

DOPC + OTAB + DNA Complexes – Effect of Ionic Strength and Surface Charge Density on DNA Condensation

Research paper

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Abstract The effect of ionic strength and surface charge density of the lipoplexes formed from the cationic surfactant octadecyltrimethylammonium bromide (OTAB) and the neutral phospholipid 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) on DNA condensation was studied using fluorescence spectroscopy. The structure of lipoplexes was examined by small- and wide-angle X-ray scattering (SAXS/WAXS). The efficiency of DNA condensation was monitored by relative changes in the intensity of the emission of the fluorescence probe ethidium bromide (EtBr). A high OTAB/DNA charge ratio of 10:1 is required for efficient condensation at all ionic strengths. The increasing mole ratio of OTAB/DOPC supports DNA condensation, while the process is significantly hindered by a high ionic strength. Only ~55% of DNA was found to be condensed by OTAB/DOPC = 1 mol mol⁻¹ complexes in 150 mmol l⁻¹ of NaCl. A condensed fluid lamellar phase (L_a^c) was detected in the DOPC + OTAB + DNA complexes. The SAXS patterns show regular DNA–DNA packing only in complexes prepared in a medium of low ionic strength (<50 mmol l⁻¹). Interestingly, free DNA in the supernatant of DOPC + OTAB + DNA complexes was not detected by UV/Vis spectrometry, indicating its trapping in the lipoplexes.

Keywords DNA – DOPC – octadecyltrimethylammonium bromide – ionic strength – fluorescence spectroscopy – SAXS/WAXS

INTRODUCTION

Gene therapy is a rapidly growing field. According to Chancellor et al. (2023), there are more than 100 different gene, cell, or RNA therapies worldwide, with more than 3700 in clinical and preclinical development.

The global outbreak of coronavirus disease 2019 (COVID-19) has validated the potential of mRNA vaccines for infectious diseases, which use similar approaches to gene therapy. In 2020, emergent authorization of the COVID-19 mRNA vaccines was approved by the Food and Drug Administration due to their excellent protective effect against COVID-19 infection (Gu et al., 2024). In hundreds of millions of people, mRNA vaccines have been used to induce an immune response against epitopes of the acute respiratory syndrome-coronavirus-2.

Gene therapy will eventually become a standard component of treatment for a wide range of diseases, revolutionizing health care by addressing the root causes of genetic disorders such as genetic diseases, cancers, acquired immunodeficiency syndrome (AIDS), Alzheimer's, etc. This procedure utilizes viral

and non-viral particles to deliver DNA or RNA to a patient's target cells (Durymanov & Reineke, 2018; Sung & Kim, 2019; Wirth et al., 2013). Friedman and Roblin first introduced the concept in 1972. In 2012, the European Medicine Agency (EMA) approved the first *in vivo* therapy against lipoprotein lipase deficiency under the product name Glybera (Büning, 2013). However, the manufacturer of Glybera did not apply for a renewal of the EMA approval. This led to its withdrawal from the community register of orphan medicinal products in Europe in 2017. In 2016, an *ex vivo* therapy against adenosine deaminase (ADA) deficiency and severe combined immunodeficiency (ADA-SCID) was made available on the European market. Both therapeutics are based on viral vectors. Viral vectors achieve a relatively higher efficiency in transfecting host cells compared to non-viral methods, but have serious disadvantages, such as immunogenic potential, expensive manufacturing, low cargo capacity, random integration of genes within the host genome, activation of oncogenes, and inactivation of tumor suppressor genes (Ramamoorthi & Narvekar, 2015; Sharma & Paschalis, 2022; Tassler et al., 2019).

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The limitations of viral vectors can be potentially overcome by using non-viral delivery methods. Non-viral vectors, formed mostly by cationic polymers, cationic lipids, or cationic surfactants, are an alternative approach that is usually used to form complexes with the DNA polyanion, which results in DNA condensation. Condensed DNA is partially protected from degradation by enzymes (Rolland, 1998) and improves cellular uptake of DNA (Nie et al., 2019). Cationic lipids as gene delivery vectors were used for the first time in 1987 and have been intensively studied as a biocompatible and biodegradable drug delivery platform (Bose et al., 2015; Felgner et al., 1987; Hubčík et al., 2018; Khan et al., 2012; Pattni et al., 2015; Perrone et al., 2013; Tassler et al., 2019; Wasungu & Hoekstra, 2006; Zhang et al., 2022).

