Cationic lipids and cationic ligands induce DNA helix denaturation: detection of single stranded regions by $KMnO_4$ probing

T.K. Prasad, Vijaya Gopal*, N. Madhusudhana Rao*

Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

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Abstract Cationic lipids and cationic polymers are widely used in gene delivery. Using 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) as a cationic lipid, we have investigated the stability of the DNA in DOTAP:DNA complexes by probing with potassium permanganate ($KMnO_4$). Interestingly, thymidines followed by a purine showed higher susceptibility to cationic ligand-mediated melting. Similar studies performed with other water-soluble cationic ligands such as polylysine, protamine sulfate and polyethyleneimine also demonstrated melting of the DNA but with variations. Small cations such as spermine and spermidine and a cationic detergent, cetyl trimethylammonium bromide, also rendered the DNA susceptible to modification by $KMnO_4$. The data presented here provide direct proof for melting of DNA upon interaction with cationic lipids. Structural changes subsequent to binding of cationic lipids/ligands to DNA may lead to instability and formation of DNA bubbles in double-stranded DNA.

Key words: Cationic lipid; DNA condensation; DNA unwinding; $KMnO_4$ probing; Transfection; Cationic lipid:DNA complex

1. Introduction

The delivery of nucleic acids into cells as complexes of cationic lipids has been extensively investigated [1–3]. Lipids shield the negative charges of the nucleic acids and render them permeable to the cell membrane. The simplicity of preparation, low toxicity, satisfactory transfection efficiencies, and low immunological response have attracted extensive efforts to improve cationic lipid-mediated gene delivery [4–6]. Unlike earlier efforts, which were targeted mainly at improving the transfection efficiency, current efforts are more knowledge-based in terms of designing the lipid formulations with firm understanding of transfection-related mechanisms at the molecular and self-assembled levels [7,8]. It is being increasingly realized that certain physical features of the cationic lipid:DNA complex (CLDC) such as charge-neutralized complexes or complexes that dissociate at low pH are more transfection-competent [9,10].

Formation of CLDC is primarily driven by electrostatic interactions between negative charges (phosphate) of nucleic acid and positive charges (usually the quaternary nitrogen) of the lipid. The complex is stabilized by the hydrophobic interaction between acyl chains of the lipid, which would result in smectic phases of the lipids around the long strands of the DNA [11]. Complexation is an endothermic process resulting in dehydration of the DNA [12]. The long-range order in lipoplexes studied using small-angle X-ray diffraction revealed a supramolecular assembly, which is a multilayer assembly of DNA sandwiched between bilayer membranes, a 2D smectic phase of DNA chains coupled to a 3D smectic phase of lipid bilayers. In the presence of non-bilayer-preferring lipids such as L-α-dioleoyl phosphatidylethanolamine (DOPE), the diffraction pattern was consistent with a 2D columnar inverted hexagonal structure in which the DNA molecules are surrounded by a lipid monolayer, with the DNA–lipid inverted cylindrical micelles arranged on a hexagonal lattice [7,13]. Circular dichroism (CD) and Fourier transform infrared (FTIR) studies of the lipoplexes revealed condensation of the DNA, reduction of the winding angle resulting from electrostatic interactions and dehydration of the lipoplex [14]. Prasad et al. have recently showed that transcription at a DNA template was enhanced on interaction with cationic lipids [15]. This observation was further confirmed by hyperchromicity, due to partial unwinding, and by studies on susceptibility of lipoplexes to micrococcal nuclease and S1 nucleases. These studies also suggest that the interaction of lipid with DNA extends beyond direct electrostatic contact to interactions between head groups and DNA bases. While the long-range interactions have been described above, information on the short-range alterations in DNA structure upon interaction with the cationic lipids is lacking. Changes in the winding angle of DNA or transcriptional activation suggest partial unwinding of the DNA in a lipoplex [14,15]; however, details or extent of these structural changes have not been investigated.

DNA demonstrates rich structural polymorphism upon interaction with various cationic ligands and proteins, by adopt-

*Corresponding author. Fax: (91)-40-27160591.
E-mail addresses: vijaya@ccmb.res.in (V. Gopal), madhu@ccmb.res.in (N.M. Rao).

Abbreviations: CD, circular dichroism; CLDC, cationic lipid:DNA complex; CTAB, cetyl trimethylammonium bromide; DC-Chol, 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]cholesterol; DDAB, dimethyldioctadecylammonium bromide; DMRIE-C, 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide-cholesterol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, L-α-dioleoyl phosphatidylethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride; FTIR, Fourier transform infrared; N/P charge ratio, positive charge of tertiary ammonium/negative charge of phosphate

