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Real-time Observation of Lipoplex Formation and Interaction with Anionic Bilayer Vesicles

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Abstract. A novel development has allowed for the direct observation of single, pairwise interactions of linear DNA with cationic vesicles and of DNA-cationic lipid complexes with anionic vesicles. A new cationic phospholipid derivative, l,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine, was used to prepare giant bilayer vesicles and to form DNA-cationic lipid complexes (lipoplexes). The cationic vesicles were electrophoretically maneuvered into contact with DNA, and similarly, complexes were brought into contact with anionic phospholipid vesicles composed of dioleoylphosphatidylglycerol (DOPG; 100%), DOPG/dioleoylphosphatidylethanolamine (DOPE; 1:1) or DOPG/dioleoylphosphatidylcholine (DOPC; 1:1). Video fluorescence microscopy revealed that upon contact with phospholipid anionic vesicles, lipoplexes exhibited four different types of behavior: adhesion, vesicle rupture, membrane perforation (manifested as vesicle shrinkage and/or content loss), and expansion of DNA (which was always concomitant with membrane perforation.) In one instance, the lipoplex was injected into the target vesicle just prior to DNA expansion. In all other instances, the DNA expanded over the outer surface of the vesicle, and expansion was faster, the larger the area of vesicle over which it expanded. Given the likelihood of incorporation of cellular anionic lipids into lipoplexes, the expansion of the DNA could be important in DNA release during cell transfection. Upon contact with naked DNA, giant cationic vesicles usually ruptured and condensed the DNA into a small particle. Contact of cationic vesicles that were partially coated with DNA usually caused the DNA to wrap around the vesicle, leading to vesicle rupture, vesicle fusion (with other attached vesicles or lipid aggregates), or simply cessation of movement. These behaviors clearly indicated that both DNA and vesicles could be partly or fully covered by the other, thus modifying surface charges, which, among others, allowed adhesion of DNA-coated vesicles with uncoated vesicles and of lipid-coated DNA with uncoated DNA.

Key words: Cationic lipid — EDOPC — Lipoplex — Video microscopy — Liposomes

Introduction

Cationic lipids (CL) have proven to be effective in the delivery of DNA into cells both in vivo and in vitro [5, 23]. The mechanism of DNA transfection is still unclear, however, and its elucidation has been the goal of a considerable amount of biophysical and biochemical research. Recent studies suggest that DNA/CL complexes (lipoplexes) enter the cell through endocytosis [6, 25]. An important subsequent step is the release of DNA from the CL [18]. This is thought to occur in the endosome, where destabilization of its membrane may play an important role in DNA release and expansion [9, 26]. Uncomplexed DNA is released into the cytoplasm, whereby it may eventually enter the nucleus [6], or, possibly, the complex escapes from the endosome and finds its way to the nucleus where it dissociates. A few studies have suggested a relationship between the structure and mode of formation of the DNA/CL complex on one hand and its efficiency of DNA release from cationic lipid [12] and its overall efficiency in cell transfection [11, 27] on the other. Given the above developments, two important steps in and their effects on the process of transfection are currently receiving attention: a) the mechanism of DNA complexation by CL [4, 8, 11, 17] and b) mechanism of DNA release from CL upon contact with anionic liposomes that simulate the inner monolayer of endosomes [1, 24]. The latter possibility would be consistent with previous reports of cationic-anionic bilayer fusion [20, 21].

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A recently developed compound, *O*-ethylphosphatidylcholine (EDOPC), was prepared from phosphatidylcholines through alkylation of the phosphate oxygen by reaction with alkyl trifluoromethylsulfonates [15, 16]. Not only is this compound an effective transfection agent, but it is also stable in serum for prolonged periods of time and, because it consists of only natural cellular metabolites with ester bonds, it exhibits low toxicity [15, 16]. EDOPC is thus a promising gene therapeutic agent and it appears to warrant further studies; in particular, characterization of its fusogenic and bilayer-formation properties [19] would seem especially relevant to understanding CL-mediated cell transfection. A recent study has already reported on certain factors involved in DNA complexation using EDOPC [11]. There have been several investigations of DNA release from CL complexes upon contact with anionic membranes [1, 24], however, there have been no examinations of such release at the level of individual lipoplexes with the exception of one figure in Koltover et al. [12].

Because it takes advantage of the presence of opposite electronic charges on the objects of interest, a method that was previously developed for the direct examination of pairwise encounters between oppositely charged phospholipid bilayers [19] proved useful for investigation of lipoplex formation from DNA and cationic lipid vesicles as well as of interactions of lipoplexes with anionic vesicles, a process that may relate to the escape of DNA from the endosome in the cell transfection process. Using this electrophoretic manipulation method, it has been possible to observe in real time the formation of CL-DNA complexes and the fusion of lipoplexes with an anionic bilayer.

We have carried out two kinds of experiments: a) direct observation of interactions between large EDOPC vesicles and pieces of λ-DNA and b) direct observation of interactions between large anionic liposomes (as models of endosome membranes) and DNA/EDOPC complexes. For experiment type b), the charge and lipid composition of the anionic vesicles were varied in order to determine whether these properties have any profound effects on the interactions between target membranes and complexes. An additional set of experiments was carried out in which the anionic vesicles (DOPG/DOPC) were filled with a green-fluorescent aqueous dye – calcein – to test their membrane-barrier properties during interactions with complexes.

Materials and Methods

Reagents

EDOPC, dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), dioleoylphosphatidylethanolamine (DOPE) and N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (R-PE) were obtained from Avanti Polar Lipids (Alabaster, AL). YOYO-1 iodide was obtained from Molecular Probes (Eugene, OR) and N-N-dioctadecyloxacarbocyanine (DiO) was obtained from Eastman Kodak (Rochester, NY). Calcein was obtained from Hach (Ames, Iowa). All of the above lipids were stored at 4°C in chloroform solutions. λ-DNA in 10 mM Tris-HCl (pH 7.4), 5 mM NaCl and 0.1 mM EDTA buffer was obtained from Life Technologies (Gaithersburg, MD).

Vesicle Preparation

Dispersions of mostly unilamellar vesicles were prepared from the following lipid compositions: 100% DOPG, DOPG/DOPE (1:1 wt/wt), DOPG/DOPC (1:1) and 100% EDOPC, All liposomes were labeled with 5% R-PE (red florescent dye) and/or filled with 1 mM calcein. The lipid stock solutions were mixed in the appropriate ratio at room temperature and bulk chloroform was evaporated under a stream of argon gas. The lipids (total weight, 50 μg) were then placed under high vacuum for a minimum of 30 minutes. 500 μl of deionized water was used to hydrate the lipid to a total concentration of 0.1 mg/ml. The dispersions were left without agitation for one hour, then swirled gently and stored at 4°C overnight until use. For calcein-filled vesicles, lipid was hydrated in 250 μl of 1 mM calcein and left without agitation for one day at room temperature. 10 μl of the vesicle dispersions was put into each end of the observation chamber and slowly suctioned into the chamber using a 200-μl air-displacement pipette.

Naked-DNA Preparation

λ-DNA was labeled with YOYO-1 iodide at a ratio of 2 nanomoles of dye per 10 μg of DNA (total volume, 50 μl). The DNA solution was centrifuged at $14{,}000 \times g$ for 15–20 minutes. Upon removal of the supernatant, a small green pellet was left at the bottom of the centrifuge tube. 200 μ1 of deionized water was used to resuspend the pellet. This pellet consisted of small bundles of DNA strands that had not completely separated during hydration. Single molecules of DNA were not resolvable with our equipment. 10-μl portions of the resuspended pellet solution were injected into one side of the observation chamber.

