Lipoplex Formation under Equilibrium Conditions Reveals a Three-Step Mechanism

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ABSTRACT Cellular transfection can be accomplished by the use of synthetic amphiphiles as gene carrier system. To understand the mechanism and hence to improve the efficiency of transfection, insight into the assembly and properties of the amphiphile/gene complex is crucial. Here, we have studied the interaction between a plasmid and cationic amphiphiles, using a monolayer technique, and have examined complex assembly by atomic force microscopy. The data reveal a three-step mechanism for complex formation. In a first step, the plasmids, interacting with the monolayer, display a strong tendency of orientational ordering. Subsequently, individual plasmids enwrap themselves with amphiphile molecules in a multilamellar fashion. The size of the complex formed is determined by the supercoiled size of the plasmid, and calculations reveal that the plasmid can be surrounded by 3 to 5 bilayers of the amphiphile. The eventual size of the transfecting complex is finally governed by fusion events between individually wrapped amphiphile/DNA complexes. In bulk phase, where complex assembly is triggered by mixing amphiphilic vesicles and plasmids, a similar wrapping process is observed. However, in this case, imperfections in this process may give rise to a partial exposure of plasmids, i.e., part of the plasmid is not covered with a layer of amphiphile. We suggest that these exposed sites may act as nucleation sites for massive lipoplex clustering, which in turn may affect transfection efficiency.

INTRODUCTION

Delivery of genes into mammalian cells, the purposes of which range from applications in cell biology in vitro to the development of gene therapy in vivo, can be accomplished by various techniques. Viral vectors usually display the highest efficiency of transfection. However, particularly in vivo, this application suffers from various disadvantages, such as a possible immune response and virus-associated toxicity. In recent years, a number of cationic polymer and amphiphile delivery systems have been developed, collectively known as non-viral vectors, which, in spite of a lower efficiency of delivery, offer some advantages over the viral systems. Thus, the use of cationic delivery systems as transfection agents is usually accompanied with a diminished immunological reaction, if any, whereas their handling is relatively easy.

Thus far a host of amphiphiles has been synthesized and shown to be effective in gene delivery to various extents (Felgner, 1996; Lee et al., 1996; Van der Woude et al., 1997). The choice of their synthesis is usually dictated by trial and error experiments. An obvious prerequisite is the cationic charge, which provides a means for electrostatic interaction with the DNA phosphate, resulting in compaction of the nucleic acid by and complexation with the amphiphile, thereby causing its shielding from attack by exogenous and endogenous DNase. The eventual behavior of the amphiphile/DNA complex is, among others, dependent on the charge ratio and the size of the particle. Toxicity of the complex, as described for many of such complexes, may depend upon the nature of the aggregate formed (Lawrence, 1994). Pertinent to sustaining such toxicity effects may be their biodegradability and the cell's capacity to eliminate a particular surfactant. It is therefore important to obtain insight into the mechanism by which these complexes are internalized by cells and deliver the gene of interest. Crucial to the mechanism of delivery is likely the relative ease by which the gene and amphiphile dissociate (Zabner et al., 1995; Dowty et al., 1995, Szoka et al., 1996, Xu and Szoka, 1996, Zanta et al., 1999). It would appear that such dissociation requires a critical timing, as the probability that free DNA efficiently finds its way to and into the nucleus is rather slim. Insight into parameters that determine stabilization and destabilization of these complexes, which are intricately related to their efficiency in gene delivery, requires an understanding of factors that govern amphiphile-DNA interaction and subsequent complex formation. As a first step toward revealing a structure-function relationship of a lipoplex, we have investigated the interaction and complex formation between SAINT-2, a highly efficient and virtually nontoxic amphiphile in cell transfection (Van der Woude et al., 1997), and a plasmid. Here we visualized the single steps in complex formation, by employing a film balance technique, which allows complex formation under equilibrium-like conditions. Single steps of the complex formation were then visualized with high resolution by atomic force microscopy. Our data demonstrate that the size of the complex is determined by the size of the supercoiled plasmids, and that a plasmid can enwrap itself with several lamellae. Fusion of such individually wrapped

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plasmids appears to give rise to the eventual assembly of the DNA/amphiphile complex.

MATERIALS AND METHODS

Plasmid preparation

pCAT plasmids (Promega, Madison, WI, 4.7 kb) were purified from *Escherichia coli* using a Qiagen Maxi-Prep Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. pGFP Plasmids (Clontech, Palo Alto, CA) were prepared and handled similarly as described above for the pCAT plasmids.

Water was used for the storage of the plasmids so as to avoid interactions between buffer salts and cationic amphiphile during the complex formation.

The purity of the plasmid preparation was examined by agarose gel electrophoresis. 0.5 μg pCAT DNA (stock solution 0.87 μg/ml in TE buffer) was diluted in 25 μl Hanks' balanced salt solution and loaded on a 0.8% agarose gel containing 1.25 μM ethidium bromide. The electrophoresis was performed for 1 h at a current of 40 mA in TBE buffer. DNA bands were subsequently visualized under UV light and photographed.

