 20 μm, then zooming at 10, 5 and 2 μm. The quality of the images, in terms of resolution, signal-to-noise ratio and topography reliability, was achieved through the optimal choice of multiple setting parameters for each test. Scan rate was lower than 0.6 Hz (lines per second), the set point did not exceed 20 nN and the gain of the feedback loop was $\leq 0.5$.

## 3. Results and discussion

In this study, purified P-gp molecules were incorporated in model membranes deposited onto solid surfaces, by opening proteoliposomes on hydrophobic substrates. In order to verify the successful P-gp

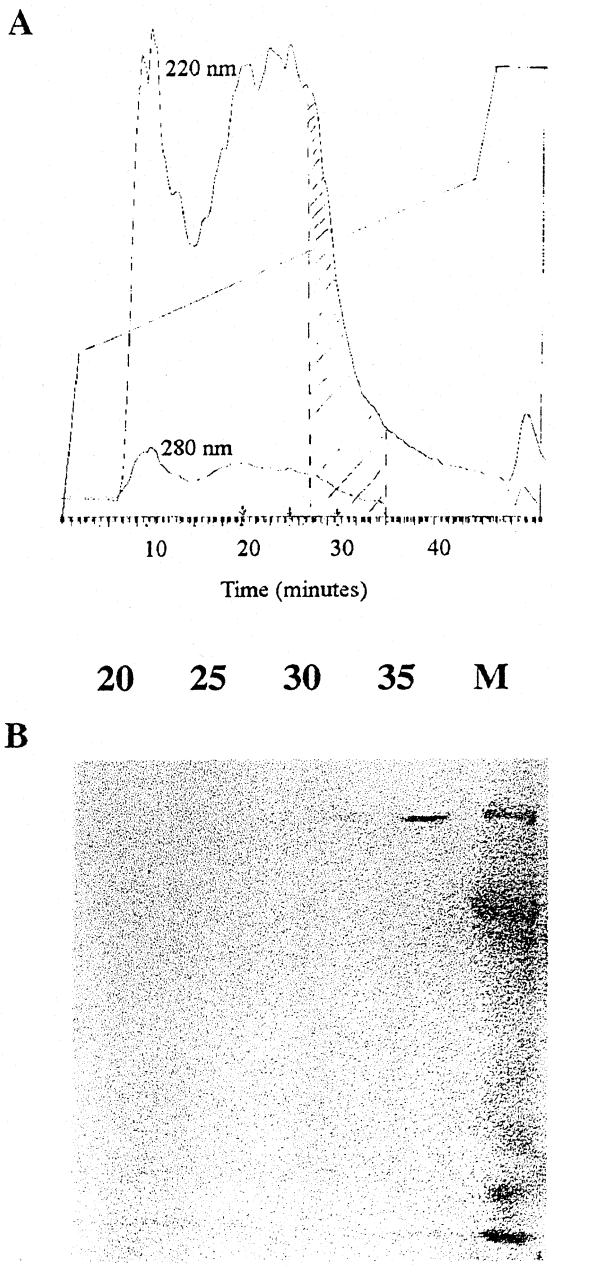


Fig. 1. (A) Chromatographic profile of P-gp purification on a Tosohaas TSK hydroxihapatite column. Fractions containing P-gp (striped area) have been collected and re-purified on the same column, after changing the detergent to 0.1% SDS. Elution profiles shown refer to the 280 and 220 nm absorption of the proteins, as indicated in the figure. (B) Immunoblot of the chromatographic fractions from TSK column. Numbers refer to the single fractions; M refers to molecular weight markers (from 18 to 200 kDa).

purification and reconstitution, both biochemical and structural experiments on liposomes were performed.

