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# Oligonucleotide delivery by a cationic derivative of the polyene antibiotic amphotericin B
# I: Interaction oligonucleotide/vector as studied by optical spectroscopy and electron microscopy

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**Abstract**

Antisense strategy requires efficient systems for the delivery of oligodeoxyribonucleotides (ODN) into target cells. Cationic *amphiphiles* have shown good efficiency in vitro and a lot of attention is currently paid to their interaction with nucleic acids. In the present study, this interaction was, for the first time, analysed at the molecular level, taking advantage of the spectroscopic properties of the positively charged chiral polyene molecule amphotericin B 3-dimethylaminopropyl amide (AMA), the efficiency of which, as delivery system, has been demonstrated [Garcia et al., Pharmacol. Ther. (2000), in press]. By UV-visible absorption and circular dichroism (CD) we studied its self-association properties in pure water, saline and RPMI medium. Drastic changes were observed upon ODN addition, stronger in pure water than in media of high ionic strength. At low AMA concentration ($< 10^{-6}$ M), the strong increase of the CD signal, characteristic of self-association, indicated condensation of AMA on the ODN molecules. At a higher concentration ($10^{-4}$ M), and for a nucleic acid negative charge/AMA positive charge ratio higher than 1, spectra were interpreted as a reorganisation of free self-associated AMA species into smaller ones 'decorating' the nucleic acid molecule. Electron microscopy data were interpreted according to this scheme. 

*Keywords:* Cationic amphotericin; Oligodeoxyribonucleotide; Vectorization; Delivery; Polyene antibiotic

Abbreviations: ODN, oligodeoxyribonucleotide; AMA, amphotericin B 3-dimethylaminopropyl amide; CD, circular dichroism; CTAB, cetyltrimethylammonium bromide; DDAB, didodecyldimethylammonium bromide; DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammoniumchloride; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl ammonium methylsulfate; DOSPA, 2′-(1″,2″-dioleoyloxypropyldimethyl-ammonium bromide)-*N*-ethyl-6-amidospermine tetra trifluoroacetic acid salt; MDR, multi-drug resistance; DTAB, dodecyltrimethylammonium bromide; DTAC, dodecyltrimethylammonium chloride; DODAC, dioctadecyl dimethylammonium chloride; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; DOGS, dioctadecylamidoglycylspermine

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## 1. Introduction

The interaction between cationic surfactants and various DNA is well documented. It induces condensation of the DNA into compact dense structures, the resulting precipitation being used for DNA extraction, concentration and counting. Considerable interest in these complexes, also called 'lipoplexes', has been generated by the observation that some of them are efficient vehicles for the delivery of foreign DNA or oligonucleotides in eukaryotic cells. Cationic lipids used for this purpose can be classified as quaternary ammonium salt lipids or lipoamines. In the first case, bound to the nitrogen atom can be found either one or two acyl chains: respectively, cetyltrimethylammonium bromide (CTAB) and didodecyldimethylammonium bromide (DDAB) for instance, or a linker bearing two acyl chains, as *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium-chloride (DOTMA) or *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl ammonium methylsulfate (DOTAP), or in addition to the preceding groups, a polyamine, for example 2′-(1″,2″-dioleoyloxypropyldimethyl-ammonium bromide)-*N*-ethyl-6-amidospermine tetra trifluoroacetic acid salt (DOSPA).

For the first time, this report gives direct information at the molecular level on the behaviour of a cationic amphiphile in the presence of nucleic acid and in conditions relevant to biological activity. Indeed, we present data on a cationic amphiphile representative of a series of cationic derivatives of the polyene antibiotic amphotericin B, the amphotericin B 3-dimethylaminopropyl amide (AMA). As its parent compound AmB, the AMA molecule presents a rigid elongated skeleton containing a polyene part (Fig. 1), which imparts to it the property of insertion into membranes and the formation of pores [2–5]. Moreover, numerous authors report its selectivity for ergosterol-containing membranes (fungal cells) as regards to cholesterol-containing ones (mammalian cells) [5–7]. On the other hand, due to its net positive charge (instead of zero for AmB) and its water-soluble character [8,9], AMA is able to interact with negatively charged nucleic acids. Finally, as compared to cationic lipids, the potential interest of AMA is that it may form transient transmembrane pores at low concentration, with a high specificity for fungal cells. Therefore, the nucleic acids/AMA interaction could enable the creation of high local concentrations of membrane active molecules, dragging nucleic acids close to the target cells.

Successful results of utilisation of AMA as a vector of nucleic acids on G185-MDR-3T3 cells have been recently reported [1]. Indeed, these authors have demonstrated that this dicationic AmB-derivative enhanced uptake and efficacy of an anti-MDR1 phosphorothioate oligodeoxyribonucleotide (ODN). They have also demonstrated that this new cationic ODN delivery required an excess of negative charges for better efficacy.