Complex formation

As cationic lipid, we employed N-methyl-4(dioleyl)methylpyridiniumchloride (SAINT-2), which was synthesized as previously described (Van der Woude et al., 1997; Meekel et al., 2000).

On a film balance of the Wilhelmy type, 20 μl SAINT-2, solubilized in chloroform at a final concentration of 1 μM, were spread over a subphase of ultrapure water. After 60 s, the chloroform was evaporated and a monolayer of SAINT-2 was formed, its isotherm being consistent with its fluid phase properties (Fig. 1 *a*). The monolayer was then compressed at 25°C to a surface tension of 25 mN/m. After injecting 20 μg plasmid with a Hamilton syringe through the amphiphile monolayer into the subphase, the surface tension changed to lower pressures showing a crystallization and a loss of cationic lipid to the subphase. After approximately 30 min, the system reached an equilibrium-like state, characterized by a stabilized surface tension (Fig. 1 *b*).

To monitor complex formation, the monolayer with interacting plasmid was transferred by the Langmuir-Schaefer technique (Hagting et al., 1999) to a silicon wafer. After cooling for 3 min on air in a box of dry ice, the sample was examined by atomic force microscopy in the Tapping Mode (Digital Instruments, Santa Barbara, CA).

Atomic Force Microscopy

The measurements were done with a Digital Nanoscope IIIa Dimension 5000 (Digital Instruments, Santa Barbara, CA). The microscope is vibration-damped and installed in an acoustic isolated steel box. Conventional pyramidal Si_3N_4 tips on a cantilever with a length of 100 μm were used. Sample examination was performed in the Tapping Mode (Hansma et al., 1993) to prevent damage to the sample surface, but allowing repeated examination of the same sample region. The tapping frequency was about 9 kHz, and the oscillation amplitude of the tip was set at 20 nm. The force applied to the sample was between 100 and 300 pN. The scan speed was proportional to the scan size and the scan frequency was between 0.5 and 1.5 Hz. Images were obtained by displaying the amplitude signal of the cantilever in the trace direction, and the height signal in the retrace direction, both signals being simultaneously recorded. The results were visualized either in height or in amplitude modus.

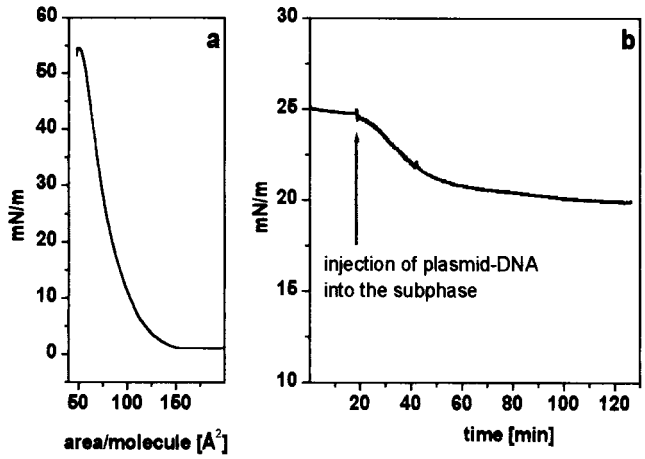

FIGURE 1 Isotherm of SAINT-2 and change in surface pressure of a SAINT-2 monolayer DNA/amphiphile interaction. (*a*) Isotherm of a SAINT-2 monolayer taken at 25°C. The shape of the curve reveals a fluid state of the cationic lipid from the liftoff at 150 Å^2/molecule until the collapse of the monolayer at 51 Å^2/molecule. Note that due to the pyridinium ring in the headgroup, the minimal area per molecule is slightly larger than that of a fluid phospholipid, e.g., DOPC. Based upon this isotherm, the monolayer was compressed to a lateral pressure of 25 mN/m (*b*). Subsequently, pCat plasmid was injected into the subphase. An instant drop of the lateral pressure is caused by a local clustering of cationic lipids and/or the loss of lipids (upon interaction with pCat) into the subphase (see text). After approximately 30 min, the lateral pressure reached an equilibrium and subsequently changed only marginally.

Preparation of vesicles from the cationic amphiphile SAINT 2

Small unilamellar vesicles (SUV) were prepared by ultrasonication of a multilamellar vesicle (MLV) suspension. Briefly, to a dried film of 1 mM SAINT-2-amphiphile 1 ml ultrapure water was added followed by vigorous vortexing at room temperature. The resulting MLVs were then sonicated in an ultrasonication bath for 20 min, when a clear solution of small unilamellar vesicles was obtained (see Fig. 2 *d* in Results; Van der Woude et al., 1997).

Lipoplex formation with vesicles in bulk phase

A 500-μl volume of 3 mM SUVs from pure SAINT-2 and 500 μl 0.5 mM pCAT plasmid (3:1 charge ratio) were mixed and left at room temperature for various time intervals, as indicated.

