The regiochemical distribution of positive charges along cholesterol polyamine carbamates plays significant roles in modulating DNA binding affinity and lipofection

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Abstract We have quantified the effects of the regiochemical distribution of positive charges along the polyamine moiety in lipopolyamines for DNA molecular recognition. High affinity binding leads to charge neutralisation, DNA condensation and ultimately to lipofection. Binding affinities for calf thymus DNA were determined using an ethidium bromide displacement assay and condensation was detected by changes in turbidity using light scattering. The in vitro transfection competence of cholesterol polyamine carbamates was measured in CHO cells. In the design of DNA condensing and transfecting agents for non-viral gene therapy, the interrelationship of ammonium ions, not just their number, must be considered.

Key words: Cholesterol polyamine carbamate; DNA binding affinity; DNA uptake efficiency; Lipopolyamine; Lipoplex; Cationic lipid

1. Introduction

Naturally occurring di- and polyamines such as the diamine putrescine (1,4-diaminobutane) 1, triamine spermidine 2 and tetraamine spermine 3 are known to effect, at high concentrations, the condensation of DNA into rod-like or toroidal-shaped structures [1–7]. In order for this process to occur, the free energy that opposes condensation has to be overcome. This energy barrier includes: the loss of entropy in going from a random-coil to a condensed form, the energy required to bend the stiff helix or cause local melting or kinking, and the electrostatic repulsion of the charged strands [4]. The condensation of DNA can become thermodynamically favourable under certain DNA-solvent conditions [8], or when the free energy of compacted DNA is lowered by the binding of various molecules including polylysine and polyamines [9]. In bacteriophage, the dominant force that opposes DNA condensation is electrostatic repulsion, and this is countered by the binding of polyamines [10]. DNA condensation in gene delivery is a rapidly expanding area of research for the design of non-viral vectors for use in gene therapy [7,11–15]. For leading references and selected reviews of both DNA and RNA delivery to cells, see [11–15].

Natural polyamines are essentially fully protonated at physiological pH. The charge distribution is clearly important for molecular recognition, but also the hydrophobic polymethylene backbone confers structural flexibility and the possibility of important secondary binding interactions. There are multiple parallel protonation pathways for the basic centres of the partially protonated species which may account for many of the biochemical functions of these molecules [16]. DNA condensation is dependent upon three characteristic properties of natural or synthetic polyamines: firstly, the number of positive charges which therefore influence the local ionic strength [17,18]; secondly, the regiochemical distribution of these charges whose pK_as are intimately dependent upon their cooperativity [16,18,19]; thirdly, the local salt concentration [4,18]. Although Wilson and Bloomfield [4] predict, using Manning's polyelectrolyte theory [20], that condensation will occur when the polyamine:DNA charge ratio approaches 1:1 (when typically ~90% of the DNA polyanionic charge has been neutralised), in practice, the off-rate of binding of simple polyamines is so large as to require a several-fold charge excess of polyamine to DNA (polyammonium ions to phosphate) in order to effect efficient condensation of DNA. The condensation process with biogenic amines is also salt dependent, the amount of polyamine required to effect DNA condensation increasing with ionic strength [18,21,22]. At physiological concentrations, polyamines enhance the binding of several proteins to DNA, but inhibit others, the degree of enhancement correlating with the cationic charge [23]. It has been postulated that charge neutralisation of intracellular polyanions, e.g. DNA or RNA, may be among the most important physiological roles of these compounds [23]. Stabilisation of specific DNA conformations may be important for processes such as nucleosome formation [24], chromatin condensation [25] and gene expression [26]. Evidence from the crystal structures of various DNA sequences in the presence of spermine [27–29], and especially the work of Sundaralingam and co-workers [30–32], indicates that this linear polyamine adopts a wide variety of binding modes, each of these slightly different polyamine-induced DNA conformations may then correlate with different biophysical properties e.g. bending and condensation.

