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Comparison of two lipid/DNA complexes of equal composition and different morphology

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ABSTRACT

Two types of complexes were prepared from a cationic cholesterol derivative, dioleoylphosphatidylcholine and DNA. Depending on the preparation procedure complexes were either dense snarls of lipid covered DNA (type A) or multilayer liposomes with DNA between layers (type B). The transfection efficiency of the snarl-shaped complexes was low but positive. The transfection efficiency of the liposome-shaped complexes was zero, while DNA release upon their interaction with anionic liposomes was 1.7 times higher. The differences in transfection efficacy and DNA release could not be ascribed to the difference in resistance of complexes to decomposition upon interaction with anionic liposomes or intracellular environment since the lipid composition of complexes is the same. Instead the complexes in which lipoplex phase is more continuous (type A) should require more anionic lipids or more time within a cell for complete decomposition. Prolonged life time should lead to the higher probability of DNA expression.

1. Introduction

While lipid mediated transfection meets growing application, the efficiency is still a challenge to be improved. This has caused hundreds of new cationic lipids to be designed. Shortly, it became evident that molecular structure of lipid is not the only factor that affects transfection efficiency and it does not affect transfection efficiency directly. The phase state of lipids [1], size [2] and morphology [3] of complexes should be considered among other factors. The correlation between molecular structure, lipoplex phase state, transfection efficiency was shown for various cationic lipids (see [4–6] and other works by groups of Hoekstra, Koynova and MacDonald). The main conclusion from these papers is that cationic lipids which promote formation of inverted hexagonal phase are most efficient transfection agents. On the other hand the morphology issues which do not involve the comparison of lipid phases are somewhat tricky to target, because of the difficulty to obtain reliably different morphologies from the same lipid mixtures. In several papers different morphologies were obtained varying lipid/DNA ratio. In these case not only morphology but a cationic lipid/DNA charge ratio is also affected [3,7]. The aim of our work was to prepare complexes with different morphology, keeping lipid composition and lipid-to-DNA ratio constant, in order to explore how the factor could affect transfection. The improvement of the transfection efficiency was put aside.

2. Materials and methods

Lipids used in this study are depicted in Fig. 1. *N*-[4-(Cholest-5-en-3β-yloxycarbonyl)butyl]pyridinium bromide (Chol-PB) was synthesized [8] and it's transfection efficiency was described previously [9]. 1,2-Dioleoyl-sn-glycero-3-phosphoetanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) was supplied by Avanti Polar Lipids (Alabaster, USA). Chloroform and other general solvents were from "Ekos-1" (Moscow, Russia). Cholesterol (Chol), sodium salt of calf thymus DNA (CT-DNA), the fluorescent probe, ethidium bromide (EtBr), D_2O, and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid and its sodium salt (HEPES) were from Sigma–Aldrich (Missouri, USA). Triton-X100 were from Merck (Darmstadt, Germany). Plasmid DNA pUC-19 (~2700 b.p.) was kindly provided by Dr. Elena Sycheva. Plasmid encoding red fluorescent protein pDsRed-Exp-N1 (~4500 b.p.) were supplied by Clontech (California, USA). Lipofectamine2000® was from Invitrogen (California, USA). Solutions were prepared by mass with deionized water.

2.1. Complex preparation

All complexes contain Chol-PB, DOPE and DNA. Chol-PB content is 37% of total lipid, and the cationic lipid to DNA charge ratio (+/−) is

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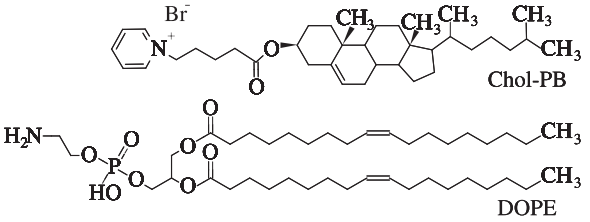

Fig. 1. Structures of cationic lipid Chol-PB (*N*-[4(Cholest-5-en-3β-yloxycarbonyl)butyl]pyridinium bromide) and lipid-helper DOPE (1,2-dioleoyl-sn-glycero-3-phosphoetanolamine).

6. High (+/−) ratio and high lipid-helper content insures that there is no unshielded DNA surface [10]. This is extremely important for intercalation assay performance since incomplete DNA binding by lipids may have great impact on ethidium bromide fluorescence.

DNA/lipid complexes based on pUC-19 plasmid were used in dynamic laser light scattering (DLS), transition electron microscopy (TEM) and steady-state fluorescence studies. Complexes based on fluorescent plasmid were used for transfection efficiency determination. CT-DNA based complexes were used in NMR experiments. DNA concentrations were determined by absorbance at 260 nm ($\varepsilon_{b.p.}$ = 6600 M^{-1} cm).

2.1.1. Lipid drops (precursors of type A complexes)

Aliquots of lipids (0.294 μmol Chol-PB, 0.501 μmol DOPE) in organic solvents were mixed, evaporated, and dried. 1000 μl of water was than added and lipids were allowed to swell overnight. Ten freeze–thaw cycles were followed by extrusion through 100 nm pore polycarbonate membranes using an Avanti Mini extruder. 50 μl were collected for TEM experiments and 450 μl was diluted with water to the total volume of 2.5 ml and used for DLS.

