# Mode of Formation and Structural Features of DNA–Cationic Liposome Complexes Used for Transfection†

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ABSTRACT: Complexes formed between cationic liposomes and nucleic acids represent a highly efficient vehicle for delivery of DNA and RNA molecules into a large variety of eukaryotic cells. By using fluorescence, gel electrophoresis, and metal-shadowing electron microscopy techniques, the factors that affect the, yet unclear, interactions between DNA and cationic liposomes as well as the structural features of the resulting complexes have been elucidated. A model is suggested according to which cationic liposomes bind initially to DNA molecules to form clusters of aggregated vesicles along the nucleic acids. At a critical liposome density, two processes occur, namely, DNA-induced membrane fusion, indicated by lipid mixing studies, and liposome-induced DNA collapse, pointed out by the marked cooperativity of the encapsulation processes, by their modulations by DNA-condensing agents, and also by their conspicuous independence upon DNA length. The DNA collapse leads to the formation of condensed structures which can be completely encapsulated within the fused lipid bilayers in a fast, highly cooperative process since their exposed surface is substantially smaller than that of extended DNA molecules. The formation of the transfecting DNA–liposome complexes in which the nucleic acids are fully encapsulated within a positively-charged lipid bilayer is proposed, consequently, to be dominated by mutual effects exerted by the DNA and the cationic liposomes, leading to interrelated lipid fusion and DNA collapse.

Negatively-charged "classical" liposomes have been used for more than a decade as vehicles for gene transfer into eukaryotic cells grown in culture, as well as for *in vivo* transfection processes. This method presents, however, some basic difficulties associated with a low efficiency of nucleic acid encapsulation, a sonication-induced DNA degradation, and the requirement to separate the DNA–liposome complexes from "ghost" vesicles (Cudd & Nicolau, 1984; Nicolau et al., 1987; Bertling et al., 1991). In order to circumvent these drawbacks and to increase the overall efficiency of nucleic acid delivery into the cells, positively-charged liposomes were designed and used as transfection agents (Felgner et al., 1987; Ballas et al., 1988; Behr et al., 1989; Pinnaduwage et al., 1989; Felgner & Holm, 1989). Since cationic vesicles interact avidly and spontaneously with the negatively-charged nucleic acids, complete entrapment of the DNA or RNA molecules is achieved even at low liposome-to-nucleic acids ratios, thus obviating the difficulties related to the presence of large amounts of empty liposomes in the transfection mixtures. The problem of sonication-induced DNA or RNA degradation is also eliminated, as the complexes used for transfection are prepared by a mere mixing of the polynucleotides with *preformed* cationic liposomes. Fusion between cationic vesicles and cell surfaces results in delivery of the entrapped nucleic acids directly across the membrane (Felgner et al., 1987), thus preventing lysosomal degradation processes which occur when anionic liposomes are used, and leads to a reduced efficiency of these species as DNA carriers (Bertling et al., 1991).

Indeed, cationic liposome-mediated transfection has been shown to allow efficient delivery of both DNA and RNA into a wide variety of eukaryotic cell types and to result in relatively high levels of expression of the exogenous nucleic acids (Felgner et al., 1987; Ballas et al., 1988; Felgner & Holm, 1989; Malone et al., 1989). As such, this method represents significant progress in the ability to perform genetic manipulations and to understand their physiological consequences (Wang & Huang, 1987). In light of these considerations, it is quite surprising that the structural characteristics of the transfection vehicles, namely, the cationic liposome–nucleic acid complexes, remain unclear. It is generally assumed that, in contrast to the negatively-charged liposomes, the cationic vesicles do not encapsulate or entrap the DNA (or RNA) but bind it at their surface while maintaining their original size and shape (Behr, 1986; Felgner & Ringold, 1989; Bertling et al., 1991; Maccarrone et al., 1992). This hypothetical model is inconsistent with the observations presented in the current study that point toward the occurrence of DNA-induced fusion of the cationic vesicles into elongated lipid bilayers which encapsulate the nucleic acids. The membrane fusion is accompanied by cooperative, liposome-induced DNA collapse which plays a crucial role in facilitating and enhancing the encapsulation processes. Significantly, agents which promote DNA packaging are shown to promote encapsulation and may, as such, increase the overall efficiency of the cationic liposome-mediated transfection.

## MATERIALS AND METHODS

Phosphatidylethanolamine (PE), ethidium bromide, sodium poly(L-glutamate) (poly-Glu), and poly(L-lysine) (poly-Lys; degree of polymerization, 90) were purchased from Sigma. DNase I (grade II, lyophilized, 2000 units/mg) from bovine pancreas was purchased from Boehringer-Mannheim; the

