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Review

Lipoplex morphologies and their influences on transfection efficiency in gene delivery

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Abstract

Cationic lipid-mediated gene transfer is widely used for their advantages over viral gene transfer because it is non-immunogenic, easy to produce and not oncogenic. The main drawback of the application of cationic lipids is their low transfection efficiency. Many reports about transfection efficiency of cationic lipids have been published in recent years. In this review, the current status and prospects for transfection efficiency of different morphologies of lipoplexes are discussed. High transfection activity will be acquired for H^C_{II} structure when membrane fusion is dominant, but when serum is present L^C_{α} lipoplexes show great superiority for their inhibition dissociation by serum during lipoplexes transporting. Increasing DOPE often gains high activity for the H^C_{II} structure promoted by DOPE. High lipofection will be gained from large lipoplexes when endocytosis is dominant, because large particles facilitate membrane contact and fusion. We suggest morphologies of lipoplex should be characterized at two levels, lipoplex size and self-assemble structures of lipoplexes, and understanding these would be very important for scientists to prepare novel cationic lipids and design novel formulations with high transfection efficiency.
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Keywords: Lipoplex morphology; Gene delivery; Transfection efficiency; Cationic lipoplexes

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1. Introduction

As a promising strategy for the treatment of many inherited and acquired diseases, gene therapy which is defined as the genetic modification of cells for therapeutic benefit has attracted many researchers during the past several decades [1]. The aim for gene therapy is to deliver healthy exogenous genic drugs such as plasmid DNA and single-strand oligonucleotides to replace a missing gene that otherwise have a normal makeup so as to cure genetic diseases, for instance, cystic fibrosis, malignant melanoma, and gaucher's disease [2,3]. Based on these factors, reliable and efficient vectors delivering exogenous genes into target cells are urgently awaited. Non-viral vectors are widely used for their advantages over viral vectors in recent years, because they are safe and cheap, easy to manufacture in lager scale, and they can also deliver large pieces of DNA [4–8].

During the past years, designing new non-viral system for DNA transfer has become an interesting field attracting more and more researchers [9]. Among non-viral vectors, since cationic lipid/DNA complex (lipoplex) was first introduced by Felgner et al. [10] who used *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trime-thylammonium chloride (DOTMA) to carry out a DNA-transfection protocol, cationic lipids and polycations used for DNA transfer have been widely investigated [3,11–16]. Some helper lipids (such as dioleyl phosphatidylethanolamine (DOPE), cholesterol or dioleoyl phosphatidyl choline (DOPC)), usually neutrally charged, are often employed with cationic lipids in order to gain high transfection efficiency [17]. The primary reason for current failure and frustration is the inadequacy of efficiency of cationic lipids. Tremendous efforts are currently paid worldwide to elucidate this problem, including the continuous synthesis of new cationic lipids and new approach of complexes formulations [18–25]. For a given cell type, the transfection efficiency is dependent on the morphological appearance (or on the packing morphology), besides structural parameters of the lipoplexes [26]. Cationic lipoplexes are often heterogeneous with respect to shape, size and composition. Many types of morphologies have been reported, including beads on a string structure [11], spaghettis or meatballs structure [27], multilamellar structure L^C_α and inverted hexagonal phase structure H^C_{II} [28]. In addition, a map–pin structure [29] and a sliding columnar phase [30] have also been identified. The low transfection efficiencies of current cationic lipids may be largely due to the result of incomplete understanding about transfection-related morphology of lipoplex.

Niculescu-Duvaz et al. [18] expatiated in great details the relationship between the structure of the cationic lipid and the transfection activity. Influence of plasmid DNA topology [31] and liposomal formulation [32] on the transfection properties of lipoplexes was discussed elsewhere. In this review we pay our attention to the effects of lipoplex morphologies on transfection activity. We will describe the process of lipoplex formation, including the driving force of lipoplex formation, DNA condensation, and various structures formed due to these factors. The parameters governing the preferred spatial geometry focusing on L^C_α and H^C_{II} and transfection efficiency of different structures will also be discussed. Lipoplex size is another parameter of morphology influencing on transfection efficiency, which should be paid much attention, too. We hope the knowledge of transfection activity of lipoplexes with different morphologies occurring in lipoplexes formation would provide valuable insights into the designing of novel cationic lipids and formulations with high transfection efficiency, thus yielding important information for the development of novel vectors for in vivo applications.

