The Potential Role of Proteoglycans in Cationic Lipid-mediated Gene Delivery

STUDIES OF THE INTERACTION OF CATIONIC LIPID-DNA COMPLEXES WITH MODEL GLYCOSAMINOGLYCANs*

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Recent evidence supports a role for proteoglycans in polycation-mediated gene delivery. Therefore, the interaction of glycosaminoglycans with cationic lipid-DNA complexes (CLDCs) has been characterized using a combination of biophysical approaches. At low ionic strength, CLDCs bind to heparin-derivatized Sepharose particles, with the ratio of cationic lipid to DNA controlling the binding. Incorporation of the helper lipids cholesterol or 1,2-dioleoyl-phosphatidylethanolamine increases the amount of bound CLDC. Heparin also induces the aggregation of CLDCs, with cholesterol reducing this effect. Isothermal titration calorimetry demonstrates an endothermic heat for the binding of heparin to CLDCs at low ionic strength, whereas circular dichroism studies suggest a heparin-stimulated release of DNA from CLDCs at a greater than 20-fold charge excess. Increasing the ionic strength to 0.11 reduces CLDC binding to heparin beads, and greatly enhances the release of DNA from CLDCs by heparin. The ability of the cell surface glycosaminoglycan heparan sulfate to release DNA from CLDCs is more sensitive than heparin to the incorporation of the cholesterol or 1,2-dioleoyl-phosphatidylethanolamine. Titration calorimetry reveals an exothermic heat for the interaction glycosaminoglycans with CLDCs at higher ionic strength. These results are consistent with the direct involvement of proteoglycans in transfection.

Complexes of DNA and various polymers such as cationic lipids, polypeptides, amino-dendrimers, and polyethyleneimine are able to transfect cells and are currently being evaluated as gene delivery vehicles for various clinical indications. The mechanism by which such complexes enter cells is, however, unclear. These agents are able to efficiently deliver DNA to a variety of very different cell types (1). This suggests a general entry mechanism rather than one requiring highly specific membrane receptors. Recent evidence suggests cell surface proteoglycans have a significant impact on polycation-mediated gene delivery. Proteoglycans are a diverse class of molecules consisting of a protein core and one or more anionic glycosaminoglycan chains, which include heparan, dermatan and chondroitin sulfates (2). They can exist as both integral and GPI-linked membrane proteins at the cell surface. Proteoglycans are fundamental components of basement membranes and the extracellular matrix, playing important roles in cell proliferation and differentiation (3). Their physical and chemical composition is highly dependent upon cell type, stage of differentiation, and cell cycle phase (2). More provocatively from the perspective of this work, they also appear to act as receptors or co-receptors for several viruses such as herpes simplex virus-2 (4), adeno-associated virus-2 (5), human immunodeficiency virus-1 (6), and adenovirus (7), all of which have been employed as efficient gene delivery vehicles.

Several roles for proteoglycans in nonviral gene delivery have been suggested. Recent studies have found that the alteration of proteoglycans on the cell surface by inhibition of polylsaccharide sulfation with chlorate, their enzymatic removal, or the use of genetically altered cells that possess reduced cell surface GAGs, greatly reduced transfection efficiency (either in terms of number of cells transfected or amount of protein expressed) for both cationic polymer (8) and lipid-based delivery complexes (8, 9). Furthermore, the presence of HSPGs and GAGs in the extracellular media has been shown to decrease the transfection efficiency of cationic polymer and lipid-DNA complexes in vitro (10, 11). In addition to the proposed role for cell surface HSPGs as nonspecific "receptors" for polycation/DNA complexes, Belting and Petersson (12) have provided evidence that proteoglycans may also play a protective role in nonviral gene delivery by sequestering cationic lipids and possibly other polycations, thereby reducing their cytotoxicity. These results provide indirect but compelling evidence that HSPGs play a significant role in nonviral gene delivery, the precise role of which is yet to be determined.

Several studies have examined the effect of polyanions on the physical properties of polycation-DNA complexes. Szoka and colleagues (13–15) have demonstrated that several polyanions, including anionic liposomes and the GAG, heparin, can release DNA from its association with cationic lipid, as demonstrated by the reversal of fluorescent dye exclusion from DNA. Subsequently, several authors have described similar results for a variety of polyanions as well as several polycations using related fluorescence-based assays (10, 16). Wong et al. and Kol-
tover et al. have described the binding of CLDCs to anionic surfaces of latex beads (17) and giant unilamellar vesicles (18), respectively. With such considerations in mind, we have conducted an analysis of the interaction of two GAGs, heparin and heparan sulfate with CLDCs, in an effort to characterize the physical nature of the interaction as well as the influence of CLDC composition on any observed complexion. Studies were conducted at two different ionic strengths to further define the interaction.

