J. Membrane Biol. 200, 35–45 (2004)
DOI: 10.1007/s00232-004-0689-4

The Journal of
Membrane
Biology

Mechanisms of Lipoplex Formation: Dependence of the Biological Properties of Transfection Complexes on Formulation Procedures

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Received: 20 January 2004/Revised: 12 May 2004

Abstract. Phospholipid-DNA complexes were made of the cationic triester derivative of phosphatidylcholine, EDOPC (1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine), by varying conditions of complex formation, in particular, the rate and direction of mixing, as well as by changing the mode of dispersing the lipid (extrusion or vortexing). The biological effects of variations in the formulation procedure were assessed by measuring transfection activity and cell association in cultures of BHK cells. Formulation procedures generally had little effect on cell association, but had marked effects on transfection efficiency. Transfection varied from effectively nil to extremely efficient with what appeared to be modest changes in formulation procedure. Formulation procedures also had significant effects on average sizes and size distributions of lipoplexes as determined by dynamic light scattering. Among the four possibilities of rapid or slow mixing combined with the two possible directions of mixing, slow addition of DNA to lipid gave results that differed significantly from the other three modes. In the case of vortexed lipid, the latter procedure was much less satisfactory than the other three, whereas in the case of extruded lipid, it was the only mode that produced satisfactory transfection. The factors that determine the difference in lipoplex properties can be identified as both geometric and physical. The geometric factor has to do with the symmetries of the participating units. There are three physical factors that are critical: the difference in vesicle stability upon interaction with DNA, the time dependence of interdiffusion of the components relative to that of vesicle rupture, and difference in input concentrations. These factors determine lipoplex size and, as already also shown by others, lipoplex size influences transfection efficiency.

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Key words: EDOPC — Lipoplex — Gene delivery — Cationic phospholipid — Non-viral vector

Introduction

In recent years, vector development for gene therapy has been concentrated on non-viral vectors as safer and capable of carrying larger gene inserts but less efficient than viruses. Cationic lipids, which readily form complexes (lipoplexes) with negatively charged DNA [10, 12, 18, 35], have become the most widely used non-viral vectors [17].

The interaction between DNA and cationic liposomes to form lipoplexes is incompletely understood. The lipids can condense DNA into chemically and physically diverse aggregates, the structure and/or transfection activity of which depends on the nature of the lipid, the method of lipid dispersion, the strength of the buffers and the method of mixing [10, 35, 40, 42]. Although macroscopically, a variety of structures have been described, the microscopic structure, as revealed by X-ray diffraction, most commonly found is a multilamellar array of DNA strands intercalated between lipid bilayers [4, 6, 16, 26]. Hexagonal arrays [4, 15, 28] and bilayer-coated strands [35] have also been observed.

It has become clear that lipid-DNA aggregates can be very heterogeneous in size [11, 32, 39]. The effect of size on transfection efficiency of lipid-DNA complexes has been investigated in several laboratories with divergent results. Some investigators have reported that lipoplex size is not related to transfection activity [33, 38]. Others have found that smaller lipoplexes were superior to larger particles [1, 36]. Recently, evidence has tended to favor the conclusion that larger lipid-DNA complexes are more effective transfection agents [9, 22, 30, 37, 41, 44]. Clearly, differences in lipid structure and formulation procedures could account for different observations

on size optima. An additional uncertainty arises because most size measurements depend upon averages derived from dynamic light scattering and this procedure becomes highly approximate when heterogeneous populations are involved.

Divergent observations have also been reported on the relationship between cell adhesion of transfection complexes and their actual transfection efficiency. A clear relationship between size, adhesion and transfection was observed when lipoplex size was controlled by arresting aggregation [30]. Furthermore, others have suggested that the low transfection observed with small particles [22] or with complexes prepared from sonicated lipids, are due to reduced transport of the smaller complexes from the medium to the cell surface [9]. On the other hand, some investigators have reported no relationship between uptake and transfection efficiency [8]. The presence of serum can have differential effects on adhesion and transfection [8, 24]. Thus, differences in procedures may account for lack of agreement that uptake is a good indication of efficient transfection.

We have previously investigated the mechanism of lipid-DNA complex formation between DNA and alkyl triesters of phosphatidylcholine [14, 19, 28]. Many of these lipid derivatives form typical bilayer membranes and most of them are efficient transfection agents when optimally formulated with DNA [21, 29]. These cationic amphipaths are structurally very closely related to natural phospholipids (in the case of the derivative used here, the only difference from phosphatidylcholine is an ethyl group) and they exhibit low in vitro toxicity [21]. We observed that the formation of EDOPC-DNA complexes can be controlled by the conditions of combination of lipid and DNA such that particles of different sizes can easily be prepared [14]. Here we report on the extension of those studies to include transfection and adhesion of complexes to cells. We explored how different degrees of lipid dispersion and different modes of mixing DNA and lipid influenced transfection efficiency. We also determined the size of lipoplexes prepared in different ways. The formulation procedure was found to have enormous effects on transfection efficiency, most of which could be ascribed to size effects, and which, in turn, could be rationalized in terms of what is known about the physical chemistry of the DNA-lipid interactions.

