Roles of lipid polymorphism in intracellular delivery

Ismail M. Hafez, Pieter R. Cullis*

Department of Biochemistry and Molecular Biology, University of British Columbia, 2146 Health Sciences Mall, Vancouver, British Columbia V6T 1Z3, Canada

Abstract

Lipids, which adopt nonbilayer phases, have fascinated researchers as to the functional roles of these components in biomembranes. In particular, lipids capable of adopting the hexagonal $H_3$ phase have received considerable attention because of the observation that such lipids can promote membrane fusion. In the rational design of lipid-based delivery systems, $H_3$ phase lipids have been employed to endow systems with fusogenic, membrane-destabilizing properties. We will outline the molecular basis for the polymorphic phase behavior of lipids and highlight some of the uses of nonbilayer lipids in the preparation of lipid-based delivery systems. In addition, a distinction will be drawn between lipid-based systems which rely on the inclusion of nonbilayer lipids for activity, and systems which contain components which actively promote formation of nonbilayer structure within biological membranes. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Lipid-based delivery systems; pH-sensitive liposomes; Inverted hexagonal phase; Membrane fusion; Bilayer destabilization

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1. Introduction

Enclosed bilayer membranes, or liposomes prepared from amphiphilic molecules dispersed in aqueous media have long been recognized as simple models of cell membranes and for their potential
utility as vehicles for drug delivery (for a historical perspective see Ref. [1]). The study of isolated lipid components of biological membranes in simplified model membrane systems has allowed for the characterization of lipids, which adopt a variety of mesoscopic phases. Steps towards understanding the functional roles of nonbilayer lipid components of biomembranes [2–4] have been paralleled by efforts in exploiting the polymorphic phase behavior of lipids for the rational design of lipid-based intracellular delivery systems [5]. A simple example will illustrate how knowledge of biological membrane properties can lead to the rational design of a triggered lipid-based delivery system.

The organization of lipid molecules in most biological cell membranes is that of a bimolecular layer of lipid molecules, or a bilayer [6]. The lipid bilayer structure of biomembranes also encompasses an extra level of complexity in its relatively simple arrangement. Biomembranes are asymmetric in composition. For example, the inner lipid monolayer of the erythrocyte membrane and indeed most eukaryotic membranes is composed of phosphatidylserine (PS) and phosphatidylethanolamine (PE), while the outer monolayer harbors most of the phosphatidylcholine (PC) and sphingomyelin (SM). This inner membrane monolayer of the erythrocyte is not stable in the presence of high Ca$^{2+}$. In isolation, model bilayer membranes prepared from the inner monolayer lipids PE and PS exhibit fusogenic and polymorphic phase behavior under conditions of elevated Ca$^{2+}$ [7] or reduced pH [8]. Model membranes composed of PE and PS were therefore formulated as pH-sensitive fusogenic liposomes [8]. These lipid vesicles can be considered prototypes for an entire class of pH-triggered liposomal systems which rely on a mixture of nonbilayer lipid which is conditionally stabilized by ionizable amphiphils [9]. pH-sensitive liposomes are only one class of lipid-based delivery systems which rely on the control of bilayer to nonbilayer phase transition for activity.

In this review, lipid polymorphism and its role in the design of lipid-based intracellular delivery systems will be discussed. Emphasis is placed on mechanisms, which may be used to modulate the structure of lipid assemblies and promote destabilization of liposomal vectors and cellular membranes.

2. Lipids can assemble into a variety of structures

Upon dispersion in water, amphiphilic molecules can self-assemble into a variety of different structures. Many reviews have been written on the polymorphic phase behavior of lipids [2,4,10,11]. Lipids such as PC adopt bilayer phases upon hydration, whereas fatty acids and lysolipids can adopt a micellar arrangement in water (Fig. 1). Of particular interest are lipids such as unsaturated PE which comprises a significant proportion of the lipids in biomembranes and in isolation adopts the inverted hexagonal (H$_{II}$) phase. For example, dioleoylphosphatidylethanolamine (DOPE) forms a bilayer phase below 10°C, while at elevated temperatures DOPE adopts the H$_{II}$ phase [12]. Formation of the H$_{II}$ phase is promoted by increasing acyl chain unsaturation and increasing temperature [13].