EXPERIMENTAL PROCEDURES

Materials—Plasmid DNA (pMB113, 9.1 kilobase pairs, >95% supercoiled) was provided by Valentis, Inc. (Burlington, CA). DOTAP, DOPE, cholesterol, and Rh-DOPE were purchased from Avanti (Alabaster, AL). Heparin (H3393, Sigma) and heparan sulfate (H9637, Sigma) were used as supplied. Heparin-Sepharose beads, “heparin beads,” having an average diameter of 90 μm were purchased from Amersham Pharmacia Biotech. All other chemicals were obtained from Sigma.

Liposome Preparation—DOTAP or a 1:1 mixture of DOTAP and the helper lipids cholesterol or DOPE (1:1 molar ratio) was deposited as a thin film on the walls of a glass vial by evaporation of the chloroform solvent under nitrogen. The dried film was stored under vacuum for at least 2 h prior to hydration with 10 mM Tris, pH 7.4, containing either 0 or 100 mM NaCl. After brief vortexing to obtain a lipid suspension, the sample was extruded 10 times through a 100-nm polycarbonate filter (Nucleopore; Whatman, Clifton, NJ). The resulting liposomes were stored at 4 °C until use. Liposomes used for the heparin bead binding studies were labeled with 0.25 mol% Rh-DOPE. Typically, the liposome concentration of 200 mM. Methanol and chloroform were added sequentially to the sample cell, and an oscillating electric field was applied to the sample by a set of electrodes. The phase shift in scattered light, relative to laser light not passed through the sample, was measured. Software optimization of the frequency of electric field inversion was based on the measured conductivity of the samples. The electric field strength was typically between 14 and 16 V/cm. Ten cycles were collected for each experiment, with three runs performed and averaged for each sample.

CLDC Preparation—Various amounts of DNA and lipid were prepared in equal volumes of 10 mM Tris, pH 7.4, containing either 0 or 100 mM NaCl. Depending on the desired ratio of lipid to DNA, the substance of lesser quantity was rapidly added to the other, with stirring. All ratios were given as the ratio of the positive charge of the lipids to the negative charge of the DNA phosphates. The complexes were allowed to equilibrate for 30 min prior to use.

Heparin Bead Binding Isotherms—Samples were prepared in 10 mM Tris, pH 7.4, containing either 0 or 100 mM NaCl by adding increasing amounts of liposomes or CLDCs to a solution containing 1.25% v/v heparin beads in 2-mL polypropylene microcentrifuge tubes. The molar concentration of heparin negative charge (150 mN/m) was determined, given that there are 2 mg of heparin for every 1 mL of drained gel, the average molecular weight of the repeating disaccharide unit is 535 Da, and there are an average of 2.4 sulfates and 1 carboxylate per disaccharide. The samples were placed on a rotating mixer (5 rpm) for 1 h and then centrifuged at 2,300 g for 2 min in a microcentrifuge.

Heparin bead binding studies were labeled with 0.25 mol% Rh-DOPE. Typically, the liposome concentration of 200 mM. Methanol and chloroform were added sequentially to the sample cell, and an oscillating electric field was applied to the sample by a set of electrodes. The phase shift in scattered light, relative to laser light not passed through the sample, was measured. Software optimization of the frequency of electric field inversion was based on the measured conductivity of the samples. The electric field strength was typically between 14 and 16 V/cm. Ten cycles were collected for each experiment, with three runs performed and averaged for each sample.

RESULTS

CLDCs Bind to Heparin Covalently Attached to a Surface at Low Ionic Strength—Understanding the interaction of CLDCs with cell surface-associated GAGs is of great importance to the implementation of polycation-mediated gene transfer. Thus, a model surface was desired that would allow for more quantitative assessment of CLDC binding than cells in culture. The use of heparin gel affinity media to measure the binding of proteins and viruses to heparin has been described previously (4, 23–25). To simulate the binding of CLDCs to a cell surface, increasing amounts of CLDCs were added to heparin beads and binding isotherms generated (Fig. 1). The binding of lipid vesicles and CLDCs resulted in complete binding after 0.5 h at 25°C by themselves scatter light poorly, and the measured scattering intensity at these concentrations is not significantly different from that of Tris buffer alone.

