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New effects in polynucleotide release from cationic lipid carriers revealed by confocal imaging, fluorescence cross-correlation spectroscopy and single particle tracking

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Abstract

We report on new insights into the mechanisms of short single and double stranded oligonucleotide release from cationic lipid complexes (lipoplexes), used in gene therapy. Specifically, we modeled endosomal membranes using giant unilamellar vesicles and investigated the roles of various individual cellular phospholipids in interaction with lipoplexes. Our approach uses a combination of confocal imaging, fluorescence cross-correlation spectroscopy and single particle tracking, revealing several new aspects of the release: (a) phosphatidylserine and phosphatidylethanolamine are equally active in disassembling lipoplexes, while phosphatidylcholine and sphingomyelin are inert; (b) in contrast to earlier findings, phosphatidylethanolamine alone, in the absence of anionic phosphatidylserine triggers extensive release; (c) a double-stranded DNA structure remains well preserved after release; (d) lipoplexes exhibited preferential binding to transient lipid domains, which appear at the onset of lipoplex attachment to originally uniform membranes and vanish after initiation of polynucleotide release. The latter effect is likely related to phosphatidyleserine redistribution in membranes due to lipoplex binding. Real time tracking of single DOTAP/DOPE and DOTAP/DOPC lipoplexes showed that both particles remained compact and associated with membranes up to 1–2 min before fusion, indicating that a more complex mechanism, different from suggested earlier rapid fusion, promotes more efficient transfection by DOTAP/DOPE complexes.

Keywords: Lipoplex; Endosomal membrane; Release mechanism; Giant unilamellar vesicles; Fluorescence microscopy; Single particle tracking

1. Introduction

Following decades of extensive research, lipid-based delivery systems have entered the mainstream as carriers of nucleic acids, either for plasmid delivery or as agents for carrying short antisense single strand oligonucleotides (ODN) to express or to down-regulate target genes in therapeutic applications. With many of the early problems related to carrier toxicity, biodegradability and stability recently ameliorated, a notorious drawback of the lipid-based vehicles remains poor efficiency [1–7].

Formation of the lipoplexes and their interaction with cellular components, leading to intracellular access of the

Abbreviations: GUVs, giant unilamellar vesicles; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; DOTAP, dioleoyltrimethylammonium propane; DOPE, di-oleoyl phosphatidylethanolamine; DOPC, di-oleoyl phosphatidylcholine; DPPS, di-palmitoyl phosphatidylserine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine; DiO, 3,3′-dihexadecyloxacarbocyanine perchlorate ($DiOC_{16}$ (3)); ITO, indium tin oxide; HPLC, high performance liquid chromatography; LSM, laser scanning confocal fluorescence microscopy; FCCS, fluorescence cross-correlation spectroscopy; SPT, single particle tracking; PCR, polymerase chain reaction

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carrier, lipoplex disassembly and polynucleotide release into cell cytosol have been extensively investigated using various techniques [6–28], aiming to determine a composition–structure–function relationship and to develop a rationalized approach in designing efficient carriers. It has been proposed that the delivery potential of the carrier is determined by the internal nano-structure of the complex [12,13,16,19], its total surface charge [10,11,14] along with lipid charge density [21]. However, it remains to be fully understood how the lipoplexes are disassembled as they are taken up by cells, and by what mechanisms and to what degree (fully or not) DNA/ODN dissociate from the carrier lipids during this process.