Materials and Methods

Materials

1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine triflate salt (EDOPC) was synthesized by reacting dioleoylphosphatidylcholine with ethyl trifluoromethylsulfonate in chloroform [21]. It is available from Avanti Polar Lipids (Alabaster, AL) as the somewhat less effective chloride salt. The bright and photobleach-resistant fluorophore, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphate, diammonium salt (BODIPY-HPA), is a phosphatidic acid containing a BODIPY-labeled fatty acid and was purchased from Molecular Probes (Eugene, OR). N-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (RhPE) was purchased from Avanti. Lipids were stored at −20°C in chloroform. A plasmid with the β-galactosidase gene (pCMV-β-gal; Life Technologies, Rockville, MD) was propagated in DH5 cells and purified with a Plasmid Mega Kit from Qiagen (Valencia, CA). Tissue culture reagents were from Life Technologies (Rockville, MD). BHK cells (CCL-10) were obtained from American Type Culture Collection (Rockville, MD).

Preparation of Lipid-DNA Complexes

Aliquots of EDOPC dissolved in chloroform were transferred to vials where the bulk of the solvent was evaporated with a gentle stream of argon gas. Then the vial was placed under a high vacuum for at least 30 min per mg of lipid to remove residual chloroform. Next, Dulbecco's phosphate-buffered saline (D-PBS; with calcium and magnesium) was added to produce either a 0.3 mg/ml or 1 mg/ml concentration and the vials were gently vortexed. According to dynamic light scattering determinations previously reported [19], vortexed preparations consisted predominantly of a 150 nm diameter population (1/3 of the scattering intensity) and an 800 nm population (2/3 of the scattering intensity). To generate 100 nm diameter extruded EDOPC vesicles, the vortexed dispersion was processed using a small-volume extrusion apparatus (Avanti Polar Lipids) as described [20], except that a single filter was used. At the concentrations used, the fluorescence of labeled EDOPC was the same after extrusion as before, verifying that extrusion did not change the EDOPC concentration. In this regard caution is advised, for significant proportions of cationic lipid preparations can adsorb to extrusion filters if the lipid is at very low concentrations (micromolar). Plasmid DNA was diluted in D-PBS to 0.33 mg/ml or 0.1 mg/ml.

EDOPC-DNA complexes were all prepared at a lipid: DNA mass ratio of 3:1 and a molar ratio of 1.03:1 (i.e., a 3% overall positive charge excess) in a total volume of 130 μl, using four different procedures (*see* also Table 1, column 1):

A. 30 μl of a EDOPC dispersion (1 mg/ml) was rapidly (< 1 sec) added to 100 μl of DNA solution (0.1 mg/ml).

B. 30 μl of a EDOPC dispersion (1 mg/ml) was added to a 100 μl of a magnetically stirred DNA solution (0.1 mg/ml) at a rate of approximately 1 μl/sec.

C. 30 μl of a DNA solution (0.33 mg/ml) was rapidly (< 1 sec) added to 100 μl of a EDOPC dispersion (0.3 mg/ml).

D. 30 μl of a DNA solution (0.33 mg/ml) was added to 100 μl of a magnetically stirred EDOPC dispersion (0.3 mg/ml) at a rate of approximately 1 μl/sec.

These methods of lipid-DNA complex formation were used with vortexed (preparations 1A-1D) as well as with extruded EDOPC (preparations 2A-2D). After mixing EDOPC and DNA, the complexes were incubated 20 min at room temperature before being applied to the cells.

DNA concentrations were determined by measuring absorption at 260 nm. The concentration of phospholipid in stock chloroform solution was determined by a standard phosphate assay [3].

Cell Transfection and Fluorescence Microscopy

For transfection and adhesion experiments, BHK cells were cultured in Glasgow minimum essential medium (GMEM) without phenol red, supplemented with 10% fetal bovine serum, 2% tryp-

Table 1. The size for different preparations of EDOPC-DNA complex

Complex preparation protocol	Identification	Difference in mean diameter calculated from cumulants and NNLS and mean values of each	
		% difference	Mean values (NNLS, Cum.)
EDOPC → DNA, quickly	1A	−4	436, 416
EDOPC → DNA, slow	1B	0	480, 480
DNA → EDOPC, quickly	1C	−4	639, 616
DNA → EDOPC, slow	1D	+41	409, 576
Extruded EDOPC → DNA, quickly	2A	+3	209, 224
Extruded EDOPC → DNA, slow	2B	−6	235, 227
DNA → extruded EDOPC, quickly	2C	−1	229, 227
DNA → extruded EDOPC, slow	2D	−7	432, 402

tose phosphate and 50 mg/l Gentamycin. The cells, in 96-well plates, were seeded in 100 μl of medium at densities to give approximately 80% confluence at the time of addition of lipid-DNA complex. Complete medium was replaced with serum-free medium and EDOPC-DNA complex, containing 3 μg of cationic lipid and 1 μg of DNA in volume of 13 μl of D-PBS, was pipetted into each well. After 4 hours of incubation at 37°C, 1/10 volume of fetal bovine serum was added to the cells. Replicates of three were used in each experiment.

Cells were tested for β-galactosidase activity 20 hours after transfection using a microplate fluorimetric assay with the substrate fluorescein digalactoside [27].

For fluorescence microscopy, BHK cells were transfected with the various preparations of the RhPE-EDOPC-DNA complex, then the cells were washed, fixed and photographed under an epifluorescence microscope using filters appropriate for rhodamine.

