The Structure of DNA−Liposome Complexes

Danilo D. Lasic,*,† Helmut Strey,‡ Mark C. A. Stuart,§ Rudolf Podgornik,‡ and Peter M. Frederik§

MegaBios Corporation, Burlingame, California
Division of Computer Research and Technology
Laboratory for Structural Biology
National Institutes of Health, Bethesda, Maryland
Department of Pathology, Electron Microscopy Unit
Limburg University, Maastricht, The Netherlands

Received August 5, 1996

Extremely rapid developments in molecular biology are making gene therapy—a new medical treatment with a potential to cure diseases on the molecular level—a promising new therapeutical modality. While appropriate plasmids (genes) can be prepared in large quantities, their efficient and safe delivery into appropriate cells *in vivo* seems to be the main obstacle in successful medical applications.[1] Cationic liposomes were shown to be a promising gene delivery system.[2] Despite numerous studies and commercially available liposome kits, however, the structure of DNA−cationic liposome complexes is still not yet well understood. Several electron microscopy studies have shown either larger aggregates surrounded by thin fibers[3,4] or condensed DNA coated by a lipid bilayer.[5] Hexagonally packed DNA coated by lipid was also proposed.[6]

We have investigated the structure of these complexes using high-resolution cryo electron microscopy (EM) and small angle X-ray scattering (SAXS). Complexes were prepared by rapid mixing of DNA and liposome solutions at room temperature.[7] Precipitation behavior of DNA−cationic liposome mixtures was studied in a phase space of DNA and cationic lipid concentration. Typically, complexes around charge neutralization and for lipid concentrations above 0.1 mM precipitate. Figure 1 shows phase diagram of DODAB/Chol (dioctadecyldiammonium bromide/cholesterol, 1:1 mol/mol) liposomes (130 nm in 5% dextrose) complexed with a 4.7 kb DNA plasmid.

Figure 2 shows cryo EM micrographs of two different DNA−cationic liposome systems with the negative/positive charge ratio of $\rho = 0.5$. Typically heterogeneous particles in the size range

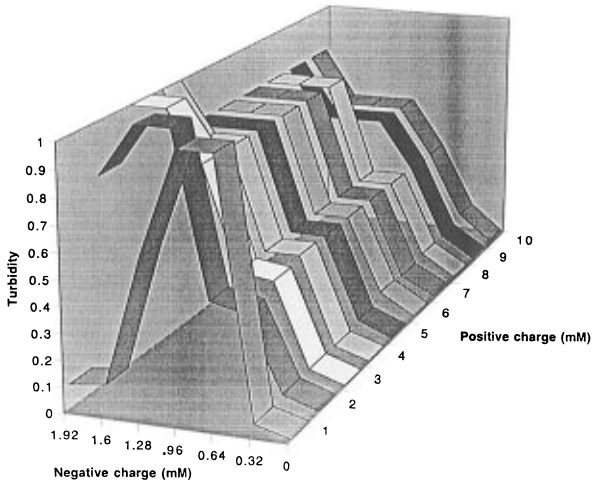

Figure 1. Phase diagram of DODAB/chol (1:1) DNA−cationic liposome (4.7 kb) system (20 °C, ionic strength <1mM). Small vesicles (130 nm, prepared in 5% glucose by extrusion) were complexed with DNA at specified ratios. On the "electroneutrality" diagonal ($\rho = 1$) precipitation occurs. Colloidally soluble complexes exist on the left side of the diagonal (anionic complexes) and on the right side (cationic complexes). At higher lipid or DNA concentrations precipitation occurs. DNA charge was calculated from 2 charges per base pair (660 Da). Values below turbidity of 0.4 indicate soluble complexes. Precipitated complexes were assigned a turbidity of 1, while values 0.8, 0.6, and 0.4 were assigned to flocculation, slight, and very slight flocculation.

