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Changes of conductance and compressibility of bilayer lipid membranes induced by oligonucleotide-cationic polyene antibiotic complexes

Tibor Hianik [a,*], Lukas Ostatnik [a], Vladimira Polohova [b], Jacques Bolard [c]

[a] *Department of Nuclear Physics and Biophysics, Faculty of Mathematics, Physics and Computer Science, Comenius University, Mlynská dolina F1, 842 48 Bratislava, Slovak Republic*
[b] *Institute of Animal Biochemistry and Genetics, Slovak Academy of Sciences, Moyzesova 61, 900 28 Ivanka pri Dunaji, Slovak Republic*
[c] *BioMoCeTi, CNRS UMR 7033, Université Pierre et Marie Curie, Genopole Campus 1, 5 rue Henri Desbruères, 91030 Evry Cedex, France*

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Abstract

The positively charged polyene molecule amphotericin B 3-dimethylaminopropylamide (AMA) is an efficient agent for the delivery of antisense oligodeoxyribonucleotides (ODN) into target cells. In the present study, bilayer lipid membrane (BLM) conductance, elasticity modulus perpendicular to the membrane plane, surface potential and electrical capacitance were measured by conductance and electrostriction methods in the presence of AMA, pure or complexed to 20-mer single stranded ODN at different ratios. Pure AMA did not induce changes in conductance of cholesterol-containing BLM, but did induce an increase in elasticity modulus and surface potential. ODN/AMA complexes changed BLM properties depending on the charge ratio. The most pronounced effect on membrane conductance was observed for positively charged ODN/AMA complexes (charge ratio $\rho-/+=0.1$), while for negatively charged complexes these changes were less marked/apparent, correlating to substantially lower binding constants. The effect of ODN/AMA complexes on elasticity modulus and charge potential was biphasic. After an increase in both values, a decrease was observed for higher incubation times and ODN/AMA concentrations. These results are interpreted as indicating that the membrane property changes result from the large AMA aggregates induced by the presence of the negatively charged ODN, which condensate on these aggregates. It is suggested that the decrease of elasticity modulus and surface potential in the presence of increasing incubation time and AMA concentration result from desorption of the complexes in the complex-free compartment of the BLM cell, or appearance of a non-linear conductance of the lipid bilayer. The first alternative would explain the AMA-induced transmembrane transfer of ODN.
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Keywords: Oligonucleotide; Amphotericin; Bilayer lipid membranes; Conductance; Electrostriction; Compressibility; Surface potential

1. Introduction

Antisense strategy is the blockade of protein synthesis by binding of an appropriate single-strand oligodeoxyribonucleotide (ODN) to mRNA (see [1] and references therein). It implies, as an essential step, internalisation of the ODN into target cells. The molecular weight of this nucleic acid and its negative charge prevent its passive diffusion across the cell membrane. To enable it, numerous delivery systems are under current study. Nearly all of them rely on an endocytic uptake mechanism. The limitation of the endosomal process is that once in the endosomes, the ODNs are degraded by enzymes and still have to reach the cytoplasm. Solutions have been proposed to solve this problem [2], in particular the use of pore-forming molecules which would allow transmembrane passage of ODNs. Indeed, AMA (Fig. 1), the positively charged 3-dimethylaminopropyl amide derivative of the polyene antibiotic amphotericin B (AmB) has been shown to enhance the intracellular uptake of a 5′ fluoresceinated anti-MDR1 20-mer into NIH-MDR-G185 cells [3]. Furthermore, the unlabelled phosphorothioate form of the ODN, complexed to AMA, inhibited P-glycoprotein expression of these cells. On the other hand, AMA did bind to a plasmid DNA containing the green fluorescent protein (GFP) reporter gene, coding for the GFP, and permitted expression of this plasmid in 3T3 cells, as seen by fluorescence microscopy [4].

Some information has been gained on the mechanism of ODN transmembrane permeabilisation by AMA, but supplementary

* Corresponding author. Tel.: +421 2 60295683; fax: +421 2 65426774.
E-mail address: hianik@fmph.uniba.sk (T. Hianik).