Besides the cationic lipids, the research also focused on lipoplexes with cationic surfactants or divalent metal ions (Caracciolo et al., 2007; Hubčík et al., 2014; Jing et al., 2004; Liskayová et al., 2017; Pullmannová et al., 2012; Ramamoorth & Narvekar, 2015, 2015; Uhríková et al., 2005, 2007, 2009) or ionizable lipids that can modulate its change based on pH (Han et al., 2021; Liu et al., 2025). The structure and polymorphic behavior of lipoplexes have been studied systematically for systems with different compositions (Uhríková et al., 2002, 2005, 2007, 2009). Many experiments showed that besides the composition, the microstructure of lipoplexes is influenced also by other factors, such as the ionic strength of the aqueous medium and the surface charge density (Hubčík et al., 2014, 2018; Kolašinac et al., 2019; Madeira et al., 2008; Pullmannová et al., 2012; Silanteva et al., 2020).

Quaternary alkylammonium bromides are cationic surfactants that, either in aqueous binary systems or as a part of ternary mixed systems, have been widely studied. In particular, those with more than eight carbon atoms in the alkyl chain have specific pharmaceutical applications as a result of their antibacterial function (Rodríguez-Pulido et al., 2009; Siddiqui et al., 2022; Silanteva et al., 2020; Trabelsi et al., 2007). Previous experiments with quaternary alkylammonium salts showed only limited transfection efficiency compared to commercially available liposomal transfection vectors, mainly because of their high cytotoxicity (Pinnaduwaage et al., 1989). However, the high cytotoxicity can be mitigated by the introduction of neutral phospholipids into the mixture. However, the transfection activity can be increased by chemical modification with substitution of one methyl group for another long alkyl group on the same ammonium group (Silva et al., 2012) or linking of two alkyl ammonium groups through a hydrocarbon spacer (Badea et al., 2005; Hubčík et al., 2018; Muñoz-Úbeda et al., 2011). Despite the inferior transfection efficiency of alkylammonium salts, their low cost and wide availability made them a good model for the study of the physicochemical interactions of cationic surfactants with DNA (Dias et al., 2000; Kawashima et al., 2006; Marchetti et al., 2006; Nakanishi et al., 2007; Rodríguez-Pulido et al., 2009).

This work is focused on the effect of the surface charge density of lipoplexes formed from the cationic surfactant

octadecyltrimethylammonium bromide (OTAB) and the neutral phospholipid 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and the effect of the ionic strength of the medium on DNA condensation and the structure of formed complexes. The surface charge density was regulated through the OTAB/DOPC mole ratio of used liposomes. Five ionic strengths of hydration medium in the range of 5–150 mM NaCl solution were selected for the measurements. DNA condensation efficiency was monitored by fluorescence spectrometry through relative changes in the emission intensity of the fluorescent probe ethidium bromide (EtBr). The condensation of DNA leads to displacement of intercalated EtBr detected as a decrease of fluorescence intensity (Eastman et al., 1997; Izumrudov et al., 2002). The structure of the formed complexes was studied by small and wide-angle X-ray scattering (SAXS/WAXS).

MATERIALS AND METHODS

Chemicals

Synthetic neutral phospholipid DOPC was purchased from Avanti Polar Lipids (Alabaster, Alabama, USA) and used without further purification. Herring testes DNA (sodium salt), highly polymerized (average M_r of nucleotides = 308), was obtained from Sigma Aldrich (St. Louis, Missouri, USA). EtBr was purchased from Merck (Darmstadt, Germany). Cationic surfactant OTAB was from Sigma-Aldrich Chemie (Steinheim, Germany). NaCl of analytical purity was purchased from Lachema (Brno, Czech Republic). Chemical formulae of used chemicals are shown in Fig. 1.

Methods

Preparation of DNA solutions

The solutions of DNA were prepared by dissolving the required amount of DNA in 5, 15, 50, 100, and 150 mmol l⁻¹ NaCl. The precise concentrations of DNA were determined using ultraviolet/visible (UV/Vis) spectrometry (SPECORD 200 PLUS; Analytik, Jena, Germany), according to $c_{\text{DNA}} = A_{260}/6600$ (mol l⁻¹), where A_{260} is the absorbance at wavelength $\lambda = 260$ nm. The concentrations of DNA solutions were in the range of 7.5–8.4 mmol l⁻¹. The number of moles refers to the moles of DNA bases.

Preparation of cationic liposomes

OTAB and DOPC, respectively, were dissolved in methanol (Slavus, Bratislava, Slovakia) to form homogeneous solutions. Mixtures of OTAB/DOPC = 0.2, 0.3, 0.4, 0.5 and 1 mol mol⁻¹ were prepared by pipetting appropriate amounts of stock solutions. Every sample was prepared 5 times and dried under a stream of gaseous nitrogen. The residues of methanol were removed under a vacuum. The dry lipid films were hydrated

