FEBS Letters 356 (1994) 361–366

FEBS 14928

New structures in complex formation between DNA and cationic liposomes visualized by freeze–fracture electron microscopy

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Received 2 November 1994

Abstract Structures formed during interaction of cationic liposomes and plasmid DNA were studied by freeze–fracture electron microscopy and their morphology was found to be dependent on incubation time and DNA concentration. These structures were formed with liposomes composed of DC-Chol and DOPE after 30 min incubation at DNA : lipid concentrations encompassing maximal transfection activity. They resembled liposome complexes (meatballs) and additionally bilayer-covered DNA tubules (spaghetti), whereby the DNA-tubules were found to be connected to the liposome complexes as well as occurring free in the suspension. At later times and higher DNA-to-liposome ratios the complexes grow larger while their membranes become discontinuous, allowing the self-encapsulation of the DNA. The relative transfection potency of the various morphologically distinct structures is discussed.

Key words: Cationic liposome; Plasmid DNA; Transfection; Freeze–fracture electron microscopy

1. Introduction

Gene therapy and genetic engineering require reliable and efficient systems for delivery of exogenous genes into target cells. Among other non-viral vectors, liposomes have been widely used as delivery agents for DNA and other polynucleotides (for reviews see [1,2]). Thereby, liposomes offer several advantages over viral vectors, including the absence of viral components, the protection of the DNA/RNA from inactivation or degradation, and the possibility for cell-specific targeting. Negatively charged liposomes, used for gene delivery in vitro to mammalian cells [3,4] or plant protoplast, however, do not deliver more effectively than other simpler methods, despite various improvements such as pH-sensitive liposomes [5] or virosomes [6].

An important breakthrough in this field was the design of positively-charged liposomes, or cationic liposomes, as transfection agents [7–14]. Since nucleic acids are highly negatively charged molecules they can interact spontaneously with the cationic liposomes. Complete complexation is achieved (even at low liposome-to-DNA ratios) simply by mixing the polynucleotides with preformed cationic liposomes. This simple and mild but still effective method is based on a complex formation between plasmid DNA and vesicles composed of synthetic cationic surfactants such as DOTMA [7], or DC-Chol [12] in combination with a helper lipid, e.g. DOPE [2,7,9,11,12,14]. Most of the cationic surfactants form micelles but not liposomes, and the addition of a phospholipid having a strong tendency to adopt the inverted hexagonal structure (H_{II} phase), such as DOPE, was found to be important [2]. DOPE helps in forming liposomes with a significantly increased transfection efficiency [15] while it reduces the cytotoxicity of the cationic surfactants [13]. A variety of different quaternary ammonium surfactants with alkyl-, ether- and ester-links to various backbones have been investigated in the search for an optimal transfection agent that is less toxic and metabolized by the cells [9–12]. For a given cell confluence, DC-Chol liposomes, for instance, have been found to be at least fivefold less toxic than that of lipofectin, which contains DOTMA [12]. More recently, cationic liposome–DNA complexes have been used successfully to express heterologous genes in vivo, after administration intravenously [16], intra-arterially [17], intra-tracheally [16], or by aerosol inhalation [18].

Despite their extensive use as transfection agents the mechanism of DNA interacting with cationic liposomes and the structure of the resulting complexes are still poorly understood. It is generally assumed that, in contrast to the negatively charged liposomes, there is no true encapsulation of the DNA by the cationic vesicles, but rather binding at their surface while the size and shape of the vesicle are maintained [8]. Electron micrographs of a recent study [19] suggest that the cationic liposomes are attached like beads on a string, gradually covering the DNA chain until, at a certain lipid to DNA ratio, a complete lipid coating of the DNA is reached composed of multilayered liposomes. It seems that this lipid coating is able to protect the DNA, to enhance the uptake by recipient cells, possibly via endocytosis and/or fusion, and possibly also to deliver material into the nucleus [20].

In this present study, we have investigated the structure of the complexes formed between plasmid DNA and preformed cationic liposomes composed of DC-Chol/DOPE, by freeze–fracture electron microscopy, and looked for the structural modifications of the complexes and their dependence on varying DNA concentration and incubation time. Although naked DNA is not visible by freeze–fracture electron microscopy, this technique is very useful for studying the interaction between DNA and cationic liposomes because the structure of the DNA is enhanced during this process by lipid coating, probably consisting of a single bilayer tubule.