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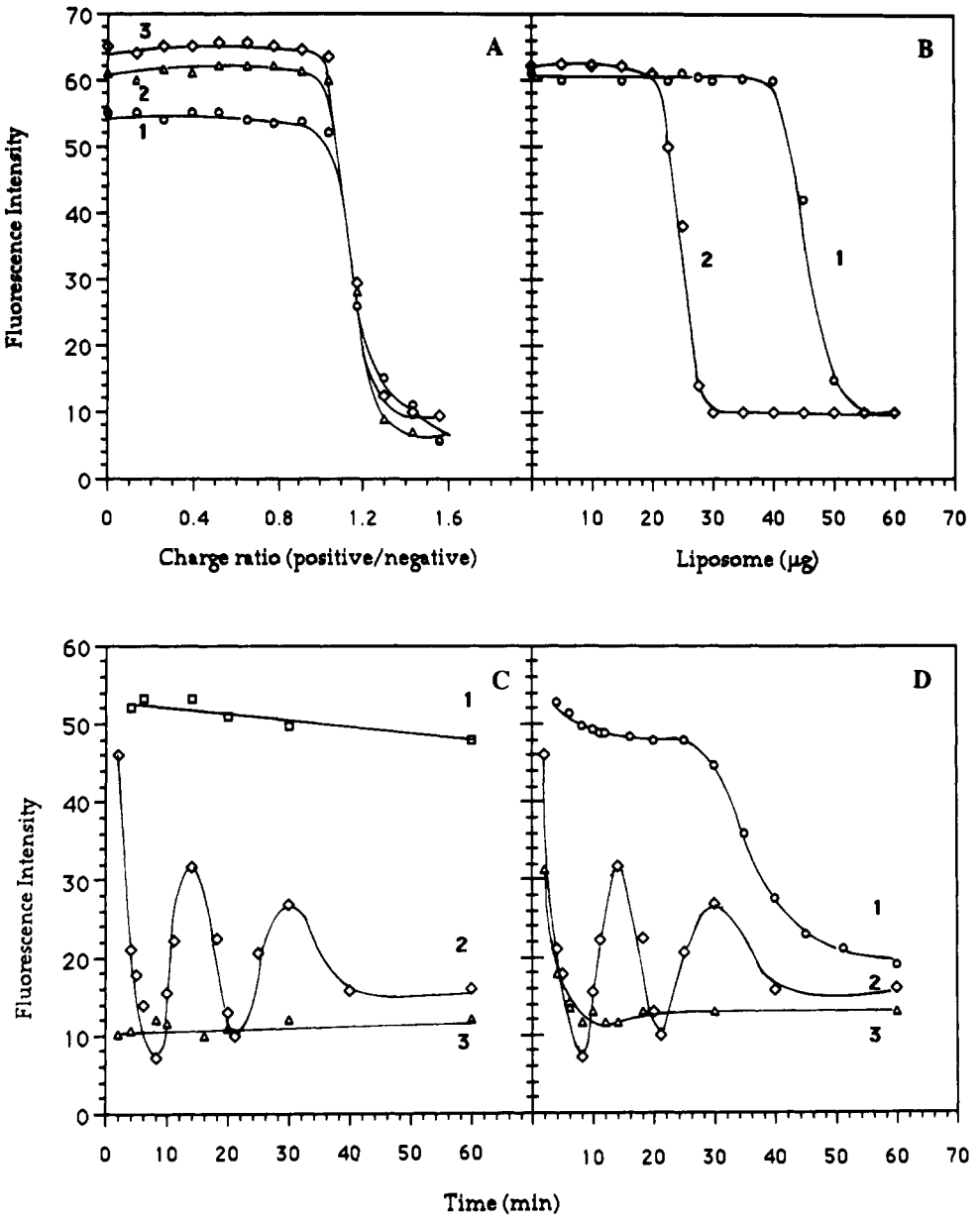


FIGURE 1: Effects of DNA length and liposome-to-DNA ratio (in terms of positive to negative charge ratio) on the fluorescence intensity. (A) Length of DNA segments (in base pairs): (1) 100–300; (2) 500–8000; (3) 23 000. (B) Different cationic liposomes: (1) DOTMA/PE (1:1 molar ratio); (2) DOTMA. (C) Time dependence of the fluorescence intensity at "extreme" positive (DOTMA) to negative (nucleotides) charge ratios: (1) 0.5; (2) 1.1; (3) 1.5. (D) Time dependence of the fluorescence intensity at charge ratios near the fluorescence quenching point: (1) 1.0; (2) 1.1; (3) 1.2. DNA concentration, $2.5 \times 10^{-5}$ M (in nucleotides); ethidium bromide (1:50 molar ratio of probe to nucleotides) was added immediately prior to the measurements.

lyophilized enzyme was dissolved in 50 mM Tris (pH 7.7), 10 mM DTT, and 30% glycerol. The solution (100 000 units/mL) was stored at −20 °C.

*N*-[1-[2,3-Bis(oleoyloxy)]propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) was synthesized following the procedure of Felgner et al. (1987).

*Nucleic Acids.* Highly polymerized calf thymus DNA (type I, Sigma) was dissolved in 20 mM Tris buffer, pH 7.5, and sonicated for 4 × 30 s using an Ultratip Labsonic System (Model 9100) sonicator from Lab-Line Instruments Inc. DNA fragments were loaded on a Sephacryl S-400 (Pharmacia LKB Biotechnology, Inc.) and eluted with 20 mM Tris (pH 7.5)/0.25 M NaCl solution. Fractions of 5 mL were collected, and the size distribution of the fragments was determined by 0.75% agarose gel electrophoresis. Samples were extensively dialyzed against 5 mM Tris buffer/5 mM EDTA and concentrated by ultrafiltration. DNA concentrations were determined by measuring the absorption at 260 nm, applying the relationship: 1.0 OD = 40 μg/mL. The *Bluescript* plasmid used in this study was prepared according to Sambrook et al. (1989).

*Liposome Preparation.* The liposomes used in this study were prepared either from DOTMA or from an equimolar mixture of DOTMA and PE. Dry PE and DOTMA were dissolved in chloroform. The solvent was evaporated under a stream of nitrogen followed by 30-min exposure to high vacuum. The resulting lipid films were resuspended in 20 mM NaCl (in DDW) by vortex mixing and sonicated in a

[1] Abbreviations: DOTMA, *N*-[1-[2,3-bis(oleoyloxy)]propyl]-*N*,*N*,*N*-trimethylammonium chloride; LUV, large unilamellar vesicle(s); *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; PE, phosphatidylethanolamine; poly-Glu, sodium poly(L-glutamate); poly-Lys, poly(L-lysine); SUV, small unilamellar vesicle(s).