We and others have recently begun to establish structure-activity relationships for polyamine binding and condensation of DNA, indicating that appropriately modified polyamines could be ideally suited for use in gene therapy [7,9,14,17,33–36]. These interactions are however readily reversible under physiological conditions, and covalent attachment of a lipid is one method of reinforcing these interactions [14]. However, we have shown that the nature of both the polyamine and the lipid moiety has profound effects upon the binding affinity of these molecules to DNA [14,19,35]. Synthetic cationic lipids

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have been used extensively to deliver DNA both in vivo and in vitro [37–41]. These cationic lipids possess common structural similarities: a hydrophobic moiety (e.g. two hydrocarbon chains or a steroid), a positively charged head group, and a linker functional group such as an ester, amide or carbamate to bind these two moieties together covalently. Despite their potential in gene therapy, little work has focused on the design of the optimum polyammonium head group to interact with the DNA. We have therefore designed and synthesized a series of polyamine carbamates of cholesterol 4–9 (Fig. 1) [19], where both the charge and its regiochemical distribution have been varied along the polyamine moiety. These molecules have been fully characterized and their pK_as determined potentiometrically and the net cationic charge at pH = 7.4 calculated using the Henderson–Hasselbach equation (Fig. 1) [19]. In this paper, we report our study of the fundamental requirements of DNA binding affinity, DNA condensation and transfection dependence as a function of positive charge distributed along the polyamine moiety using the cholesterol polyamine carbamates 4–9.