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Abbreviations: DC-Chol, 3β[*N*-(*N'*,*N'*-dimethylaminoet hane)-carbamoyl]cholesterol; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOTMA, *N*-[1-[2,3-dioleyloxy)propyl]-*n*,*n*,*n* -trimethylammonium chloride.

SSDI 0014-5793(94)01315-2

2. Materials and methods

2.1. DNA, surfactant, lipid, and preparation of the liposomes

Plasmid pRSV-LUC, containing a luciferase gene driven by the Rouse sarcoma virus promoter [21] was prepared by a CsCl/EtBr gradient method [22]. The plasmid DNA was of the B-type (2.37 nm) and existed predominantly in the supercoiled state. DOPE was purchased from Avanti Polar Lipids Inc. DC-Chol was synthesized according to the method of Gao and Huang [12]. DC-Chol and DOPE were mixed in a 3:2 molar ratio, evaporated in a stream of nitrogen to form a thin film, and vacuum desiccated for several hours to remove any residual chloroform. The lipid film was rehydrated in distilled deionized water which had been autoclaved. The liposomes were allowed to hydrate overnight. DC-Chol liposomes were prepared by microfluidization using a M-110S microfluidizer (Microfluidics Corp.) to an average diameter below 200 nm. The particle size was measured by using a Coulter sub-micron particle analyzer, using a uni-modal analysis. The sample was counted for 200 s. The liposomes were filtered through a 0.2 μm filter to provide sterilization. The liposomes were diluted to a final concentration of 2 μmol/ml (1.2 mg/ml of total lipid).

2.2. Formation of the DNA–liposome complexes

To a volume of 10 μl HEPES buffer (20 mM, pH 7.5) 20 μl of DC-Chol liposomes (2 μmol/ml) with varying amounts of pRSV-LUC (1 mg/ml) were mixed at room temperature by a Hamilton syringe, to obtain final DNA-to-lipid ratio ranging from 1–10 μg of DNA to 20 nmol DC-Chol liposomes. Thereby, the total volume of the sample was increased, resulting in concentrations ranging from 31.25–200 μg of DNA/ml. The concentration of the suspensions, chosen for freeze–fracture electron microscopy was approximately 15- to 100-fold-higher than what is used in transfection because it is always a problem to refine tiny little structures such as interaction events in too dilute suspensions at the microscope.

DNA–DC-Chol complexes were investigated by freeze–fracture electron microscopy at room temperature, after different incubation times, ranging from 10 min to 24 h, and at various DNA-to-lipid ratios, ranging from 1–10 μg of DNA per 20 nmol DC-Chol liposomes.

2.3. Freeze–fracture electron microscopy

DC-Chol liposomes, naked DNA, and DNA–liposome complexes were quenched rapidly for freeze–fracture electron microscopy using the sandwich technique and liquid propane (cooling rate $>10^4$ K/s). The cryofixed specimens were fractured and shadowed in a Balzers BAF 400D freeze–fracture device at −120°C and 2×10^{-6} Torr. The cleaned replicas were examined in a transmission electron microscope (Jeol JEM 100B or Zeiss CEM 902 A) [23].

3. Results and discussion

We have investigated the complex formation between plasmid DNA and preformed cationic liposomes by freeze–fracture electron microscopy and studied the structural modifications of both components, depending on DNA concentration- and incubation time.

3.1. Liposome control

Freeze–fracture electron micrographs of liposomes, made of DC-Chol/DOPE (6:4 by molar ratio; 2 μmol lipid/ml; 'liposome control') and prepared by microfluidization, showed well-defined, mainly small liposomes which were well separated from each other in the ice (Fig. 1A). The liposomes were fairly uniform in size, showing mean diameters below 200 nm, which is in good agreement with laser light scattering measurements. Due to the surface charge on the liposomes, contributed by the positively charged DC-Chol component, there is a repulsive force between the liposomes, preventing aggregation.

3.2. DNA control

In freeze–fracture electron micrographs of the pRSV-LUC DNA (0.67 mg DNA per ml HEPES buffer, 20 mM, pH 7.5; 'DNA control') it was not possible to clearly visualize the naked plasmid DNA (Fig. 1B, some very weak features are marked by an arrow head). With a maximal width of 2.37 nm of the DNA strands, these structures are at the resolution limit of freeze–fracture technique (about 2 nm for periodical structures [23]). This is also true of another cryo-technique, cryo-electron microscopy, where some effort is needed to visualize the structure of naked DNA in detail [24]. Typically, DNA samples for electron microscopy are prepared by the Kleinschmidt method of DNA spreading followed by metal rotary shadowing [25]. One drawback of this method in visualization of the DNA together with liposomes is that the Kleinschmidt methodology does not allow a size comparison of these species, since DNA molecules are detected as cytochrome *c*–DNA complexes and appear much bigger than their naked form [19].