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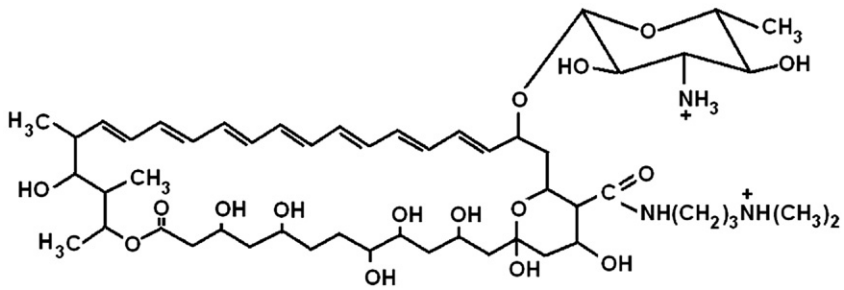

Fig. 1. Chemical formula of AMA.

studies are still necessary to get a better understanding thereof. The formation of ODN/AMA complexes was studied by optical spectroscopy and electron microscopy, and stressed the importance of the electrostatic interactions between the positively charged AMA and the negatively charged ODN [5]. The characteristics of the ODN/AMA complexes adsorption onto monolayer lipid membranes were determined using surface/pressure measurements [6].

It is, however, not yet clear whether the ODN/AMA complexes could translocate through the lipid bilayer (although two-sided addition of cationic derivatives of AmB has been shown to induce permeability in planar bilayer lipid membranes (BLM) [7]) or how this translocation depends on the charge ratio ODN/AMA. If such a translocation exists, its mechanism is not clear, considering in particular the large size of the ODN/AMA complexes. To obtain further insight in this problem, the conductance and electrostriction properties of BLM in the presence of AMA or ODN/AMA complexes at different charge ratios were also examined. Conductance could provide information about the translocation of the complexes, while electrostriction measurements provide information on membrane compressibility and surface potential.

2. Experimental

2.1. Reagents

AMA was kindly provided by Edward Borowski (Technical University, Gdansk, Poland). A 10^{-3} M AMA stock solution was prepared daily by dissolution in Millipore water (Elix 5, Millipore, El Paso, USA). The ODN was a 20-mer phosphodiester of sequence: 5′-TGT ATA GCT AAA GTA GGC GC-3′, purified by HPLC and supplied by Generi Biotech (Czech Republic). ODN was dissolved in Millipore water at a final concentration of 10^{-4} M. ODN/AMA mixed solutions were prepared by mixing the stock solutions in the desired charge ratio (ρ−/+ = molar ratio of negative charges of nucleic acid over the positive charges of AMA). The charge ratio was calculated as the 20-mer ODN molecule having 20 negative charges and AMA two positive charges. Then the ODN/AMA mixture was diluted in order to obtain the desired final concentration of ODN/AMA. The interaction of both AMA and ODN/AMA complexes of a charge ratio ρ−/+ = 0.1, 0.44 and 4.0 with BLM were studied. The buffer used was 150 mM NaCl + 10 mM Tris–HCl and 1 mM EDTA (pH 7.6) (where the pH was adjusted by addition of small amounts of 0.1 M NaOH).

2.2. Preparation of BLM

Bilayer lipid membranes (BLM) were prepared from a mixture of diphytanoylphosphatidylcholine (DPhyPC) (Avanti Polar Lipids, USA) with cholesterol (Fluka, Switzerland) (4:1 w/w) dissolved in *n*-decane (Fluka) at a concentration of 20 mg/ml. BLMs were formed according to Mueller et al. [8] on a circular aperture of a 0.5 mm diameter in a Teflon partition separating the Teflon cup into two identical compartments with a volume of 3 ml each. Briefly, after accurate cleaning of the cell with detergent, washing with distilled water and drying at 80 °C, the aperture was pre-treated with a small amount of lipid solution (for pre-treatment *n*-heptane was used instead of *n*-decane because of its superior evaporation characteristics). After drying of this solution and formation of a thin lipid layer at the aperture, the cell was filled with a buffer. Then a Pasteur pipette was immersed into the lipid solution and placed at the bottom part of the aperture, where the bubble developed. The bubble first covered the aperture and then rose. The small amount of lipid film that remained attached to the aperture was sufficient for formation of the BLM. The film was first rather thick (several hundreds of nm) as indicated by the appearance of coloured Newton circles. However, as soon as the self assembly thinning process started (almost immediately after appearance of the Newton circles), the black-bilayer regions appeared, rapidly expanding over the aperture. The formation of the membrane was observed optically by means of a stereo microscope and electrically by measuring the electrical capacitance.

