ELSEVIER

Biochimica et Biophysica Acta 1464 (2000) 309–321

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

Oligonucleotide delivery by a cationic derivative of the polyene antibiotic amphotericin B
II: study of the interactions of the oligonucleotide/cationic vector complexes with lipid monolayers and lipid unilamellar vesicles

Isabelle Blanc, Michèle Saint-Pierre Chazalet *

Laboratoire de Physicochimie Biomoléculaire et Cellulaire, CNRS ESA 7033, Université Pierre et Marie Curie, case 138, 4 place Jussieu, F-75252 Paris Cedex 05, France

Received 22 September 1999; received in revised form 26 January 2000; accepted 31 January 2000

Abstract

We report a study of the behavior of oligodeoxyribonucleotide (ODN)/amphotericin B3-(*N′*-dimethylamino)propylamide (AMA) complexes, in the presence of lipid monolayers and large unilamellar vesicles. This study follows the recent discovery of the capacity of AMA, as a new cationic vector, to enhance ODN cellular uptake and efficacy. It aims at investigating the internalization mode of a nucleic acid by AMA. A first study at the air–water interface of AMA and AMA/ODN by surface pressure measurement shows that only free AMA would adsorb at the air–water interface. Second, in the presence of zwitterionic phospholipid- and sterol-containing mixture, ODN–AMA interactions in solution would be higher than lipid–AMA interactions at the interface. In monolayer or with large unilamellar vesicles, AMA monomers adsorb mainly at the phospholipid interface. These results favor a crossing mechanism through AMA channel formation, despite the size of ODN.

Keywords: Cationic amphotericin; Oligodeoxyribonucleotide; Phospholipid; Monolayer; Vesicle

1. Introduction

The formation of a stable phosphodiester oligodeoxyribonucleotide (ODN)/amphotericin B3-(*N′*-dimethylamino)propylamide (AMA) complex and the enhancement of the intracellular uptake of an anti-MDR1 phosphorothioate ODN by AMA into G185-MDR-3T3 cells have been previously reported in a recent article [1] and have therefore justified our new approach of nucleic acids vectorization by a dicationic AmB-derivative. In a first publication [2], we have reported the preliminary study of the interactions between AMA and a chosen ODN in solution, revealing the existence of complexes of different structures, according to the physical state of AMA in solution and to the relative concentrations of the two components. We have also shown that the structure of the resulting complexes was governed by a cooperative binding between both molecules.

Abbreviations: ODN, oligodeoxyribonucleotide; AMA, amphotericin B3-(*N′*-dimethylamino)propylamide; MDR, multidrug resistance; LUV, large unilamellar vesicle; SUV, small unilamellar vesicle; POPC, palmitoyloleoylphosphatidylcholine; EPC, egg phosphatidylcholine

* Corresponding author. Fax: +33-1-44-27-75-60;
E-mail: stpierre@lpbc.jussieu.fr

0005-2736/00/$ – see front matter
PII: S0005-2736(00)00161-9

As already demonstrated in previous publications [3,4], the potential interest of AMA, besides its capacity to interact with nucleic acids, is that it forms transient transmembrane pores at low concentration, as its parent compound amphotericin B (AmB). Numerous authors related the membrane-permeabilization properties of AmB to its physical state into the membrane. Thus, Fujii suggests that the appearance of the channel activity coincides with the transition from the monomer to the aggregated form of the polyene [5]. Another interesting parameter is the well-known selectivity of the polyene for fungi cells (membranes with ergosterol) as regard to the mammalian cells (membranes with cholesterol), demonstrated by numerous authors for the AmB and also for its derivatives [3,6,7].

The following question could then be asked: how does the AMA permit the enhancement of the ODN intracellular uptake; in other words, by which mechanism does the AMA allow the crossing of cell membrane by the nucleic acid, and does the AMA permit a cellular targeting? Recent results obtained on the vectorization of an antisense ODN by AMA tend to show that the cell membrane crossing would not occur by an endocytotic mechanism (data not shown). Taking into account the conditions of formation of the ODN/AMA complexes in solution and their various structures depending on their relative concentrations, developed in our first article [2], we were interested in the possible affinity of these complexes to lipid membrane and also in the influence of the sterol-composition (cholesterol or ergosterol) of simplified membrane models.

Therefore, our aim was to underline the existence of interactions between the complexes and the lipids (phospholipid and sterol) and eventually identify their mechanisms in order to give some hypothesis of membrane-crossing way. If many membrane models are used to study the interaction of drug with cells, the results have to be examined with caution because these models are generally very simple. Important parameters like the nature of the molecules employed, the presence of sterol and proteins, the curvature of the surface and also the experimental protocol used to put the drug in contact with the membrane model have to be carefully considered. The most currently used models are lipid vesicles (large unilamellar vesicles, LUV, or small unilamellar vesicles, SUV), planar lipid bilayers, black films and lipid monolayers. Study of AmB solutions by surface pressure measurements has already been performed [8] and has demonstrated that the amphiphilic molecule was able to adopt two different positions at the interface, the first with its polyene-axis parallel to the interface at low surface pressures and the second with its axis perpendicular to interface at high pressures. Monolayer studies have also allowed confirmation of the higher affinity of AmB for ergosterol as compared to cholesterol, a characteristic already largely documented by vesicles studies for AmB solutions [7,9,10], and also for AMA solutions [3]. However, it must be remarked that the different techniques did not agree on the stoichiometry of the AmB:sterol complexes, the vesicles studies gave a 1:1 stoichiometry [6,9,11] whereas the monolayer ones indicated a 2:1 stoichiometry [8].

Finally, this report presents a study of the interactions of AMA and various ODN/AMA mixed solutions with planar lipid monolayers on the one hand and unilamellar vesicles on the other hand, both chosen as simplified membrane models. In both cases, sterol was incorporated into the lipids in order to get closer to the real cell membranes. As a first step of this analysis, we have studied the behavior of AMA and ODN/AMA solutions at the air–water interface.

2. Materials and methods

2.1. Reagents

2.1.1. Amphotericin

Amphotericin B3-(*N*′-dimethylamino)propylamide (AMA) [2], an amphiphilic molecule derived from amphotericin B, was kindly provided by Edward Borowski (Technical University, Gdansk, Poland). Solutions of AMA were daily prepared in Millipore water or dimethylsulfoxide/ethanol (DMSO/EtOH) (1/9) at an initial concentration of 10^{-3} M. Dimethylsulfoxide and ethanol were purchased from Sigma (St. Louis, MO, USA) and were 99% pure and used without further purification.

