# Analysis of Cationic-Lipid–
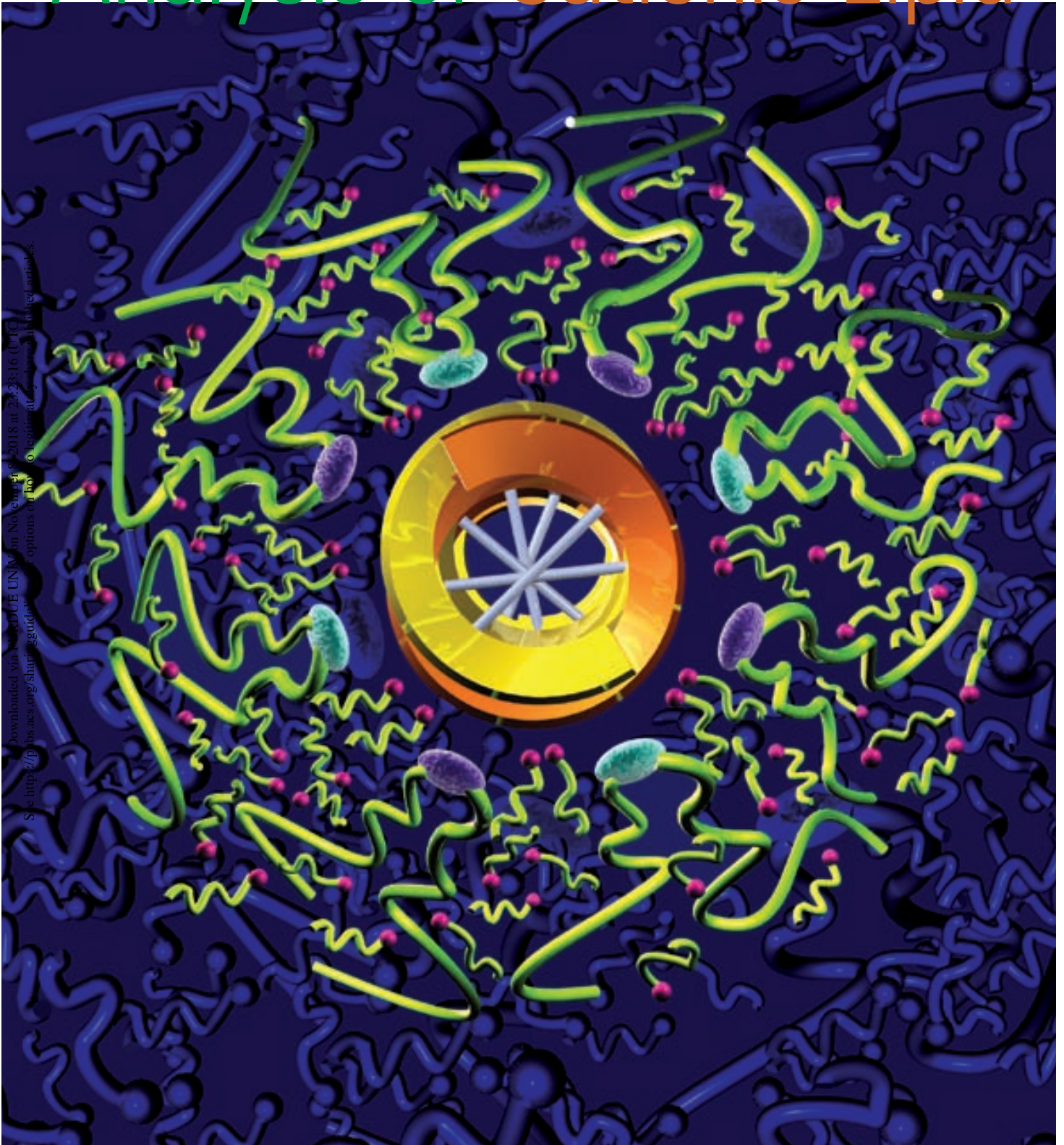
# –Plasmid-DNA Complexes

**Complexes between plasmid DNA and various cationic lipids are promising vehicles to deliver genetic information into cells for gene therapy or vaccines.**
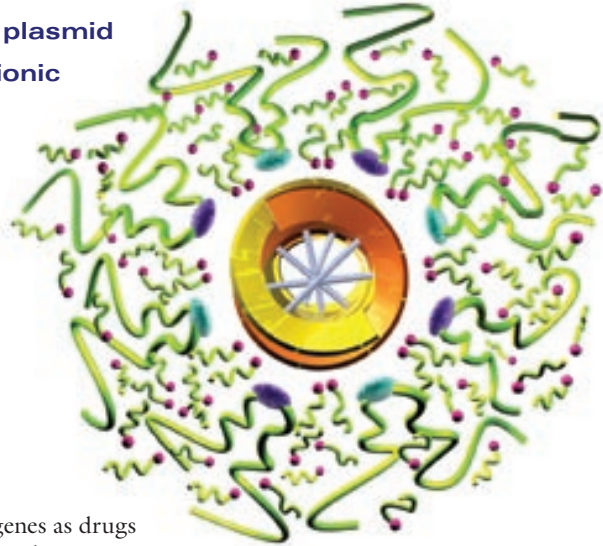
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The idea of using genes as drugs is both exciting and controversial. In most gene therapies, the proteins that are coded for by the DNA have a therapeutic effect, but the genetic material itself is delivered to the patient, generally in two different ways. The first uses modified viruses that contain the gene(s) of interest. This takes advantage of the fact that viruses have evolved over hundreds of millions of years to efficiently deliver their genomes into cells and have their content expressed. This can work fairly well, but the problems of immunogenicity and toxicity, even to the extent of death, have led to serious concerns about this approach (*1, 2*).

The second method attempts to build a synthetic virus—an approach that provides better control of the delivery system's properties. These nonviral vectors usually are created by combining a bacterial plasmid containing the gene(s) of interest with a positively charged (usually polymeric) partner to form a molecular complex. Plasmids themselves are manipulated easily to contain any gene of interest as well as on-and-off elements and other regulatory signals. Preparation of large quantities in a pure form is also straightforward. Positively charged complexing agents that have been used so far include basic peptides, polyethylenimine, amino dendrimers, various synthetic block copolymers, and cationic lipids (CLs) (*3–7*). Anionic lipids in combination with multivalent cations have been explored as an