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Factors affecting DNA binding and stability of association to cationic liposomes

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ABSTRACT

Lipoplexes are complexes formed between cationic liposomes (L^+) and polyanionic nucleic acids (P^-). They are commonly used in vitro and in vivo as a nucleic acid delivery system. Our study aims are to investigate how DOTAP-based cationic liposomes, which vary in their helper lipid (cholesterol or DOPE) and in media of different ionic strengths affect the degree, mode of association and degree of condensation of pDNA. This was determined by ultracentrifugation and gel electrophoresis, methods based on different physical principles. In addition, the degree of pDNA condensation was also determined using the ethidium bromide (EtBr) intercalation assay.

The results suggest that for cationic lipid compositions (DOTAP/DOPE and DOTAP/cholesterol), 1.5 M NaCl, but not 0.15 M NaCl, both prevent lipoplex formation and/or induce partial dissociation between lipid and DNA of preformed lipoplexes. The higher the salt concentration the greater is the similarity of DNA condensation (monitored by EtBr intercalation) between lipoplex DNA and free DNA. As determined by ultracentrifugation and agarose gel electrophoresis, 30–90% of the DNA is uncondensed.

SDS below its critical micellar concentration (CMC) induced "de-condensation" of DNA without its physical release (assessed by ultracentrifugation) for both DOTAP/DOPE and DOTAP/cholesterol lipoplexes. As was assessed by agarose gel electrophoresis SDS induced release of 50–60% of DNA from the DOTAP/cholesterol lipoplex but not from the DOTAP/DOPE lipoplex.

This study shows that there are conditions under which DNA is still physically associated with the cationic lipids but undergoes unwinding to become less condensed. We also proved that the helper lipid affects level and strength of the L^+ and DNA^- electrostatic association; these interactions are weaker for DOTAP/cholesterol than for DOTAP/DOPE, despite the fact that the positive charge and surface pH of DOTAP/cholesterol and DOTAP/DOPE are similar.

1. Introduction

Cationic lipids are the most commonly used transfection reagents for nucleic acid (NA) delivery. Cationic lipid–DNA complexes (lipoplexes) (Felgner et al., 1997) have attracted major attention in recent years due to their potential use as nucleic acid deliver systems, which makes them a promising alternative to viral vectors (Simberg et al., 2004; Gao et al., 2007). Currently, despite the research on lipoplex-mediated nucleic acid such as plasmid DNA (pDNA), oligonucleotide (ODN), and small interfering RNA (siRNA), efficiency of these delivery systems is lower than desirable. Improving our understanding of structure–activity relationship of lipoplexes in NA delivery in vitro (cell culture) and in vivo may aid in optimizing lipoplex performance.

Cationic liposomes used for DNA delivery typically include a neutral "helper lipid" in order to increase the transfection efficiency (Kerner et al., 2001; Boktov et al., 2007). Helper lipids affect lipoplex electrostatics as well as the form of lipid self-assembles (micellar, lamellar, hexagonal, vesicular, etc.); the level of hydration; and DNA secondary and tertiary structures (Hirsch-Lerner and Barenholz, 1999; Zuidam et al., 1999a). For in vitro transfection, a favorable helper lipid is DOPE, which enables better matching charge density of the lipid surface to the DNA helices. DOPE also facililitates counterion release from the lipid surface by DNA (Zuidam and Barenholz, 1997, 1998) and decreases lipid hydration (Hirsch-Lerner and Barenholz, 1998, 1999). On the other hand, for in vivo transfection, cholesterol is a better helper lipid than DOPE (Templeton et al., 1997; Sternberg et al., 1998; Li et al., 1999; Zuhorn et al., 2002b). The differences between the in vitro and in vivo situation are related to difference in lipoplex stability in the in vitro (cell culture) media and in vivo (in whole blood) (Simberg et al., 2003, 2004). In general, it seems that in order to obtain efficient

Abbreviations: DOTAP, *N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; PC, phosphatidylcholine; DCP, dicetylphosphate; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; SDS, sodium dodecyl sulfate; EtBr, ethidium bromide; LR-PE, lissamine rhodamine-phosphatidylethanolamine pH-sensitive probe; K_{SV}, Stern–Volmer coefficient; LUV, large unilamellar vesicles; MLV, multi-lamellar large vesicles; UHV, unsized (non-extruded) heterogeneous vesicles.

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transfection in vitro (Zuidam et al., 1999b; Kerner et al., 2001) and in vivo (Templeton et al., 1997; Mahato et al., 1998; Simberg et al., 2003), the lipoplex should remain cationic and should have enough membrane defects to cause – size instability (Zuidam et al., 1999b; Simberg et al., 2001). However, too much instability results in loss of transfection productivity. Among the variety of physical interactions involved in lipoplex formation and maintenance, the electrostatic interactions play a major role in various steps of the transfection process. Lipoplexes are formed spontaneously by electrostatic neutralization. The driving force for such complex formation is the removal of small counterions (cations from DNA and anions from the cationic liposome surface) (May and Ben-Shaul, 1997; Templeton et al., 1997; Sternberg et al., 1998; Wagner et al., 2000). Release of small counterions depends on the liposome lipid composition, i.e., the type of cationic lipid and type of helper lipid present in the assembly (Zuidam and Barenholz, 1998; Zuidam et al., 1999b) and on the medium composition (Simberg et al., 2004).