Measurement of Lipoplex Association with Cells

EDOPC was labeled with 0.05 mol% of fluorescent BODIPY-HPA and complexed with plasmid DNA as described above. BHK cells were treated with EDOPC-DNA complexes under the same conditions as used for transfection. For these experiments, replicates of eight were initially prepared. The cells of four replicates were lysed immediately after applying the EDOPC-DNA complex. The cells from the other four replicates were lysed four hours after addition of EDOPC-DNA complex. In each case we treated the medium separately from the cells (one pair of duplicates), and also as cell and medium together (second pair of duplicates). The former pair was used to establish the amount of fluorescence associated with the cells or with the medium and the latter was used to verify that the total fluorescence was indeed equal to the sum of that from cells and medium and that fluorescence was not lost through the cell washing procedure. The whole-well sample was treated by addition of 200 μl of 2% Triton in D-PBS. For lysis of adherent cells, the medium was aspirated and cells were washed by D-PBS, then placed in 100 μl of GMEM without phenol red or supplements and mixed with 200 μl of 2% Triton in D-PBS. Aspirated medium from cell samples was added to 200 μl of 2% Triton in D-PBS. Thus, all samples, the whole-well samples, cell samples, and medium samples, were at the same volume of 300 μl, containing 100 μl of GMEM (lacking phenol red and serum) and 200 μl of lysis buffer. Fluorescence was measured on AlphaScan spectrofluorometer (PTI, Princeton, NJ). Samples were excited at 495 nm and fluorescence intensity was measured at 520 nm with bandwidths of 4 nm for both excitation and emission.

Size Characterization of Cationic Phospholipid Vesicles and Their Complexes with DNA by Dynamic Light Scattering

Dynamic light scattering measurements were done with a Brookhaven Instruments BI-200SM goniometer and a BI-9000 digital correlator (Brookhaven, NY). A 3-W argon laser (Lexel 95; Lexel Laser, Fremont, CA) was the source of 514 nm wavelength light.

Lipoplex preparations used for size determinations were the same as those used for the transfection experiments of Fig. 1., i.e., the preparations were the same for both sets of experiments and only manipulations specific to transfection or sizing were different. Dilutions for light scattering were such that the final sample volume of 200 μl (D-PBS) contained 10 μg lipid. Measurements were made in 5 × 60 mm borosilicate glass culture tubes.

The NNLS (non-negatively least squares) algorithm provided with the instrument was used to obtain particle size distributions. In order to reduce artifacts, up to 20 correlation curves were obtained for each sample (data collection time was 5 min per curve) and the corresponding distribution functions were averaged. The mean diameters were then calculated from each of the averaged distributions. (It should be emphasized that the distributions obtained by this procedure are *intensity* distributions. Since the size range represented by our samples is that in which Rayleigh and Mie scattering overlap and because we do not know the structure of all the contributing particles, we cannot determine what the distribution of particle number or particle mass is. Furthermore, is must be understood that scattering methods, when applied to heterogeneous samples, are approximate methods. Given the care we have taken to average large numbers of measurements, we believe that our results are about as accurate as can be expected from the method, but we emphasize that the means and especially the distributions are approximations of the true properties of the population). Mean particle sizes were also determined from the cumulants method. The latter includes contributions from particles too large to be dealt with by the NNLS method, so the presence of very large (several microns) particles is indicated by a difference in the mean diameter calculated by the two procedures.

Measurement of the Integrity of Vesicles after Their Interaction with DNA

Rupture of vesicles due to interaction with DNA was determined by measuring the extent of release of entrapped terbium dipicolinate into an external phase containing ethylenediamine tetraacetic acid (EDTA). When such vesicles break, the terbium dipicolinate,

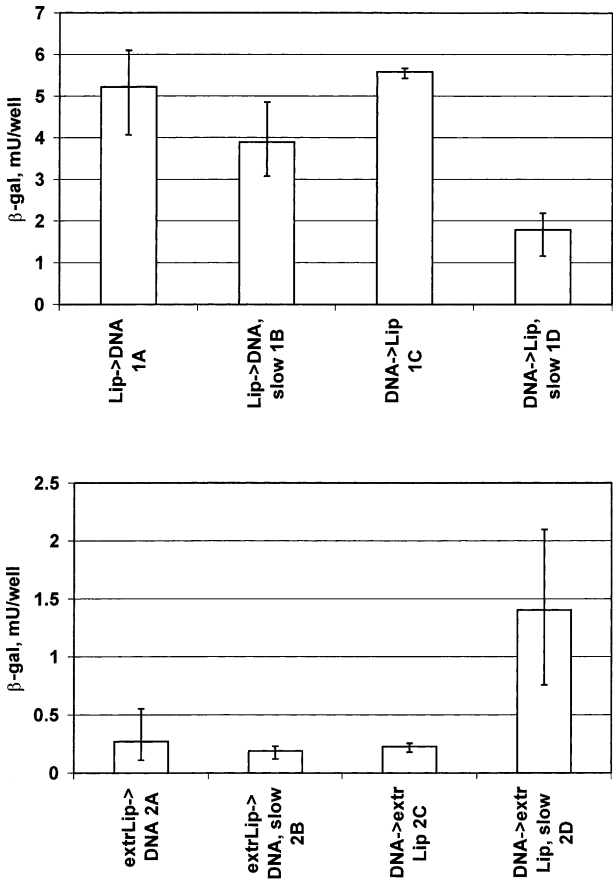

Fig. 1. Transfection efficiency of different formulations of EDOPC-DNA complexes. BHK cells were transfected with lipid-DNA complexes prepared from vortexed EDOPC (*top*) and from extruded EDOPC (*bottom*). Error ranges indicate the highest and the lowest values of replicates.

which is highly fluorescent, dissociates in the presence of EDTA to form the EDTA chelate of terbium, which is only very weakly fluorescent. This reduction in fluorescence is easily measured in the spectrofluorometer. The procedure has been described in detail elsewhere [14]. In addition to measuring peak fluorescence as previously, for the present investigation we also determined the area under the peak in wavelength scans and found entirely consistent results for the two procedures. For the present investigation, measurements were made from immediately after (1–2 min manipulation time) to several hours after mixing DNA and vesicles.