alternative to CLs (*8*). The CL-containing vectors are, however, the most thoroughly investigated. They have been shown to have some efficiency in the clinic, although it is significantly less than that of their viral counterparts; cytotoxicity and immunogenicity are still problematic (*9, 10*). Reviews have addressed characterization and the biological aspects of CLs for gene delivery (*11–13*).

Traditionally, gene delivery vectors have been analyzed in terms of their biological effects, such as their ability to enter cells and direct the expression of selected encoded genes. This process is known as transfection, and its measurement lacks the accuracy and precision expected for the analysis of a material that will be used as a drug in humans. Furthermore, the complexity of biological fluids makes physical characterization of vectors in such environments difficult. Characterization
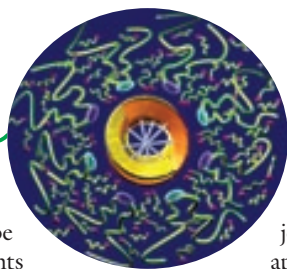
of vectors in situ typically requires the vector to be radio- or fluorescently labeled. Thus, measurements are performed most often on pure, buffered solutions of DNA–polycation complexes. The vectors themselves are large, complex, and usually very heterogeneous in structure. This makes their physical and chemical characterization difficult, even in a pure state.

Recent attempts have been made to treat such vectors with rigorous physicochemical methods to produce stabler, more effective, and safer gene-based drugs. In this article, we take CL–DNA complexes (CLDCs), or "lipoplexes", as prototypical examples and discuss the progress and future of their physical and chemical analysis. Originally described in 1987 (*7*), CLDCs are the most thoroughly studied nonviral gene delivery agents and serve as a good introduction to the analytical problems involved in their characterization.