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ing different structures such as kinked, bent, looped or toroidal structures, when complexed with the cationic molecules [16]. Structural transitions in DNA are also influenced or induced by divalent cations [17,18] and histones [19]. Cations having less than three charges screen the charges on DNA but do not condense the DNA [18]. Cationic lipids carrying a single charge, excluding the polycationic lipid 2,3-dioleyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate and a few others, behave similar to polyvalent cations due to their self-aggregating properties [16]. To describe the interaction of cationic lipids with DNA further it is important to understand fine structural changes in DNA affected by cationic lipids. It is also important to compare these changes with other polymeric molecules that interact with DNA, such as polylysine, polyethyleneimine, spermine, spermidine, etc. Enzymatic probes seldom detect fine structural changes due to the largeness of these probes, but chemical probes such as $KMnO_4$ can detect strand separations even to a base level with high sensitivity [20]. Preference to modify thymine residues in single-stranded DNA allowed extensive usage of $KMnO_4$ to detect and characterize distorted DNA structures in a variety of contexts including transcription [20], elongation [21], drug–DNA binding [22], chromatin positioning and recombination [23] and on interaction with cationic amphiphiles [24]. $KMnO_4$ can reveal sequence-dependent distortion on DNA alone and any sequence deviating from the B-form is a model candidate for $KMnO_4$ probing. $KMnO_4$ was also used for the purpose of sequence determination and selective degradation of thymidine residues specifically in the single-stranded region of nucleic acids [25]. $KMnO_4$ specifically oxidizes pyrimidine residues at the 5,6 double bond to form pyrimidine glycol residues. These residues are sensitive to piperidine, which cleaves the phosphodiester backbone [26]. We have investigated the effects of cationic lipid/ligand binding to DNA using $KMnO_4$ and report here a novel observation that cationic lipids/ligands induce partial melting of DNA.

2. Materials and methods

2.1. Chemicals

1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP), DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), dimethyldioctadecylammonium bromide (DDAB), and 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) were purchased from Avanti Polar Lipids. 1,2-Dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide-cholesterol (DMRIE-C) was from Gibco BRL. Cholesterol, spermine, spermidine, polyethyleneimine, cetyl trimethylammonium bromide (CTAB), and β-mercaptoethanol were obtained from Sigma-Aldrich. Acrylamide and bis-acrylamide were from Sisco Research Laboratories, Mumbai, India. T4 polynucleotide kinase was from New England Biolabs. [γ-^{32}P]ATP was from Bhabha Atomic Research Centre, Mumbai, India. All other chemicals including $KMnO_4$ were of analytical grade quality.

2.2. Preparation of double-stranded DNA probe for $KMnO_4$ probing

DNA duplex, containing the A1 promoter (499 bp long), from the plasmid pAR1435 [15], was obtained by polymerase chain reaction (PCR), using the following primers. Primer 1, sequence ID A1435F, 5′-TTAATGCGGTAGTTTATCACA-3′ and primer 2, sequence ID A1435R, 5′-AACCGCACCTGTGGCGCCGG-3′, correspond to the forward and reverse primers. The reverse primer was labeled with [γ-^{32}P]ATP using polynucleotide kinase according to the standard protocol [27]. Radiolabeled double-stranded DNA was obtained by carrying out PCR using radiolabeled A1435R corresponding to the reverse primer and unlabeled forward primer A1435F using standard procedures. Qiagen PCR purification kit was used to purify the radiolabeled 499 bp PCR product according to the manufacturer's protocol, which was run on a 2% agarose gel and quantitated by ethidium bromide staining to determine the concentration of the DNA.

2.3. Preparation of cationic liposomes/ligands

Cationic liposomes of various compositions were prepared by drying their chloroform stock solutions under nitrogen to make a thin film. Later this was dried under vacuum for 3 h. Then by adding sterilized MilliQ water these lipids were rehydrated overnight. Lipids were then sonicated to get a clear solution of small unilamellar vesicles. The following stocks of liposome formulations DOTAP (1 mM), DOTAP:DOPE (1 mM:1 mM), DOTAP:DOPC (1 mM:1 mM), DOTAP:Chol (1 mM:1 mM), DOPC (1 mM), 1 mg/ml solution of DDAB:DOPE (1:2.5 w/w), DC-Chol:DOPE (1 mM:1 mM) were prepared which were later diluted according to the requirement. CTAB was prepared in sterilized MilliQ water to obtain 1 mM stock. Spermidine, spermine, polyethyleneimine, poly-L-lysines, and protamine sulfate were prepared by dissolving in sterilized MilliQ water to obtain the desired concentrations. Lipofectamine, DMRIE-C and lipofection were diluted from the commercial preparation to obtain the desired concentrations.

2.4. $KMnO_4$ reaction

Cationic lipids/ligands and radiolabeled DNA complexes were prepared at the indicated ratios of positive charge of tertiary ammonium/negative charge of phosphate (N/P charge ratio). Complexes were incubated at room temperature for 15 min and $KMnO_4$ probing was done according to standard protocols [28]. Equal counts of the reaction mixture were loaded in each lane on an 8% denaturing polyacrylamide gel (19:1, acrylamide:bis-acrylamide; PAGE) in 1×TBE buffer pH 8.0. Gel runs were calibrated by running a G-ladder prepared from the same DNA fragment according to the standard protocol [27]. After the run the gel was exposed to Fuji phosphor imaging plate and scanned in a Fuji phosphorimager (FLA-3000).