2.3. BLM conductance

BLM conductance was studied based on the measurement of current flow across BLM at a constant dc potential of 50 mV, by means of a Keithley 6512 (USA) programmable electrometer connected on line with an IBM PC 486 DX via a KPC-488.2AT Hi Speed IEEE-Interface board. The potential was applied from a dc source to the BLM by means of Ag/AgCl electrodes placed into a plastic tube filled with agar gel. The agar gel was prepared using 3 M KCl and served as a salt bridge. The experiments were performed at 20 ± 1 °C.

2.4. BLM electrostriction

The electrostriction method enabled simultaneous measurement of the elasticity modulus in the direction perpendicular to the membrane plane, $E_{\perp}$, the electrical capacitance, C, and the

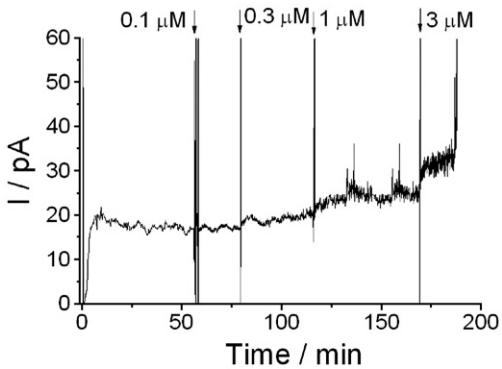

Fig. 2. Time dependence of the current flowing across BLM after one-sided addition of ODN/AMA complexes (charge ratio $\rho-/+=0.1$) at various concentrations. Addition of ODN/AMA complexes is shown by arrows.

surface potential difference, $\Delta\Phi$ of the bilayer. Briefly, an alternating voltage $U=U_0\sin 2\pi ft$ of an amplitude $U_0=50$ mV and frequency $f=1$ kHz was applied to the BLM through the reference Ag/AgCl electrode. Due to the non-linear dependence of capacitance on the voltage ($C=C_0(1+\alpha U^2)$, where C_0 is the capacitance at $U=0$ and α is the electrostriction coefficient), second and third current harmonics with frequencies $2f$, $3f$ and amplitudes I_2, I_3, respectively are generated in addition to the basic first current harmonic of an amplitude I_1 and frequency f. The measurements of these amplitudes allowed us to determine the elasticity modulus:

$$E_{\perp} = 3I_1C_sU_0^2/(4dI_3) \quad (1)$$

where C_s is the specific capacitance of the membrane ($C_s=C/A$, A is the membrane area) and d is the thickness of the hydrophobic part of the membrane. The specific capacitance was determined from electrical capacitance and area of BLM. The area was estimated by means of stereomicroscope. For BLM used the $C_s=(4.0\pm1.0)\times10^{-3}$ F/m^2. The thickness of the BLM was determined by equation $d=\varepsilon\varepsilon_0/C_s$, where $\varepsilon\cong2.1$ is relative dielectric permittivity of the hydrophobic part of the membrane and $\varepsilon_0=8.85\times10^{-12}$ F/m is the dielectric permittivity of the vacuum. Thus, $d=(4.6\pm1.1)$ nm. The surface potential difference can be determined from the following equation:

$$\Delta\Phi = -U_1 + U_0I_2/(4I_3), \quad (2)$$

where U_1 is the external dc voltage. The value $\Delta\Phi$ represents the sum of Gouy–Chapman potential, $\Delta\Phi_{GC}$, and dipole potential $\Delta\Phi_d$. ($\Delta\Phi=\Delta\Phi_{GC}+\Delta\Phi_d$). The electrical capacitance is proportional to the amplitude of the first current harmonics, I_1, as follows:

$$C = I_1/(2\pi fU_0) \quad (3)$$

(see [9] for details of the method).

3. Results and discussion

3.1. Changes in BLM conductance induced by the addition of ODN, AMA, or ODN/AMA complexes

An example of the kinetic changes of BLM conductance induced by the addition of the ODN/AMA complexes (charge ratio $\rho-/+=0.1$) at different concentrations is shown on Fig. 2. The current flowing through an unmodified BLM is relatively small, around 16 pA, which correspond to a membrane conductance of 1.6×10^{-3} S m^{-2}. This agrees with the previously reported values of BLM conductance [10] associated with the insulating properties of the lipid bilayer. Addition of relatively small concentrations of the ODN/AMA complexes (0.1 μM) did not cause significant changes of membrane conductance. However, conductance started to increase following addition of higher concentration of the complexes. We should note that addition of pure AMA or ODN, up to 3 μM did not result in changes of BLM conductance. The fact that AMA does not induce conductance of BLM when added only to one side of the membrane is not surprising. It is known that cationic derivatives of amphotericin B form channels only when they are added from both sides of the BLM [7].