The protein was purified following the method of Dong et al. [13] after SDS extraction from cellular membranes (Fig. 1). The purification procedure allowed us to obtain a functional protein. The ATPase activity of P-gp, following its insertion into liposomes, was tested by fluorimetric assay. As reported in Fig. 2, the protein showed a basal ATPase activity

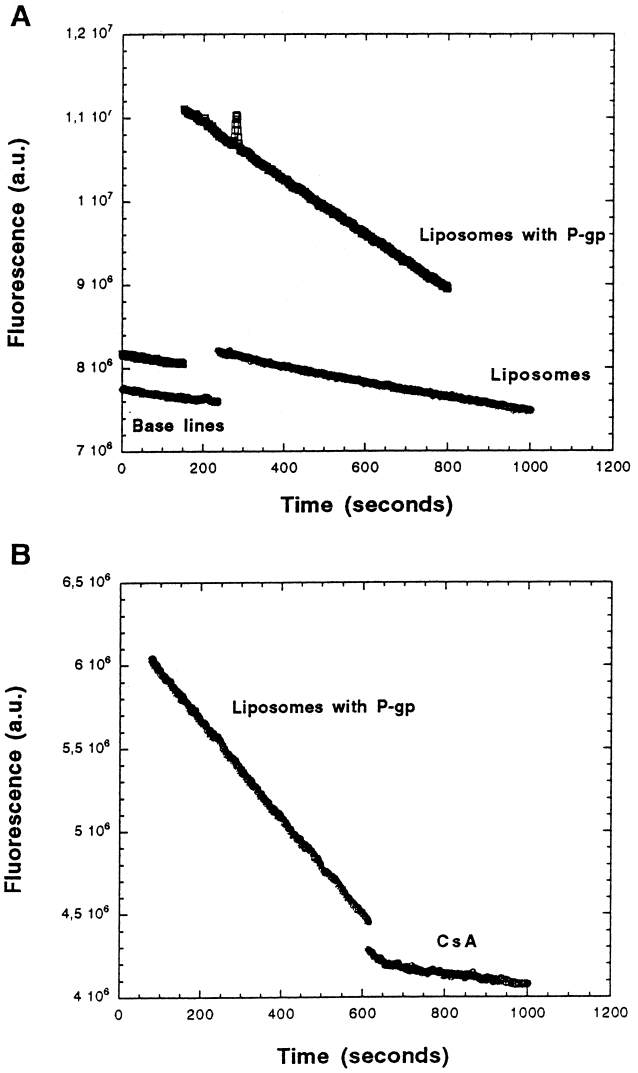


Fig. 2. Enzymatic ATPase activity of P-gp, revealed by fluorescence assay. (A) Liposomes with or without purified P-gp (see Section 2 for the preparation) were added to the reaction mixture. (B) Inhibitor effect of CsA on the P-gp ATPase function. Its effect is clearly visible in the slope of the corresponding curve; the reactions were followed at $\lambda_{em.} = 460$ nm ($\lambda_{ex.} = 340$ nm).

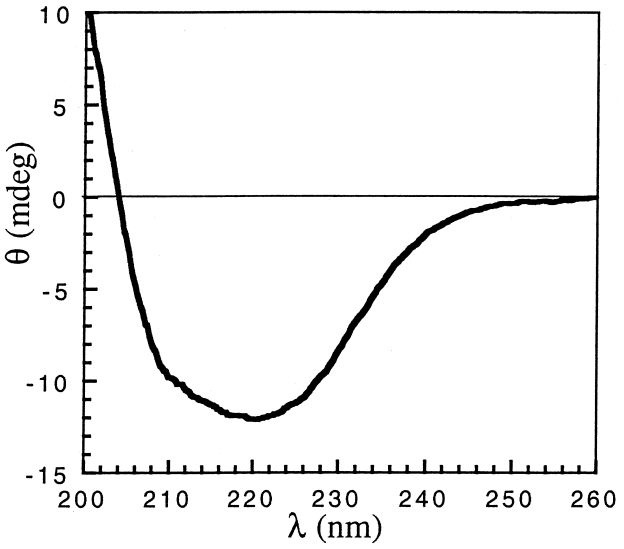


Fig. 3. CD spectra of liposomes with P-gp inserted in the lipid bilayer. The broad negative band centered around 220 nm indicates the presence of a well-conformed protein.

that was clearly inhibited by the presence of CsA [17].