0.2−0.5 μm are observed, and their shapes vary from stacks of bilayers, which can be flat, concentric, or bent, to amorphous aggregates. Both systems (Figure 2A,B) show lamellar structures with a periodicity of 6.5 nm. In some micrographs, a second periodicity around 3.5 nm can be observed also (arrows in Figure 2B). Exactly the same periodicities were observed by SAXS (Figure 3). A very strong reflection is observed around 6.5 nm, and second- and third-order reflections can be easily detected at positions clearly indicating lamellar symmetry, in which reflections occur at *d/n* (*d*-spacing, *n*-order of reflection). Using the Warren−Gaussian approximation[8] for analyzing the line shape of the first-order reflection, we determined an average domain size of the lamellar DODAB/Chol−DNA complex ($\rho = 0.5$) to be 36 nm, corresponding to about six repeat spacings and consistent with cryo EM micrographs. A weak reflection with shorter periodicity of 3.6 nm can be also observed, in agreement with EM (Figure 2B, arrow).

As controls, unreacted liposomes, unsonicated dispersion of lipid, and naked DNA did not give any reflections. Plasmid condensed by polyethylene oxide and salt showed typical hexagonal structure[9] with an interhelical periodicity of 2.5 nm. In a similar system, atomic force microscopy of DNA deposited on a supported cationic lipid bilayer has shown that DNA adsorbs in a single layer in the form of aligned helices, without any knots and crossings resembling a two-dimensional (2D) nematic phase.[10]

From these data, the structure of these complexes can be estimated. We believe that DNA is adsorbed between cationic bilayers as a single layer of parallel helices with average in-plane separation consistent with the short periodicity observed by SAXS and EM. Stacks of alternating cationic lamellae and 2D DNA yield long periodicity of 6.5 nm, which is consistent

* Author to whom correspondence should be addressed: D.D. Lasic, Liposome Consultations, 7512 Birkdale Dr., Newark, CA 94560.
† MegaBios Corporation.
‡ National Institute of Health.
§ Limburg University.

(1) (a) *Handbook of Nonmedical Applications of Liposomes: From Gene Delivery and Diagnostics to Ecology*; Lasic, D. D., Barenholz, Y., Eds.; CRC Press: Boca Raton, FL, 1996; Vol. IV. (b) Lasic, D. D. *Liposomes in Gene Delivery*; CRC Press: Boca Raton, FL, In press.

(2) (a) Felgner, P. L.; Gadek, T. R.; Holm, M.; Roman, R.; Wenz, M.; Northrop, J. P.; Ringold, M.; Danielsen, H. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7413. (b) Behr, J. P. *Acc. Chem. Res*. **1993,** *26*, 274. (c) Gao, X.; Huang, L. *Biochem. Biophys. Res. Commun.* **1991**, *179*, 280. (d) Solodin, I.; Brown, S. C.; Bruno, M. S.; Chow, S.; Jang, E.; Debs, R.; Heath, T. D. *Biochemistry* **1995**, *34*, 13537. (e) Templeton, N. S.; Lasic, D. D.; Roberts, D. D.; Frederik, P. M.; Pavlakis, G. Submitted.

(3) Sternberg, B.; Sorgi, F.; Huang, L. *FEBS Lett.* **1994**, *356*, 361.

(4) Gustafsson, J.; Almgrem, A.; Arvidson, G. *Biochim. Biophys. Acta* **1995**, *1235*, 305.

(5) See: Minsky, A.; Ghirlando, R.; Gerhson, H. in ref 1, pp 7−30.

(6) See: Felgner, J. H.; Tsai, Y.; Felgner, J. H. in ref. 1, pp 43−57.

(7) Complex formation is, in addition to thermodynamic factors, kinetically controlled. Slow mixing at lipid concentrations >0.1 mM caused precipitation. Also, anionic complexes must be prepared by adding liposomes into DNA and vice versa for cationic ones. Using the phase diagram shown in Figure 1, we can see that that by doing so the crossing of the solubility gap (around $\rho = 1$ diagonal) is avoided. Quick mixing assures good dispersal and growth of many small complexes as opposed to the growth of a smaller number of larger ones for slow mixing, which results in precipitation. This is analogous to crystallization and preparation of inorganic colloidal particles where reactions far from equilibrium conditions ("burst of nucleation embrii") yield the smallest particles. (See: Lasic, D. D. *Bull. Chem. Soc. Jpn*. **1993**, *66*, 709.) Equivolumetric mixing and the use of small unilamellar vesicles offer the quickest reaction and best dispersal, respectively.

