FEBS Letters 409 (1997) 188–194 FEBS 18653

Structure of in-serum transfecting DNA–cationic lipid complexes

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Received 27 March 1997

Abstract Noticeable modifications of in-serum transfection efficiency of dioctadecylamidoglycyl-spermine (DOGS)–DNA complexes are observed, depending on DNA condensation conditions. The structures of the complexes are studied, keeping in mind the variability of lipid polymorphism, by cryo-transmission electron microscopy and X-ray diffraction. By increasing both pH and ionic strength, well-organised lamellar structures with a period of 65 Å replace supramicellar aggregates. A relationship between the structures and their in-vitro transfection activity is established. Efficiency in the presence of serum is maintained when a lamellar arrangement is involved.

Key words: Transfection efficiency; Cationic lipid; DNA condensation; Cryo-transmission electron microscopy; X-ray diffraction; Lamellar structure

1. Introduction

Synthetic cationic lipids and the complexes they form with DNA constitute the most promising alternative to the use of viruses for gene therapy. Despite high transfection efficiencies, viruses indeed exhibit intrinsic drawbacks such as immunogenicity, recombinogenicity, cytopathicity and scaling-up difficulties [1–3] which endanger widespread therapeutic applications. Cationic lipid–DNA complexes associate DNA with cationic detergents [4], cationic lipids [5,6], such as lipopolylysines [7,8], cholesterol derivatives [9–11] or lipopolycations [12–14]. A neutral colipid such as dioleylphosphatidylethanolamine (DOPE) is frequently added [4–10].

Only a few authors have characterised the structures of the complexes, despite their possible importance on gene transfer. These structures are difficult to elucidate because of their variability as a function of composition and preparation conditions [15–19]. Indeed, the excess ionic charges carried by the complexes resulting from the association of the DNA polyanion and the self-assembling cations, directly influences their properties and severely complicates their structural study. However, multilamellar structures made of a mixture of cationic lipid, neutral colipid and DNA, sometimes called 'DNA–liposome complexes', have been evidenced very recently [20,21].

A net positive charge, and therefore a cationic lipid excess, has been observed to be needed for optimal in vitro transfection efficiency, likely because of the net negative charge exhibited by the plasmic membrane of the target cell [7,8,10,13]. Lipospermines are synthetic lipidic derivatives of spermine, a natural polycation which exhibit electrostatic interactions with DNA, leading to its collapse [22–25]. They have been found to be efficient cationic amphiphiles for gene delivery, even in the absence of colipids [14,26–28]. DOGS (dioctadecylamidoglycyl-spermine) is a well-known representative of this class of compounds. It has been assumed, following transmission electron microscopy (TEM) observations to form hexagonal structures in association to DNA [18].

The need for a lipid excess led us to postulate that lipid self-assembly should govern, at least partly, the structure of the complexes. Then, the organisations of the particles formed by self-association of DOGS in the absence of DNA and their evolution versus time have been first studied as a function of pH, ionic strength, counterion nature and temperature [29]. Actually, the formation of variable shaped micelles and of lamellar and hexagonal phases was observed. However, addition of DNA to these structures should modify the global organisation of the association, as shown for other cationic lipids [20,21]. Additional parameters such as lipid/DNA ratio [16] and complex concentration, should also interfere on adopted structures.

Most of the successful reported transfection studies are achieved in vitro and without serum [4,13,14,30,31], probably because of the low DNA uptake reached in the presence of serum. In fact, very few in vitro transfection studies succeeded in 10% fetal calf serum (FCS) [7,32,33] and none of the authors gave any reasons why it had been feasible. Moreover, none of the above-listed structural studies have related them to transfection efficiency.

In the present study, the influence of DNA introduction on DOGS polymorphism is examined through the physical characterisation of DOGS–DNA complexes, mainly by cryo-transmission electron microscopy (cryo-TEM) and X-ray diffraction. A relationship between these structures and gene transfer activity is established in vitro. The influence of FCS on transfection efficiency is also addressed.

2. Materials and methods

2.1. Synthesis of lipospermines

DOGS was synthesised as described by Behr [12]. The lipid was freeze-dried from a water/acetonitrile (75:25, v/v) mixture. DOGS purity was checked by ^{1}H NMR and mass spectrometry. Elementary microanalysis results and their computation through an in-house developed optimisation software allowed an estimation of the average molecular composition of DOGS/trifluoroacetate (TFA)/water (1:4.4:2.1) for the resulting product.