2. Formation of cationic lipoplexes

2.1. Driving force and DNA condensation during lipoplex formation

Though the transfection efficiency of more and more novel cationic lipoplexes has been enhanced compared with traditional non-viral delivery systems, the mechanisms of lipoplex formations are still being investigated all the time. Felgner et al. [33] concluded that the formation of lipoplexes was a result of the electrostatic interaction between cationic charge from lipids and anionic charges from DNA. Kreiss et al. [34] reported that lipoplex morphologies were determined by the competitive interactions between electrostatic forces and elasticity forces driven by the lipid hydrophobic moiety. A single plasmid is surrounded by sufficient cationic lipids to completely neutralize the negative charges of DNA and provide a complex with a net positive charge that can associate with the negatively charged surface of cells, which may be correlated with effective transfection [10]. To understand the interaction between lipoplexes and cells, it is important to understand the thermodynamics underlying lipoplex formation.

In fact, lipoplex formation is a highly dynamic event, involving the relatively uncontrolled interaction between liposomes and plasmid DNA [17,24]. Pozharski et al. [35] proposed that lipoplex formation was endothermic with less than 1 kcal absorbed per mole of lipid or DNA charge, and DNA–DNA repulsion dominated the formation enthalpy of cationic lipid/DNA complex, simultaneously the lipoplex formation was driven by an increase of entropy associating with the release of tightly bound counterions from DNA and the lipid bilayers [36,37].

Two processes occur during the formation of cationic lipoplexes, namely, DNA-induced membrane fusion and liposomes-induced cooperative DNA collapse. The latter is a key event, and leads to clusters bound to the DNA molecules structure (spherical shape of vesicles can be discerned yet) and short rod-like structure in which condensed DNA are completely

encapsulated within a smooth lipid bilayers [38]. A concentric "ring-like" pattern, arising from cationic liposome-induced DNA compactation, was recognized by Scarzello et al. [39]. DNA molecules undergo a dramatic condensation to a compact, usually highly ordered toroidal structure, such as so-called ψ-DNA, a separate phase that is organized very much like liquid cholesteric crystals, with left-handed chirality [40], because of the condensation and modification of the cationic lipids [41]. During the complex formation, both lipid and DNA undergo a complete topological transformation into compact quasispherical complex particles with ~0.2 mm diameter, and they are easy to form string-like colloidal aggregates, inside of which the complexes have an ordered multilamellar structure (L^{C}_{α}), when the complexes are neutrally charged [16,42].

2.2. Lipoplex structures

Two fundamentally different types of models have been employed in order to interprete the cationic lipoplex structures, an "external" model, in which DNA is adsorbed onto the surface of cationic liposomes, and an "internal" model, in which the DNA is surrounded or "coated" by a lipid envelope [43]. In one model of lipopelx structures, as exemplified by Felgner et al. [11], the DNA is bound electrostatically to the outside of the cationic lipid vesicles, i.e., it is adsorbed onto the vesicle, gaining a beads on a string structure. Cry-TEM applied to DOTAP or DDAB and DNA complexes containing DOPE revealed entrapment of DNA into aggregated multilamellar structures at low lipid-DNA ratios. An excess of cationic surfactant to DNA in terms of charge, leads to entrapment of the DNA molecules between the lamellas in clusters of aggregated multilamellar structures, independent on the choice of surfactant [44]. By a three-step mechanism, several single bean-like structures are aggregated in a parallel or perpendicular orientation in the end, and their diameters match the length of single plasmid DNA (approximately 200 nm), implying that the condensing effect is fairly small [45]. Vesicles made from pure pyridinium-based lipids analog SAINT-2 (Fig. 1) associate rapidly with plasmid DNA, leading to the formation of ellipsoid-shaped particles. When vesicles consisting of a mixture of SAINT-2 and DOPE (1:1 molar ratio) were incubated with plasmids, round-shaped lipoplexes bearing smoother appearance were formed, whereas free DNA was not visible [46].

A recent study revealed that lamellar structure was present during the condensation and transport of the DNA, whereas a more aggressive inverted hexagonal structure was formed upon contact with the cell membrane [47]. The appearance of the lipoplexes is often a highly ordered tubular structure when they are endocytosed by cells and assume perinuclear localization in these endosomes [48]. The observed ordered cationic lipoplexes mainly have multilamellar structure L^{C}_{α} (Fig. 2 a) and hexa-

Fig. 1. Molecular structure of SAINT-2.