Upstream of this study, we were interested in the mechanism of ODN transport by AMA through membrane models and in the primary interaction between the two molecules.

In this first report, we present a study of the interaction between AMA and ODN, taking advantage of the spectroscopic properties of the polyene molecule. Indeed, AMA shares with AmB a high extinction coefficient and an exquisite sensitivity of its CD and absorption spectra to changes in environment and self-association, enabling the study of its physicochemical properties at the concentrations used for transfection. Our strategy of study was therefore to follow the changes on AMA absorption and CD spectra when varying several physico-chemical parameters as the ionic strength of the solution, the final concentration in AMA and for each of them several concentrations in ODN. This also permitted study of the influence of the charge ratio (between the negative charges of the nucleic acid and the positive charges of the polyene) on the characteristics of the ODN/AMA mixtures. Some preliminary results concerning the structure of the complexes, obtained from electron microscopy measurements, are also presented as an illustration of our conclusions.

## 2. Materials and methods

### 2.1. Reagents

#### 2.1.1. Amphotericin

AMA, an amphiphilic molecule derived from amphotericin B (Fig. 1), was kindly provided by Edward Borowski (Technical University, Gdansk, Poland). Solutions of AMA were daily prepared by

dissolution in Millipore water at a $10^{-3}$ M concentration.

*2.1.2. Oligonucleotide*

The ODN was a 27-mer phosphodiester, the sequence of which was TGTGTTTTTGTTTTGTTGGTTTTGTTT. It was synthesised, desalted but not purified, by Genosys (Cambridge, UK). It was dissolved in Millipore water (pH $\approx$ 5.5) at an initial concentration of $10^{-4}$ M.

*2.1.3. Medium*

Red phenol-free RPMI was obtained from Polylabo (Paris, France).

*2.2. Spectroscopic measurements*

Circular dichroism spectra were recorded with a Jobin-Yvon Mark V dichrograph. Spectral wavelengths are given $\pm 0.5$ nm. $\Delta\varepsilon$ is the differential molar dichroic absorption coefficient ($10^3$ $cm^{-1}$ $M^{-1}$). Electronic absorption spectra were recorded with a Hewlett Packard 8452A spectrophotometer.

Electronic absorption and CD spectra of AMA in aqueous medium, alone or in the presence of ODN, were recorded at room temperature. AMA concentrations were varied between $10^{-3}$ and $2\times10^{-7}$ M by dilution from a $10^{-3}$ M stock-solution in water. The ODN/AMA mixed solutions were prepared, unless otherwise specified, by first mixing the stock-solutions ($10^{-4}$ M in molecule of ODN) in the desired ratio ('$\rho-/+$' = molar ratio of the negative charges of the nucleic acid over the positive charges of the amphotericin; the charge ratio was calculated as the 27-mer ODN molecule having 27 negative charges and each cationic amphotericin two positive charges per molecule), then diluting the mixture at the proper AMA final concentration with the chosen medium. Spectra were immediately recorded after mixing. Three series of measurements were done: in pure water, in 150 mM NaCl-containing water ('saline') and in RPMI.

*2.3. Electron microscopy*

Five different solutions were studied by this method, all of them were prepared in 0.1 M ammonium acetate: a $10^{-4}$ M AMA solution, a $3\times10^{-5}$ M ODN solution, three ODN/AMA mixtures of constant AMA concentration ($10^{-4}$ M) and three ODN concentrations of $7.5\times10^{-7}$, $3\times10^{-6}$ and $3\times10^{-5}$ M, corresponding, respectively, to $\rho-/+$ = 0.1, 0.4 and 4. Electron microscopic specimens were prepared as follows. A 10 μl drop of the solution to be observed was applied to the electron microscopic grid covered with a collodion-carbon film. The preparations were contrasted by 1% uranyl acetate staining. All samples were analysed with a 410 Philips electron microscope operating at 80 kV.

## 3. Results

*3.1. Spectroscopy measurements*

Between 250 and 450 nm, a vibronic progression was expected to be observed both for AMA and ODN/AMA solutions, similar to that observed with the parent compound AmB [10] and due to the system of conjugated double bonds of the polyene.

*3.1.1. AMA solutions*

The same general features as with AmB were observed.

*3.1.1.1. In saline and RPMI.* The absorption spectra of AMA at low concentration ($2\times10^{-7}$ and $10^{-6}$ M) showed four absorption bands representative of monomers at 409, 385, 365 and 347 nm, with high values of $\varepsilon$ (Fig. 2). At high antibiotic concentration ($10^{-4}$ M), one band located at 337 nm superseded the absorption bands of the monomers. Similarly to the case of AmB, this band is assigned to an excitonic coupling resulting from the self-association of the polyene molecules. It could be noted that the proportion of monomers in solution was higher at $10^{-6}$ M than at $10^{-4}$ M.