DNA/CL Complex Preparation

λ-DNA (unlabeled or labeled with YOYO-1) was added to hydrated EDOPC in deionized water (0.1 μg/μl) at a weight ratio of 1:6 DNA/CL (1:2 charge ratio). EDOPC vesicles were either unlabeled, labeled with 5% R-PE or labeled with 5% DiO. A 3:1 wt ratio (lipid:DNA) corresponds to a 1:0.97 charge ratio.

Observation of Vesicle Interactions

To facilitate the preparation of giant vesicles, experiments were done in deionized water. The exciting filter in the incident light fluorescence microscope was a BG12, which is a broadband filter that provides efficient excitation of YOYO-1, calcein, DiO and R-PE. Images were captured through a 50× oil immersion objective (NA = 0.80). Because it was necessary to survey a large number of interacting objects (vesicles, lipoplexes or clusters of DNA), the only practical approach was to videotape most of our manipulations and convert them to digital still image format subsequently. When possible, frame averaging was used to enhance the quality of single images, however, image intensity was often limited by photobleaching during selection and positioning of the chosen

particles. Consequently, to illustrate more clearly events that could be seen on videotape, we usually increased the contrast and brightness of the image as well as adjusted color intensities using the image enhancement capabilities of Adobe Photoshop. Even with such image processing, however, a set of still images falls far short of conveying what can be appreciated when the entire sequence of events is viewed in real time. Thus, what happened in many important kinds of interactions is not obvious from the images alone, and we have therefore provided a set of drawings (Figs. 1′–13′) to illustrate processes that were clearly evident after examination of the videotapes.

For readers interested in a more detailed view of some of the interactions described here, video clips may be found at the Internet site: http://www.biochem.northwestern.edu/MacDonald/home/home_contents.html

Results

DNA and Cationic Liposome Interactions

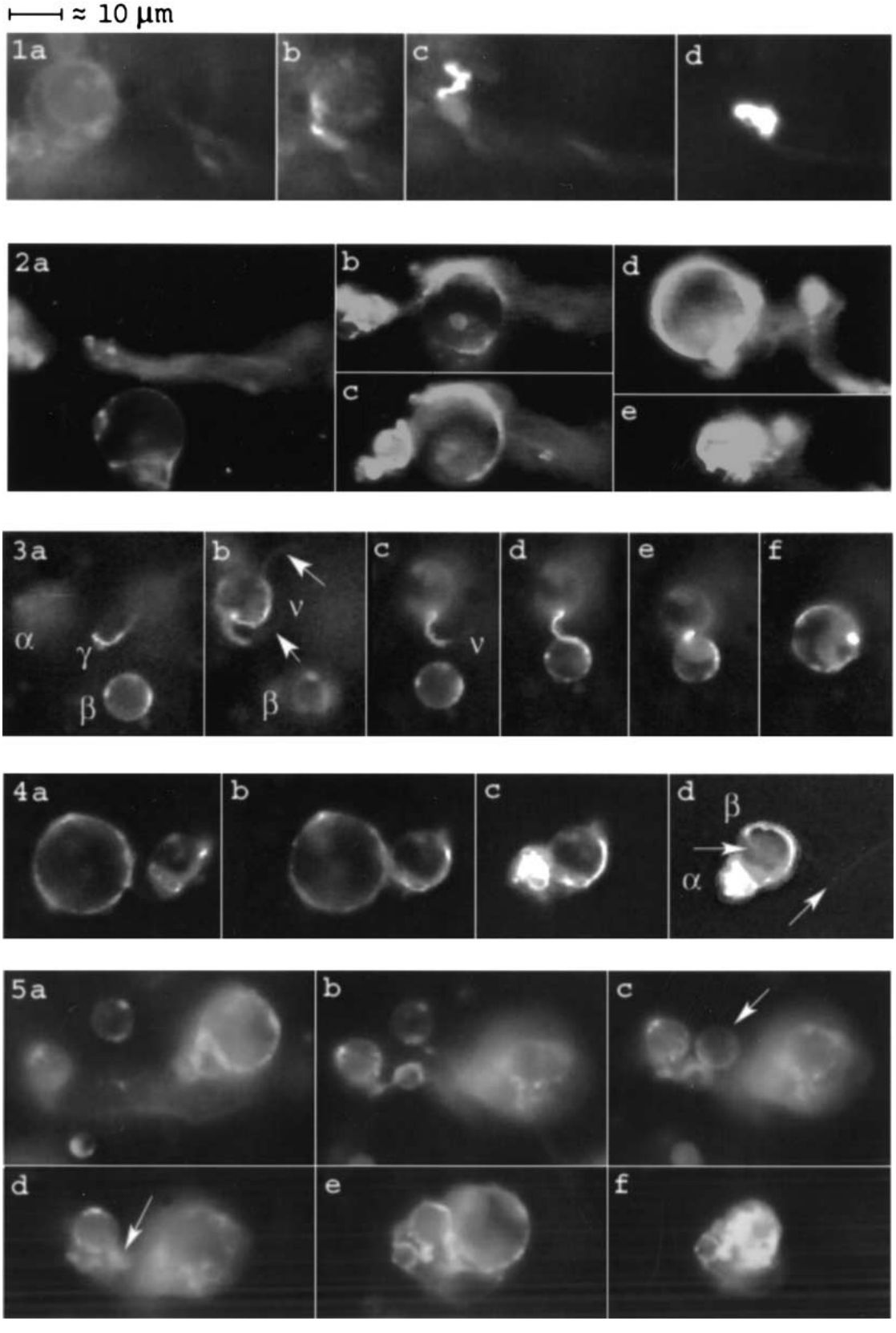
Clusters of DNA (seen as green fluorescence), ranging in length from 10 to 250 μm, appeared as loose, stringy bundles, or (more rarely) rigid and tightly packed sheaves. These two forms occurred sometimes on the same piece of DNA. Regions of stringy bundles had diameters from 1 to 10 μm, whereas the sheaf-like regions were as thick as 20 μm. It may be that the former are largely individual strands of DNA, which, at a few points, are more tightly associated with each other, perhaps as in the sheaves. Some bundles consisted of intricate branches and coils, whereas others were quite linear. In any case, both forms presented an anionic DNA surface that was convenient for bringing into contact with cationic vesicles. Cationic vesicles (seen as red fluorescence) ranged in size from 5 to 30 μm in diameter. A total of 28 clusters of DNA were recorded interacting with cationic vesicles.

The interaction of stringy DNA with cationic vesicles led to several different immediate outcomes, which depended on whether the vesicle had any previous coating of DNA. If the vesicle was visibly clean (i.e., it had not previously interacted with any DNA) then contact with the DNA led to immediate rupture of the vesicle and rapid condensation of the DNA (Fig. 1). This occurred very quickly, namely within one video frame, or 30 msec. However, when vesicles had some DNA coating[1] (indicated by areas of yellow or white arcs along the vesicle rim) they did not rupture immediately upon contact but instead rolled along the DNA. Alternatively, the DNA became wrapped around the vesicle. Usually, these interactions persisted for a few seconds and then the vesicle ruptured. On many occasions (roughly 40%), though, vesicles did not rupture and either slowed down dramatically or simply came to a stop, remaining stable long enough for additional manipulations (Figs. 2 and 3). In such cases, it was usually possible to bring a second vesicle into contact with the stable vesicle or with the portion of DNA that was not wrapped onto the stable vesicle. Upon contact with the DNA-coated vesicle, the second cationic vesicle sometimes fused with it (3 cases out of 8 observations), but more frequently the second vesicle simply adhered to the DNA-wrapped vesicle, with the eventual rupture and collapse of one or both of the vesicles. These events of adhesion and fusion indicate that the vesicles had acquired a coating of DNA, for otherwise cationic vesicles exhibited no interactions with one another.