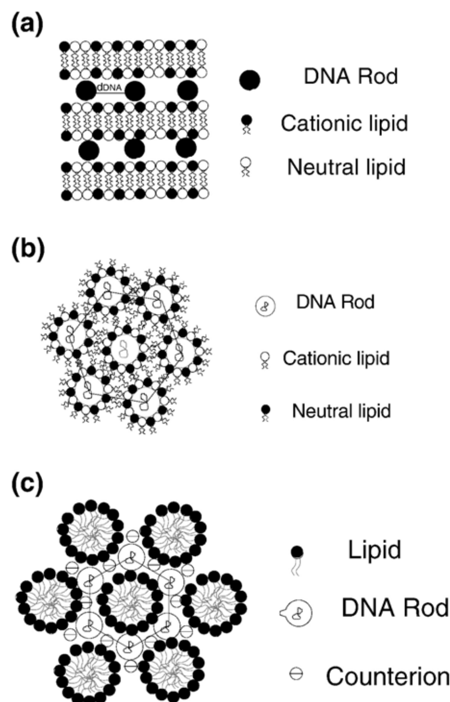

Fig. 2. Schematic of (a) the lamellar structure (L^{C}_{α}) of cationic lipid/DNA lipoplexes, where the DNA rods are sandwiched between the lipid bilayers; (b) the inverted hexagonal structure (H^{C}_{II}), where DNA rods are coated with lipid monolayer arranged on a hexagonal lattice; and (c) the intercalated hexagonal structure (H_I), where DNA rods are covered by three honeycombs of lipid micelles that are arranged on a hexagonal lattice [49,50].

gonal structures containing inverted hexagonal H^{C}_{II} (Fig. 2 b) and hexagonal H_I (Fig. 2 c). Most complexes assume lamellar phase (L^{C}_{α}) structures with DNA sandwiched between the cationic lipids [19,51,52]. A report proposed that a transient spaghetti-like structure existed between L^{C}_{α} and H^{C}_{II}, and it possibly served as a precursor to the phase H^{C}_{II} [53]. Cryo-electron images of L^{C}_{α} and H^{C}_{II} structures can be seen in Fig. 3.

Formation of lipoplexes, even if it may seem very simple from its concept, could determine the morphology and transfection potential of lipoplexes. In a mechanism of two-step lipoplex formation processes, the first step is attributed to the electrostatic binding of DNA to the liposome surface, and the second one involves fusion and rearrangement of the liposomes [54]. Though lipoplex morphologies are influenced by the second step, it is not the dominant factor governing lipoplex morphologies during lipoplex formation.

3. Factors affecting phase behavior

Now it is known that lipoplex structure and stability depend on many parameters, including the molecular characteristics of the cationic and helper co-lipids [55] and ratios of liposomes to DNA [38]. Some thermodynamic properties of lipoplex structures limiting to the two primary structures (L^{C}_{α} and H^{C}_{II}) and relating to their phase diagram have been described previously [53]. Now we will discuss the parameters governing the preferred spatial geometry focusing on L^{C}_{α} and H^{C}_{II} in the following sections.

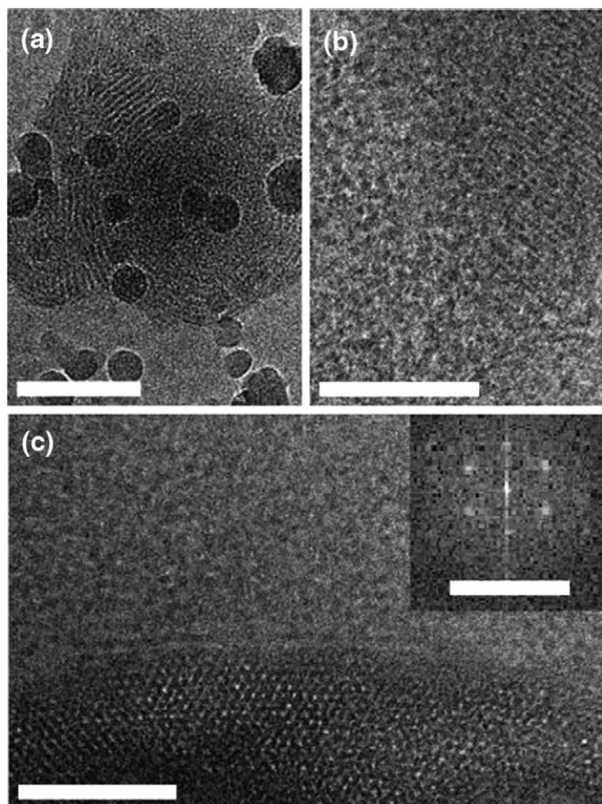

Fig. 3. Cryo-electron microscopy images of lipoplexes. (a) condensed lamellar phase; (b) inverted hexagonal (H^{C}_{II}) structure; (c) Fourier transform pattern derived from (b), (b) and (c) shown that HCII structure was present in different orientations, and it highlights the columnar characteristics and the hexagonal order, respectively. Reprinted with permission from J. Am. Chem. Soc. 2003, 125, 1551–1558. Copyright 2003 American Chemical Society.