## Primary structure and composition

When highly negatively charged DNA is mixed with virtually any polycation, some degree of condensation of the polynucleotide occurs. In solution, plasmid DNA usually exists as a highly supercoiled, covalently closed circle of DNA with small amounts of open circular (nonsupercoiled) and linear (cleaved in both polynucleotide chains) contaminants. If you take a rubber band and twist it several times, you will observe its collapse into a much smaller volume. This is essentially what happens when the negative charges on the phosphate groups of the DNA are neutralized by polycations, which relieve the electrostatic repulsive forces within the DNA and subsequently reduce its volume.

In the original bacterial cell used to produce the plasmid, the DNA is collapsed by an energy-dependent enzymatic reaction in which the strands are twisted into a more condensed, highly tensioned state. The lipids in the complex are thought to be in a bilayer form with the apolar lipid tails on the inside and the cationic head groups on the outer surface. The question is what happens when the DNA and CL are combined. Regular and irregular particle-like structures are formed with a size range of a few tens to a few hundreds of nanometers, but the resultant structures display significant size (and, presumably, compositional) heterogeneity. Whether such mixtures can be analyzed, even in principle, by any type of rigorous procedure is certainly a reasonable question. We will argue that they can be, although ambiguities in interpretation are significant.

The actual composition of lipoplex formulations is more difficult to define than expected. Although only two macromolecular components may be present (e.g., DNA and CL), they can be present either as pure species or as complexes of unknown composition. Furthermore, both molecules are sub-

ject to a variety of chemical changes. The simplest approach is to separate the DNA from the CL by methods based on size, charge, or density. This can work only if the complexes can be dissociated quantitatively. Because the interactions between the CL and DNA are primarily electrostatic in nature, in principle, separation can be achieved by adding salt, but the presence of additional, less clearly defined interactions makes this difficult if not impossible.

If separation can be accomplished, analysis of the individual components is easy with conventional methods. For example,
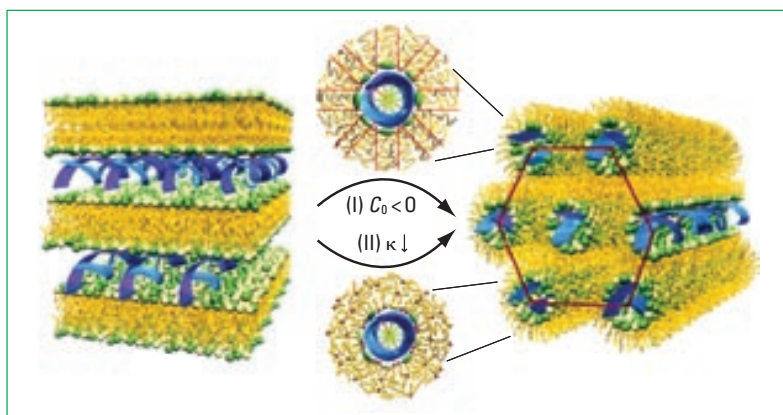


**FIGURE 1.** A depiction of two distinct pathways from the (left) lamellar phase to the (right) columnar inverted hexagonal phase of CLDCs.

(Left) Helical DNA strands (blue ribbons) between layers of CLs (green head groups with yellow aliphatic chains) and neutrally charged helper lipids (white head groups with yellow aliphatic chains). (Center top) Along pathway I, the natural curvature $C_0$ of the CL monolayer is driven negative by the addition of the helper lipid DOPE. The CL DOTAP is cylindrical, whereas DOPE is conical, thus leading to the negative curvature. (Center bottom) Along pathway II, the transition is induced by adding helper lipids consisting of mixtures of DOPC and the cosurfactant hexanol, which reduces the membrane bending rigidity κ. (Adapted with permission from Ref. 17.)

