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Physico-chemical aspects of the interaction between DNA and oppositely charged mixed liposomes

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Abstract

The mechanism of complex formation between DNA and oppositely charged dioctadecyldimethylammonium bromide/dioleoyl phosphatidylethanolamine (DODAB/DOPE) and 1,2-dioleoyl-3-trimethylammonium propane (DOTAP)/DOPE mixed liposomes, as well as the physico-chemical properties of DNA–mixed liposome complexes, were examined. Fluorescence microscopy showed that the interaction between DNA and oppositely charged mixed liposomes started at very low liposome concentrations and induced a discrete coil–globule transition in individual DNA molecules. The DNA size distribution was bimodal in a wide range of liposome concentrations. The critical concentration of the cationic lipid needed for the complete compaction of single DNA molecules depended on the composition of the charged mixed DODAB/DOPE and DOTAP/DOPE liposomes. Cryogenic transmission electron microscopy (cryo-TEM) observations of DNA complexes with mixed liposomes revealed that the lamellar packing of lipid molecules was typical for the complexes formed from the cationic lipid-enriched mixtures, while inverted hexagonal arrays were found for the neutral lipid-enriched complexes. The microstructures of the complexes were also examined with the use of the small-angle X-ray scattering (SAXS) technique, which confirmed the results obtained by cryo-TE microscopy and enabled the quantitative characterization of lipid packaging in the complexes with DNA macromolecules. We also found that the introduction of the neutral lipid into the complexes between DNA and oppositely charged lipids, DODAB and DOTAP, moderately increased the thermal stability of the complexes and changed the quantitative characteristics of the melting profiles of the complexes.

Keywords: DNA–mixed liposomes complexes; DNA compaction and condensation; Cryogenic transmission electron microscopy; Fluorescence microscopy; X-ray scattering; DNA–lipid complexes; Melting

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1. Introduction

Complexes between DNA and cationic liposomes are considered as potential vehicles for the gene transfer [1,2]. Despite some recent reports on the cytotoxicity of cationic liposomes [3], they are widely used for the DNA transfection in vitro and in vivo. Also, some of the cationic liposome products, which frequently consist of both cationic and neutral lipids, have firmly established themselves on the pharmaceutical market [4,5]. Unfortunately, the efficiency of transfection in the presence of positively charged liposomes is often unpredictable and dramatically depends on various factors, such as the composition of liposomes, i.e. the ratio between cationic and neutral lipid, preparation conditions, the way of the introduction of DNA–liposome complexes to the cell cultures, etc. [1,2,4–8]. One of the important factors that affects the efficiency of DNA delivery through the biological membranes, is a choice of a neutral helper lipid and its role in the transfection process.

Recent studies have demonstrated that the introduction of a neutral lipid into the cationic liposomes substantially increases the fluidity of the patient cell membrane and, therefore, facilitates the penetration of genetic material into the cell [9]. At the same time, the effect of cationic liposome composition on the morphology of lipid mixtures in aqueous solutions was investigated by using cryo-transmission electron microscopy (cryo-TEM), and a tendency toward polymorphism of the lipid mixtures was reported [10]. Recently, besides the empirically established particularities, Koltover et al. [9] reported that the novel structure of DNA–mixed cationic liposomes complexes was formed for the lipid mixtures with a high neutral lipid content. Such inversed hexagonal structures of complexes were found to be more efficient for the transfection of mammalian cells, compared to the previously reported lamellar complexes [11–13].

The dynamic behavior of individual DNA molecules in extremely diluted aqueous solutions in the presence of mixed cationic dioctadecyldimethylammonium bromide/dioleoylphosphatidyl ethanolamine (DODAB/DOPE) liposomes is also largely affected by the mixed liposome composition. We have recently shown with the use of the fluorescence microscopy technique that the compaction of single large linear dsDNA molecules strongly depends on the composition of the cationic mixed liposomes, i.e. molar ratio between cationic DODAB and neutral DOPE in the liposomes [13]. However, the fluorescence microscopy, which allows the observation of the dynamics of single DNA molecules in aqueous solutions and, therefore, provides the information that is unattainable from any other experiments, has certain limitations. First, since the size of liposomes is often much less than the wavelength of the fluorescence light, the fluorescence microscopy could not be applied for the monitoring of mixed liposomes in the solution, even if the liposomes are stained with a fluorescent marker. Also the results from the fluorescence microscopy experiments do not answer the question on the microscopic structure of complexes between DNA and mixed liposomes. Taking into account these limitations of the fluorescence microscopy method, a more detailed study on the peculiarities of DNA–mixed liposome interaction is needed for the better understanding of physicochemical properties of DNA–mixed cationic liposomes systems with respect to their transfection efficiency.

In the present study we examined the physicochemical properties of complexes between DNA and oppositely charged mixed liposomes, as well as the peculiarities of the complex formation, with respect to the understanding of the role of the liposome composition in the enhancement of the efficiency of DNA–liposome complexes for the gene transfection. The mechanism of interaction and the effect of liposome composition on the coil–globule transition in individual DNA molecules were studied in extremely diluted solutions with the fluorescence microscopy technique. The microstructures of formed complexes between DNA and cationic liposomes were observed by cryo-TEM, whereas small-angle X-ray scattering was applied to determine the structure of lipid aggregates in the complexes. Melting behavior of DNA–liposome complexes, i.e. the he-

lix–coil transition in double-stranded DNA, and effect of neutral lipid content on the thermal stability of complexes were studied with the use of UV spectroscopy technique. Finally, we discuss the differences in physico-chemical properties of the cationic liposome-based gene-delivery systems at various compositions of cationic and neutral lipid mixtures with respect to their applications.

2. Materials and methods

2.1. Materials

The neutral (zwitterionic) lipid, dioleoyl phosphatidylethanolamine (DOPE) and cationic lipids, 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and dioctadecyldimethylammonium bromide (DODAB), were used as purchased (Avanti Polar Lipids, Alabaster, AL, USA). Solutions were made using deionized water that was further purified with a Milli-Q Plus filtration system with a pore size of 22 μm (Millipore Corp., Bedford, MA, USA). The cationic liposomes of mixed lipids at different molar ratios between neutral and charged lipids were generated by sonication of aqueous solutions of mixed lipid films, processed from lipid stock solutions in chloroform, at 50°C for DOTAP/DOPE mixtures and at 60°C for DODAB/DOPE mixtures, until clear. Taking into account that the neutral lipid, DOPE (unsaturated, 16:1c9/16:1c9), undergoes $L_\alpha \rightarrow H_{II}$ transition at 43°C [14], the liposomes enriched with DOPE were never heated at temperatures > 35°C to avoid the phase transformations in the lipid mixtures. Cationic liposomes of mixed lipids were qualitatively and quantitatively characterized by cryo-TEM.

Salmon sperm DNA (Gibco BRL, Life Technologies, Inc., Gaithersburg, MD, USA) used for the cryo-TE microscopy and UV-spectroscopy experiments was purified by the extraction from 1:1 (vol:vol) phenol:chloroform mixtures. Bacteriophage T4 DNA (1.1×10^8 Da, approx. 167 kb) was purchased from Sigma and used without further purification. DNA concentration was determined by measuring the absorbency of its stock solution at a wavelength of 260 nm; the molar extinction coefficient for DNA bases at this wavelength was equal to 6600 M/cm. The ratio of the absorbance of a DNA stock solution at 260 nm to that at 280 nm was determined to be 1.80 for salmon sperm DNA and 1.85 for bacteriophage T4 DNA. For the fluorescence microscopy experiments bacteriophage T4 DNA was labeled with a fluorescence dye, 4′,6-diamidino-2-phenylindole (DAPI). DAPI and an antioxidant for the fluorescence microscopy, 2-mercaptoethanol (ME), were from Sigma.