To better explore the biological state of the protein, the structural features of P-gp reconstituted in liposomes was investigated by CD measurements. In Fig. 3 the CD spectrum of P-gp inserted in liposomes is reported. A broad negative band centered on 220 nm, typical of a well-conformed protein (non-random-coil), is evident. The shape of the spectrum indicates the presence of both α-helix and β-secondary structures. In fact, the CD spectrum of a pure α-helix

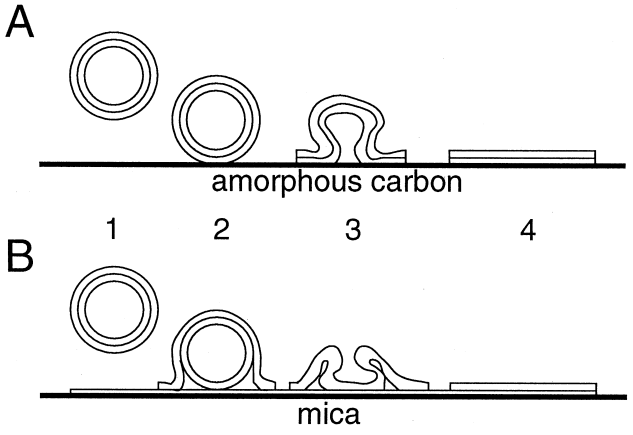


Fig. 4. Mechanisms of bilayer formation from opening and fusion of proteoliposomes on two different hydrophobic planar substrates: (A) amorphous carbon film; (B) DPPC L–B film deposited onto mica.