3.1. Characteristics of cationic lipid

The relationship between the phase behavior of cationic lipids and the final morphologies after DNA complexation is especially important for the prediction of transfection efficiencies on the basis of the molecular structure of the carriers [39]. A curvature theory has been reported in which the "shape" of the molecule that determines the natural curvature of the membrane, Co = 1/Ro, will also determine the actual curvature, $C = 1/R$ in the lipid systems. Herein "R" denotes the actual radius and "Ro" the natural radius of curvature of the lipid monolayer. The actual curvature describes the structures of lipoplex, for example, when $C = 0$ lamellar L^{C}_{α} structure is favored, Co < 0 an inverted hexagonal H^{C}_{II} structure is preferred, and Co > 0 hexagonal H_I structure is dominant [56]. In the H_I phase, tubular lipid micelles are arranged on a hexagonal lattice and the DNA rods, depending on the extent of hydration, are arranged on a honeycomb lattice in the interstices of the lipid micelle arrangement. Space of surplus in the H_I structure is filled with the headgroups, water, and counterions [50].

Using sunfish amphiphiles Scarzello et al. [39,55] concluded, differing only in their hydrophobic domain, the dependence of the aggregate morphology on the size of the hydrophobic region of an amphiphile could be expressed by the dimensionless packing parameter, $P = V/a \cdot l$, in which "V" denotes the hydrophobic chain volume , "a" the optimal cross-sectional headgroup area and "l" the length of the hydrophobic tails (e.g. $1/2 < P \leq 1 \rightarrow$ lamellar organization; $P > 1 \rightarrow$ inverted structures) [57,58]. Lipid containing four alkyl chains exhibits high ability to induce H^{C}_{II} phases for their large acyl chain cross-section [24]. Limiting to pyridinium amphiphiles, the lipoplex tends to assume a well defined H^{C}_{II} phase structure with the increase of lipid alkyl chain length. It is established that the introduction of a double bond leads to increased preference for the H^{C}_{II} organization, which may attribute to an increase in the cross-sectional area in the hydrocarbon chain region [59,60], but variations in the position of the unsaturation do not lead to any morphological differences [55].

3.2. Ionic strength and temperature

The L^{C}_{α} phase keeps stable in a wide range of ionic strength and temperatures. The mobility of the lipid chains which is believed related to the transition from L^{C}_{α} phase to H^{C}_{II} phase increases when temperature is elevated [55]. At high temperature, it results in decrease of "a" as a consequence of the decrease in the hydration shell and increase of hydrophobic chain volume "V" due to the increased mobility of the alkyl chains [39]. Upon an increase in ionic strength, mixtures aggregate into a relatively stable, dispersed cubic phase and finally undergo a quantitative transition to the H^{C}_{II} phase upon DNA complexation. With increasing of ionic strength (such as presence of NaCl), the main consequence is the screening of the electrostatic repulsion between the headgroups of the lipids, then it leads to a decrease of cross-sectional headgroup area "a" and, consequently, an increase of packing parameter "p", consistent with the transition to the H^{C}_{II} phase [39,55]. Recently, using a Nile Red-based assay, Wasungu et al. [61] showed that lipoplexes made from sugar-based gemini surfactants GS1 and GS2 (Fig. 4) underwent a lamellar phase to non-inverted micellar phase (H_I) transition upon decreasing the pH from neutral to mildly acidic, differing from that of SAINT-2/DOPE, a classical H^{C}_{II} phase forming system. The two lipoplexes of GS1 and GS2 are capable of remaining a good colloidal stability in salt and in serum at physiological pH owing to their lamellar organization consistent with a prolonged stability in vivo [62].

3.3. Helper lipid

The presence of the helper lipid DOPE could increase the average packing parameter and lead to mixed bilayers [55]. Most

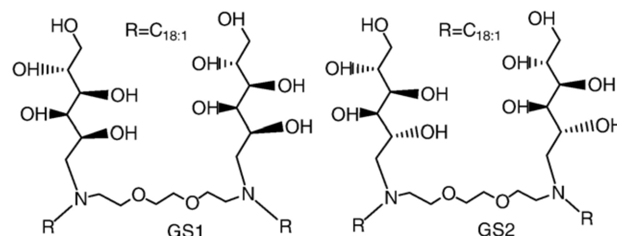

Fig. 4. Molecular structures of GS1 and GS2.

studies have shown that lipoplexes containing the non-bilayer-phase-preferring lipid DOPE or cholesterol would promote H^C_{II} organization [17]. A transition from the L^C_α phase to the H^C_{II} phase could be expected by increasing weight fraction of DOPE, via controlling the spontaneous radius of curvature "Ro" of the lipid layers, favored by the elastic free energy [56]. On the other hand, the ability of DOPE to control the structure of the cationic lipoplexes also depends on the molecular shape of the cationic lipid [63], which is determined by the ratio of head group area over hydrophobic area [64]. Conversely, it would be expected that lipids such as dioleoylphosphatidylcholine (DOPC) would hinder the ability of cationic lipids to induce non-bilayer structure [60]. By interaction with anionic vesicles, to simulate lipoplexes–endosomal membrane interaction, SAINT-2/DOPE lipoplex showed a perfect hexagonal phase, whereas SAINT-2/DPPE lipoplexes formed a mixed lamellar–hexagonal phase when containing lamellar-phase-forming dipalmitoylphosphatidylethanolamine (DPPE) [17].