2.2. Plasmid

The *Photinus pyralis* luciferase encoding plasmid PXL2784 con-

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PII S0014-5793(97)00505-X

tained the ColE1 origin of replication. The luciferase gene was under the control of pCDNA3 plasmid cytomegalovirus (CMV) promoter. Supercoiled plasmid DNA was cloned in *Escherichia coli* and purified by alkaline lysis and cesium chloride gradient centrifugation according to techniques already described [34].

2.3. Cationic lipid preparation and complex formation

For transfection studies, 2 mM DOGS ethanolic solution or aqueous suspension in 0.15 M NaCl was added to a 10 μg/ml DNA solution. The latter was obtained by solubilising DNA (i) in water (pH 4.5), (ii) in 0.15 M NaCl solution at a pH of 4 adjusted by trifluoroacetic acid or (iii) in 1 mM Tris(hydroxymethyl)aminomethane buffer (Trizma buffer) containing 0.15 M NaCl at pH ranging from 6 to 8 adjusted by 1 N NaOH. The suspensions of complexes were gently vortexed. At this stage, the DOGS concentration was 6.10^{-2} mM and the lipid/DNA ratio was 8.1:1 (w/w). The corresponding theoretical charge ratio is 6 when three of the four amino groups of DOGS are ionised [13]. The pH of complex suspensions was measured by using a pH microprobe (3 mm diameter). Immediately after preparation, the suspensions of complexes were added on cell culture. It is worth noting that the culture medium pH ($\approx$8.0) was not significantly modified upon addition of condensation medium.

For cryo-TEM study, DOGS concentration was 6 mM and DNA concentration was 1 mg/ml, ensuring the same lipid/DNA ratio as for the transfection study. DNA condensation was performed from a 100 mM DOGS aqueous suspension. Contact between DNA and lipid was achieved either in water (pH=4), immediately before vitrification by freezing or in 0.15 M NaCl at pH 8 (adjusted by NaOH 1 N), 3 h before. The 3-h maturation at pH 8 has been found not to modify substantially transfection efficiency [29].

For X-ray studies, DOGS concentration was, in each case, 8% (w/w_{total}), i.e. 59 mM. For examination of DOGS–DNA complexes, DNA concentration was 1% (w/w_{total}). The DOGS/DNA ratio is therefore the same as for the transfection studies. The samples were analysed either in water (pH $\approx$4) or in 0.15 M NaCl at pH 8 (adjusted by 1 N NaOH), 1 week after preparation.

2.4. Transfection assays

NIH 3T3 cells (mouse fibroblast) were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL ref. 41965) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 50 000 IU/l penicillin, 50 000 IU/l streptomycin, in which 10% FCS (Gibco-BRL) was added. Cells were seeded in 24-well culture plates with 2.10^4 cells per well the day before transfection and incubated at 37°C in 5% CO_2. Just before transfection, cells were washed twice in 0% FCS. Then, 50 μl of resuspended pre-formed complexes was added to each well. This 50 μl correspond to 3 nmol lipid and 0.5 μg DNA in 250 μl of supplemented culture medium (with or without FCS) per well. Dishes were placed at 37°C in 5% CO_2. After 4 h of transfection step, complex suspensions were replaced by 0.5 ml of supplemented culture medium containing 10% FCS. After 48 h culture, cells were washed twice with 250 μl phosphate-buffered saline solution. A volume of 250 μl lysis buffer (25 mM Tris-phosphate, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, 10% glycerol and 1% Triton X-100) was then added and dishes were gently shaken for 20 min. Luciferase expression was quantified on 5 μl of centrifuged lysate supernatant, using a luciferase assay kit (Promega). Light emission was measured by integration over 10 s at 25°C using a luminometer Lumat LB 9501 (EG&G, Berthold, Evry, France). Relative light units (RLU) were calculated versus background activity. The total protein concentration was also measured by the BCA protein assay reagent (Pierce) to make sure that variations in luciferase activity were not to be attributed to cellular survival or to complex toxicity. Its influence on transfection efficiency was found negligible, so that RLU measurements were not standardized according to this parameter.