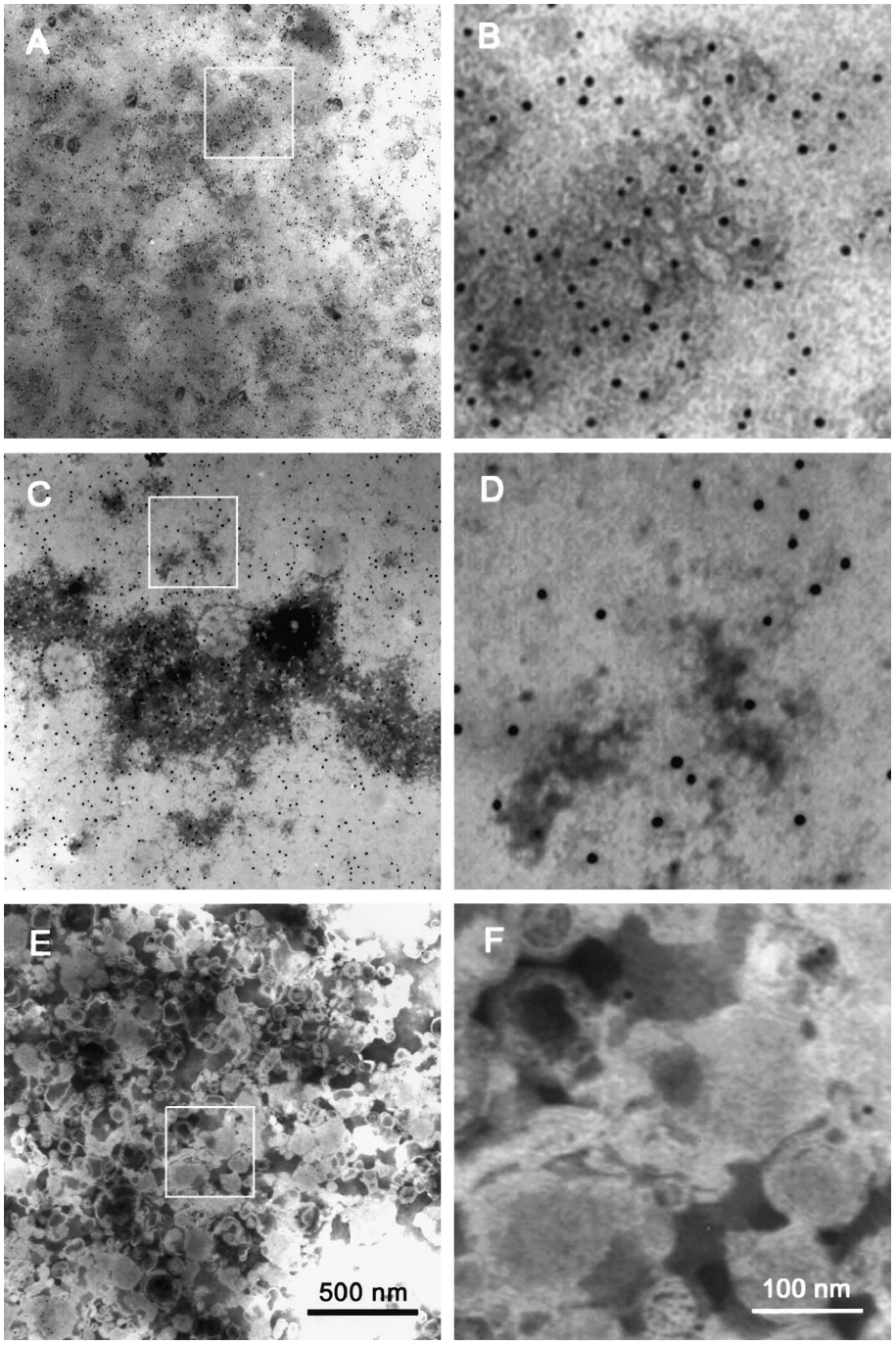


Fig. 5. TEM images (negative staining) of (A,B) liposomes containing P-gp deposited onto thin amorphous carbon film and labeled with MM4.17 antibody; (C,D) liposomes containing P-gp deposited onto thin amorphous carbon film and labeled with MRK-16 antibody; (E,F) plain liposomes (negative control) deposited onto thin amorphous carbon film and labeled with MM4.17 antibody. (B,D,F) are 5× magnification of the areas in the boxes (A,C,E), respectively.

peptide is characterized by two minima at 222 and 208 nm, while the spectrum of a pure β-structure peptide shows a minimum at 217 nm. In our case the spectrum can be considered as a combination of these two principal contributions. This finding suggests that P-gp inserted in liposomes maintains a non-random conformation, according to data based on attenuated total reflection Fourier trans-

form infrared measurements, where P-gp is described as constituted by α-helices and β-structures [3,18]. The CD spectrum relative to liposomes without P-gp did not show any particular CD signal in the same spectral region (data not shown).

The mechanisms of opening and fusion of liposomes onto several kinds of hydrophobic surfaces, were studied in detail and are summarized in Fig. 4 [9,19]. As it can be observed the deposition of liposomes onto hydrophobic surfaces, as amorphous carbon (paths A) or L–B film (path B), leads to the formation of a lipid double layer following two different mechanisms.

The first substrate we used was amorphous carbon, generally prepared as a very thin film (about 50 nm), suitable for TEM analysis. Fig. 5A,B shows TEM images of liposomes containing P-gp after the deposition onto amorphous carbon surface. Typical round-shaped liposomes were not visible, suggesting that they opened and fused each other, thus leading to the formation of a lipid double layer. In this sample, P-gp was labeled employing the highly reactive specific monoclonal antibody MM4.17, and revealed by goat anti-mouse immunoglobulins conjugated with 5 nm gold particles. A large number of gold particles (black dots in the figure), labeling the protein molecules incorporated in the lipid bilayer can be observed. This result clearly indicates that the reactivity of epitope, recognized by mAb MM4.17 and localized on the fourth loop of the P-gp molecule [11], was not compromised by the mechanism of liposome opening and fusion. This result was confirmed by the labeling of the same sample by mAb MRK-16 (Fig. 5C,D). This antibody recognizes epitopes located on the first and fourth loops of P-gp [12] and was revealed by goat anti-mouse immunoglobulins conjugated with 5 nm gold particles.

Even though in this figure it is possible to observe few whole liposomes (about 250 nm diameter), the background looks like the lipid bilayer in Fig. 5A,B. In fact, the only difference between the two samples is the antibody used for labeling. A negative control, performed on liposomes containing P-gp labeled with an irrelevant antibody, did not display gold particles (data not shown). Labeling performed by highly specific mAbs MM4.17 and MRK-16 clearly indicated that, at least in the surroundings of their reactive epitopes, P-gp maintained its structural features. It is noteworthy that both mAb MM4.17 and MRK-16 recognize P-gp in living cells.

The lower number of gold particles detected in the sample labeled with mAb MRK-16 as compared to the sample labeled with mAb MM4.17 suggests that the epitopes recognized by mAb MRK-16 were less accessible to the antibody. The lower reactivity of mAb MRK-16, when compared to that of mAb MM4.17, was previously reported in P-gp labeling experiments of living MDR tumor cells [8].

