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Aspects of nonviral gene therapy: Correlation of molecular parameters with lipoplex structure and transfection efficacy in pyridinium-based cationic lipids

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ABSTRACT

This study seeks correlations between the molecular structures of cationic and neutral lipids, the lipid phase behavior of the mixed-lipid lipoplexes they form with plasmid DNA, and the transfection efficacy of the lipoplexes. Synthetic cationic pyridinium lipids were co-formulated (1:1) with the cationic lipid 1,2-dimyristoyl-sn-glycero-3-ethylphosphocholine (EPC), and these lipids were co-formulated (3:2) with the neutral lipids 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) or cholesterol. All lipoplex formulations exhibited plasmid DNA binding and a level of protection from DNase I degradation. Composition-dependent transfection (beta-galactosidase and GFP) and cytotoxicity was observed in Chinese hamster ovarian-K1 cells. The most active formulations containing the pyridinium lipids were less cytotoxic but of comparable activity to a Lipofectamine 2000™ control. Molecular structure parameters and partition coefficients were calculated for all lipids using fragment additive methods. The derived shape parameter values correctly correlated with observed hexagonal lipid phase behavior of lipoplexes as derived from small-angle X-ray scattering experiments. A transfection index applicable to hexagonal phase lipoplexes derived from calculated parameters of the lipid mixture (partition coefficient, shape parameter, lipoplex packing) produced a direct correlation with transfection efficiency.

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1. Introduction

Biotherapeutic agents such as antisense oligonucleotides, knock-in of DNA and short interfering RNA (siRNA) are among the most important therapeutic advancements for genetic diseases in decades (Patil et al., 2005). The goal of nonviral gene delivery is to correct a gene defect by intracellular delivery of nucleic acids. Gene delivery using synthetic, nonviral reagents continues to motivate research efforts since the first report by Felgner et al., 1987 on the use of the cationic lipid *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium (DOTMA) as a vehicle for nucleic acid

Abbreviations: AI, amphipathic index; a_0, lipid head group area; β-gal, β-galactosidase; Chol, cholesterol; CHO-K1, Chinese hamster ovarian (K1) cells; CR, charge ratio of cationic lipid N to anionic DNA P; Di16:0, 3,5-bis((hexadecyloxy)carbonyl)-1-methylpyridin-1-ium; Di16:1, 3,5-bis((hexadec-15-en-1-yloxy)carbonyl)-1-methylpyridin-1-ium; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; EPC, 1,2-dimyristoyl-sn-glycero-3-ethylphosphocholine; f_{lat}, f_{cyl}, filling factors of the lattice and cylinder unit cell, see equation 6; GFP, green fluorescent protein; HGS, headgroup size; l_c, l_{lip}, critical chain length of the hydrocarbon portion of a lipid, overall length of the lipid including the head group; LDS, lipophilic domain size; LI, lipofection index, see equation 1; $\log P_{sub}$, octanol-water partition coefficient, subscript indicates mole weighted average value of mixed lipids (mix), cationic lipids (+), or neutral lipids (0); n_{exp}, molar amount of lipid in the experiment with respect to the unit cell; n_{lat}, n_{cyl}, optimum molar amount of a lipid to fill the unit cell of a hexagonal lattice or a cylinder outside of the volume occupied by DNA; *p*DNA, plasmid DNA; QSAR, Quantitative structure–activity relationship; R, ratio of cationic lipid to neutral lipid; S, lipid shape parameter, see equation 2; S_+, S_{mix}, mole weighted average value of S for cationic lipids or mixed lipids; SAXS, small-angle X-ray scattering; TI, transfection index computed according to equations 3 to 6; V_C, V_{lip}, V_{mix}, partial molar volume of the hydrocarbon portion of a lipid, the overall lipid molecule including a counterion if required, mole weighted average value of a mixture.

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delivery. Synthetic lipids, due to their low toxicity, relative ease of large-scale production and purification, coupled with their ability to exist in many self-assembled macromolecular shapes under varying conditions of formulation provide a versatile material for lipofection. The majority of synthetic nonviral lipids for transfection contain a positively charged headgroup, typically tertiary or quaternary ammonium groups or polyamines, and a hydrophobic domain based on either alkyl chains or cholesterol (Lonez et al., 2012). Protection (from endogenous nucleases) and cellular delivery of the genetic cargo is achieved by packaging the inherently negatively charged nucleic acid with the cationic lipids into a compacted particle (a lipoplex), driven by electrostatic and hydrophobic interactions (Mintzer and Simanek, 2009; Zuhorn et al., 2007). The structural nature of the lipid hydrophobic domain as well as the functional nature of the charged headgroup have a significant impact on the particle integrity and morphology, and hence on transgene efficiency and cytotoxicity.

Several studies have revealed the importance of the lipid-nucleic acid complex morphology towards transfection efficiency, and how lipid domain assembly depends on headgroup and tail structure of the synthetic lipid. Examples of lipid systems that contain conformationally strained (or restricted) polar functional groups in their headgroup region have been reported(Huang et al., 2008; Majeti et al., 2004; Midoux et al., 2009; Mukthavaram et al., 2009). Pyridinium-based cationic amphiphiles are another class of nonviral lipid vectors possessing a cyclic moiety near the polar headgroup, and have shown much promise as gene delivery vectors (Zuhorn et al., 2007; Adrian et al., 2010; Audouy et al., 2000; Ilies et al., 2002, 2004, 2005, 2006, 2011; Ilies and Balaban, 2001; Pijper et al., 2003; Scarzello et al., 2005a,b; Shi et al., 2001; Smisterova et al., 2001; van der Gun et al., 2007; van der Woude et al., 1997; Wasungu et al., 2006; Zhu et al., 2008). The success of these gene transfer vectors, characterized by a heterocyclic polar headgroup, has been attributed to a large degree to the delocalized positive charge which gives a favorable electrostatic interaction (Ilies et al., 2011). Specifically, this diffuse charge offers a desirable balance between binding the nucleic acid cargo (necessary for compact and charge neutralization of the negative nucleic acid) and subsequent cargo release (to allow endosomal escape). If the electrostatic binding interaction is too strong, or conversely too weak, this can significantly impact the overall transgene efficiency. Pyridinium lipids tend to produce hexagonal phase lipoplexes which may confer additional advantages as the lipoplex releases cargo in the endosome (Ma et al., 2007).