For atomic force microscope (AFM) examination, complexes were transferred onto silicon wafers, which were used as sample holders, by a dipping procedure. Handling and examination of the samples were performed as described above.

Lipid mixing assay

Lipid mixing was monitored by an assay based on resonance energy transfer between two lipid probes (Struck et al., 1981). SAINT-2 vesicles (50 nmol) labeled with both 0.5 mol% N-NBD-phosphatidylethanolamine (N-NBD-PE) and 0.5 mol% N-Rhodamin-phosphatidylethanolamine (N-Rh-PE) were diluted with 200 nmol unlabeled SAINT-2 vesicles in 1.5 ml ultrapure water. After addition of 1.3 nmol DNA, the increase in NBD fluorescence was measured, which reflects the lipid mixing between the

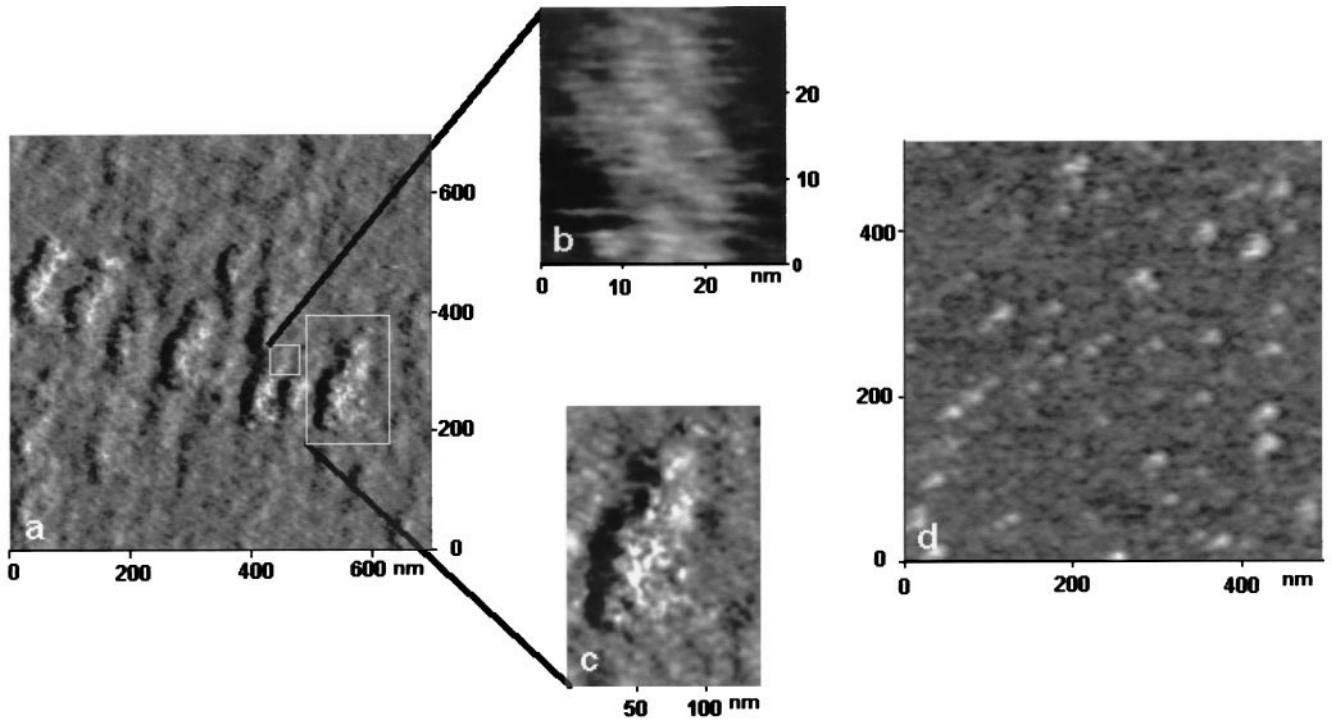

FIGURE 2 pCAT plasmids and vesicles, composed of SAINT-2, as visualized by AFM. The plasmids (*a* and *c*, zoom), prepared on silicon supports as described in Materials and Methods, show a typical supercoiled appearance with a length of approximately 200 nm and a width of 40 nm. In (*b*), the detailed structure of one plasmid is shown, revealing two double-stranded helices of DNA forming again a helix. (*d*) SAINT-2 vesicles are shown, most of which display diameters of approximately 50 nm. Note their fairly homogeneous distribution.

two vesicle populations. The 100% lipid mixing was determined by measuring fluorescence after adding 20 μl 10% Triton X100 solution, which was corrected for detergent quenching of NBD fluorescence, and taking into account the ratio of labeled versus unlabeled vesicles.