The AMA concentration dependence of the changes of the ratio of conductance induced by AMA or AMA/ODN complexes at various charge ratios is shown in Fig. 3. We can see that practically no changes of conductance took place as a result of increasing concentrations of pure AMA. However, with ODN/AMA complex at the charge ratio $\rho-/+=0.1$, i.e. with a positively charged complex, a considerable increase in conductance took place. For a less positively charged complex of ODN/AMA ($\rho-/+=0.44$) the changes of conductance were less well-defined. However, even for a negatively charged complex ($\rho-/+=4$) a BLM conductance increase was still observed. It should be noted that the internalisation of a fluorescein-labelled oligonucleotide into NIH-MDR-G185 cells follows a similar tendency [3]: the intracellular fluorescence intensity obtained by confocal laser microspectrofluorimetry was 1.7 times higher for a ratio $\rho-/+=2$ than for a ratio $\rho-/+=4$. An additional increase in conductance was observed following addition of Mg^{2+} ions at a final concentration of 5 mM to both sides of the BLM. The increase in conductance in the presence of Mg^{2+} ions is likely due to partial shielding of the negative charges of the complexes. The addition of Mg^{2+} ions alone, at a final

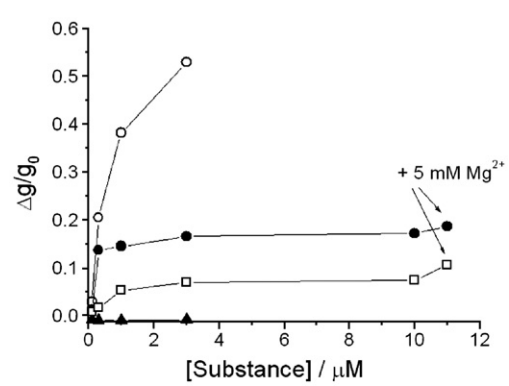

Fig. 3. The changes of the ratio of conductance of BLM, $\Delta g/g_0$, as a function of the concentration of AMA or ODN/AMA complexes at different charge ratios $\rho-/+$($\Delta g=g-g_0$, where g_0 is the BLM conductance prior to, and g the conductance after addition of AMA or ODN/AMA complexes) and following addition of Mg^{2+} ions at both sides of the membrane at a final concentration of 5 mM. Substance: ▲ — AMA, ○ — ODN/AMA ($\rho-/+=0.1$), ● — ODN/AMA ($\rho-/+=0.44$), □ — ODN/AMA ($\rho-/+=4$).

concentration of 5 mM, did not result in significant changes of BLM conductance.

The analysis of the binding of AMA to a membrane can be performed by Hill plot. The binding constant, that characterize the interaction of ligand, L with a binding site at the membrane, can be expressed as [11,12]:

$$K_c^m = \frac{[\mathrm{LB}]}{[B]c^m} \tag{4}$$

where, K_c^m is binding constant, [LB] is the concentration of the ligand-binding site complexes, $[B]$ is the concentration of binding sites, c is the concentration of free, unbounded ligands (i.e. ODN/AMA complexes) and m is the Hill coefficient. The Hill coefficient is an indicator of cooperativity of the binding. For $m=1$, the binding is non-cooperative and all binding sites are equivalent. When $m \neq 1$ the binding is more complex and the binding is either positively cooperative: $m>1$ or negatively cooperative: $0<m<1$. For the fraction of bound ligand $f=[\mathrm{LB}]/([\mathrm{LB}]+[B])$ and considering that $\Delta g/g_0$ is proportional to f, from Eq. (4) we obtain:

$$K_c^m = \frac{\Delta g/g_0}{(1-\Delta g/g_0)c^m} \tag{5}$$

and in a logarithmic form:

$$\log\frac{\Delta g/g_0}{1-\Delta g/g_0} = m\log(K_c/M) + m\log(c/M) \tag{6}$$

The plot of $\log\frac{\Delta g/g_0}{1-\Delta g/g_0}$ as a function of $\log c$ allowing to determine both the binding constant and the Hill coefficient. An example of this plot for ODN/AMA complexes of charge ratio $\rho-/+=0.1$ (i.e. the complexes are positively charged) is shown on Fig. 4. From the square least fit of this linear dependence we obtain the Hill coefficient: $m=1.02\pm0.23$ and binding constant $K_C=(5.4\pm1.3)\times10^5\ \mathrm{M}^{-1}$.