A stock solution was immediately used for measurements. Like the parent molecule AmB, AMA is a polyene macrolide composed of two parts, one hy-

drophilic and the other hydrophobic, which confers its tensioactive properties. This amphiphilic molecule is soluble in water but can form monolayers at the air–water interface.

2.1.2. Oligonucleotide

Oligonucleotide (ODN) was a 27-mer phosphodiester, d(TGTGTTTTTGTTTTGTTGGTTTTGTTT). It has been synthesized by Genosys (Cambridge, UK). It was dissolved in Millipore water at an initial concentration of 10^{-4} M. The ODN molecule is totally soluble in aqueous solutions and then not tensioactive.

2.1.3. ODN/AMA mixed solutions

The ODN/AMA mixed solutions were prepared as described previously [2], by mixing the two aqueous stock-solutions (10^{-3} M for AMA, 10^{-4} M for ODN) in the desired charge ratio (ρ = ratio between the negative charges of the ODN and the positive charges of the AMA). The mixed solutions were used immediately after preparation. The AMA final concentration in the Langmuir trough was always 2.4×10^{-7} M; the chosen ODN final concentrations were 2×10^{-9}, 7×10^{-9} and 7×10^{-8} M corresponding to $\rho = 0.1$, 0.4 and 4, respectively.

2.1.4. Lipids

Palmitoyloleoylphosphatidylcholine (POPC, a zwitterionic phospholipid), egg phosphatidylcholine (EPC), cholesterol and ergosterol were purchased from Sigma (St. Louis, MO, USA) and were 99% pure and used without further purification. For the surface pressure measurements, POPC, cholesterol and ergosterol were dissolved in an ethanol/chloroform 1/1 (v/v) mixture at a concentration of 10^{-3} M. For the studies with large unilamellar vesicles, EPC, cholesterol and ergosterol were dissolved in chloroform at concentrations of 130 μM for EPC and 20 μM for the sterols. The ratio lipid/amphotericin was 0.25 for these experiments.

2.2. Monolayer study

Monolayers were prepared using a Teflon trough provided by Riegler (Riegler and Kirstein, Wiesbaden, Germany). The trough ($6.2\times26.3\times0.5$ cm) was filled with an NaCl solution (150 mM, pH = 5.5) or pure water (pH = 5.5). The surface pressure was measured using the Wilhelmy method, by means of a very thin plate of filter paper. An electronic device enabled us to keep the monolayer pressure constant by monitoring the displacement of the barriers. This system was used during penetration experiments. All experiments were performed at 21 ± 1°C. The speed of compression and decompression of the barriers (3×10^{-2} cm s^{-1}) was kept constant during the experiments.

Two methods were used to study the behavior of AMA and AMA/ODN solutions at the air–water interface. In the first case, we spread 20 μl AMA stock solution dissolved in DMSO/EtOH (1/9) at the interface. The compression isotherms were recorded immediately or 70 min after spreading. In the second case, 20 or 40 μl AMA stock solution dissolved in pure water was injected into the subphase of the trough and we studied the adsorption of AMA at the air–water interface at constant surface area. We have recorded the variation of surface pressure during 10 or 70 min, and then compression isotherms of the resulting molecules at the interface were obtained. In the same way we also studied the adsorption of ODN/AMA solutions at the air–water interface.

For the study of the behavior of AMA and ODN/AMA solutions in the presence of a lipid monolayer, the following principle was used. A phospholipid monolayer (POPC/sterol) was spread at the interface and compressed to 5 or 25 mN/m. This pressure was kept constant and aqueous solutions of AMA or ODN/AMA were injected under the monolayer at a final concentration of 2.4×10^{-7} M in AMA, with charge ratios of 0.1, 0.4 and 4 for the ODN/AMA solutions. If an interaction occurs between the molecules of the subphase and the monolayer, the barriers moved back to keep the pressure to 5 or 25 mN/m, respectively, and we recorded the variation of the surface area versus time during the 10 min of the experiments. Then the molecules remaining at the interface (phospholipids and molecules of the subphase eventually adsorbed and/or penetrated) were compressed; if an adsorption and/or a penetration has occurred, the recorded P/A isotherm was shifted to higher areas as compared to the pure phospholipid monolayer.

These experiments were done with POPC/choles-

terol (80–20 mol%) and POPC/ergosterol (80–20 mol%) monolayers.

2.3. LUV preparation and circular dichroism measurements

For the study of the interactions between ODN/AMA mixed solutions and LUV, the LUV were prepared as previously described [12]. Briefly, the lipid film containing EPC and sterol (80–20 mol%) was dispersed by sonication into 20 mM K_2SO_4/KH_2PO_4, pH = 7.2. The dispersion was subjected to rotary evaporation during 50 min. The ODN/AMA mixed solutions were added to aliquots of LUV dispersed into 150 mM NaCl. Immediately the circular dichroism spectra were recorded. The measurements were done on a Jobin-Yvon Mark V dichrograph. Spectral wavelengths are given ±0.5 nm. $\Delta\varepsilon$ is the differential molar dichroic absorption coefficient (10^3 l cm^{-1} mol^{-1}).

3. Results

3.1. Behavior of AMA and ODN/AMA solutions at the air–water interface

3.1.1. Behavior of AMA at the air–water interface

We used two techniques to study the AMA behavior at the air–water interface: first, we spread AMA from an organic solvent at the interface, second we injected an aqueous solution of AMA in the trough and then recorded its adsorption at the interface.

3.1.1.1. AMA spread at the interface. AMA dissolved in DMSO/EtOH (1/9) was spread at the air–water interface, the trough containing a 150 mM NaCl solution. Fig. 1 shows an isotherm obtained with compression performed just after the spreading (curve a), immediately followed by two additional compressions (curves b and c). We observed a slow increase of the molecular compressibility at the interface until a pressure of 10 mN/m, followed by a sudden rapid increase of this compressibility. After three compressions, the mean molecular areas remained constant at high pressures. The shape of the compression isotherm performed 70 min after spreading (Fig. 1, curve d) differed from the others but reached areas a little smaller at high pressures. The mean molecular area calculated with the hypothesis that all the spread molecules stay at the interface is small (10 $Å^2$). This is probably due to a loss of molecules during the first compression, while the different shapes of these following curves would be indicative of a reorganization of the molecules finally staying at the interface.

3.1.1.2. AMA adsorbed at the interface. In this part, the molecules were injected into the trough whose barriers were totally extended. After 10 or 70 min, the molecules adsorbed at the interface were compressed. The recorded resulting 'adsorption' isotherms were then compared (Fig. 2). The AMA adsorption at the air–water interface appeared to depend on three parameters: the AMA final concentration in the trough, the saline concentration of the subphase and the adsorption time.