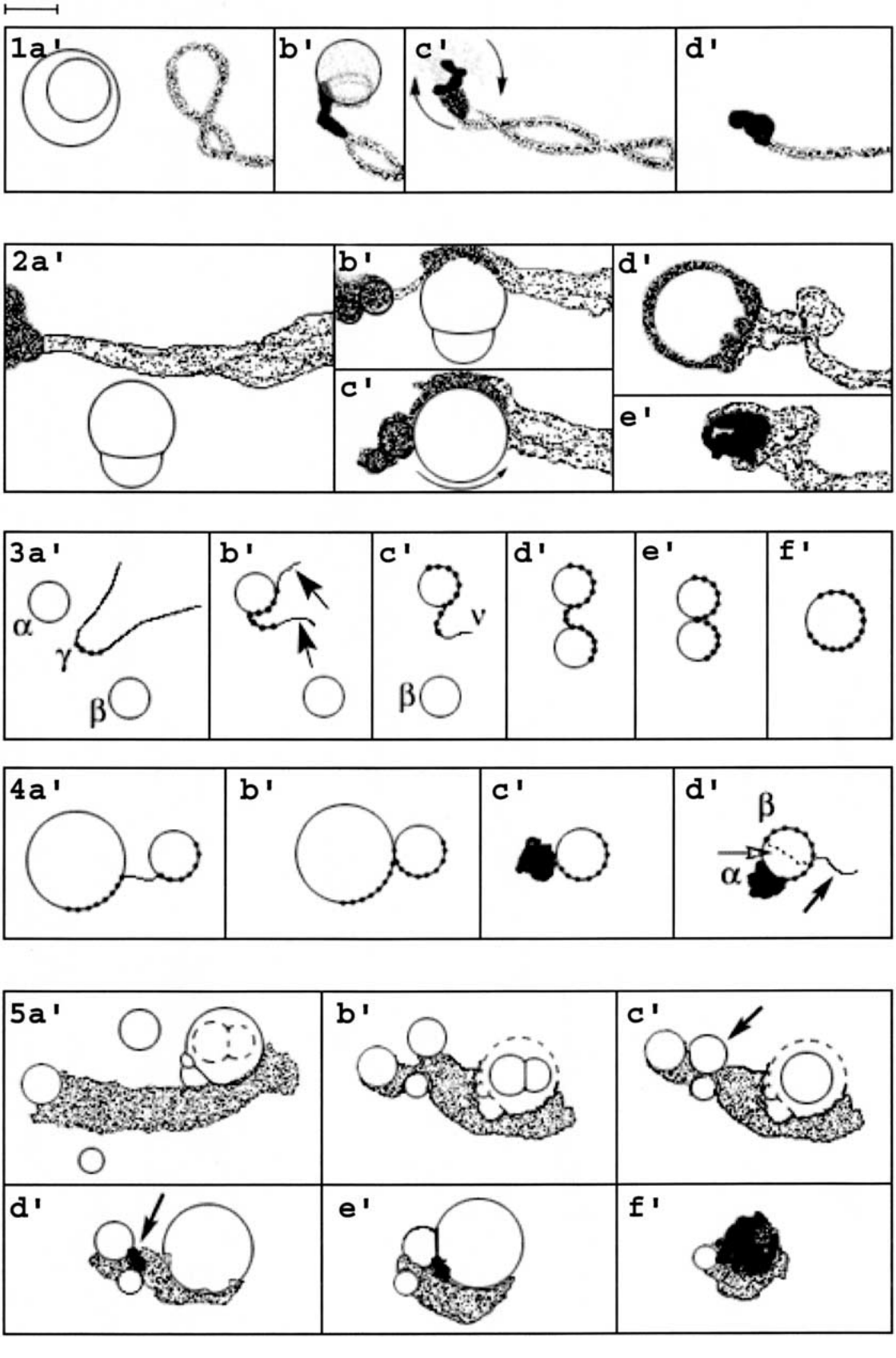
As vesicles interacted with the DNA, they not only acquired a coating of DNA, but also some lipid was released to the DNA, as was indicated by the change in the color of fluorescence of the DNA (it became yellow due to the overlap of the two fluorophore emissions, or even white due to video phosphor saturation at high light intensity). Frequently, a vesicle that had rolled and ruptured on a bundle of DNA left a coating of lipid on only a portion of the bundle, while other regions remained free of lipid, at least for the duration of the observation period. When other vesicles were brought into contact with the uncoated portion of DNA, the response was the same as in the case of DNA that had undergone no interactions with cationic vesicles. In one interesting instance, a rolling vesicle slowed down dramatically and remained intact when it neared a region of DNA already coated with lipid by contact with a previously ruptured vesicle. A second DNA-coated vesicle was then brought into contact with the uncoated DNA on the far side of the coated region of DNA. Likewise, this second vesicle rolled along the DNA and slowed down as it neared the lipid-coated region of DNA. The interaction continued until the DNA brought the two vesicles into contact, whereupon they fused (Fig. 3). Evidently the second vesicle was still largely positively charged while the first vesicle had become coated with DNA. This instance was unusual, however, for typically, when multiple vesicles on a single piece of DNA came together, aggregation and rupture were the most common occurrences (Figs. 4 and 5). Typical event pathways of DNA-cationic vesicle interactions are reviewed below in the Discussion.

When DNA-coated cationic vesicles interacted with the thicker, sheaf-like regions of DNA (rigid and flat clusters of DNA that did not coil), they rarely ruptured immediately and instead of rolling, they remained attached to the DNA for up to tens of seconds. Vesicles induced some bending of these clusters of DNA, but much less so than in the case of loosely bundled, stringy DNA, which is evidently more flexible. After adhering to the DNA, vesicles remained intact for several seconds and then collapsed. In two cases, the collapsing vesicles resembled

[1]Presumably, such partial coating came from small pieces of DNA that had previously become attached to the vesicle.



Figs. 1–5.



Figs. 1′–5′.

a slowly deflating balloon on the surface of the DNA and they coated only the region of DNA that was immediately adjacent to them. In other cases, vesicles on a sheaf-like region of DNA collapsed only after contacting several other vesicles. A typical situation involved two vesicles attached at opposite ends of a sheaf of DNA (Fig. 5). They slowly came into contact as two additional vesicles bound to and induced bending in the middle of the DNA. One of the middle vesicles collapsed, causing further bending of the DNA until the remaining three vesicles became aggregated. Two of these remaining vesicles ruptured simultaneously, while the third remained intact and stably adhered to the complex for at least 10 seconds.

Lipoplex Interactions with Anionic Liposomes

Adhesion, the prerequisite for all lipoplex-vesicle interactions, was characterized by the formation of a brightly fluorescent contact zone where the lipoplex adhered to the vesicle. This bright fluorescence is evidently due to the overlap of the fluorescence of the two participants and was unmistakable, becoming immediately apparent as soon as the lipoplex and vesicle surfaces came into contact. Subsequent events generally led to a compromise in vesicle membrane integrity, but the paths leading to such a state varied considerably.

There were several distinguishable consequences of lipoplex/anionic vesicle contact: (*a*) immediate rupture of the anionic vesicle, (*b*) simple adhesion of the complex to the vesicle, which remained intact and did not shrink, (*c*) adhesion of the complex to the vesicle, leading to DNA expansion with simultaneous vesicle shrinkage, (*d*) adhesion of the complex, leading to a localized breakage of the anionic membrane and shrinkage of the vesicle and (*e*) adhesion of the complex, leading to a localized perforation of the anionic membrane, shrinkage of the vesicle and subsequent expansion of DNA.