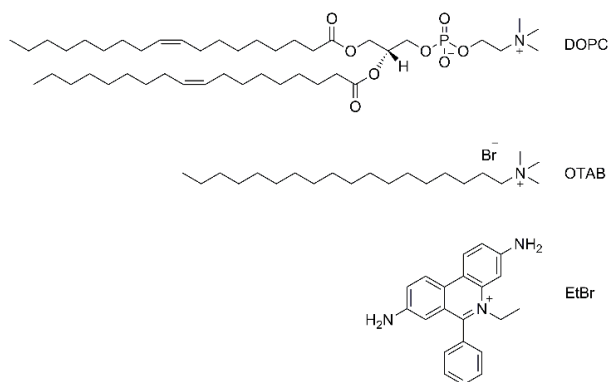


Figure 1. Structures of DOPC, OTAB and EtBr.
 DOPC: 1,3-bis(sn-3'-phosphatidyl)-sn-glycerol, OTAB: octadecyltrimethylammonium bromide, EtBr: 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide

by NaCl medium of different ionic strengths (5–150 mmol l⁻¹). The samples were homogenized by repeated vortexing, freezing, and thawing in an ultrasonic bath (+40 °C).

Fluorescence spectrometry

The samples of DOPC + OTAB + DNA complexes for fluorescence experiments were prepared in the range of mole ratios $0.2 \leq \text{OTAB/DOPC} \leq 1$ for five studied NaCl concentrations. DNA solutions were mixed with fluorescence probe EtBr. For the condensation experiments, the concentrations of DNA (2.5 μmol l⁻¹) and EtBr (0.5 μmol l⁻¹) in the samples were kept constant. Cationic liposomes were added to the samples at the desired mole ratio, and the volume was adjusted to 3000 μl with the corresponding NaCl solution. The fluorescence intensity of the samples was measured 30 min after preparation using the Fluoromax-4 spectrofluorometer (HORIBA Jobin Yvon, Lille, France) at the laboratory temperature. The fluorescence emission intensity of EtBr was measured at $\lambda_{\text{em}} = 597$ nm with exciting wavelength $\lambda_{\text{ex}} = 513$ nm. The emission intensity of each sample was corrected for the background fluorescence of EtBr in the absence of DNA and then normalized to the EtBr fluorescence of the sample containing DNA without any DOPC + OTAB liposomes (OTAB/DNA = 0), following the equation

$$I_{\text{norm}}(\%) = \frac{I_i - I_{\text{blank}}}{I_{\text{max}} - I_{\text{blank}}} \cdot 100\% \quad (1)$$

where I_{norm} is the normalized fluorescence intensity of the sample, I_i is the fluorescence intensity of the sample, I_{blank} is the fluorescence intensity of EtBr in the absence of DNA, and I_{max} is the EtBr fluorescence of the sample containing DNA without liposomes.

X-ray scattering

The structure of DOPC + OTAB + DNA complexes was investigated using SAXS/WAXS experiments, which were performed at BL11-NCD-SWEET beamline at the ALBA synchrotron (Barcelona, Spain) using a monochromatic radiation of wavelength 0.12 nm. Samples were prepared in advance following the above procedure at two selected mole ratios OTAB/DOPC = 0.3 and 1. Liposomes containing 8 mg DOPC were mixed with DNA solutions with the respective ionic strength to achieve the mole ratio OTAB/DNA = 10. The volume of the samples was made 1 ml with the corresponding NaCl solution. The samples were stored at ~4 °C for 24 h and then homogenized by several vortexing and three freezing–thawing cycles in the temperature range from –20 °C to +37 °C, which is well below the denaturation temperature of double-stranded DNA. The homogenization was carried out after DNA addition to promote uniform distribution of DNA within the lipid mixture. Keeping neutral pH (Galyuk et al., 2009) and few repetitions of freezing–thawing (Krajden et al., 1999), we do not assume any significant damage of DNA. After homogenization, the samples were centrifuged at 13,000 rpm for 2 min using a Minispin centrifuge (Eppendorf, Hamburg, Germany). The resulting sediment was carefully transferred into thin-walled quartz capillaries (Hilgenberg, Malsfeld, Germany) with 1.5 mm diameter and closed with plasticine. The capillary samples were positioned vertically in a temperature-controlled capillary holder. Samples were measured at 20 °C and 50 °C. Scattering intensity was recorded over a duration of 1.5 s. The samples were measured repeatedly to exclude possible radiation damage due to a selected exposure time. SAXS data were detected on a Pilatus 1 M detector calibrated using AgBh (Huang et al., 1993). WAXS data were detected on an LX255HS Rayonix detector calibrated using Cr₂O₃ (certificate SRM 674b; NIST, Maryland, USA). The 2D scattering patterns were azimuthally integrated into 1D data using the pyFAI python library (Kieffer & Wright, 2013). Intensity profiles of the raw data were normalized to the intensity of the incident beam. Data analysis was performed using OriginPro 2021 software. The WAXS pattern of all measured samples exhibited one wide, diffuse scattering peak in the range of $q \approx 10\text{--}20$ nm⁻¹, characteristic of liquid-like carbon chains of phospholipid. The diffraction peaks of the SAX region were fitted with Lorentzian functions and linear background (Nagle & Tristram-Nagle, 2000). The repeat distance d of bilayer stacking was calculated using the equation

$$d = 2\pi/q \quad (2)$$

where q is the position of the first lamellar SAXS peak.