In agreement with other studies, it can be hypothesized that the presence of proteins in the liposome perturbs the original unstressed organization of the lipid bilayer [20,21] and facilitates liposome opening and fusion processes. This hypothesis appears to be supported by our TEM observation, under the same experimental conditions, of P-gp-free liposomes. Fig. 5E,F shows the presence of many unopened liposomes that can be easily recognized by their rounded shape. This finding is consistent with a structural stability of protein-free liposomes higher than that of P-gp-containing liposomes. As a further negative control of labeling experiments, liposomes without P-gp were labeled with mAbs MM4.17 and/or MRK16. As expected, the presence of very few gold particles (Fig. 5E,F) was revealed, indicating that neither a specific nor aspecific bond occurred in the sample. The isolated gold particles were very likely due to residual secondary antibodies remaining after the washing procedure.

The second hydrophobic substrate we used was the surface of DPPC L–B monolayer deposited onto mica, extremely flat and suitable for AFM analysis. AFM allows us to accurately characterize the lipid surface, not only in the plane ($x$–$y$ direction) but even in the $z$-direction. Fig. 6 shows AFM images of lipid bilayers formed after the fusion of liposomes with the L–B monolayer.

Fig. 6A,B refers to samples containing P-gp labeled with mAb MM4.17 and 10 nm gold particles. In this figure, the presence of white spots of about 30 nm diameter is clearly evident. These spots appear considerably larger than the nominal diameter of gold particles: this could be due both to the presence of the antibody molecules and the AFM tip convolution effect. Like in TEM images (Fig. 5A,B) many gold particles (white spots) can be observed, confirming the presence of P-gp in the lipid bilayer. Two

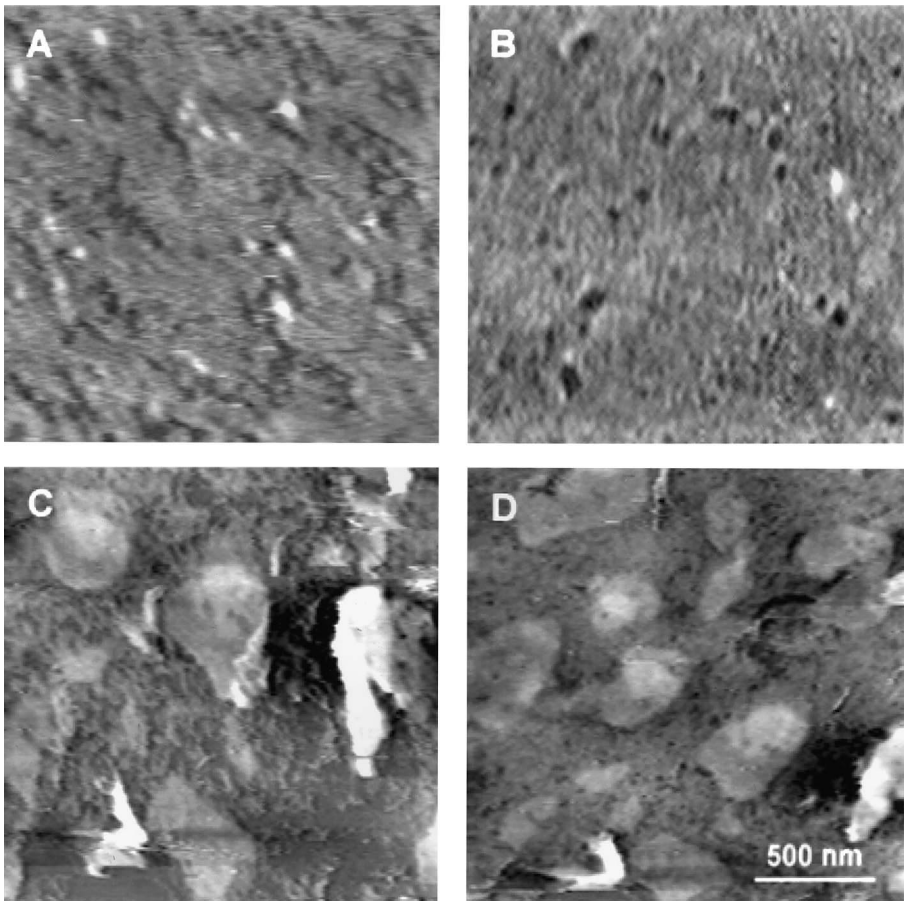


Fig. 6. AFM images relative to (A,B) liposomes containing P-gp deposited onto L–B DPPC monolayer and labeled with MM4.17 antibody; (C,D) plain liposomes (negative control) deposited onto L–B DPPC monolayer and labeled with MM4.17 antibody.