3. Results

3.1. $KMnO_4$ probing of promoter DNA in a lipoplex

Several lines of spectroscopic and biochemical investigations on the physical state of DNA in a CLDC suggest alterations in the duplex stability, probably changes in the base stacking [14]. To investigate the structural basis we probed the fine structural changes in the template DNA in CLDC, especially formation of single strands, using $KMnO_4$. CLDC were prepared and probed by the ability of $KMnO_4$ to detect single-stranded regions in vitro, as induced by a variety of cationic lipids. A 499 bp PCR-amplified DNA fragment containing the A1 promoter region, and the flanking sequences of the plasmid construct pAR1435, was obtained to make the CLDC. DOTAP was chosen as the cationic lipid, since it has been the model cationic lipid and considerable information was available on the lipoplexes formed between DOTAP and DNA [1]. Here, we used $KMnO_4$ as a structure-specific probe by taking advantage of its property to modify unpaired T residues in DNA partially melted by cationic lipids. The extent of reactivity generated in the DNA template varied as a function of the concentration of cationic molecules as seen from the following results.

3.1.1. DNA melting in a DOTAP:DNA complex. The partial sequence of the A1 promoter, as derived by PCR amplification from the plasmid pAR1435, and which was used throughout this study, is shown in Fig. 1. Labeled DNA was prepared by labeling the bottom strand as described in Section 2, DOTAP:DNA complexes were prepared at various N/P charge ratios prior to the addition of permanganate. The products of such a reaction, resolved on an 8% denaturing polyacrylamide gel, generated a pattern indicative of the extent of reactivity of the residues in the DNA template.

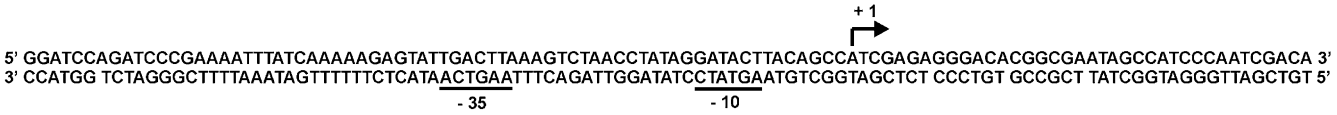

Fig. 1. Partial sequence of the double-stranded DNA template used in the $KMnO_4$ probing experiments. −35, −10 promoter regions of the DNA template are underlined. +1 arrow marks the transcription start site.

$KMnO_4$ probing followed by piperidine cleavage of the modified DNA template, in the presence of DOTAP, is shown in Fig. 2A. The band pattern obtained in lane 1 shows the positions of the modified Ts of the labeled bottom strand, in the absence of DOTAP. The band intensity increases with the increase in N/P charge ratio, indicating that these Ts are more susceptible to modification by $KMnO_4$. There is a clear preference for T residues of TA or TG dinucleotides, indicating conformational flexibility of pyrimidine/purine (YR). In the absence of DOTAP, the basal level reactivity of Ts, as seen by piperidine cleavage, occurs at positions +36, +22, +19, +11, +7, +5, −10, −12, and −15, in all the lanes, which indicates small fluctuations in some of the bases of template DNA. In the presence of DOTAP, at N/P charge ratios of 0.3:1, 0.5:1, 1:1, 3:1, and 9:1, the DNA undergoes structural changes which result in increased susceptibility of T residues most discernible at positions +36, +32, +26, +22, +20, +13, +11, +1, and −4, indicating flexibility of YR steps which occur at each of the mentioned positions with the exception of +22 and −4, which are pyrimidine/pyrimidine (YY) residues. The T residues that are exposed in the presence of increasing amounts of DOTAP, thereby becoming increasingly reactive to $KMnO_4$, are clearly discernible from bands that are absent in the DNA control lane. This indicates the intrinsic propensity towards DNA distortion at the T residues which are followed by purines, i.e. YR dinucleotides. There is an increased local helix distortion at the YR steps, which occurs with greater frequency in regulatory sites in DNA, as induced by increasing concentrations of the cationic lipids. One important observation here is that susceptible Ts were not restricted to the promoter region (−10 to −35) and were present at other positions along the DNA template studied. Density scans of the gel clearly demonstrate the concentration-dependent susceptibility of the Ts, especially at po-