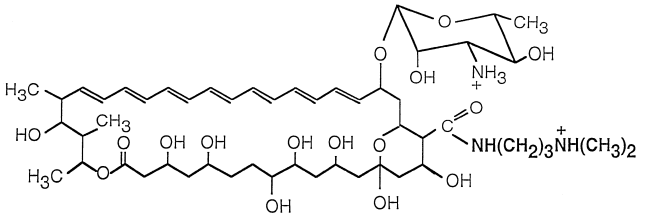


Fig. 1. Chemical formula of amphotericin B3-(*N'*-dimethylamino)propylamide (AMA).

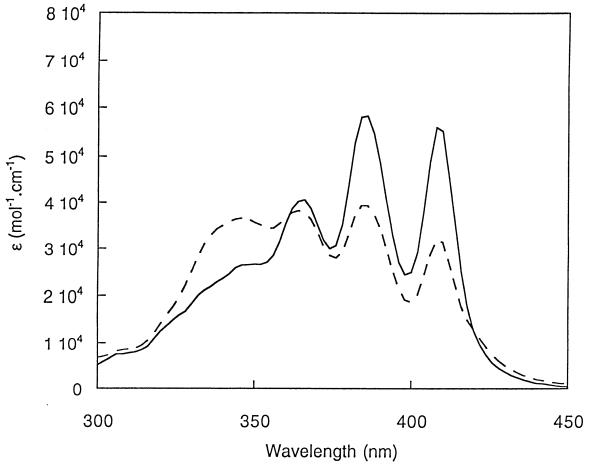


Fig. 2. AMA absorption spectra in a 150 mM NaCl solution. (dashed line) $10^{-4}$ M; (solid line) $10^{-6}$ M.

The CD spectrum of $10^{-4}$ M AMA solutions exhibited a very intense dichroic doublet centered at 340 nm ($\Delta\varepsilon \approx 225$) (Fig. 3). Stepwise dilution led to a decrease of the signal (Fig. 4). The dichroic doublet clearly visible at high AMA concentrations corresponds to the 337 nm band on the absorption spectra and is then also attributed to an excitonic coupling resulting from the self-association of AMA molecules. During our study, we took advantage of this large circular dichroic signal to follow the modifications of the polyene self-associated species by studying the changes on the circular dichroic doublet,

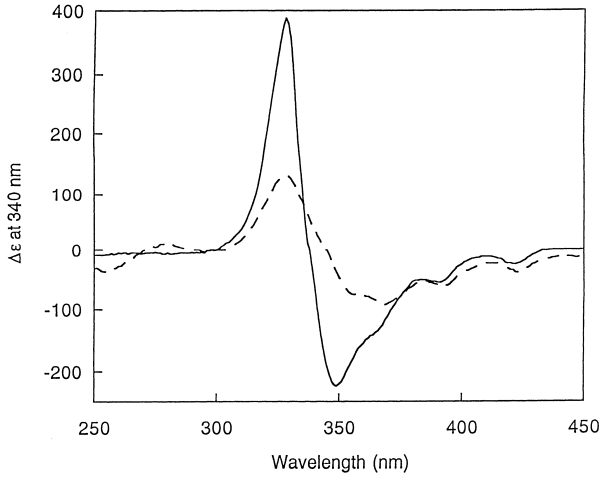


Fig. 3. AMA and ODN/AMA solution ($\rho = 4$) circular dichroism spectra in a 150 mM NaCl solution. AMA final concentration: $10^{-4}$ M; (solid line) AMA; (dashed line) ODN/AMA; $\rho = 4$.

whereas the presence of monomer species was followed by recording the absorption spectra of the solutions.

*3.1.1.2. In pure water.* The absorption and CD spectra showed the same global shape as in saline but the intensity of the bands attributed to self-associated species were much lower than in saline: whatever the AMA concentration, the dichroic doublet intensity appeared always lower than it was in saline (Fig. 4). Therefore, it seems that the AMA would have less ability to self-associate in water than in saline.

*3.1.1.3. Influence of the ionic strength.* To study the influence of the ionic strength on the state of the AMA molecules in solution (monomeric or self-associated species), we recorded the absorption and CD spectra of $10^{-4}$ M AMA in solutions of increasing NaCl concentrations (0–1 M) in water. A strong decrease of the monomer absorption band at 409 nm was observed; the dichroic doublet intensity increased from 80 to 330, without wavelength shift (Fig. 5). These results confirmed that the self-association of the polyene molecule was greatly favoured by increasing ionic strengths, probably due to the ionic environment and the lowering of repulsion forces between identical charges.