Cationic liposome-DNA complexes used for the cryo-TEM and UV-melting experiments were prepared by mixing aqueous solutions of DNA and cationic liposomes of mixed lipids in a 1:5 DNA/cationic lipid molar ratio, which resulted in positively charged complexes avoiding a precipitation [12]. Complexes between DNA and cationic liposomes used for the small-angle X-ray scattering (SAXS) were prepared at nearly equimolar ratio of components, with a cationic lipid concentration above 0.1 mM that led to the formation of precipitates. All experiments were made within 24 h from the DNA–liposome complex preparation.

2.2. Melting temperature measurements

Melting temperatures (T_m) for free DNA and DNA–liposome complexes were measured by following the changes in absorption at 260 nm (A_{260}) as a function of temperature in a DMS 100 UV-Vis spectrophotometer (Varian, Australia). The absorbance intensities at 260 nm were plotted against individual temperatures and the midpoints of the inflection region in the temperature-A_{260} curves were taken as the corresponding T_m values. The corresponding mixed lipid dispersions of the same concentrations as those in the measured cuvette have been used as reference solutions to compensate the effect of melting of lipid mixtures.

2.3. Cryogenic transmission electron microscopy (cryo-TEM)

Specimens for electron microscopy were pre-

pared in a controlled environment vitrification system (CEVS) to ensure fixed temperature and to avoid water losses from the solution during sample preparation. The specimens were prepared as thin liquid films, < 0.3 mm thick, on lacey carbon films, supported by a copper grid and quenched into liquid ethane at its freezing point. The technique was described in detail by Bellare et al. [15]. The technique leads to vitrified specimens, so that component segmentation, rearrangement and water crystallization are prevented and original microstructures are preserved during thermal fixation. The vitrified specimens were stored under liquid nitrogen and transferred to the electron microscope (Philips CM 120 BioTWIN) equipped with a post-column energy filter, using an Oxford CT3500 cryoholder and its workstation. The acceleration voltage was 120 kV and the defocus was approximately 1 μm. Magnification of 55 000 allowed a pixel width of 5 Å. Images were collected under low dose conditions, with the dose being < 0.1 electron per nm^2. The images were recorded digitally with CCD camera (Gatan MSC791).

2.4. Fluorescence microscopy

The fluorescence microscopy samples containing DNA aqueous solutions in the presence of mixed cationic liposomes were prepared as follows: DNA stock solutions were diluted with the 10 mM Tris–Cl buffer (pH 7.6) containing 4% (v/v) ME, a free-radical scavenger, and a fluorescent dye, DAPI. The resulting solution was gently mixed with the aqueous liposome solutions and was then kept for 2 h before the observation. The final concentrations were as follows: DNA in nucleotide units, 0.5 μM; and DAPI, 0.5 μM. Under these conditions, the binding number of DAPI per 1 DNA base pair in an aqueous buffer solution was estimated to be equal to 0.05 and the persistence length of DNA chain was expected to remain nearly the same as in the absence of DAPI [16,17].

A fluorescence microscopy study was performed as follows: the samples were illuminated with a UV-mercury lamp; the fluorescence images of single DNA molecules were observed using a Zeiss Axioplan microscope, equipped with a 100 × oil-immersed objective lens, and digitized on a personal computer through a high-sensitive SIT video camera and an image processor, Argus-20 (Hamamatsu Photonics, Japan). The apparent long-axis length of the DNA molecules, L, was defined as the longest distance in the outline of the fluorescence image of single DNA. Images of the dynamic motion of single DNA molecules in aqueous solutions were taped with a conventional S-VHS video recorder. The observations were carried out at 25°C. Special care was taken to clean the microscope glasses (No. 0, Chance Propper, England) thoroughly before the observation to prevent DNA degradation, as well as precipitation to the glass surface [18].

2.5. Small-angle X-ray scattering (SAXS)

SAXS measurements were performed on a Kratky compact small-angle system equipped with a position-sensitive detector (OED 50M, MBraun, Austria) containing 1024 channels of width 53.0 μm. Cu Kα radiation of wavelength 1.542 Å was provided by a Seifert ID-300 X-ray generator, operating at 50 kV and 40 mA. A 10-μm thick Ni filter was used to remove the Kβ radiation, and a 1.5-mm W filter was used to protect the detector from the primary beam. The sample-to-detector distance was 277 nm. To minimize the scattering from air, the camera volume was kept under vacuum during the measurements. A Peltier element controlled the temperature within 25°C. The obtained Bragg peaks were relatively sharp, assuring that in each case the peak position can be evaluated from the slit-smeared SAXS data.

3. Results

3.1. Fluorescence microscopy

To understand better the effect of a neutral lipid, DOPE, on the peculiarities of the compaction of individual DNA molecules in extremely diluted solutions, the fluorescence mi-

croscopy observations were carried out on aqueous solutions of bacteriophage T4 DNA in the presence of mixed DODAB/DOPE and DOTAP/DOPE liposomes.

First, the dependence of the higher order structure of single DNA molecules on the concentration of positively charged mixed DODAB/DOPE liposomes was examined. We found that the interaction between DNA chains and oppositely charged liposomes started at the extremely low lipid concentrations in the solution, below the concentration of DNA used in our experimental conditions. When the mixed liposomes were introduced into the aqueous buffer DNA solution, the single DNA molecules exhibited two limiting conformational states even at the lowest studied liposome concentrations. They were characterized by substantially different linear dimensions and fluorescence intensities and corresponded to an extended coil and a compact globule conformations (Fig. 1A). When the liposome concentration reached a critical value, the conformational distribution of DNA chains became unimodal and all DNAs were found to be in a collapsed globular conformation (Fig. 1B). Our present observation implied that in our experimental system the disproportionation mechanism dominated [19]. Therefore, even at lowest concentrations of mixed liposomes there was no binding of minor lipid fractions onto all DNA molecules in the solution. Oppositely to that, some DNA molecules were entirely occupied with the oppositely charged lipid molecules, while some DNAs remained free. This result proved high cooperativity of the lipid binding to DNA molecules in aqueous solutions and agreed well with the earlier results on the DNA interaction with the oppositely charged synthetic surfactants [20–22].