DNA can be hydrolyzed, and the nucleotide composition can be determined by LC and MS methods. A number of techniques exist to analyze specific degradation products as well (*14*). Perhaps surprisingly, however, the composition of CLDCs is not usually determined at all—rather, the composition is simply assumed to be defined by the identity and amounts of the components originally introduced. In the future, however, as gene therapy agents approach pharmaceutical reality, more rigorous criteria likely will be required.

## Secondary, tertiary, and quaternary structure

*Microscopy.* Under certain conditions, polymers almost always manifest local interactions within their chains that lead to highly organized structure. The best known examples of this in biochemical systems are the α-helices and sheets of proteins, the various forms of the DNA double helix, and the bilayer structure of amphipathic lipids. The latter two structures are the most relevant. The structures of DNA and CL bilayers have been observed both separately and in complex by X-ray

diffraction; small-angle X-ray scattering; and transmission electron, atomic force (AFM), and scanning tunneling (STM) microscopies (*15–20*).

From such work, a fairly uniform picture has emerged in which aligned strands of the helical DNA are sandwiched between alternating monolayers of CLs (Figure 1, left; *16*). When neutrally charged helper lipids (so called because they enhance transfection efficiency) are included, a remarkable transformation takes place and an inverted hexagonal phase of the CL part of the complex is formed (Figure 1, right; *17*). In this state, the previously buried apolar side chains of the lipids now extend outward from the DNA chains, forming a uniform apolar coat around each strand of DNA. Although this second form of the complex has been accepted to transfect more efficiently, research has shown that factors other than structure may account for differences in efficiency of gene delivery. In one case, increasing the charge density was shown to increase the transfection efficiency of lamellar complexes to levels observed with the inverted hexagonal complex (*21*). A separate study showed that an optimum charge density may exist that balances the structural stability of the complex with the release of the DNA (*22*). Unfortunately, the methods used to achieve these insights into CLDC structure are not applicable to complexes in pharmaceutical formulations. They do, however, clearly establish the helical nature of the DNA and the local structure of the CLs.

A quite different problem occurs when imaging methods are used to probe the structure of lipoplexes. Whether negative staining combined with electron microscopy or AFM or STM methods are used, one almost always sees images containing a collection of amorphous entities described as anything from nebulous globs to structures that resemble spaghetti and meatballs (*19*). At present, the utility of such data for rigorous analysis remains to be established. Although the helical nature of the DNA can sometimes be seen, typically little further detail is apparent.

***Dynamic light scattering (DLS).*** In addition to direct imaging methods, the size and shape of lipoplexes can be characterized by DLS. In this method, the fluctuations in the intensity of scattered light due to the Brownian motion of particles are analyzed by autocorrelation methods to yield a diffusion constant and ultimately a size in the form of a hydrodynamic diameter. Although the polydispersity of the complexes formed by CLs and plasmid DNA makes a rigorous analysis of the resulting data somewhat difficult, a mean diameter can still be obtained or the data deconvoluted in such a way as to obtain distinct populations of particles as a function of approximate size ranges.

When DLS is used in the presence of an electromagnetic field, an estimate can be made of the charge on the particle or the $\zeta$ potential (the voltage at the surface of shear). The intensity of the scattered light as a function of the angle of detection

also can be used to directly measure the molecular weight of the particle as well as its radius of gyration (which is based on the distribution of mass of the particle rather than its hydrodynamic behavior). The ratio of the radius of gyration to the experimentally determined or calculated hydrodynamic radius provides a measure of the asymmetry of the particle.

DLS studies have identified CL–DNA particles in the size range 50–300 nm and confirmed their intrinsic heterogeneity (*23–25*). Their $\zeta$ potentials range from the highly negative (an excess of DNA) to the highly positive (an excess of CL). One of the few correlations between the physical properties of particles and their ability to transfect cells is based on DLS measurements where it has been found that smaller (90–150 nm), positively charged lipoplexes generally produce good gene expression. This probably reflects the mechanism by which such particles enter cells; initially, the mechanism appears to involve electrostatic interactions with highly negatively charged cell-surface proteoglycans (*26, 27*).