3.3. DNA–liposome complexes

Freeze–fracture electron micrographs of DNA–liposome complexes, made after a short incubation time (10 min, Fig. 1C, left panel) or low DNA-to-lipid ratio (1 μg/20 nmol DC-Chol, Fig. 1C, right panel), showed semi-fused liposomes where the number of liposomes involved was low (mainly 2–3 liposomes, Fig. 1C) and their size was roughly the same compared to the control liposomes as shown in Fig. 1A. As described in the figure legends, the bars represent 100 nm for all the electron micrographs. The reason for choosing a 2-fold (in Fig. 1C, left panel) and a 3-fold higher magnification (in Fig. 1C, right panel) compared to Fig. 1A is to demonstrate possible fusion structures. Indeed, in Fig. 1C (left panel), lipid particles (marked by arrows) were visible at the fusion area. The negatively charged plasmid DNA (not visible) seems to act as a fusogenic agent, drawing together the positively charged liposomes and forming semi-fused liposomes (liposome–DNA aggregates, Fig. 2).

At longer incubation times (30 min–24 h) and at higher DNA-to-lipid ratios (2–10 μg of DNA to 20 nmol of DC-Chol liposomes), some proportions of the DNA clearly became visible, as shown in Fig. 1D–G and Fig. 2. Obviously, its structure was enhanced, presumably by lipid-coating. The diameter of these tubular spaghetti-like structures (Fig. 1D; some of the