Phase Analysis Light Scattering—The colloidal properties of complexes of heparin with CLDCs were characterized by measuring their light scattering intensities. The dynamic light scattering (DLS) results were fit by the method of cumulants to yield the average diffusion coefficient of the complexes (20). Data are reported for a quadratic fit. The effective hydrodynamic diameter was obtained from the diffusion coefficient, using the Stokes-Einstein equation (21). GAGs by themselves scatter light poorly, and the measured scattering intensity at these concentrations is not significantly different from that of Tris buffer alone.

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FIG. 1. Binding isotherms for CLDCs of various compositions to heparin beads at low ionic strength. Heparin beads were present at 1.25% (w/v) corresponding to 150 μM negative charge. A, 1:1 DOTAP CLDCs; B, 2:1 DOTAP CLDCs; C, 2:1 DOTAP:cholesterol CLDCs; D, 2:1 DOTAP:DOPE CLDCs. The x axis corresponds to the concentration of the DNA phosphate from the CLDCs added to the beads. The moles of DOTAP bound per mole of heparin negative charge are represented by closed circles, and the moles of DNA phosphate bound per mole of heparin negative charge are represented by open circles. The S.E. for three experiments was typically less than 10% for all data points but is not shown for visual clarity.

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of a 2:1 DOTAP CLDC (Fig. 1B), somewhat greater binding was seen when complexes were added up to a DNA concentration of 75 μM phosphate, with roughly 0.23 mol of DNA and DOTAP bound/mol of heparin negative charge. Above this level, however, the amount of DNA bound to the heparin beads began to decrease, while the amount of DOTAP bound manifested additional binding with a maximum of 0.4 mol/mol of negative charge of heparin bound at higher CLDC concentrations.

Since the incorporation of cholesterol or DOPE into DOTAP CLDCs has often been shown to increase transfection efficiency, the effect of these helper lipids on the binding of DOTAP CLDCs to heparin beads was also examined (Fig. 1, C and D). For 2:1 DOTAP:cholesterol CLDCs, binding of the DNA to the beads saturated at 0.13 mol/mol of heparin negative charge (Fig. 1C). The lipid manifested saturation binding with 0.5 mol of DOTAP bound/mol of heparin negative charge. For 2:1 DOTAP:DOPE CLDCs, DNA binding saturated at ~0.25 mol/mol of negative charge of heparin (Fig. 1D). The amount of lipid bound was similar to the amount of DNA bound in the low concentration region of the isotherm. Above 75 μM DNA phosphate added, however, the amount of lipid bound increased and did not appear to show saturation within the CLDC concentration range studied.

Increased Ionic Strength Reduced CLDC Binding to Heparin Beads—The influence of ionic strength on the binding of CLDCs to a model heparin surface is an important aspect of such interactions. Unfortunately, the study of this interaction using the techniques employed in this investigation at physiological ionic strength (e.g. 150 mM NaCl) is quite difficult due to the colloidal instability of CLDCs at the atypically high concentrations necessary for these studies. Additionally, colloidal instability of the complexes at this higher ionic strength prevented the study of CLDCs at charge ratios greater than 1:1. Thus, the interaction was studied by increasing the ionic strength of the solution by the addition of 100 mM NaCl to explore trends in ionic strength effects. When the ionic strength was increased, binding of 1:1 DOTAP CLDCs to heparin beads was reduced by about one-fourth to 0.04 mol of DNA or DOTAP/mol of heparin negative charge (Fig. 2A). Incorporation of cholesterol into the CLDC doubled the amount of DNA and lipid bound (0.08 mol of DNA or DOTAP/mol of heparin negative charge; Fig. 2B). Incorporation of DOPE into the CLDCs also resulted in an increase in CLDC binding to the beads with no apparent saturation in binding apparent over the concentration range studied (Fig. 2C).

Heparin Caused Aggregation of CLDCs at Low Ionic Strength—Given the ability of CLDCs to bind to heparin covalently attached to a surface, further studies were conducted with GAGs free in solution. DLS and PALS were used to examine the colloidal properties of CLDCs in the presence of unconjugated heparin in solution. Since the interaction between CLDCs and heparin appeared to be dominated by electrostatics, the ratios of heparin to CLDC are presented in terms of the total negative charge (as contributed by both heparin and DNA) versus the total positive charge from the lipid. This value increased with increasing heparin concentration. In contrast, the CLDCs are described by the inverse ratio (e.g. x).