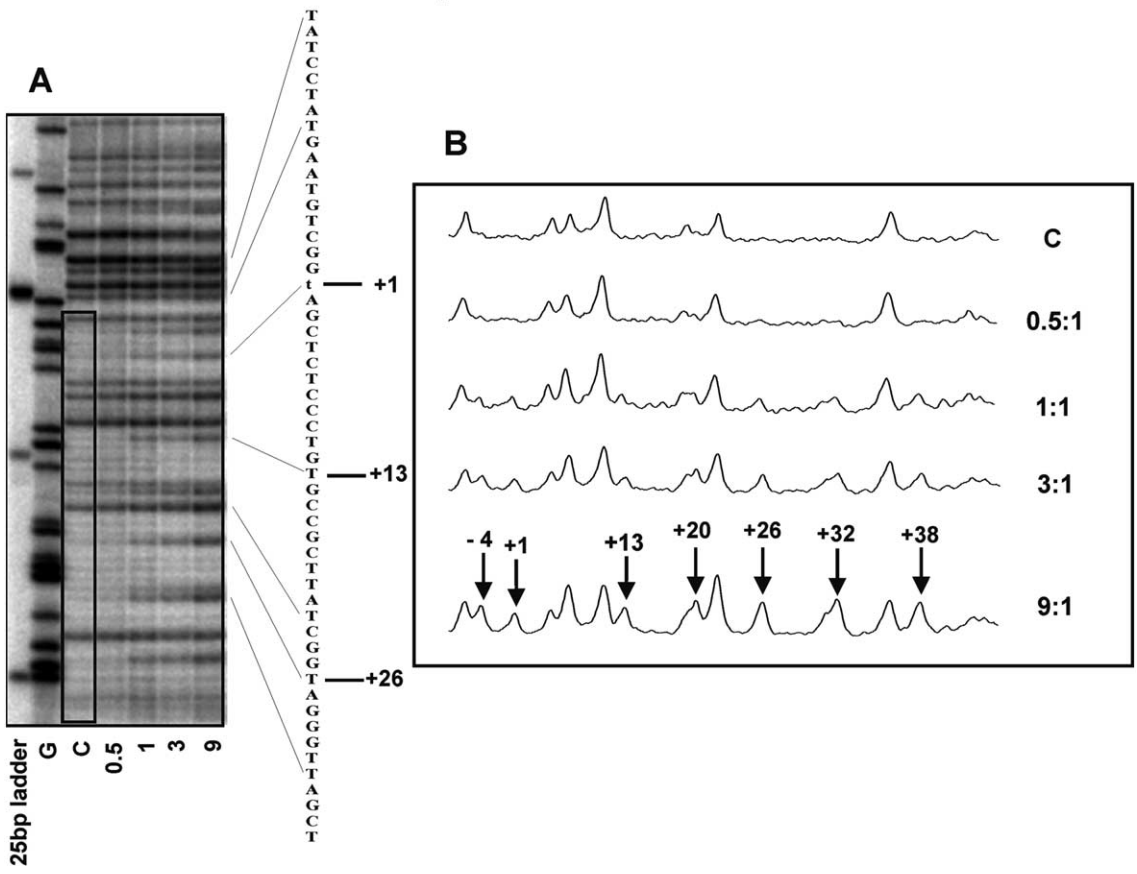

Fig. 2. $KMnO_4$ probing of DOTAP:DNA complexes. The DNA template was complexed with different amounts of DOTAP. Either complexed or uncomplexed DNA was treated with $KMnO_4$ and modified T residues were cleaved with piperidine and resolved on 8% urea-PAGE as described in Section 2. A: Each lane of the gel is labeled at the bottom; 0.5, 1, 3 and 9 indicate N/P ratios. 'C' is control lane (only DNA template). 'G' is the G-ladder, prepared by using the same labeled DNA for calibrating the gel as described in Section 2. '25 base pair ladder' was run as a molecular weight marker with the most intense band corresponding to 125 nt. B: Density scan tracing of a small selected area from the gel shown in A. Each trace is labeled alongside to indicate the N/P charge ratio of DOTAP/DNA. Positions of some hyperreactive T residues are marked.