The linear dependences for Hill plot were obtained also for ODN/AMA complexes with other charge ratios ($\rho-/+=0.44$ and 4, respectively), but the Hill coefficient was lower then 1 (Table 1) in these cases, which suggest negative cooperativity. This means that the binding of ligand at one binding site interfere with another binding site. The situation when $m<1$ is much less understood in comparison with positive cooperativity

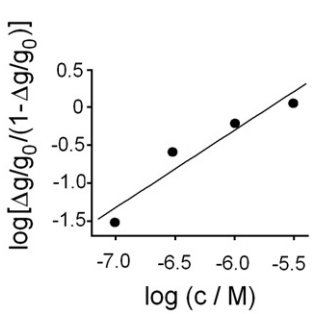

Fig. 4. The Hill plot according to Eq. (6) for ODN/AMA complexes with a charge ratio $\rho-/+=0.1$. The curve is the fit of experimental results (symbols) using least square method.

Table 1
The Hill coefficient, m and binding constant, K_C, determined from the Hill plot according to Eq. (6) for ODN/AMA complexes at various charge ratios, $\rho-/+$. Results represent mean ± SD from 3 independent experiments

Charge ratio of ODN/AMA complexes	$\rho-/+=0.1$	$\rho-/+=0.44$	$\rho-/+=4$
m	1.02 ± 0.23	0.42 ± 0.07	0.44 ± 0.09
K_C, M^{-1}	$(5.4\pm1.3)\times10^5$	491 ± 171	507 ± 239

processes. This is particularly due to the fact that also the heterogeneous binding of ligand, i.e. mixture of non-identical binding site yield to $m<1$ [13]. However, according to Ref [12] it is possible to estimate minimum number of interacting binding sites, N, as $N\geq 1/m$. Thus, for charge ratio $\rho-/+>0.1$ the number of interacting binding site is $N>2$. In this case, however, also binding constant K_C was substantially lower in comparison with positively charged ODN/AMA complex ($\rho-/+=0.1$). Thus, the binding constant substantially decreases with increasing the negative charge of the ODN/AMA complexes. This is also consistent with the results of the conductance changes. The maximal changes of $\Delta g/g_0$ decreased with increasing the negative charge of the ODN/AMA complexes. The decrease of binding constant with increasing ODN/AMA ratios $\rho-/+$ is in agreement with those of Blanc et al. [6] from lipid monolayer surface-pressure measurements studies and under experimental conditions similar to ours (0.24×10^{-6} M AMA and 150 mM NaCl-containing medium) these authors found that the adsorption onto the monolayers was much lower for the ODN/AMA complexes than for AMA alone, and furthermore it decreased when ODN concentration increased. An interpretation based on these and spectroscopic data has been proposed [5,6]. It assumed that amphotericin B and its derivatives are present in aqueous media under three concentration-dependent forms: monomers, water-soluble self-associated species and non water-soluble aggregated self-associated species. Monomers are assumed not to induce permeability through cholesterol-containing membranes [14]. In the presence of the negatively charged ODN, according to Manning's popular model, the cation AMA becomes territorially bound to the oligonucleotide backbone. At low $\rho-/+$, large AMA aggregates are induced, on which oligonucleotides are compacted (species A). With increasing $\rho-/+$, the large aggregates reorganize into smaller ones, which distribute along the ODN skeleton, "decorating it"(species B) (Fig. 5). From our results it appears that not only monomeric AMA (M), — as proposed by Blanc et al. [6] — binds, but also another species interact with the BLM, because permeability is induced (as said above, monomeric polyene antibiotics are considered as not forming transmembrane pores). Species A are the better candidates since their amount decrease with increasing $\rho-/+$, as do permeability inducement and K_C.