As a next step, DNA conformational behavior in the presence of mixed DOTAP/DOPE liposomes was studied. In this case, the interaction also started at very low liposome concentrations in the solution, similarly to the trend in DODAB/DOPE-DNA systems. For all of the studied liposome compositions the regions of the existence of compact DNA globules followed by the coexistence region, where DNA coils and globules were observed simultaneously. Fig. 2 exemplifies the phase diagram of single DNA molecules in the presence of DODAB/DOPE and DOTAP/DOPE liposomes with various molar ratios between the cationic and neutral lipids. It can be

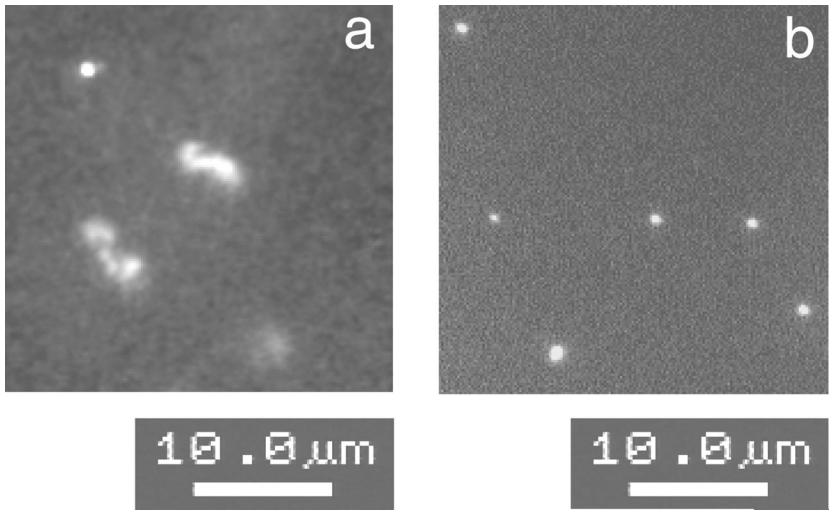

Fig. 1. Fluorescence microscopy images of individual T4 DNA molecules in the presence of DODAB/DOPE liposomes. (A) Coexistence of coiled and globular T4 DNA in the presence of 1:1 mol/mol DODAB/DOPE liposomes, [DODAB] = 1.0×10^{-6} M; (B) compacted T4 DNA chains in the presence of 1:5 mol/mol DODAB/DOPE liposomes, [DODAB] = 1.0×10^{-4} M.

seen from the phase map in Fig. 2 that the DNA–liposome interaction in the presence of DOTAP/DOPE liposomes also was highly cooperative, and the disproportionation mechanism dominated under these conditions. The width of the coexistence region was quite substantial, compared to that reported from studies of DNA-cationic surfactant interactions [23–25]. The results correlated well, both with the recent data from studies of DNA conformational behavior in the presence of DODAB [26] and with the modern theory of polyelectrolyte collapse [27,28]. It can be also clearly seen from Fig. 2 that the concentration of mixed liposomes expressed in terms of the molar concentration of cationic lipid, which corresponded to complete DNA collapse in the solution, depended on the liposome composition, i.e. on the molar ratio between cationic and neutral lipids for both DODAB/DOPE and DOTAP/DOPE liposomes. However, there were clear differences between DODAB and DOTAP. First, the concentrations of mixed liposomes, which corresponded to the complete DNA collapse in the solution, were much higher for DOTAP/DOPE liposomes, than those for DODAB/DOPE liposomes (Fig. 3). This observation concurred well with the recently published results on DNA–lipid interaction in concentrated solutions [8]. As a result, the slope of the dependencies of C_{cr} (the critical concentration of cationic lipid needed for complete DNA collapse in the sample) on the concentration of positively charged lipid in the system was quite different, i.e. the C_{cr} decreased much more with the increase of the Y-parameter for DODAB/DOPE liposomes.

It is commonly accepted that the interaction between cationic liposomes and DNA in aqueous solution, which leads to the formation of condensed structures, occurs at nearly equimolar ratio of components in the solution [6]. However, our results showed that with the increase of the neutral lipid content in a liposome, the amount of cationic lipid for complete DNA collapse also increased, significantly in the presence of DODAB and slightly in the presence of DOTAP. Therefore, this should be helpful for the rational development of a delivery system for therapeutic purposes, since it allows to predict the optimal amount of both cationic lipid and neutral helper lipid necessary for the DNA delivery. It should also be noticed that this result can hardly be perceived with the use of other experimental methods, since only FM allows the observation of single-molecular DNA dynamics in the solution.

3.2. Cryo-TE microscopy

The fluorescence microscopy technique, which gives unique information on the dynamics of

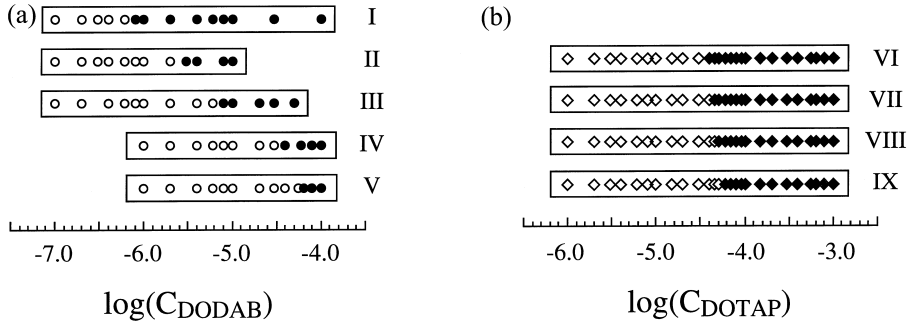

Fig. 2. (a) Phase diagram of T4 DNA–DODAB/DOPE liposome system at 25°C in 10 mM TE buffer (10 mM Tris–Cl, 1 mM EDTA, pH 7.6) obtained with the fluorescence microscopy at various DODAB/DOPE molar ratios: (I) $Y = 1.00$; (II) $Y = 0.80$; (III) $Y = 0.50$; (IV) $Y = 0.17$; (V) $Y = 0.09$, where $Y(\text{DODAB}) = [\text{DODAB}]/([\text{DODAB}] + [\text{DOPE}])$. (b) Phase diagram of T4 DNA–DOTAP/DOPE liposome system at 25°C in 10 mM TE buffer (10 mM Tris–Cl, 1 mM EDTA, pH 7.6) obtained with the fluorescence microscopy at various DOTAP/DOPE molar ratios: (VI) $Y = 1.00$; (VII) $Y = 0.80$; (VIII) $Y = 0.50$; (IX) $Y = 0.20$, where $Y(\text{DOTAP}) = [\text{DOTAP}]/([\text{DOTAP}] + [\text{DOPE}])$. C_{DODAB} and C_{DOTAP} correspond to the molar concentrations of DODAB and DOTAP in the mixed liposomes.

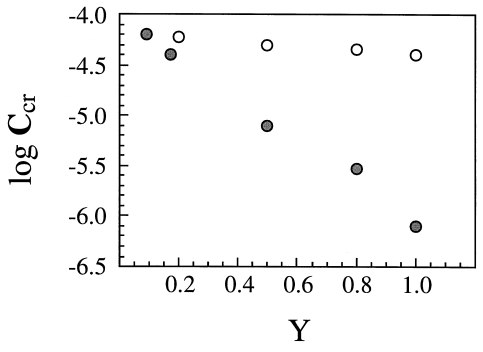

Fig. 3. Dependence of the critical concentration of cationic lipid (C_{cr}) in the DODAB/DOPE liposomes (filled circles) and DOTAP/DOPE (opened circles), corresponding to complete DNA compaction in the sample solution, on the liposome composition *Y*.