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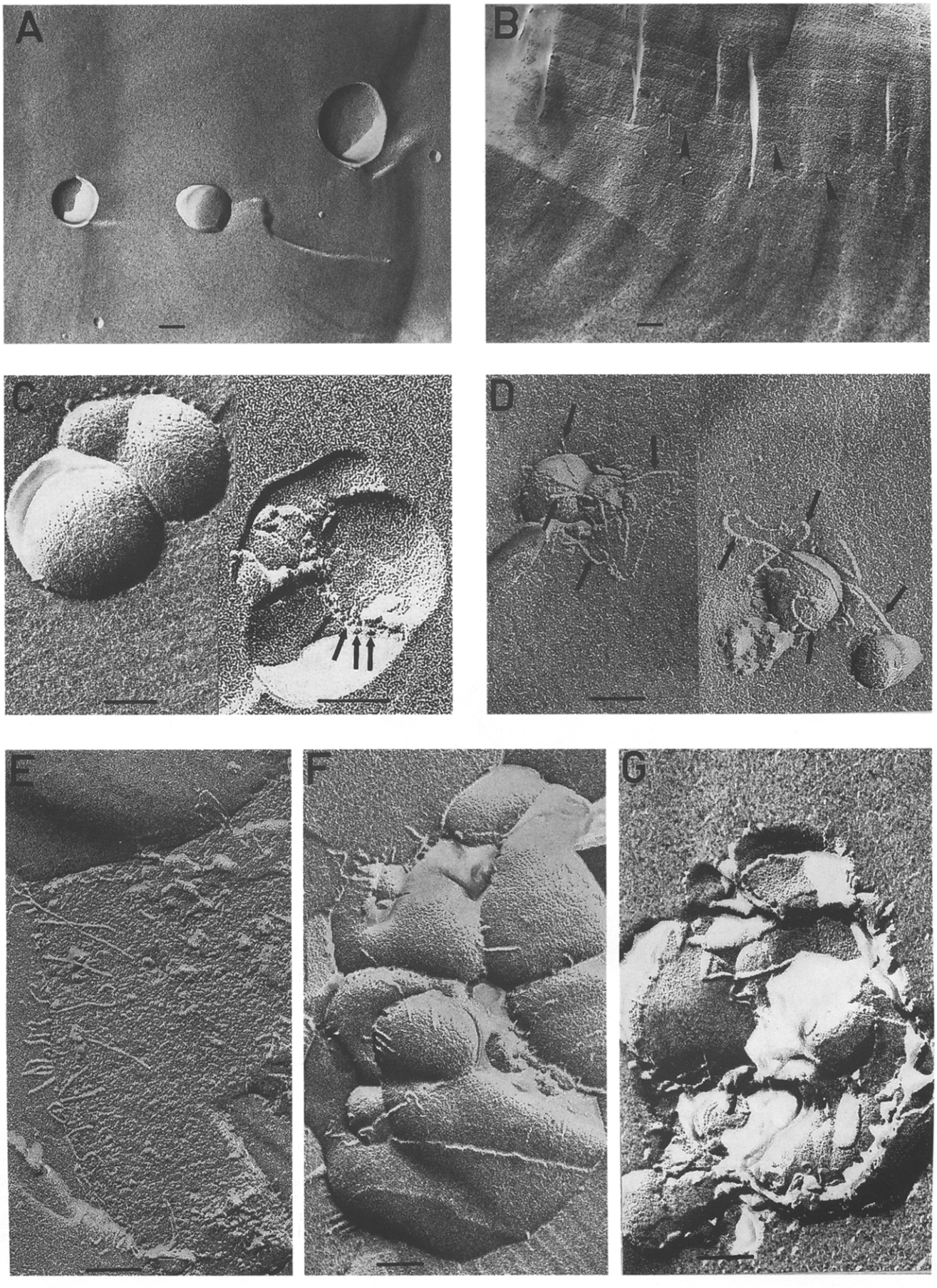
Fig. 1. Freeze–fracture electron micrographs of (A) liposomes, made of DC-Chol/DOPE (6:4 by mol; liposome control); (B) naked pRSV-Leu DNA (DNA control, some of the very weak features are marked by an arrow head); and (C–G) DC-Chol–DNA complexes, at various DNA-to-lipid ratios and incubation times: (C) fused liposomes without spaghetti-like structures at 10 min incubation time and 4 μg DNA/20 nmol DC-Chol in the left panel and at 24 h incubation time and 1 μg DNA/20 nmol DC-Chol in the right panel (some lipidic particles are marked by arrows); (D) spaghetti–meatball complexes at 24 h incubation time and 2 μg DNA/20 nmol DC-Chol (some spaghetti, connected with the liposomes, are marked by an arrow); (E) 'free' spaghetti-like structures, attached to the metal foil of the sandwich at the same incubation time and concentration as in D; (F) spaghetti–meatball assembly at 30 min incubation time and 4 μg DNA/20 nmol DC-Chol, and (G) spaghetti–meatball assembly with partly disrupted liposome membranes at the same DNA-to-lipid ratio as in F but at 60 min incubation time. The bar on all electron micrographs represents 100 nm and the shadow direction is running from bottom to top.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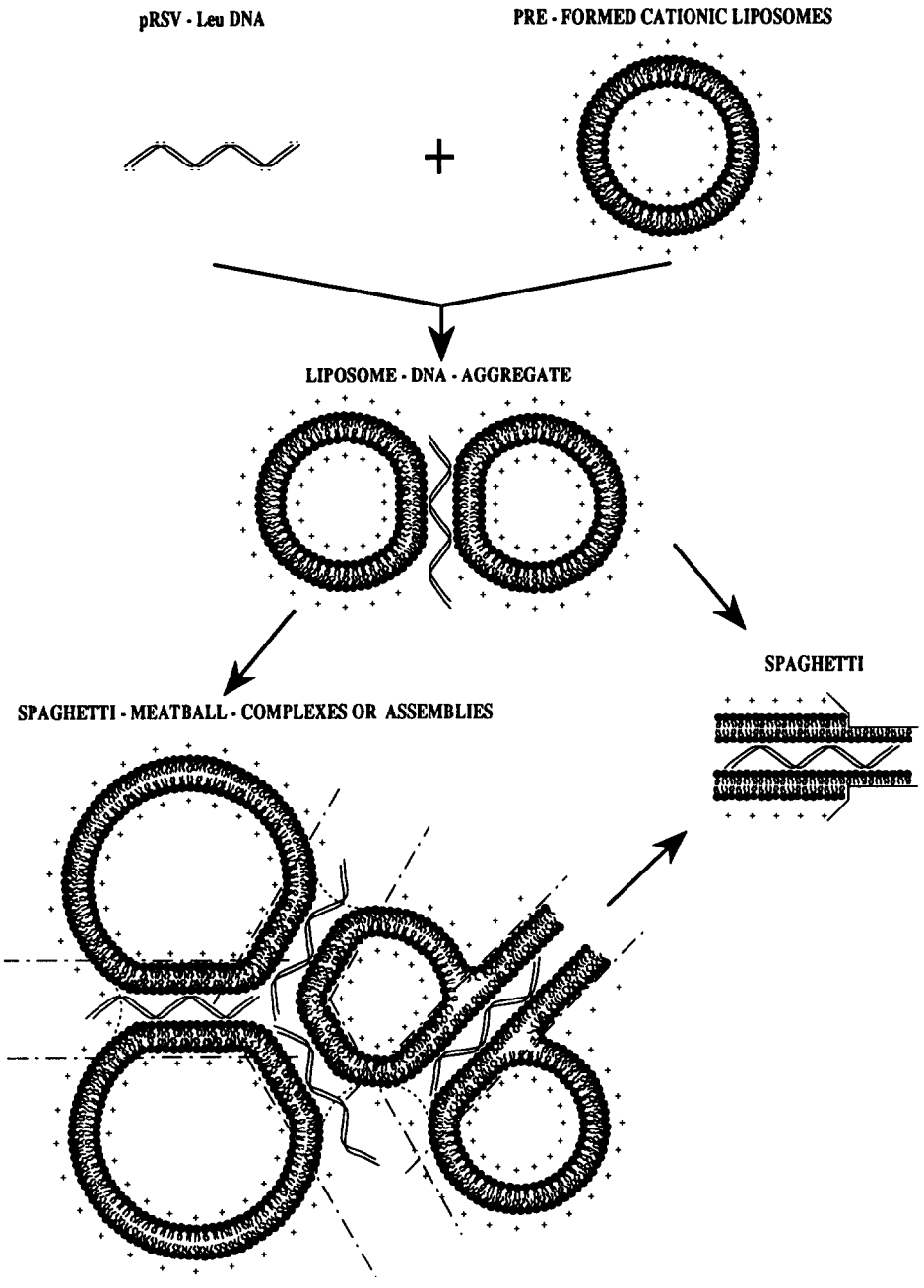