Results

Transfection Efficiency of EDOPC-DNA Complexes Prepared by Different Methods and their Distribution in the Cells

PC-DNA complexes were prepared from vortexed as well as 100 nm extruded EDOPC (labeled with 0.05% BODIPY-HPA) and plasmid DNA (carrying the β-galactosidase gene) by rapid and slow mixing. Complexes were made both by adding DNA to lipid and lipid to DNA. The various procedures and their associated designations, 1A through 2D, are listed in column 1 of Table 1 (results in the remaining columns of the figure are described in a subsequent section). To examine the effect of the mixing procedure on transfection efficiency, we applied the various preparations of EDOPC-DNA complex to BHK cells at a dose of 3 μg of EDOPC combined with 1 μg of plasmid β-gal. This was previously determined to be optimal for transfection with EDOPC in 96-well plates [21]. The BODIPY label used to assess the extent of association of lipoplexes with the cells (*see* next section) did not affect transfection by EDOPC, for fluorescent and unlabeled lipid exhibited the same transfection efficiency (*data not shown*).

As shown in Fig. 1 (*top*) there is no significant difference among complexes prepared from vortexed EDOPC, except in the case of complex 1D. That complex, prepared by dropwise addition of DNA to lipid, was only 1/2 to 1/3 as effective as complexes prepared by the other three methods. The lipoplexes prepared by those methods all exhibited high transfection efficiency (5–4 mU of β-gal per well).

In contrast to the complexes produced from vortexed lipid, most of those prepared from extruded EDOPC (Fig. 1, *bottom*), gave very weak transfection (note different vertical scales in the two panels of the figure). Indeed, transfection mediated by three of the four extruded EDOPC complexes was very close to background. The complex produced by dropwise addition of DNA to extruded EDOPC (complex 2D) behaved differently from the other complexes prepared from extruded lipid and exhibited approximately the same transfection efficiency as complex 1D, which was prepared in the same way, but from vortexed lipid.

Given the observed large differences in transfection it was of interest to determine whether complexes prepared from vortexed lipids distributed throughout cells in a different way than those prepared from extruded vesicles. To compare the cell distribution of complexes prepared from vortexed and extruded lipids we doped lipoplexes with the fluorescent phospholipid derivative, RhPE. In these experiments, BHK cells were treated using four of the versions of EDOPC-DNA complex described above (1A, 1D, 2A, 2D).

The transfection-efficient complex 1A gave bright intracellular fluorescence staining. In many cases, large aggregates were seen associated with a number of cells, and in many cases, occupied much of the volume of the cell. Less efficient in transfection, complexes 1D, 2A and 2D gave relatively weak fluorescence staining. The fluorescent particles were small and distributed throughout the cytoplasm of the cells (*data not shown*).

Cell Association of Various Preparations of EDOPC-DNA

The fluorescence of BODIPY-labeled EDOPC associated with BHK cells was measured 4 hours after addition of lipid-DNA complex. The amount of complex associated with BHK cells for the first three complexes prepared from vortexed EDOPC (Fig. 2, *top*) was about 25–35% of the total complex added to the wells. Lipoplex 1D exhibited divergent behavior; in the case of this complex, 70% of fluorescence was recovered from lysed cells.

All four complexes prepared from extruded EDOPC exhibited similar levels of cell association (Fig. 2, *bottom*). Preparation 2D associated with cells somewhat more than preparations 2A–C, but in all cases, the proportion of applied fluorescence found in cell lysates was the range of 20–32%. These levels of cell association were very similar to all but the fourth (1D) of the complexes prepared with vortexed EDOPC.

Comparison of Sizes of Different Preparations of EDOPC-DNA Complex

The distributions of particle sizes as obtained from the non-linear, least-square minimization procedure (NNLS) are shown in Fig. 3 for the various preparation procedures examined. Immediately apparent is that most of the complexes prepared from vortexed EDOPC contain a significant population of particles with diameters approaching a micron (*A*, upper 4 panels), whereas the complexes prepared from extruded vesicles contain predominantly smaller particles (*B*, lower 4 panels). The exceptions to this trend are preparations 1D and 2D. Although they contain approximately similar amounts of large particles, it's relatively fewer than for preparations from vortexed lipids and noticeably more than for lipoplexes from extruded lipids.

All of the vortexed lipid preparations produced lipoplexes in which there was a significant light scattering contribution in the smaller particle sizes, comparable to the predominant sizes in lipoplexes formed from extruded lipids. This is perhaps not unexpected given that the vortexed lipid dispersion itself contains two populations of particles, one group nearly as small as the extruded vesicles we have used, and a second population that is about 800 nm in diameter. The former population could well represent the precursors of the small lipoplexes.