Of a total of 37 pairwise interactions that were observed between DNA/CL complexes and DOPG vesicles, 11 (30%) led to DNA expansion. Of 51 observed interactions between DNA/CL complexes and DOPG/DOPE (1:1) vesicles, 20 (39%) led to DNA expansion. Of a total of 45 interactions between DNA/CL complexes and DOPG/DOPC (1:1) vesicles, 17 (38%) led to DNA expansion (these included 12 interactions with calcein-filled vesicles, 5 (42%) of which led to DNA expansion.) DNA expansion was thus seen in about 1/3–2/5 of the interactions.

DNA expansion was characterized by the sequence: 1, the bright green complex adhered to the surface of the anionic vesicle; 2, the complex became very bright (usually white due to high local light intensity); 3, the complex expanded into a green, amorphous body that was typically 5–10 times the size of the original complex and always remained attached to the target anionic vesicle. The rate of DNA expansion varied according to how it expanded; DNA expansion *along* the membrane surface was usually complete in seconds (Fig. 6), whereas ex-

See figures on pp. 102 and 103.

Fig. 1. DNA-cationic vesicle interaction leading to immediate rupture of vesicle and condensation of DNA. Uncoated cationic vesicle (100% EDOPC) containing an internal vesicle was brought into contact with a long, stringy bundle of DNA. Upon contact, the outer vesicle ruptured (*b*), which was immediately followed by DNA contact with and rupture of the inner vesicle (*c*). After rolling along the full length of DNA shown in (*c*), the collapsed lipid came to a stop, leaving some DNA that remained free of lipid (*d*). The scale bar at top left indicates 10 μm, and the magnification for figures 1–5 is approximately (±10%) the same. Figures 1–13, reproduced in black and white from the original color video images, are accompanied by replicate drawings (Figs. 1′–13′) on the opposite page. These drawings are intended to further clarify processes that may not be readily apparent in the images alone.

Fig. 2. DNA-vesicle interaction leading to rolling, fusion (within aggregate) and subsequent vesicle rupture. A pair of hemifused, DNA-coated cationic vesicles (100% EDOPC) were brought into contact with a large piece of DNA (*b*). The vesicles fused together and the DNA wrapped around the product vesicle (*c*), which remained stable for several seconds (*d*). Subsequently (*e*), DNA and vesicle suddenly condensed into a compact wad.

Fig. 3. DNA-mediated cationic vesicle fusion. A cationic vesicle (100% EDOPC, α) was brought into contact with a strand of DNA (γ) that contained a small region already coated with cationic lipid (black dots in drawing). In (*b*), the upper strand of DNA (*top arrow*) bound completely to the vesicle, whereas the bottom strand (*bottom arrow*) was inhibited from further binding to the vesicle by the lipid coating (γ). This resulted (*c*) in a dangling strand of naked DNA (*v*), which was brought into contact (*d*) with a second cationic vesicle (*β*). The coated portion of DNA slowly brought the two vesicles together (*e*) which resulted in vesicle fusion (*f*).

Fig. 4. DNA-mediated vesicle aggregation and rupture. Vesicles (100% EDOPC) attached to both ends of a piece of DNA (barely visible) came together by interactions with the DNA (*b*). The vesicle on the left collapsed onto the DNA while the vesicle on the right remained intact (*c*). The resulting aggregate, which consisted of collapsed lipid-DNA (α), DNA bound to vesicle (*β*) and naked DNA (barely visible, diagonal arrow), remained stable for at least 10 seconds (*d*). The naked portion of DNA extends from where it is attached along the back side of the vesicle (*dashed line in d′*; horizontal arrow).

Fig. 5. Interactions of sheaf-like DNA with several vesicles. A coated vesicle and a vesicle aggregate were attached to the left and right ends of a thick sheaf of DNA. The two free-floating vesicles (middle) came into contact with the mid-section of the DNA (*b*). In (*b*) the plane of focus has been shifted to the two hemifused vesicles on the vesicle aggregate, which fused to each other (*c*). This fusion product subsequently fused with the larger vesicle of the aggregate (*d*), while the upper, middle vesicle (*arrow*) collapsed and further condensed the DNA. The vesicles on opposite ends of the DNA come together (*e*) and subsequently collapsed (*f*).

pansion *away* from the surface of the vesicle usually took tens of seconds (Fig. 7). In perpendicular expansion, the contact areas between DNA and anionic membranes were much smaller than in surface expansion cases. In all instances, DNA expansion was also accompanied by vesicle shrinkage, i.e., the target vesicle measurably decreased in diameter. This occurred through either pathway (*c*) or (*e*) above. In (*e*), perforation of the vesicle membrane by the complex occurred first (usually immediately upon contact as revealed by rapid shrinkage), and subsequently DNA became expanded. In these few cases (1 for DOPG, 5 for DOPG/DOPE, 2 for DOPG/DOPC), the vesicles visibly shrank to about 30–60% of the original diameter before the DNA began expanding, and the membrane usually became flaccid and exhibited Brownian motion. By contrast, in the vast majority of DNA expansion events (*c*), shrinkage was not as drastic as in event (*e*) and it occurred *as* the DNA was expanding (i.e., it was visibly apparent only after the DNA had fully expanded.) There was a rough correlation between the size of the expanded DNA and the extent of vesicle shrinkage; when DNA complexes were unusually small or had expanded only 2–4 times their original size, vesicle diameters decreased 5–10%, whereas when the complexes were unusually large or had expanded 10–15 times their original size, the vesicle diameters decreased by at least 20% and up to 70% in one case. The vesicles (which always remained spherical and exhibited no Brownian motion) ceased shrinking at about the time the DNA reached its final expanded state.

In most cases in which DNA expansion was observed, the DNA remained on the outer surface of the vesicle. However, one notable exception involved the interaction of a DNA/EDOPC complex with a DOPG/DOPE (1:1) vesicle (Fig. 8). This case seemed to follow the course of events denoted above as (*e*),

See figures on pp. 106 and 107.

Fig. 6. "Surface" expansion of a lipoplex upon contact with an anionic vesicle. A DNA/EDOPC complex (1:2 charge ratio) interacted with a DOPG/DOPC (1:1 charge ratio) vesicle. The complex (right side of vesicle) initially bound to the vesicle membrane (*b*) and began expanding after 2–3 seconds. About 5 seconds later, the DNA expanded to its fullest extent over the surface of the anionic vesicle (*c*). The contact region between the DNA and the vesicle encompassed the entire length of expanded DNA. The scale bar at top left indicates 10 μm, and the magnification for figures 6–13 is approximately (±10%) the same.

Fig. 7. "Perpendicular" expansion of lipoplex upon contact with anionic vesicle. A DNA/EDOPC complex interacted with a DOPG/DOPC (1:1 charge ratio) vesicle. The colors of the complex and vesicle were seen to have mixed, appearing white, and (*b*) was reached about 20 seconds after initial binding of the complex to the anionic vesicle. After an additional 40 seconds, the DNA expanded to its final form (*c*). The narrow contact zone is indicated by an arrow.

Fig. 8. Membrane perforation, lipoplex injection and subsequent DNA expansion inside an anionic vesicle. A DNA/EDOPC complex (1:2 charge ratio) was brought into contact with a DOPG/DOPE (1:1) anionic vesicle. Upon contacting the vesicle, the complex breached the membrane and entered the vesicle, which immediately shrank to about 85% of the vesicle's original diameter (*b*). Subsequently, slow vesicle shrinkage and DNA expansion occurred simultaneously for about 15 seconds (*c*). The rate of vesicle shrinkage and simultaneous DNA expansion increased for about 10 seconds until slowing down again (*d*). After 20 more seconds, the two processes ceased completely, and most of the expanded DNA was contained inside the shrunken vesicle (*e*).