3.2. Electrostriction

The electrostriction method was used in order to gain further insight into the interaction of AMA and ODN/AMA complexes with BLM. This method enables simultaneous determination of

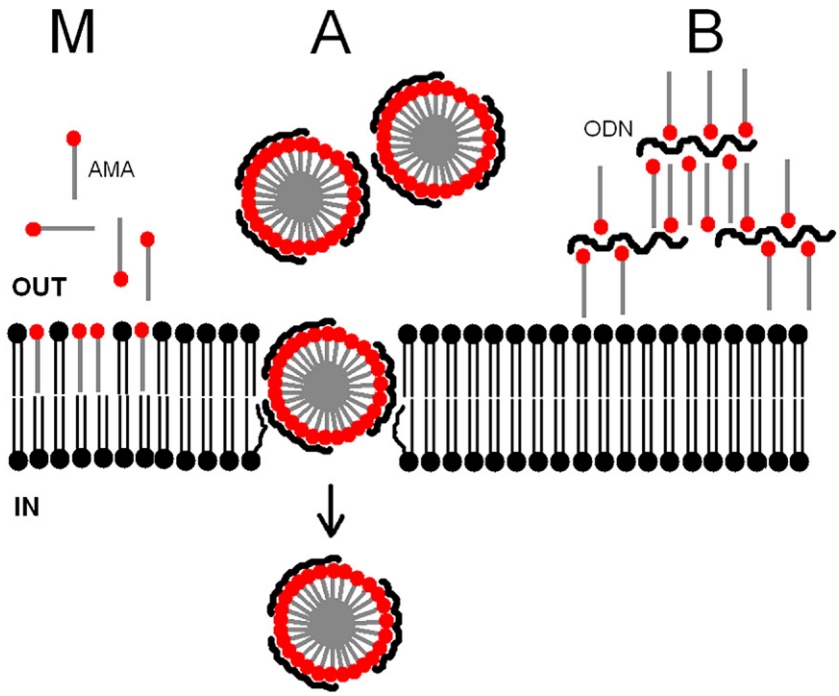

Fig. 5. Schematic representation of incorporation of monomers of AMA (M) into the outer monolayer of BLM, translocation of AMA aggregates with adsorbed ODNs at low charge ratio ($\rho-/+=0.1$) through lipid bilayers (A) and ODNs decorated by AMA at higher charge ratio ($\rho-/+=0.44$ or 4) (B).

the membrane capacitance, C, the compressibility modulus in the direction perpendicular to the membrane plane, $E_{\perp}$, and the surface potential, $\Delta\Phi$. The elasticity modulus is rather sensitive to the structural changes of BLM, while the surface potential is a measure of the changes of both Gouy–Chapman and dipole potential at the surface of the BLM. The changes of the above values following addition of pure AMA into the buffer at one BLM side are presented in Fig. 6. It appears that, while membrane capacitance practically does not change, the elasticity modulus and surface potential increase with increasing AMA concentration. The increase in surface potential is obviously due to the positive charges of the AMA molecules. Thus one side of BLM becomes positively charged and this causes the appearance of differences in the surface potential between both BLM sides. The increase in elasticity modulus suggests an ordering effect of AMA on the BLM structure, most probably due to the formation of tightly packed AMA-cholesterol complexes in the outer BLM monolayer. Our results are in agreement with those obtained by Blanc et al. by surface-pressure studies [8]: AMA molecules were shown to penetrate into the cholesterol/phospholipid monolayers.

Analogous changes of the capacitance, elasticity modulus and surface potential for ODN/AMA complexes of a different charge ratio $\rho-/+=0.1$, 0.44 and 4 are presented on Figs. 7, 8 and 9, respectively. For the positively charged ODN/AMA complex ($\rho-/+=0.1$) the changes of the measured values were similar to those of pure AMA, although higher amplitude of noises was observed. However, for ODN/AMA complexes with charge ratios $\rho-/+=0.44$ and 4, the changes of elasticity modulus and surface potential were different (Figs. 8 and 9). Below the complex concentration of 1 μM both elasticity modulus and surface potential increased with increasing concentration of ODN/AMA complexes. However, at higher concentration, a sharp time-and concentration-dependent decrease of these