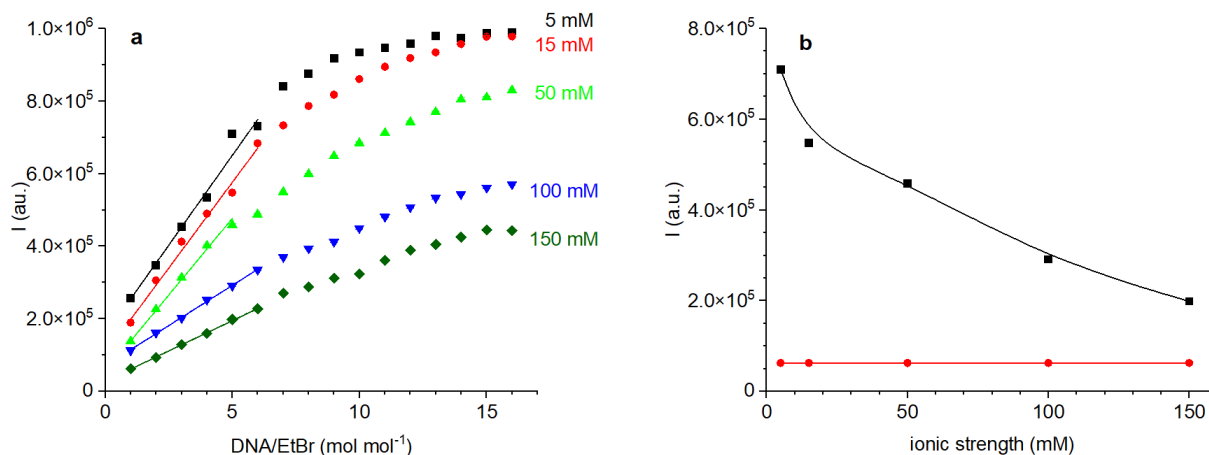


Figure 2. (a) Dependences of the emission fluorescence intensity I of complex DNA + EtBr on DNA/EtBr mole ratio at different NaCl ionic strengths. Error bars are within the size of the symbols. Lines represent the best linear fits of respective experimental data. (b) Dependences of the fluorescence intensity I of DNA/EtBr = 5:1 mol mol^{-1} complex (*) on the ionic strength compared to pure EtBr (*). Curves are a guide to the eyes only.

UV/Vis spectrometry

UV/Vis spectrometry was used to investigate the binding capacity of OTAB + DOPC liposomes for DNA. The fraction of DNA bound into the aggregates was determined from the supernatant of the samples prepared for SAXD experiments as the difference between the total amounts of DNA added to the sample and the free DNA in the supernatant. Measurements were performed in a quartz cuvette with an optical path length of 1 cm using the diode array spectrophotometer (Hewlett Packard 8452A). The samples were measured at room temperature. The absorbance of DNA was monitored at the wavelength $\lambda = 260$ nm.

RESULTS AND DISCUSSION

Fluorescence study of DNA + EtBr complexes

EtBr is a cationic fluorescent probe that intercalates between the bases of uncondensed DNA. In solution, EtBr molecules transition from the excited state to the ground state mainly through nonradiative processes that involve the transfer of a proton from the amino group to the solvent molecules. However, when EtBr is intercalated, it is isolated from the solvent, blocking the nonradiative decay pathway. This leads to a dramatic increase in the EtBr fluorescence intensity upon intercalation (Izumrudov et al., 2002).

We performed a set of measurements at 15 different DNA/EtBr mole ratios to determine the optimal ratio. The effect of ionic strength on this ratio was verified for five hydration media in the range of 5–150 mmol l^{-1} NaCl. A linear increase in emission fluorescence intensity was recorded approximately up to a DNA/EtBr mole ratio of 4–6 depending on the ionic

strength (Fig. 2a). In the linear part, the binding sites of the DNA for EtBr are fully saturated. At higher mole ratios, the dependences are no longer linear due to excess DNA. For further measurements, we chose a constant mole ratio DNA/EtBr = 5 for all studied ionic strengths. This finding is consistent with the results of other authors (Barreleiro & Lindman, 2003; Eastman et al., 1997; Lengyel et al., 2011). The ionic strength significantly influences the fluorescence intensity of EtBr. We detected a nonlinear decrease in fluorescence intensity with increasing ionic strength (Fig. 2b). The DNA + EtBr complex had up to 15.3-fold higher fluorescence than the probe alone in 5 mM NaCl solution, but in 150 mM NaCl, only a 3.8-fold increase in fluorescence intensity was detected. This is mainly caused by the shielding of electrostatic interactions between DNA and EtBr by the solution at high ionic strength, resulting in reduced affinity of EtBr for DNA-binding sites (Vardevanyan et al., 2001, 2016). Ionic strength also influences the conformation of DNA, stabilizing the double helix structure and preventing unwinding. These changes are making intercalation energetically less favorable as it requires significant changes in the double-helix structure (Kas'yanenko, 2006; Manning, 2015; Zipper et al., 2004).