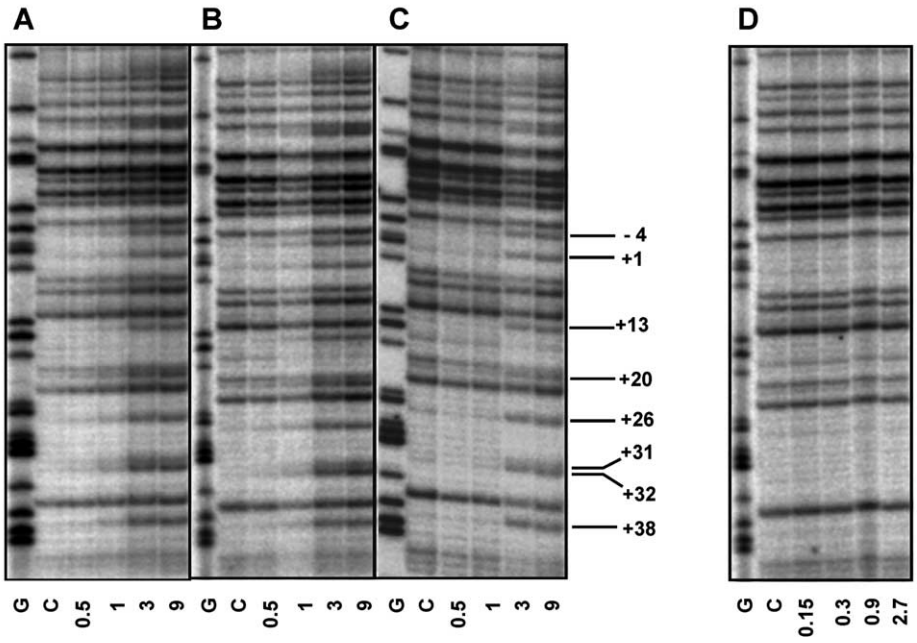

Fig. 3. $KMnO_4$ probing of DOTAP with co-lipids/DNA. The DNA template was complexed with different amounts of DOTAP+co-lipids. Either complexed or uncomplexed DNA was treated with $KMnO_4$ and modified T residues were cleaved with piperidine and resolved on 8% urea-PAGE as described in Section 2. A: DOTAP:DOPE. B: DOTAP:DOPC. C: DOTAP:Chol. Each panel shows the $KMnO_4$-modified and piperidine-cleaved T residues of DNA template complexed with DOTAP:co-lipids at various N/P ratios. Each lane of the gel is labeled at the bottom. 0.5, 1, 3 and 9 indicate N/P ratios. 'C' is control (only DNA template) and 'G' is the G-ladder prepared from the same DNA template. D: T cleavage pattern of DNA template complexed with the zwitterionic lipid DOPC. Each lane of the gel is labeled at the bottom. 'C' is control (only DNA template) and 'G' is the G-ladder prepared from the same DNA template. 0.15, 0.3, 0.9 and 2.7 indicates nmol of DOPC mixed with DNA in each reaction. These amounts were similar to the amount of DOTAP used to get N/P ratios of 0.5, 1, 3, and 9 respectively. Positions of some hyperreactive T residues are marked.

sitions −4, +1, +13, +20, +26, +32, and +38 (Fig. 2B). We also probed the non-template strand (top strand of Fig. 1) of the same DNA template complexed with the cationic lipid DOTAP, with $KMnO_4$. The pattern obtained with the top strand had a similar trend with cleavage occurring at T residues (data not shown).

As a transfection agent, DOTAP was used in combination with co-lipids, which enhance the efficiency of transfection. DOTAP tested with other co-lipids such as DOPE (Fig. 3A), DOPC (Fig. 3B) and cholesterol (Fig. 3C) also yielded similar reactivity at the T residues, corresponding to the melted regions observed with DOTAP alone. The transfection efficiency of DOTAP with DOPC as co-lipid was very insignificant compared to co-lipid DOPE. However, the cleavage pattern with DOTAP:DOPC was similar to the pattern obtained with co-lipids DOPE or cholesterol. This observation suggests that the ability of DOTAP to alter the structure of template DNA was not dependent on the nature of the co-lipid present in the formulation. In the presence of the zwitterionic lipid DOPC, the cleavage pattern was similar to the control indicating this lipid does not bring about the melting of DNA (Fig. 3D). DOPC does not condense the DNA nor is it transfectionally competent. Presence of a positive charge on the ligand/lipid was essential to bring about changes in the template DNA.

3.1.2. DNA melting by other cationic lipids/ligands. Condensation of DNA favors several biological processes. In non-viral gene therapy, condensation of DNA is a primary requisite for optimal internalization, without much degradation, via the endocytotic pathway, and recent investigations have shown that transfection efficiency correlates with the condensed phase DNA [8]. Having observed enhanced transcription with cationic lipids, cationic detergents and polyethyleneimine [15], we also extended our study by probing DNA complexed with other popular cationic lipids and their formulations such as lipofectamine, lipofectin, DMRIE-C, DDAB:DOPE, and DC-Chol:DOPE at 1:1 N/P charge ratios (Fig. 4A). In this study we also included other small molecular DNA-condensing cationic ligands, spermine and spermidine. Compared to the other lanes, lipofectamine (lane 1) and spermine (lane 7) showed the highest reactivity pattern. Notably a strikingly intense band pattern was observed for both lipofectamine and spermine at +38, +32, +26, +20, +13, +1, and −4. Fig. 4B shows the density scan traces of selected lanes from Fig. 4A. Interestingly, these are all YR dinucleotide residues, with the exception of −4, which is a TC (YY) dinucleotide. On comparison of the intensity pattern observed at one N/P charge ratio (1:1), it is apparent that cationic lipids/ligands with multiple charges, especially lipofectamine (spermine-containing lipid) and spermine, demonstrate strong ability to alter the template DNA structure. Among these ligands the effects of DMRIE-C and DC-Chol were the weakest (Fig. 4A, lanes 2 and 5 respectively).

3.1.3. DNA melting in CTAB:DNA complexes. Similar to cationic lipids, electrostatic interactions mediate the formation of a CTAB:DNA complex. Unlike cationic lipids, CTAB, a cationic detergent, possesses a single hydrophobic chain, hence its aggregating properties would be different from cationic lipids. However, several physical studies have demonstrated the presence of DNA:CTAB aggregates at low detergent concentrations [29]. As shown in Fig. 5, in the presence of increasing amounts of CTAB, melting of DNA was re-