*3.1.1.4. Comparison with AmB.* Even if the same general features were kept for AMA solutions when

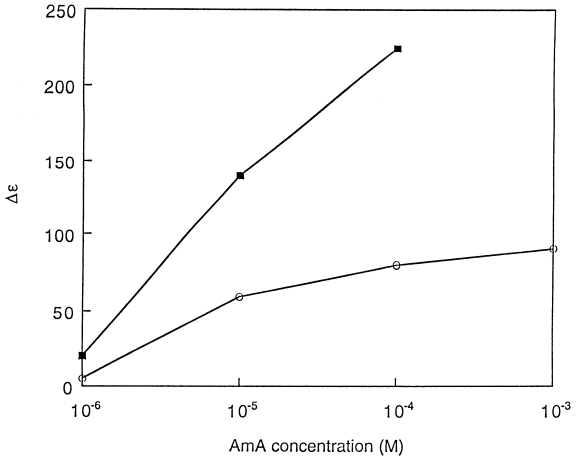


Fig. 4. Evolution of the intensity of the dichroic doublet of AMA solutions as a function of the AMA final concentration and the medium; in 150 mM NaCl (■), in pure water (○).

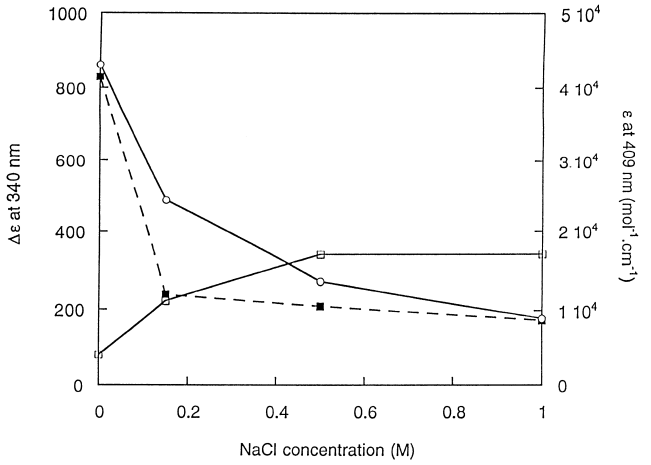


Fig. 5. Influence of the NaCl concentration on the intensity of the 409 nm absorption peak of a $10^{-4}$ M AMA solution and on the intensity of the dichroic doublet (340 nm) of $10^{-4}$ M AMA and AmB solutions. AMA, $\varepsilon_{409}$ (○) and $\Delta\varepsilon_{340}$ (□); AmB, $\Delta\varepsilon_{340}$ (■).

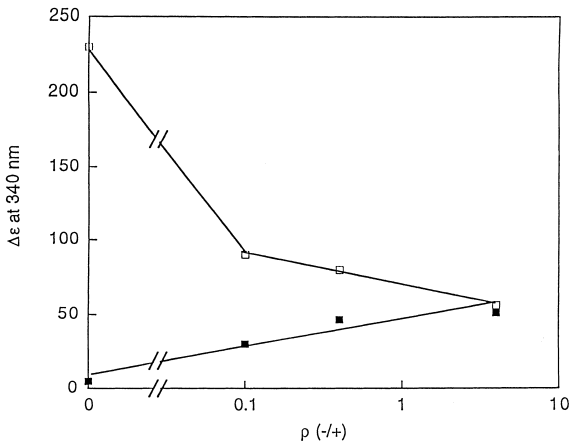


Fig. 6. Intensity of the AMA dichroic doublet of ODN/AMA solutions in a 150 mM NaCl solution, as a function of the AMA final concentration and on the charge ratio ($\rho$−/+). AMA final concentration: $10^{-6}$ M (■), $10^{-4}$ M (□). The ODN/AMA solutions were prepared as described in Section 2.

compared to AmB solutions, two important differences should be noted. First, the intensity of the excitonic bands around 340 and 420 nm (absorption and CD spectra also), compared to that of the monomers at 409, 386 and 356 nm, appeared lower for AMA solutions in water than for AmB solutions. Consequently, the characteristic bands of monomer species were always present on the spectra of AMA solutions in water, whatever the concentration, whereas they were absent on spectra of AmB solutions of high concentration. Second, the influence of the ionic strength on the polyene state in solution appeared opposite for the two molecules: increasing NaCl concentration led to a strong decrease of the excitonic doublet of AmB solutions (accompanied by a 10 nm red-shift), whereas the reverse was observed with AMA (without wavelength-shift) (Fig. 5). This confirms the importance of the charge environment on the self-association mechanism.

### 3.1.2. ODN/AMA mixed solutions

Mixing AMA stock solution with ODN stock solution before dilution to the desired AMA final concentration resulted in important changes in the AMA absorption and CD spectra. Studies were carried out in saline or in RPMI and in pure water, for four different AMA final concentrations ($2\times10^{-7}$, $10^{-6}$, $10^{-5}$ and $10^{-4}$ M). In Figs. 6 and 7 the variation of intensity is shown of the dichroic doublet of ODN/AMA solutions, respectively, in saline and in pure water, for two AMA final concentrations ($10^{-6}$ and $10^{-4}$ M) and increasing ODN concentrations.