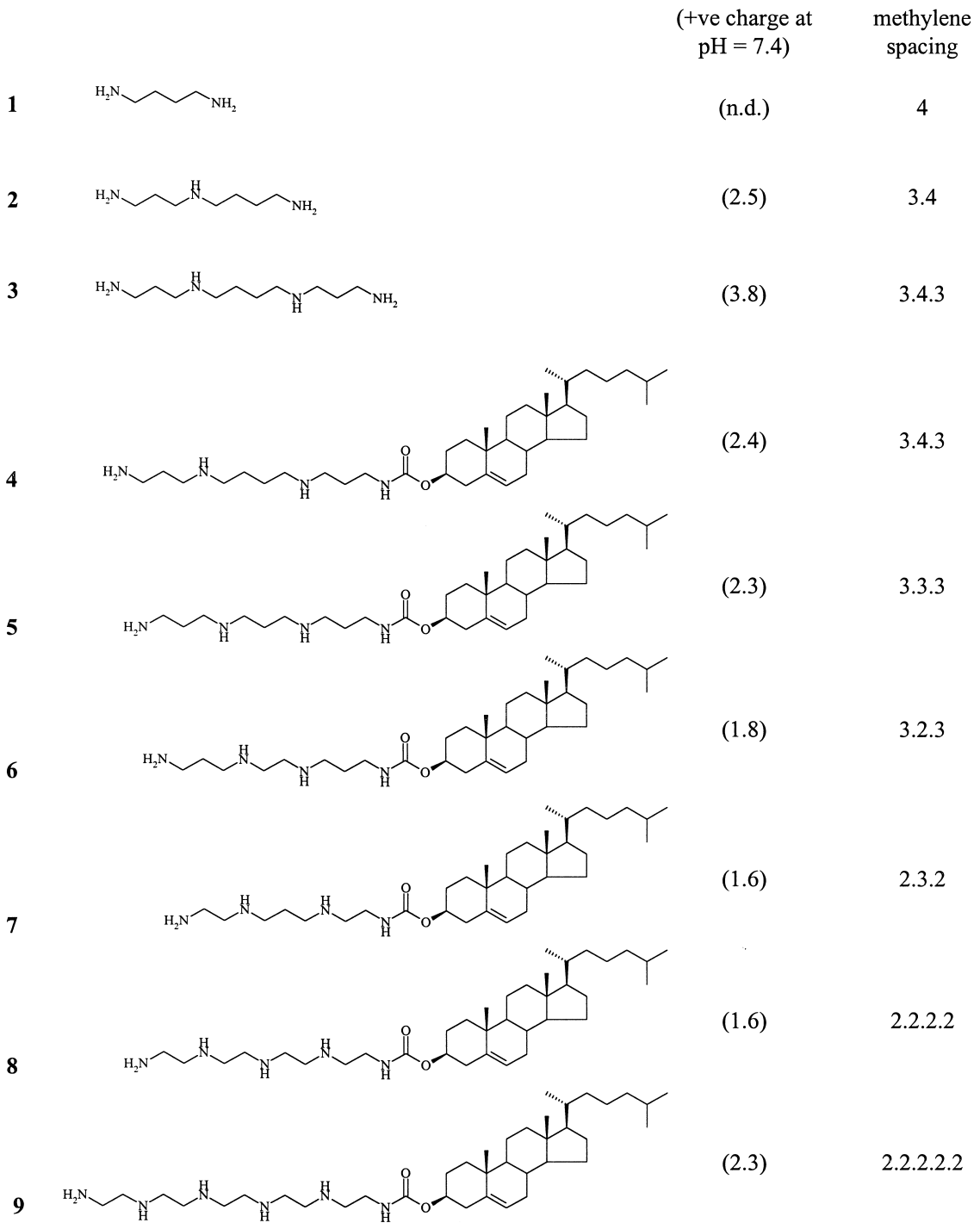

Fig. 1. Structures of di- and polyamines and of target cholesterol polyamine carbamates 4–9, their net positive charge at pH = 7.4 (calculated using the Henderson–Hasselbach equation), and the polyamine methylene spacing.

2. Materials and methods

2.1. Materials

Plasmid pEGlacZ containing the bacterial β-galactosidase gene under the control of the human cytomegalovirus immediate early promoter was constructed using standard molecular cloning techniques. The 7676 base pair (bp) plasmid was derived by cleavage of pGFP-N1 (Clontech) with *Hin*dIII and *Bcl*I to remove the reporter gene fragment. The β-galactosidase coding region from pSV-β-galactosidase (Promega) was then inserted via a *Hin*dIII to *Bam*HI fragment. Plasmid DNA was then purified using an anion-exchange column (Qiagen Ltd). The average molecular weight per base pair was calculated to be 620.84 Da (310.5 Da for a monophosphorylated nucleotide). The amount of negative charge on the plasmid was then determined on the basis that a single negative charge is associated with each nucleotide. Linear double stranded calf thymus DNA was purchased as the sodium salt (Sigma) and, prior to use, was dissolved in buffer (20 mM NaCl, 2 mM HEPES, pH 7.4). The quantity and purity of DNA were determined using triplicate spectrophotometric readings at 260 and 280 nm with a Milton Roy Spectronic 601 spectrometer. Cholesterol polyamine carbamates 4–9 were synthesized and characterized chromatographically and spectroscopically as previously described [19]. Their pK_as were determined and the net positive charge of the polyammonium moiety was calculated at physiological pH (7.4), using the Henderson–Hasselbach equation.

2.2. Ethidium bromide displacement assay

The DNA binding affinities of the target compounds were measured using an ethidium bromide fluorescence quenching assay based upon the displacement of ethidium bromide (1.3 μM) from calf thymus DNA (6 μg, [DNA base pair] = 3.0 μM) [11,15], adapted from the work of Cain et al. [42]. The method is rapid and involves the addition of microlitre aliquots of polyamine conjugate to a 3 ml solution of ethidium bromide (1.3 μM) and calf thymus DNA (6 μg, [DNA base pair] = 3.0 μM) in buffer (20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) with the decrease in fluorescence monitored [11] (λ_{excit} = 260 nm, λ_{emiss} = 600 nm; 1 cm path length glass cuvette) recorded after each addition (1 min equilibration time).

2.3. Light scattering assay

The target compounds (TFA salts) were dissolved in MilliQ water. The buffer solution (20 mM NaCl, 2 mM HEPES, 10 μM EDTA) was pH adjusted to 7.4 with NaOH. A stock solution of calf thymus DNA of approximately 1 mg/ml (3 ml) was made in this HEPES buffer and its concentration determined spectroscopically. DNA (60 μg) was diluted to 3 ml with buffer in a glass cuvette with a micro-flea and its concentration determined spectroscopically. Aliquots (5 μl) of the cholesteryl carbamate (0.25 mg/ml) were then added to the stirring solution and the absorbance (light scattering) at 320 nm was measured after 1 min stirring to reach equilibrium (1 cm path length, 3 ml glass cuvette).

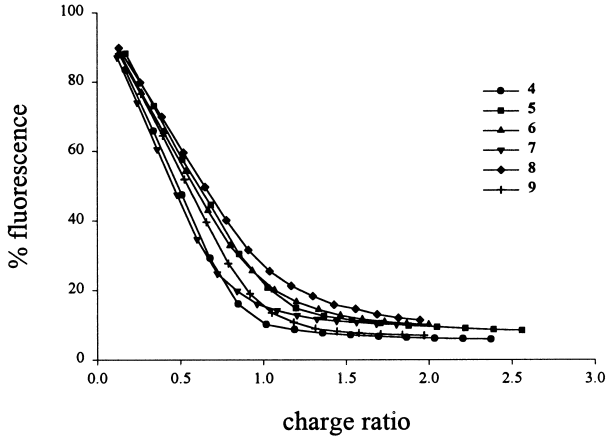

Fig. 2. Ethidium bromide displacement assay of cholesterol polyamine carbamates 4–9 in 20 mM NaCl.