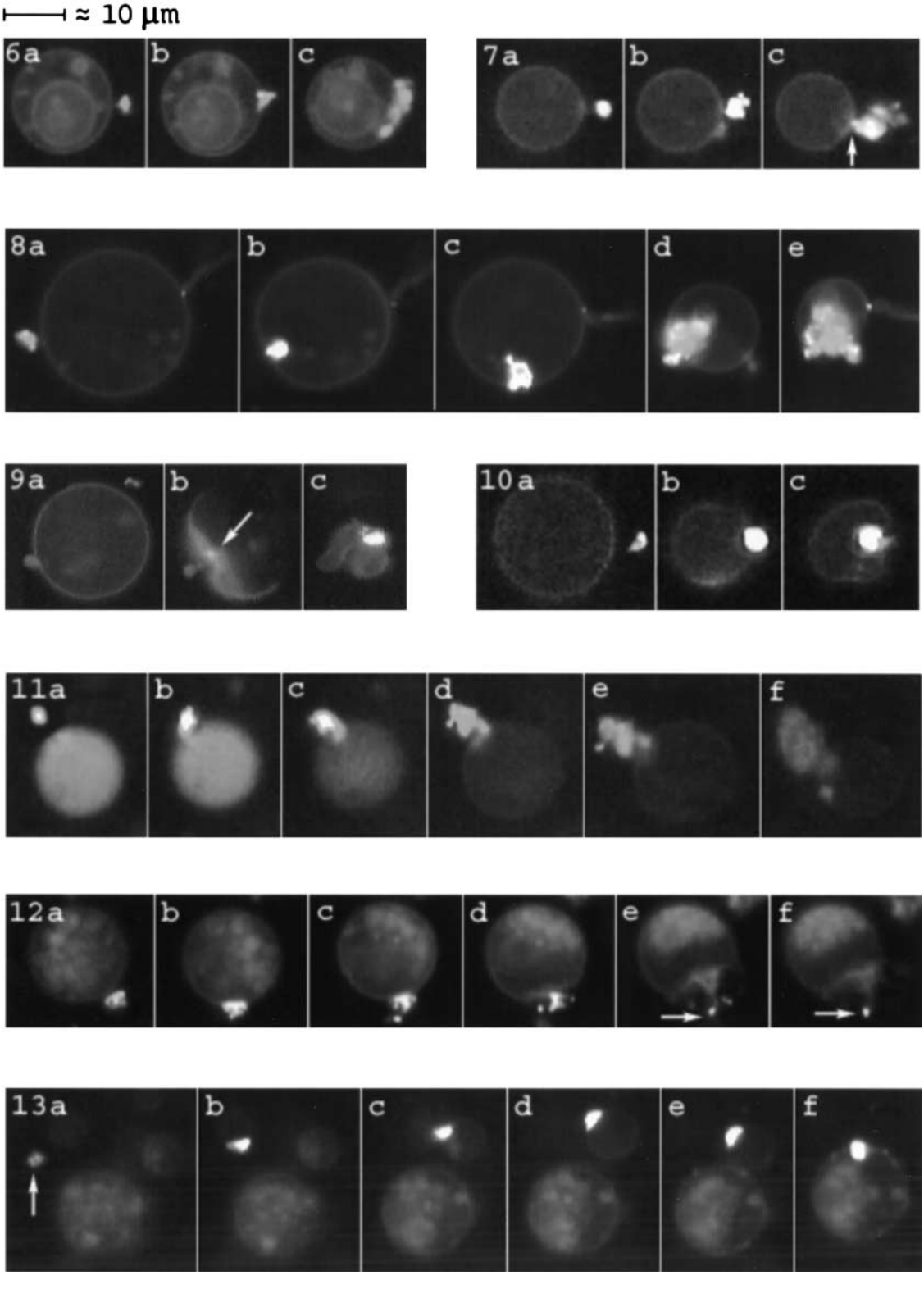
Fig. 9. Lipoplex-induced rupture of an anionic vesicle. A DNA/EDOPC complex (upper right; charge ratio 1:2) was brought into contact at approximately the one o'clock position on a DOPG (100%) anionic vesicle, which resulted in immediate bursting of the vesicle (*b*). Arrow indicates position of the complex immediately after rupture. After several seconds, the lipid reorganized into flaccid blobs that swirled around each other under the application of an electric field (*c*). The object at the lower left of the vesicle in the image in *a* and *b* is an attached ball of lipid, which was incidental to the lipoplex-vesicle interaction and is not shown in the drawings.

Fig. 10. Membrane perforation and rapid shrinkage of an anionic vesicle induced by lipoplex adhesion. A DNA/EDOPC complex interacted with a DOPG/DOPE (1:1) vesicle. The vesicle broke and shrank rapidly (*b*). About 6 seconds later, the membrane became flaccid (*c*).

Fig. 11. Membrane perforation (without rapid shrinkage) induced by DNA expansion. A DNA/EDOPC complex (1:2 charge ratio) labeled with R-PE was brought into contact with a calcein-loaded DOPG/DOPC (1:1) vesicle with an unlabelled membrane. After adhering to the vesicle (*b*), the complex began expanding away from the vesicle as the calcein leaked out and lipid from the complex diffused into the target vesicle membrane (*c*). Within seconds, the calcein escaped completely (*d*) and the DNA continued to expand as the target membrane (which was seen to be orange due to R-PE that diffused from the complex) began to shrink (*e*). The DNA reached its final expanded state after about 45 seconds, at which time the target vesicle had shrunk but had remained spherical (*f*).

Fig. 12. DNA expansion with formation of lipid tubular projections. A DNA/EDOPC complex (charge ratio 1:2), in which the lipid was labeled with DiO and the DNA was unlabelled, was brought into contact with a DOPG/DOPE (1:1) vesicle (*b*). After a few seconds, the fluorescence due to the lipid in the complex began disappearing concomitant with DNA expansion (*c*), which flattened the surface of the vesicle (*d*), indicating where the DNA was attached to the vesicle. Regions of DNA still covered by lipid are highlighted by arrows in (*e*) and (*f*). After 8 seconds, most of the lipid was released from the DNA and tube-like projections emanated from the vesicle (*f*). The vesicle contained smaller vesicles (not shown in the drawings), which were displaced as the DNA expanded, possibly due to negative-negative electrostatic repulsion between the DNA and the vesicles.