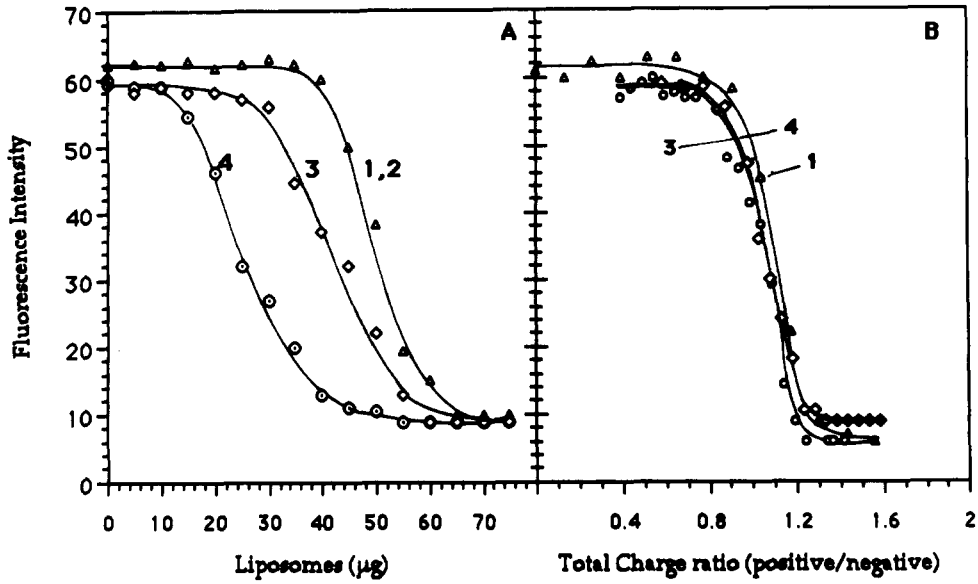


FIGURE 2: Effects of poly-Lys on the fluorescence of DNA–liposome complexes as a function of (A) increasing amounts of liposomes and (B) total positive (poly-Lys + DOTMA) to negative (nucleotides) ratio. (1) No poly-Lys included; (2) monomeric lysine residues ($2.5 \times 10^{-5}$ M); (3, 4) poly-Lys ($1.25 \times 10^{-5}$ and $2.5 \times 10^{-5}$ M in amino acid residues, respectively). DNA concentration was kept at $2,5 \times 10^{-5}$ M, in nucleotides.

bath-type sonicator (Ultratip-Labsonic-System, Model 9100 from Lab-Line Inc.) until turbidity had cleared.

*Fluorescence Studies.* Fluorescence measurements were carried out on a Shimadzu RF-540 spectrofluorophotometer, using a 1-cm light path cell with slits of excitation and emission of 5 nm. Fluorescence was monitored immediately following addition of ethidium bromide to the various DNA–liposome mixtures ($\lambda_{ex}$ = 260 nm, $\lambda_{em}$ = 600 nm) using a 395-nm filter. Measurements were conducted in 20 mM NaCl solution.

*Lipid Mixing.* Lipid mixing was determined by using the resonance energy transfer (RET) methodology (Struck et al., 1981). Fluorescently labeled PE/DOTMA (1:1, in molar ratio) vesicles, containing 1 mol % each of *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (energy donor, $\lambda_{ex}$ = 467 nm and $\lambda_{em}$ = 534 nm) and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (energy acceptor, $\lambda_{ex}$ = 560 nm and $\lambda_{em}$ = 585 nm) (*N*-NBD-PE and *N*-Rh-PE, respectively), were prepared by the same procedure as the nonlabeled liposomes. Lipid mixing determinations were conducted on mixtures of 1:9 labeled and nonlabeled liposomes by following the changes in fluorescence intensity at 530 nm ($\lambda_{ex}$ = 467 nm) and using 2-nm filter slits to reduce light scattering interference. Total fluorescence (equivalent to minimal energy transfer or dequenching) was obtained by solubilizing the liposomes with 0.2% Triton X-100 and correcting for the quenching effect of Triton X-100, i.e., ×1.5 (Beigel et al., 1988). Zero percent lipid mixing (background fluorescence) was taken as the fluorescence intensity of the liposome mixtures in the absence of DNA.

*Electron Microscopy Studies.* Samples for electron microscopy were prepared by the Kleinschmidt method of DNA spreading followed by metal rotary-shadowing (Kleinschmidt et al., 1959). Aliquots of 10 μL containing DNA (at 35 μg/mL) and liposome mixtures were made up to 100 μL with 0.5 M ammonium acetate, 0.1 M Tris (pH 8.0), and 2.5 mM EDTA. To this solution was added 10 μL of cytochrome *c* (2.5 mg/mL), and samples were spread on the surface of a solution of the above buffer diluted in a ratio of 1:20. The resulting monolayers were lifted onto parlodion-coated grids which were stained for 1 min in ethanolic uranyl acetate, washed, and blotted dry. Grids were subsequently rotary-shadowed at an angle of 8° with platinum:palladium (80:20) and visualized in a Phillips 410 electron microscope operated at 80 kV.

*Agarose Gel Electrophoresis.* (*a*) *DNA–Liposome Samples.* Samples containing 2.0 μg of calf thymus DNA and liposomes in various liposome-to-DNA ratios were incubated at room temperature for 1 h and loaded on a 1.5% agarose gel. Samples were run at 3.5 V/cm with TBE buffer and stained by exposure to 10 μg/mL ethidium bromide.

(*b*) *Samples Digested with the Nuclease DNase I.* Samples containing 6.0 μg of DNA (500–2000 base pairs) and liposomes in various liposome-to-DNA ratios were incubated at room temperature for 1 h in a buffer containing 100 mM Tris (pH 7.5) and 1.0 mM $MnCl_2$. Each mixture (corresponding to a given liposome-to-DNA ratio) was divided into two samples, and 20 units of DNase I was added to one of the two samples. The mixtures were incubated for 1 min at room temperature and then quenched with phenol (added to all samples). Phenol was extracted with 2 × 400 μL of chloroform/isoamyl alcohol (24:1) solution, which also solubilizes the liposomes and extracts the lipids. Following ethanol precipitation, all samples were loaded on a 1.5% agarose gel.