isolated DNA molecules in the presence of mixed oppositely charged liposomes, at the same time has certain limitations. One of them is the lowest limit of the size of the observed particles, which should be of the same order as the length of the fluorescence light. Also, a suitable fluorescence marker should be introduced to the studied objects. Therefore the information on the dimensions of mixed cationic liposomes should be obtained with the use of another technique. In this connection, vesicles prepared by sonication of lipids mixtures at different molar ratios between cationic and neural lipids were characterized with the use of the cryo-TEM technique. The range of compositional changes in cationic/neutral lipid mixtures was based on the data reported by Koltover et al. [9]. They have shown that until the weight fraction of the neutral lipid, DOPE, does not exceed 0.85 in mixture with the cationic lipid, DOTAP, that corresponds to the approximately 1:6 molar ratio of cationic to neutral lipid, the separate H_{II} phase of pure DOPE has not been formed and both lipids (cationic and neutral) participate in the formation of complex with DNA. In our experiments the morphology of liposomes varied from elongated tubules (for the high contents of neutral lipid, Fig. 4A,B) to nearly spheroidal particles (for the high contents of cationic lipid, Fig. 5). Some deviations from the spherical shape of the liposomes for DODAB/DOPE mixtures might have been influenced by the presence of DODAB in these mixtures at the temperatures below the melting temperature (T_m) of this lipid (Fig. 5). Polymorphisms of the DOPE-based lipid blends in aqueous dispersion has been studied in detail by Gustafsson et al. [10], and the tendency of these lipid mixtures to form the inverted hexagonal structures was found to increase with the ratio of DOPE in the mixture. The appearance of an ordered inverted phase was also found in our experiments for the compositions with high contents of DOPE (Fig. 4C), but the dominant population was attributed to highly polydispersed liposomes. Since the existence of inverted structures in lipid mixtures at high contents of DOPE was not dominant in our experimental conditions, we strongly presuppose that it is correct to interpret the interactions in these systems as DNA–liposome interactions.

Introduction of oppositely charged mixed liposomes to DNA aqueous solutions leads to the condensation of DNA, disintegration of liposomes and formation of DNA–lipid complexes. Imaging of these systems by cryo-TEM presents some experimental problems, since complexes between DNA and lipids are extremely beam-sensitive. Therefore, the low-dose EM technique with energy filtering to reduce the radiation damage, which recently has been proposed by Battersby et al. [29], was applied for imaging. The quality of the patterns was greatly enhanced by using the energy filtering coupled with a high-magnification cryo-TEM. Moreover, the samples were viewed within 24 h after the preparation of the complexes, which facilitated the high-contrast imaging due to the reduction of DNA–lipid complex aggregation over time. Otherwise, if the complex aggregates grow substantially, the probability of finding complexes in the sufficiently thin vitreous ice is severely reduced. Taking into account all these precautions we succeeded in imaging of DNA–mixed cationic liposome complexes. The observations with cryo-TEM technique were carried out at the same concentrations of DNA and lipids in aqueous solutions as those utilized for the fluorescence microscopy studies of this system. They should also display the condensation

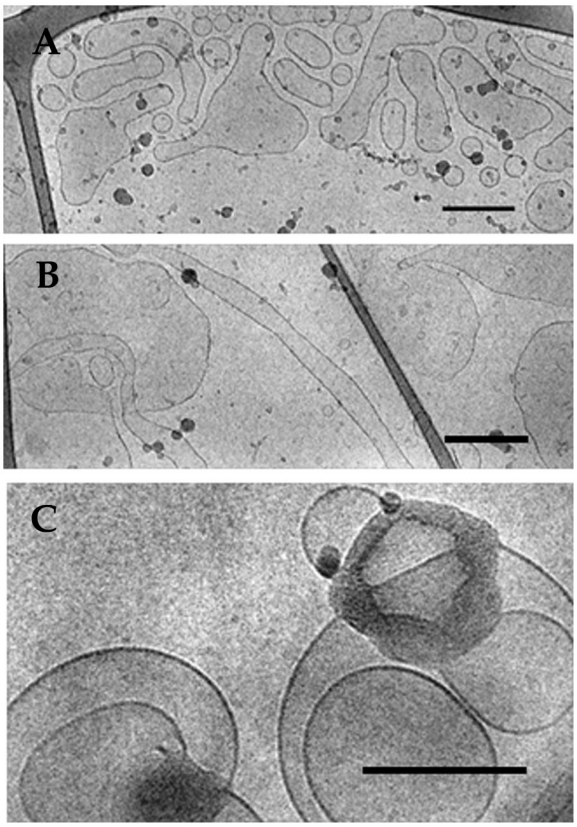

Fig. 4. Cryo-TEM images of lipid mixtures enriched with a neutral lipid. Compositions of mixed liposomes: (A), (B) DODAB:DOPE (1:6 molar ratio); (C) DODAB:DOPE (1:4 molar ratio). Scale bar 300 nm.

process of DNA in the presence of cationic liposomes as a discrete coil–globule transition in individual DNA chains. No intact liposomes were found in the sample at high DNA–lipid molar ratio, and the coexistence between free DNA chains and DNA–lipid complexes was also observed by the cryo-TEM technique. Fig. 6A shows compact DNA chains in lamellar complexes with lipids (dark round particles on Fig. 6A, right), which coexisted with free DNA chains in aqueous solution (dark lines around condensed particles). On the other hand, at low DNA-cationic liposome molar ratios there were no free DNA molecules in the solution, and the complexes (or condensed DNA molecules) coexisted with intact liposomes, as is demonstrated in Fig. 6B. The tendency of free and complexed DNA to concentrate toward the edge of the hole as thicker part of the vitrified film can be seen in both figures (Fig. 6A,B right).

With regard to DNA–mixed liposome complexes with different cationic-to-neutral lipid molar ratio, it should be mentioned that besides the lamellar structures of DNA–mixed lipid complexes, which have been observed by [10,11,29] for different compositions of complexes, we succeeded in imaging of hexagonal structures of complexes. Fig. 7 shows the typical hexagonal arrangement of DNA complexed with lipid mixtures enriched with neutral lipid, DOPE. Application of a Fourier transform (FT) to the image breaks up the latter into its Fourier components, e.g. each component corresponds to a regular pattern of a certain direction and periodicity, and was successfully used for direct measurements of

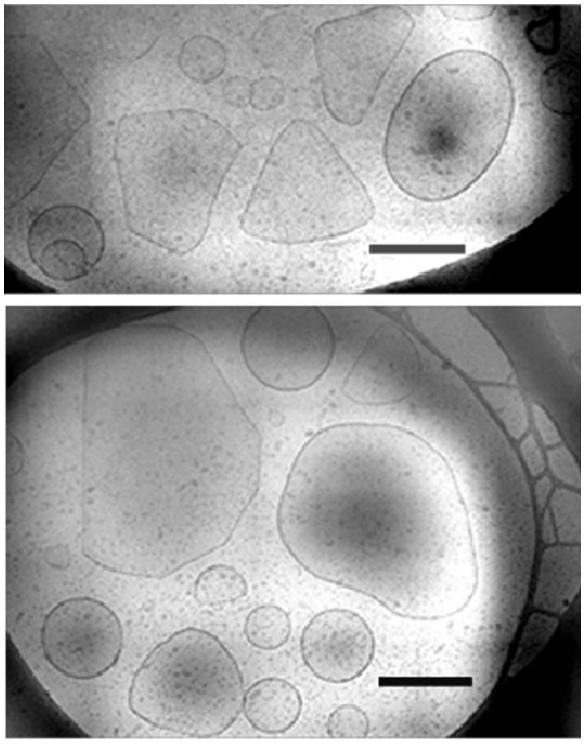

Fig. 5. Cryo-TEM images of lipid mixtures enriched with a cationic lipid. Composition of mixed liposomes–DODAB:DOPE (4:1 molar ratio). Scale bar 300 nm.