Fluorescence study of DOPC + OTAB + DNA + EtBr complexes

When DNA interacts with cationic liposomes, cationic polymers, or multivalent cations, it leads to the formation of complexes in which the opposite charges are canceled out. The driving force for the formation of the complexes is the high entropic gain from the release of small counterion during the mutual neutralization of the opposing charges

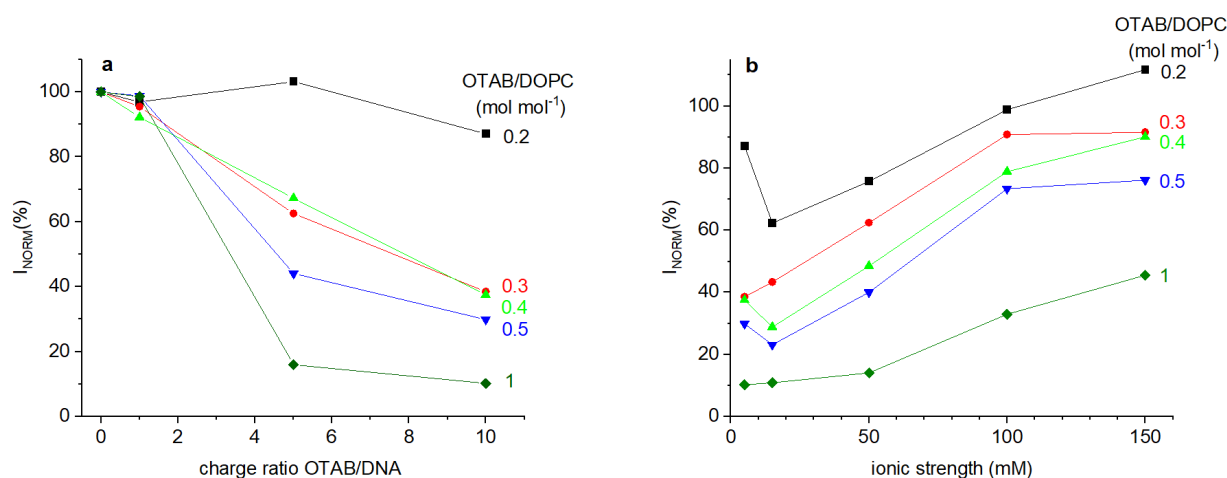


Figure 3. (a) Dependence of normalized fluorescence intensity I_{norm} of DOPC + OTAB + DNA + EtBr complexes on the OTAB/DNA charge ratio at different OTAB/DOPC mole ratios in 5 mmol l⁻¹ NaCl. The points are connected to guide the eyes. Error bars are smaller than the symbol size. (b) Dependence of normalized fluorescence intensity I_{norm} of DOPC + OTAB + DNA + EtBr complexes on ionic strength at a charge ratio of OTAB/DNA = 10 and at different OTAB/DOPC mole ratios.

(Flock et al., 1996; Rädler et al., 1997). Cancellation of more than 90% of the negative charge of the DNA phosphate groups leads to condensation of DNA, during which DNA changes its conformation from coil to tightly packed toroidal or globule particles (Bloomfield, 1991). Condensed DNA is no longer accessible to intercalating dyes such as EtBr, which will lead to the displacement of intercalated EtBr molecules and a subsequent drop in fluorescence intensity (Eastman et al., 1997; Izumrudov et al., 2002). This enables us to use fluorescence spectroscopy to evaluate the ability of DOPC + OTAB liposomes to form complexes with DNA.

In our experiment, we modulated the surface charge density of liposomes through the OTAB/DOPC mole ratio and the ionic strength of the solution by the addition of NaCl. At first, we determined the ability of DOPC + OTAB liposomes to condense DNA in 5 mM NaCl (Fig. 3a). For most of the OTAB/DOPC compositions, the increase in the concentration of cationic liposomes expressed as the charge ratio OTAB/DNA leads to a significant drop in the fluorescence intensity, reflecting the extent of DNA condensation. However, the liposomes prepared at OTAB/DOPC = 0.2 mol mol⁻¹ seem to be ineffective for DNA condensation. We detected only ~13% of condensed DNA at the highest studied OTAB/DNA charge ratio. The inability of liposomes with OTAB/DOPC = 0.2 mol mol⁻¹ to efficiently condense DNA even at a high OTAB/DNA charge ratio demonstrates that the surface charge density of cationic liposomes is an important factor for successful DNA condensation. With a small increase in the OTAB/DOPC ratio to 0.3 mol mol⁻¹, the ability of cationic liposomes is significantly enhanced, reaching almost 60% at a OTAB/DNA charge ratio of 10:1. However, only liposomes with OTAB/DOPC = 1 mol mol⁻¹ were able to exceed 90% of condensed DNA.