## RESULTS

*Fluorescence Studies.* The fluorescence experiments were performed by exposing DNA molecules to positively-charged liposomes, followed by addition of ethidium bromide, which—upon intercalation in between the DNA base pairs—acts as a fluorescence probe. The fluorescence properties which are revealed by a given concentration of DNA segments exposed to increasing amounts of cationic liposomes (DOTMA/PE, 1:1, Felgner et al., 1987) are presented in Figure 1A as function of both the DNA length and the positive to negative charge ratios (in terms of DOTMA to nucleotide molarities). The fluorescence intensity is not affected by increasing concentrations of the cationic liposomes until a specific liposome-to-DNA ratio is reached, upon which a large and very sharp decrease of the intensity is observed. The specific liposome-to-DNA ratio at which the fluorescence quenching occurs, corresponding to a positive to negative

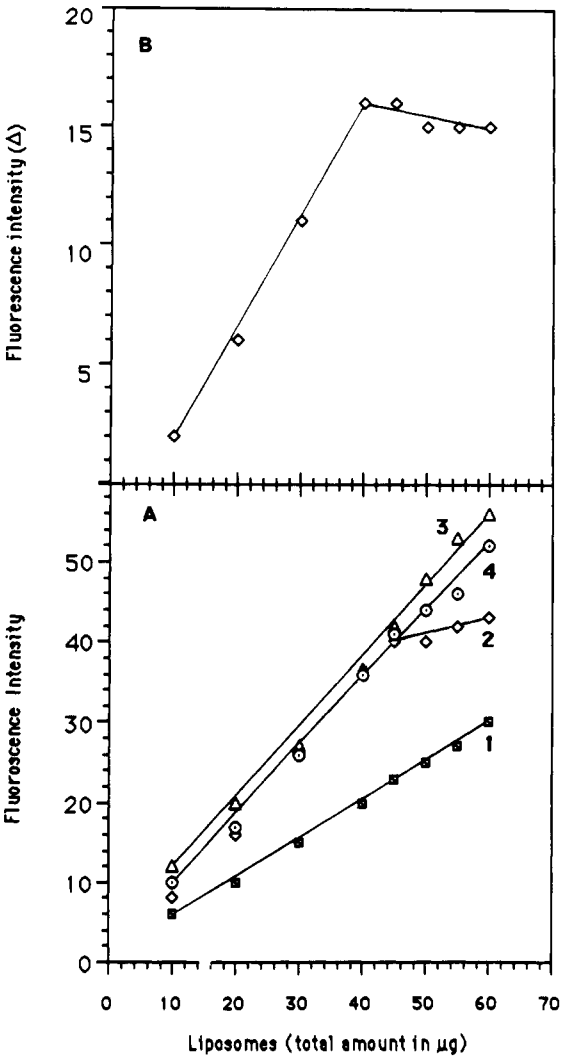


FIGURE 3: Lipid mixing of labeled vesicles induced by anionic polymers. (A) (1) Background fluorescence of labeled liposomes; (2) exposure to DNA ($2.5 \times 10^{-5}$ M, in nucleotides); (3) exposure to Triton X-100 (0.2%, w/v); (4) exposure to poly-Glu ($2.5 \times 10^{-5}$ M in amino acid residues). (B) Specific effects of the DNA obtained by subtracting the background fluorescence from the fluorescence values exhibited in the presence of DNA. Experimental details are as described under Materials and Methods.

charge ratio of 1.1, is found to be independent of DNA size, in the range of 100–23 000 base-pairs (Figure 1A). Neither is this value affected by the *absolute* concentrations of DNA and liposomes: a decrease of the concentrations of both substances by a factor of 5, or an increase by a factor of 2 relative to the concentrations shown in Figure 1A, did not modify the step-function shape of the fluorescence curves (data not shown).

The notion that the properties of the liposome–DNA system crucially depend upon the positive to negative charge ratios is supported by the fluorescence characteristics exhibited by DNA segments which are exposed to liposomes that are composed *only* of the positively charged lipid DOTMA. In this case, precisely half the amount of lipid molecules is required to affect the fluorescence quenching (Figure 1B). Notably, this amount corresponds to the *same positive to negative charge ratio* that is exhibited by the PE/DOTMA mixed-lipid liposomes at the fluorescence quenching point. Kinetic fluorescence measurements of DNA–liposome interactions were performed by addition of ethidium bromide to DNA–liposome mixtures of various charge ratios, following different incubation times. At low positive to negative charge ratios, namely, those exhibiting large fluorescence values, no time dependence is observed, and the fluorescence remains high and constant. At high liposome-to-DNA ratios, characterized by low fluorescence values, the quenching of the fluorescence intensity to a background level is found to be very fast and to occur within few seconds (Figure 1C). At intermediate ratios of liposomes-to-DNA, corresponding to the value at which the fluorescence decrease is affected, a clear fluctuation of the fluorescence intensity as a function of time is observed, culminating in background fluorescence values (Figure 1D). Fluorescence measurements of a closed-circular-supercoiled plasmid incubated with the cationic liposomes exhibited very similar fluorescence parameters.

The effects of positively charged species upon the fluorescence properties of the liposome–DNA complexes were studied by including lysine or poly(L-lysine) (poly-Lys) in the vesicle–DNA systems. The presence of poly-Lys—but not of the monomeric lysines—results in a shift of the liposome-to-DNA ratio at which the fluorescence quenching is obtained toward lower values: as the concentration of poly-Lys is gradually increased, lower amounts of liposomes are required to affect the quenching (Figure 2A). Significantly, the value of overall positive (DOTMA + poly-Lys) to negative (DNA) charge ratio at which the fluorescence decrease occurs is found to be constant and equal to that observed in the absence of poly-Lys. Thus, in order to induce fluorescence quenching, the increase in the concentration of positive charges supplied by poly-Lys equals and compensates for a decrease in the positive charges derived from the liposomes, as clearly indicated in Figure 2B. In the presence of liposomes, the fluorescence of DNA–ethidium bromide complexes is found to be unaffected by poly-Lys, thus indicating that the observed effects of the polypeptide are not associated with its mere interference with the fluorescence properties.