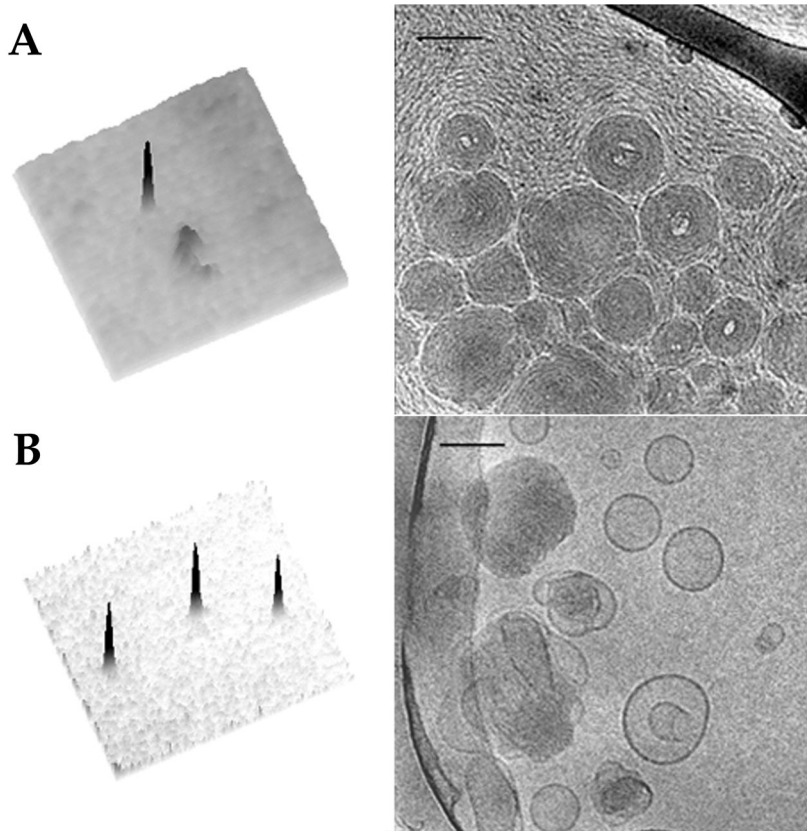

Fig. 6. Condensation of DNA in the presence of lipid mixtures, as visualized by fluorescence microscopy (left) and cryo-TEM (right). (A) coexistence of condensed and unfolded DNA in the DODAB:DOPE (1:1 molar ratio) liposome solution, DNA:cationic lipid molar ratio is equal to 15:1. (B) Coexistence of condensed DNAs and liposomes in the presence of excess of mixed liposomes at DNA:cationic lipid molar ratio 1:10. Composition of lipid mixture-DOTAP:DOPE (1:4 molar ratio). Fluorescence microscopy photographs area is 10×10 micrometers. Scale bar on the cryo-TEM images is 80 nm.

multilamellar structures of DNA/lipids complexes [29]. The FT in Fig. 7B reflects six bright spots in the array, which correspond to hexagonal packing of lipid in the complex with characteristic distance of approximately 68 Å. This distance was very close to lamellar spacing determined for cationic lipids' enriched complexes (64 Å, data not shown). Thus, to distinguish the hexagonal pattern from a lamellar one, we searched for the areas with perpendicular orientation of DNA–lipid inverted cylindrical micelles relatively to the surface of the grid (or parallel to the electron beam). Such structures as shown in Fig. 7 were not found in lipid mixtures at high DOPE content or in solutions of free neutral lipid without any added DNA. Therefore, the appearance of hexagonal structures in the presence of DNA molecules in the solution could be attributed to the formation of DNA–lipid complexes in these systems. It is known [30] that the increase of temperature > 43°C, or the acidification of DOPE dispersions at ambient temperatures [31], induces the $L_\alpha \rightarrow H_{II}$ transition in this lipid. Since special care was taken to keep the temperature below the transition point for lipid mixtures enriched by neutral lipid, the appearance of inverted hexagonal structures in the presence of DNA in lipid mixtures with high DOPE content, could also, to some extent, be attributed to the latter process which might have resulted in a phase transition of lipid mixtures accompanying by the formation of DNA–lipid complexes.

3.3. Small-angle X-ray scattering

Comparative exploration of precipitated complexes between DNA and mixed DODAB/DOPE liposomes with the use of SAXS technique also proved the $L_\alpha \rightarrow H_{II}$ phase transition, as it was observed for DOTAP/DOPE mixtures [9]. Fig. 8 shows three SAXS diffraction patterns for different compositions of DNA–lipid complexes. Complexes formed by mixing DNA and DODAB:DOPE (3:1 molar ratio) solutions gave three diffraction maxima at 0.097, 0.174 and 0.192 $Å^{-1}$ (Fig. 8A), where the first and the third diffraction maxima indicated the lamellar structure ($d = 64.8$ Å), while the second maximum corresponded to the in-plane spacing between parallel DNA helices ($d_{DNA} = 36.1$ Å). The intermediate composition of DNA–lipid complexes ([DODAB]:[DOPE] = 2:3, Fig. 8B) showed the coexistence of two phases: one lamellar phase with the same characteristic distances as described above (DNA diffraction maxima at 0.174); and one hexagonal phase with diffraction maxima at 0.11, 0.188 and 0.22 $Å^{-1}$, which were consistent with two dimensional inverted hexagonal structure with unit cell spacing of 66.7 Å. Complexes enriched with neutral lipid ([DODAB]/[DOPE] = 1:3, Fig. 8C) demonstrated the hexagonal array with spacing 68.2 Å (diffraction peaks at 0.108, 0.186 and 0.215 $Å^{-1}$). Similarly to the results of Koltover et al. [9] for DOTAP/DOPE solutions, we attribute the observed hexagonal structures to the H_{II} phase of DNA–DODAB/DOPE complexes, but not to the hexagonal phase of pure neutral lipid. Instead, this forms a separate phase at higher contents of DOPE with a unit cell spacing of 74.4 Å, according to [9]. On the other hand, direct cryo-TEM observations of complexes enriched with neutral lipid (Fig. 7) gave us a value close to that of hexagonal spacing, approximately 68 Å. There was still a weak peak at 0.174 $Å^{-1}$ in Fig. 8C attributed to parallel DNA helices within lamellar phase, which might be the indication of some remaining lamellar phase of DNA–mixed lipids complex.