Influence of the AMA final concentration in the trough. The adsorption isotherms recorded 70 min after injection of 20 or 40 μl of the AMA stock solution into the trough (AMA final concentration in the trough: 2.4×10^{-7} and 4.8×10^{-7} M, respectively) show that the increase of the AMA final concentration did not result in a two-fold increase of the

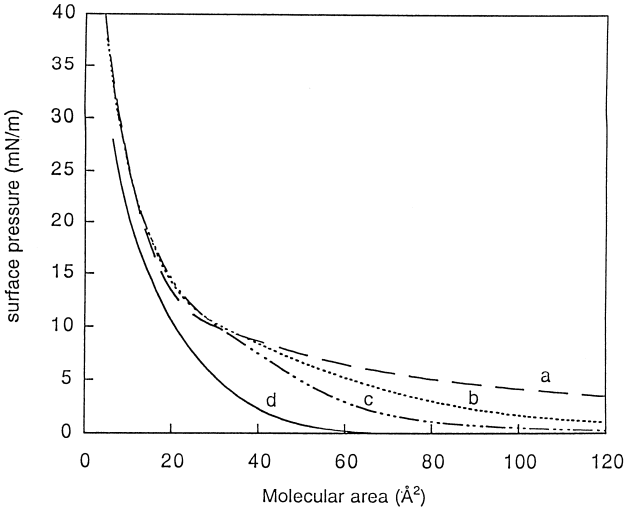

Fig. 1. Isotherms of AMA solution (in DMSO/EtOH 1/9) spread at the air–water interface. First compression recorded immediately after spreading (a), second compression (b), third compression (c) and isotherm recorded 70 min after spreading (d). (In the subphase: [NaCl] = 150 mM, pH = 5.5, $T = 21 \pm 1$°C.)

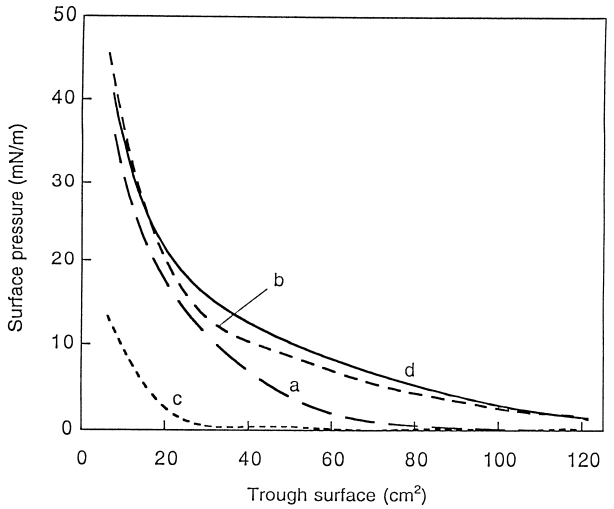

Fig. 2. Isotherms of AMA solution (in water) recorded after adsorption at the air–water interface. Adsorption time: 70 min; in the subphase: [AMA] = 2.4×10^{-7} M, [NaCl] = 150 mM (a), adsorption time: 70 min; in the subphase: [AMA] = 4.8×10^{-7} M, [NaCl] = 150 mM (b), adsorption time: 10 min; in the subphase: [AMA] = 2.4×10^{-7} M, Millipore water (c), and adsorption time: 10 min; in the subphase: [AMA] = 2.4×10^{-7} M, [NaCl] = 150 mM (d) (pH = 5.5, $T = 21\pm1$°C).

surface area occupied by the polyene molecule, as one could expect (Fig. 2, respectively, curves a and b). Then, saturation probably occurred at the interface.

Influence of the saline concentration of the subphase. If we compare the adsorption isotherms recorded 10 min after injection of AMA stock solution into the trough (at a final concentration of 2.4×10^{-7} M) containing pure water (curve c) or 150 mM NaCl (curve d), it appeared that the adsorption was greatly enhanced by the presence of salt in the subphase. This difference could be explained by an increase of the solubility of the AMA molecules in pure water and consequently also a lower tensioactive character, as compared with NaCl solution.

Influence of the adsorption-time. The compression isotherms obtained 10 or 70 min after injecting aqueous AMA stock solution into the trough (final concentration: 2.4×10^{-7} M) show that the surface occupied by the adsorbed molecules at the interface decreased with increasing adsorption-time (Fig. 2, curves a and d). Moreover, kinetics of adsorption recorded during 10 min at a reduced surface area (48 cm^2, Fig. 3, curve a) confirmed this adsorption profile, with a first rapid step of adsorption of some AMA molecules at the interface, followed immediately by their progressive slow desorption.

From these different data and the comparison between the two methods used, spreading and adsorption, we can propose an interpretation of the AMA behavior at the air–water interface.

As for the parental molecule AmB [8], and due to its hydrophobic and hydrophilic properties, the AMA molecule could adopt two different positions at the interface, depending on the orientation of its long polyene-axis: parallel to the interface or perpendicular to it. A molecular modeling study enabled us to estimate the molecular area of AMA in these two positions. First, the cross section according to the long polyene-axis of the molecule was considered; in this geometry the molecular area was at the most 200 Å^2. Second, the cross section of the polar head according to the carboxyl group and the amino-sugar moiety was taken into account; in this case the molecular area was 90 Å^2.

Knowing the number of spread (at the air–water interface) or injected (in the subphase) AMA molecules, $n = C\times V\times N$, where C is the concentration of the AMA stock solution (10^{-3} M), N the Avogadro number and V the volume of AMA solution spread or injected (20 or 40 μl), the mean molecular area

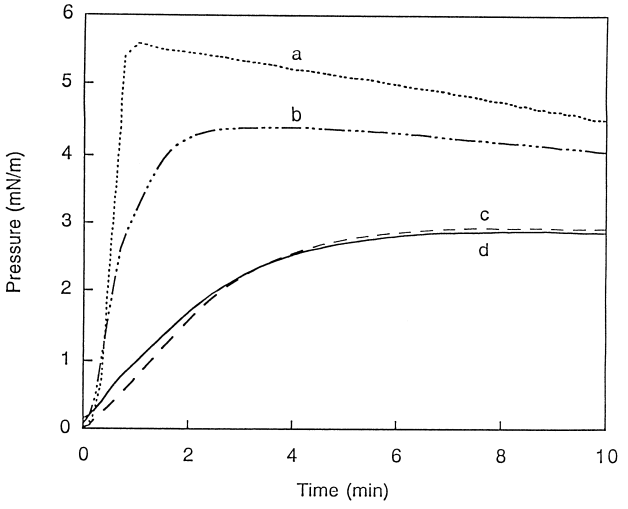

Fig. 3. Adsorption kinetics ($P = f(t)$) of AMA solution and ODN/AMA mixed solutions of different charge ratio ODN/AMA (ρ) at the air–water interface. The surface area of the trough was kept constant at 48 cm^2. AMA (a), $\rho = 0.1$ (b); $\rho = 0.4$ (c) and $\rho = 0.4$ (d). (In the subphase, [AMA] = 2.4×10^{-7} M, [NaCl] = 150 mM, pH = 5.5, $T = 21\pm1$°C.)

occupied by each molecule at the interface for each surface pressure should be calculated by $A = S/n$ (A in $Å^2$), where S is the surface of the trough at the corresponding pressure.