When heparin was titrated into 1:1 DOTAP CLDCs at low ionic strength, a gradual increase in the apparent hydrodynamic size was observed from 165 to 185 nm (Fig. 3B). PALS data indicate that these CLDCs have a negative ζ-potential near −55 mV (Fig. 3E). Surprisingly, no significant change in the ζ-potential was observed when increasing amounts of heparin were added to these complexes. In the case of 2:1 DOTAP CLDCs (Fig. 3A), complex size increased dramatically from an initial value of 200 nm to particles greater than 1 μm (too large to be measured by DLS) with phase separation occurring as heparin was added to the point of complete neutralization of the excess DOTAP present in the CLDC. When heparin was added in charge excess, the size immediately dropped to 220 nm as heparin coated the surface of the CLDCs. When more heparin was added, the size showed a further increase. PALS analysis of these positively charged CLDCs revealed a rapid decrease in the ζ-potential from the initial value of +50 mV, as charge neutrality approached −60 mV when heparin was added in charge excess (Fig. 3D).

The helper lipid-containing DOTAP CLDCs were somewhat larger than the 2:1 DOTAP CLDCs (250 nm (DOPE) and 270 nm (cholesterol) versus 200 nm, Fig. 3A). In the case of cholesterol containing CLDCs, the relative increase in size when
cholesterol CLDCs, but also manifested a positive potential, the value of which is not significantly different than that of 2:1 DOTAP CLDCs (Fig. 3E). Again, the same charge reversal that was observed for the DOTAP CLDCs was seen when of the helper lipids cholesterol and DOPE were included in the complex.

Isothermal Titration Calorimetry (ITC) Revealed an Entropically Driven Association of Heparin with CLDCs at Low Ionic Strength—The thermodynamic parameters of the interaction of heparin with CLDCs were studied by ITC. Fig. 4A shows the apparent enthalpy of interaction ($\Delta H_{app}$) for the titration of heparin into DOTAP CLDCs at various charge ratios. The apparent titration end point for each curve correlated well with the point at which complexes became electrically neutral and precipitate from solution (arrows, Fig. 4A). $\Delta H_{app}$ taken at this end point was endothermic and found to be similar for 2:1 and 4:1 DOTAP CLDCs (1.1 ± 0.07 and 1.2 ± 0.05 kJ/mol of DOTAP, respectively). $\Delta H_{app}$ was significantly lower when heparin was titrated into DOTAP liposomes alone (0.8 ± 0.01 kJ/mol of DOTAP).

Release of DNA from CLDCs at Low Ionic Strength—Circular dichroism was used to follow the changes in the packing of DNA in the CLDCs upon titration with heparin at low ionic strength. Representative CD spectra of DNA, CLDCs, and CLDCs in the presence of a large excess of heparin are shown in Fig. 5. The spectrum of the plasmid alone is consistent with that of the canonical B-form, containing positive peaks at 275, 222, and 190 nm (the latter peak not shown) along with negative troughs at 245 and 210 nm. The spectra below ~230 nm of CLDCs complexed with heparin is largely due to the signal from the N-acetyl moiety of heparin, which displays negative ellipticity at 210 nm and a positive signal below 200 nm (26).

As DOTAP was complexed to DNA at a 2:1 charge ratio, a decrease in the intensity and an apparent red-shift in all of the peaks and troughs was observed. The trough at 245 nm increased in magnitude while the peak at 275 nm was shifted upward 10 nm and the intensity was reduced by approximately half. Upon addition of a large excess of heparin, the original shape and intensity of the peak at 275 and trough at 245 nm was almost completely restored to that of DNA alone. Similar results were observed for DOTAP CLDCs containing cholesterol and DOPE (data not shown).

Fig. 6A illustrates quantitatively the changes seen in the molar ellipticity at 275 nm as increasing amounts of heparin were added to the CLDCs. Up to a 5-fold excess of negative charge, the molar ellipticity decreased, with the extent of this decrease dependent on the composition of the cationic vesicles used to prepare the CLDCs. Cholesterol-containing CLDCs showed the largest decrease. This decrease was also accompanied by a further red shift in the peak, which primarily accounts for the decrease in the intensity at a fixed wavelength. This shift may be due, in part, to absorption flattening phenomena as the size of the particles dramatically increases over this range of heparin concentration (see Fig. 3A). Above this charge ratio, the spectra began to return to that of the unperturbed B-form. This presumably reflects the release of DNA from the CLDCs. IC$_{50}$ values suggest that, although DOTAP CLDCs require only a ~20-fold charge excess of heparin to release the DNA from the complex, CLDCs containing the helper lipids cholesterol and DOPE require ~30-fold heparin charge excess for 50% release.