2.5. Cryo-TEM studies

Copper or nickel electron microscope grids (3 mm) were used as specimen support for vitrification. These grids were covered by a perforated plastic film (holes from 1 to 10 μm) coated with carbon on both sides. Just before specimen deposition, the grids were made hydrophilic by glow discharge in 0.1 T air, in order to obtain optimum wetting of the support film. A small (5 μl) droplet of the suspension was then applied to the grid and blotted with a filter paper to reduce the thickness of the liquid film to about 30–200 nm, forming menisca on the holes. The grid was then immediately plunged in liquid ethane at a temperature ranging from −184 to −188°C. Blotting and plunging were performed in a continuous stream of water saturated air to prevent evaporation. The ultra-fast cooling allows complete vitrification of the specimen, which can be checked by the absence of ice crystals reflections in the film on electron diffraction patterns. Just after vitrification, grids were stored and transferred, in liquid nitrogen, onto a GATAN 626 cryo-specimen holder for TEM observation, using a dedicated cryo-transfer device. The TEM used was a Philips CM 12 equipped with a low-dose facility which reduces electron damage to a minimum. Specimens were equilibrated in the microscope column at a temperature close to −160°C, ensuring no structural changes. They were examined at 120 kV using original magnification not greater than 35 000×.

2.6. High-resolution X-ray experiments

Measurements were performed on D22 beam line at LURE (Orsay, France). Sample-to-detector distances were 625 mm (for DOGS alone) or 1690 mm (for DOGS–DNA complexes). Data were collected for 1200, 1800 or 2400 s, by means of a 1024-channel position-sensitive linear Xe-filled detector. Samples were introduced in calibrated thickness polyimide discs after preparation. The patterns were normalised according to the incident beam intensity, sample transmission and acquisition time. For each pattern shown in this paper, continuum pattern was subtracted from sample's one.

3. Results

The conditions of DNA condensation by DOGS are critical for the structural organisation of the mixed aggregates they form. This mainly arises from the polyelectrolytic nature of both compounds and from the amphiphilic character of DOGS molecules. Direct determination of the impact of all these conditions on aggregate structures does correspond to an extensive work. Hence, in a preliminary study, transfection efficiency was measured as a function of various physicochemical parameters chosen during DNA condensation: DOGS and DNA respective concentrations, pH, temperature, ionic strength, counterion nature, presence or absence of ethanol and durations of the different steps of DNA/lipid complex preparation protocols [29]. In the present work, conditions for complex formation used were chosen for both reproducibility and significant differences of the transfection results. On the other hand, considering that DOGS is usually introduced in the condensation medium by means of an ethanolic solution [12,13,18], the influence of ethanol was examined in parallel.

Fig. 1 shows the influences on transfection efficiency, with or without FCS, of the presence of NaCl and ethanol in the condensation medium as well as the pH of the latter. In the absence of FCS, no significant difference in transfection efficiency is noticed whatever the condensation protocols, except in the case of pure water. In contrast, when FCS is present in the transfection medium, a great variability of transfection efficiency is evidenced according to the DNA condensation conditions. Transfection rates, at least as high as those found without FCS, can be maintained in presence of serum when condensation is performed in 0.15 M NaCl at pH 8.3. Lower condensation pH led to an important decrease in gene transfer. Indeed, transfection is reduced by two orders of magnitude when condensation is performed in pure water at a native pH of 4.5.

In FCS, the protocols using initial ethanol DOGS solution (Fig. 1a) instead of non-vesicular DOGS aqueous dispersion (Fig. 1b) show similar effects of condensation pH. However,