Fig. 13. Lipoplex-induced fusion of several anionic vesicles. A DNA/EDOPC complex (1:2 charge ratio) (*arrow*) was first brought into contact with the small vesicle above it. The complex bound to the vesicle (*b*). This vesicle subsequently contacted the small vesicle on the right (*c*), to which it fused (*d*). The fusion product then contacted the large vesicle (containing several internal vesicles) beneath it (*e*) leading to a second fusion event, the product vesicle of which is shown in (*f*). Anionic vesicles were DOPG/DOPE (1:1 charge ratio).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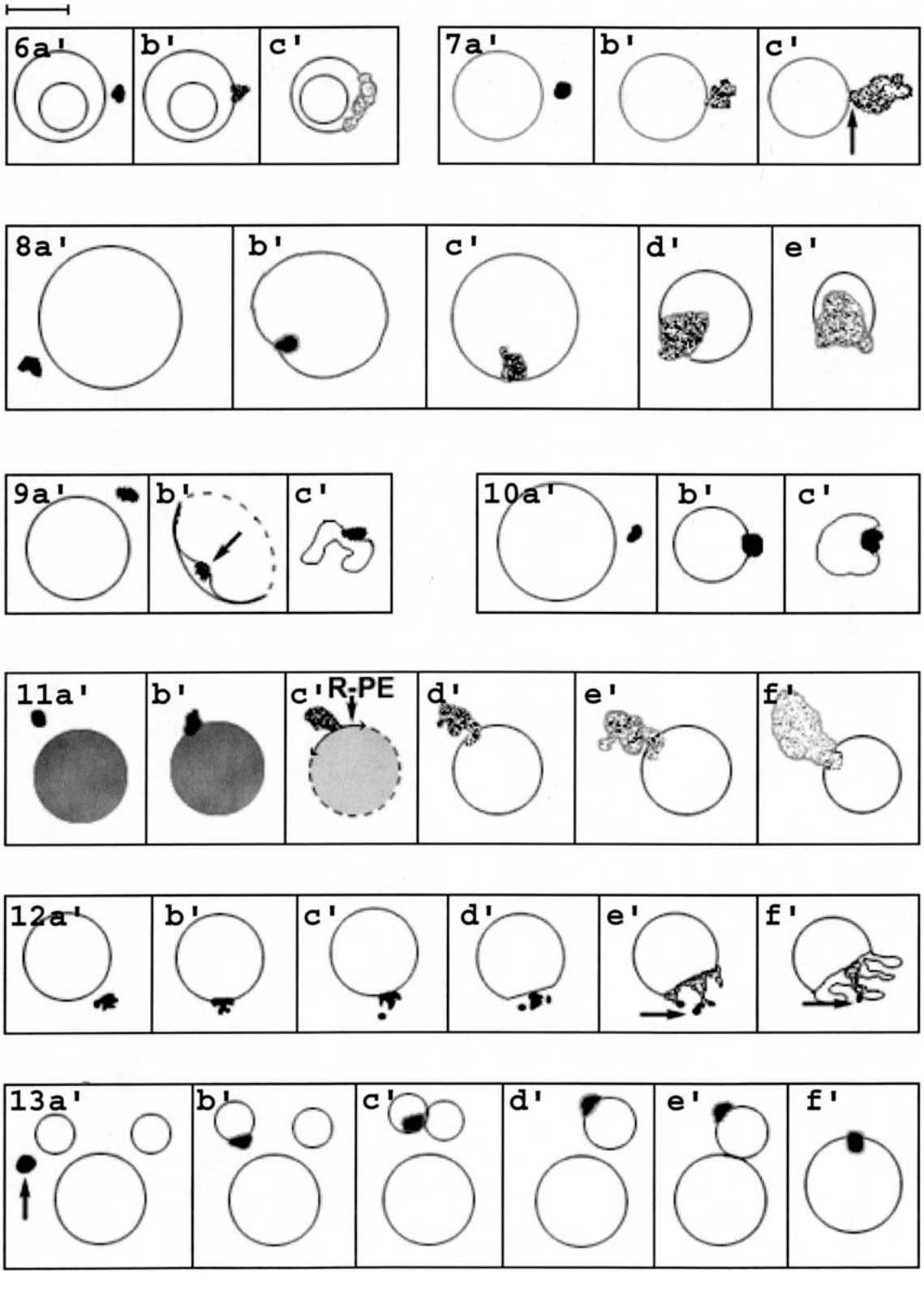
Figs. 6–13.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Figs. 6′–13′.

with the additional feature that upon contact the complex was injected *into* the vesicle through the perforation in its membrane, and the DNA expanded inside the vesicle. That the expanded DNA was inside the vesicle was evident from the fact that the rim of the vesicle and the expanded DNA were each most clearly in focus at the same focal plane.

Two thirds to three fifths of the contacts led to no DNA expansion. Bursting of the vesicle induced by the complex (event *a*) was frequent for DOPG (Fig. 9) and DOPG/DOPC vesicle compositions (35% and 23% respectively), but not for DOPG/DOPE vesicles (only 2%). DOPG/DOPE vesicles were instead much more prone to stable adhesion (event *b*, frequency 51%), while stable adhesion with DOPG and DOPG/DOPC vesicles occurred about as frequently as rupture (32% for both.) Barrier property loss induced by complex adhesion (events *d*, *e*) was relatively infrequent, but observed for all three compositions (6% DOPG, 18% DOPG/DOPE, 11% DOPG/DOPC.) In roughly half of these cases, the DNA subsequently expanded, following vesicle shrinkage (event *e*). Events (*d*) and (*e*), for all composition types, were often accompanied by loss of membrane integrity, as indicated by the appearance of flaccidity in the vesicle membrane (Fig. 10).

To assess whether perforation of the anionic vesicle membrane was the typical result of adhesion to the lipoplex, even in those cases where rupture of the vesicle did not ensue, a number of experiments was carried out in which the vesicle was loaded with the fluorescent dye, calcein. Release of calcein from an otherwise intact vesicle would indicate formation of a passageway for calcein to escape through the vesicle bilayer. In those cases involving DOPG/DOPC (1:1) vesicles (calcein experiments were not run for DOPG or DOPG/DOPE vesicles), lipoplex adhesion almost always occurred without content leakage. In all the events in which DNA expanded, the calcein was lost from the vesicle as the DNA began expanding. DNA expansion was also accompanied by vesicle shrinkage as was observed in the non-calcein experiments.

For some experiments, the fluorescent probe was omitted from the vesicle membrane and the complex was labeled with R-PE. As usual, DNA was labeled with YOYO-1. These experiments allowed us to determine whether and if so, when, lipid from the lipoplex became transferred to the vesicle bilayer. We observed that, during both adhesion and DNA expansion, the target vesicle membrane became visibly red, indicating that lipid from the complex had indeed diffused into the vesicle membrane and that this transfer could occur even in the absence of DNA expansion. Given this observation, it was not surprising that R-PE transfer was also observed in cases where the DNA underwent expansion (Fig. 11).

The lipid "lost" during DNA expansion (anionic lipid during vesicle shrinkage and cationic lipid released from DNA) must be accounted for somehow. Fig. 12 depicts a complex containing unlabelled DNA and DiO-labeled cationic lipid interacting with a DOPG/DOPE anionic vesicle (labeled with R-PE.) Although the DNA is invisible, it is clear from the disappearance of green fluorescence of the cationic lipid that the DNA had already expanded by the time of panel 12b. Toward the end of the expansion, flaccid, tube-like lamellar formations could be seen projecting from the vesicle along the DNA-vesicle contact area. These most likely account for at least some of the transferred lipid during DNA expansion and were most visible when the DNA was unlabelled.

Fusion between multiple DOPG/DOPE (1:1) vesicles in which one of them adhered to a complex was also observed (Fig. 13).