Fig. 2. Scheme showing our personal suggestion, based on freeze–fracture electron micrographs, about the interaction of negatively charged DNA with the cationic DC-Chol-containing liposomes and the formation of liposome–DNA aggregates without spaghetti-like structures, occurring at short incubation times and low DNA-to-lipid ratios, and spaghetti–meatball complexes, spaghetti–meatball assembles and spaghetti-like structures, connected with the semi-fused liposomes but also occurring 'free' in suspension at longer incubation times and higher DNA-to-lipid ratios.

spaghetti are marked by arrows) was approximately 7 nm. Both convex DNA strands (shadow behind) as well as concave DNA furrows (shadow in front, one of them is marked by an arrow in Fig. 3A) are found. These findings support the possibility of a bilayer tubule covering the DNA strands. During freeze–fracturing, the fracture plane usually follows the hydrophobic interior of a bilayer, whether it is surrounding a cell or a liposome [26], or in this case, while surrounding a DNA strand. This produces a theoretical diameter of 6.37 nm for the spaghetti-like structure by the summing of 2 nm of each of the two fluid bilayer halves and 2.37 nm of the diameter of the plasmid DNA (Fig. 3B). Measured and theoretical diameters of the spaghetti are in good agreement and support the hypothesis of a single bilayer tube coating the DNA.

The strong charge interaction between the positively charged DC-Chol and the negatively charged DNA may stabilize the high curvature of the bilayer tubule around the DNA strand, and the high content of DOPE may also assist in the stabilization of the spaghetti structure. Based on its wedge-shaped molecular structure, DOPE can adopt highly curved structures such as H_{II} tubules, at excessive lipid and/or high temperatures [27], or other non-bilayer structures [28]. Freeze–fracture elec-