For a 150 mM NaCl solution in the trough and whatever the method used (spreading or adsorption), the mean molecular area at high pressure (25 mN/m) calculated with all the molecules standing at the interface was between 9 and 17 $Å^2$. These areas appear very weak, compared to the molecular size given by molecular modeling. Then, even if the molecules are oriented with their polyene-axis perpendicular to the interface, which is the optimal molecular position for a maximal adsorption at the interface (according to the steric hindrance), only 10 to 15% of the injected or spread molecules would stand at the interface at 25 mN/m after the first compression. The small shoulder observed on the spreading isotherms recorded after two or three compressions would result from a reorientation of the AMA molecules at the interface, which have their polyene-axis parallel to the interface at low pressures and perpendicular at high pressures. The low percentage of molecules finally remaining at the interface is in good agreement with the high solubility of AMA in the aqueous phase.

The study of the saline concentration influence on AMA adsorption can give some information about the species remaining at the interface by this method. Indeed, we have seen that very little adsorption occurred at the air–water interface when the trough contained pure water, whereas the isotherm recorded with a 150 mM NaCl solution gave surface pressures twice to three times higher for the same molecular areas (Fig. 2). If we refer to the results of the study of AMA in solution [2], we must note that for a same final low concentration of 10^{-6} M, the AMA molecules are more aggregated in saline than in water. Due to the mechanism of self-association of the polyene, resulting from the association of polyenic chains of several AMA molecules while the hydrophilic heads remain in contact with the medium [13], these self-associated AMA species appear more soluble than the monomeric ones. Then, the adsorption recorded at the interface should be attributed to the monomers of AMA, whereas the self-associated species would stay in the subphase. The lower monomer adsorption noted in the case of pure water subphase, as compared with a saline subphase, could be the consequence of their greater solubility in water than in NaCl solution.

A question that can be asked is why after several successive compressions of spread AMA, the isotherms are modified. As far as the AMA molecules are known to easily self-associate, like the AmB [6], we can suppose that the first compression involves the dissolution of the majority of the spread AMA molecules, whereas it involves also the self-association of the minority staying at the interface. Once the molecules are self-associated at the interface, no more dissolution could occur and the shoulder observed on isotherms following the first one (Fig. 1, curves b and c) would traduce the reorientation of packets of self-associated molecules at the interface. However, when the isotherm is recorded 70 min after spreading (Fig. 1, curve d), the areas noted at the beginning of the compression isotherm are very low, indicating that the AMA molecules dissolved before the compression. It suggests that some time is needed to reach equilibrium at the interface. When it is reached, the molecules remaining at the interface would be in a self-associated state with their polyene-axis standing perpendicular to the interface and no more reorientation would happen. It is important to note that these results are valid for both 150 mM NaCl solution and pure water in the subphase.

The difference between the areas finally noted at 25 mN/m on the isotherms recorded 10 or 70 min after spreading (Fig. 1) are not so significant for our study and, to overcome the problems of instability of the AMA stock-solutions, all our measurements will be done after 10 min adsorption.

Under these conditions we will study the behavior of mixed ODN/AMA solutions at the air–water interface.

3.1.2. *Behavior of mixed ODN/AMA solutions at the air–water interface*

We first verified that ODN molecules did not adsorb at the interface under the conditions of our experiments (adsorption during 10 min and ODN concentration in the trough inferior to 7×10^{-8} M).

3.1.2.1. First case: 150 mM NaCl solution in the subphase. As in the previous study in solution [2], mixed ODN/AMA solutions were prepared by add-

ing the ODN stock-solution (10^{-4} M in water) to the AMA stock-solution (10^{-3} M in water) in the desired charge ratio ($\rho = 0.1$, 0.4 and 4). These solutions were then injected in the trough at the AMA final concentration of 2.4×10^{-7} M, whatever ρ was.

The surface area was kept constant (48 cm^2) and the pressure variations were recorded during 10 min (Fig. 3). As already noted, AMA alone adsorbs rapidly at the air–water interface and progressively desorbs (Fig. 3, curve a). It appeared that the adsorption at the interface slowed down in the presence of ODN, likewise the desorption tended to disappear with increasing ODN amounts, corresponding to an increase of the charge ratio (Fig. 3, curves b, c and d).

The compression isotherms of the resulting adsorbed species, presented in Fig. 4, showed a decrease in surface pressures when increasing the charge ratio (or the ODN amount), while the general shape of the curves remained unchanged.

3.1.2.2. Second case: pure water in the subphase. Due to the good solubility of the AMA monomers in pure water, we have observed that a low adsorption was recorded after injecting AMA solution in the trough. In the case of ODN/AMA solutions, the adsorption decreased at the interface with increasing ODN concentration in the trough and the resulting surface pressures appeared too low to be evaluated (data not shown).

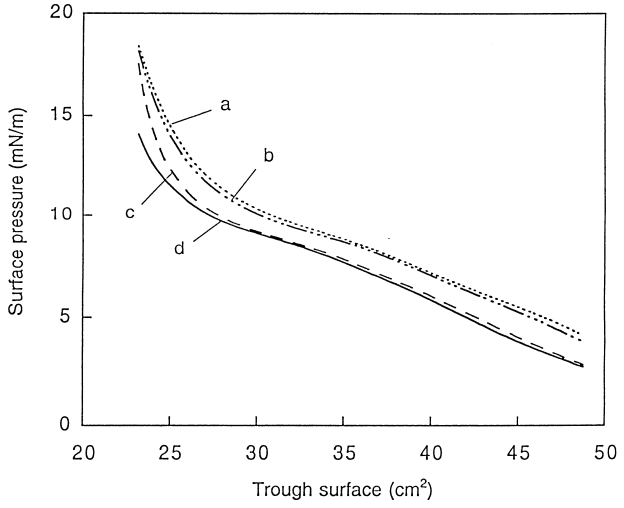

Fig. 4. Isotherms of AMA solution and ODN/AMA mixed solutions of different charge ratio ODN/AMA (ρ), recorded after 10 min adsorption at the air–water interface. AMA (a), $\rho = 0.1$ (b), $\rho = 0.4$ (c) and $\rho = 4$ (d). (In the subphase, [AMA] = 2.4×10^{-7} M, [NaCl] = 150 mM, pH = 5.5, $T = 21 \pm 1$°C.)