*3.1.2.1. In saline or RPMI.* The CD spectra of the mixed solutions are very different from the spectra of AMA alone. For mixed solutions of low AMA concentration ($10^{-6}$ M, Fig. 6), increasing ODN concentrations resulted in a continuous increase of the dichroic doublet centered at 340 nm, as compared to

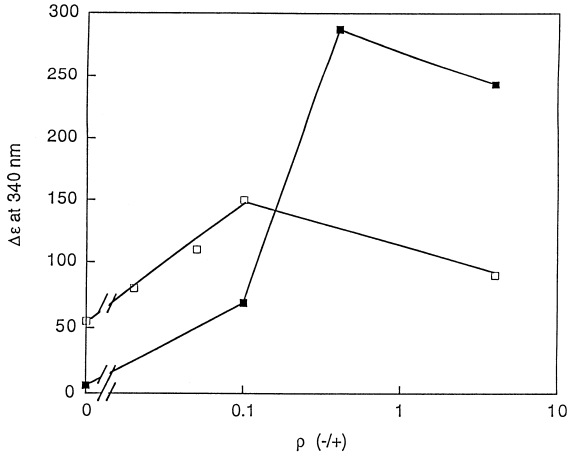


Fig. 7. Intensity of the AMA dichroic doublet of ODN/AMA solutions in Millipore water, as a function of the AMA final concentration and on the charge ratio ($\rho$−/+). AMA final concentration: $10^{-6}$ M (■), $10^{-4}$ M (□). The ODN/AMA solutions were prepared as described in Section 2.

that of the AMA solution at the same concentration, without any wavelength shift. These modifications suggested that the self-association of the polyene molecule was induced by the ODN. In contrast, for mixed solutions of high AMA concentration ($10^{-4}$ M), a three-fold decrease of the circular dichroic doublet intensity was observed for $\rho-/+ = 0.1$, as compared to the spectrum of AMA alone at this concentration. For $\rho-/+ = 4$, a new dichroic doublet appeared centered on 260 nm, which is representative of the ODN molecule (Fig. 3). At the same time, a 10-nm red-shift was observed for the AMA dichroic doublet. This small red-shifted signal remained approximately constant whatever $\rho$ is. The absorption spectra of these solutions showed an intensity decrease of the bands characteristic of AMA monomers for increasing $\rho-/+$, together with the increase of the intensity of those attributed to the associated species. At the same time, a new band appeared at 420 nm; this new band is attributed to ODN/AMA 'complex' entities.

*3.1.2.2. In water.* ODN/AMA mixed solutions of $2\times10^{-7}$ and $10^{-6}$ M AMA final concentrations presented for $\rho-/+ = 4$ a huge *increase* of the AMA dichroic doublet centered at 340 nm: the signal was multiplied by 55 (Fig. 7). Furthermore, the presence of ODN induced a red-shift of the doublet (more than 10 nm at $\rho-/+ = 4$). Correspondingly, in the absorption spectra, the bands characteristic of monomeric AMA almost disappeared, while new bands appeared at 350 and 420 nm (Fig. 8). Then, the effect of the presence of ODN on the AMA association seems to be higher in water than in saline and also probably not of the same nature, as indicated by the high wavelength-shift observed in water. This could emphasise the importance of the electrostatic interactions and of the ionic composition of the solution on the interactions between the two components.

For mixed solutions of $10^{-4}$ M AMA final concentration, the dichroic doublet intensity increased for increasing $\rho-/+$ up to $\rho-/+ = 0.1$ (Fig. 7); then for higher $\rho-/+$ it decreased, together with a progressive red-shift. Thus, the dichroic doublet intensity for $\rho-/+ = 4$ was hardly higher than that of AMA alone, but with a 10-nm red-shift. This result suggested again a modification of the AMA aggregation state, induced by the ODN molecule.

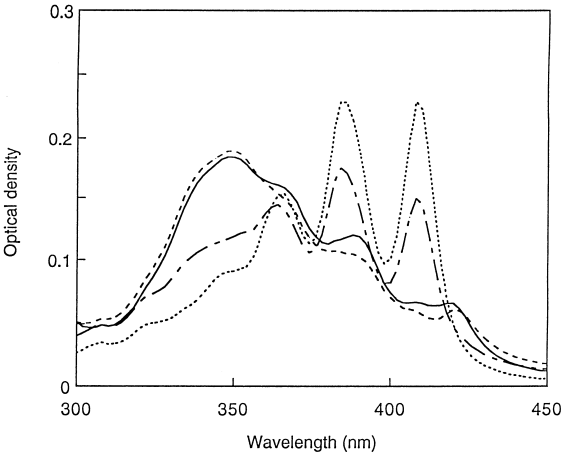


Fig. 8. Absorption spectra of ODN/AMA mixed solutions in water. AMA final concentration: $10^{-6}$ M, trough thickness: 5 cm. AMA alone (dotted line); $\rho$: 0.1 (dot-dashed line), 0.4 (solid line) and 4 (dashed line). The ODN/AMA solutions were prepared as described in Section 2.