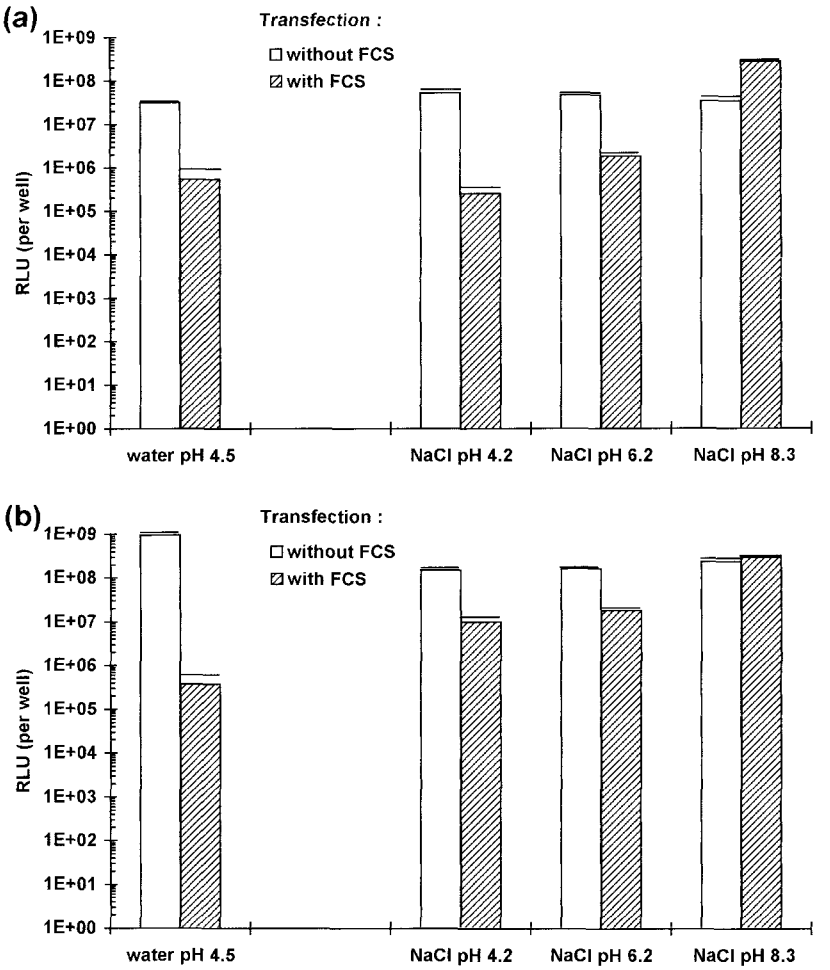

Fig. 1. Influence on transfection efficiency of pH and ionic strength (NaCl addition) during the compaction step, starting from a 2 mM ethanolic DOGS solution (a) and non-ethanolic suspension (b). Contact between lipid and DNA is achieved in 0.15 M NaCl at pH ranging from 4.2 to 8.3 or in pure water (pH = 4.5). Transfection step is performed in the presence (hatched bars) or the absence (open bars) of fetal calf serum (FCS). DNA uptake by cells is expressed in relative light units per well (RLU/well). The reported transfection efficiencies correspond to four measurements. The SD is represented by a horizontal bar at SD+mean value.

the decreases of transfection rates observed with decreasing pH are more pronounced in the presence of ethanol (three orders of magnitude for pH changes from 8.3 to 4.2). At a pH close to 4, transfection efficiency is not influenced by the presence of NaCl in condensation medium when ethanol is used, whereas this salt favours gene transfer by more than one order of magnitude in the absence of ethanol.

The structures of the complexes displaying high shift between their transfection efficiency in the presence of FCS, i.e. prepared in water at native pH and in 0.15 M NaCl at pH 8, were studied and compared. Fig. 2b,d shows their respective cryo-TEMs. The nucleolipidic particles have been identified by comparing micrographs of similarly prepared samples containing no DNA (Fig. 2a,c). Pure DOGS structures will be described in a future paper [29].

DNA condensation in water at a low pH gives rise to particles the transfection ability of which is partly inhibited in the presence of FCS. Cryo-TEM of this sample (Fig. 2b) shows large micrometric aggregates composed of roughly spherically shaped particle units, attributed to individual nucleolipidic complexes. These individual particles display a size ranging from 40 to 200 nm. A continuous covering of pure spherical micelles ($\approx$3 nm) is also observed in the background. The comparison of Fig. 2a and 2b allows to identify these micelles as DOGS excess. Some vesicles are observed but they only represent a small fraction of the whole lipidic material.