***Analytical ultracentrifugation.*** This method can be used in one of two modes. In equilibrium sedimentation, material is sedimented at such a speed that equilibrium is reached in the centrifuge tube. The resultant distribution of mass of the solute in the tube is then optically monitored. This distribution can be analyzed and converted to an absolute molecular weight, which can in turn be used to calculate a size on the basis of assumptions about the density and shape of the particles. Densities (partial specific volumes) can be measured experimentally with oscillating U-tubes or pycnometers. In the second approach, the rate at which the particles sediment is measured (sedimentation velocity); this rate permits an estimate of hydrodynamic size to be made. Unfortunately, the usual heterogeneity of lipoplexes has made it difficult to apply either method.

A simple but less informative method is based on the sedimentation of lipoplexes in preformed density gradients of inert solutes, such as sucrose or dextrose (*28, 29*). This method has been used to provide empirical measures of particle behavior that are not related simply to their physical properties. As the homogeneity of pharmaceutical preparations of lipoplexes increases, both sedimentation velocity and equilibrium experiments have the potential to play important roles as analytical tools.

***Gel electrophoresis.*** Although sufficiently porous size-exclusion matrices exist, this method has yet to be widely applied to the structural characterization of lipoplexes. The gel retardation assay is occasionally used to determine the positive-to-negative charge ratios that minimize the amount of unbound DNA (*30*). Because of the relative charge and size differences between lipoplexes and plasmid DNA, electrophoresis of the two produces widely separated bands. The gel retardation assay shows that with increasing charge ratios the amount of unbound DNA decreases and the quantity of lipoplexes retarded
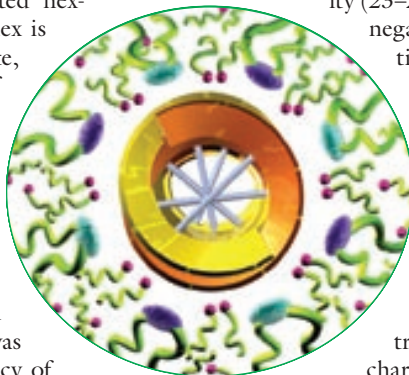
near the top of the gel increases. Complete retardation of DNA often does not occur until an excess of positive lipid charge to negative DNA charge exists; this probably indicates that not all of the positive charge associated with the lipid is involved in the formation of the complex. Farhood et al. attributed this observation to steric hindrance between the two species due to the bulky nature of the liposome and superhelical DNA (*31*).
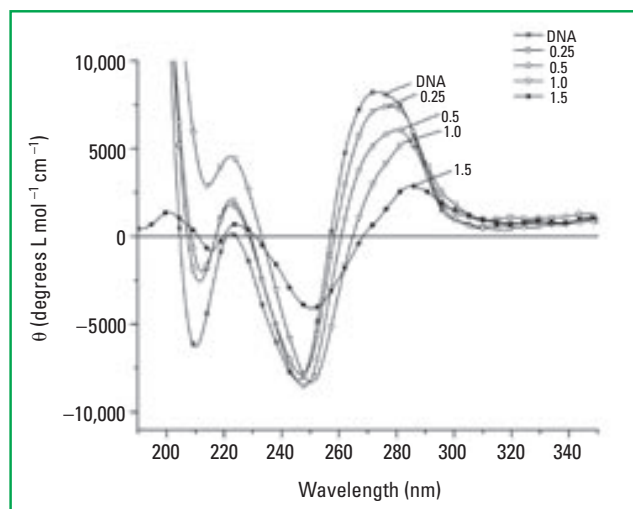


**FIGURE 2**. Representative CD spectra of DOTAP–DNA complexes.

The charge ratios are indicated. Open symbols are CLDCs below charge neutrality, and the closed symbol is a CLDC above charge neutrality. (Adapted with permission from Ref. 22.)