For intermediate $\rho-/+$, between 0.1 and 4, a strong flattening of the absorption spectra accompanied by a strong decrease of the dichroic doublet occurred, probably corresponding to a condensation of the structures formed at these charge ratios.

*3.1.3. ODN/AmB mixed solutions*

A remarkable point is the absence of change of AmB solutions spectra (CD and absorption) when AmB and ODN stock solutions were mixed in the same way as described before with AMA. This fact could be attributed to the absence of electrostatic interactions between the AmB and the ODN.

*3.2. Electron microscopy*

We studied by electron microscopy a $3\times10^{-5}$-M ODN solution (Fig. 9a) and ODN/AMA mixtures of constant AMA concentration ($10^{-4}$ M) and variable ODN concentration: $3\times10^{-5}$, $3\times10^{-6}$ and $7.5\times10^{-7}$ M corresponding, respectively, to $\rho-/+ = 4$ (Fig. 9b), 0.4 (Fig. 9c) and 0.1 (Fig. 9d).

*3.2.1. ODN solution*

Dense structures of sizes varying between 50 and 100 nm were observed (Fig. 9a), composed of round-shaped entities which have the tendency to form necklace-like structures. Each round-shaped entity

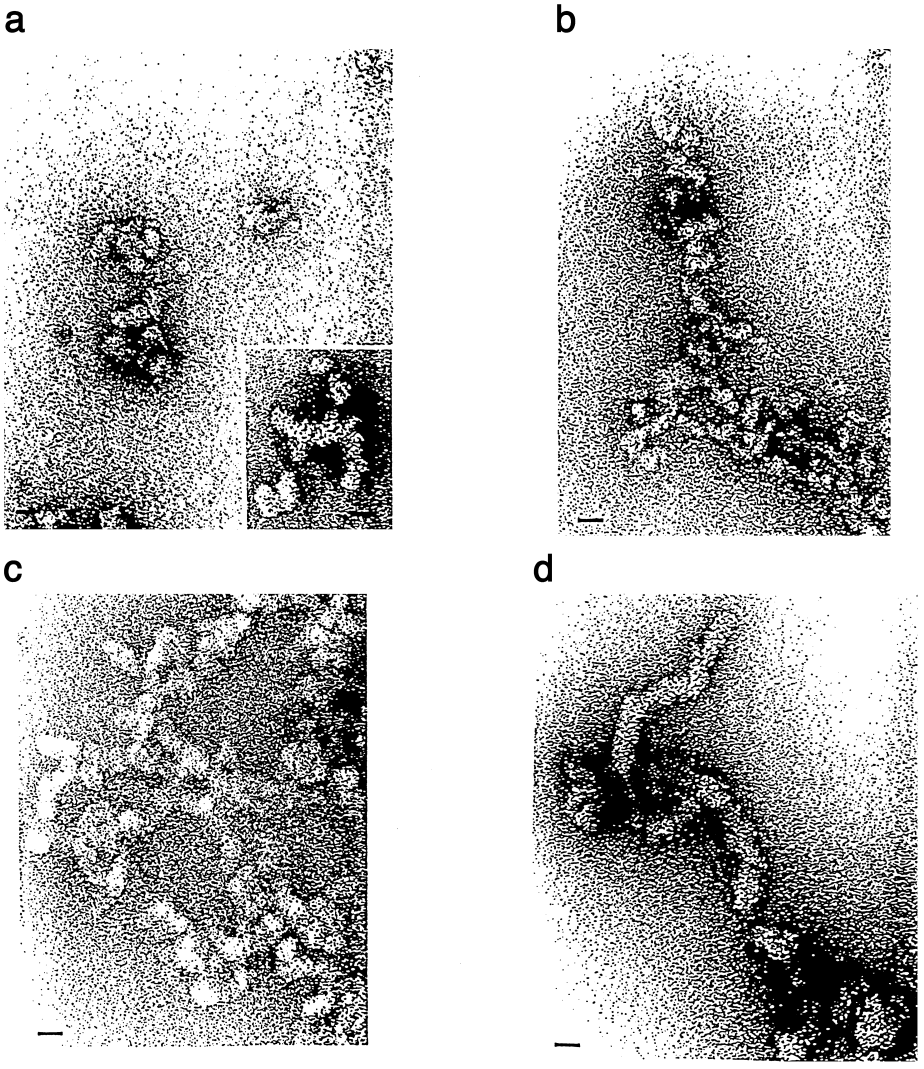


Fig. 9. Electron micrographs of ODN and ODN/AMA solutions ([AMA] = $10^{-4}$ M) obtained by negative staining with 1% uranyl acetate. (a) ODN alone ([ODN] = $3\times10^{-5}$ M); (b) ODN/AMA mixture, $\rho-/+=4$ ([ODN] = $3\times10^{-5}$ M); (c) ODN/AMA mixture, $\rho-/+=0.4$ ([ODN] = $3\times10^{-6}$ M); (d) ODN/AMA mixture, $\rho-/+=0.1$ ([ODN] = $7.5\times10^{-7}$ M). Magnifications 255 000× (a), 241 500× (b), 204 000× (c), 270 000× (d). Bar represents 26 (a), 27 (b), 33 (c) and 25 nm (d).