From these results it clearly appeared that ODN affects the AMA adsorption at the air–water interface. The questions were, how does ODN modify the adsorption and which species are present at the interface?

The tendency of the resulting adsorbed AMA molecules to desorb would result from equilibrium between the hydrophobic interactions into the AMA aggregates and the hydrophobic interactions between AMA monomers at the interface.

Since the mean molecular area decreased when ODN concentration increased (Fig. 4), it could be concluded that ODN limits the adsorption of molecules at the interface. Moreover, in so far as the shape of compression isotherms remained unchanged in the presence of ODN, and because of the total solubility of ODN in aqueous media, one could assume that only the AMA molecules were present at the interface. The results established in a previous report [2] showed that ODN induces the aggregation of AMA molecules, leading to 'complex' entities in aqueous solution; these observations confirm the fact that only the monomers of AMA could adsorb at the interface (whatever the nature of the subphase was), while the AMA self-associated species would form in the subphase a soluble complex with the ODN molecules. Results of absorption spectroscopy measurements and electron microscopy measurements [2] allow the following hypothesis to be proposed. For complexes of low ODN concentration, we could assume that some AmA monomers, engaged in the AMA aggregates in interaction with the ODN but located at their periphery and therefore far away from the ODN strand, could be attracted by the air–water interface by hydrophobic interactions. As they are not directly engaged in electrostatic interactions with the ODN, these external monomers could escape from the AMA aggregates and consequently adsorb at the air–water interface. When increasing ODN concentration in the subphase, each AMA monomer could be engaged in the complex due to electrostatic interactions with the ODN on the one hand, hydrophobic interactions with the surrounding AMA monomers on the other hand. The consequence would be a progressive stronger trapping of the AMA monomers into the complexes with in-

creasing ODN amounts, hence the progressive adsorption and also the disappearance of desorption at the air–water interface.

However, another hypothesis could be formulated, if we consider that not only the free AMA adsorbs at the interface, but also the AMA bound to ODN. In this case, we could suggest that the interaction between AMA and ODN produces a rather hydrophobic complex by electrostatic interactions, which could then adsorb at the interface. As the compression isotherms still have the same shape, the AMA molecules engaged in the complex would stand at the interface while the bound ODN would stay just underneath it. In this way, only the AMA bound molecules would get involved in the compression movement, the ODN molecules forming a screen just underneath the interface and preventing AMA desorption. By following this hypothesis, the dramatic decrease of the adsorption rates with increasing ODN concentrations would be the consequence of the adsorption of complex species, which is slower than free-AMA adsorption, probably because of their larger size. A saturation would occur at the interface beyond a charge ratio of 0.4, then limiting the adsorption, probably due to the complex size and a steric hindrance at the interface. Yet it can be remarked that this saturation does not induce desorption from the interface at this charge ratio, whereas some desorption is noted for the lower ratio of 0.1. In this case the desorption can be attributed to free AMA molecules present at the interface together with some complex species formed at this charge ratio.

Taking into account the results of the study in solution (complexes more hydrophilic than AMA monomers, see [2]), the first hypothesis seems, however, to be the most valid to explain the behavior and the mode of adsorption of AMA and ODN/AMA mixed solutions.

3.2. Behavior of AMA and ODN/AMA solutions in presence of planar lipid monolayers and large unilamellar vesicles (LUV)

The previous study enabled us to precisely describe the adsorption mode of AMA and ODN/AMA solutions at the air–water interface. It was a preliminary step before studying the interactions of these solutions with lipid monolayers and bilayers.

As a first membrane model, we chose planar monolayers composed of POPC and sterol, as far as the interactions of AMA with the external layer of the cell membrane appear determining. The interactions of AMA and ODN/AMA solutions with POPC/sterol monolayers were investigated by studying the surface variations of the lipid monolayer after injecting AMA and ODN/AMA solutions into the subphase at constant pressure (see Section 2). For the reasons explained before, all adsorption experiments were done during 10 min. On account of the well-known selectivity of AMA in favor of fungal cell membranes (ergosterol-containing), as compared to mammalian cell membranes (cholesterol-containing), we have realized two series of experiments, depending on the nature of the sterol spread at the interface: in the first one, the monolayer was composed of POPC and cholesterol (80–20 mol%), in the second of POPC and ergosterol (80–20 mol%).

Large unilamellar vesicles (LUV) composed of EPC/cholesterol or EPC/ergosterol were used as a bilayer model. The interactions of AMA and ODN/AMA solutions with LUV were studied by recording the circular dichroism spectra of AMA and AMA/ODN solutions before and after addition of LUV.

3.2.1. Interaction of AMA and ODN/AMA solution with a POPC/sterol (80–20 mol%) monolayer

3.2.1.1. POPC/cholesterol monolayer. After the spreading of POPC/cholesterol at the air–water interface, the pressure was kept constant at 5 mN/m and an aqueous solution of AMA was injected in the subphase to a final concentration of 2.4×10^{-7} M. We observed immediately a moving back of the trough barriers to maintain a constant pressure and an increase of the surface occupied by the molecules (Fig. 5, curve a); these observations indicated that AMA molecules of the subphase adsorbed and/or penetrated the POPC/cholesterol layer. This adsorption was immediately followed by stabilization and low desorption. After 10 min, the resulting molecules at the interface (phospholipids and molecules of AMA remaining adsorbed and/or penetrated) were compressed and an isotherm shifted to higher areas as compared to the POPC/cholesterol isotherm (Fig. 6, curves a and b). The area difference (ΔA) of the isotherms recorded before and after adsorption of