RESULTS

Characterization of amphiphilic vesicles and plasmids by AFM

AFM provides excellent possibilities for obtaining detailed insight at the structural and even molecular level (Hansma, 1996). The advantage is particularly apparent in case of size limitations and sensitivity to preparation methods. Thus AFM is able to visualize samples with high resolution, and when applied in the Tapping Mode, in which the tip of the AFM touches the sample only slightly, structural damage can be avoided. Such an approach also allows for repeated scanning of the sample for detailed investigation. A further improvement of the resolution can be obtained by applying higher tapping forces. To be able to increase the tapping forces without damaging the sample and to prevent the material from sticking to the AFM tip, which would make visualization impossible, we hardened the material by cooling the sample to approximately 0°C, taking care to avoid generating water crystals.

To properly interpret the various steps in DNA-amphiphile complex assembly, we first examined the appearance of amphiphilic vesicles and plasmids separately. Fig. 2, *a* and *c*, shows the self-assembled pure extract of the plasmids on silicon, as revealed by AFM. The sizes of these pure plasmids are about 200 nm in length and 50 nm in width, while they are oriented in a parallel fashion on the surface of the silicon wafer. Note that most of the plasmids are in the supercoiled form and little if any open circular or broken plasmids are detectable in these samples. Entirely consistent with their microscopic appearance was their chromatographic behavior upon separation on agarose gels. As shown in Fig. 3, the purified plasmid also reveals a supercoiled form (>95%), implying that the underivatized silicon does not affect the structure of the plasmids, which is a priori anticipated since both are hydrophilic and negatively charged. Interestingly, the supercoiled plasmid displays a fairly compacted structure, because between single strands only small grooves are visible. Occasionally in these compact structures the helix of two double-stranded DNA helices can be distinguished (Fig. 2 *b*). After measuring the helix properties we calculated an eightfold cording of the plasmid DNA to form the visualized supercoiled plasmids.

In Fig. 1 *d*, the appearance of the other partner in lipoplex formation, i.e., the cationic amphiphile SAINT-2, is shown,

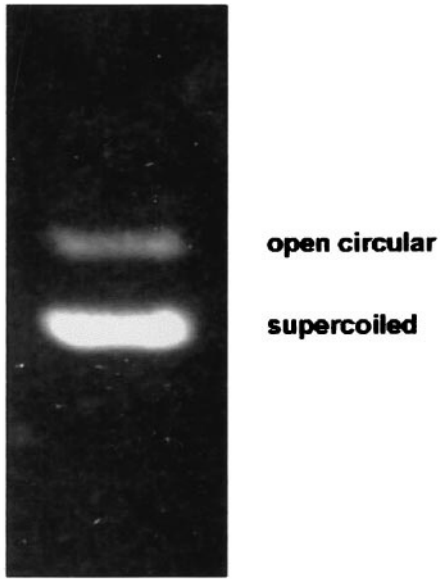

FIGURE 3 Analysis of purified pCat plasmid by agarose gel electrophoresis reveals the almost exclusive presence of supercoiled plasmid. After purification with a Qiagen Midi Prep, the plasmid preparation was analyzed for purity and secondary structure. On a 0.8% agarose gel, labeled with ethidium bromide, two fluorescent bands appear. The upper band reflects the presence of open circular DNA, the lower band that of supercoiled DNA. Note that the pCat plasmid is present almost exclusively in the supercoiled form.

of which sonicated vesicles were prepared as described in Materials and Methods. To prepare a vesicle sample for inspection by AFM, a silicon wafer was dipped into the vesicle solution. The micrographs of this sample reveal that the vesicle distribution is very homogeneous, showing a vesicle diameter of around 50 nm. The vesicle population is stable, because neither vesicle clustering nor a size increase due to fusion was seen, when the sample was kept at room temperature for 48 h. It is relevant to note that the size of the vesicles is much smaller than the size of the plasmid. The size of the plasmid, rather than that of the vesicles, will therefore determine the size of the resulting lipoplex.

Complex formation under equilibrium conditions

To monitor the formation of the DNA-amphiphile complex, experimental conditions were required such that its formation could be monitored under controlled conditions. Such conditions would differ from bulk phase vesicle-plasmid interactions, the kinetics of which are too fast to allow distinct steps to be visualized. Application of a film balance appeared to be a suitable alternative, as complex formation can take place at a surface boundary phase at near-equilibrium conditions. Kinetically, the latter conditions can then be regarded as monitoring complex formation by slow motion, when compared to the kinetics in bulk phase. Thus a monolayer of the cationic amphiphile SAINT-2 was formed on a film balance, and the plasmid was subsequently injected into the subphase, as described in detail in Materials and Methods (Fig. 1). Surfacing DNA is then able to interact with the amphiphile in a quasi-equilibrium state. As a result, cationic lipid is locally clustered when interacting electrostatically with the plasmid at the interface, and the ensuing amphiphile/DNA complex may subsequently enter the aqueous subphase. In either case a decrease in monolayer surface tension is anticipated (Fig. 1 *b*, *arrow*). In conjunction with visualization of this event with AFM, we were able to distinguish the different stages of the assembly of the transfection complex by this approach. As demonstrated in Fig. 4 *a*, plasmids interacting with the monolayer

