Biochimica et Biophysica Acta 1808 (2011) 2156–2166

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Synthesis and characterization of degradable multivalent cationic lipids with disulfide-bond spacers for gene delivery

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ARTICLE INFO

Article history:
Received 29 December 2010
Received in revised form 4 April 2011
Accepted 12 April 2011
Available online 24 May 2011

Keywords:
Gene delivery
Cationic lipid
Cationic liposome
Nonviral

ABSTRACT

Gene therapy provides powerful new approaches to curing a large variety of diseases, which are being explored in ongoing worldwide clinical trials. To overcome the limitations of viral gene delivery systems, synthetic nonviral vectors such as cationic liposomes (CLs) are desirable. However, improvements of their efficiency at reduced toxicity and a better understanding of their mechanism of action are required. We present the efficient synthesis of a series of degradable multivalent cationic lipids (CMVLn, n = 2 to 5) containing a disulfide bond spacer between headgroup and lipophilic tails. This spacer is designed to be cleaved in the reducing milieu of the cytoplasm and thus decrease lipid toxicity. Small angle X-ray scattering demonstrates that the initially formed lamellar phase of CMVLn–DNA complexes completely disappears when reducing agents such as DTT or the biologically relevant reducing peptide glutathione are added to mimic the intracellular milieu. The CMVLs (n = 3 to 5) exhibit reduced cytotoxicity and transfect mammalian cells with efficiencies comparable to those of highly efficient non-degradable analogs and benchmark commercial reagents such as Lipofectamine 2000. Thus, our results demonstrate that degradable disulfide spacers may be used to reduce the cytotoxicity of synthetic nonviral gene delivery carriers without compromising their transfection efficiency.

1. Introduction

Gene therapy has the potential to address a wide variety of diseases at their root cause. These include cancer, hereditary disorders, and many others [1–4]. However, despite massive efforts to the contrary, including numerous clinical trials worldwide [5,6], widespread therapeutic success has remained elusive. A key challenge for gene therapy is to develop efficient and safe gene delivery methods. Viral vectors, such as engineered adenoviruses and retroviruses, are very efficient (particularly in vivo) and have resulted in gene therapy's first successes [7], but they suffer from a number of drawbacks. A primary concern is vector safety: in clinical trials, viral vectors have led to serious adverse effects, owing to immunogenicity and non-specific integration of their genetic cargo into the host genome [8–11]. Other limitations are the small size of viral genomes and the comparatively difficult vector production. Synthetic nonviral vectors, on the other hand, lack immunogenic protein components, do not impose a size limit on the nucleic acid to be transferred, and are more easily prepared and modified. Their main limitation is their lower transfection efficiency (TE, a measure of the amount of successfully transferred and transcribed DNA) and the onset of toxicity with increasing cationic lipid/nucleic acid mole ratios, which need to be improved in order to compete with viral vectors [12–15].

Cationic liposome (CL)–nucleic acid (NA) complexes are one of the main classes of nonviral vectors [13,16–20]. They form spontaneously when cationic liposomes (typically containing a neutral lipid (NL) as well as a cationic lipid) are combined with NAs. A large number of cationic lipids have been synthesized [21–23] to enhance the efficiency of CL–DNA complexes and uncover structure–TE relationships, albeit with limited success. Thus, further development of cationic lipid-based vectors is required to match the TE of viral

Abbreviations: Boc, *tert*-butoxycarbonyl; CL, cationic liposome; DEAD, diethylazodicarboxylate; DIEA, *N,N*-diisopropylethylamine; DLS, dynamic light scattering; DOB, 3,4-di(oleyloxy)benzoic acid; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; DOTAP, 2,3-dioleoyloxypropyltrimethylammonium chloride; DTT, dithiothreitol; EtBr, ethidium bromide; GSH, glutathione; L/D, cationic lipid/DNA weight ratio; NA, nucleic acid; NL, neutral lipid; RLU, relative light units; SAXS, small-angle X-ray scattering; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TE, transfection efficiency; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; TL/D, total lipid/DNA weight ratio; Φ_i, mole fraction of lipid i in the lipid mixture; σ_M, membrane charge density

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doi:10.1016/j.bbamem.2011.04.020

vectors. Among the chemical approaches to improve the TE of CL–DNA complexes, the synthesis of cationic lipids with multivalent head groups has been particularly promising [24–30].

The development of cationic lipids with low cytotoxicity is an important objective, especially for applications such as gene silencing which require higher cationic lipid/nucleic acid ratios [27]. The toxicity of CL–NA complexes is known to be due to the cationic lipid component of the complex [27,31]. Thus, we hypothesized that triggered disintegration of an appropriately designed, degradable multivalent cationic lipid component of the CL–DNA complex upon entry into the cytoplasm should reduce cytotoxicity. At the same time, such lipids should retain high TE levels comparable to those of recently synthesized non-degradable multivalent lipids [24–30] because the key steps of lipid-mediated DNA delivery prior to cytoplasmic release—DNA compaction, complex uptake and endosomal escape [32,33]—will not be compromised by the lipid degradation process.

In order to test this hypothesis and potentially decrease CL-induced cytotoxicity of lipid vectors, we prepared a series of cationic lipids designed to quickly degrade in the cytoplasm. As we describe, degradable multivalent lipids exhibit reduced toxicity with increasing cationic lipid/nucleic acid mole ratio while maintaining the high TE levels of previously synthesized multivalent lipids [24–30], consistent with our hypothesis.