*Circular dichroism (CD).* This straightforward technique focuses primarily on the helical structure of DNA by measuring the difference in absorption of left- and right-handed circularly polarized light. Because of the regular helical array of the DNA bases, very strong optical activity is present within DNA molecules. For the most common type of DNA, known as B-form, a very characteristic spectrum displays a strong positive peak near 275 nm and a negative signal of similar intensity at ~245 nm. When CLs are added to DNA, a red shifting of both peaks and a fairly dramatic reduction in intensity of the 275 nm feature are seen (Figure 2). These large spectral changes were originally thought to be due to a change from B-form DNA (10 bp/turn) to C-form (9 bp/turn). Later studies, however, based on IR and Raman measurements and molecular dynamics simulations, strongly suggest that the changes seen are due to small alterations in the interactions between the bases while the DNA essentially remains in the canonical B-form (*32*). What is important from an analytical point of view is that the CD spectra of the complexes are very sensitive to their structure and that therefore CD can monitor the state of the DNA in lipoplexes and polyplexes (cationic polymer-based complexes; *33–35*).

*FTIR.* This method can characterize the structure of both the DNA and CL components of complexes (*36*). A disadvantage of this technique is that 10–20-fold higher concentrations are required for solution studies; an advantage is that very concentrated and even dried materials can be examined easily (*37, 38*). In such studies, peak positions corresponding to vibrational modes of a wide variety of molecular groups, including vibrations of the DNA bases and phosphate groups, as well as peak positions corresponding to lipid methylene and carbonyl stretching bands are measured as a function of CL-to-DNA ratios.

Titration of one component into the other clearly reveals distinct peak positions for various stages of complex formation—this supports the use of this method for structural characterization and verification. Figure 3 presents two examples in which the CLs DOTAP and DDAB were titrated into a solution of plasmid DNA. During these titrations, the positions of peaks corresponding to various vibrational modes and stretching bands were monitored. The data show shifts in the peak positions occurring over a range of CL-to-DNA weight ratios; these correspond to distinct structural states in the complexes. Similar, but preliminary, studies have been undertaken with Raman spectroscopy (*32*), but that approach is limited because of the higher concentrations needed to obtain spectra.

*Fluorescence spectroscopy.* This technique has been used to examine various aspects of lipoplex structure. The most commonly used method is the simple displacement of fluorophores bound to plasmid DNA. Typically, dyes are either intercalated between the bases of the DNA or bound within the minor groove. The normally solvent-quenched quantum yield of fluorescent dyes is dramatically enhanced when they are bound to DNA. When a polycation such as a CL micelle is added to such labeled DNA, the fluorescent dye often is displaced through competition with the former CL. This displacement of the dye leads to a reduction in fluorescence quantum yield that is easily measured. If a combination of dyes is used along with titration experiments, lipoplex-induced structural (especially topological) changes in the DNA can be detected, and changes in the solvent accessibility of the minor groove are seen (*39*). Thus, simple measurements of dye fluorescence intensity and wavelength emission maximum can be used to obtain at least comparative structural information. The major disadvantage of this approach is that, unlike CD and FTIR studies, a label is needed. Furthermore, the label is usually added to the DNA before complex formation occurs. Therefore, this method cannot be used to characterize an intact lipoplex directly.

Another fluorescence-based method that suffers from some of the same problems but is of significantly higher resolution is fluorescence resonance energy transfer (FRET). When two fluorescent groups have the property that the emission spectrum of the donor overlaps the absorption spectrum of the acceptor, it is possible to excite the donor but see emission from the acceptor (or quenching of the donor; *40*). By measuring the efficiency of such events, one can estimate the distance between the donor(s) and acceptor(s).

FRET is a versatile technique that is increasingly seen in a wide variety of applications (*41*). In the case of lipoplexes, it has been possible to label the DNA by either intercalation of a dye between the bases or binding of a dye to the minor groove (donors). Then, a different dye can be placed within

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