3.4. DNA concentration (or lipid to DNA ratio)

Globular condensates are formed generally at high lipid/DNA ratio in terms of charge [38,44,65]. With the addition of DNA, larger chain-like structures emerge subsequently and they are linked by an invisible thread revealed by Brownian motion of these globules [66]. Large unilamellar vesicles (LUV) and sedimented multilamellar vesicles (sMLV), opposed to small unilamellar vesicles (SUV), formed lipoplexes that existed as a single phase over a relatively broad range of mixing (+/−) ratios, which was revealed by sedimentation in sucrose density gradients [67]. Precompaction of DNA with the single-tailed cationic surfactant CTAB, followed by complexation with DMTAP: DOPE (1:1) liposomes, led to ternary complexes with a multilamellar organization proved by electron microscopy data. The study about complexes formed by dsDNA with CTAB, in the presence of the cosurfactant hexanol (a membrane soluble cosurfactant) [28], using small angle X-ray diffraction, revealed an intercalated hexagonal (H_I)→lamellar (L^C_α)→inverted hexagonal (H^C_{II}) transformation on increasing hexanol content at low DNA concentrations. A H^C_{II}→L^C_α transformation was observed as increasing of DNA concentration at higher hexanol content. Thus, an L^C_α→H^C_{II}→L^C_α reentrant phase transitions was obtained as DNA concentration increasing at higher hexanol content [68]. Detailed parameters of two hexagonal structures H_I and H^C_{II} were described further using quantitative analysis of the diffraction data elsewhere [69]. A report revealed that cationic lipoplex structures changed with various lipid to DNA charge ratio but were not affected at above isoelectric point, where the charges on the DNA exactly matched those on the cationic lipids [37,70].

3.5. Zeta potential

Zeta potential is an indirect measurement of the vesicle surface charge, and it can be used to evaluate the extent of interaction of the liposomal surface cationic charges with the anionic charges of DNA [71–73]. In general, zeta potential is a function of lipid to DNA ratios, and the structure of lipoplexes with a positive zeta potential is different from lipoplexes with a negative zeta potential [72]. Lipoplexes with positive zeta potential might correspond to the aggregated multilamellar structure (L^C_α) [66]. Negative zeta potential leads to free plasmids or protruding DNA-strings. It is also possible that the "external" structure is formed at low lipid: DNA charge ratios, in which the majority of the DNA is bound to the exterior of the complex and not encapsulated within a lipid coat [44,72,74]. However, zeta potential is just one fact affecting transfection activity.

There are still many other parameters affecting lipoplex morphologies, including processing parameters [75,76], formulation procedure [77], and the ambient conditions [43] during lipoplexes formation and DNA incubation time [27]. Although influences of these parameters aren't neglected, we speculate molecule shape of cationic lipid, helper lipids (especially the nonbilayer-phase-preferring lipid DOPE and lamellar-phase-preferring DPPE) and lipid to DNA ratio (or charge ratio of positive and negative) are three key factors governing lipoplex structures during their formation. The most general principle that arises from above is that if one can correlate the efficiency of transfection with the morphology of the lipoplex, one will be able to make lipofection predictions based on known parameters governing lipoplex morphologies.

4. Relationship between transfection efficiency and lipoplex morphologies

Lipofection of the lipoplexes may occur though several stages: (a) protection of DNA (prevention dissociation by serum); (b) transporting of lipoplexes to target tissues; (c) cellular uptake; (d) fusion of an internalized cationic lipoplex with the endosome membrane; (e) escape of DNA from the endosome; (f) transporting of DNA to the nuecleus; (g) nuclear entry and (h) release of DNA from lipoplexes [18,78,79]. Transfection for some of the above stages can be enhanced by some structures or sizes of lipoplexes, and other stages may be promoted by lipoplexes with other structures or sizes.

4.1. Lipoplexe structure-transfection efficiency

In vivo, highly stabilized lipoplexes, in particular, those that have some projections, map–pin structures lead to high transfection efficiency [29,78]. Worm-like structures or tubes of lipoplexes containing DOTAP/MOG, DOTAP or DOTAP/PC, and DOTAP/DOPE were observed in freeze-fracture electron micrographs. The tubes are extremely short and appear bead-like in lipoplexes containing DOTAP/MOG, slightly longer in those containing DOTAP or DOTAP/PC, and extensively elongated in DOTAP/DOPE lipoplexes. The Extensively elongated tubular structures are not required for their inability in terms of transfection activity [74]. Small Angle X-Ray Scattering and Fluorescence Correlation Spectroscopy measurements also confirmed the existence of worm-like or tube structures [80]. The spaghetti-like structures, occurring at DNA: lipid concentrations which are typically used during transfection (2 μg of DNA to 20 nmol DC-Chol liposomes) and their diameter comes closest to the diameter