Cells maintain a high redox potential gradient between the intracellular (reducing) environment and extracellular (oxidizing) space. The main intracellular reducing agent is the short peptide glutathione (GSH), which is continually recycled [34]. The intracellular concentration of GSH varies for different tissues but can reach up to 5 mM (e.g. in the liver) [35,36]. The redox potential gradient can be exploited to trigger, e.g., the cleavage of disulfide bonds in molecules once they reach the cytoplasm. This mechanism is used by biological toxins [37] as well as in drug delivery and diagnostic imaging [38–42]. A few studies have also applied the concept to gene delivery vectors [43–46], including CL–DNA complexes [47–50].

We report the convenient and efficient synthesis of a series of new degradable multivalent cationic lipids, termed CMVLn (n = 2 to 5). These CMVLs contain a reductively cleavable disulfide bond in the spacer between the hydrophobic moiety and the cationic head group. Reducing conditions result in separation of the hydrophobic tail from the headgroup. Except for the spacer, the CMVLs are identical to a previously synthesized series of multivalent lipids [32]. Of these, MVL5 in particular is a highly efficient vector for both DNA and siRNA [26,27,32]. The headgroup charge of the CMVLs was measured using an ethidium bromide (EtBr) displacement assay. CL–DNA complexes prepared from mixtures of the CMVLs with neutral 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) were studied using small-angle X-ray scattering (SAXS) under reducing and non-reducing conditions, as well as measurements of cytotoxicity and TE. The CMVLs were compared with commercially available 2,3-dioleoyloxypropyltrimethylammonium chloride (DOTAP) and Lipofectamine 2000 as well as with non-degradable MVL5. All CMVL/DOPC–DNA complexes form lamellar phases (L_α^C) which can be completely disassembled by addition of reducing agents such as GSH and DTT. Importantly, the complexes of CMVLn (n = 3, 4, 5) exhibit high TE at reduced cytotoxity when compared to non-degradable analogs and commercial lipid reagents.

2. Materials and methods

2.1. Materials and reagents

MVL5 was prepared as described [26]. Opti-MEM cell culture medium and Lipofectamine 2000 were purchased from Invitrogen. The lipids DOTAP and DOPC were purchased from Avanti Polar Lipids. The chemical structures of MVL5, DOTAP and DOPC are shown in the Supplementary data, Fig. S1. For X-ray samples and ethidium bromide displacement assay, highly polymerized calf thymus DNA (Sigma-Aldrich) was used. Luciferase plasmid DNA (pGL3 Control Vector, Promega) for microscopy, dynamic light scattering, cytotoxicity, and transfection assays was propagated in *E. coli* and isolated using a Qiagen Giga Kit. Other chemicals were purchased from Sigma-Aldrich unless otherwise stated and were used as received.

2.2. General methods

NMR spectroscopy was carried out on Bruker Avance instruments (200 MHz and 500 MHz). Detection of the spots in thin-layer chromatography was achieved by UV absorption and ninhydrin reagent (300 mg in 95 mL of 2-propanol and 5 mL of acetic acid) or phosphomolybdic acid in ethanol (10 wt%). Silica gel (Merck) with a mesh size of 200–425 was used for flash chromatography [51].

2.3. Lipid suspensions

Lipid stock solutions in chloroform (for DOTAP and DOPC) or chloroform/methanol (9:1, v/v; for MVL5 and all CMVLs) were combined at the appropriate ratios in glass vials. The obtained lipid solutions were dried, first by a stream of nitrogen and subsequently in a vacuum for 12 h, to form a thin lipid film. Sterile high resistivity (18.2 MΩ cm) water was added and the mixture was incubated at 37 °C for at least 12 h to give suspensions of a final concentration of 50 mM for X-ray samples. For transfection, aqueous lipid suspensions were prepared at 0.5 mM. Lipid mixtures containing higher mole fractions of DOPC formed opaque suspensions which were tip-sonicated to clarity (Vibra-cell, Sonics Materials). The aqueous lipid suspensions were stored at 4 °C until use.

2.4. Ethidium bromide displacement assay

Wells in a 96-well plate were preloaded with a mixture of 2.4 μg of DNA and 0.28 μg of EtBr in 100 μL of water. To the wells, varied amounts of a 0.2 mM aqueous lipid solution (at a lipid mole fraction of DOPC (Φ_{DOPC}) = 0) were added, preparing at least duplicate samples for each data point. Fluorescence was measured on a 1420 Multilabel counter Victor 3 V (Perkin Elmer) plate reader. The results were normalized to the fluorescence reading in the absence of lipid. To calculate the lipid headgroup charge, Z, the following equation was used: at the isoelectric point (determined from the data as described [32]), $\frac{N^+}{N^-} = 1 = \frac{m_{CL} / M_{CL}}{m_{DNA} / M_{DNA-bp}} \frac{Z}{2}$, where N^+ and N^- are the number of cationic charges on the lipid and of negative charges on the DNA, respectively; m_{CL} and m_{DNA} are the mass of cationic lipid and of DNA respectively; and M_{CL} and $M_{DNA\text{-}bp}$ are the molecular weight of cationic lipid and DNA basepair, respectively.

2.5. Optical microscopy

For imaging of complexes with optical microscopy, 0.2 wt.% (of total lipid) DHPE-Texas Red (Avanti Polar Lipids) was added to the lipid mixture (before evaporation of the organic solvent). This fluorescent dye has an excitation/emission peak at 585/615 nm. Cy5 (Mirus Bio LLC), with an excitation/emission peak at 605/670 nm, was conjugated to DNA using the manufacturer's protocol. Complexes were then prepared as for transfection and imaged in Opti-MEM, 20 min after complex formation. Fluorescence intensity profiles of particles were obtained with the ImageJ Color Profiler software tool.

2.6. Dynamic light scattering (DLS)

Complexes for DLS were prepared as for transfection but at a final concentration of 2 μg/mL of DNA (twice the concentration used for transfection, to achieve a sufficient particle concentration for the DLS