Increased Ionic Strength Facilitates Heparin-mediated DNA Release from CLDCs—Light scattering revealed that CLDCs were larger in higher ionic strength media and carried a reduced $\xi$-potential compared with complexes at low ionic strength (Fig. 3, B and E). Titration of 1:1 DOTAP CLDCs with heparin found a maximum in the effective diameter around an overall charge ratio of 1.4 (−/+/), decreasing at higher heparin concentrations. 1:1 DOTAP:cholesterol CLDCs produced a similar trend, whereas 1:1 DOTAP:DOPE CLDCs showed a maximum at a charge ratio of 2 (−/+). The $\xi$-potential did not change significantly at any concentration of heparin (Fig. 3E).

In ITC experiments, titration of heparin into DOTAP liposomes at high ionic strength revealed an endothermic heat of binding, with the titration end point occurring when only 40% of DOTAP was neutralized by the negative charge of heparin.
The mechanism by which nonviral gene delivery vehicles enter cells remains controversial. Several previous studies have addressed a potential role for HSPGs and GAGs in nonviral gene delivery (8–12). Although these studies show an increase in transfection efficiency when HSPGs are present on the cell surface and a corresponding decrease when HSPGs are

The Interaction of Heparan Sulfate with CLDCs Differed from That of Heparin—Dynamic light scattering revealed a similar profile when the cell-surface GAG heparan sulfate was titrated into 1:1 DOTAP CLDCs at high ionic strength compared with heparin (Fig. 3, compare C with B). The $\zeta$-potential of the CLDCs showed a slight decrease in magnitude upon addition of heparan sulfate. Titration of 1:1 DOTAP:cholesterol and DOTAP:DOPE CLDCs with heparan sulfate resulted in a significant increase in the effective size, which does not decrease over the concentration range studied. The $\zeta$-potential of either of these complexes does not differ significantly over this range of heparan sulfate concentrations (Fig. 3F).

Calorimetry experiments at higher ionic strength demonstrated several differences in the interaction of heparan sulfate with DOTAP liposomes and 1:1 DOTAP CLDCs compared with heparin (Fig. 4, B and C). Binding to DOTAP liposomes resulted in an endothermic heat of binding with an overall $\Delta H_{\text{app}}$ of 0.3 ± 0.02 kJ/mol of DOTAP. The stoichiometric end point of the titration occurred when 20% of the DOTAP was neutralized by heparan sulfate. Titration of heparan sulfate into 1:1 DOTAP CLDCs again produced an exothermic heat of interaction, however considerably less in magnitude than that observed for heparin (−0.3 ± 0.01 versus −2.4 ± 0.2 kJ/mol DOTAP, Fig. 4C). Additionally, the relative affinity of this interaction appeared visually to be significantly less than that for heparin, as indicated by the sharpness of the breakpoint (Fig. 4C, arrows). The observed stoichiometry of the interaction suggests that heparan sulfate neutralized ~50% of the DOTAP in the CLDC.

Circular dichroism measurements suggest that heparan sulfate also caused the release of DNA from 1:1 DOTAP CLDCs at the higher ionic strength (Fig. 6C). When the helper lipids cholesterol and DOPE were incorporated into the CLDCs, however, the ability of heparan sulfate to release DNA from the complex was greatly diminished. The $IC_{50}$ value for DNA release from 1:1 DOTAP CLDCs was 1.8, whereas no appreciable heparan sulfate-induced release of DNA from helper lipid-containing CLDCs was observed up to a 50-fold charge excess.