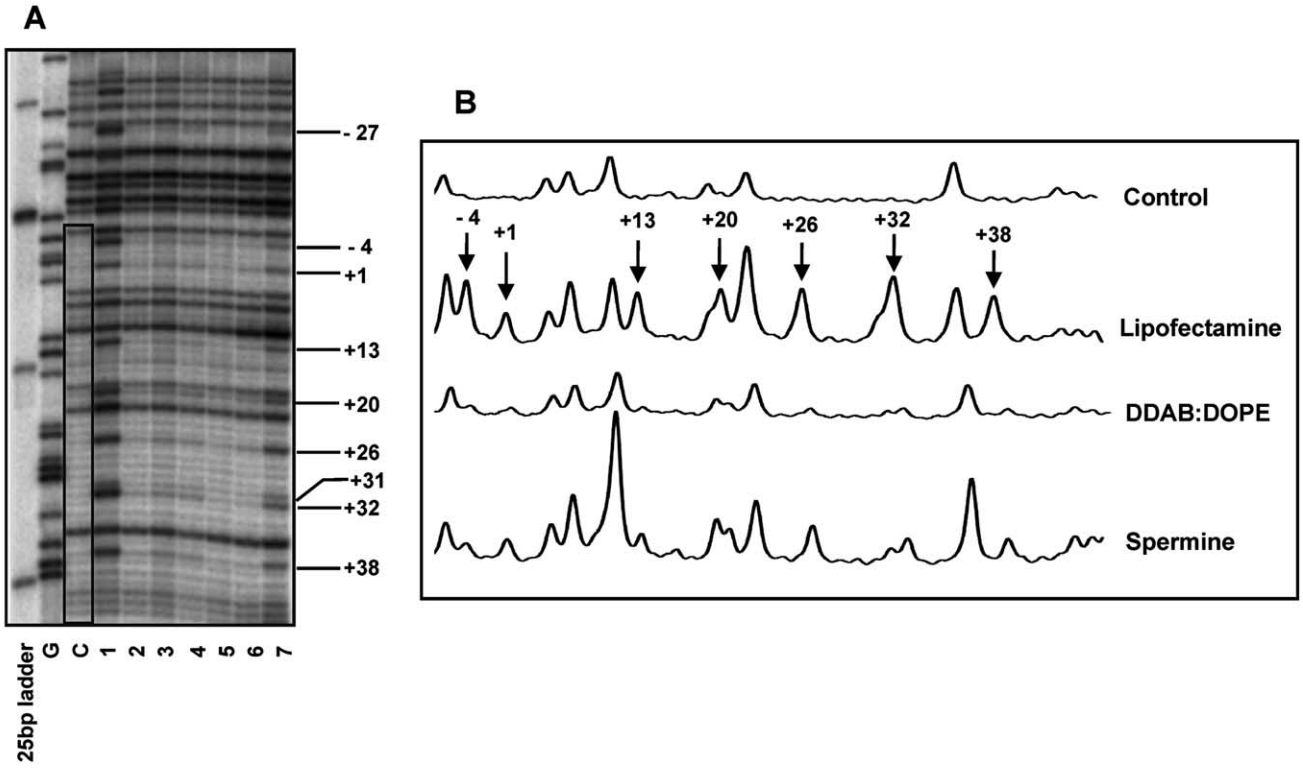

Fig. 4. $KMnO_4$ probing of DNA complexed with various cationic lipid formulations/ligands. Either complexed or uncomplexed DNA was treated with $KMnO_4$ and modified T residues were cleaved with piperidine and resolved on 8% urea-PAGE as described in Section 2. A: T cleavage pattern of (lane 1) lipofectamine; (lane 2) DMRIE-C; (lane 3) lipofectin; (lane 4) DDAB:DOPE; (lane 5) DC-Chol:DOPE; (lane 6) spermidine; (lane 7) spermine. 'C' is control (only DNA template) and 'G' is the G-ladder prepared from the same DNA template. '25 base pair ladder' was run as a molecular weight marker with the most intense band corresponding to 125 nt. B: Density scans of control, lipofectamine, DDAB:DOPE and spermine from lanes marked 'C', 1, 4, and 7 (encompassing the area marked by a rectangle from a selected region as shown in lane 'C') on the gel in A. Positions of some hyperreactive T residues are marked.

vealed by increased band intensities at positions similar to those observed for DOTAP (Fig. 2A).

3.1.4. DNA melting by water-soluble polymeric cations. Polylysine and protamine sulfate have been extensively used as gene delivery agents due to their ability to condense the DNA and protect it from nucleases [30,31]. Based on the ability of polyamines to cause changes in DNA structure, we tested polylysines of different molecular weights. The ability of poly-L-lysine to deliver DNA into cells depends on its molecular weight. $KMnO_4$ reactivity patterns of template DNA in the presence of poly-L-lysine 3 kDa, poly-L-lysine 30 kDa, poly-L-lysine 120 kDa and protamine, an arginine-rich basic protein, are presented in Fig. 6A–D. T reactivity pattern was observed with all these polymeric cations, all along the DNA template. At N/P charge ratios of 1 and above, the pattern was strikingly intense. Unlike cationic lipids, these basic proteins do not show appreciable effects at a charge ratio of 0.1. The effects were similar to both arginine- and lysine-rich proteins, which also agrees with their ability to condense the DNA.

Polyethyleneimine and its derivatives are extensively used either directly or in combination with cationic lipids as a gene delivery agent [32,33]. Obtaining branched polyethyleneimine or performing chemistry to link ligands to polyethyleneimine was convenient [34,35]. Polyethyleneimine also alters the T cleavage pattern, but higher amounts are required to bring about the same effect (data not shown).

3.2. Conformational transitions of DNA in a CLDC: a model

Our studies clearly demonstrate that the interaction of DNA with cationic lipids/ligands result in partial melting of duplex DNA. The model that we propose is applicable for any double-stranded DNA. Binding of cationic lipids to B-form DNA is preceded by displacement of the counter ions from DNA. Electrostatic interactions between the alkylammonium cations and the DNA phosphates and subsequent stabilization of the lipids due to hydrophobic effects stabilize the CLDC [36]. The binding alters the winding angle resulting in DNA conformation away from the B-form and close to the C-form [14,15]. Local opening of the double helix and formation of denaturation bubbles, which now may be energetically favorable, may relieve the altered strain in the DNA on lipid binding. These bubbles may localize at AT-rich regions owing to their inherent susceptibility to breathing when compared to GC-rich regions [37]. Such DNA structures formed in a CLDC are potential sites for modification by $KMnO_4$ resulting in the band patterns reported by us.