When compaction is performed in 0.15 M NaCl at pH 8 (Fig. 2d), lamellar aggregates corresponding to the nucleolipidic complexes are observed with a well-organised internal packing. The apparent complex density evidenced by a higher contrast, by comparison with the previous samples, indicates that increasing the pH and ionic strength induces a stronger condensation of the particles. Interestingly, in the absence of DNA, vermicular structures are predominantly viewed (Fig. 2c). In fact, a 3-h incubation period does not allow the lipid to reach equilibrium structure. Indeed, a much slower evolution towards a lamellar phase has been shown [29]. Thus, DNA would accelerate the process of lamellar phase formation (Fig. 2d).

The small angles X-ray scattering study, performed on both types of complexes, allowed to characterise the internal structure of the particles. Again, for each analysis, the nucleolipidic structures were compared to those of samples prepared from pure DOGS, strictly under the same conditions. X-ray diffraction pattern of DOGS–DNA complexes of low efficiency in the presence of FCS (prepared in water at native pH) is not

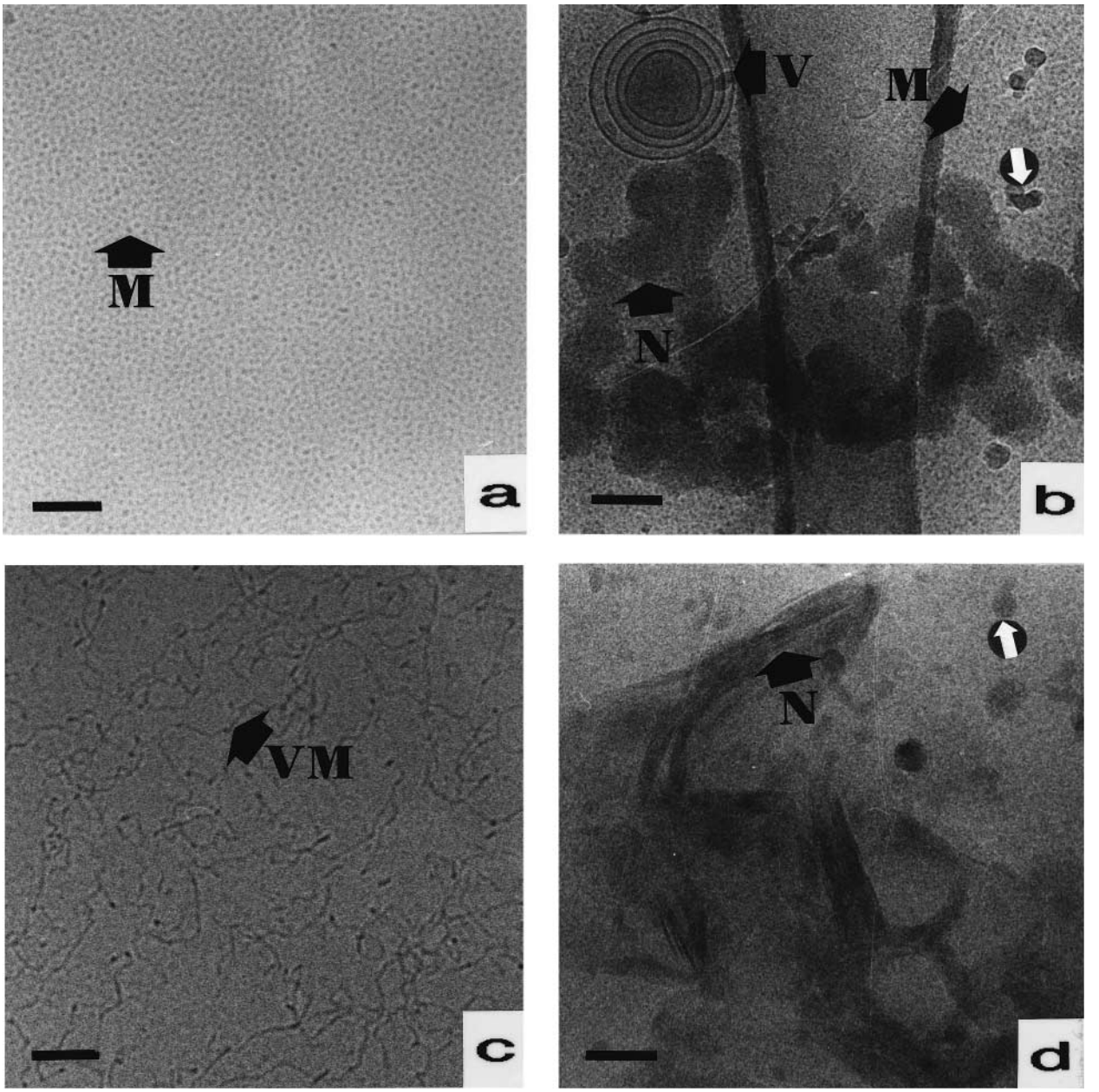

Fig. 2. Cryo-transmission electron microscopy of DOGS (a,c) and DOGS/DNA complexes (b,d). a,c: DOGS in water at native pH = 4 (a) and in a 0.15 M NaCl solution at pH = 8 (c). DOGS concentration is 6 mM. b,d: DOGS/DNA complexes in water at native pH = 4 (b) and in a 0.15 M NaCl solution at pH = 8 (d). DOGS and DNA concentrations are, respectively, 6 mM and 1 mg/ml. Filled arrows: DOGS/DNA complexes (N), DOGS micelles (M), DOGS vermicular micelles (VM) and vesicles (V). Open arrow: ice. Bar: 100 nm.