**DISCUSSION**

FIG. 3. Light scattering analyses of CLDCs in the presence of various amounts of heparin or heparan sulfate. Panels A–C represent the effective hydrodynamic diameter determined by DLS, and panels D and E represent the $\zeta$-potential of the same complexes. The x axis represents the ratio of the total negative charge from both heparin and DNA in the sample to the positive charge from the DOTAP. A and D, low ionic strength, titration with heparin; 2:1 DOTAP CLDCs (open circles), 1:1 DOTAP:cholesterol CLDCs (open squares), and 2:1 DOTAP:DOPE CLDCs (open triangles). B and E, titration with heparin; 1:1 DOTAP CLDCs at low ionic strength (closed circles), 1:1 DOTAP CLDCs at high ionic strength (closed diamonds), 1:1 DOTAP:cholesterol CLDCs at high ionic strength (closed triangles), 1:1 DOTAP:DOPE CLDCs (closed squares), and 2:1 DOTAP:DOPE CLDCs (closed triangles). C and F, high ionic strength, titration with heparan sulfate; 1:1 DOTAP CLDCs (closed diamonds), 1:1 DOTAP:cholesterol CLDCs (closed squares), and 1:1 DOTAP:DOPE CLDCs (closed triangles). D and F, high ionic strength, titration with heparan sulfate; 1:1 DOTAP CLDCs (closed diamonds), 1:1 DOTAP:cholesterol CLDCs (closed squares), 1:1 DOTAP:DOPE CLDCs (closed triangles), and 2:1 DOTAP:DOPE CLDCs (closed triangles). E and F, high ionic strength, titration with heparan sulfate; 1:1 DOTAP CLDCs (closed diamonds), 1:1 DOTAP:cholesterol CLDCs (closed squares), 1:1 DOTAP:DOPE CLDCs (closed triangles), and 2:1 DOTAP:DOPE CLDCs (closed triangles). The overall $\Delta H_{\text{app}}$ was still endothermic but was half the value for a similar titration at lower ionic strength (0.4 ± 0.01 kJ/mol of DOTAP versus 0.8 ± 0.01 kJ/mol of DOTAP). This is in agreement with the inability to neutralize all of the DOTAP. Titration of heparin into 1:1 DOTAP CLDCs at low ionic strength resulted in endothermic heat of binding (0.3 ± 0.03 kJ/mol of DOTAP, Fig. 4C). Heparin titration into 1:1 DOTAP CLDCs at high ionic strength produced an exothermic heat of interaction, which saturated at a ratio of heparan negative charge to DOTAP positive charge near unity (Fig. 4C). An overall $\Delta H_{\text{app}}$ of −2.4 ± 0.2 kJ/mol of DOTAP was observed.

CD experiments found that heparin released DNA from 1:1 DOTAP CLDCs with an $IC_{50}$ of 1.4 (Fig. 6B), corresponding to that where the maximum size is observed by light scattering. Again, incorporation of cholesterol and DOPE into CLDCs resulted in an increased amount of heparin necessary for release of the DNA with $IC_{50}$ values at 1.8 and 1.9 observed for 1:1 DOTAP:cholesterol and DOTAP:DOPE CLDCs, respectively.

The mechanism by which nonviral gene delivery vehicles enter cells remains controversial. Several previous studies have addressed a potential role for HSPGs and GAGs in nonviral gene delivery (8–12). Although these studies show an increase in transfection efficiency when HSPGs are present on the cell surface and a corresponding decrease when HSPGs are
added to the cell culture medium, whether this involves a direct interaction between cell-surface HSPGs and nonviral gene delivery vehicles is unknown. This study presents the first attempt to quantitatively describe the interaction of CLDCs with GAGs bound to a surface and free in solution. Although this is very much a model study, the use of such a well defined system permits the quantitation of physical interaction to be directly addressed. The inherent heterogeneity of these CLDCs, however, makes a more detailed interpretation of results difficult. Instead, a global average description of the interaction is reported. By examining this interaction under a variety of solution conditions and using several analytical approaches, however, we are able to directly demonstrate the interaction of cationic lipid-DNA complexes with model proteoglycans and three major aspects of this interaction: 1) the ionic strength dependence of the interaction, 2) the effect of helper lipids on the interaction, and 3) the effect of glycosaminoglycan structure on the interaction.

**Effect of Ionic Strength on the Interaction of GAGs with CLDCs**

Variations in ionic strength are expected to have significant effects on interactions that are primarily electrostatic in nature. Unfortunately, the actual ionic strength at any cell surface is poorly defined. Since we are dealing with highly charged surfaces, the "apparent" ionic strength of the surface is much higher than the bulk media. For instance, if we assume the surface has a \(\Phi_{25}\) potential of \(\pm 100\) mV, then the ionic strength at the surface would be 0.17 for a bulk ionic strength of 0.11 (e.g., these experiments) from a simple Boltzmann calculation. Thus, using lower ionic strength media (e.g., \(I = 0.11\)) to characterize the interaction of charged surfaces (e.g., cell surfaces and the surface of a cationic lipid-DNA complex) might, in fact, be fairly representative of physiologic ionic strength. Due to the uncertainty in defining the actual ionic strength at the cell surface, we studied the interaction between CLDCs and model proteoglycan systems at two ionic strengths. Although these studies could not be performed at physiological ionic strength (i.e., 150 mM NaCl) due to the instability of the CLDCs at the high concentrations necessary for biophysical analysis, clear trends are apparent upon increasing the ionic strength from 0.01 to 0.11.