3.4. UV-melting spectroscopy

Another experimental source of information on DNA–mixed liposome interaction is the examination of UV-melting transitions of DNA–lipid complexes. Fig. 9 shows the melting curves for free DNA and DNA–lipid complexes at different liposome compositions, i.e. ratios between cationic and neutral lipids. Salmon sperm DNA in aqueous solution has a melting temperature of 52°C. This means that with the increase of temperature the double-helical structure of DNA is disturbed,

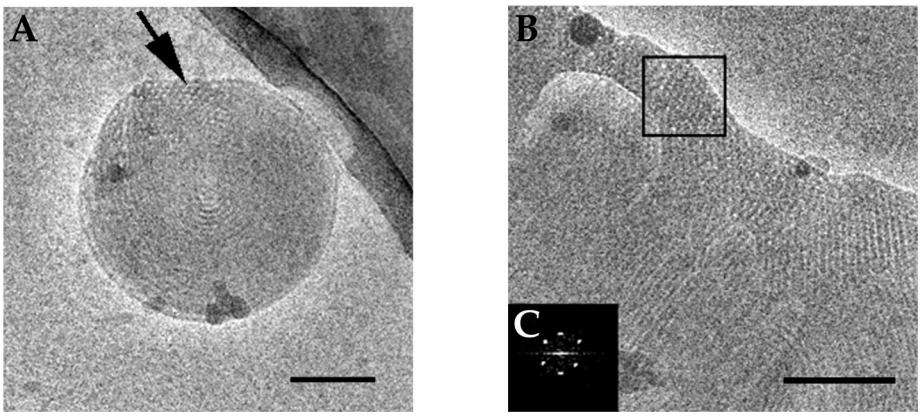

Fig. 7. Cryo-TEM images of DNA–mixed lipids complexes enriched with a neutral lipid at DODAB:DOPE molar ratio equal to 1:4. (A) Arrow points to the inverted hexagonal pattern of complex particle; (B) Inverted hexagonal pattern of DNA–mixed lipids complex; C-Fourier transform of detail from Fig. 7B. The diffraction spots correspond to the 6.8 nm periodicity, which we attribute to the inverted hexagonal phase of DNA–mixed lipids complexes. Scale bar 80 nm.

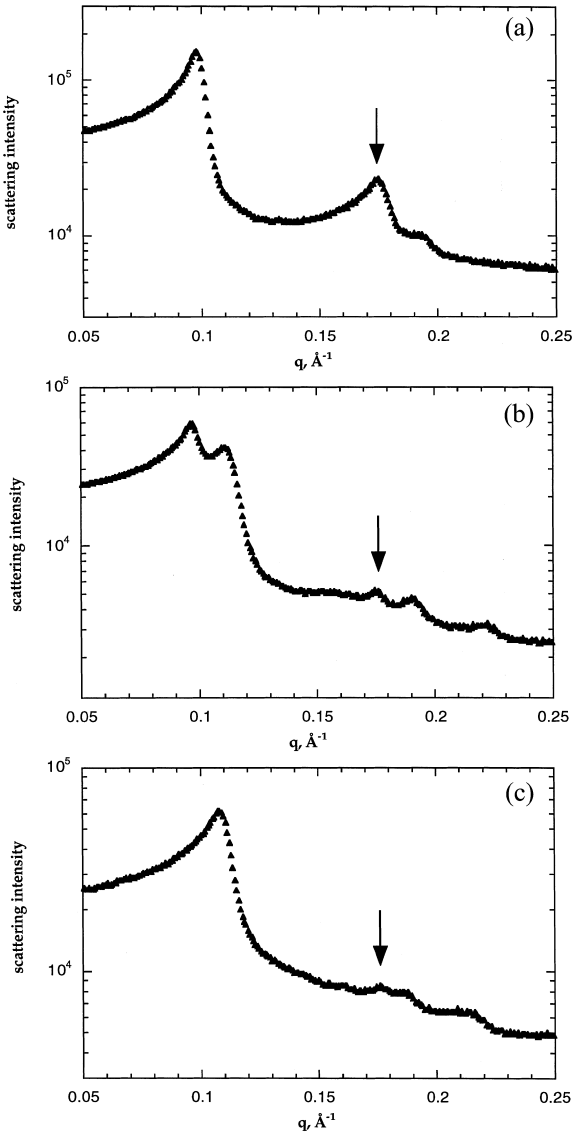

Fig. 8. SAXS diffractograms of salmon sperm DNA–mixed lipids complexes at different compositions of mixed liposomes. Compositions of mixed liposomes, DODAB:DOPE molar ratio: (A) 3:1; (B) 2:3; and (C) 1:3. Arrows point to diffraction maxima corresponding to DNA chains intercalated between lipid layers.

and DNA melts, or undergoes a helix–coil transition of its secondary structure. DNA complexed with pure cationic lipid DODAB showed biphasic behavior with the main sharp transition at temperature very close to T_m of free DNA, while a second transition started at approximately 76°C. This type of melting behavior was also found by Chaires [32] for DNA complexed with a synthetic cationic surfactant, cetyltrimethylammonium bromide (CTAB). The model developed by McGhree [33] for DNA melting in the presence of binding ligands has been used for a detailed analysis of DNA–CTAB binding. Taking into account numerous similarities in the binding behavior of synthetic cationic surfactant and cationic lipid to a negatively charged DNA chain, this model can be also applied to the DNA–mixed liposome interactions as a case of a cooperative binding of ligands. According to that model, the first transition could be associated with melting around the uncomplexed base pairs of DNA helix accompanied by rearrangement of the bound ligands to allow the DNA to melt in larger loops. Since cationic lipids are bound to DNA in a cooperative way, such vacant areas should have the melting behavior very similar to that of the DNA helix and a melting profile that is very steep. As demonstrated by Chaires [32], the magnitude of the first transition depends on the DNA:lipid ratio in the complexes and should decrease with increase of the lipid concentration. The quite flat middle region following the first steep transition then ended when the bound lipids were expelled from their complexes with DNA. We were not able to reach the final temperature of this second melting region because of instrumental limitations, but up to 87°C the continuous increase of absorbance was observed. Introduction of neutral lipid dramatically changed the melting behavior of DNA–mixed lipids complexes. Fig. 9B shows that the increase of absorbance at 260 nm, which corresponds to denaturation of DNA within mixed lipids complexes with different lipid compositions, started at slightly higher temperatures and continued evenly up to the achieved temperature maximum. Such melting behavior clearly indicated more complex processes in these systems.