The intensity distributions of Fig. 3 are very useful for assessing the heterogeneity of the different preparations, but these distributions suffer the disadvantage that, not only are they not readily convertible to number or mass distributions, but also, the NNLS method necessarily requires specifying a cut-off in maximum size. This is not true of the cumulants

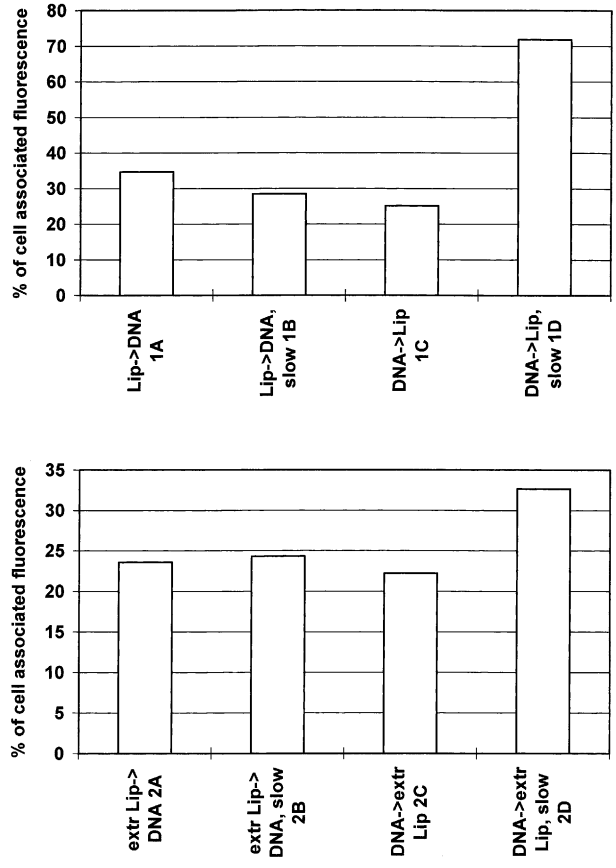

Fig. 2. Association of fluorescent EDOPC-DNA complexes with cells after 4 hours of incubation. BHK cells were treated with lipid-DNA complexes prepared from vortexed EDOPC (*top*) and from extruded EDOPC (*bottom*), in which the EDOPC was labelled with 0.05 mol% BODIPY-HPA. Replicates of two were used in this experiment and variation between them was less than 5%.

method, and to test for presence of particles too large for detection by the NNLS method, we calculated mean diameters by the two methods and compared them in column 3 of Table 1. As may be seen, the two procedures gave similar means in all cases except 1D. In that case, the mean diameter from the cumulants method was over 40% larger than that obtained from the NNLS procedure. This discrepancy indicates that preparation 1D is unusual in that it contains significant numbers of particles larger than several microns.

Relative Stability of Extruded and Vortexed Liposomes upon Contact with DNA

EDOPC-DNA mixtures were prepared using vesicles, both vortexed and extruded, loaded with terbium dipicolinate. The decrease of terbium fluorescence, attributable to the release of vesicle inner contents and subsequent dissociation of terbium ion from the dipicolinate, was monitored for a few hours after complex preparation. These measurements were done for the four rapid mixing