Lipids can also adopt some interesting non-vesicle bilayer structures. PS, for example, forms cochleate cylinders in the presence of calcium [14] while the galactosylcerebroside (GalCer) lipids can adopt bilayers which assemble into helical ribbons and nanotubes [15]. Lipid structures such as the nanotubes hold promise for rapid protein crystallization and structure determination using electron microscopy techniques [16].

3. Theory of lipid polymorphism

“Molecular shape” arguments have been used to rationalize the phase behavior of lipids [10]. Lipids with a large headgroup area and a small hydrocarbon area have a cone-like geometry, self-assemble into micelles and are said to exhibit positive membrane curvature (Fig. 1A). Lipids, which are cylindrical in shape, having nearly equal headgroup to hydrocarbon area, self-assemble into lipid bilayers (Fig. 1B). Alternatively, lipids with small headgroup areas adopt “inverted” lipid phases such as the inverted hexagonal (H$_{II}$) phase or cubic phases and are said to exhibit negative membrane curvature (Fig. 1C). Thus, complementary mixtures of nonbilayer micellar lipids and nonbilayer H$_{II}$ phase preferring lipids can adapt bilayer phases [17,18]. In addition, mixtures of oppositely charged surfactants, which form
micellar structures in isolation, can spontaneously assemble into bilayer vesicles [19]. The behavior of mixed anionic and cationic surfactant systems can be rationalized as arising from the reduction in surfactant headgroup size and increase in hydrophobic area following formation of cationic–anionic di-acyl zwitterions which have a molecular shape compatible with the formation of bilayer structure. The effective molecular shape and consequently lipid phase behavior can also be modulated by changes in hydration, state of ionization, presence of divalent cations and temperature [2].

4. Nonbilayer lipids and membrane fusion

Membrane fusion is a ubiquitous process in biological systems and involves the union of two opposing bilayers in order to complete processes such as exocytosis or viral infection. A local departure from the bilayer structure must take place in order to allow two lipid membranes to merge into one. Little is known about the structure of these membrane intermediates, which are involved in membrane fusion in biological systems. However, the study of membrane fusion in model lipid systems has provided a guide to understanding some of the factors, which may underlie membrane dynamics in biological fusion events.

Lipidic particles observed by freeze–fracture were first interpreted to be inverted micelles formed at the junctions between lipid bilayers undergoing membrane fusion [20] (Fig. 2). Alternatively, the lipidic particles observed by freeze–fracture techniques may be related to the formation of the “stalk” intermediate of membrane fusion as defined by Markin et al. [21] and later developed by Chernomordik and Zimmerberg [22] and Siegel [23]. In the stalk theory of membrane fusion, two apposed bilayers undergo a union of the contacting monolayers through the
formation of a semi-toroidal lipidic structure called the stalk (Fig. 2). It has been proposed that the expansion of the stalk intermediate produces a transmonolayer contact (TMC) which ruptures due to increasing mechanical tension to produce the fusion pore. Time-resolved cryoelectron microscopy has been used to directly visualize TMC-like structures formed in the early stages of pure lipid vesicle fusion [24].

The geometry of the stalk intermediate favors the incorporation of lipids, which exhibit negative membrane curvature. Lipids such as unsaturated phosphatidylethanolamine which has a cone, or wedge structure have compatible shape to incorporate into the highly bent stalk intermediate. Conversely, micellar lipids, which exhibit positive membrane curvature, have a shape, which is incompatible with the orientation of lipids proposed in the stalk structure. Indeed, a correlation is observed between the shapes of lipids in the contacting monolayers and membrane fusion. Inverted hexagonal phase-adopting lipids such as DOPE [12] or protonated PS [25] promote fusion of lipid vesicles [26], while micellar lysolipids inhibit fusion of large unilamellar vesicles (LUVs) and virosomes when applied to the outer lipid monolayers [27] lending indirect support to the stalk mechanism of membrane fusion. Chernomordik et al. have demonstrated the inhibitory effect of lysolipids on biological membrane fusion events. Addition of lysolipids to the contacting membrane
monolayers inhibited sea urchin egg cortical exocytosis, mast cell degranulation, rat liver microsome–mitochondrion fusion, and viral fusion. This indicates that membrane fusion in biological and model systems is highly dependent on the physical properties of the contacting lipid monolayers.