The balance between lipoplex stability, cellular uptake and endosomal escape (Zuhorn et al., 2007; Ma et al., 2007; Crook et al., 1996; Hoekstra et al., 2007; Tarahovsky et al., 2004; Zhang et al., 1997) must ultimately reflect structural features of the synthetic lipid and any co-lipids, and the stoichiometric factors in a given formulation. One of the key factors that influences gene delivery is the final macromolecular shape of the lipid-DNA complex (Felgner et al., 1987; Smisterova et al., 2001; Gershon et al., 1993; Pitard et al., 1999; Sternberg, 1998). Of particular importance is the role of lamellar and inverted hexagonal lipid phases in controlling different stages of the process.

There are a very large number of structural and operational variables, each of which contributes to the transfection. Ultimately we would like to be able to predict the transfection efficacy of a given lipid structure and formulation from fundamental parameters. An analysis of *quantitative structure–activity relationships* (QSAR) in transfection data published up to 2004 focused on two key structural factors(Horobin and Weissig, 2005). Overall lipid hydrophobic character or lipophilicity was represented by use of tabulated octanol–water partition coefficients ($\log P$) and calculated amphipathic indexes (AI) as related solely to the non-polar region of the amphiphiles. Overall lipid shape was represented by headgroup size (HGS), lipophilic domain size (LDS), and critical chain length (l_c). These factors map onto the lipid shape parameter (S; also called the lipid packing parameter), which is typically derived from experimental data (Israelachvili, 1992; Kumar, 1991). For predictive and QSAR purposes direct computation of HGS and LDS from atomic composition is a superior approach, but such parameters do not directly give S. From the literature data it appears that these parameters ($\log P$ or AI with HGS/LDS) define a zone of interest for effective transfection, but this analysis did not include stoichiometric variables (Horobin and Weissig, 2005). Following this analysis, the efficacy of a series of steroidal lipids was correlated using a defined lipofection index (LI) (Gruneich and Diamond, 2007):

$$\mathrm{LI} = \mathrm{CR}\left(\frac{R}{R+1}\right)(\log P_{\mathrm{mix}})\left(\frac{\log P_{+}}{|\Delta \log P|}\right) \quad (1)$$

For a given formulation, CR is the charge ratio of cationic lipid cations to DNA phosphate anions, R is the molar ratio of neutral co-lipid to cationic lipid, $\log P_{\mathrm{mix}}$ is the molar weighted average $\log P$ of the cationic and neutral lipid, $\log P_{+}$ is the $\log P$ of the cationic lipid, and $|\Delta \log P|$ is absolute difference in $\log P$ between the cationic lipid and the neutral lipid.

The lipofection index LI encompasses several of the known variables which affect transfection. The first two terms of Eq. (1) directly relate to the lipoplex size and indirectly influence the internal morphology (lamellar/hexagonal lipid phase), the third term relates to the overall lipophilicity of the formulation, and the final term reflects the ability of the components to migrate from the lipoplex to the endosomal wall as the lipoplex unravels in the endosome (Gruneich and Diamond, 2007). Factors pertaining to the lipid shape parameter (HGS, LDS) were not considered, as these are closely similar within the series of compounds investigated. In short, the effect of cationic lipid shape on the lipoplex was approximately constant.

Transfection efficiency as a function LI gives a bell-shaped profile with a maximum at some optimal value of LI. Arguing from a different perspective, Safinya and co-workers (Ewert et al., 2010; Zidovska et al., 2009) showed a Gaussian relationship of transfection efficiency with membrane surface charge density. This approach applies only to lipids in lamellar-phase lipoplexes; hexagonal-phase lipoplexes gave poor correlation. Neither LI nor the surface potential approach using structural and operational parameters is directly predictive because the optimum value is a fitted parameter not defined by the analysis.

The inherent advantages of pyridinium lipids, and the potential to use quantitative tools to correlate structure to transfection efficacy prompted us to explore the activity of the pyridinium-based lipid vectors, **Di16:0** and **Di16:1** (Fig. 1) chosen for their ability to bind, protect and deliver plasmid DNA. The saturated compound has been previously reported as significantly active (Pijper et al., 2003); the terminal unsaturation in **Di16:1** was seen as a site to expand the diversity of the structure. Formulations of our lipids contained an equimolar amount of the commercial cationic lipid, 1,2-dimyristoyl-sn-glycero-3-ethylphosphocholine (EPC), to facilitate complete hydration, along with a co-lipid, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) or cholesterol (Fig. 1), both of which are known to enhance the transfection efficiency of cationic lipids.

In this study, the synthesis and chemical characterization of pyridinium-based cationic lipid vectors is described, together with the structural characterization of the lipid-DNA complexes from small-angle X-ray scattering (SAXS) and the *in vitro* lipid-DNA lipoplex delivery into Chinese hamster ovarian-K1 (CHO-K1) cells. These results then provide the basis from which to explore correlations with specific structural parameters, the goal being to develop a predictive structure-activity correlation.

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