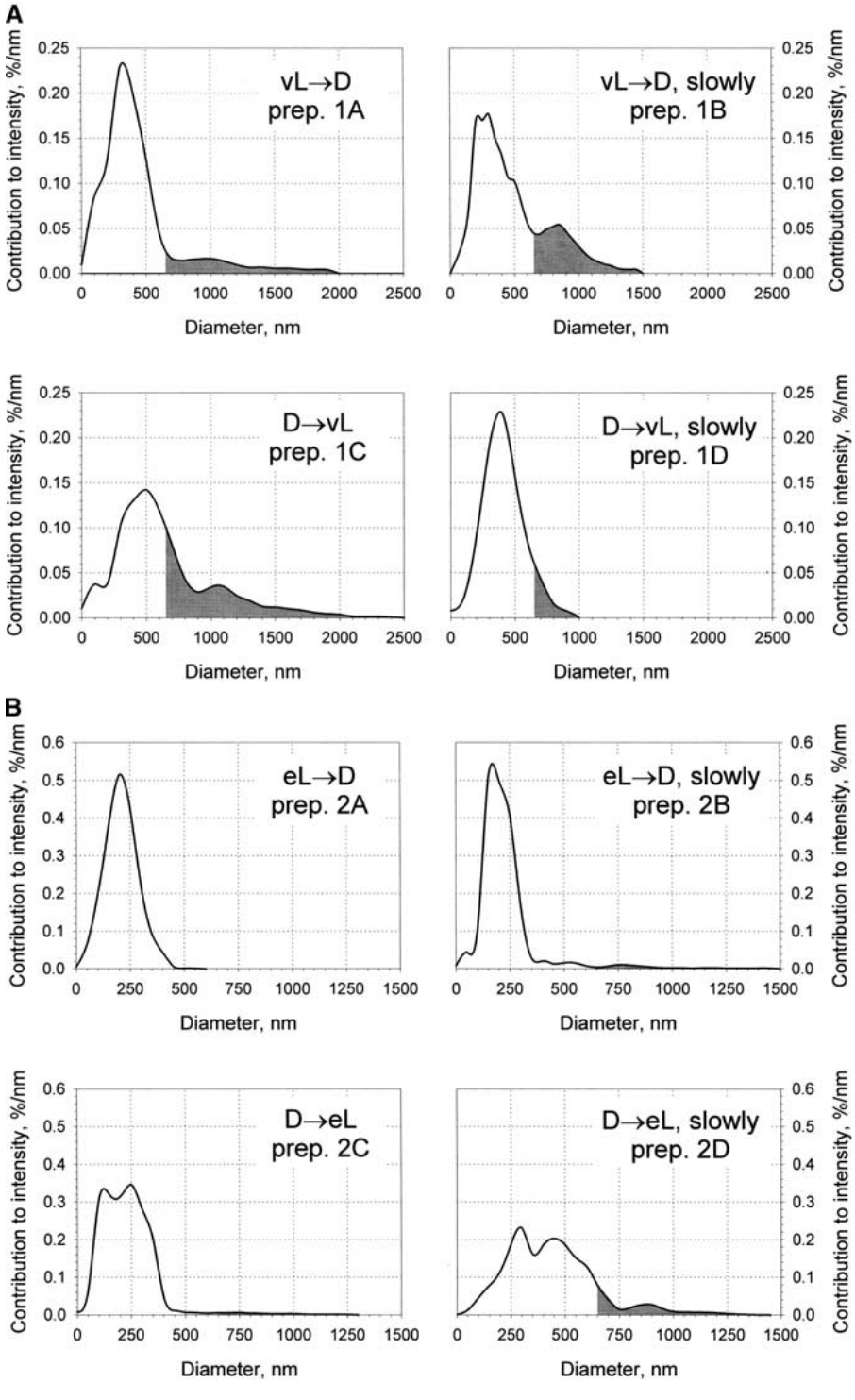

Fig. 3. The size distribution of particles in EDOPC-DNA preparations. The complexes were prepared as for transfection. Multiple (10–20) size measurements were performed with a dynamic light scattering instrument and the results averaged for increased reliability. The distributions were calculated using the non-linear least-squares (NNLS) minimization algorithm. The shaded areas of the plots represent the regions of the distributions containing particle diameters above 650 nm, according to the NNLS method. Please note that the size scales are different for (*A*) and (*B*).

protocols, for which the measurement is quite straightforward. In general, addition of DNA to lipid leads to more vesicle rupture than does the reverse mixing procedure and vortexted lipid preparations are considerably more susceptible to rupture by DNA than are extruded preparations. Measurement immediately after addition of lipid to DNA revealed that 1/2 of the vortexed vesicles ruptured (procedure 1A), in contrast to only 30% of the extruded vesicles (procedure 2A). Similar differences were seen when DNA was added to vesicles, although rupture was always greater for this formulation procedure due to the asymmetric forces generated when two or more vesicles adhere to the

same region of DNA (*see* Discussion); 3/4 of the vortexed vesicles (procedure 1C) immediately released their contents, whereas only half of the extruded vesicles (procedure 2C) were ruptured. At longer times, namely 2–3 h, only 10–15% the vortexed vesicles remained intact, but three times as many extruded vesicles were still intact.

Discussion

We have examined eight different modes of preparing DNA-EDOPC complexes that have a small excess of positive charge and observed that three of the four preparations generated from vortexed lipids were much more effective transfection agents than those prepared from extruded lipids. When DNA was slowly added to either vortexed or extruded lipid, the resultant lipoplexes showed approximately the same transfection efficiency: however, it was lower than that of vortexed preparations and higher than that of extruded preparations. These relatively minor changes in formulation procedures had disproportionate influences on transfection efficiency. As revealed by lipoplex size measurements, formulation procedures have large effects on lipoplex size and optimal transfection requires complexes with diameters in the region of 650–1500 nm, with smaller as well as very large complexes being ineffective.

A growing literature concerns the structures produced by mixing DNA and cationic amphipiles [5, 7, 10–14, 16, 26, 28, 31, 34, 43] and it now appears possible to rationalize the formation of different lipoplexes based on how the two components interact under different combination conditions. To assist the reader in following the explanation of the effects of formulation procedures, diagrams of the major steps in lipoplex formation are provided in Fig. 4. Underlying the different sequences are the following physical considerations: 1. Upon initial contact, all of the DNA is available for interaction, but, until the vesicle breaks, only the lipid in the external monolayer is available. 2. When mixing is slow, the component being titrated is in excess except at the end stages of mixing (assuming formation of a final complex that is near charge neutrality). 3. Vesicles may acquire a coating of DNA and persist metastably; the stability of such vesicles is inversely dependent upon size. Points 1 and 2 are evident upon analysis of the combination process, whereas point 3 is derived both from the present work and from our earlier study [14], which revealed vesicle differential stability, depending upon titration direction, as well as from the cryomicroscopy study of Huebner et al. [13], which showed that small vesicles can be stably coated with DNA. As recently pointed out, the relative number of charges on the DNA and on an average vesicle can affect the relationship between the input ratio of components and the actual composition of the lipoplex [11].

Preparations 1A and 2A

These preparations both involved rapid addition to DNA of vortexed lipid preparations in the case of 1A, and of extruded preparations in the case of 2A. When the vesicles are small (preparation 2A) such vesicles are stable for some hours, whereas if they are as large as typical MLVs (preparation 1A), they break on the order of a few minutes. The less stable vortexed vesicles rupture extensively even when vesicles are added to DNA rapidly (preparation 1A). In contrast, the more stable extruded vesicles rupture more slowly and probably only some time after having been coated with DNA. Since the DNA is initially in excess (half of the lipid is inaccessible in the inner monolayer), electrostatic repulsion among the DNA-coated vesicles prevents aggregation. That the particles are much smaller in the extruded preparation (2A) than in the vortexed preparation (1A) is clear from size measurements (Fig. 4).

Preparations 1B and 2B

When lipid is titrated into DNA, the vesicles encounter an excess of DNA (until near the endpoint), and so become coated with DNA. The small vesicles are more stable and remain intact longer than large vesicles. This means that the vortexed lipid undergoes rupture, leading to formation of large complexes, whereas the extruded lipid vesicles become coated with DNA without rupture. Thus, preparation 2B contains little material above 650 nm—the region that seems to provide lipoplexes that are most efficient in transfection, whereas the distribution of intensity of 1B is quite significant in the region above 650 nm.