Our results agree well with those of the published literature. An increase in surface charge density generally leads to better efficiency in condensing the DNA up to a certain point at which the maximum efficiency is reached. In addition, at least the critical surface charge density of the cationic liposomes is required to condense DNA. This critical surface charge density can vary depending on the lipids used and the ionic strength of the solution (Ryhänen et al., 2003; Wang et al., 2001).

To evaluate the effect of ionic strength on DNA condensation by DOPC + OTAB liposomes, we chose the maximal tested concentration of liposomes with OTAB/DNA charge ratio = 10. The dependence of the normalized fluorescence intensity I_{norm} of DOPC + OTAB + DNA + EtBr complexes on the ionic strength at the studied OTAB/DOPC mole ratios is shown in Fig. 3b. The I_{norm} increases with increasing ionic strength for all studied OTAB/DOPC mole ratios. The minimal values of fluorescence intensity were recorded for the highest mole ratio, OTAB/DOPC = 1 mol mol⁻¹, in all studied NaCl solutions. At low ionic strength (5 mM NaCl), DNA condensation reached 90%, while only ~55% of DNA was condensed in 150 mM NaCl (I_{norm} = 45.6%). For the least efficient OTAB/DOPC mole ratio of 0.2, we even recorded I_{norm} over 100% in 150 mM NaCl, indicating that the addition of liposomes with low surface charge density in physiological ionic strength caused a conformational change in DNA that increased its accessibility for EtBr.

The general trend of the ionic strength reducing the ability of cationic liposomes or polymers to condense DNA was observed through the exclusion of intercalated fluorescent dyes and is well documented in the literature (Coelho et al., 2021; Eastman et al., 1997; Even-Chen et al., 2012; Hirsch-Lerner et al., 2005; Hubčík et al., 2014; Jing et al., 2004). It

is attributed to the screening of electrostatic interactions between DNA and cationic liposomes by the small ions in the solution.

A high concentration of small ions in the solution also reduces the entropy gain from the counterion release during the formation of the complexes (Harries et al., 2013), which reduces the condensation efficiency.

Other techniques such as titration calorimetry also show suppression of DNA condensation by cationic liposomes in physiological ionic strength compared to 5 mM NaCl. In the work of Pullmannová et al. (2012), titration calorimetry was used to assess the endpoint of the condensation process with cationic liposomes containing DOPC and the cationic surfactant ethane-1,2-diylbis(dodecyldimethylammonium bromide) (C2GS12) in 5 and 150 mM NaCl. While at low ionic strength, the condensation endpoint was reached at the charge ratio of 2:1, at physiological ionic strength, it was reached at the charge ratio of 9:1. The molar enthalpy per mole of DNA base pairs for DNA condensation was reduced from 15.1 kJ mol⁻¹ in 5 mM NaCl to 4.7 kJ mol⁻¹ in 150 mM NaCl. This indicates that even with the higher required ratio of cationic lipid to DNA, the condensation was still more than three times less efficient in 150 mM NaCl. This aligns with our systems with the mole ratio OTAB/DOPC ≤ 0.5. Liposomes with a higher surface charge density were less affected by the increase in ionic strength.

Structure study of DOPC + OTAB + DNA complexes

At the investigated temperatures, fully hydrated DOPC forms a well-defined liquid-crystalline lamellar L_a phase (Nagle & Tristram-Nagle, 2000; Uhríková et al., 2005). In the SAXS pattern (Fig. 4), we observed two reflections corresponding to regularly packed bilayers. The repeat distance of the lipid bilayer stacking, including the thickness of the bilayer and the water layer, was determined from the position of the first lamellar peak L(1) according to Eq. 2.

We found the repeat distance $d = 6.28 \pm 0.01$ nm in 5 mM NaCl and $d = 6.40 \pm 0.01$ nm in 150 mM NaCl at 20 °C (Fig. 4a), which is in agreement with the literature (Nagle & Tristram-Nagle, 2000; Pullmannová et al., 2012; Uhríková et al., 2005; Želinská et al., 2020). After heating to 50 °C (Fig. 4b), an increase in repeat distance was observed, which reached $d = 6.34 \pm 0.01$ nm in 5 mM NaCl and $d = 6.49 \pm 0.01$ nm in 150 mM NaCl. The water layer thickness increases more rapidly with increasing temperature, compared to the decrease of bilayer thickness, which ultimately manifests itself in increased repeat distance (Nagle & Tristram-Nagle, 2000).