*Lipid Mixing Experiments.* Lipid mixing experiments, which enable assessment of the occurrence of liposome fusion processes and differentiation of such processes from mere aggregation, were conducted in order to evaluate the effects exerted on the cationic vesicles by the DNA molecules. The methodology is based on the resonance energy transfer which occurs between two fluorescence probes that are in close physical proximity if the emission band of the energy donor overlaps the excitation band of the energy acceptor. Dilution of the probes due to membrane fusion or to solubilization of the membranes results in dequenching of the donor emission band, being the monitored parameter (Struck et al., 1981; Beigel et al., 1988). The fluorescence exhibited by *N*-NBD-PE- and *N*-Rh-PE-labeled liposomes, mixed at a 1:9 ratio with nonlabeled liposomes to final vesicle concentrations identical to those used in the above described experiments, was initially studied in the absence of DNA (Figure 3A, curve 1). The values thus obtained, which did not change after an incubation of 6 h, serve as background fluorescence and correspond to maximal quenching of the donor emission. A given amount of DNA molecules was added to a set containing increasing concentrations of labeled liposome mixtures. A substantial increase in the fluorescence intensities at 530 nm (i.e., fluorescence dequenching) relative to the background fluorescence values obtained in the absence of DNA is noted (Figure 3A, curve 2), indicating the occurrence of fusion processes. The DNA-induced membrane fusion is found to be fast, occurring within 1 min after addition of the DNA molecules. Notably, the increase of the fluorescence intensity as a function of increasing ratios of liposome-to-DNA is not linear. As shown in Figure 3B, in which the difference between

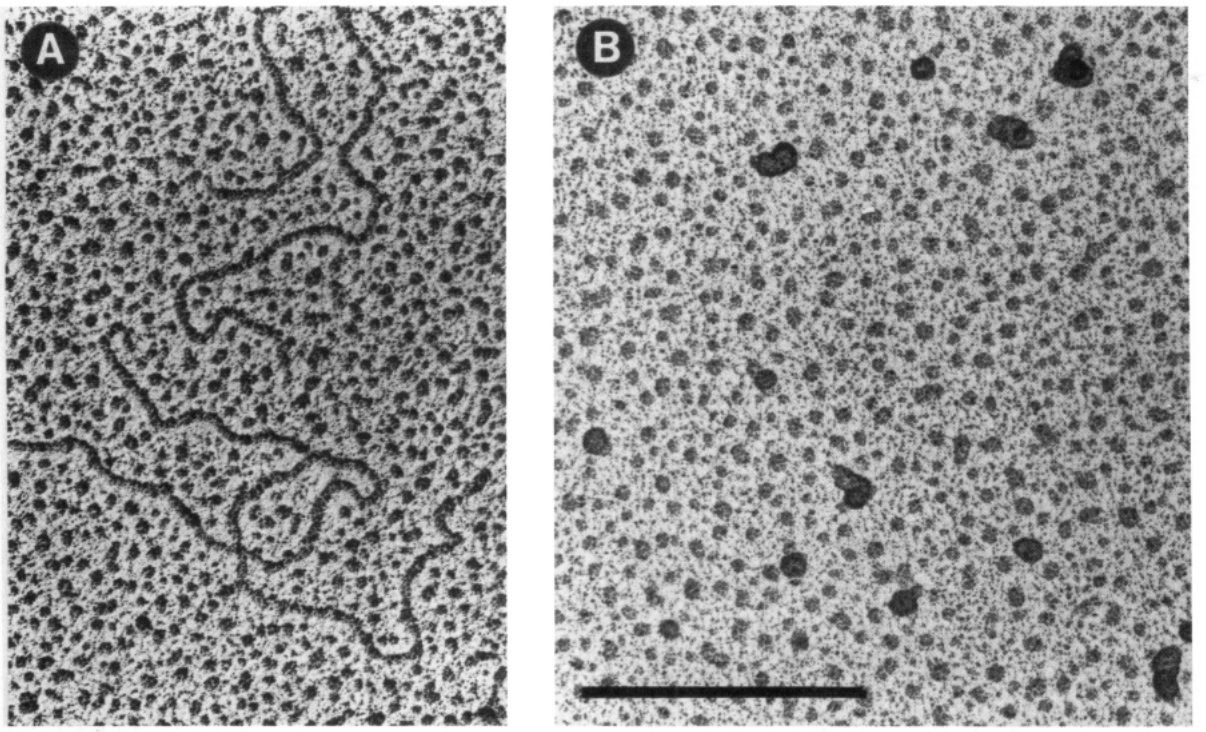


FIGURE 4: Electron microscopy of metal-shadowed samples of (A) DNA molecules (3.5 μg/mL) and (B) PE/DOTMA liposomes. Spreading and shadowing procedures are detailed under Materials and Methods. It should be noted that visualization of the DNA and liposomes by the Kleinschmidt methodology does not allow a size comparison of these species, since DNA molecules are detected as cytochrome *c*–DNA complexes. Scale bar represents 0.5 μm.

the fluorescence of the labeled liposome mixture in the presence and absence of DNA is plotted, a clear change in the slope occurs at the specific ratio of liposome-to-DNA which has been found to affect the sharp fluorescence quenching in the liposome–DNA–ethidium bromide complexes (i.e., positive to negative charge ratio of 1.1). Total fluorescence dequenching values (Figure 3A, curve 3), corresponding to total lipid mixing, were obtained by exposing the labeled–unlabeled liposome mixtures to the liposome-solubilizing agent Triton X-100 (Beigel et al., 1988).

