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Transfection efficiency boost by designer multicomponent lipoplexes

Giulio Caracciolo [a], Daniela Pozzi [a], Ruggero Caminiti [a,*], Cristina Marchini [b], Maura Montani [b], Augusto Amici [b], Heinz Amenitsch [c]

[a] Department of Chemistry, University of Rome "La Sapienza", P.le A. Moro 5, 00185 Rome, Italy
[b] Genetic Immunization Laboratory, Department of Molecular Cellular and Animal Biology, University of Camerino, Via Camerini 5, 62032 Camerino (MC), Italy
[c] Institute of Biophysics and Nanosystems Research, Austrian Academy of Sciences, Schmiedelstrasse 6, A-8042 Graz, Austria

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Abstract

Cationic liposome–DNA complexes (lipoplexes) have emerged as leading nonviral gene carriers in worldwide gene therapy clinical trials. Arriving at therapeutic dosages requires the full understanding of the mechanism of transfection. We investigated the correlation between structural evolution of multicomponent lipoplexes when interacting with cellular lipids, the extent of DNA release and the efficiency in transfecting mouse fibroblast (NIH 3T3), ovarian (CHO) and tumoral myofibroblast-like (A17) cell lines. We show, for the first time, that the transfection pattern increases monotonically with the number of lipid components and further demonstrate by means of synchrotron small angle X- ray scattering (SAXS) that structural changes of lipoplexes induced by cellular lipids correlate with the transfection efficiency. Specifically, inefficient lipoplexes either fused too rapidly upon interaction with anionic lipids or, alternatively, are found to be extremely resistant to solubilization. The most efficient lipoplex formulations exhibited an intermediate behaviour. The extent of DNA unbinding (measured by electrophoresis on agarose gel) correlates with structural evolution of the lipoplexes but DNA-release does not scale with the extent of transfection. The general meaning of our results is of broad interest in the field of non-viral gene delivery: rational adjusting of lipoplex composition to generate the proper interaction between lipoplexes and cellular lipids may be the most appropriate strategy in optimizing synthetic lipid transfection agents.

Keywords: Cationic liposome; DNA; Lipoplex; Gene delivery; Transfection efficiency; SAXS

1. Introduction

Synthetic cationic liposomes (CLs) form stable complexes with polyanionic DNA [1]. CL–DNA complexes, named lipoplexes, have recently emerged as leading nonviral vectors in worldwide gene therapy clinical trials. Unfortunately, their present low transfection activity severely compromises their systematic use both *in vitro* and *in vivo*. Improving transfection efficiency (TE) of lipoplexes requires answers to some specific questions: what is the mechanism of formation of lipoplexes, what is the correlation between the physical attributes of lipoplexes and their functional activity? Usually, lipoplexes are organized in multilamellar structures (L_{α}^{C} phase) with DNA embedded with cationic lipid membranes [2–4]. Less frequently, an inverted hexagonal H_{II}^{C} phase comprised of lipid-coated DNA strands arranged on a hexagonal lattice has been observed [4]. Even though some earlier studies suggested superiority in transfection of hexagonal lipoplexes with respect to lamellar ones [4], experimental evidence negated a correlation between structure and activity [5–7]. However, recent experiments have disputed this suggestion [7,8], but there is general consensus that a direct correlation between initial lipoplexes structure and transfection efficiency does not exist [7,9].

Unfortunately, the mechanism of interaction of lipoplexes with cell membranes remains poorly understood. As a result, the structural evolution of lipoplexes upon interaction and mixing with cellular lipids has been attracting a great number of scientists in the field of non-viral gene delivery. Hereby the structural changes of lipid carriers resulting in DNA release may be the key step in lipid-mediated DNA delivery (lipofection) [9–13]. At the same time, more efficient synthetic reagents are highly desirable.

* Corresponding author. Tel.: +39 06 4991 3661; fax: +39 06 490631.
E-mail address: r.caminiti@caspur.it (R. Caminiti).

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To this end, we prepared multicomponent lipoplexes incorporating from three to six lipid components within the lipid bilayer [14,15]. Recently, we have demonstrated the superiority in transfection of multicomponent lipoplexes with respect to binary ones usually employed for gene delivery [16]. For instance, the four-component lipid system incorporating cationic lipids 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and (3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl])-cholesterol (DC-Chol) and neutral helper lipids dioleoylphosphocholine (DOPC) and dioleoylphosphatidylethanolamine (DOPE) transfected DNA into mouse fibroblast (NIH 3T3) and tumoral myofibroblast-like (A17) cell lines more efficiently than DOTAP−DOPC and DC-Chol−DOPE cationic liposomes separately.

It is currently accepted, and several evidences exist, that lipoplexes are internalized into the cytoplasm by endocytosis and that fuse with the negatively charged cellular membranes [6]. It is a central point since fusogenicity is considered to contribute significantly to cytoplasmic delivery of DNA [17]. Many investigators have shown that lipid mixtures are more fusogenic than single lipids. Such a phenomenon has been related to nonideal mixing of lipid components [18,19], formation of lipid rafts [19] (that may play a role in membrane recognition) and higher probability for packing defects in mixtures relative to single component bilayers [18]. Furthermore, preparation of liposomes incorporating very different lipid headgroups and/or aliphatic chains has been shown to produce asymmetric vesicles [20]. Such an asymmetry is expected to increase the biocompatibility and flexibility of vesicle drug delivery systems [20]. According to all these suggestions, multicomponent lipoplexes may be more fusogenic, biocompatible and flexible gene carriers than binary lipoplexes.