4. Discussion

Understanding the structure of CLDC has been a challenge, since these complexes do not have regular morphology, are strongly dependent on the N/P ratio and do not lend themselves easily to the application of optical spectroscopic techniques. Interest in these complexes is primarily due to the

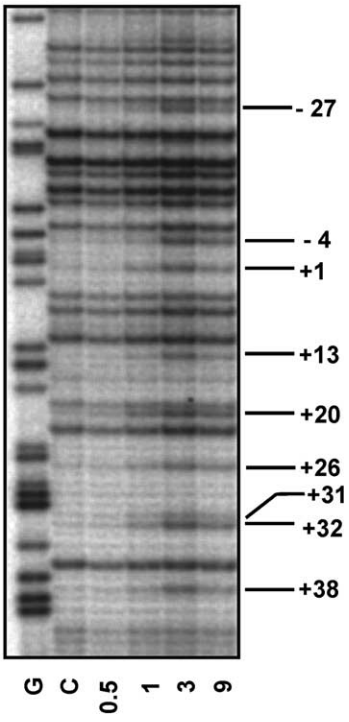

Fig. 5. $KMnO_4$ probing of DNA complexed with the monoacyl chain cationic detergent CTAB. Either complexed or uncomplexed DNA was treated with $KMnO_4$ and modified T residues were cleaved with piperidine and resolved on 8% urea-PAGE as described in Section 2. Figure shows the T cleavage pattern where 'C' is control (only DNA template) and 'G' is the G-ladder prepared from the same DNA template. 0.5, 1, 3 and 9 indicate N/P ratios. Positions of some hyperreactive T residues are marked.

ability of these complexes to ferry the DNA into the cells [3]. Due to the absence of regularity in these complexes, techniques that address long-range order were often used. Small-angle X-ray studies on these complexes were very useful in providing the detail of these complexes [11]. These analyses suggest mesophases where DNA is enclosed in lamellar sheets or inverted hexagonal phases. Submicron microscopy, especially cryotransmission electron microscopy and atomic force microscopy, provided the morphology of these complexes. The present investigation was addressed to look at the fine structural changes, especially interactions between bases, in the DNA present in such complexes.

Upon binding to cationic lipids, DNA undergoes a structural change resulting in opening of the base stacks making them susceptible to oxidative cleavage of the phosphodiester backbone. In duplex DNA, stereochemical hindrance from base stacking prohibits reactivity of double-stranded B-form DNA. The data presented in this report demonstrate for the first time partial unwinding of DNA on interaction with cationic lipids. Two lines of evidence support our observation. The recent report of Prasad and co-workers suggested partial unwinding of DNA in a CLDC based on an increase in hyperchromicity, micrococcal nuclease activity of DNA with increasing N/P ratio, and enhancements in transcription [15]. All three observations would be aided by perturbation of base stacking of DNA in complexation with cationic lipids. Secondly, CD studies of several labs suggest the DNA in a lipoplex is in the C-form. [15,38,39]. Braun and co-workers have re-examined the structure of DNA in a CLDC using FTIR as well as Raman spectroscopy [14]. In addition, molecular dynamics simulations showing an alteration in the local helicity and perturbation of stacking interactions of the bases with the overall helicity being maintained were carried out using a positively charged phospholipid to mimic a cationic lipid surface on a 10 bp double-stranded DNA [14]. It thus seems clear that lipid/DNA interactions progress through an initial step of direct charge–charge interaction wherein the cationic lipid/ligand interacts with the phosphate backbone which in turn results in changes in base stacking which result in a decrease in the 273 nm peak. The observation of single-stranded regions in DNA in CLDC as reported here provides additional and direct evidence for perturbed base stacking in DNA in CLDC.

The physical properties of CLDC vary with change in the N/P ratio [40,41]. At N/P charge ratios above 1:1 the particles are uniform, positively charged and show high transfection efficiency and low DNase I susceptibility. In contrast to this behavior the cleavage intensity at any T residues was observed to increase linearly with increase in N/P ratio. With increase in lipid either more DNA was becoming part of the CLDC and/or the perturbation in the base stacking increased. The partial unwinding ability of the cationic lipid was independent of the co-lipid used. However, these co-lipids do not alter the DNA unwinding ability of DOTAP. It is also apparent that the ability to melt DNA is independent of the physical properties of the cationic lipids/ligands.