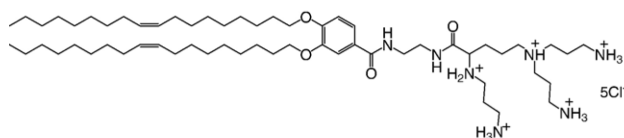

Fig. 5. Molecular structure of MVL5.

of the nuclear pores, may be the active cationic lipoplex. The reason for their high activity is likely attributed to that spaghetti, similar to microvilli, is extremely curved structure with very small radii (especially at the ending tip) and therefore is able to adhere and fuse to flat cells easily. Meanwhile, residual positive charges on their surfaces enhance the interaction and fusion with cell and probably nuclear membranes, thereby promoting the transfer of DNA into the cytoplasm and eventually into the nucleus of cells [27].

The structure-efficiency relationships studied widely recent years mainly focus on L^{C}_{α} structure and H^{C}_{II} structure, and the conclusions are not consistent either. It is proposed that the absence of a propensity for transition to the H^{C}_{II} phase may result in a lower transfection potential compared to the lipoplexes that exhibit a higher-order inverted hexagonal structures [28,55,64]. The difference of transfection efficiency between L^{C}_{α} phase and H^{C}_{II} phase comes from the reason that H^{C}_{II} lipoplexes fuse and release DNA when in contact with anionic vesicles, which are cell-free models of cellular membranes, in particular, anionic endosomal vesicles, but L^{C}_{α} lipoplexes remain stable when in contact with vesicle membranes, revealed by optical microscopy [28]. On the other hand, H^{C}_{II} phase facilitates the interaction with the cell membrane and/or enables it to escape from the endosome [47]. The ability of cationic lipids to promote inverted hexagonal phase structures can at least in part lead to facilitate its fusion with the endosomal membrane and disruption of the endosomal membrane following uptake of nucleic acid–cationic lipid lipoplexes into cells, thus facilitating cytoplasmic release of the plasmid or oligonucleotide into the cytosol [17,60,76,81]. So Zuhorn et al. [17]concluded that inverted hexagonal phase formation in lipoplexes was not only a prerequisite for nucleic acid release from the complex, but also highly critical for accomplishing efficient translocation of nucleic acids across the endosomal membrane into the cytosol for transport to the nucleus. But H^{C}_{II} structures formed from DDAB/DNA/DOPE lipoplexes, have high transfection activity in vitro, and low in vivo either due to serum instability or a high clearance rate [29].

Interestingly, novel ternary detergent/DNA/lipid lipoplex was found to be more efficient in the presence of serum, for their highly dense and bent lamellar structure adopted by these lipoplexes which prevented their dissociation by serum proteins and allowed subsequent efficient internalization in the target cells [82]. Dioctadecylamidoglycyl-spermine(DOGS)-DNA lipoplex also could maintain transfection efficiency in the presence of serum when the complexes adopted a lamellar arrangement [83]. For L^{C}_{α}, DOPC/DOTAP-DNA lipoplexes shows a strong dependence on the molar fraction of neutral lipid DOPC (ΦDOPC) and therefore membrane charge density σ_M. The transfection efõficiency starts low for $0.5 < \Phi DOPC < 0.7$ and increases dramatically to a similar value, at $\Phi DOPC = 0.2$, with H^{C}_{II} lipoplex achieved by the DOPE/DOTAP-DNA [37]. For L^{C}_{α} structure of multivalent cationic lipid MVL5/DOPC/DNA lipoplex (Fig. 5), the transfection efficiency increases exponentially with a linear increase of σ_M [51]. But others found that the curve of transfection efficiency versus σ_M assumed a bell-shape with increasing σ_M using classes of MVL cationic lipids recently [84]. In L^{C}_{α} phase at high concentrations of cationic lipid, the enhanced transfection efficiency due to the formation of pores opening in the membranes induced by the large electrostatic pressure through which the DNA molecules may escape the complex into cytoplasm was supported by a Meso-scale computer modeling of cationic lipid lipoplexes [85]. In conclusion, most reports approximately consider that membrane charge density σ_M is a universal parameter governing the transfection efficiency of L^{C}_{α} lipoplexs [19,51,52,84,86].

A recent study for lipoplex of dendritic lipid MVLBG2/DOPC/DNA, with a composition around 25 mol% MVLBG2 (Fig. 6), showed that lipoplex in the H_I phase efficiently transfected mouse and human cells in culture. The high efficiency may be ascribed to the existence of a continuous DNA substructure which likely facilitates release of the DNA cargo, as in principle all DNA is accessible once a part of it is exposed to the inside of the cell [50].