At present, it is widely accepted that lipoplexes enter cells via endocytosis [23–28]. To carry out its function, DNA has to escape from endosomal compartments while simultaneously detaching itself from the carrier lipids and be released into the cytosol—a complex mission, whose fate and driving mechanisms are not fully understood. Considering that phospholipid content is an important factor in determining biophysical properties of the membranes [29,30], understanding phospholipid roles in interaction with the lipoplexes has been highly significant for uncovering release mechanisms. Indeed, phospholipids have been regarded as major species involved in interaction with the lipoplexes [26,31–33], while cholesterol showed no effect on DNA/ODN release [32]. A current understanding of the release mechanism, as suggested by Szoka et al [26,31,32], proposes that due to interaction with the lipoplexes, anionic phospholipids from the cytoplasmic-facing leaflet of the endosomal membrane enter into its internal leaflet, resulting in the formation of a charge-neutral ion pair between anionic cellular lipids (phosphatidylserine, PS) and cationic lipids. Such a coupling of anionic PS to cationic lipids leads to DNA displacement from the complex and release, due to electrostatic forces. In fact, it has long been postulated that perturbation of lipid asymmetry in the membrane bilayer, involving transmembrane re-distribution of aminophospholipids (PS and phosphatidylethanolamine, PE) in endosomal membranes, is a general aspect of endocytosis [34–37]. Recent experimental studies have given new evidence for this hypothesis, generally pointing toward a more active role of lipid re-arrangement in inducing membrane invagination during endocytosis [38–41]. Still, it remains questionable whether energy costly lipid flip-flop, which would require several hours [42], can happen within the time scale of a continuous endocytosis process, and whether the cationic complexes can be promoters of trans-leaflet lipid mixing in the endosomal membranes. Also, the exact role of cellular PE lipid along with usage PE as a “helper” lipid in cationic complexes remain intriguing and need better understanding [43–47].

In this work, we model endosomal membranes by giant unilamellar vesicles (GUV) and investigate the distinct roles of major cellular phospholipids (PC, PE, PS and sphingomyelin, SM) in interaction with the cationic lipid-DNA/ODN complexes, aiming to gain a deeper insight into the release mechanisms. In contrast with small liposomes, used in the previous studies, the GUV system not only allows visualization of the release process, but also investigation of the state of the target membrane during and after the interaction with lipoplexes. We examine the extent of release of short single and double stranded DNA across model membranes by laser scanning confocal imaging. Also, we use fluorescence cross-correlation analysis of DNA diffusion to assess whether DNA has been detached from the carrier lipids and if the double-stranded DNA has dissociated into single strands upon release. In addition, we apply single particle tracking methodology to monitor in real time and at the level of single lipoplexes the course of lipoplex fusion with the membranes. The observed effects are discussed in the context of interactions between cationic lipid-DNA/ODN complexes and cellular membrane lipids.

2. Materials and methods

2.1. Materials

Lipids included cationic DOTAP (dioleoyl-trimethylammoniumpropane), anionic DPPS (dipalmitoyl-phosphatidylserine), DOPS (1,2-dioleoyl-sn-glycero-3-phospho-L-serine) and neutral DOPE (dioleoyl-phosphatidylethanolamine), DOPC (dioleoyl-phosphatidylcholine), SM (sphingomyelin). All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). The lipid analog fluorescent dye 3,3′-dihexadecyloxacarbocyanine perchlorate (DiO) was received from Molecular Probes (Eugene, Oregon).

A 66-nt single strand oligonucleotide with the sequence as described elsewhere [48] was labeled at its 5′ end with Alexa 488 and its complementary strand with Cy-5. They were annealed by the standard procedure to obtain a 66-nt double-stranded double-labeled (Alexa 488 and Cy-5 at the ends) DNA. Double-stranded 368-nt DNA labeled with Cy-5 at the 5′-end was generated by PCR using TaKaRa Ex Taq Polymerase. All oligonucleotides were custom synthesized, HPLC purified and labeled by IBA GmbH. (Goettingen, Germany). PCR reaction products (1 ml) were purified and concentrated using the MiniElute PCR-Purification Kit (Qiagen, Hilden, Germany). Further purification was done by extraction from 1.5% (w/v) agarose gel (NucleoSpin extract, Macherey and Nagel GmbH, Duren, Germany) and ethanol precipitation. DNA was dissolved in LichroSolv HPLC water (Merck, Darmstadt, Germany) at a final concentration of ~100 nM. Note, henceforward to clarify what type of oligonucletotide is discussed in the specific experiments we additionally specify ss-ODN or ds-DNA when relevant.

2.2. Preparation of liposomes and lipoplexes

Chloroform solutions of lipids (DOTAP/DOPE and DOTAP/DOPC at various mole fractions) were dried under