Thus, we have extended our previous study to investigate how transfection changes with increasing number of lipid components the synthetic carrier is made of. With the aim of providing new insights into the mechanism of transfection, we also investigated the structural evolution of lipoplexes upon interaction with cellular (anionic) lipids by means of synchrotron small angle X-ray scattering (SAXS). First, the existence of different regimes of stability was demonstrated: inefficient complexes were either easily disintegrated by anionic lipids (regime of instability) or definitely too resistant (regime of high stability). The most efficient lipoplexes exhibited intermediate 'optimal stability'. Secondly, to correlate structural changes of lipoplexes and DNA release, we also measured the extent of DNA release by electrophoresis after addition of negatively charged lipids to preformed lipoplexes. The extent of DNA unbinding correlates strictly with the instability of lipoplexes, but we note that the DNA release is not proportional to the transfection efficiency. On the basis of our results, here we advance the concept that the structural stability of lipoplexes against anionic lipids is the critical factor regulating transfection efficiency by lipoplexes.

2. Materials and methods

2.1. Cationic liposomes preparation

Cationic 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and (3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl])-cholesterol (DC-Chol), anionic dioleoylphosphatidylglycerol (DOPG) and neutral dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphocholine (DOPC), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. DOTAP–DOPC, DOTAP–DLPC, DOTAP–DOPE, DC-Chol–DOPE and DC-Chol–DMPC cationic liposomes were prepared according to standard protocols [21]. In brief, each binary mixture, at a molar fraction of neutral lipid in the bilayer X_N=(neutral lipid/total lipid) (mol/mol)=0.5, was dissolved in chloroform and the solvent was evaporated under vacuum for at least 24 h. The obtained lipid films were hydrated with the appropriate amount of Tris–HCl buffer solution (10^{-2} M, pH 7.4) to achieve the desired final concentration (10 mg/ml for the X-ray samples). The obtained liposome solutions were stored at 30 °C for 24 h to achieve full hydration. Indeed, we have recently found evidence that lipid hydration is important to achieve the equilibrium structure of lipoplexes [22]. The same protocol was followed to prepare anionic liposomes (AL) made of DOPG. Negatively charged liposomes mimicking membrane lipid composition were also prepared (MM=DOPC:DOPE:DOPG, 1:1:1 molar ratio).

2.2. Lipoplexes preparation

Calf thymus Na-DNA was purchased from Sigma-Aldrich (St. Louis, MO). DNA was dissolved in Tris–HCl buffer (5 mg/ml) and was sonicated for 5 min inducing a DNA fragmentation with length distribution between 500 and 1000 base pairs, which was determined by gel electrophoresis. Plasmid DNA (pGL3 which codifies for firefly luciferase) was purchased from Promega (Madison, WI). By mixing adequate amounts of the DNA solutions to suitable volumes of liposome dispersions, self-assembled DOTAP–DOPC/DNA, DOTAP–DLPC/DNA, DOTAP–DOPE/DNA, DC-Chol–DOPE/DNA and DC-Chol–DMPC/DNA binary lipoplexes were obtained. To examine the effect of DNA kind (linear or plasmid) on the structure of lipoplexes, both CL-linear DNA and CL-plasmid DNA complexes were prepared. Multicomponent lipoplexes were prepared by adding DNA to mixed lipid dispersions made of distinct populations of CLs. For instance, DOTAP–DOPC (1:1) plus DOTAP–DLPC (1:1) loaded with DNA results in DOTAP–DOPC–DLPC/DNA complexes (2:1:1). In such a manner, multicomponent lipoplexes, incorporating from three to six lipid species, were formed in a self-assembled manner [14,15]. Further all samples were prepared with the same cationic lipid/DNA ratio (mol/mol), i.e. ρ=(cationic lipid (by mole)/DNA base)=3.2. The chosen charge ratio (positively charged lipoplexes) guarantees maximum DNA load, which is not the case for isoelectric complexes (ρ=1). In Table 1 the lipoplexes are listed as a function of increasing membrane charge density that was calculated according to ref. [6].

After storage for 3 days at 4 °C, allowing the samples to reach equilibrium, they were transferred to 1.5 mm diameter quartz X-ray capillaries (Hilgenberg, Malsfeld, Germany). The capillaries were centrifuged for 5 min at 6000 rpm at room temperature to consolidate the samples.

Table 1
Cationic liposomes listed as a function of increasing membrane charge density, σ_M

Lipid composition	σ_M 10^{-3} (e/Å^2)
DOTAP–DOPC (1:1)	7.6
DOTAP–DOPC–DLPC (2:1:1)	7.6
DOTAP–DLPC (1:1)	7.9
DOTAP–DC-Chol–DOPC (1:4:5)	8.1
DOTAP–DC-Chol–DOPC (5:2:3)	8.2
DOTAP–DC-Chol–DMPC–DLPC (1:1:1:1)	8.4
DOTAP–DC-Chol–DOPC–DOPE–DMPC–DLPC (2:2:1:1:1:1)	8.5
DOTAP–DC-Chol–DOPC–DOPE (1:1:1:1)	8.5
DOTAP–DC-Chol–DMPC–DLPC (1:1:1:1)	8.7
DOTAP–DC-Chol–DOPE–DLPC (1:1:1:1)	8.9
DOTAP–DC-Chol–DOPE–DMPC (1:1:1:1)	9.4
DC-Chol–DMPC (1:1)	9.4
DC-Chol–DOPE–DMPC (1:1)	9.6
DC-Chol–DOPE (1:1)	9.8