2.1.2. Type A complexes

The 500 μl of lipid drops solution (see above) were mixed with 10-μl aliquot of pUC-19 solution and incubated for 1 h at room temperature. 50 μl were collected for TEM experiments and the rest of the solutions were diluted with water to the total volume of 2.5 ml and used for DLS.

2.1.3. Type B complexes

Aliquots of lipids (0.147 μmol Chol-PB, 0.250 μmol DOPE) in organic solvents were mixed, evaporated, and dried. Then 500 μl of water and 10 μl of pUC-19 solution were added. The system was allowed to swell overnight and afterwards was subjected to 10 freeze–thaw cycles followed by extrusion through 100 nm polycarbonate membranes. 50 μl was used for TEM experiments and the rest of the solutions were diluted with water to the total volume of 2.5 ml and used for DLS.

2.2. DLS measurements

DLS was performed on Brookhaven 90Plus analyzer operated at room temperature. Immediately before sample dilution, deionized water was additionally filtered through 0.2 μm polycarbonate membranes. Final lipid concentration in sample was 0.11 mg/ml.

2.3. Transmission electron microscopy

The DNA free solid drops and complexes of both types were studied under Zeiss LIBRA®120 PLUS with OMEGA-type energy filter. A droplet of a sample was added on a copper carbon coated grid and left for 2 min. Then excess of a liquid was removed carefully by the edge of a filter paper and the grid was stained with a droplet of 2% uranyl acetate aqueous solution for 2 min. After removal of the stain droplet the grid was air-dried. The transmission electron microscopy was performed using acceleration voltage of 80 kV and magnification in the range of 20,000–200,000. An empty carbon coated grid stained with uranyl acetate was used as negative control, the structures observed in the control were considered artefacts. The image grey value profiles were obtained with ImageJ software freely available from NIH (http://rsb.info.nih.gov/ij/).

2.4. ^{31}P NMR

Bruker DPX-300 instrument was used for NMR spectra acquisition. Since the rotational diffusion of particles less than 0.5 μm affects phosphatidylcholine's ^{31}P signal width, it is not correct to compare directly lipid packing in type A and B complexes by means of the method. Meanwhile DNA influence on lipid packing was studied using large multilayer vesicles. Samples were prepared by swelling of lipids with 10% D_2O or CT-DNA solution in 10% D_2O followed by cyclical freeze-thawing. Total lipid concentration in NMR samples was 25 mg/ml.

2.5. Ethidium bromide exclusion assay

Fluorescence intensity of EtBr increases approximately 30 times upon its intercalation between base pairs of double-stranded DNA. Upon complex formation the layer of cationic lipids prevents EtBr intercalation into DNA. Consequently, its fluorescence intensity decreases [11]. Biomembrane-mimicking anionic liposomes made of dioleoylphosphatidylcholine (DOPC), DOPE, dioleoylphosphatidylserine (DOPS), and cholesterol (45:20:20:15 by mass) were shown to effectively release DNA from lipoplexes [12] and therefore were used for comparison of DNA accessibility for EtBr intercalation in complexes of different types.

Ethidium bromide solution in 40 mM HEPES pH 7.4 ([EtBr] = 7.92 μM) was used instead of water to prepare complexes of both types, anionic liposomes, free DNA, and Triton-X100 solutions to keep its concentration constant during all experiments, therefore no correction for dilution was needed. To avoid the inner filter effect, the absorbance of EtBr solution used for complex preparation was kept below 0.05. DNA/EtBr ratio was 10/1 (1 ethidium bromide molecule per 10 nucleotides). The lipid complexes contained 0.0950 μmol Chol-PB and 0.162 μmol DOPE. Initially 200 μl of suspension was put into the cuvette. Anionic liposomes were prepared by mixing and drying lipid aliquots, followed by hydration of the lipid film, freezing-thawing, and extrusion through 100-nm polycarbonate membrane filters.

Fluorescence was measured using a HITACHI F-4000 fluorescence spectrometer. The measurements were performed in a quartz cuvette, 5 × 5 mm, upon constant stirring at room temperature. The fluorescence was excited at 520 nm. At this wavelength free and DNA-associated EtBr has the same molar extinction coefficient. Emission was reordered at 620 nm. Both slits were 10 nm. The fluorescence intensity of each sample was corrected for the background fluorescence and normalized to maximum fluorescence intensity, obtained after Triton-X100 addition.

2.6. Transfection

The human fibrosarcoma cell line (HT1080) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. The cells were plated in 24-well culture plates (1.2×10^5 cells per well) and allowed to adhere overnight. Immediately before transfection the complete medium was replaced by serum free DMEM (200 μl/well). Then DNA/lipid complexes of type A or B were added in amount equivalent to 0.5 μg of DNA per well. After 4 h of incubation the medium was replaced