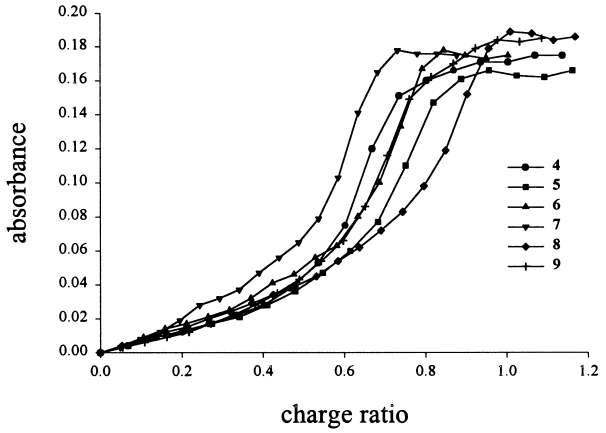

Fig. 3. Light scattering assay (absorbance at λ = 320 nm) of cholesterol polyamine carbamates 4–9 in 20 mM NaCl.

2.4. DNA-cholesterol polyamine carbamate complex preparation and transfection

Transfection complexes were prepared at three different charge ratios (ammonium:phosphate 0.5:1, 1:1, 4:1) in 20 mM HEPES buffer at pH 7.4. An equal volume of a 120 μg/ml solution of plasmid DNA was added to the polyamine solution at the appropriate concentration. Chinese hamster ovary (CHO) cells were seeded into 24-well plates at 100 000 cells per well 24 h before the experiment. The adherent cells were washed once in Opti-MEM (Gibco BRL) prior to transfection. Washed medium was removed and replaced with 0.5 ml of Opti-MEM to which 167 μl of the transfection complex was added (5 μg DNA). Cells were incubated for a further 4 h at 37°C in 5% CO_2 before removal of the medium and non-cell associated complex and addition of 1 ml of fresh medium (Dulbecco's modified Eagle's medium (DMEM), plus glutamate, asparagine, adenosine, guanosine, cytidine, uridine, thymidine and 10% dialysed foetal calf serum (Gibco)). Cells were cultured for a further 72 h before harvesting. The medium was then aspirated and the cells were washed twice with 1 ml phosphate buffered saline. Cells were lysed by the addition of 200 μl per well of lysis buffer (Promega) and the plate was agitated on a orbital mixer for 15 min. The lysates were transferred to individual Eppendorfs, taking care to remove attached cells by scraping with a pipette tip prior to removal. Cell debris was removed by centrifugation in a microfuge for 5 min followed by transfer of the supernatant into clean Eppendorfs.

Levels of β-galactosidase (β-gal) activity were determined using an enzyme assay system (Promega) as follows: cell extract (50 μl) was incubated with the provided buffer and substrate *O*-nitrophenyl-β-D-galactopyranoside (ONPG) and the optical density measured spectroscopically. The transfection competence was critically compared for compounds 4–9, levels of β-gal expression were quantified by reference to a standard curve and related to the amount of protein in the extract, measured using a BCA assay kit (Pierce), to give final values expressed as mU of β-gal per mg of protein.

3. Results and discussion

Measurement of the ability to displace the intercalator dye ethidium bromide has been shown to be valid for comparisons of DNA binding affinity within a series of intercalative or non-intercalative ligands [11,17–19,21,42,43]. Thus, the observed decreases in fluorescence are critically compared for carbamates 4–9 as a function of their charge ratio (Fig. 2). These data show slight, but reproducible differences in the binding affinity for DNA of these lipopolyamines. Significantly, the only structural differences in these molecules are designed to be in their polyamine moieties. The changes in methylene spacing and in the number of nitrogens impart a different net positive charge and a different charge distribution

to the molecules, which we have shown to have an effect on the molecules' ability to induce DNA conformational changes. These variations in binding affinity for DNA may influence lipoplex formation and stability, which would have profound effects on transfection competence.