4. Discussion

In our preliminary report on the DNA single-

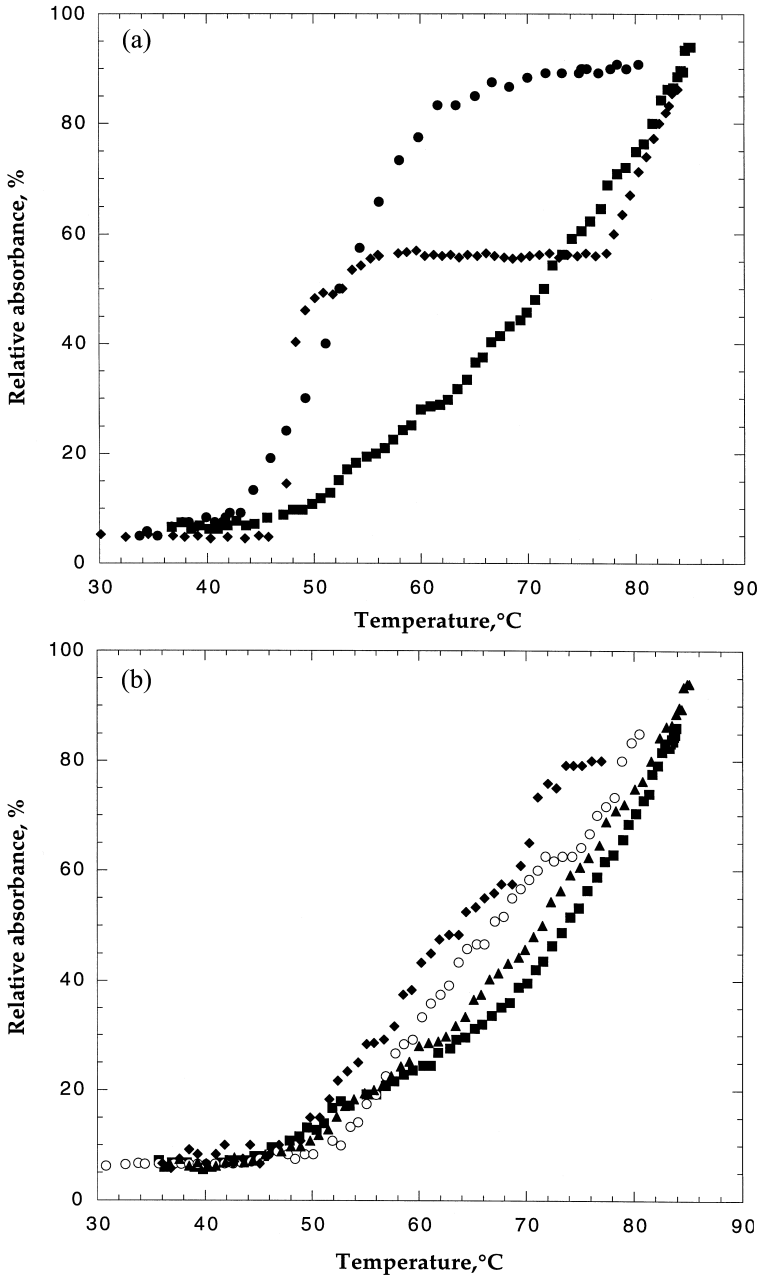

Fig. 9. Melting profiles of salmon sperm DNA in the presence and absence of mixed lipids. (a) represents the melting profiles of free salmon sperm DNA (0.16 mM) (●), in presence of 0.8 mM DODAB (♦), in presence of DODAB/DOPE (1:1 molar ratio) liposomes (■). Concentration of cationic lipid, DODAB, 0.8 mM in all samples. (b) Effect of neutral lipid on DNA melting profile. Compositions of mixed lipids (DODAB:DOPE, molar ratio): (○) 4:1; (■) 2:1; (▲) 1:1; and (♦) 1:4. Concentration of cationic lipid, DODAB, 0.8 mM in all samples.

molecular compaction in the presence of oppositely charged mixed liposomes [13] we noticed that the interaction between DNA and oppositely charged mixed liposomes strongly depends on the

liposome composition, i.e. the ratio between charged and neutral lipids. In the present study DNA compaction in the presence of mixed DODAB/DOPE and DOTAP/DOPE liposomes was examined with the use of a fluorescence microscopy method. We found that even at the lowest liposome concentrations individual elongated DNA chains coexisted with the compacted DNA–lipid complexes, as could be clearly seen through the direct visualization of DNA molecules. In the coexistence region both linear dimensions and fluorescence intensities of DNA chains were substantially different, as is demonstrated in Figs. 1 and 6. The coexistence of the two different conformational states of the stiff DNA chains in thermodynamic equilibrium in the presence of oppositely charged liposomes implied that under these experimental conditions the difference between the free energies of single coiled and globular DNAs did not exceed the thermal energy kT [24]. Our observations of the coexistence of two different conformational states of DNA at the same concentration of the liposome were in a good agreement with the report by Lasic and Templeton [6]. They recently reported the existence of fully condensed particles, partially condensed structures, as well as unreacted DNAs in the mixtures of plasmid DNA and cationic liposomes [6].

Another interesting peculiarity of interaction can be seen from Figs. 2 and 3. The concentration of mixed liposomes expressed as the molar concentration of cationic lipid, which corresponded to complete DNA collapse in the solution, depended on the liposome composition, i.e. on the molar ratio between cationic and neutral lipids. This effect was much more pronounced for DODAB/DOPE liposomes, compared to DOTAP/DOPE liposomes. A difference was expected considering differences in the individual properties of DODA and DOTAP cations [34]. It is commonly accepted that the interaction between cationic liposomes and DNA in aqueous solution, which leads to the formation of condensed structures, occurs at nearly equimolar ratio of components in the solution [1–6]. However, our results showed that with the increase of the neutral lipid content in a liposome, the amount of cationic lipid for complete DNA collapse also increased significantly. In our recent report [13] we were unable to make an indisputable description of the observed phenomenon, since our experimental results were obtained with FM and, therefore, mainly provided a qualitative description of DNA–liposome interaction. We believed that at least two different processes might have been of importance [13].

First, the positively charged lipids could have been incorporated into mixed liposomes with the neutral lipids and concurrently could also have been forming complexes with DNA. This hypothesis agreed well with our previous observation of the formation of complexes between DNA and DODAB in the presence of neutral L-α-phosphatidylcholine (PC) liposomes [26,35]. In those studies it was concluded that the introduction of neutral PC liposomes to an aqueous solution of DNA–DODAB compact globules leads to the unfolding of DNA chains due to the partial transfer of lipid molecules from the bulk to the liposome.

Second, the liposome surface charge density may also have been important for the explanation of the interaction mechanism. It has clearly been shown by Dubin et al. [36–38] that the interaction between polyelectrolytes and oppositely charged mixed micelles strongly depends on the molar fraction of ionic surfactant in the micelle. However, we mentioned that his hypothesis about the surface charge density effect can be convincing if the liposome structures did not undergo significant changes during the formation of DNA–liposome complexes.

Our new results obtained with the use of cryo-TEM and SAXS demonstrated clearly that the mixed cationic liposomes underwent a dramatic change in their structure during the interaction with DNA macromolecules. For instance, with the use of cryo-TEM it was proved that mixed cationic liposomes, which clearly could be detected in the solution in the absence of DNA, underwent disintegration followed after the addition of DNA stock solution. This process led to the formation of DNA–lipid complexes with regular packing of lipid molecules. Moreover, the microscopic structure of lipid aggregates in the complexes with

DNA could be determined with the use of SAXS technique, which indubitably showed that the lipid arrangement was changed from lamellar to the inversed hexagonal upon the addition of DNA to the mixed oppositely charged liposomes. Therefore, our hypothesis on the concurrent binding of cationic lipid by DNA and neutral lipid, which has a much lower interaction constant, but which is in much higher concentration than DNA, is credible. With respect to the application of these systems to DNA delivery, it means that the interaction of DNA and cationic lipid may be driven by the tuning of the ratio between cationic and neutral lipids in the mixed liposomes. This can help to avoid the addition of the unnecessary amount of liposomes to DNA in transfection protocols.

Considering the above results, one may conclude that under our experimental conditions positively charged DODA or DOTAP ions formed complexes with DNA polyanions stabilized due to the strong electrostatic attraction and hydrophobic interactions between hydrocarbon chains of DODAB. At the same time the cationic lipid molecules might have been incorporated into the mixed liposomes with DOPE owing to the hydrophobic interactions between hydrocarbon lipid moieties. In other words, the partitioning of cationic lipid molecules between mixed liposomes with DOPE and DODAB–DNA (or DOTAP–DNA) complexes took place in the system. With the increase of DOPE concentration in the sample, when the neutral lipid concentration was several orders of magnitude higher than that of DNA polyanions, the effect due to the presence of DOPE in the solution was quite substantial. Obviously, the free fraction of positively charged lipid ions in the solution, which has a determining influence on the coil–globule transition in large individual DNA, decreased [13]. Support of our explanation is clearly presented in Fig. 3, which reflects the dependence of C_{cr} on the ratio between DODAB and DOPE in the initial liposomes, Y. As is seen in Fig. 3, the C_{cr} value underwent an almost two orders of magnitude increase, while Y was changed from 1.00 down to 0.09 for the DODAB/DOPE–DNA system.