It was reported that the OTAB forms complexes with DNA exhibiting a hexagonal structure (Kawashima et al., 2006). The use of DOPC as a helper lipid in DOPC + OTAB + DNA complexes preserve the lamellar phase at both tested OTAB/DOPC mole ratios (Fig. 5). However, the peaks in the SAXS region are broad, which indicates a significant decrease in the positional ordering of the stacked bilayers. The lamellar repeat

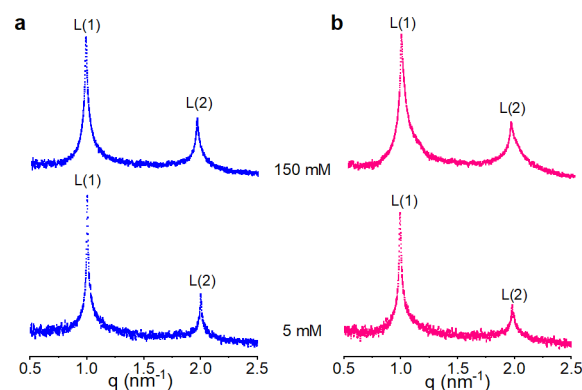


Figure 4. The SAXS patterns of DOPC in 5 and 150 mM of NaCl at 20 °C (a) and 50 °C (b). Intensity is in a logarithmic scale.

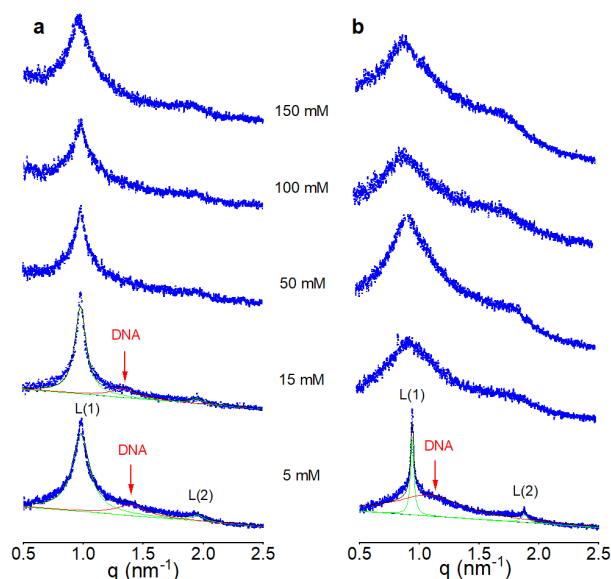


Figure 5. The SAXS patterns of DOPC + OTAB + DNA complexes at different ionic strengths and mole ratios OTAB/DOPC = 1 (a) and 0.3 (b) at 20 °C. For illustration, the data points are simulated with the best Lorentzian fit curves. Intensity is in a logarithmic scale.

distance d for the condensed lamellar phase of complexes was found in the range 6.21–6.51 nm for OTAB/DOPC = 1 mol mol⁻¹ and 6.48–6.83 nm for OTAB/DOPC = 0.3 mol mol⁻¹ (Fig. 6). The peaks that are typical for regularly ordered condensed DNA in between the lipid bilayers are visible only at low ionic strengths. At the mole ratio OTAB/DOPC = 1, the peaks indicating evidence of DNA–DNA organization were detected at $q = 1.40$ nm⁻¹ (5 mM NaCl) and $q = 1.37$ nm⁻¹ (15 mM NaCl). The resulting interhelical DNA–DNA distance was $d_{\text{DNA}} = 4.5 \pm 0.1$ nm at 5 mM NaCl and 4.6 ± 0.1 nm at 15 mM NaCl. At the mole ratio OTAB/DOPC = 0.3, we determined the DNA–DNA distance $d_{\text{DNA}} = 5.8 \pm 0.1$ nm. The peak of DNA is not distinguishable at higher ionic strengths, which means

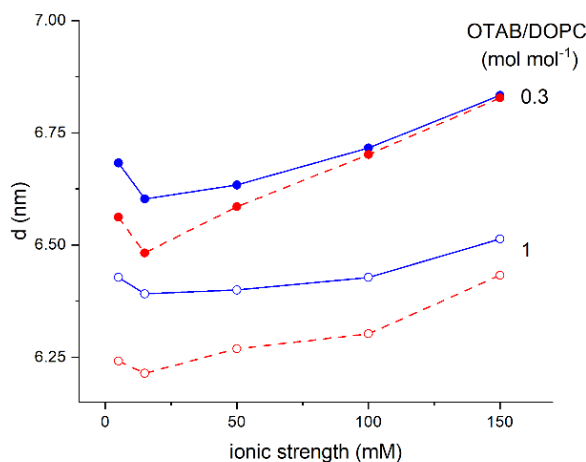


Figure 6. Dependence of the repeat distance d of the lipid bilayers stacking in the DOPC + OTAB + DNA system on ionic strength at the mole ratios OTAB/DOPC = 0.3 (dots) and 1 (circles) at 20 °C (blue line) and 50 °C (red line).

that DNA is weakly ordered between the lipid lamellae. The weak ordering of DNA at high ionic strength is in agreement with the condensation experiments which showed a large decrease in the fraction of condensed DNA with increased NaCl concentration.