Preparations 1C and 2C

Let us consider first highly efficient in transfection preparation 1C formed after rapid addition of DNA to vortexed lipid. It is expected from their known high affinities for each other that when a DNA strand entering the solution contacts a lipid vesicle, it will maximize its area of contact with the bilayer surface and hence quickly coat the vesicle.[1] Subsequently, DNA-coated vesicles interact with virgin vesicles, producing clusters of vesicles glued together with DNA [12]. This permits maximal lipid-DNA interactions in the form of a multi-decker sandwich-like

[1] Bundles of hydrated DNA wrapping around giant cationic vesicles can easily be observed by fluorescence microscopy [22].

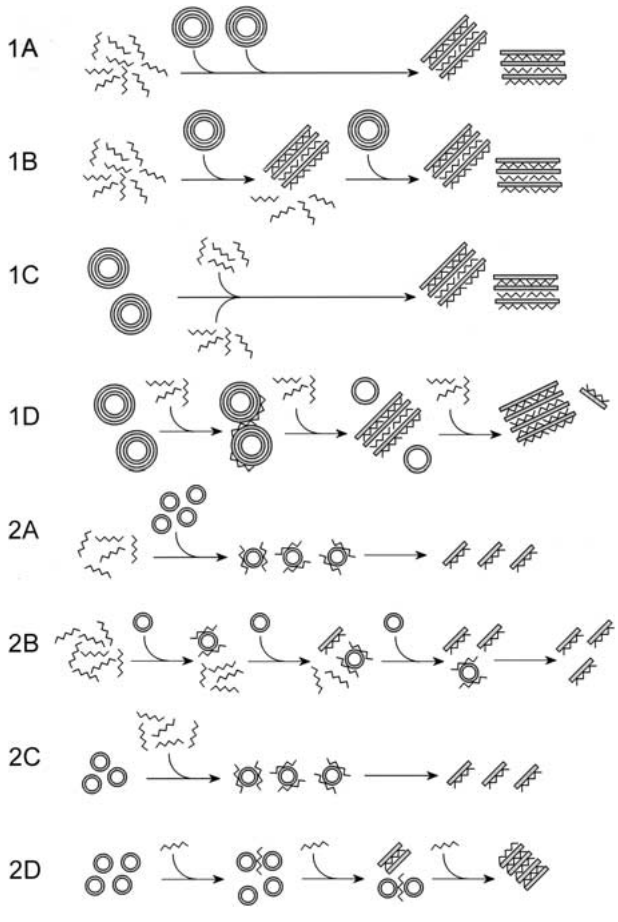

Fig. 4. Pathways of formation of lipoplexes. The panels describe likely processes of lipoplex formation for the preparations labeled A–D in Table 1. For simplicity, vortexed preparations (upper 4 diagrams) are shown as homogeneous, two-lamellar vesicles. *1A*. Addition of vortexed vesicles to DNA, rapidly. When mixing is rapid, the vortexed vesicles become coated with DNA, which is temporarily in excess, but then rupture on their own, giving rise to lipoplexes consisting of lipid from one or a small numbers of vesicles. *1B*. Addition of vortexed vesicles to DNA, slowly. Vesicles become coated with DNA and rupture, giving rise to lipoplexes originating largely from single vesicles. *1C*. Addition of DNA to vortexed vesicles, rapidly. Because the mixing is rapid relative to lipid rearrangement, this process is similar to *1A*. *1D*. Addition of DNA to vortexed vesicles, slowly. Until the end of the titration, lipid is in excess and hence more than one vesicle binds to one piece of DNA. The asymmetric forces lead to progressive rupture of the vesicles, releasing their lipid, which is free to adhere to other vesicles or lipoplexes as additional DNA enters the suspension. The final portions of DNA added lead to aggregation of available cationic surfaces, which hence generates a proportion of very large lipoplexes, along with smaller lipoplexes. *2A*. Addition of extruded vesicles to DNA, rapidly. Because the vesicles do not immediately rupture, DNA is in excess (half of the lipid is internal) and the vesicles become largely coated with DNA and hence do not form large aggregates. When they rupture, the lipoplexes so formed remain small. *2B*. Addition of extruded vesicles to DNA, slowly. Until the very end of the titration, each vesicle is coated and then breaks, acquiring further DNA, but still as individual particles. *2C*. Addition of DNA to extruded vesicles, rapidly. Vesicles acquire coating of DNA, but because only half of the lipid is initially exposed, DNA is in excess and when the vesicles do rupture, they remain largely isolated. *2D*. Addition of DNA to extruded vesicles, slowly. As DNA enters dispersion, it encounters an excess of vesicles and induces their adhesion. The aggregates grow and rupture as the DNA addition progresses.

structure in which lipid and DNA form alternate layers [6, 19, 26].

To prepare lipoplex 2C, the DNA was added rapidly to extruded vesicles. Because of their stability, extruded vesicles would remain intact until well after the DNA had all been added, so that the DNA charge would exceed the lipid charge due to the unavailability of the inner monolayer lipid. Thus, the overall vesicle charge is negative and this net charge prevents aggregation. The size of lipoplexes remains small and this preparation is a very weak transfection agent.