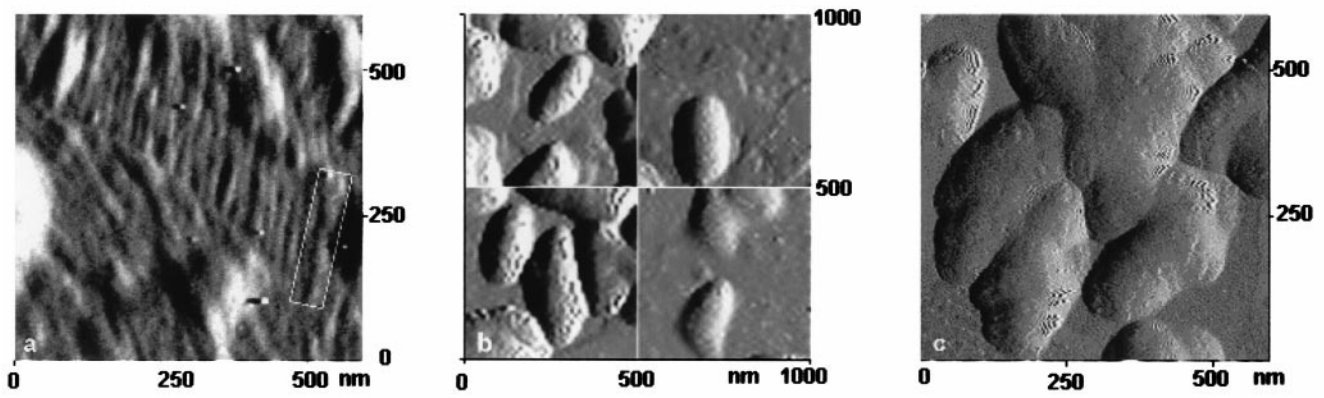

FIGURE 4 Assembly of plasmid-amphiphile complexes according to a three-step mechanism monitored in a monolayer system. (*a*) The plasmids (square) are ordered according to a parallel orientation, when interacting with the SAINT-2 monolayer. Subsequently (*b*), the amphiphilic molecules are wrapped around single plasmids, the outer layer presumably being a bilayer (see text). Note a typical bean-like appearance of the plasmid/SAINT-2 complexes. In a third step (*c*), several single bean-like structures are aggregated in a parallel or perpendicular orientation. Smooth boundary phases suggest that assembly of the larger complexes may involve a fusion step.

are ordered in the same way as the free plasmid, present in dispersion (Fig. 2 *a*). The plasmids are still supercoiled but show a more stretched appearance, as reflected by an increase in the average size from 200 nm to approximately 300 nm in length, whereas the width decreases slightly to 30 nm. Also in this case a strong parallel ordering of the plasmids, relative to each other, is apparent.

Since DNA is highly water-soluble, it is evident that not all plasmids surface simultaneously. As a consequence, complex formation at different stages of progress can be distinguished in the sample, which is reminiscent of the process of complex formation when vesicles interact with DNA in bulk phase. Hence, in the same sample (Fig. 4 *b*), bean-like complexes with a smooth surface are seen, of a size that is slightly smaller than that of the pure plasmids. Thus, in this case, the mean length of the lipoplexes is approximately 200 nm, whereas the width now ranges between 80 and 100 nm. In this sample, distinct plasmid structures are no longer visible, suggesting that essentially all DNA structures have been covered with a bilayer of lipids. Accordingly, the assembled lipoplexes did not remain localized at the air/water interphase, but rather submerged into the aqueous subphase. Note that in the presence of an organic phase, such as that described in Reimer et al. (1995), the lipids would likely only form a monolayer and partition into the hydrophobic environment, as reported.

Intriguingly, the bean-like structures cannot be considered as stable end products of the interaction of plasmid and amphiphile, since, as revealed in Fig. 4 *c*, single complexes tend to aggregate. Also the aggregation process appears to occur in well-ordered manner, as the "beans" aggregate parallel to each other or show a perpendicular orientation. Interestingly, at boundary phases within the aggregates, distinct contours of individual "beans" are no longer apparent; the smoothness of the plane of interaction suggesting that fusion may have taken place. Indeed, when preparing a mixture of unlabeled SAINT-2 vesicles and SAINT-2 vesicles containing 0.5 mol% each of N-Rh-PE and N-NBD-PE followed by addition of plasmid, lipid mixing (Struck et al., 1981) was observed (Fig. 5), indicating the potential of the SAINT-2 system to undergo fusion. The degree of lipid mixing is consistent with the DNA/vesicle ratio present in the incubation mixture. Also note, that the increase in size causes a slow sedimentation of the complexes, as reflected by the gradual decrease in the fluorescence tracings. Finally, when fluorescently labeled SAINT-2/plasmid complexes, prepared as described in Materials and Methods, were mixed with an excess of unlabeled SAINT-2, no significant lipid dilution was observed over a time interval of 30 min (not shown), the fluorescence development being essentially indistinguishable from the baseline, shown in Fig. 5. These data thus suggest that lipid mixing occurs largely during the initial stages of complex assembly.