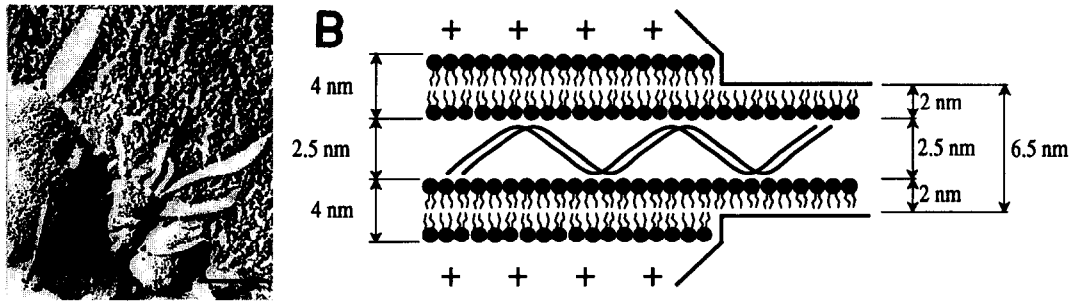

Fig. 3. (A) Cut-out of the freeze–fracture electron micrograph of Fig. 1F showing enlarged convex DNA strands and a concave DNA furrow, marked by an arrow. The bar represents 100 nm and the shadow direction is running from bottom to top. (B) Cross-fraction of a spaghetti.

tron microscopy is one of the best methods of distinguishing between bilayer and non-bilayer structures [23] and to visualize hexagonal tubules [23,28]. However, no hexagonal tubules were visible at any time in our investigation of DNA–cationic liposome systems, containing excess water, as observed in DNA–lipid systems with excess lipid [29].

Spaghetti-like structures were found to be still connected to the liposomes (spaghetti–meatball complex, Fig. 1D; spaghetti–meatball assemblies, Fig. 1F,G; scheme of Fig. 2), but were also found separated from the liposomes, 'free' in suspension (Figs. 1E and 2). Possibly due to the residual positive charge at their surfaces, spaghetti-like structures are often found in contact with the metal foil of the sandwich which was used for the rapid freezing of the samples (Fig. 1E).

With higher DNA concentrations and longer incubation times (beginning with 4 μg of DNA per 20 nmol DC-Chol liposomes at 30 min) a proportion of the DNA–liposome complexes were observed as larger structures containing several semi- or totally fused liposomes (spaghetti–meatball assemblies; Figs. 1F,G and 2). Most of the liposomes involved in the spaghetti–meatball assemblies had grown in size in comparison to the control liposomes (Fig. 1A), presumably by fusion of these liposomes (Figs. 1F,G and 2). During the formation and growth in size of the spaghetti–meatball assemblies the membranes of the liposomes appeared discontinuous, allowing for self-encapsulation of the DNA (Fig. 1G). All distinct structures, such as spaghetti–meatball complexes, spaghetti–meatball assemblies, as well as 'free' spaghetti, were found at incubation times from 30 min to 24 h and at DNA-to-lipid ratios from 2 to 10 μg of DNA per 20 nmol of liposomes. However, the proportion of different structures were different. With increasing DNA-to-lipid ratios, the content of spaghetti–meatball assemblies increased whereas the content of spaghetti–meatball complexes decreased.

From an examination of the optimal DNA-to-lipid ratio for transfection and their dimension, it seems likely that the spaghetti-like structures may be the active DNA–lipid complex. The spaghetti-like structures occur at DNA:lipid concentrations which are typically used during transfection (2 μg of DNA to 20 nmol DC-Chol liposomes) and their diameter comes closest to the diameter of the nuclear pores [30]. However, calculating more precisely the diameter of the DNA–lipid tubules surrounded by one full bilayer, which is about 10 nm (Fig. 3B), and the diameter of the nuclear pores (7 nm; [30]) even the spaghetti are too thick to pass these pores freely. On the other hand, spaghetti, similar to microvilli, are extremely curved structures with very small radii (especially at their ending tips) and therefore are able to adhere and fuse to flat cells easily [31]. Additionally, spaghetti-like structures as well as spaghetti–meatball complexes and assemblies, which were observed during our investigation, may still bear residual positive charges on their surfaces. This may also lead to an interaction and fusion with cell- and probably nuclear membranes, thereby promoting the transfer of the DNA into the cytoplasm and eventually into the nucleus of the cells.

Acknowledgements: We thank Mrs. I.-M. Hermann and Mrs. R. Kaiser for technical assistance in freeze–fracturing, Mrs. G. Engelhardt and Mrs. G. Vöckler for their phototechnical work, and PhD student U. Strohbach for designing Figs. 2 and 3B on the computer. We are grateful to Professor D. Papahadjopoulos for helpful discussions and reading the manuscript. The original work in the laboratory of Leaf Huang was supported by NIH Grants CA 59327, HL 50256, DK 44935, and CA 64654. The work here is supported by the Deutsche Forschungsgemeinschaft under the grants Sonderforschungsbereich 197/B8 and Ho-13877/1-1-896/92.

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