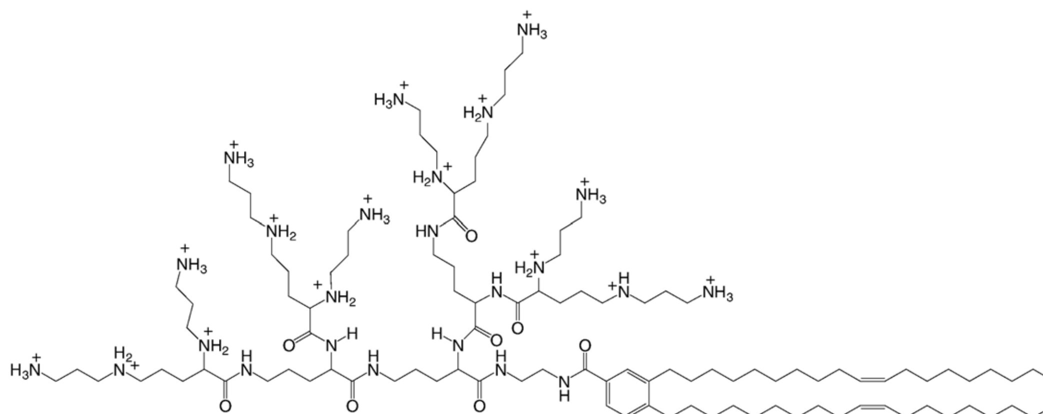

Fig. 6. Molecular structure of MVLBG2.

The behavior of lipoplexes containing univalent lipids and multivalent lipids may be different, and the transfection efficiency of the same structure can not maintain consistent in vivo and in vitro [28,29]. It is widely accepted that the main entry route of lipoplex to mammalian cells is endocytosis, so any structure facilitating membrane fusion, DNA release from lipoplexes and escape from endosome could acquire high transfection efficiency, and H^C_{II} is a representative in these aspects. High transfection efficiency from L^C_α mainly benefits from their stability which can restrain dissociation of serum during lipoplexes transporting. Therefore, it will be much important to find out more how formulations affect lipoplex structures so that we can better predict the character of the lipoplex supramolecular assemblies with high transfection efficiencies.

4.2. Lipoplex size-transfection efficiency

Another parameter of morphologies affecting transfection efficiency is lipoplex size, for the important role of lipoplex size in determining the nature of the entry pathway by endocytosis [76]. The cationic lipoplex stability depends on the charge ratio of lipid to DNA [16,26,87]. Lipoplex size and the heterogeneity of the structures both appear to increase along with the increasing of lipid to DNA ratio [43]. Condensing ability of cationic lipids (especially monovalent lipids) is another role affecting complex size [88]. However, size effect of lipoplexs on the transfection activity has not been unified so far. Some consider that there is no apparent correlation of the size of lipoplexes with transfection efficiency [89–91], others suggest that lipoplex size influence [77,92] transfection efficiency, even lipoplex size is a major factor [93,94] in terms of lipofection efficiency.

Lipoplex size is very important for gene transfer to actively endocytosing cells [93]. The influences on the transfection efficiency of lipoplexes by cationic lipid:DNA ratio, types of liposomes, incubation time in polyanion containing media, and time of serum addition, are channeled mostly through their influences on lipoplex size. For example, lipofection inhibition by serum is largely due to the serum inhibition of lipoplex size growth, and may be overcome by using large, stable lipoplexes [95]. Lipoplexes of less than 250 nm in size measured by dynamic light scattering show efficient transfection only in the absence of serum. Conversely, lipoplexes of over 700 nm mean diameter induce efficient transfection in the presence or absence of serum [87]. It was reported that the particle size may be one of the factors that were contributed to serum resistance of EDL (ethanol-dried lipid-DNA) lipoplexes, and the large cationic lipoplexes may delay the dissociation of DNA with lipid, thereby enhancing DNA transfection efficiency [96]. Konopka et al. [97] proposed that serum decreased the size of lipoplexes but was essentially not inhibitory to transfection activity.

Large lipoplexes size is more efficient to transfer gene because large particles taken up by cells lead to the formation of large intracellular vesicles which are more easily disrupted, thus releasing DNA into the cytoplasm [98]. In addition, the advantageous effect of large particles upon lipofection has been attributed to maximum contact with cells [99], increased phagocytic activity accompanied by endosomal escape [74] and faster sedimentation and better cellular trafficking [100]. However, highly effective lipoplex for gene transfer was obtained in NIH 3 T3 cells from RPR120535/DNA lipoplex (Fig. 7) characterized by their small diameter (50 nm) [16]. A recent report also supported that particles with smaller size would gain high transfection efficiency [101]. The requirement for efficient transfection may be different in vivo and in vitro. Small particle size (40–80 nm) is required for high efficiency in vivo delivery owing to traversal of the capillary network (e.g. in the lungs), while 200–400 nm is the optimal size for lipoplexes in vitro [78,99,102].