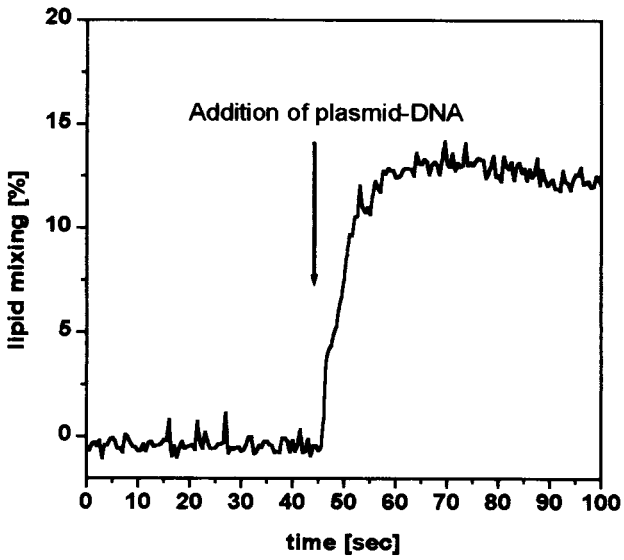

FIGURE 5 Lipid mixing of SAINT-2 vesicles induced by plasmid DNA. 50 nmoles SAINT-2 vesicles were prepared as described in Materials and Methods, containing 0.5 mol% each of N-NBD-PE and N-Rh-PE, and mixed with 200 nmoles unlabeled SAINT-2 vesicles. After adding of 6.6 nmoles plasmid (*arrow*), an increase in NBD fluorescence reflects the lipid mixing between the two vesicle populations.

Lipoplex formation with cationic liposomes

Next, it was of interest to determine whether there was any difference in the structural features of amphiphilic vesicles interacting with plasmids in bulk phase, and those observed under equilibrium conditions. Fig. 4 *a* illustrates the result of such a vesicle-induced bulk phase complexation. Immediately after complexation, spherical lipoplexes with a diameter of approximately 200 nm can be distinguished. Interestingly, the size distribution of the lipoplexes is fairly homogenous, and appears to be closely related to the length of single plasmids. For several hours the size of the particles remained unaltered. However, when left for 24 h, larger complexes can be distinguished, showing sizes up to 2.5 μm in diameter (Fig. 6b). Nevertheless, a substantial fraction of the original small lipoplexes remained. These particles were still present as single complexes and did not show a tendency to aggregate. Note that occasionally, supercoiled DNA-stretches appear as worm-like structures on the surface of these lipoplexes, their size suggesting that they are only partly covered by the amphiphile.

DISCUSSION

Thus far, little insight has been obtained into the structure-function relationship of lipoplexes. Their appearance has been discussed in terms of multilamellar aggregates of DNA (Gustafsson et al., 1995; Radler et al., 1997), rod-shaped structures (Gershon et al., 1993), DNA coating on the sur-