significantly modified as compared to that of pure DOGS structures under the same conditions (Fig. 3a,b). For both pure DOGS and DOGS–DNA complex samples, the absence of a narrow diffraction peak and the presence of a broad signal, located at about 0.12 $Å^{-1}$ and attributable to particle scattering, suggest the existence of small aggregates. This is in agreement with the observation of spherical micelles by cryo-TEM (Fig. 2a,b) and of an isotropic solution for pure DOGS or a slightly turbid one for DOGS–DNA mixture. Moreover, in this concentration range, the interactions between micelles lead to the appearance of a structure factor corresponding to the first peak located at 0.046–0.047 $Å^{-1}$. DNA compaction would only lead to some kind of agglomeration of the micelles, through DNA bridging, forming the observed DNA–DOGS particles. Both shape and size of the micellar unit appear to be unaltered since the position of the form factor is quite unchanged. The apparent increase in intensity of the micelle signal through DNA addition (see Fig. 3b) is likely due to the small angle scattering of very large aggregates that have been depicted by cryo-TEM (see Fig. 2b).

The structure of complexes keeping their transfection efficiency in the presence of FCS (prepared in NaCl 0.15 M at pH 8) is clearly of lamellar type (Fig. 3d). Two long spacings corresponding to the first (64.5 Å) and second (32.4 Å) orders of a multilayered structure are evidenced. This is in agreement with the cryo-TEM pictures (Fig. 2d) and the high turbidity of the samples. In 0.15 M NaCl at pH 8, pure DOGS also forms lamellar structures (Fig. 3c and [29]) evidenced by two weak