Preparations 1D and 2D

If DNA is added to vortexed lipid slowly (preparation 1D), the situation differs from the rapid mixing case because the lipid is in excess during the initial stages of the addition process [14]. Each aliquot of DNA added to the dispersion leads to vesicle-vesicle adhesion and rupture; however, when the addition is stepwise and slow, the lipid is in excess until the end of the titration is approached [14]. In the initial stages of addition, particle size is limited by the electrostatic repulsion of the cationic particles; however, in the later stages of the titration, the added DNA neutralizes the remaining lipid, but since much DNA has already been incorporated into complexes, the amount is limited. Thus, these final portions of DNA lead to aggregation of a only a portion of the complexes. As a consequence, a slow titration generates a small number of very large complexes and a large number of rather small complexes. Evidence for large particles was found in the difference in average diameter produced by the cumulant and NNLS methods (Table 1, column 3). The presence of very large particles in this sample would also account for the unusually high cellular adhesion levels seen with this sample (Fig. 2).

Among the samples prepared from vortexed lipid, this procedure was clearly the worst in terms of transfection. The fact that the proportion of particles in the size range immediately above 650 nm was also much smaller than that in the other vortexed samples suggests that this size range is important for efficient transfection. The large particles, alluded to in the previous paragraph, are apparently too large to be taken up by cells.

The preparation 2D was the only lipoplex preparation produced from extruded vesicles that was efficient in transfection. This lipoplex preparation is the only one from extruded vesicles with a significant

population of particles larger than 650 nm. As in the case of 1D, internal lipid (here, the inner monolayer) is released during addition of the DNA so there is rough neutralization of complexes as they form and that absence of net charge leads to greater particle aggregation. The difference from the 1D case is that there the vesicles were large to begin with and the lipoplexes grew to become too large to be effective transfection agents, whereas in the case of the extruded vesicles of 2D, the lipoplexes became larger than the typical extruded lipoplex and many moved into the effective size range for transfection.

Cellular Adhesion Comparisons

The amount of complex associated with cells did not differ greatly among the various preparation methods with the exception of 1D. As indicated above, the presence of some very large particles, absent from the other preparations, may explain the large amount of measured adhesion by 1D. There are two ready explanations of why the complexes prepared from extruded and vortexed lipids differed little in cell adhesion even though they differed enormously in transfection activity. 1. At the levels used, which were chosen on the basis of optimization of transfection, the binding sites on the cell surface were saturated by all preparations. 2. The truly effective complexes are such a small proportion (perhaps the population from about 650 nm to about 1500 nm) of the total population that they are simply overwhelmed by the amount of ineffective complexes that are bound to the cells. Others have observed little difference in DNA uptake but large differences in expression and suggested a third reason, namely that the effective complexes have a sufficiently different structure that they readily release DNA to the nucleus [8]. Recently it was shown that lipoplex efficiency is not correlated with the changes in lipoplex-cell interactions, but mainly with lipoplex size [2].

High Transfection Activity Correlates with the Presence of Complexes >650 nm and <1500 nm

The general correlation between transfection efficiency and the presence of particles larger than about 650 nm in diameter suggests there is a lower threshold in lipoplex size for efficient transfection of BHK cells.[2] The size estimation clearly reveals that the smaller and the larger populations are quite ineffective. As indicated in the introduction, this conclusion is consistent with that already published by a number of other laboratories; however, this particular size estimate is based on BHK cells and other cells may optimally respond to lipoplexes of different sizes. Of course, in vivo size optima are likely to be different still. In any case, we would emphasize that our primary conclusion is not that there is an optimum size for lipoplexes, but rather that the sizes of most lipoplex formulations vary as a natural consequence of the physical and geometric properties of DNA, lipids and liposomes. The analysis presented here is likely to hold for lipoplexes that are formed from vesicular preparations other than the cationic phospholipid derivative used, although some variations are expected, depending upon the rate at which the vesicles of different amphipaths rupture and disintegrate upon contact with DNA. On the other hand, lipoplexes formed from micellar cationic amphipaths will necessarily be generated through a somewhat different mechanism.

If particle sizes in the region of 650 nm and somewhat larger are, in fact, optimal for transfection of BHK cells, then it would appear from the distributions of Fig. 3, that the best preparations are far from optimal. Even the best preparation, 1C, contains a significant proportion of material with diameters below 500 nm. It thus appears that there remains much more to be done in terms of generating homogeneous and optimal transfection complexes. Ideally, one would like to be able to fractionate a given preparation and determine the transfection efficiency of each size fraction. Unfortunately, the potentially feasible procedures for fractionation or size modification have, at least in our hands, serious drawbacks. Centrifugation causes aggregation, and sonication and extrusion can damage DNA. In addition, sophisticated analytical methods will be required to determine the actual amount of the components in each particle. With respect to the latter challenge, however, we have made progress. Currently we are using flow cytometry to elucidate individual single particle structure and composition. This approach has been found to be very powerful and to permit access to critical physical characteristics of lipoplexes not available through other methods [25]. It should allow very accurate analysis of distributions of size and compositions of formulations having particular average transfection efficiencies.

[2]The value 650 nm is somewhat arbitrary. Values between 600 and 900 nm were examined graphically and statistically. Statistically, 850 nm provided a marginally better correlation between size and activity, but 650 gave a graphically more obvious correlation. In any case, it is clear that there is a threshold size in this range, and it is doubtful that it can be established more precisely than 200 nm, given the inherent uncertainty in light scattering procedures when applied to heterogeneous samples.

This research was supported by NIH grant GM 52329. We thank Ruby MacDonald for help in the laboratory in numerous ways. We are grateful to Spiro Pantazatos for his excellent rendition of Fig. 4.

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