Transfection involves lipoplex formation and uptake by the cells. Surface electrical charge properties of both cell membrane and lipoplex are critical in determining the efficiency of DNA uptake by the cell (Lasic, 1997; Dass, 2002; Zuhorn and Hoekstra, 2002). Optimum transfections in vitro (Felgner et al., 1987; Kerner et al., 2001) and in vivo (Song et al., 1997; Eliyahu et al., 2002) occur at an excess of positive charge (cationic lipid) to anionic NA phosphate charge, i.e., a ratio of >1.0). This has been explained on the basis that the cationic particles interact with the anionic cell membrane, and this interaction induces adsorptive endocytosis (Lasic, 1997; Dass, 2002; Zuhorn and Hoekstra, 2002; Zuhorn et al., 2002a), followed by endosomal membrane destruction. Therefore, transfection medium properties (ionic strength, counterion species, and pH) that affect cell surface and lipoplex electrostatics are very important.

NaCl is an important component of most biological fluids, including serum. Therefore, a better understanding of the lipoplex fate in biological milieu requires understanding the effect of NaCl and separating it from the effect of high molecular weight components (mainly proteins). Theoretically, the implication of the effect of NaCl on ease of lipid–DNA separation could be important in transfection because too tightly bound DNA may not be released inside the cells.

This study is aimed to investigate the association of DNA with cationic liposomes (DOTAP/DOPE and DOTAP/cholesterol in different concentrations of medium (NaCl). The effect of anionic detergent (SDS) and anionic liposomes (egg PC/DCP) on DNA lipoplex/medium partition and DNA level of condensation were also investigated.

2. Materials and method

2.1. Materials

N-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride (DOTAP), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE), and egg phosphatidylcholine (PC) were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesterol and dicetylphosphate (DCP) were obtained from Sigma.

Sodium dodecyl sulfate (SDS) was obtained from Bio-Rad (Hercules, CA). SYBR Green I and SYBR Gold DNA dyes and lissamine rhodamine were obtained from Molecular Probes (Eugene, OR) and ethidium bromide (EtBr), from Sigma. pH-sensitive lissamine rhodamine phosphatidylethanolamine (LR-PE) probe was prepared by Dr. A. Dagan and Dr. R. Cohen of our department, and water-soluble pH-sensitive lissamine rhodamine ethanolamine (LR-Eth pH-sensitive probe) was synthesized and purified by Dr. R. Cohen (Barenholz et al., 2001) Fluorescence measurements were performed using the LS50B luminescence spectrometer (PerkinElmer, Norwalk, CT), using excitation and emission slits as described in each of the experiments.

2.2. DNA determination and purity

Plasmid pCi/LS, expressing sequences coding for both L (large) and S (small) proteins of hepatitis B virus, in Tris-borate EDTA buffer (TBE) pH 8.0, was a gift from Prof. Jörg Reimann (University of Ulm, Germany). The plasmid was purified using the QIAGEN plasmid Mega kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. No chromosomal DNA or RNA was present. The ratio of A260/A280 was 1.88, indicating the absence of protein contamination (Maniatis et al., 1982). Qualitative agarose gel (1%) electrophoresis of pCi/LS based on the use of the DNA dyes SYBR Green I or SYBR Gold (Even-Chen and Barenholz, 2000) showed that pCi/LS was mostly in the super coiled topoisoform.

The absolute concentration of DNA was quantified by determination of organic phosphorus (Barenholz and Amselem, 1993; Zuidam et al., 1999b) and expressed as equivalent concentration of organic phosphate, which represents level of DNA negative charge (Felgner et al., 1997).

2.3. Liposome preparation

Cationic large (~100 nm) unilamellar vesicles (LUV) were prepared by mixing DOTAP and DOPE (1:1 mole ratio), or DOTAP and cholesterol (1:1 mole ratio) in *tert*-butanol, followed by freeze-drying overnight. The lyophilized cake was hydrated with 20 mM Hepes (pH 7.4) or pure H_2O and vortexed for several minutes to form cationic unsized heterolamellar vesicles (UHV) ~500 nm (mostly uni- or oligolamellar vesicles) (Simberg et al., 2003). LUV were downsized from UHV using the extrusion system LipoSofast (Avestin, Ottawa, Canada), which is based on MacDonald et al. (1991) 11 times through a 0.4-μm pore-size filter and 11 times through a 0.1-μm pore-size filter (Poretics, Livermore, CA), successively to get ~100-nm LUV.

Anionic multilamellar vesicles (MLV) of size (0.5–2.5 μm) were prepared using a mixture of neutral lipid egg PC and the anionic lipid DCP (1:1 mole ratio) in 20 mM Hepes (pH 7.4) using a procedure similar to the above, except for the downsizing steps.

Particle size distribution of the liposome dispersions was determined at 25 °C by dynamic light scattering (DLS) using the Coulter model N4SD instrument (Coulter Electronics, Hialeah, FL) (Barenholz and Amselem, 1993).

2.4. Quantification of lipoplex and lipoplex-associated DNA

2.4.1. Method I: ultracentrifugation

DOTAP/DOPE and DOTAP/cholesterol cationic liposomes were incubated for 10 min at room temperature with 15 nmol pCi/LS DNA at $DOTAP^+$: DNA^- charge ratios of 1.0 and 2.0. To each sample, 2.5 nmol ethidium bromide was added, and samples were incubated at room temperature for an additional 10 min. Lipoplex and lipoplex-associated DNA were separated by ultracentrifugation at 100,000 × *g* for 30 min at 4 °C using Beckman Ultracentrifuge TL 45. At these conditions, >99% of lipoplexes precipitated and less than 1% is unassociated DNA. Level of lipoplex-unassociated DNA in the supernatant was determined as lipid phosphorus (Barenholz and Amselem, 1993; Zuidam et al., 1999b).

The state of DNA condensation was evaluated from the EtBr fluorescence intensity in the supernatant. The level of lipoplexes in the supernatant, was determined by 90° static light scattering (SLS) using a PerkinElmer LS50B luminescence spectrometer at the wavelength of excitation = emission of 600 nm. DNA alone under