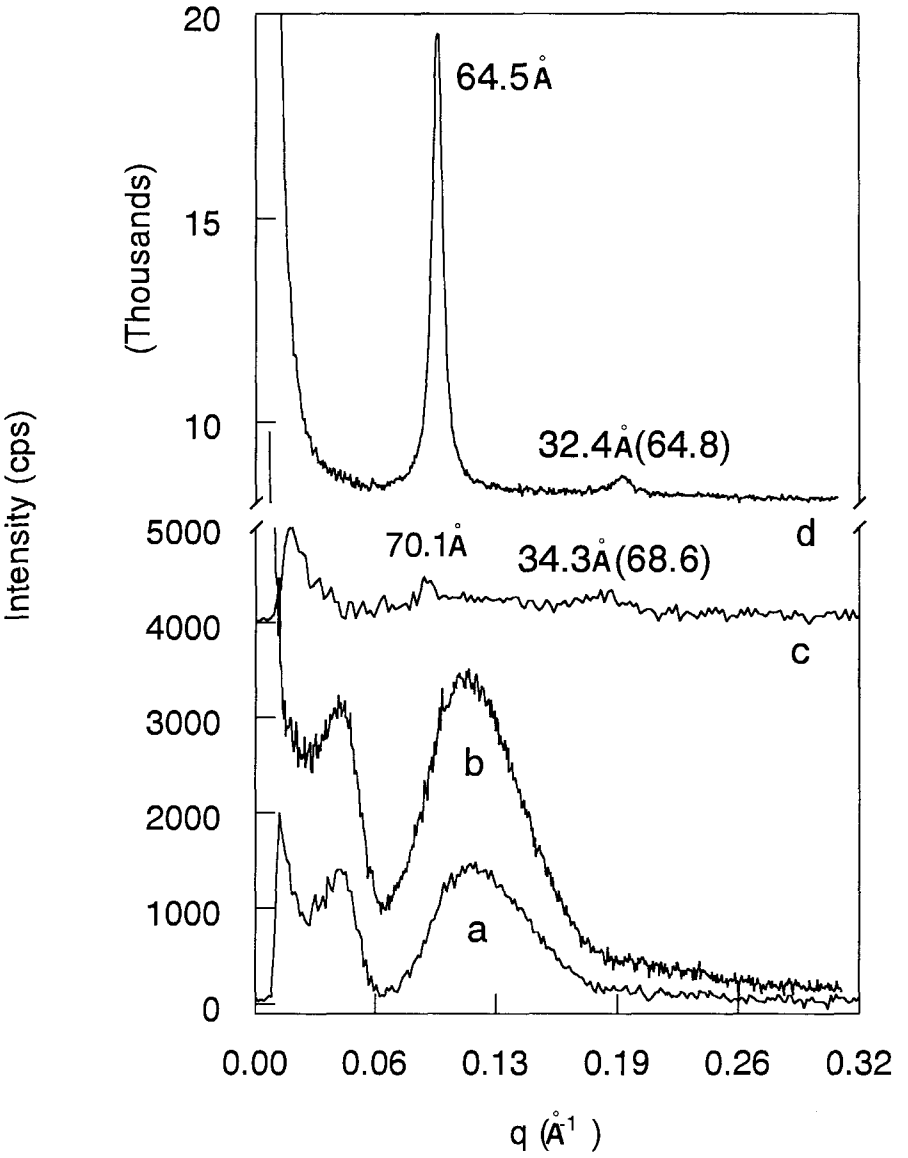

Fig. 3. Small-angle X-ray diffraction patterns of DOGS (a,c) and DOGS/DNA complexes (b,d). a,c: DOGS in water at native pH = 4 (a) and in a 0.15 M NaCl solution at pH = 8 (c). DOGS concentration is 8% (w/w_{total}). The diffraction intensity has been multiplied by two in (c). b,d: DOGS/DNA complexes in water at native pH = 4 (b) and in a 0.15 M NaCl solution at pH = 8 (d). DOGS and DNA concentrations are, respectively, 8% w/w_{total} and 1% w/w_{total}.

diffraction peaks corresponding to a period of about 70 Å. This last lamellar organisation seems not to be regular enough to reach narrow diffraction peaks. Possible reasons for the decrease in diffraction intensity might be a change in electronic contrast between the two types of aggregates, i.e. with and without DNA, explained by a variation of the mean composition of the aqueous layers and a better long-range organisation of the bilayers through DNA–DOGS interactions.

4. Discussion

Phosphate residues, distributed all along the DNA molecule backbone, induce strong intramolecular electrostatic repulsions responsible for its expanded structure. Their neutralisation by water-soluble polycations, like spermine and its polyamine analogues, results in DNA condensation. However, the water solubility of this type of cationic compounds jeopardises the stability of the complex formed by favoring its dissociation in physiological conditions. Linkage of these hydrophilic polycations to long aliphatic chains circumvents this drawback [18] by decreasing drastically their water solubility and stabilising complex formation through its phase separation. Moreover, the self-associating properties of the cationic lipids lead to the formation of polycationic macrostructures which persist in the presence of DNA, enforcing the plasmid condensation. The combination of these effects is complex and the resulting structures are highly dependent on the mixing conditions of both types of components, especially when long-chain lipospermines, like DOGS, are implicated. The variations of parameters such as concentration, pH, temperature, ionic strength and kinetic have been shown to induce

strong structural changes in DOGS polymorphism [29]. At pH lower than 4.6, all amino groups are protonated and DOGS behaves as a surfactant forming spherical micelles, whereas at pH 8 nearly two amino groups remain ionised allowing a lamellar arrangement.