Lipid mixing processes induced by poly-Glu were studied by exposing the labeled liposome mixtures to the negatively-charged polypeptide at the same positive to negative charge ratios (DOTMA to amino acid molarities) used in the liposome–DNA experiments. As indicated in Figure 3A, curve 4, the presence of poly-Glu results in a fluorescence dequenching that is virtually identical to that affected by Triton X-100. Specifically, no change in the slope of the curve describing the fluorescence dequenching is observed, in clear contrast to the effect exerted by the DNA molecules.

DNA–liposome complexes were probed by agarose gel electrophoresis (results not shown). In samples prepared at low liposome-to-DNA ratios, all the DNA loaded on the gel could be detected following exposure to ethidium bromide, whereas no ethidium bromide staining of DNA was observed in mixtures containing high liposome-to-DNA ratios. Thus, both fluorescence and gel studies of the DNA–liposome complexes point toward the existence of a specific, critical ratio of vesicles to DNA below which the nucleic acids are fully accessible to the fluorescence probe, whereas above it they are completely sequestered. Notably, both techniques indicate that the efficiency of DNA encapsulation is independent of its length. The susceptibility of the DNA molecules *within* the DNA–liposome complexes toward enzymatic degradation was probed by exposing these species, obtained at various liposome-to-DNA ratios, to the nuclease DNase I. Complete DNA cleavage is observed in samples containing low liposome-to-DNA ratios whereas total protection of the DNA molecules against the nuclease activity is obtained above the critical ratio.

*Electron Microscopy Studies.* The shape of the DNA–liposome complexes, formed at various liposome-to-DNA ratios, was studied by using the Kleinschmidt method for DNA spreading (Kleinschmidt et al., 1959). Spread and metal-shadowed DNA molecules (500–2000 base pairs), as well as uncomplexed liposomes, are shown in Figure 4. As liposomes are added in increasing amounts to a given concentration of DNA, gradually increasing regions along the DNA molecules become covered by liposome aggregates (Figure 5). At a 1:1 liposome-to-DNA ratio, approximately half of the DNA molecules are detected as liposome-bound species, and upon a very slight further increase of this ratio, almost all the DNA appears to be covered. It should be noted that whereas at low liposome-to-DNA ratios the DNA-bound liposomes exhibit, in most cases, distinct, roughly spherical shapes, at high ratios the DNA–liposome complexes are detected as smooth rodlike structures. Very similar patterns are obtained upon exposing closed-supercoiled plasmid DNA to increasing liposome concentrations. DNA–poly-Lys complexes (1:1 molar ratio in terms of nucleotides and amino acids) exposed to increasing amounts of liposomes are shown in Figure 6. DNA–poly-Lys samples devoid of liposomes reveal structures similar to those characterizing the "naked" DNA species. Addition of small amounts of liposomes leads, initially, to the formation of "spider-like" forms (Figure 6A), in which the thicker regions correspond to DNA–poly-Lys species covered by liposomes and the thin coils represent unbound, "free" DNA. As the liposome-to-DNA ratio is increased, the thick segments are elongated while the thin regions progressively disappear (Figure 6B,C).