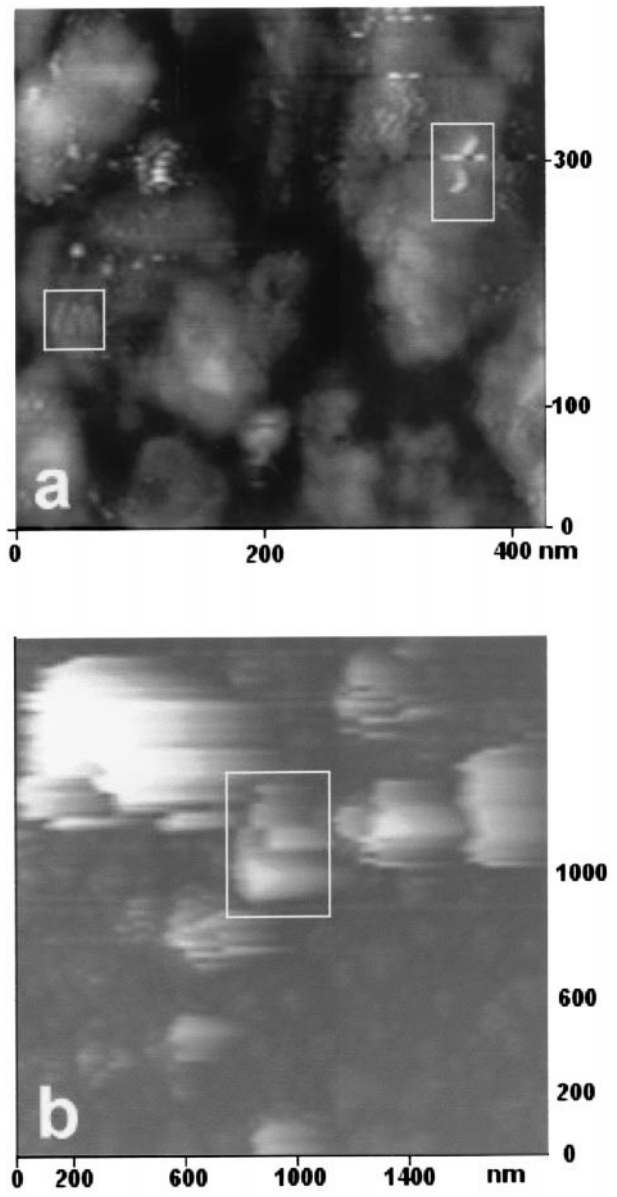

FIGURE 6 Lipoplex formation in bulk phase, as visualized by AFM. Twenty nmoles of SAINT-2 vesicles were mixed with 1 μg of plasmid DNA in water. A sample for examination by AFM was prepared immediately by dipping the silicon wafer in the solution (*a*). Note the occasional presence (*square*) of plasmid structures on the surface of the complex. After 48 h in solution, kept at room temperature, large lipoplex structures are seen (*b, square*), although complexes of the size seen immediately after preparation (*a*) are also still visible.

face of cationic liposomes (Eastman et al., 1997), or bead-on-string-like complexes (Sternberg et al., 1994). Evidently, differences in morphological appearance affect transfection efficiency, implying that insight into structural parameters that govern complex assembly and packing morphology is highly relevant.

By AFM, the pCAT plasmid appeared almost exclusively as supercoiled DNA, which, in the absence of external forces, is the most stable and most condensed structure for a plasmid. From the difference between the size of pure supercoiled DNA and the size of the complexes, we conclude that the condensing effect is not significant during the complexation of the plasmid with SAINT-2-amphiphiles. This seems reasonable in the context of the notion that DNA condensation only becomes possible when the secondary structure changes. Accordingly, these considerations emphasize the importance of characterizing the initial structural state of the plasmid DNA, i.e., before the addition of the cationic lipid, in order to appropriately evaluate and appreciate the condensing effect of the surfactant. In this regard it should be noted that the mechanism of condensation of an open circular plasmid differs considerably from that of a supercoiled structure (Golan et al., 1999).

Condensing effects are often measured by using a fluorescent dye, which intercalates into the plasmid (Gershon et al., 1993). The decrease of fluorescence is then interpreted as DNA condensation, causing release of the dye from the helices. However, it is uncertain whether this conclusion is justified, because the decrease of fluorescence may be a reflection of a substitution of charged molecules, rather than a change of the size of the molecule. Our observations thus call for careful interpretation, indicating that the nature of the starting plasmid material should be taken into account (Ogris et al., 1998), when claiming condensation as a first and necessary step in the mechanism of plasmid/cationic lipid complex formation.

Under near-equilibrium conditions, using a film balance approach, we were able to visualize lipoplex assembly and to define distinct steps in the mechanism of plasmid/amphiphile complex formation. Our results suggest a three-step mechanism. In a first step, supercoiled plasmid DNA interacts electrostatically with a monolayer of the cationic amphiphiles. Apparently, the nature of this interaction forces the plasmid into a parallel orientation under the monolayer, which a priori is not unexpected, given the predicted long-range orientational correlation between DNA helices (Leforestier et al., 1995). The charge interactions also led to a slightly stretched form of the plasmid, when compared to its appearance on the (low-charged) silicon. Such a stretching, induced by charge interactions, would be consistent with the effect of more prominently charged mica surfaces on the plasmids, which leads to a single-stranded appearance of plasmids (Hansma et al., 1998).

In a second step, bean-like structures arise. Given the relatively smooth surface of the lipoplexes and their dimensions, their appearance is interpreted to result from the coating of single supercoiled plasmids with a (bi)layer of cationic lipids, the charged amphiphile head groups facing the hydrophilic environment in which the complex is formed. The more compact form of the pure plasmid DNA evidently dictates the size. The width of the lipoplex is larger and in fact, the increase in width from 30 to 50–70 nm would suggest that the plasmid is surrounded by 3–5

bilayers of the amphiphile (Fig. 7). In this process, it is likely that the asymmetric localization of the plasmid will affect the packing properties of the aligned bilayers, the reaching of which likely depends on the number of lamellae surrounding the plasmid. Thus local defects may arise, the high free energy of which can be dissipated upon merging with a similarly perturbed structure, present on an adjacent complex. This event is indeed visualized as a concomitant and third step in lipoplex assembly, after initial coating of individual plasmids. In several studies (Escriou et al., 1998; Behr, 1986) this phenomenon has been described, but not visualized.

It should be noted that such sequential steps are not readily distinguished during bulk phase formation, i.e., upon mixing of plasmid and vesicles, the normal procedure for the preparation of these complexes. In this case, addition of the plasmid immediately leads to the aggregation of several vesicles, followed by rupture and reorganization of the bilayer structure, as revealed by our lipid mixing experiments. As a result, globular complexes are formed with a diameter that matched the length of single plasmids (approximately 200 nm; Fig. 6 *a*). Thus, as observed in the monolayer experiments, also in bulk phase the size of the lipoplexes, was dependent on the size of the plasmids, implying that the condensing effect was fairly small.