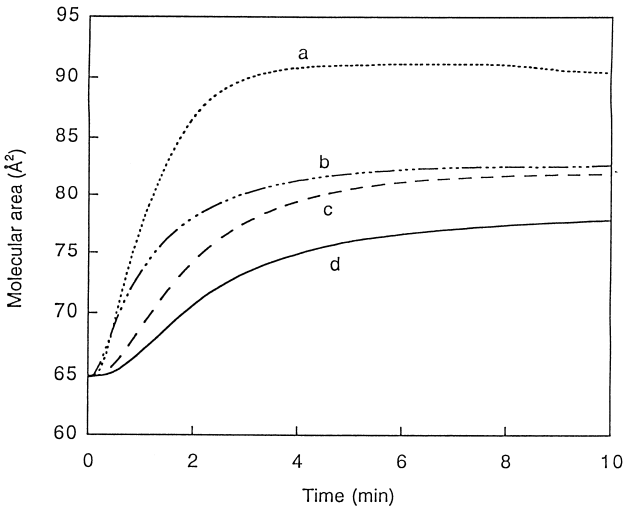

Fig. 5. Adsorption kinetics ($A = f(t)$) of AMA and ODN/AMA mixed solutions of different charge ratio ODN/AMA (ρ), in a POPC/cholesterol (80–20 mol%) monolayer. The pressure of the phospholipid monolayer during the adsorption was kept constant at 5 mN/m. AMA (a), $\rho = 0.1$ (b), $\rho = 0.4$ (c) and $\rho = 4$ (d). (In the subphase, [AMA] = 2.4×10^{-7} M, [NaCl] = 150 mM, pH = 5.5, $T = 21 \pm 1$°C.)

AMA in the phospholipid monolayer varied from 26 to 3 Å^2 (which corresponds to a trough surface area ΔS of 30 to 3.5 cm^2) for surface pressure increasing from 5 to 40 mN/m; that indicates that the adsorbed AMA molecules progressively desorb and/or stand up with their axis perpendicular to the interface when compressed. It appears that the AMA adsorption in the lipid monolayer is lower than that recorded at the air–water interface (ΔS varying between 80 and 8 cm^2). Two explanations should be consequently proposed: first, the presence of lipids at the interface would reduce the place available for exogenous molecules, hence a low adsorption of the AMA from the subphase. Second, even if AMA molecules adsorb into the phospholipids, they do not have the possibility to self-associate as easily as they did at the air–water interface and consequently the major part would desorb.

As for the interactions of ODN/AMA mixed solutions with the lipid monolayer, Fig. 5 shows that contrary to the case of the AMA solution, no desorption occurred, whatever the ODN concentration (curves b, c and d). Moreover, the adsorption decreased when ODN concentration increased: ΔA varied from 18 to 14 Å^2 for, respectively, $\rho = 0.1$ to 4 and was in all cases much lower than noted in the case of AMA solution (26 Å^2) (Table 1). From these observations it appeared that AMA and ODN/AMA solutions would adopt a behavior in the presence of a lipid monolayer very similar to their behavior at the air–water interface. Then, as at the air–water interface, the ODN would limit the adsorption of AMA molecules in a POPC/cholesterol monolayer maintained at the constant surface pressure of 5 mN/m. The same explanation could then be proposed in the two cases: the progressive trapping of AMA monomers by the ODN molecule into 'complex' entities whose structures were investigated in the previous report [2].

In order to be closer to the physiologic conditions, a similar adsorption experiment was realized with a POPC/cholesterol monolayer maintained at a surface pressure of 25 mN/m, instead of 5 mN/m. A very low adsorption was noted for AMA alone whereas no adsorption can be measured for ODN/AMA mixed solutions. Little adsorption could occur, without any visible area variation. It should be assumed that at this surface pressure, there is no sufficient place in the phospholipids to permit the adsorption of AMA molecules.

As far as the polyene is known to have a good affinity for ergosterol as compared with cholesterol, it could therefore be asked if the composition of the

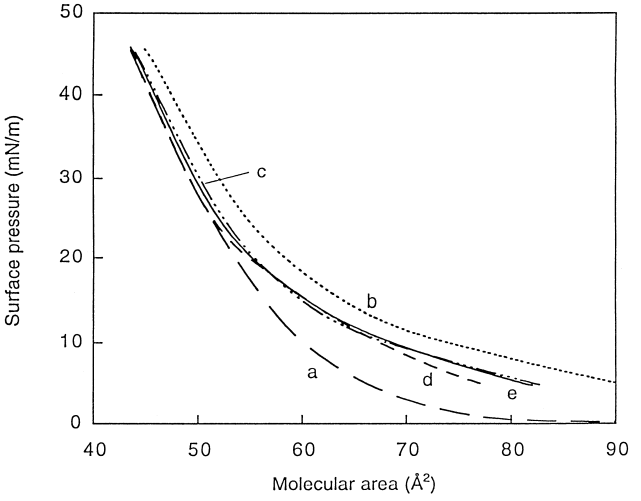

Fig. 6. Isotherms POPC/cholesterol (80–20 mol%) before and 10 min after adsorption of AMA and ODN/AMA mixed solutions of different charge ratio ODN/AMA (ρ) into the lipid monolayer maintained at $P = 5$ mN/m. Pure lipids (a), AMA (b), $\rho = 0.1$ (c), $\rho = 0.4$ (d) and $\rho = 4$ (e). (In the subphase, [AMA] = 2.4×10^{-7} M, [NaCl] = 150 mM, pH = 5.5, $T = 21 \pm 1$°C.)

sterol in the lipid monolayer could influence the AMA adsorption. In order to answer this question, we have realized the same experiments with a POPC/ergosterol (80–20 mol%) spread monolayer.

3.2.1.2. POPC/ergosterol monolayer. The increasing area (ΔA) resulting from the adsorption of molecules into the POPC/ergosterol (80–20 mol%) monolayer are summarized in Table 1. First, we observed that the qualitative adsorption behavior remains the same, whatever the sterol associated to the lipids: the adsorption decreased with increasing ODN concentration. As in the precedent case (POPC/cholesterol monolayer), molecule adsorption decreased with increasing ODN concentration. Moreover, the adsorption and/or penetration of AMA and ODN/AMA solutions were in all cases higher in the POPC/ergosterol than in the POPC/cholesterol monolayer.

The same experiments were performed when the lipid monolayer was maintained at a constant surface pressure of 25 mN/m. Whereas no adsorption was visible under these conditions in a POPC/cholesterol, positive results were recorded in the case of a POPC/ergosterol monolayer. Indeed, as shown in Table 1, the injection of AMA and ODN/AMA solutions in the subphase led to an increase of the surface occupied by the molecules at the interface of 1.5, 1, 1 and 1 Å^2, respectively.

In both cases, these differences should be attributed to the higher affinity of the polyene for the ergosterol, as compared to cholesterol.