The rapid growth of highly organised lamellar DNA–lipid structures apparently only occurs when the repulsions between lipid polar heads are decreased. Therefore, different protocols can lead to the structure of nucleolipidic complexes. Considering the polyamine nature of DOGS, the easiest way to obtain the desired organisation is to increase slightly the pH of the condensation medium up to 8 and therefore to decrease the average ionisation of DOGS. The use of a saline solution is also a good means of facilitating the multilayer packing by the shielding effect provided by the solubilised salts (NaCl).

In the conditions of the present study, DOGS polymorphism is preserved upon addition of DNA. Cryo-TEM and X-ray diffraction results suggest that DNA added to DOGS micelles in acidic medium does not modify the latter but would wrap around them originating the observed nanometric supramicellar assemblies. Similarly, at pH 8, comparison of the two lamellar structures, with and without DNA, shows that DNA obviously improves the organisation of DOGS bilayer stacking, even accelerating it, as seen by the combined observations of cryo-TEM and X-ray diffraction. In addition, conservation of the lipid lamellar periodicity in the presence of nucleic acid indicates that the DNA molecules are likely positioned in the aqueous spaces, parallel to the bilayers. In both conditions studied, the formation of nucleolipidic complexes proceeds from a *mutual condensation* of the two components.

Gene transfer via cationic lipids has been reported to be reduced markedly when the transfection media contained serum [7,32,33]. Our study has established conditions that maintain transfection efficiency in the presence of serum, authorising promising improvements in in-vivo application. Such conditions involve a lamellar organisation of the internal structure of the complexes, probably alternating condensed DNA layers and lipid bilayers, as also was observed for other cationic lipids [21,22]. This organisation seems to be one of the prerequisites needed to overcome the effect of serum inhibition, although further optimisation should be reached to succeed in in-vivo transfection.

In DMEM containing no serum, transfection efficiency does not vary according to compaction conditions. Indeed, whatever the starting DOGS structural organisation (micellar or lamellar), the DMEM pH ($\approx$8) and its ionic composition lead to a lamellar phase at the transfection step (data not shown). Therefore, all nucleolipidic particles become efficient on gene transfer. In contrast, in culture media containing FCS, the fact that the transfection efficiency of micellar complexes preformed in water at native pH is significantly reduced may suggest that their evolution towards DOGS–DNA lamellar particles is hindered. This can be due to interaction of the particles with serum proteins among which albumin is well known for its colloidal protection properties (emulsion and suspension stabilising agent [35,36]) and its high affinity for polycations [37]. This drawback is avoided when lamellar structure is adopted from DNA condensation step at 0.15 M NaCl at pH 8.

The correlations between lipid organisation and transfection efficiency depicted for DOGS could explain why cationic surfactants that are only able to form micellar structures are inefficient [4,14] except when mixed with a lipid 'helper' able to form lamellar phases in association with them [4,16].

The formation of lamellar structures, when favourable conditions for transfection are encountered, is confirmed by cryo-TEM and by X-ray diffraction. This is in agreement with the phases previously observed by cryo-TEM [38,39], by recent X-ray studies [20,21] and by the use of alkaline pH for stabilisation of DOPE lamellar phases [40]. Although, they do not agree with a recent TEM study concluding, also for DOGS–DNA complexes, to the hexagonal phase formation [18]. The cryo-TEM technique used in our study allows the preservation of structures [41] and avoids artefacts due to sample drying or staining. Moreover, the lamellar structure is confirmed by X-ray diffraction.

The present study points out that the DOGS–DNA association is strongly governed by lipid polymorphism. Gene delivery is allowed when a multilayered lamellar arrangement of the complex is formed. Understanding of mutual condensation of DNA and DOGS and control of the transfection mechanisms need a systematic examination of the physicochemical parameters influencing DOGS aggregative behaviour and polymorphism. Such experiments are underway.

Acknowledgements: This work was part of the 'BioAvenir' program supported by Rhône-Poulenc with the participation of the French Ministry of Research and the French Ministry of Industry. We thank Bruno Pitard and Anne-Marie Lachagès for plasmid supplying, Gerardo Byk for DOGS synthesis, Pierre Lesieur for help in X-ray diffraction, Bertrand Schwartz, Catherine Dubertret, Ravi Rangara, Gabrielle Jaslin for their support in transfection studies. Daniel Scherman, Michel Veillard, Didier Bazile, Gilles Spenlehauer and Joël Crouzet are gratefully acknowledged for receiving this research program in their laboratories.

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