main levels in the background were observed: a deep level (dark), corresponding to the DPPC L–B monolayer and a superior level (at about 4 nm) (gray) that can be attributed to the lipid bilayer. The lipid bilayer (gray) seems to be widely formed, though not perfectly homogeneously because of the presence of small fissures and holes allowing us to see the deeper level (dark).

Fig. 6C,D, relative to the control without P-gp, clearly shows flattened rounded liposomes, not completely fused to the underlying lipid monolayer. In Fig. 6C we cannot observe a uniform background like in the other images of Fig. 6. In this sample, only isolated regions of lipid bilayers have formed. However, the two levels ascribable to the monolayer and the bilayer can still be distinguished. Fig. 6C,D is in agreement with Fig. 5E,F (TEM image), demonstrating that liposomes without P-gp are more stable during the fusion process and tend to maintain their original rounded shape. As expected, gold particles are not detectable in P-gp-free control samples, confirming TEM observations.

In general, while the P-gp-containing samples (Fig. 5A–D from TEM, and Fig. 6A,B from AFM) show the formation of a rather uniform lipid layer in which the protein is inserted, the P-gp-free samples exhibit many unopened liposomes (Fig. 5E,F from TEM and Fig. 6C,D from AFM).

On the basis of ellipsometric measurements, Puu and Gustafson [9] suggested that liposomes containing proteins form stable lipid structures on solid supports more readily than plain liposomes do, and that the protein should be considered an 'intrinsic' fusogenic agent that promotes the transfer of lipids to the solid surfaces. This is a reasonable hypothesis if we consider that a protein inserted in the lipid bilayer of the liposome perturbs lipid organization and packing, rendering the liposome less stable [20]. The TEM

and AFM observations reported in this paper confirm this hypothesis. The absence of P-gp in the control samples hinders the fusion process between liposomes deposited onto carbon film (TEM) or between liposomes and the L–B-covered mica substrate (AFM). Our observations clearly show that liposomes without P-gp keep their original close structure in both cases, demonstrating the essential role of the protein in the fusion process.

Moreover, the comparison between results obtained by TEM and AFM prompts some considerations. In TEM micrographs, the presence of a high number of gold particles reveals that the P-gp epitopes (Fig. 5A,B), localized in the loops protruding from the cellular membrane [5], maintain their native orientation during the fusion process between liposomes on carbon film, and remain available for the reaction with the specific monoclonal antibody. On the contrary, the lower number of gold particles observed in AFM images (Fig. 6A,B) could be associated with a different behavior of the protein to maintain its conformational state when carrier liposomes interact with a lipid L–B monolayer. This can be ascribed to a different opening mechanism of the liposomes onto carbon film and L–B film. It could be hypothesized that the double-layer formation occurring in the presence of a well-packed and -ordered lipid L–B film, is a more difficult mechanism in which the protein transfer from the liposome to the forming supported lipid bilayer is not favored.

## 4. Conclusions

Both enzymatic assays and CD measurements indicate the successful P-gp purification and reconstitution in liposomes. Moreover, TEM and AFM allowed us to obtain useful information about the mechanisms of opening and fusion of liposomes onto two different hydrophobic substrates: amorphous carbon and DPPC L–B monolayer. Our data are in good agreement with the mechanisms proposed in literature, suggesting that the presence of P-gp plays an important role in stability of the liposome. P-gp probably acts as a fusogenic agent, decreasing the lipid organization and packing.

The epitope reactivity, studied by immunogold labeling, is preserved after P-gp insertion in the supported lipid bilayer formed onto both substrates. Finally, information about P-gp aggregation state can be obtained by observing the distribution of the gold particles. In all the figures relative to samples containing P-gp inserted in lipid bilayers, performed by TEM (Fig. 5A–D) and AFM (Fig. 6A,B), gold particles appear as isolated units. The absence of couples or clusters of gold particles in the micrographs allows us to rule out the presence of P-gp dimers or oligomers.

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