(8) Dutta, P.; Sinha, S. K. *Phys. Rev. Lett*. **1981**, *47*, 50.

(9) (a) Maniatis, T.; Venable, J. W.; Lerman, S. *J. Mol. Biol*. **1974**, *84*, 37. (b) Podgornik, R.; Strey, H.; Rau, A.; Parsegian, A. D. *Biophys. Chem.* **1995**, *55*, 11.

(10) Yin, J.; Wang, L.; Cammerini-Otero, R. D. Submitted.

S0002-7863(96)02713-8 CCC: $14.00

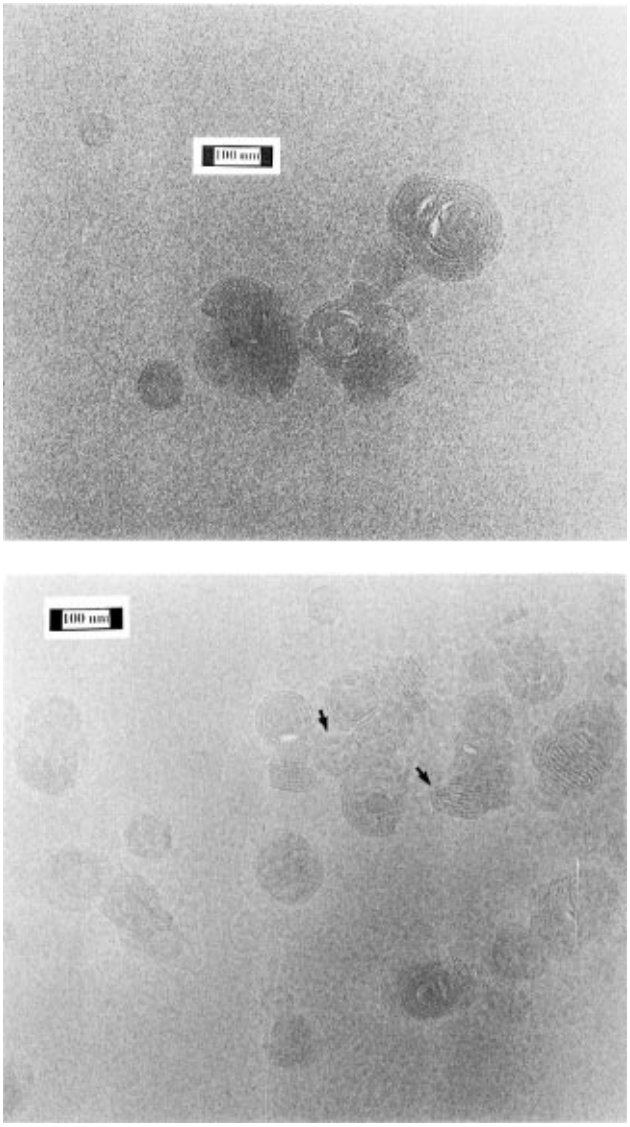

Figure 2. Cryo electron micrograph of (A) DODAB/Chol−DNA (ρ = 0.5, DODAB concentration 1.8 mM) colloidal particles and (B) DOIC/Chol−DNA (ρ = 0.5, DOIC, 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride, concentration 1.8 mM) complexes. Complexes were prepared by rapid mixing of the DNA solution into liposomes. More reproducible results, as measured by turbidity and size, were obtained when the liposome solution was stirred in a vial and DNA was injected through a thin needle at 2 mL/s, as opposed to commonly used pipette mixing.

with a lipid bilayer thickness of 4 nm[11] and hydrated (one-water shell) DNA helix of 2.5 nm.[10] This indicates that the binding is atmospheric and not site-specific, which would cause the dehydration of charges, and one would expect a smaller overall spacing: first, because of the missing water layer and second, because the absence of water would freeze the hydrocarbon chains causing molecular tilt within bilayer thus reducing the periodicity to 4.5−5 nm.[11]

Preliminary analytical ultracentrifugation experiments have shown a picture consistent with a very heterogeneous population of complexes. A continuous pelleting of complexes is observed from very dense aggregates with sedimentation coefficient $S >$ 800 Sv to the band of trailing liposomes with its characteristic S, which can range, depending on lipid composition, from negative values (floating) to $S = 20$ Sv. In anionic complexes ($\rho = 2$), the picture is similar, only the trailing band can be