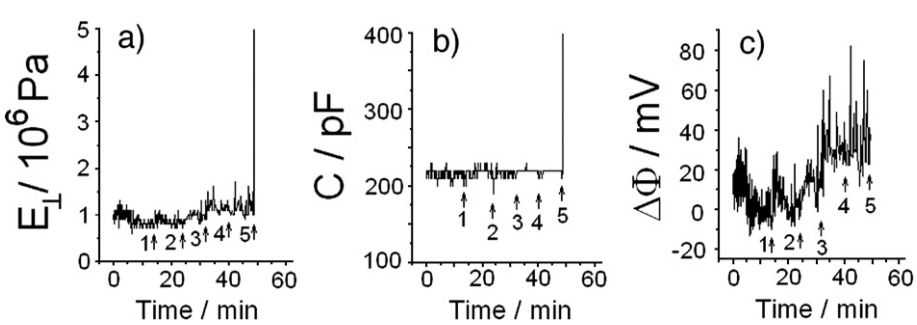

Fig. 6. Changes of (a) elasticity modulus, $E_{\perp}$, (b) electrical capacitance, C, and (c) surface potential, $\Delta\Phi$, of a BLM following one-sided addition of AMA at a final concentration of (1) 0.1 μM, (2) 0.3 μM (3) 1 μM (4) 3 μM (5) 10 μM.

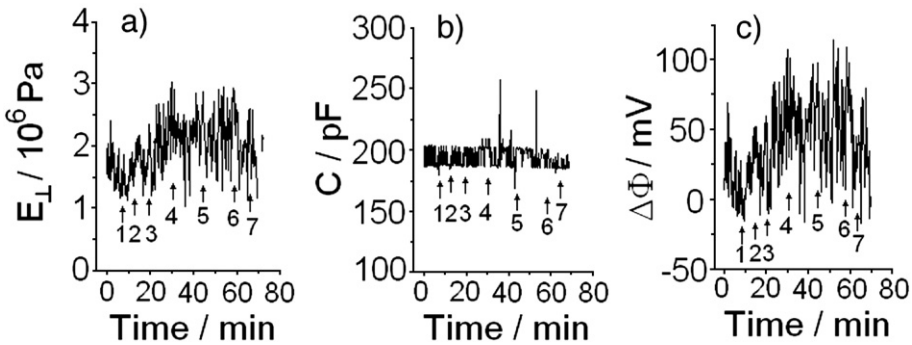

Fig. 7. Changes of (a) elasticity modulus, $E_{\perp}$, (b) electrical capacitance, C, and (c) surface potential, $\Delta\Phi$, of BLM following one-sided addition of ODN/AMA complexes (charge ratio $\rho-/+=0.1$) at a final concentration of (1) 0.1 μM, (2) 0.3 μM, (3) 1 μM, (4) 3 μM, (5) 10 μM, and of Mg^{2+} at a final concentration of (6) 5 mM and (7) 12.1 mM.

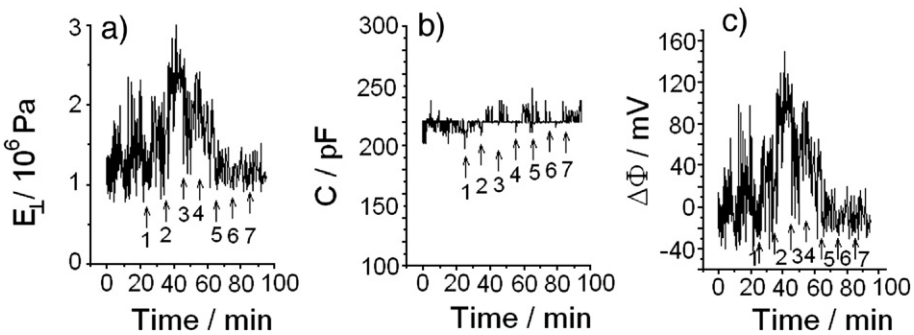

Fig. 8. Changes of (a) elasticity modulus, $E_{\perp}$, (b) electrical capacitance, C, and (c) surface potential, $\Delta\Phi$, of BLM following one-sided addition of ODN/AMA complexes (charge ratio $\rho-/+=0.44$) at a final concentration of (1) 0.1 μM, (2) 0.3 μM, (3) 1 μM, (4) 3 μM, (5) 10 μM, and of Mg^{2+} at a final concentration of (6) 5 mM and (7) 12.1 mM.

values was observed. These changes were not accompanied by a modification of electrical capacitance. The initial increase in elasticity modulus suggests that adsorption of ODN/AMA complexes occurred and resulted in a more rigid membrane in agreement with surface-pressure measurements [8].

The corresponding increase in surface potential supports our interpretation, according to which it is AMA or low $\rho-/+$ ODN/AMA complexes (species A) that bind to the membrane. However, binding of high $\rho-/+$ ODN/AMA complexes (species B) results in more complex changes both in surface potential and in elasticity modulus. The increase in both surface potential and elasticity modulus can be interpreted in analogy with that for pure AMA and for low $\rho-/+$ ODN/AMA complexes.