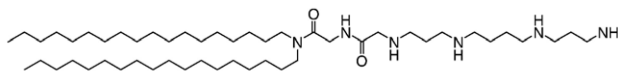

Fig. 7. Molecular structure of RPR120535.

Although conflicting reports exist upon the optimal size of lipoplexes for lipofection, there is no doubt that high lipofection would be gained from large lipoplexes when endocytosis is dominant, because large particles facilitate membrane contact and fusion. When the type of cell is not actively endocytosing cell, either small particles may have high transfection efficiency, or lipoplex size doesn't correlate with lipofection efficiency. Besides lipoplex size, different conditions (such as cationic lipids, cell types and in vivo or vitro) may result in different transfection efficiency.

5. Cytotoxicity

Nguyen et al. [103] clearly indicated that there was a synergism between cationic lipid and pDNA in causing cytotoxicity. They found that cationic lipid (Lipofectamine 2000) alone induced only a slight cellular toxicity, irrespective of the absence or presence of serum, and free pDNA did not show any cytotoxicity. However, lipoplexes induced a significant cytotoxicity toward HeLa cells, B16BL6 cells and RGC-6 cells compared to cationic lipid alone, and the cytotoxicity increased as the cationic lipid content in the lipoplex increased. Another study proposed that cationic liposomes formulated from DOPE and cationic lipids (such as DOTAP, DMTAP, DPTAP, DSTAP), whether or not they were complexed with DNA, were highly toxic in vitro toward macrophages, but not toward non-phagocytic T cells. The incorporation of DNA marginally reduced cationic liposome toxicity toward macrophages [104]. Other factors affecting cytotoxicity of lipoplexs include zeta potential, incubation time, cell type and cell density [105,106]. Toxicity of the lipoplex may depend upon the nature of the aggregates formed. For example, the same lipoplex has been shown to exhibit a significantly reduced toxicity when present in a vesicular as opposed to a micellar solution [107]. Literatures about direct relationship between toxicity and morphologies of lipoplex are very few at present, and it is worthy to study for scientists in the future.

6. Discussion

Different stages in the pathway of transfection may be promoted by different types of morphological behavior of the

lipoplexes, and different structures lead to a distinctly different mechanism of cell entry [53]. The high transfection efficiency of lipoplexes containing DOPE may be ascribed to potential transformation to an invert hexagonal structures H^{C}_{II} promoted by DOPE. A transition from the L^{C}_{α} structure to the H^{C}_{II} structure and the subsequent increasing of transfection efficiency could be expected by increasing weight fraction of DOPE. This, however does not mean that all lipoplexes containing DOPE do have high transfection efficiency, because we are not sure that H^{C}_{II} have high transfection efficiency in every gene delivery. High lipofection will be acquired for H^{C}_{II} structure when membrane fusion is dominant, but when serum is present L^{C}_{α} lipoplexes show great superiority for their inhibition dissociation by serum during lipoplexes transporting. High lipofection would be gained from large lipoplexes when endocytosis is dominant, because large particles facilitate membrane contact and fusion. When the type of cell is not actively endocytosing cell, either small particles may have high transfection efficiency, or lipoplex size doesn't correlate with lipofection.

It is difficult to say which structure or lipoplex size is better for gene delivery, because transfection efficiency of the cationic lipoplexes may depend on not only morphologies of the complexes, but also other factors, such as cell types. Different types of cells may possess different ability to the uptake and expression of DNA [108]. Therefore, a coherent and comprehensive conclusion of the morphology-efficiency relationship of cationic lipoplexes is often difficult, due to the fact that it is difficult for scientists to perform a comparison of different classes of cationic lipids and different cell types within one study.

The morphologies of lipoplex should be physicochemically characterized at two different levels, one level, which relates to size and size instability, and the other level, which relates to the self-assembled structures including all parameters influencing lipoplex structures and optimized structure with high lipofection. Clearly, more work will be required before we have a perfect understanding of the lipoplex morphology at macro level and an understanding of all the critical parameters that control and affect the lipoplex structures at micro levels. In the future, detailed data generated from these studies in the process of gene delivery will help scientists to design new generations of vectors with low toxicity and relatively high transfection efficiency. Influences of lipoplex morphologies on transfection efficiency are greatly depended on internalisation mechanisms and intracellular trafficking. Some studies have expatiated lipoplex internalisation mechanisms and intracellular trafficking [109,110], but they are currently not well documented. However, because the multiple barriers to entry of the lipoplexes into the target cell are becoming clear at the molecular level, the role of morphologies of lipoplex is becoming more and more important for scientists in vector design, formulation and molecular assembly of lipoplexes.

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