5. DOPE: the king of nonbilayer lipids

DOPE is the most commonly utilized nonbilayer lipid for the preparation of so-called “fusogenic” lipid-based delivery systems. The claim that DOPE is a “fusogenic lipid” is derived from the ability of DOPE to adopt the inverted hexagonal phase in isolation. It has been demonstrated that lipids which adopt inverted lipid phases promote fusion of lipid bilayers and structural intermediates involved in membrane fusion are similar to those involved in bilayer to H$_{II}$ phase transitions. One appealing physical parameter of DOPE is that it forms the H$_{II}$ phase above 10°C and therefore, at physiological temperatures, DOPE prefers a nonbilayer phase. However, caution must be taken when interpreting data relating to the behavior of DOPE-containing systems at low temperatures, for example for cell culture experiments performed at 4°C when DOPE prefers a bilayer phase.

The primary route of internalization of liposomes by cells is the endocytic pathway via clathrin-coated pits. Therefore, a main barrier in lipid-based drug delivery is the escape of hydrolytically sensitive material from degradation in lysosomes, which in this review will be referred to as intracellular delivery. Inclusion of DOPE into lipid-based drug delivery systems such as pH-sensitive liposomes, target-sensitive liposomes, cationic lipoplexes, stabilized plasmid lipid particles (SPLPs), and programmable fusogenic vesicles (PFVs) has been found to be a key factor for intracellular delivery. Replacement of the H$_{II}$-phase lipid DOPE with the bilayer lipid dioleoylphosphatidylcholine (DOPC) either completely inhibits or severely attenuates intracellular delivery. In addition, designer lipids such as polymer conjugated poly(ethylene glycol) (PEG)–lipids which stabilize DOPE into a bilayer also have inhibitory effects on liposome fusion and on intracellular delivery. Although the use of DOPE has proven highly successful, few studies have investigated the formulation of lipid-based delivery systems, which utilize other nonbilayer lipids such as highly unsaturated phosphatidylethanolamines or structurally dissimilar lipids such as diacylglycerol, monoolein, or monogalactosyldiacylglycerol.

6. Nonbilayer lipids and pH-sensitive liposomes

If ionizable lipids are incorporated into bilayer phases with DOPE, the stability of the bilayer is conditional on the pH, which controls the structural preferences of the ionizable lipid. The first system described as a fusogenic pH-sensitive liposome was composed of PS–DOPE (2:8 molar ratio) [8]. These vesicles were stable at neutral pH, but underwent fusion at acidic pH values. PS itself adopts a bilayer phase on hydration at neutral pH values, however, below pH 4, unsaturated PS species are known to adopt the inverted hexagonal phase [25]. Thus at acidic pH, PS–DOPE liposomes contain only lipids which prefer a nonbilayer phase, and as a result are unstable and fusogenic. A variety of different lipid combinations have been used to prepare pH-sensitive liposomes (Table 1).

The potential to use pH-sensitive liposomes for intracellular delivery was highlighted by Straubinger et al. They demonstrated that anionic liposomes are taken up by CV-1 cells through endocytosis and encounter a low pH compartment. Shortly following this discovery, pH-sensitive liposomes prepared from the nonbilayer lipid DOPE and oleic acid were shown to mediate the release of the encapsulated fluorescent dye calcein into the cytoplasm of cultured cells. pH-sensitive liposomes have since been used for intracellular delivery of a variety of macromolecules including nucleic acids such as DNA and antisense oligonucleotides, protein toxins, and antibiotics. An overview of the various macromolecules introduced into cells using pH-sensitive liposomes is presented in Table 2.