As polyamines bind to the double helix and cause condensation [1–7] an increase in absorbance due to light scattering is observed above 300 nm [4,21,22]. Therefore, in order to follow the condensation of DNA with carbamates 4–9, UV absorbance ($\lambda = 320$ nm) was also measured (Fig. 3). These data are consistent with particle formation by condensation of calf thymus DNA. Comparisons of Figs. 2 and 3 show a correlation between high DNA binding affinity and efficient particle formation. Furthermore, light scattering reaches a plateau at the same charge ratio (approximately 0.9) (Fig. 4) at which we previously observed maximal ethidium bromide displacement (Fig. 2). However, it should be noted that, due to the lack of sensitivity of this light scattering assay, the DNA concentration was in a ten-fold excess compared to that used in the fluorescence quenching assay (Fig. 2) and no intercalator dye was present.

Cholesterol polyamine carbamates 4–9 have been tested for transfection competence at three different charge ratios (0.5:1, 1:1, 4:1), calculated taking into account the average charge per molecule at pH 7.4 (Fig. 5). It is clear that carbamate 4, incorporating the 3.4.3 methylene spacing along the polyamine (spermine) moiety, has the highest transfection activity. The barriers to transfection by cationic lipids include extracellular complex stability and complex dissociation either in the endosome or in the cytoplasm in order that plasmid DNA can be localized in the nucleus and expressed [44,45]. One explanation for the release of DNA from the complex is that certain ionic molecules found in high concentration in the cell (ATP, polypeptides, RNA, spermine, histones or anionic lipids) displace the ionic interaction between plasmid DNA and the cationic lipid [44]. Polyamine DNA binding affinity has a vital role in these key aspects and small differences in binding affinity for DNA may provide an explanation for the differences seen in the transfection experiments (Fig. 5).

These cholesterol polyamine carbamate target molecules 4–9 have been designed to incorporate the same cholesteryl (lipid) moiety joined to the polyamine via a carbamate linker, but they have different methylene spacing and hence a significantly different positive charge distribution. Even though ther-

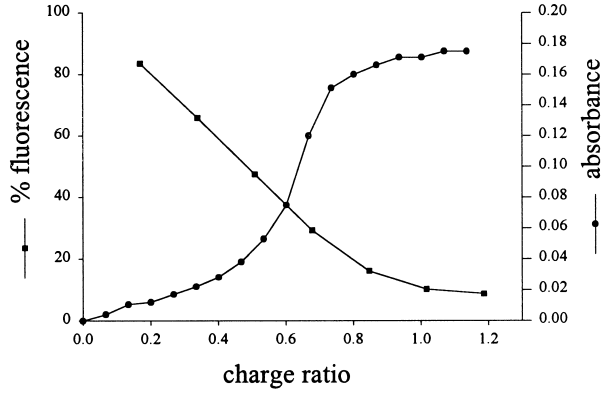

Fig. 4. Comparison of ethidium bromide displacement and light scattering assays of cholesterol polyamine carbamate 4 in 20 mM NaCl.