Our above explanation was also supported by the recent notion of Lasic [11] that during the interaction between DNA and cationic liposomes, DNA compaction is accompanied by the disintegration of liposomes. Therefore we may conclude that our explanation based on the redistribution of charged lipid molecules between a DNA–DODAB complex and mixed DODAB/DOPE liposomes was plausible. In this case the surface charge density of liposomes was changed during the process due to the strong electrostatic interactions between DNA and DODAB and preferential binding of cationic lipid onto the DNA chains.

Another conclusion can be made from our experimental observations. It is natural to anticipate that the effect due to the chemical structure of positively charged lipid will be diminished with the increase of the neutral lipid content in the liposome. Therefore, one can expect that at the highest molar ratios of neutral lipid, DOPE, in DODAB/DOPE and DOTAP/DOPE liposomes, the difference in values of C_{cr} for DOTAP/DOPE and DODAB/DOPE liposomes should decrease. This assumption is ascertained in Fig. 3. The values of C_{cr} on the positively charged lipid concentrations became almost equal at $Y = 0.20$, when DOPE was in great excess with respect to the concentration of the positively charged lipid in the liposomes. One can expect that the modification of the physico-chemical properties of the mixed liposomes by changing the chemical structure of cationic lipid should be carried out at relatively high molar ratios of positively charged lipid. Otherwise, the effect due to the cationic lipid structure will be lessened.

Besides the effect of a neutral lipid on the mechanism of interaction between DNA and oppositely charged mixed liposomes, the stability of the complexes of DNA with lipid mixtures was investigated in the present study. We found that, compared to the free DNA in aqueous solution, complexation of DNA with a cationic lipid resulted in the increase of the thermal stability of DNA. This result was in line with our previous findings obtained with the use of the fluorescence microscopy technique (Sergeyev and Mel'nikov, unpublished results). Large DNA molecules were stained with a fluorescence marker, DAPI, and observed at elevated temperatures. Since DNA

molecules could be successfully visualized with the use of DAPI, a DNA minor groove binder, if the double-helical structure remains undisturbed, this might be a criterium of the stability of DNA helix with the increase of temperature. In those experiments we found that globular complexes of DNA with the synthetic cationic surfactant, CTAB, remained visible at much higher temperatures than those corresponding to the disappearance of fluorescence emission from DNA–DAPI complexes without addition of CTAB. Therefore, those results supported our current conclusion concerning the increase of thermal stability of the DNA double helix after complexation with cationic lipid.

At the same time, the melting behavior of DNA–cationic lipid complexes differed from that of DNA–mixed lipid complexes, and no pronounced biphasic behavior was found for any compositions of mixed lipids. Most probably this can be explained by a more effective packing of DNA within these complexes leading to the absence of any kind of defects existing in the case of pure cationic lipid–DNA complexes. On the other hand, substantial broadening of the melting profile could reflect the structural complexity of such mixtures. The observed melting behavior in the case of DNA–mixed lipid complexes could be due to the presence of two types of complexes (lamellar and inverted hexagonal) or the existence of different parts of DNA–mixed lipid complexes enriched by one or another lipid with corresponding T_m. Keeping in mind that the phase behavior of charged and neutral lipids is different with an increase of temperature, we can speculate that during melting, the DNA complexes might be getting enriched with cationic lipid and phase separation of neutral lipid could occur. This behavior should be reflected in the shape of the melting curve: smooth in the beginning and steeper in the end. Such shapes have indeed been observed for compositions with high content of cationic lipid. Also some fine changes of melting profiles were found that could be attributed to the influence of other factors but have not been in the focus of the present study.

The above presented results lead to an important conclusion. It is well known from biomedical studies on DNA transfection [7,9] that the introduction of a neutral 'helper' lipid to the cationic liposomes significantly improves the DNA delivery in vitro and in vivo. There are also some studies of the effect of the neutral lipid on individual properties of both mixed liposomes and DNA–liposome complexes [9,10]. In contrast to those studies, we presented here a detailed examination of the physico-chemical peculiarities of DNA complexation with oppositely charged mixed liposomes with respect to the effect of the neutral lipid on this interaction. Applying the fluorescence microscopy technique, we received clear evidence that the interaction between isolated DNA molecules in an extremely diluted aqueous solution with the oppositely charged mixed liposomes strongly depended on the neutral lipid content. With the increase of molar ratio between neutral and cationic lipids, the complete collapse of DNA chains occurred at higher liposome concentrations. At the same time, at high contents of neutral lipid (in our experiments, [DOPE]/[cationic lipid] = 1/10) the effect of various cationic lipids on DNA compaction became quite similar, i.e. DNA compaction occurred at the same liposome concentrations for both DODAB/DOPE and DOTAP/DOPE systems.

Cryo-TEM experiments revealed the disintegration of mixed liposomes after the addition of DNA stock solutions into the aqueous liposome solutions and formation of DNA–lipid complexes with the formation of various continuos lipid structures, lamellar or inverted hexagonal. It was also shown that the manner of lipid packaging in the complexes with DNA also depended on the composition of the mixed liposomes, i.e. molar ratio of cationic and neutral lipids. Lamellar complexes were detected at high contents of cationic lipid in the liposome, whereas inverted hexagonal structures dominated at a high content of a neutral lipid.

The microstructures of complexes were also examined with the use of the SAXS technique, which confirmed the results obtained by cryo-TE microscopy and enabled the quantitative characterization of lipid packaging in the complexes

with DNA macromolecules. The melting behavior of complexes also depended on the liposome composition: introduction of neutral lipid moderately increased the thermal stability of complexes; and changed the quantitative characteristics of the melting profiles.

Therefore, we conclude that the introduction of a neutral lipid into the positively charged liposome strongly affects both the intrinsic liposome properties and the interaction of mixed liposomes with the oppositely charged DNA chains. In spite of the mainly electrostatic character of interaction between DNA and oppositely charged lipids, the addition of a neutral lipid largely influences the character of the process and properties of the resulting DNA–lipid complexes. Consequently, the increase of the transfection efficiency of mixed liposomes may be attributed to the modification of physico-chemical properties of DNA–liposome interaction in the presence of a neutral lipid.

5. Conclusions

Our data indicated that DNA condensation in mixed liposome solutions had many particularities attributed to the compositional changes in lipid mixtures. Coil–globule transition of DNA in diluted solutions of mixed lipids, as it was shown by fluorescence microscopy, depended on neutral lipid contents and DNA compaction. Thus, a key role in the process of DNA transfection, is the composition of mixed liposomes. The structures of formed complexes, as observed by cryo-TEM and SAXS measurements changed from the lamellar array for DNA–lipid complexes formed from the cationic lipid-enriched mixtures to inverted hexagonal array for the neutral lipid-enriched complexes. The melting behavior of complexes also depended on liposome composition: introduction of neutral lipid increased the thermal stability of complexes and changed the quantitative characteristics of the melting profiles.

It has been shown earlier that, with respect to the DNA transfection, only certain compositions of mixed cationic/neutral lipids–DNA complexes demonstrate the substantial effect. Our data indicated that the physico-chemical properties of such delivery systems, based on various compositions of cationic/neutral lipid mixtures, as well as the mechanisms of their formation, can be of use to explain some particularities of the mixed liposome-enhanced DNA transfection efficiency.

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