Table 1
Variation of the mean molecular area of a POPC/sterol monolayer (ΔA) obtained 10 min after adsorption of AMA and ODN/AMA mixed solutions of different charge ratio ODN/AMA (ρ)

Molecules in subphase	Constant pressure of the POPC/cholesterol monolayer (mN/m)		Constant pressure of the POPC/ergosterol monolayer (mN/m)	
	5	25	5	25
	ΔA (Å^2)		ΔA (Å^2)	
AMA	26	0.5	38	1.5
ODN/AMA, $\rho = 0.1$	18	0	32	1.5
ODN/AMA, $\rho = 0.4$	17	0	23	1
ODN/AMA, $\rho = 4$	14	0	21	0.5

The pressure of the lipid monolayer was kept constant during the adsorption at 5 and 25 mN/m. (In the subphase, [AMA] = 2.4×10^{-7} M, [NaCl] = 150 mM, pH = 5.5, $T = 21 \pm 1$°C.)

3.2.2. Interaction of AMA and ODN/AMA solutions with EPC/cholesterol and EPC/ergosterol LUV

The interactions between AMA, ODN/AMA mixtures and LUV have been studied by circular dichroism spectroscopy (CD). The experimental protocol was the following: we have successively recorded the circular dichroism spectra of AMA solution and ODN/AMA mixtures in the absence and presence of LUV (at low ratio lipid/amphotericin). In order to avoid the problems of turbidity in the solutions, due to the presence of the vesicles, we always worked at high final concentrations in AMA (10^{-4} M). As in a previous publication [14], we assigned the CD spectra recorded in the presence of the LUV to the AMA molecules remaining free in solution (not bound to the LUV) (Fig. 7) and compared the intensities of the dichroic doublet recorded with and without LUV. Thus, our results are presented as the percentage of loss of AMA molecules after interaction with the LUV (Table 2). Three principal points appeared from the results of the interactions of AMA and ODN/AMA solution with EPC/cholesterol and EPC/ergosterol LUV. First, AMA interacts with LUV the intensity of the CD doublet decreased by 33% from the spectrum of AMA alone in solution

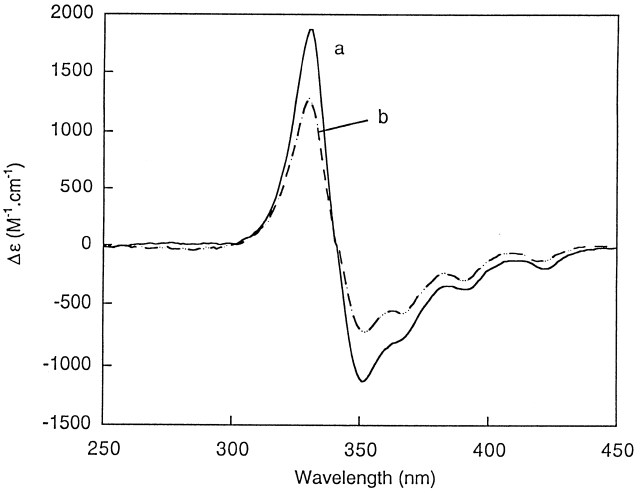

Fig. 7. CD spectra of AMA alone in solution or in the presence of POPC/cholesterol(80–20 mol%)-containing LUV. AMA alone in solution (a) and AMA in presence of LUV (b). ([AMA] = 10^{-4} M, [NaCl] = 150 mM, pH = 5.5.)

Table 2
Percentage of decrease of the CD doublet intensity of AMA and ODN/AMA ($\rho = 4$) solutions after interaction with EPC/sterol (80–20 mol%): $\%\Delta\varepsilon$

	$\Delta\varepsilon$ with POPC/chol LUVs (%)	$\Delta\varepsilon$ with POPC/ergo LUVs (%)
AMA	33	70
ODN/AMA, $\rho = 4$	9	13

([AMA] = 10^{-4} M, [NaCl] = 150 mM, pH = 5.5, $T = 21 \pm 1$°C)

to the spectrum of AMA in the presence of cholesterol-containing LUV (respectively, 70% for ergosterol-containing LUV). These results confirm the capacity of the polyene to interact with LUV, already demonstrated in a previous review [3]. Second, it appeared that this interaction was higher for ergosterol-containing LUV as regard to cholesterol-containing ones. This effect could be attributed again to the well-known higher affinity of the polyene for ergosterol, as opposed to cholesterol. Third, the interaction between AMA and LUV decreased drastically in the case of ODN/AMA mixed solutions. Indeed, the percentage of loss of AMA molecules due to their interaction with the LUV decreased from 33% for an AMA solution to 9% for the ODN/AMA solution ($\rho = 0.4$) in the case of cholesterol-containing LUV and from 70 to 13%, respectively, for ergosterol-containing ones.

The most important result is that, as in the case of planar lipid monolayers, the ODN limits the interaction between the cationic vector and the LUV by interacting with AMA. Second, it appears that the percentage of the AMA molecules engaged in complexes with the ODN which interact at the same time with the LUV is higher for ergosterol-containing LUV than for cholesterol-containing ones. Therefore the ergosterol seems to enhance the interaction of the polyene with the LUV, to the detriment of ODN.

4. Discussion

In order to investigate the mode of cellular membrane crossing by a vectorized nucleic acid (in our case the system ODN/AMA), we studied the interactions between AMA and ODN/AMA solutions with simplified membrane models: planar lipid monolayers and large unilamellar vesicles. A preliminary study of the behavior of the two molecules at the air–water interface has already allowed us to demonstrate that the AMA molecules, although water-soluble, were able to adsorb at the interface, while the presence of ODN limited this adsorption. The effect of the nucleic acid was attributed to the formation of 'complex' ODN/AMA species in solution, well documented in a previous publication [2]. Moreover, it has also been demonstrated that the adsorbed species were tensioactive monomers of AMA, while the rather hydrophilic 'complexes' would stay in the subphase; this point underlined the role of the hydrophobic/hydrophilic balance on the adsorption at the interface.

In the presence of a lipid monolayer at the interface, the same decrease in adsorption with increasing ODN concentration in the subphase was noted. Therefore, our experiments suggest that the adsorption behavior of AMA and ODN/AMA mixed solutions was qualitatively not modified by the presence of a phospholipid monolayer. However, it clearly appeared that the phospholipids quantitatively affect the adsorption, as for all solutions the adsorption was lower than at the air–water interface. Two explanations can be proposed for this: (1) the presence of lipids at the interface drastically reduces the free available place for exogenous molecules (AMA from the subphase) and therefore limits their adsorption and (2) due to the presence of lipids, the adsorbed AMA molecules cannot self-associate as they did at the air–water interface and, therefore, partially desorb. Indeed it has already been mentioned that isolated AMA monomers at the interface tended to desorb, whereas self-associated species did not desorb.