Spermidine with three charges was much weaker than spermine in its ability to alter the DNA structure. Though the condensing ability of polyamines, such as spermidine and spermine, has been investigated by several methods, to our knowledge, their ability to open up the base pairs has not been reported so far. Other cationic lipids selected were structurally different from DOTAP. DC-Chol is a cholesterol-based cationic molecule unlike DOTAP, which is a glycerol-based cationic lipid. DDAB does not have a glycerol to link the cationic charge to the fatty acids; instead the fatty acids are directly linked to the quaternary nitrogen. DMRIE is similar to DOTMA but possesses an additional ethoxy group on the quaternary nitrogen, which may alter the interaction of quaternary nitrogen with negative phosphates on DNA. In DDAB, DMRIE and lipofectamine the acyl chains are ether-linked and in the case of DOTAP, the acyl groups are ester-linked. Despite the dissimilarities in the nature of the ligands, from water-soluble polyamines to amphiphilic lipids, they all bring about similar structural changes upon binding DNA.

The interaction of CTAB with flexible elements in the structure of promoter DNA has been well characterized by Masulis and co-workers [24]. These authors have mainly looked at the interactions of DNA with CTAB by $KMnO_4$ probing and their results agree with ours obtained for CTAB. Masulis and co-workers have shown that the conformational transitions are strongly associated with YR steps [24]. In their studies, CTAB was shown to bring about helix distortions. This is in line with our studies where several of the other cationic ligands and commercial formulations used in gene delivery were tested. All cationic ligands that condense the DNA would probably induce partial DNA unwinding to various extents. Protamine sulfate induces modified B-form with appreciable base unstacking in calf thymus DNA as revealed by Raman spectroscopy [42]. High-resolution crystal data of a

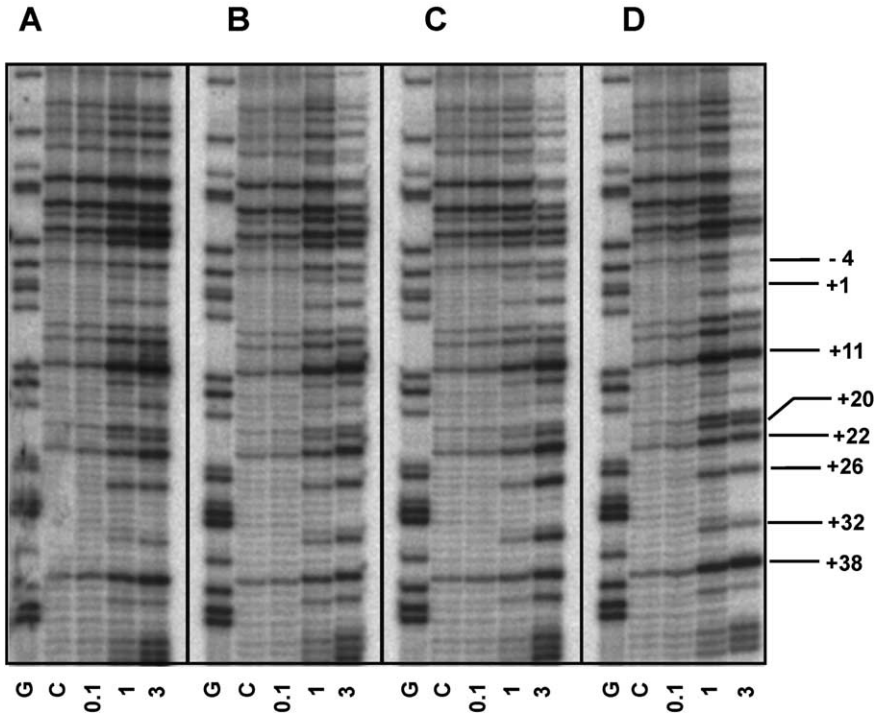

Fig. 6. $KMnO_4$ probing of DNA complexed with water-soluble polymeric cations. Complexed or uncomplexed DNA was treated with $KMnO_4$ and modified T residues were cleaved with piperidine and resolved on 8% urea-PAGE as described in Section 2. The figure shows the T cleavage pattern of DNA complexed with poly-L-lysine 3 kDa (A), poly-L-lysine 30 kDa (B), poly-L-lysine 120 kDa (C) and protamine sulfate (D) at various N/P ratios indicated at the bottom of each lane. 'C' in each panel indicates control (only DNA template) and 'G' is the G-ladder prepared from the same DNA template. 0.1, 1 and 3 indicate N/P ratios. Positions of some hyperreactive T residues are marked.

spermine:DNA complex show bending or distortion of the double helix [43].

Probes that have access to the interior of CLDC can decipher the molecular nature of DNA. Such complexes are inaccessible to other larger molecules, such as enzymes, for example nucleases. The YR dinucleotides are most easily deformed and this tendency is consistent with empirical energy predictions [44,45]. That the relative stability of DNA in a duplex is based on the DNA sequence and also primarily on the identity of the neighboring base has been well documented by several groups based on knowledge-based energy calculations of available three-dimensional models of several protein–DNA complexes. When we performed energy calculations on our template, we obtained results wherein the YR was most easily deformed and no sequence specificity was observed at the cleaved T (not shown).

Cationic lipid-based formulations are promising gene delivery agents for their simplicity and self-organizing nature. The absence of immune reactions to CLDC and the suitability to attach various moieties that can be targeted specifically make them potential vehicles of DNA. The drawback of low efficiency was largely due to the gaps in our understanding of CLDC structure and consequences of the structure on the cell biological fate of the CLDC. In addition to nearly complete information on long-range order in CLDC the present observation on fine structural changes in DNA in CLDC would help in describing the CLDC completely.

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