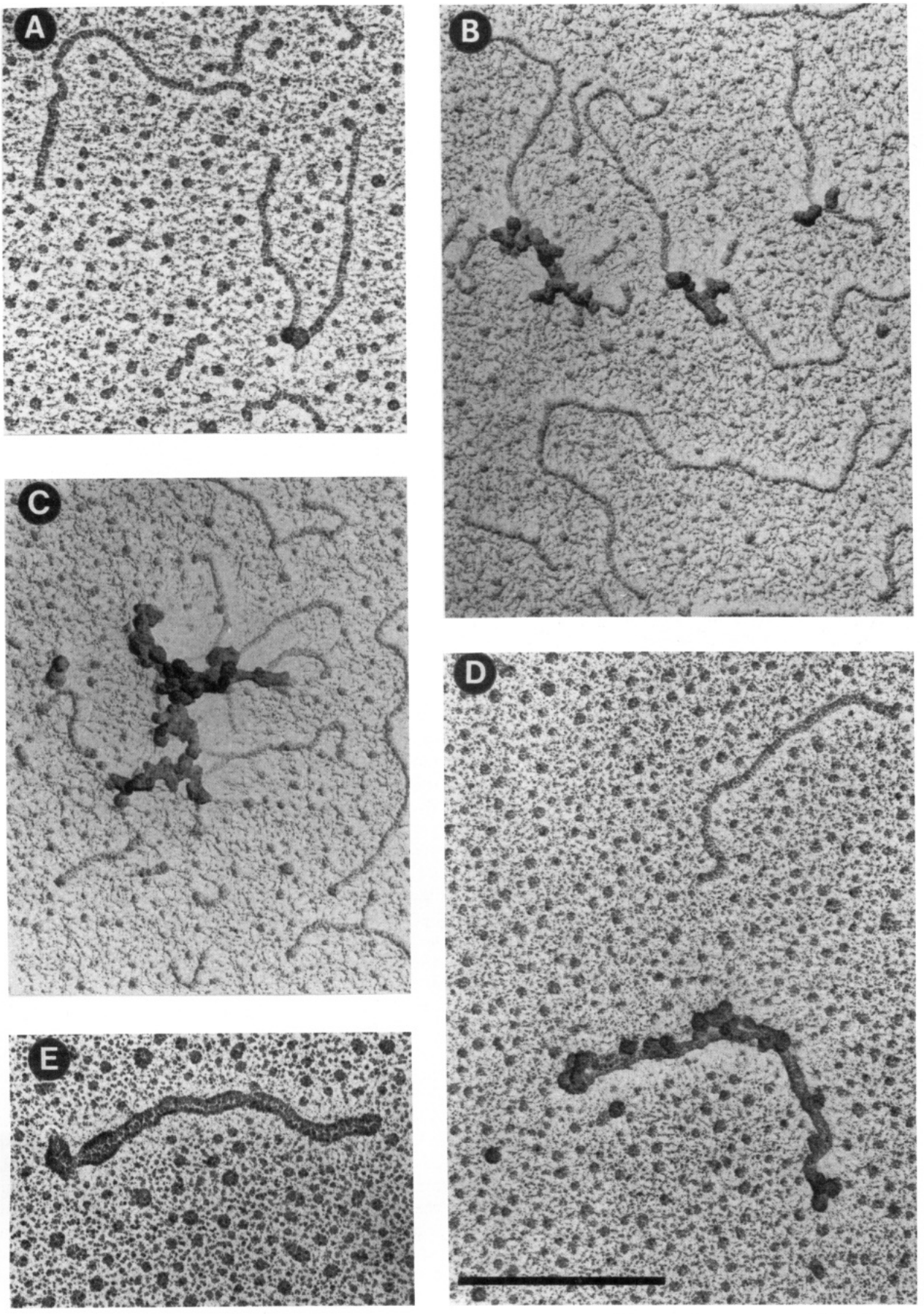


FIGURE 5: Electron microscopy of DNA–liposome complexes. (A–E) Complexes prepared from a constant amount of DNA (3.5 μg/mL) and a gradually increasing amount of cationic liposomes. Liposome-to-DNA ratios (in terms of positive to negative charges) are (A) 0.2, (B) 0.4, (C) 0.6, (D) 1.0, and (E) 1.5. Note the aggregated (B–D) versus fused (E) complexes. Scale bar represents 0.5 μm.

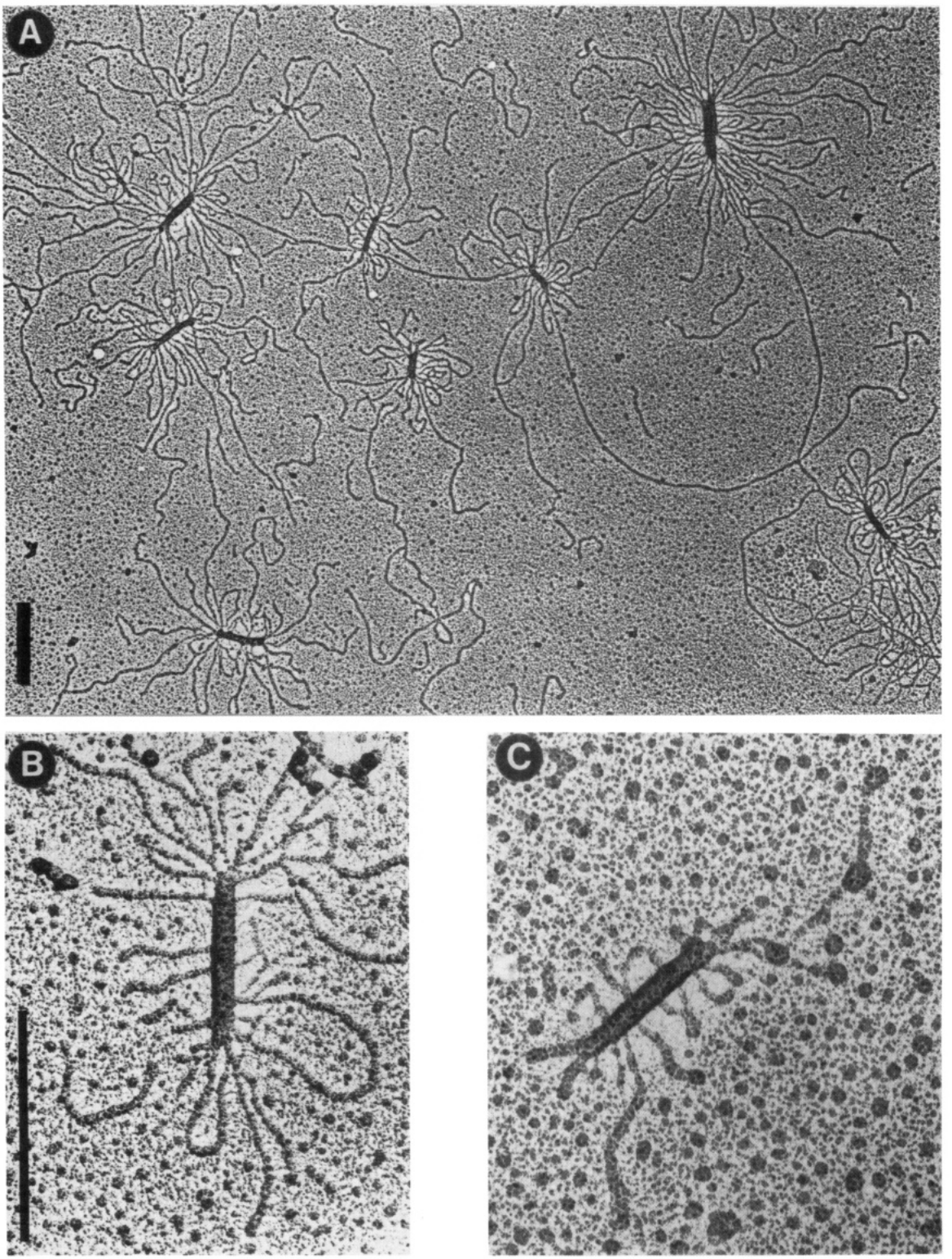


FIGURE 6: DNA–poly-Lys–liposome complexes. (A–C) Complexes prepared from constant amounts of DNA and poly-Lys (3.5 μg/mL) and a gradually increasing amount of liposomes. Liposome-to-DNA ratios (in terms of positive to negative charges) are (A) 0.2, (B) 0.4, and (C) 0.6. Scale bar represents 0.5 μm.