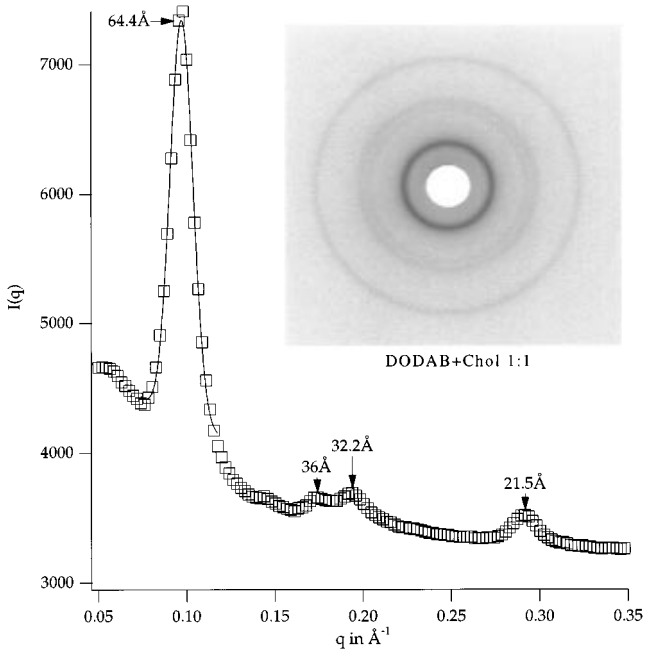

Figure 3. Plot of radially averaged scattering intensity of DODAB/Chol−DNA (ρ = 0.5) complex as a function of the scattering wave vector q. The solid lines are fits using a Gaussian line shape on top of the background, which was measured separately. In the upper right corner, the corresponding X-ray picture is shown. The DODAB/Chol−DNA complex shows three X-ray diffraction maxima of 6.44, 3.22, and 2.15 nm, indicating lamellar structure. The additional spacing of 3.6 nm could correspond to the in-plane spacing between parallel DNA helices.

associated with naked DNA which is characterized, as determined in a control experiment, by the same $S = 5$ Sv.[12]

The structure of these complexes can be therefore described as particles with a short-range lamellar order in which 2D layers of oriented DNA are sandwiched between lipid bilayers. Because we did not observe any attached fibers to the complexes, we believe that strong interaction with lipids reduces persistence length and allows high curvature. The absence of fibers can also explain colloidal stability because often complexes precipitate within 15 min[1,2c] what can be attributed to bridging flocculation of particles by coated DNA fibers. From the sizes of the complexes we estimate that between 5 and 15 plasmids are associated in a single complex. SAXS and sedimentation experiments also show that DNA is condensed and structures are characterized by lamellar (and not hexagonal[13]) symmetry.

To maximize the concentration of suspended DNA we have used small unilamellar vesicles, minimal ionic strength, and high concentration of reactants. We could prepare topologically very different complexes from the same reagents just by varying concentrations, ionic strength, liposome size, and kinetics of mixing.

In conclusion, we have shown that these DNA−lipid complexes contain condensed DNA. The size of the particles is below 0.5 μm. Complexes are heterogeneous with respect to size and shape and are characterized by a short-range lamellar symmetry. Furthermore, DNA condensation induced by cationic liposomes is another example of supramolecular chemistry. Experimentally,[13] as well as theoretically,[14] it was shown that single-valent cations cannot condense DNA. In our case, however, the self-assembled bilayer of univalent cationic lipids interacts as a polyvalent entity.

JA962713G

(11) Lasic, D. D. *Liposomes: from Physics to Applications*; Elsevier: Amsterdam, New York, Tokyo, 1993; p 555.

(12) McRorie, D.; Lasic, D. D. Unpublished results. In agreement with the observed boundary of unreacted liposomes, some cryo EM micrographs also showed some intact liposomes.

(13) Bloomfield, V. A. *Biopolymers* **1991**, *31*, 1471.

(14) Manning, G. *Q. Rev. Biophys.* **1978**, *11*, 179.