The mechanism of intracellular delivery via pH-sensitive liposomes is not well-defined. Following endocytosis it is proposed that pH-sensitive...
Table 1
pH-sensitive liposome formulations

<table>
<thead>
<tr>
<th>Nonbilayer lipid</th>
<th>Titratable lipid</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPE</td>
<td>Phosphatidylserine</td>
<td>[8]</td>
</tr>
<tr>
<td>DOPE</td>
<td>Palmitoylhomocysteine (PHC)</td>
<td>[43]</td>
</tr>
<tr>
<td>DOPE</td>
<td>Cholesteryl hemisuccinate (CHEMS)</td>
<td>[44]</td>
</tr>
<tr>
<td>DOPE</td>
<td>N-Succinyl dioleoylphosphatidylethanolamine (N-Succ-DOPE)</td>
<td>[45]</td>
</tr>
<tr>
<td>DOPE</td>
<td>Oleic acid</td>
<td>[46]</td>
</tr>
<tr>
<td>DOPE</td>
<td>Series of double-chain amphiphiles</td>
<td>[47]</td>
</tr>
<tr>
<td>POPE</td>
<td>Diacylsuccinylglycerols (SGs)</td>
<td>[48]</td>
</tr>
<tr>
<td>DOPE</td>
<td>α-Tocopherol hemisuccinate</td>
<td>[49]</td>
</tr>
<tr>
<td>DOPE</td>
<td>Sulfatide</td>
<td>[50]</td>
</tr>
</tbody>
</table>

Table 2
Intracellular delivery using pH-sensitive liposomes

<table>
<thead>
<tr>
<th>Entrapped molecule</th>
<th>Assay method</th>
<th>Lipid formulation</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcein</td>
<td>Fluorescence microscopy</td>
<td>Oleic acid/DOPE</td>
<td>[36]</td>
</tr>
<tr>
<td>Calcein</td>
<td>Fluorescence microscopy</td>
<td>PHC/DOPE</td>
<td>[51]</td>
</tr>
<tr>
<td>Arabinoside-C</td>
<td>Cell killing</td>
<td>Oleic acid/DOPE</td>
<td>[52]</td>
</tr>
<tr>
<td>Diphtheria Toxin A</td>
<td>Cell killing</td>
<td>Oleic acid/DOPE</td>
<td>[53]</td>
</tr>
<tr>
<td>CAT-Plasmid DNA</td>
<td>CAT activity</td>
<td>Oleic acid/Chol/DOPE</td>
<td>[54]</td>
</tr>
<tr>
<td>FITC-Dextran (4.2 kDa)</td>
<td>Fluorescence microscopy</td>
<td>CHEMS/DOPE</td>
<td>[55]</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>MHC class-I presentation</td>
<td>SG/DOPE</td>
<td>[56]</td>
</tr>
<tr>
<td>Oligonucleotide</td>
<td>Friend retrovirus inhibition</td>
<td>Oleic acid/Chol/DOPE</td>
<td>[57]</td>
</tr>
<tr>
<td>PolyIC RNA</td>
<td>IFN production</td>
<td>Oleic acid/Chol/DOPE</td>
<td>[58]</td>
</tr>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td>Cell-associated SOD activity</td>
<td>SG/DOPE</td>
<td>[59]</td>
</tr>
<tr>
<td>Listeriolysin/ovalbumin and HPTS</td>
<td>Fluorescence microscopy/MHC class-I presentation</td>
<td>CHEMS/DOPE</td>
<td>[60]</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>Bacterial killing</td>
<td>N-Succ-DOPE/DOPE</td>
<td>[42]</td>
</tr>
</tbody>
</table>

Liposomes undergo destabilization and leakage upon encountering an intracellular acidic stimulus. This may lead to the release of the liposomal contents within acidic endosomal compartments. Alternatively, if close proximity is achieved between the liposome and the lumenal membrane of the endosome at the time of acidification, destabilization of the endosomal membrane may result from the preference of the pH-sensitive liposomal lipids for non-bilayer phases.