## DISCUSSION

Multivalent anions such as citrate or phosphate, as well as negatively-charged polymers such as polyaspartate or even DNA, have been found to induce fusion of positively-charged liposomes (Beigel et al., 1988; Keren-Zur et al., 1989; Düzgünes et al., 1989). On the basis of these findings and on the above-described lipid mixing and electron microscopy experiments, DNA–liposome complexation is suggested to be associated with DNA-induced lipid fusion processes. The conspicuous independence of DNA encapsulation upon its length, indicated by both the fluorescence and gel electrophoresis results, supports this assumption: mere DNA entrapment within the limited space of a single cationic SUV would become progressively inefficient as the length of the DNA molecules increases.

The lipid mixing results taken on their own do not provide an interpretation for the highly cooperative mode of DNA–liposome interactions that is indicated by the very sharp

fluorescence quenching observed in the fluorescence and electrophoresis experiments. Notably, such a cooperative process is inconsistent with the currently prevailing model for DNA–liposome interactions according to which the DNA is being progressively surrounded by cationic liposomes—which maintain their original structure—as their concentration is increased. The lipid mixing results point, however, toward a different effect of DNA and poly-Glu upon membrane fusion, as the process affected by the polypeptide is continuous (compare curves 2 and 4 in Figure 3A). The difference may be attributed to a DNA structural phase modulation which occurs precisely at the liposome-to-DNA ratio leading to the sharp fluorescence quenching. It is well established that at a given degree of charge neutralization, DNA molecules collapse into packed forms in a highly cooperative process (Manning, 1980, 1981). Since charge neutralization might be affected by positively-charged vesicles, we claim that liposome-induced cooperative DNA collapse is a key event in the course of DNA–cationic vesicle complexation. The observation that the lipid concentration required to affect DNA encapsulation with liposomes composed only of the positively-charged DOTMA is precisely half that of DOTMA/PE liposomes (Figure 1B) buttresses the notion of liposome-induced DNA collapse induced at a critical positive charge density, since the different liposome concentrations of the two types of vesicles correspond to an identical charge density.

The collapsed DNA structures, whose exposed surface is substantially smaller than that of the fully extended forms, undergo particularly efficient encapsulation by the lipid bilayer. Thus, a very slight increase of the liposome concentration above the critical value required for DNA collapse is sufficient to complete the encapsulation in a fast process which is indicated by the kinetic fluorescence experiments conducted at high liposome-to-DNA ratios (Figure 1C,D). The time-dependent fluctuations of the fluorescence intensities observed at intermediate charge ratios can be attributed to the large sensitivity of DNA collapse processes to minor changes in the environmental conditions which characterize the initiation of these processes (Reich et al., 1991), and may therefore lead to an equilibrium between encapsulated and free DNA regions.

The suggested pathway for DNA–liposome interactions is corroborated by the electron microscopy observations (Figure 5). At low ratios of liposomes-to-DNA, the liposomes appear as clusters bound to the DNA molecules in which the distinct, spherical shape of the vesicles can still be discerned. The short, rodlike structures observed above the critical ratio are proposed to reflect complexes in which DNA molecules are packed (and hence the short, rodlike appearance which usually characterizes condensed DNA phases) and completely encapsulated within a smooth lipid bilayer. Moreover, at high liposome-to-DNA ratios, the overall number of rodlike shapes detected on the grid is substantially lower than the number of unbound DNA molecules, indicating that these structures represent a complex in which *several* DNA molecules are packed together within the lipid bilayer.

At significant observation concerned with the relation between DNA collapse and its encapsulation is related to the effects exerted by poly-Lys. Specifically, the overall positive charge density (poly-Lys + liposomes) required for DNA encapsulation is found to remain identical to the critical charge density which affects this process and provided *only* by the liposomes. Poly-Lys has been shown to induce DNA packaging processes (Shapiro et al., 1969; Carrol, 1972), and its presence in DNA/liposome mixtures results in the formation of packed DNA regions which facilitate DNA encapsulation. Indeed, in electron microscopy samples of DNA/liposome/poly-Lys mixtures, the formation of packed, lipid-covered regions can be observed already at low liposome-to-DNA ratios. A slight increase of the liposome concentrations results in complete encapsulation of the noncomplexed DNA segments that stem out from the packed regions (Figure 6). An additional indication of the relation between DNA collapse and its encapsulation is provided by the observation that the encapsulation pathway is determined by the overall positive to negative charge ratio—and not by the absolute concentration of the nucleic acids. It has indeed been shown that DNA packaging is not affected by its concentration over a rather broad range (Huey & Mohr, 1981) but, in the case of poly-Lys-induced packaging, by the ratio between the two polymers (Carroll, 1972).

On the basis of these observations and considerations, the following model for cationic liposome-DNA complexation is proposed. At low ratios of liposomes-to-DNA, positive vesicles are adsorbed to the nucleic acids to form aggregates that gradually surround larger segments of the DNA. As the amount of liposomes is increased, the aggregated liposomes along the DNA reach critical concentrations and charge densities at which membrane fusion and cooperative DNA collapse processes are initiated. Following an additional increase of the liposome concentration, the collapsed DNA structures are efficiently and completely covered by the lipid bilayers. A significant tenet of the proposed model concerns the mutual effects exerted by the DNA molecules and the cationic liposomes. DNA molecules induce aggregation and fusion of vesicles, and the resulting positively-charged fused lipid bilayers enable cooperative DNA packaging in a manner similar to that revealed by cationic polymers such as poly-Lys. The packed phases, in turn, facilitate and enhance the encapsulation processes of the DNA by the bilayers. Thus, DNA packaging processes, membrane fusion, and nucleic acid encapsulation represent causally related key events in the formation of DNA–liposome complexes.

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