Discussion

DNA and CL Interactions

That uncoated EDOPC vesicles in deionized water usually ruptured immediately upon contact with stringy DNA is consistent with the observations by Kennedy et al. [11] on vesicle populations. In a buffer of low ionic strength, the electrostatic forces between the cationic vesicles and anionic DNA are much more intense and hence have a higher destabilizing effect on the vesicle membrane than at high ionic strength. Even at this low ionic strength, however, vesicles were seen to remain intact after acquiring a coating of DNA. This coating was comprised of much smaller and barely visible pieces of DNA as compared to the large clusters of DNA used to capture visible interactions on tape. Vesicles from the middle and late stages of experiments usually acquired such a coating, since by then electrophoretic manipulations had mixed many of the smaller DNA particles with the cationic vesicle suspension. These pieces of DNA were small and flexible enough so that they could easily conform to the surface contour of the vesicle as they adhered to it. Thus, anisotropic forces that lead to bending (and hence destabilization) of the vesicle membrane by the larger, less flexible pieces of DNA, are probably negligible with the smaller molecules of DNA. Since DNA-coated vesicles have much of their positive charge neutralized, they would be expected to behave more like vesicles in higher ionic-strength buffer (i.e., no immediate rupture, but aggregation and then rupture). Kennedy et al. [11] suggested that cationic vesicles that do not rupture immediately upon contact with DNA could come into contact with other DNA-coated vesicles, as the anionic DNA neutralizes their cationic charge. Upon contact, DNA-coated vesicles adhere to uncoated vesicles, creating asymmetrical forces that induce rupture of

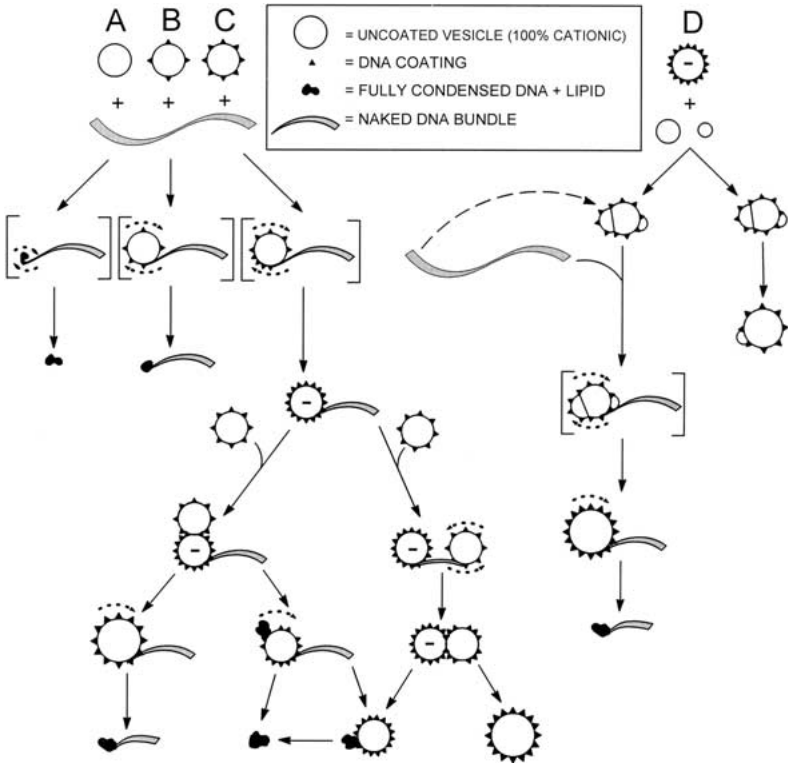

Fig. 14. Typical pathways of cationic vesicle – DNA interactions. When cationic vesicles (100% EDOPC) had no DNA coating they ruptured immediately upon contact with large pieces of DNA (*A*). When vesicles that were partially coated with DNA (but still positively charged), were brought into contact with large pieces of DNA, they rolled and twisted along the DNA for several seconds before they ruptured and collapsed onto the DNA (*B*). Heavily coated vesicles that were still somewhat positively charged rolled and twisted along the DNA until they came to a stop (*C*). The free piece of DNA or the stably wrapped vesicle was then brought into contact with another DNA-coated vesicle, to which it adhered, ruptured and sometimes fused. Vesicles that were coated with enough DNA so that their surfaces were anionic were seen to have adhered to and often hemifused with other cationic vesicles (*D*). With time, the hemifused vesicles within the aggregate sometimes fully fused with one another (right side). When these aggregates were brought into contact with large pieces of DNA, the vesicles within the aggregates often fused during rolling, and either ruptured or came to a stop and exhibited the events shown for (*C*). The density of positive charge on vesicles diminishes with increasing amounts of DNA; close-packed studs indicate that the surface is covered with DNA and hence such vesicles are negatively charged (indicated by a minus sign).

one or more vesicles. This was observed in our experiments, in which a DNA-coated cationic vesicle rolled along a strand of DNA (or induced bending in the case of sheaf-like DNA) and formed aggregates with other vesicles/lipid already present on the DNA that caused rupture of one or more of those vesicles. All the types of DNA-cationic lipid interactions that were observed are diagrammed in Fig. 14.

It is apparent that there is a limit to the extent that lipid can spread over DNA; lipid was observed to spread out over DNA beyond the region of contact, but when the supply of lipid was limited (i.e., the vesicle was small) the spreading stopped before all the DNA was covered. We suspect this occurs because the lipid cannot form a coating that is thinner than a bilayer.

Fig. 3 is significant in that it explicitly shows DNA-mediated fusion of cationic liposomes, which to date has only been demonstrated through indirect techniques that could not distinguish between lipid mixing due to vesicle collapse and lipid mixing due to vesicle fusion [10, 17, 21].

Lipoplex - Anionic Vesicle Interaction

The frequencies of the different types of lipoplex-anionic vesicle interactions were similar for DOPG/DOPC (1:1), DOPG/DOPE (1:1) and 100% DOPG anionic liposomes, with the exception of stable adhesion being significantly higher and immediate rupture lower for DOPG/DOPE (1:1). These interactions and their frequencies are described in Fig. 15. Lipoplex interactions with DOPG/DOPC (1:1), DOPG/DOPE (1:1) and 100% DOPG anionic liposomes were also similar to each other with respect to the rate and modes of DNA expansion. This result is consistent with that of another study on bulk suspensions, which found that maximum release of DNA was virtually independent of the amount of anionic amphiphiles added beyond a $-/+$ charge ratio >1 [1].

Vesicle shrinkage must be due to a loss of bilayer barrier properties and consequent loss of contents. The calcein experiments revealed that perforation of the vesicle membrane occurred even in those cases where shrinkage was not obvious. In some cases the calcein fluorescence diminished after a complex adhered to the vesicles, but in all cases involving DNA expansion, the calcein fluorescence diminished only after the already adherent complex began expanding. It is to be expected that the bilayer would be broken in some way, for otherwise it would seem that there could be no path for transfer of lipid from the vesicle to the DNA.

Injection of the lipoplex into the anionic liposome (Fig. 8) could occur through several possible pathways, 1) engulfment, 2) hemifusion or 3) pore formation. Engulfment would be due to mutual adhesion of the two membranes and can only occur when the vesicle is considerably larger than the lipo-