7. pH-sensitive liposomes composed of mixtures of anionic and cationic lipids

We have recently shown that pH-sensitive liposomes may be prepared by using a different strategy [61]. Mixtures of the anionic lipid cholesteryl hemisuccinate (CHEMS) and the cationic lipid dioleoyldimethylammonium chloride (DODAC) can be used to prepare negatively charged vesicles at slightly alkaline pH values, which undergo fusion as
the pH is reduced. The particular advantage of this system is that the pH at which membrane fusion occurs can be readily and predictably tuned by adjusting the ratio of anionic to cationic lipids [61]. In these systems there is no nonbilayer lipid component per se. However, we have found that equimolar mixtures of the anionic lipid CHEMS and the cationic lipid DODAC [61] as well as mixtures of anionic phospholipids and cationic lipids adopt non-bilayer phases such as the hexagonal H\textsubscript{II} phase [61,62]. Thus in tunable pH-sensitive liposomes, the excess anionic lipid acts to stabilize the remaining anionic--cationic lipid pairs, and fusion occurs upon neutralization of vesicle surface charge [61].

"Molecular shape" arguments can be used to rationalize the phase behavior of mixtures of anionic and cationic lipids. Separately anionic and cationic lipids adopt bilayer phases, yet in neutralized mixtures nonbilayer phases are preferred. As with mixtures of oppositely charged surfactants [19] which undergo a micelle to bilayer transition due to a reduction in spontaneous monolayer curvature, oppositely charged bilayer-forming lipids would be expected to also undergo a decrease in monolayer curvature due to the formation of cationic--anionic lipid pairs. Formation of such ion pairs would be expected to exclude counter-ions and their associated water molecules thus reducing hydration and resulting in formation of a cone-shaped zwitterion capable of adopting H\textsubscript{II} phase structure.

8. Role of nonbilayer lipids in transfection mediated by cationic lipoplexes

The interesting polymorphism observed with mixtures of anionic and cationic lipids lead us to investigate possible intracellular interactions between cationic lipids and cellular anionic phospholipids. Szoka Jr. and co-workers have previously shown that cationic lipid--nucleic acid lipoplexes release associated nucleic acids upon interaction with anionic liposomes [63,64]. Further work showed that ion-pairs are formed between anionic and cationic lipids following displacement of nucleic acid polymers from cationic lipids by anionic lipids [65].

The transfection potency of most cationic liposome formulations can be enhanced by the presence of the H\textsubscript{II} phase forming lipid DOPE [38,66--70]. We have recently demonstrated that cationic lipids are able to actively induce H\textsubscript{II} phase structure in mixtures with anionic phospholipids. Thus helper lipids such as DOPE appear to potentiate the ability of cationic lipids to induce nonbilayer structure of biological membranes (Hafez and Cullis, submitted). We suggest that the ability of cationic lipids to induce nonbilayer phases in the presence of anionic lipids is critical to the mechanism of how cationic lipids promote intracellular delivery of macromolecules such as plasmid DNA. In addition, agents which are known to promote nonbilayer phases in model membranes can also enhance transfection. Examples of these agents include calcium [71] and polylysine [67] which can enhance cationic lipoplex transfection and also induce nonbilayer phase transitions in anionic phospholipid mixtures [2,25].

Conversely, lipids, which promote bilayer or micelle formation, are found to strongly inhibit transfection. These lipids include bilayer-forming species such as DOPC [38], and micellar lipids such as PEG--PE [72] both of which are able to stabilize against the formation of the hexagonal H\textsubscript{II} phase [18,73].

A strong correlation is therefore observed between the potentiation of transfection and the inclusion of H\textsubscript{II} phase lipids in cationic lipoplexes. Cationic lipids, which themselves actively promote the formation of the H\textsubscript{II} phase in mixtures with bilayer-forming anionic phospholipids, should be considered extremely potent bilayer-destabilizing agents. In support of this, cationic lipids are often observed to promote enhanced transfection levels in the absence of helper lipids such as DOPE [74--76].

9. Conclusions

The potential of lipid-based systems lies within the diversity of lipid components that can be employed to prepare systems with a wide variety of properties [77]. A distinction should be made between systems, which rely on components to modulate the structural behavior of the carrier system and those systems, which contain agents that can actively participate in
the destabilization of cellular target membranes. Lipids such as DOPE may have activity in both capacities, while the cationic lipids used to formulate cationic–nucleic acid lipoplexes can actively modulate the phase behavior of bilayer assemblies containing anionic phospholipids. Investigation into agents that produce similar specific polymorphic effects on biomembranes such as fusogenic peptides [78] warrants continued investigation.

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