Another alternative is that a DNA peak could be overlapped with an $L(1)$ peak. In cases where the two signals are close in q -space, their individual features could merge into a single, broadened peak that might be misinterpreted or simply be less distinguishable. High salt concentrations can lead to a more heterogeneous distribution of DNA within the lipid complexes. This heterogeneity could cause the DNA correlation peak to broaden significantly, reducing its intensity below the detection limit, even though DNA is still associated with the complexes.

The absence of DNA reflection in the SAXS region of diffractograms was also reported in studies of the structure of DOPC–DNA aggregates formed in the presence of calcium and magnesium ions (Francescangeli et al., 2003; Uhríková et al., 2005). The weak ordering of the condensed fluid lamellar phase L_a^c suggests that the interaction between DNA and cationic surfactant is weak. This could be an advantage during the release of DNA, after endocytosis, into the cell medium (Mel'nikova & Lindman, 2000).

The effect of the mole ratio OTAB/DOPC and the ionic strength on the repeat distance d in the lipid bilayer stacking of the DOPC + OTAB + DNA system at 20 °C and 50 °C is shown in Fig. 6. At the higher surface charge of the bilayer (OTAB/DOPC = 1 mol mol⁻¹), the repeat distance is systematically lower by ~0.25 nm, compared to OTAB/DOPC = 0.3 mol mol⁻¹, as a consequence of stronger electrostatic interaction between DNA and OTAB. With increasing ionic strength after a small drop at 15 mM NaCl, we observed a nonlinear increase of d for both OTAB/DOPC mole ratios and both temperatures, which

suggests that the higher ionic strength screens the interaction between DNA and OTAB. As a result of the screening, the attractive force between DNA and positively charged lipid membranes that pulls the lipid lamellae closer together is weakened and the distance between the membranes is increased.

At 50 °C, we observed similar dependences; however, the d -values were systematically lower. This indicates that the temperature-induced decrease of the thickness of the lipid bilayer prevails against the diffusion of water molecules from the bulk water phase in between the lipid bilayers. The same effect was also observed in the lipoplexes with DOPC + Ca²⁺ and dilauroylphosphatidylcholine + cationic gemini surfactant (Uhríková et al., 2002, 2005).

The amount of bound DNA was verified by UV/Vis spectrometry in the supernatant after centrifugation of samples used for SAXS measurements. Surprisingly, free DNA was not detected in the supernatant (data not shown). This means that almost all DNA is bound in the lipoplexes, despite the peaks for DNA not being visible on the diffractograms and the condensation experiments showing only 55% of condensed DNA in 150 mM NaCl for the mole ratio OTAB/DOPC = 1 and less than 20% of condensed DNA for mole ratio OTAB/DOPC = 0.3. Thus, even in complexes with the majority of uncondensed DNA, almost all DNA is associated with the lipoplexes.

The apparent discrepancy between fluorescence spectrometry and UV/Vis determination of unbound DNA in the supernatant can be explained by the fact that DNA does not need to be fully condensed to be effectively removed from the supernatant. As the condensation of DNA requires 90% of its negative charge to be canceled out by the cationic lipid, a large fraction of the DNA can be associated in lipoplexes but is still partially accessible for EtBr. The EtBr assay is reliable for assessing condensation, but must be interpreted carefully alongside other data, as it does not directly measure binding efficiency.

A similar observation was also reported in the work of Even-Chen et al. (2012). The authors determined condensation of DNA below 50% in lipoplexes prepared in 150 mM NaCl, however, less than 5% of free DNA in the solution detected by UV/Vis spectroscopy in the supernatant and also by agarose gel electrophoresis.

CONCLUSIONS

The interaction between DNA and cationic liposomes is strongly influenced by the ionic strength of the solution and the surface charge density of the lipoplexes. At high ionic strength, the interaction is effectively shielded. Our experiments demonstrated that a high concentration of the cationic additive is required to achieve efficient nucleic acid condensation under physiologically relevant conditions (150 mM NaCl).

However, even the complexes with a low amount of condensed DNA have almost all DNA associated with the

cationic liposomes. DNA complexes with DOPC + OTAB liposomes create a condensed fluid lamellar phase L_a^c , where parallel DNA strands are intercalated between the lipid bilayers and protected from degradation by enzymes during transport to the cell. The interaction of DNA with OTAB is relatively weak, which can be an advantage after passing through the cell membrane, enabling the release of DNA to be easier.

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