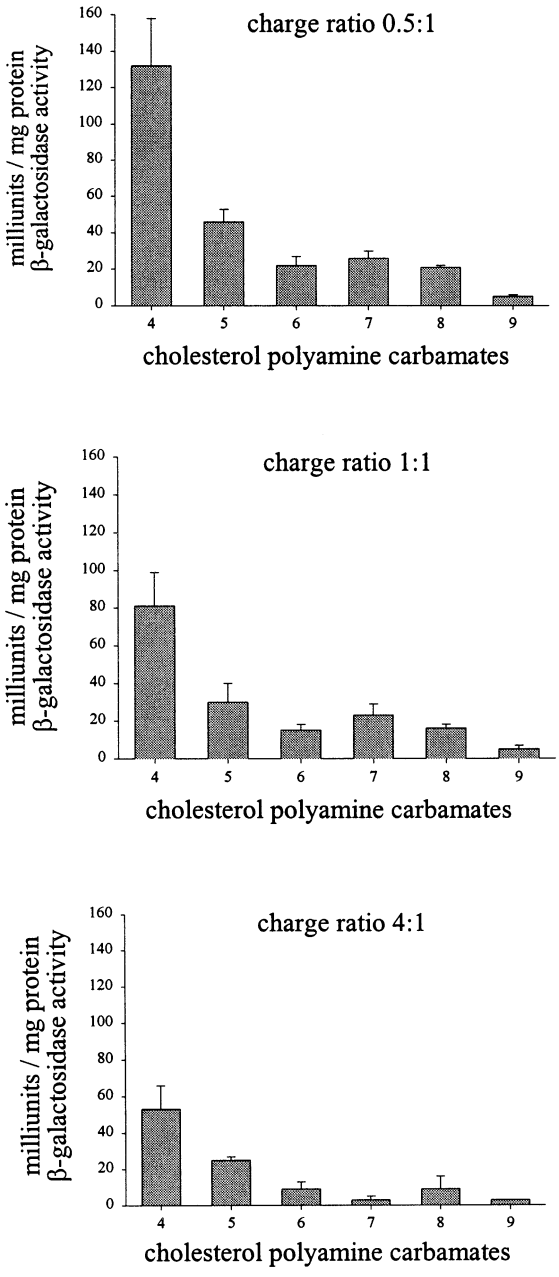

Fig. 5. Comparison of β-galactosidase activity in CHO cells following delivery of pEGlacZ complexed with cholesterol polyamine carbamates 4–9 (at charge ratios ammonium:phosphate of 0.5:1, 1:1, 4:1). Mean ± S.D. ($n = 3$).

mine carbamate 5 carries a similar charge at pH 7.4 to that on spermine carbamate 4, the spacing of these positive charges is less favourable in the former. Indeed, an important corollary is that in their recent work, Basu, Marton and co-workers [18,21] have shown that the length of the central carbon chains of free polyamines (rather than of lipopolyamine conjugates) is important for the induction of conformational changes in DNA. This aspect of DNA affinity is further underlined by our results obtained with carbamate 9 which has a poor performance in the transfection assay, yet it also carries a net cationic charge of 2.3, but with a significantly different methylene backbone. Due to electrostatic repulsion within the polyamine moiety of carbamate 9, it is likely that

the majority of the positive charge will be distributed between the primary amine and the secondary amine next to the carbamate linker, with the balance on the central secondary amine due to electrostatic repulsion. Thus, with the 2.2.2.2.2-polymethylene backbone, in this case polyethylenimine, the charge distribution on this molecule is different and this provides a possible explanation for the experimentally observed difference in transfection activity.

Some structure-activity relationship studies of the condensation of DNA by free polyamines (i.e. non-conjugated) have been reported [17,46–50]. It has been postulated that the central aliphatic chain of spermine 3 (tetramethylene) is suitable to bridge between different strands of DNA, but a trimethylene spacing is suitable to interact with adjacent phosphate groups on the same strand of DNA [45–47]. It has also been shown [51,52] that α,ω-diaminoalkanes with an odd number of carbon atoms (three and five) induce compaction of a single double strand of DNA more efficiently than those where the interamine (interammonium ion) chain is an even number of carbon atoms, but the diamine putrescine 1 (tetramethylene spacing) tends to induce aggregation between different molecules of DNA, instead of the compaction of individual molecules. Thus, even with simple diamines the spacing as well as the number of positive charges is of particular importance. Chromatin precipitation analyses have revealed that spermine 3 was several-fold more effective than spermidine 2 at condensing chromatin and that putrescine 1 had only a minor effect [49].

Binding of free polyamines causes conformational changes to DNA that are dependent on both the charge and structure of the cation, and are also related to the polyammonium ion distribution along the methylene backbone [18,33,53]. Although these free polyamines appear to be simple ligands, they do have several modes of interaction with DNA [33]. Rowatt and Williams [36] have investigated the strength of binding of polyamines to DNA and found that the presence of a butylene rather than a propylene chain is preferable for tight binding, and that spermine 3 was capable of combining with every phosphate group on the DNA [36]. We have extended these free polyamine studies to lipopolyamines that incorporate cholesteryl as the lipophilic moiety and compared DNA binding with condensation and transfection in CHO cells.

In conclusion, the four methylene (butylene) spacing found in spermine, could have significant implications for DNA polyamine association and lipoplex formation. Both the number of positive charges on the polyamine, and the distribution of charge on the surface of the molecule have profound effects on its ability to induce DNA conformational changes, which may be relevant to their biological function. Lipopolyamine binding to DNA has a vital role in lipoplex dissociation and extracellular stability, small changes in binding affinity may therefore have profound effects on these barriers to transfection. The most active carbamate 4 in the transfection experiments contains the methylene distribution found in spermine 3 and we therefore conclude that the transfection activity of these cholesterol polyamine carbamates is sensitive to both the number of positive charges and their interrelationship along the polyamine (polyammonium) backbone.

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