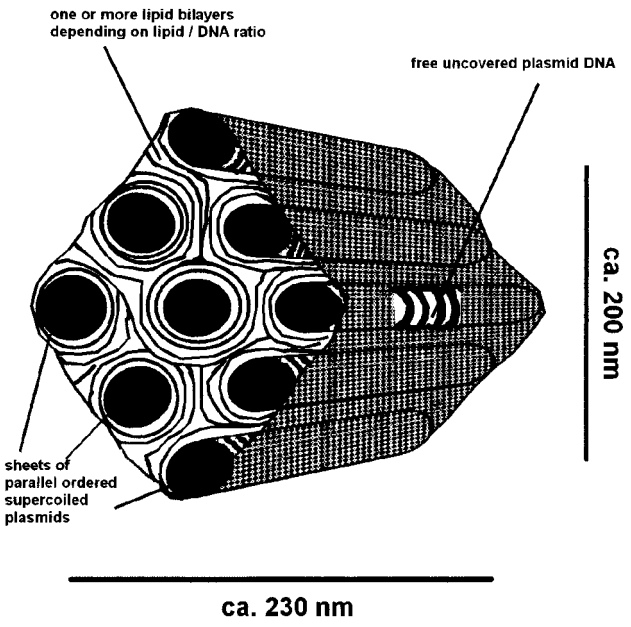

FIGURE 7 Schematic structural organization of a lipoplex, formed under bulk phase conditions. The monolayer experiments indicate the formation of single plasmids wrapped into several layers of amphiphile, which subsequently merge. Based upon bulk phase experiments, the size of the entire complex is roughly around 200 nm in diameter, which implies that the SAINT-2 lipoplexes contain approximately 9 to 16 plasmids. Occasionally parts of plasmids are exposed (cf. Fig. 4; for further details, see text).

Interestingly, distinct differences were observed in the latter system. In bulk phase, the coverage of the plasmid was less complete than in the monolayer system. Thus, taking into account the size of the plasmid, its partial exposure on the vesicle surface was often detected in the bulk phase system, a feature that has been noted before (Eastman et al., 1997; Behr, 1986). In fact, these plasmids could be labeled with ethidium bromide, but were protected from degradation by DNase (data not shown). These data suggest that sheets of bilayer, rather than recruitment of individual molecules, are wrapping the plasmids in bulk phase according to a mechanism in which entire bilayers absorb to and fold along DNA charges (Huebner et al., 1999). Their growth in size may then occur analogously as described in steps two and three for the monolayer system, thus giving rise to a multilamellar organization of amphiphile bilayers, alternating with an orientational ordering of plasmids between the sheets, as driven by long range interactions (Fig. 7; Leforestier et al., 1995). Moreover, elsewhere (Meekel et al., 2000) we have shown by cryo-electron microscopy that complexes of SAINT-2 and plasmid are superimposed with a clear fingerprint-like pattern, which is thought to originate from DNA organized into parallel helices, sandwiched between amphiphile bilayers. Indeed, original amphiphilic vesicles (diameter of 50 nm) were no longer visible after complexation. This would be consistent with the notion that (i) a total reorganization of the lipids occurs (Fig. 5), surrounding the plasmids, and that (ii) the transfection efficiency does not depend on the size of the initial vesicles employed for preparing the complex (Van der Woude et al., 1995; Mok and Cullis, 1997).

In passing, we note that similar fingerprint-like patterns and compact striated structures have been reported, using cryo-electron microscopy and x-ray diffraction, for complexes that contained the cationic lipids DODAC (dioctadecyl-dimethylammonium chloride), DOTAP (1,2 dioleoyl-3-trimethylammonium propane), or DC-Chol (3β[N-(n′,N′ dimethylaminoethane)carbamoyl] cholesterol, dioleoyl) (Lasic et al., 1997; Radler et al., 1997; Xu et al., 1999; Huebner et al., 1999). It is, therefore, tempting to suggest that in terms of lipid and DNA organization, distinct similarities exist in their mode of assembly. Obviously, further work will be needed.

An intriguing aspect of this study was that part of the initially formed lipoplexes in bulk phase are/remain unstable and eventually aggregate and/or merge further into larger complexes upon prolonged incubation. The size of these complexes largely exceeds that of the plasmid and may acquire diameters in the order of microns. It seems reasonable to suggest that this fraction will be excluded from involvement in cellular transfection, as such a size will preclude cellular uptake. Presumably, this fraction may actually originate from vesicles that in the early phase of preparation still display surface-bound plasmid, or at least some uncovered parts of DNA (Fig. 6 *a*). It also implies that

plasmids that are rapidly and completely covered with amphiphile (cf. Fig. 4) will remain in bulk solution as stable particles with little tendency to cluster, and capable of bringing about efficient transfection. It is possible, therefore, that the stability of lipoplexes may depend on the amphiphile-dependent packing efficiency of plasmids, implying a degree of flexibility that would ensure rapid and complete shielding of the DNA, thereby preventing unlimited growth of complexes, which would hamper their internalization.

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