In studying the binding of CLDCs to a model heparin-derivatized surface, increasing the ionic strength 11-fold from 0.01 to 0.11 results in a 4-fold decrease in the amount of DOTAP CLDC bound (compare Figs. 1 and 2). Both lipid and DNA
components of CLDC at a 1:1 charge ratio, which possesses a negative ψ-potential, bind equally to the heparin surface at both ionic strengths, suggesting the complex remains intact. At either ionic strength, complete neutralization of heparin negative charge is not observed, probably due to steric constraint of the immobilized species along with steric packing limitations of CLDCs at the surface. If the amount of lipid bound to the beads is converted from moles of lipid to moles of a single CLDC (see Ref. 27), however, roughly 6 × 10⁶ CLDCs bind a single heparin bead at low ionic strength whereas 1.5 × 10⁶ CLDCs bind to a single bead at higher ionic strength, suggesting a significant capacity for complexes on the heparin surface. This result is comparable to the number of copies of CLDC plasmid described previously with HeLa cells (28). Additional calculations based on the scaled particle theory of Cotter (29) lend support for an enhanced association of CLDCs with cell surface HSPGs, based on the fact that such CLDCs would have activity coefficients between 10 and 100 as a consequence of excluded volume effects of the highly concentrated proteoglycans at the cell surface (data not shown).

At low ionic strength (10 mM Tris buffer), binding isotherms of 2:1 DOTAP CLDCs suggest the presence of multiple species of complexes, as indicated by the differences in the relative amount of lipid and DNA bound when added above or below 75 μM DNA phosphate. Xu et al. (30) have demonstrated by sucrose density gradient centrifugation that a 2:1 DOTAP CLDC is composed of at least two different species. Complexes at 11% (w/v) sucrose presumably represent lipid-rich and those at 18% DNA-rich species. Our results suggest that, when 2:1 DOTAP CLDCs are added to the beads at relatively low concentrations, the DNA-rich species dominates the interaction. At higher CLDC concentrations, the less abundant lipid-containing species, possessing a greater affinity for the beads, occupies the majority of the heparin binding sites. As CLDCs represent a heterogeneous population of particles, further studies are necessary to more accurately define the apparent dichotomy observed in complex binding to a heparin surface.

Although binding of CLDCs to HSPGs at the cell surface may be the major interaction necessary for transfection, association of CLDCs with heparin free in solution may also be important in terms of release of the DNA from the CLDC either in the extracellular space or upon cell internalization. Results regarding the interaction of soluble heparin with CLDCs suggest a significant shift in the relative “affinity” of DNA for DOTAP as compared with that of heparin upon increasing ionic strength. Light scattering measurements demonstrate a heparin-induced aggregation of the DOTAP CLDCs at low ionic strength based on the observed increase in the apparent size of the complexes (Fig. 3). For positively charged CLDCs, precipitation occurs when heparin is added to the point of complete charge neutrality (Fig. 3, A and D), whereas all CLDCs showed a lesser degree of aggregation in the presence of excess heparin at low ionic strength (Fig. 3, B and E). Similar trends are not observed at higher ionic strength. The return of the apparent size to a value smaller than that observed for CLDC alone in the presence of excess heparin suggests complex disassembly (Fig. 3C). Circular dichroism, which can be used to measure the packaging of DNA in CLDCs, suggests that heparin can release DNA from CLDCs, with much greater amounts of heparin required to cause release at low compared with higher ionic strength (Fig. 6).

Further insight into the nature of the interaction between CLDCs and heparin in solution is obtained by ITC. Given the spontaneity of this interaction, its entropic nature is apparent from the unfavorable endothermic heats observed when heparin is titrated into DOTAP CLDCs at low ionic strength (Fig. 4A). This favorable entropic signature is consistent with an electrostatic character to the interaction (31, 32). Additional entropic contributions from apolar interactions (e.g. membrane fusion) may be present but are not readily separable. The ~10-fold lower ΔH_{app} of binding seen for the interaction of heparin with DOTAP compared with the interaction of DNA

![Graph](image)