ranged between 12 and 20 nm in diameter. Taking into account the reported spacing of 3.4 Å$^2$ between two bases in a nucleic acid [11], our 27-mer oligonucleotide length, in elongated state, would be approximately 10 nm, and 2 nm in diameter as a round-shaped entity. Then, one round-shaped structure could comprise 10 ODN molecules on average. Fig. 9a shows variable aggregations of the ODN alone in solution.

### 3.2.2. ODN/AMA mixtures

Aggregates up to 650 nm have been observed on the ODN/AMA mixtures of charge ratio equal to 4 (ODN concentration $3\times10^{-5}$ M) (Fig. 9b). They were composed of irregularly shaped entities forming long interconnected arrays. When round-shaped entities were identified, the average diameter accounts for 12–20 nm. When decreasing ODN concentration from $3\times10^{-5}$ to $3\times10^{-6}$ M and then to $7.5\times10^{-7}$ M, their global shape changed (Fig. 9c,d). In contrast to $3\times10^{-5}$ M (Fig. 9b), for $3\times10^{-6}$ M (Fig. 9c) the mixture contained larger aggregates of regular round-shaped or small rod-like structures interconnected forming a branched network, while the mix-

ture of $7.5\times10^{-7}$ M ODN concentration showed long arrays of rod-like structures (Fig. 9d).

From these results, we can conclude primarily that the ODN formed aggregates in solution and secondly that a complexation occurred between the ODN and its dicationic vector. Indeed, although no electron microscopy results have been recorded with AMA alone, the structure of ODN clearly changed by the presence of AMA: the structure of the mixtures, revealed by the electron microscopy study, depended on the charge ratio $\rho-/+$.

## 4. Discussion

### 4.1. AMA behaviour in solution

The strong similarity between the absorption and CD spectra of AMA and its parent compound AmB in wavelength, intensity and concentration dependence, allows the extrapolation to AMA of the structural data already proposed for AmB: upon increase of concentration, the polyene antibiotic self-associates under oligomeric form, then forms micelles [5,12,13]. In a recently described model [14], AmB dimers are stacked in helix and form a cylinder, the hydrophobic moieties gather together with the mycosamine and the carboxylate moieties turned outwards. The helical pitch governs the intensity and wavelength of the dichroic doublet resulting from the chiral stacking. For AMA we propose the same arrangement, the hydrophobic part of the molecule being the same as that of AmB. It enables the added positively charged dimethylaminopropyl group to be sufficiently remote so as not to experience electrostatic repulsion between each other and therefore not to perturb the parent structure. Moreover, our results indicate that the relative proportions in monomeric and self-associated species in solution depends at once on the concentration of the polyene and also on the saline concentration of the solution. Indeed, we have shown that the quantity of self-associated species, as regard to monomeric ones, increases with increasing NaCl concentration. However, the influence of the saline concentration of the solution on the self-association properties of AmB and AMA appears opposite, as deduced from the spectroscopic characteristics. We think that these differences may be explained by the delicate interplay of the interaction/repulsion forces and the existence of two types of interactions between the polyene molecules, resulting in two different spectra: a strong doublet centered around 340 nm on the one hand, a much weaker doublet around 350 nm on the other hand, the decrease of the specific $\Delta\varepsilon$ indicating rather a different structural relationship between the molecules than a weaker interaction. The first type of interaction is observed in water with AmB and to a weaker extent with AMA. Screening the electrostatic effects by increasing the ionic strength modifies the interaction between AmB molecules and leads to a new spectrum. The corresponding new type of self-association should involve a smaller number of interacting molecules since it is red-shifted [14]. Importantly, this spectrum is observed in several ODN/AMA mixed solutions (see below). Concerning AMA molecules, it appears that the repulsion in water between the positive charges limits their self-association. Decreasing this repulsion by increasing the ionic strength allows AMA self-association to a similar extent as that of AmB in water (similar $\Delta\varepsilon$ and doublet at the same wavelength).

### 4.2. Behaviour of the ODN/AMA mixtures in solution

#### 4.2.1. ODN/AMA interactions

The first result of our study is that the two positive charge bearing AMA interact with ODN, in contrast with the neutral AmB. Furthermore this interaction is ionic strength-dependent.

The interaction of charged polymers with their counter-ions, and in particular of nucleic acids with cations, has been widely addressed. The popular Manning's model is often considered, in which small cations such as $Na^+$ become territorially bound to the negatively charged DNA backbone.