As the adsorption difference obtained with both types of interfaces (air–water or lipid monolayer) was much lower for the ODN/AMA solutions than for AMA solution, it should be assumed that a competition between hydrophobic and electrostatic strengths occurred. Indeed, we have already shown that the 'complex' formation was first governed by electrostatic attractions, then followed by hydrophobic interactions between AMA self-associated species; besides, as far as the phospholipid used is zwitterionic, the interactions between the polyene and lipid monolayer would be principally of hydrophobic nature. Our results allowed, therefore, the proposal

of the following hypothesis. At low ODN concentration, the hydrophobic interactions between AMA molecules and lipids at the interface would induce the escape from the complexes of the AMA monomers weakly bound to them in the subphase (monomers bound to AMA aggregates by hydrophobic interactions but not electrostatically attached to the ODN); the resulting AMA monomers free in solution would immediately adsorb in the lipid monolayer or at the air–water interface. When increasing ODN concentration, the electrostatic interactions between AMA molecules and ODN would predominate and the result would be the decrease of adsorption both in the lipid monolayer and at the air–water interface.

It should be noted that the high AMA adsorption observed in the POPC/ergosterol (80–20 mol%) monolayer confirms the well-known high affinity of the polyene for the ergosterol. However, the hydrophobic interactions between the lipid monolayer and the AMA molecules in the subphase remained always lower than the electrostatic interactions with the ODN.

The study of the interactions of AMA and ODN/AMA solutions with LUV by circular dichroism led to results in good agreement with those obtained on planar monolayers. Indeed, it also appeared that the ODN limited the interactions of the polyene with the LUV, which confirms the competition between hydrophobic and electrostatic strengths. Moreover, the role of the sterol also clearly appeared since the interactions of AMA and the complex with LUV were higher in the case of ergosterol-containing LUV, as regard to cholesterol-containing ones.

5. Conclusion

In conclusion, in this first study of the interactions between membrane models and a new system of nucleic acid vectorization, it clearly appears that the nature of interaction between AMA and nucleic acid on the one hand and nucleic acid/AMA complexes and lipids on the other hand, affects the possible crossing of the nucleic acid through the cell membrane. Knowing the capacity of AMA to transiently permeabilize the cell membrane, we first made the hypothesis that the nucleic acids could enter the cell through the pores thus formed. However this mechanism appeared rather unlikely in the case of our study, our ODN being too large as compared to the reported size of the pores formed by the AmB in cell membranes [15–17]. However, this hypothesis could perhaps be available for smaller nucleic acids. In addition, let us note that the idea of nucleic acid vectorization through pores of small radius is still topical [18,19].

The hypothesis of the internalization of the whole ODN/AMA complex in the cell, thanks to membrane organization changes, has also to be considered. Indeed, the absence of affinity of these hydrophilic complexes for our planar lipid monolayers could be due to the utilization of zwitterionic lipids in our study. An interaction could perhaps be demonstrated between the same complexes (positively-charged) and negatively-charged lipids, like those found in the cell membrane.

Finally, the internalization of a nucleic acid vectorized by the AMA exists, since it has been demonstrated in a previous publication [1]. However, the passive transport at first considered as the mechanism of entry is perhaps not the only possible way, as our results seem to show.

Acknowledgements

We thank Professor E. Borowski for generously providing us with AMA. I.B. was supported by a fellowship from the French Ministère de la Recherche et Technologie and the Fondation pour la Recherche Médicale. This work was financially supported by the GDR Vectola (CNRS, France). We also thank Mrs. Maria-Helena Bueno Da Costa from the Butantan Institut (Sao Paolo, Brazil) for her kind assistance in lipid vesicle experiments.

References

[1] C. Garcia, O. Seksek, J. Grzybowska, E. Borowski, J. Bolard, Pharmacol. Ther. (2000), in press.
[2] I. Blanc, M.-H. Buena Da Costa, J. Bolard, M. Saint-Pierre Chazalet, Biochim. Biophys. Acta 1464 (2000) 299–308.
[3] M. Chéron, B. Cybulska, J. Mazerski, J. Grzybowska, A. Czerwinski, E. Borowski, Biochem. Pharmacol. 37 (1988) 827–836.

[4] A. Jarzebski, L. Falkowski, E. Borowski, J. Antibiot. 35 (1982) 220–229.
[5] G. Fujii, J.-E. Chang, T. Coley, B. Steere, Biochemistry 36 (1997) 4959–4968.
[6] S. Hartsel, J. Bolard, Trends Pharmacol. Sci. 17 (1996) 445–449.
[7] B. Cybulska, M. Hervé, E. Borowski, C.M. Gary-Bobo, Mol. Pharmacol. 29 (1985) 293–298.
[8] M. Saint Pierre-Chazalet, C. Thomas, M. Dupeyrat, C.M. Gary-Bobo, Biochim. Biophys. Acta 944 (1988) 477–486.
[9] P. Legrand, M. Cheron, L. Leroy, J. Bolard, J. Drug Target 4 (1997) 311–319.
[10] A. Vertut-Croquin, J. Bolard, M. Chabbert, C. Gary-Bobo, Biochemistry 22 (1983) 2939–2944.
[11] J. Bolard, P. Legrand, F. Heitz, B. Cybulska, J. Am. Chem. Soc. 30 (1991) 5707–5715.
[12] F.Jr. Szoka, D. Papahadjopoulos, Proc. Natl. Acad. Sci. USA 75 (1978) 4194–4198.
[13] P. Millié, J. Langlet, J. Bergès, J. Caillet, J.-P. Demaret, J. Phys. Chem. 103 (1999) 10883–10891.
[14] J. Milhaud, M.A. Hartmann, J. Bolard, Biochimie 71 (1989) 49–56.
[15] T.E. Andreoli, Ann. N. Y. Acad. Sci. 235 (1974) 448–468.
[16] R.W. Holz, Ann. N. Y. Acad. Sci. 235 (1974) 469–479.
[17] A. Marty, A. Finkelstein, J. Gen. Physiol. 65 (1975) 515–526.
[18] B. Hanss, E. Lealpinto, L.A. Bruggeman, T.D. Copeland, P.E. Klotman, Proc. Natl. Acad. Sci. USA 95 (1998) 1921–1926.
[19] P.-G. De Gennes, Proc. Natl. Acad. Sci. USA 96 (1999) 7262–7264.