Two explanations can be proposed concerning the decrease of both elasticity modulus and surface potential at higher incubation times and higher complex concentration. Firstly, desorption of the membrane-bound species can occur. This has been observed by surface-pressure measurements with AMA [8]. However by the same method, in the presence of ODN/AMA complexes no desorption was observed but it should be noted that the membrane model was a monolayer, not a bilayer, the concentration used was 2.4×10^{-7} M and the time of study was limited to 10 min. In our study on BLM, AMA concentrations and incubation times were larger. If a transmembrane movement of species A (low $\rho-/+$ ODN/AMA complexes) occurs or if the bound species spans both layers,

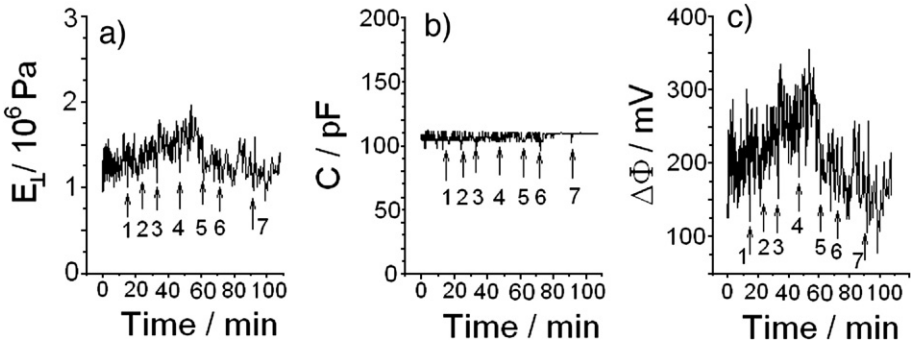

Fig. 9. Changes of (a) elasticity modulus, $E_{\perp}$, (b) electrical capacitance, C, and (c) surface potential, $\Delta\Phi$, of BLM following one-sided addition of ODN/AMA complexes (charge ratio $\rho-/+=4$) at a final concentration of (1) 0.1 μM, (2) 0.3 μM, (3) 1 μM, (4) 3 μM, (5) 10 μM, and of Mg^{2+} at a final concentration of (6) 5 mM and (7) 12.1 mM.

desorption is likely to occur due to the absence of AMA and ODN molecules in the second compartment of the Teflon cup. As a result a net transfer of oligonucleotide will be observed, to the extent that ODN molecules remain bound to AMA during the interaction with membranes.

Secondly, it had been shown earlier that higher harmonics are generated not only in the case of membrane electrostriction, i.e. due to non-linear dependence of capacitance on the voltage, but also in the case of non-linear conductance, i.e. $g=g_0(1+\alpha U^2)$. Using this phenomenon we developed the method of determination of current voltage characteristics of ionic channels, including those formed by amphotericin B [9]. Thus it would be possible to explain the observed phenomenon assuming that a non-linear conductance of the membrane appears at higher concentrations of ODN/AMA complexes. Certainly, increase in the non-linear conductance would result in an increase in the amplitude of the third current harmonic. As a result, both elasticity modulus and surface potential, which are reciprocally proportional to the amplitude of the third current harmonic, should decrease (see Eqs. (1) and (2)). Further study would, however, be necessary for more detailed understanding of the phenomena observed.

4. Conclusion

Our results show that the interaction of ODN/AMA complexes with BLM induce substantial changes in the conductance of lipid bilayers. A decrease in the charge ratio $\rho-/+$ of the complexes from 0.1 to 4 results in a lower permeability inducement accompanied by lower binding affinity to the membranes. This results that the permeabilising species would consist of ODN condensed on large AMA aggregates (species A) rather than of AMA monomers or small AMA oligomer decorated ODN (species B). On the other hand, the initial increase in elasticity modulus of BLM induced by ODN/AMA complexes and pure AMA reveals increased bilayer rigidity. For longer incubation times or higher concentrations of ODN/AMA complexes the surface potential and elasticity modulus decreases following the initial increase may be interpreted as representing a desorption of the complexes in the second compartment of the Teflon cup or the appearance of a non-linear conductance of the membrane. The first hypothesis would explain the transfer of oligonucleotide into 3T3 cells observed by fluorescence microscopy and its higher efficacy at low $\rho-/+$.

Acknowledgements

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