**Fig. 6.** Fraction of DNA released by GAGs. Increasing amounts of GAGs were added to CLDCs, and the molar ellipticity at 275 nm was monitored. The fraction of DNA released was calculated as described under “Experimental Procedures.” Panel A, titration with heparin at low ionic strength; 2:1 DOTAP CLDCs (circles), 2:1 DOTAP:cholesterol CLDCs (triangles), and 2:1 DOTAP:DOPE CLDCs (squares). Panel B, titration with heparin at high ionic strength; 1:1 DOTAP CLDCs (circles), 1:1 DOTAP:cholesterol CLDCs (triangles), and 1:1 DOTAP:DOPE CLDCs (squares). Panel C, titration with heparan sulfate at high ionic strength; 1:1 DOTAP CLDCs (circles), 1:1 DOTAP:cholesterol CLDCs (triangles), and 1:1 DOTAP:DOPE CLDCs (squares). The x axis represents the ratio of the total negative charge from both GAG and DNA in the sample versus the positive charge from the DOTAP.
with the same lipid (32) suggests distinct roles for the charge density, molecular weight, and chemical nature of the polyanion involved in the interaction. At increased ionic strength, the $\Delta H_{\text{app}}$ of binding of heparin to liposomes alone is decreased and $\Delta H_{\text{app}}$ of binding of heparin to 1:1 DOTAP CLDCs switches from endothermic to exothermic. Although the heat of binding for heparin to DOTAP is +0.4 kJ/mol DOTAP, that of DNA binding to DOTAP is $\sim$ +3 kJ/mol DOTAP (33). Assuming that 1 mol of polyanion negative charge interacts/1 mol of DOTAP positive charge, additivity of the enthalpies of binding of heparin and release of DNA (+0.4 $\sim$ (+3) kJ/mol of DOTAP) would result in an exothermic heat of $-2.6$ kJ/mol DOTAP, which is within experimental error of the heat observed in the calorimetric titration ($-2.4$ kJ/mol DOTAP, Fig. 4C). Thus, at physiologically relevant ionic strength, a significant concentration of free GAGs would be expected to facilitate release of DNA from CLDCs.

Effect of Helper Lipids on the Interaction of CLDCs with a Model GAG—Thus far, the role of helper lipids has mainly been defined in terms of the fusogenic properties of the helper lipids (34). Although it appears that this helper lipid-associated property is important for endosomal release of DNA, protection of the DNA and association of DNA in CLDCs with the cell surface could also be important properties as well. Incorporation of helper lipids into CLDCs results in enhanced heparin-head binding at both ionic strengths studied (Figs. 1 and 2). Consistent with this observation, incorporation of DOPE up to a 3:2 ratio with cationic lipid has been shown to enhance cellular association of DNA over that of cationic lipid alone (35). This enhancement presumably reflects the structural differences between CLDCs with and without helper lipids (e.g. size, relative amount of DNA per particle, etc.).

In solution, helper lipid containing CLDCs show trends similar to DOTAP CLDCs in heparin-dependent aggregation at both ionic strengths studied (Fig. 3). Circular dichroism studies reveal that by incorporating DOPE or cholesterol into CLDCs, a greater amount of heparin is required to release 50% of the DNA from these complexes (roughly 1.5 times more heparin for both ionic strengths studied). Thus, incorporation of helper lipids into CLDCs may have significant roles in transfection beyond that of facilitating endosomal release of DNA.

Influence of GAG Structure on the Interaction with CLDCs—Since heparan sulfate is more representative of cell surface GAGs than heparin, we also examined its interaction with CLDCs. Although both polysaccharides possess similar carbohydrate backbone structures and broad size distributions in the range of 4–20 kDa, they differ considerably in their distribution of sulfate groups along the polymer. Heparin is more highly sulfated (2.4 sulfate groups/disaccharide unit) with a considerably greater fraction of N-sulfated glucosamine monomers, whereas heparan sulfate possesses fewer sulfates per disaccharide (1.2) and a greater fraction of N-acetylgalactosamine monomers (36). It is apparent that these two polymers interact quantitatively differently with CLDCs. In the case of 1:1 DOTAP CLDCs, DLS and CD measurements suggest that heparan sulfate facilitates DNA release as well as heparin. Calorimetry experiments demonstrate that heparan sulfate binding to 1:1 DOTAP CLDCs is also exothermic as a result of DNA release from the CLDC. Titration of 1:1 DOTAP CLDCs with heparan sulfate, however, does not yield results that closely resemble the simple additivity of interactions of heparan sulfate and DNA with DOTAP, suggesting that there may be additional processes involved in the interaction of heparan sulfate with CLDCs. In contrast to heparin, incorporation of helper lipids into the CLDC greatly reduces DNA release from the complex by heparan sulfate.

These studies have provided quantitative insight into the interaction of model proteoglycans with CLDCs. Information is forthcoming regarding the effect of ionic strength on the interaction as well as discovery of additional potential roles for helper lipids in CLDC-cell interactions. Additionally, results from these studies suggest that differences in GAG expression can significantly affect the interaction as indicated by the comparison between heparin and heparan sulfate above. Although it is now clear that the anionic nature of HSPGs can facilitate significant CLDC association with cells, studies of CLDC interaction with GAGs upon internalization may elucidate further roles for proteoglycans in CLDC-mediated gene delivery.

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