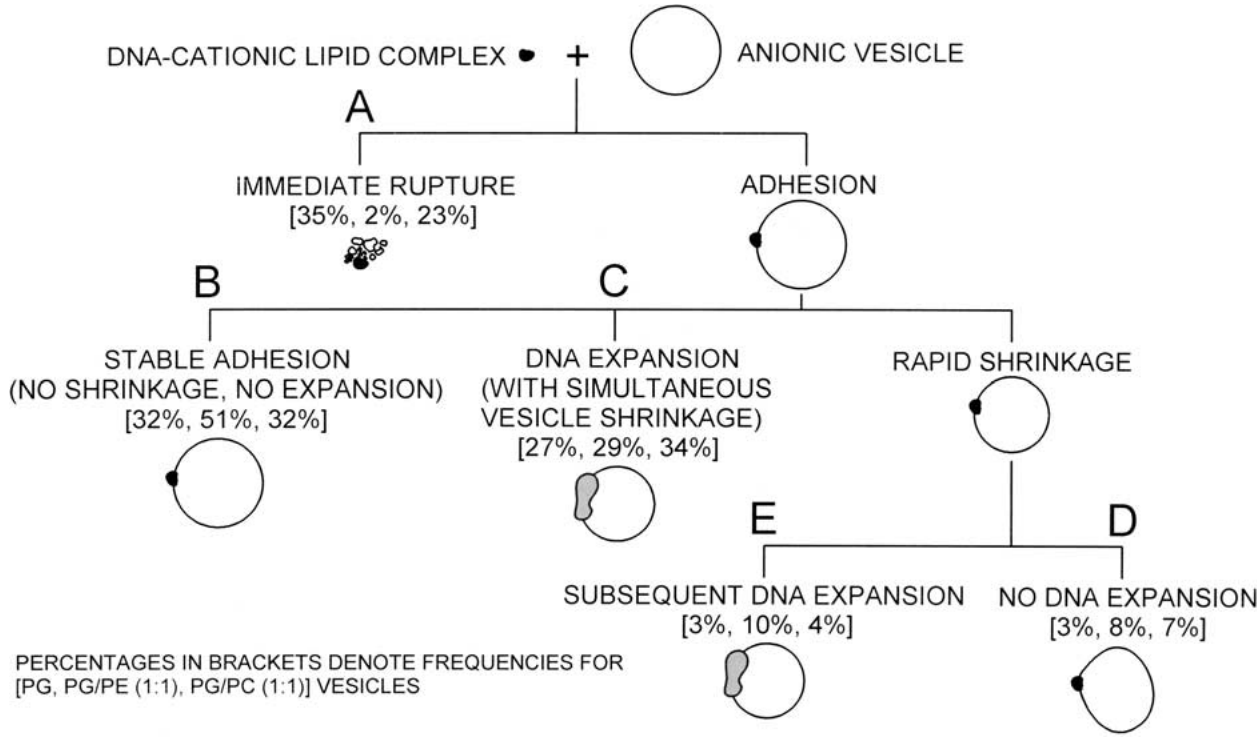

Fig. 15. Cationic lipid complex interactions with anionic vesicles. When DNA/EDOPC complexes (1:2 charge ratio) were brought into contact with anionic vesicles, they either ruptured the membrane (*A*, 35% frequency for interactions with DOPG vesicles, 2% for DOPG/DOPE (1:1) vesicles, and 23% for DOPG/DOPC (1:1) vesicles) or adhered to it. Adhesion led to three possible outcomes; stable adhesion with no vesicle shrinkage and no DNA expansion (*B*, 32% for DOPG and DOPG/DOPC vesicles, 51% for DOPG/DOPE vesicles), DNA release with simultaneous and slow vesicle shrinkage (*C*, 27% for DOPG vesicles, 29% for DOPG/DOPE vesicles and 34% for DOPG/DOPC vesicles), and rapid shrinkage caused by breaching of the membrane. The latter event took one of two subsequent courses: no DNA expansion (*D*, 3% for DOPG vesicles, 8% for DOPG/DOPE vesicles and 7% for DOPG/DOPC vesicles), or DNA expansion following breaching of the membrane (*E*, 3% for DOPG vesicles, 10% for DOPG/DOPE vesicles, and 4% for DOPG/DOPC vesicles). Both (*D*) and (*E*) exhibited membrane flaccidity and Brownian motion due to rapid increase in the ratio of membrane surface area to inner content volume, but to a higher extent when DNA was not released (*D*). 37 total interactions were observed for DOPG vesicles, 51 for DOPG/DOPE (1:1) vesicles, and 45 for DOPG/DOPC (1:1) vesicles.

plex or if the vesicle contains invisible invaginations or evaginations that would provide the extra bilayer for wrapping around the lipoplex. Decondensation of the DNA within the vesicle would require subsequent fusion or transfer of lipid by some mechanism. Hemifusion as a hypothesis would be consistent with interactions observed between oppositely charged vesicles of different sizes (Lei and MacDonald, unpublished). At typical compositions (lipid excess), the surface of the lipoplex is largely covered by a bilayer [14], so it should hemifuse in much the same way as do cationic lipid vesicles [7, 19]. When hemifusion occurs, the cationic and anionic lipids mix in the external monolayer and the change from head group repulsion to mutual attraction increases the tension in the external monolayer, effectively squeezing the core of the lipoplex into the large vesicle. Also in this case, the vesicle must be large enough or have sufficient extra membrane that its internal monolayer is able to envelop the entering lipoplex core. Neither this mechanism nor engulfment by the entire bilayer requires a breach in the vesicle, which we know occurs from the loss of calcein, although formation of a rend in the bilayer from DNA expansion would not be surprising for either mechanism. Finally, although a mechanism is not readily apparent, it may be that pore formation of some sort occurs upon contact of the lipoplex with vesicles, which leads both to calcein release and DNA entry. To the extent that an anionic vesicle resembles a membrane of the cell, any of these processes could occur during transfection.

That lipid may diffuse from the lipoplex into the anionic vesicle is apparent from electrostatic behavior. Evidence that cationic lipid from lipoplexes can not only neutralize, but even reverse the charge of the initially anionic vesicle is to be seen in Fig. 13. Following adhesion of the lipoplex with the target anionic vesicle, the latter fused with two adjacent anionic vesicles. Almost certainly, this could only occur if the first vesicle had acquired a positive charge. Of course, a net change in charge is only possible when the lipoplex has excess positive charge relative to negative charge of the vesicle. Our observations are consistent with previous findings [1, 13] of extensive lipid mixing

occurring when DNA/CL complexes were added to anionic amphiphiles, although those studies say nothing about the direction of lipid transfer.

The amount of anionic lipid (in the form of oleic acid) required to release DNA from EDOPC lipoplexes is slightly more than one equivalent of charge [15], indicating that anionic and cationic lipid neutralized each other to form a phase that is more stable (presumably because both are hydrophobic) than the cationic lipid-DNA interactions. Hence, the release of DNA will occur only after anionic lipid has neutralized all free cationic lipid. Thus, the pool of anionic lipid from a vesicle must equal the cationic lipid in ion pairs with the DNA, plus any free cationic lipid. Given the known phase behavior of mixtures of cationic and anionic lipids, [22] it is likely that cubic and hexagonal phases result, at some point, from the interaction.

The anionic vesicles we chose were roughly the same size, but lipoplexes are inherently heterogeneous, and when examined individually, varied considerably in both size and composition. It is thus to be expected that the extent to which DNA is released from a complex by anionic vesicles should vary considerably from instance to instance, ranging from barely discernable to virtually complete.

The tendency of DNA complexes to stably adhere to DOPG/DOPE (1:1) vesicles without rupturing them (as in the cases of DOPG and DOPG/DOPC (1:1) vesicles) indicates that DOPE may help stabilize anionic membranes against the disruptive effects of CL-DNA complexes. This stabilizing effect may in part account for the helper lipid properties of DOPE when used in certain CL-DNA complexes [2, 3, 13].

For convenience in preparation of giant vesicles, we have done most experiments at low ionic strength, but we have had experience with high ionic strength, both with lipoplexes and with membrane-membrane interactions [19] and the primary difference has been in rate and extent of interactions. As would be expected, higher ionic strength diminishes electrostatic interactions, so processes are slower and sometimes less extensive at high salt concentrations. The differences tend to be quantitative, however, and we judge that it was more important to employ conditions that would allow acquisition of a statistically valid selection of data and the opportunity to gain insight into processes that have not previously been investigated.

Although the resolution of the light microscope is obviously too low to elucidate molecular processes, it is clear that, given giant vesicles and the ability to manipulate them easily, a considerable amount of information can be obtained on lipoplex formation and their interactions. We anticipate that with more sensitive cameras and with quantitative measurements of fluorescent intensities it will be possible to clarify additional details of these processes.

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