For the purpose of our study, it is essential to recall the results of former studies, not underestimating the difference between DNA and ODN. Even in the presence of high amounts of $Na^+$ (e.g. 150 mM), traces of *monovalent* cationic surfactants binds to DNA. For instance, binding of DTAB or DTAC [15,16] and CTAB [17] was shown to be highly cooperative and biphasic. Complexes of DNA with DODAC, DOTMA, prepared in Bligh and Dyer monophase, could be extracted in water [18]. The

binding to helical or single strand DNA was shown to involve a two-step process: first, binding to an isolated phosphate site on the DNA strand, and second, a highly cooperative binding event that seems to involve hydrophobic interactions between hydrocarbon chains of the bound CTAB. It was shown by fluorescence microscopy that upon addition of CTAB, the higher order structure of large DNA chains changes in a discrete manner between elongated coil and compact globular state [19]. Furthermore the coexistence region of the coil and globule states almost coincides with the region of 'cooperative' binding in the binding isotherm [20]. As for the structure of DNA, no change (or only very small changes) in the circular dichroism spectra were observed upon addition of CTAB [17,21] or DOGS [22]. However, according to Akao, the conformation of DNA is forced to change by cationic lipids forming rigid bilayer membranes (not by micellar ones).

The binding of *multivalent cationic lipids* to nucleic acids has not been, as far as we know, analysed on a theoretical point of view. Data on *water-soluble* multivalent cations are, however, numerous [23,24]; they explain the origin of their strong interaction as compared to that with monovalent ions. For instance, the interaction of spermidine and spermine with DNA has been addressed [23,25]. In our case, in the presence of two types of multivalent cations: monomeric bivalent AMA and self-associated multivalent AMA; the interaction should be stronger with the latter.

For intermediate $\rho-/+$, we observed a strong flattening of the spectra, which can be attributed to the Duysens [26] effect resulting from aggregation. As a matter of fact, Lasic [27] described the precipitation of ODN/cationic lipid mixture for these ratios. This event is well documented for a DNA/cationic lipid mixture [22,25,27].

#### *4.2.2. Structures of the ODN/AMA assemblies*

Concerning the structure of the assemblies ODN/AMA, some hypothesis can be made, taking into account both spectroscopy and electron microscopy data.

The intensity increase of the AMA dichroic doublet in the presence of ODN suggests that ODN induces AMA condensation around the nucleic acid and increases formation of self-associated species identical to those observed in the absence of ODN. When the presence of ODN in the solution results in a red-shift of the dichroic doublet, we consider that self-associated species containing a smaller number of AMA molecules are formed.

For complexes of lower charge ratio, the AMA molecules are in excess when compared to the nucleic acid. Therefore, the nucleic acid offers few sites of interaction of electrostatic nature to the cationic vector. Consequently, we assumed that the species interacting with the few negative sites were large AMA aggregates. The proximity between them could induce self-assembly by hydrophobic interactions, causing at the same time the compaction of the ODN molecules. This hypothesis would explain the presence of less dense ramified structures (Fig. 9d), as compared with the ODN alone in solution (Fig. 9a). When increasing the ODN concentration of the complexes (increasing charge ratio), the number of sites of fixation of AMA aggregates on the ODN molecules grows. Then we assume that the large AMA aggregates reorganise into smaller ones, which distribute along the ODN skeleton, 'decorating' it. Since they are more spaced out than before, they would interact less with each other and therefore induce a lower compaction of the nucleic acid (Fig. 9b). The consequence would be a structure less dense and more ramified than in the case of low charge ratio (Fig. 9d). Let us note that the morphology of DOTAP/ODN complexes was addressed by freeze-fracture EM [28]. It appeared that these complexes were aggregated, but without the tubular hexagonal or spaghetti-like structures observed in the presence of DOPE and DNA [29].

## 5. Conclusion

Let us remember that the potential interest of the dicationic molecule AMA for antisense ODN delivery, as compared with other cationic lipids, is that it forms transient transmembrane pores at low concentration, with a high specificity for fungal cells. Thus it seems to offer not only the possibility to interact with a nucleic acid, but also the possibility to interact with cell membranes at the same time, perhaps thus dragging nucleic acids close to their target cells. In our study, we have characterised the conditions of

interactions of the polyene with an ODN and we have also given a hypothesis about the structures of the resulting complexes. The question which could be asked now is 'how will these entities cross the membrane?'. Taking into account the permeabilising properties of the AMA, would it be possible for the ODN to enter into the cell through the pores formed by its vector? From our results, however, this first hypothesis seems rather unlikely, our micrographs showing ODN/AMA complexes of larger size than the reported size of transmembrane pores formed by the AmB [30–32]. Another hypothesis could then be a modification of the lipid organisation of the membrane, allowing the penetration of the complexes into the cells. In order to try to answer these questions, we are interested, in the second part of our work, in the interactions of ODN/AMA mixtures with planar lipid monolayer and large unilamellar lipid vesicles, both taken as membrane models.

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