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Roles of lipid polymorphism in intracellular delivery

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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_{II} 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_{II} 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.

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

Contents

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

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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 adopt bilayer phases [17,18]. In addition, mixtures of oppositely charged surfactants, which form

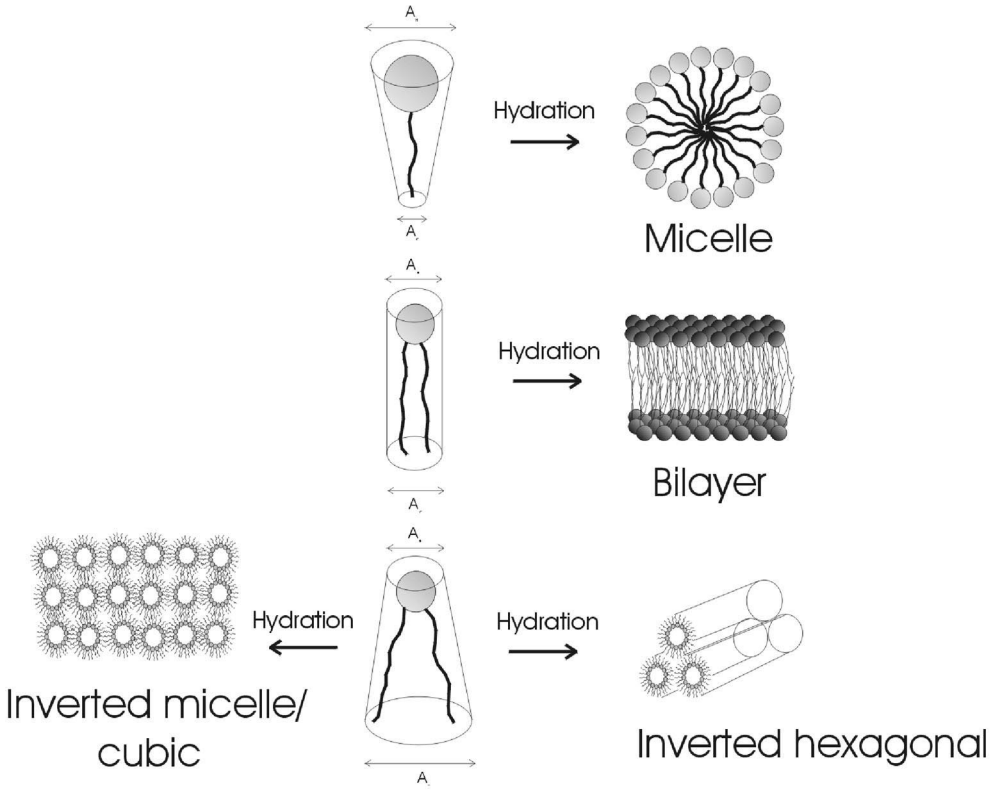

Fig. 1. Molecular geometry of lipids and the predicted self-assembly of morphologically distinct structures.

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

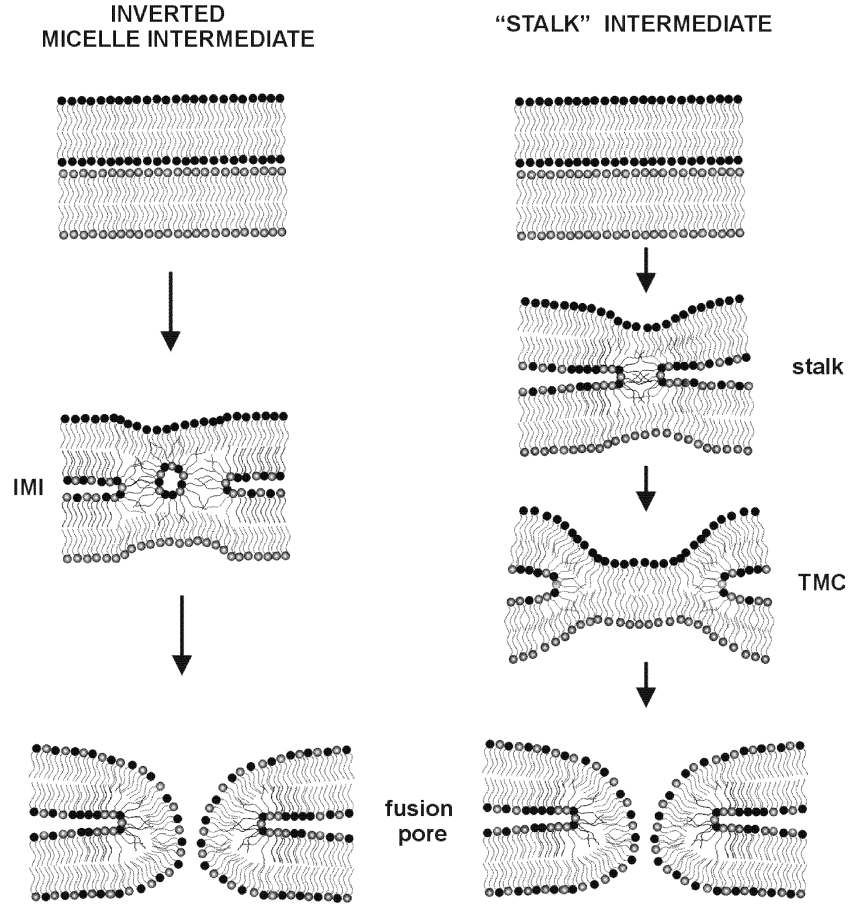

Fig. 2. Proposed intermediates of membrane fusion. Two apposed bilayers are schematically represented to undergo fusion through either an inverted micelle intermediate (IMI) or the stalk and transmembrane contact (TMC) intermediates.

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–microsome fusion, and viral fusion [28]. 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 [5]. The claim that DOPE is a ''fusogenic lipid'' is derived from the ability of DOPE to adopt the inverted hexagonal phase in isolation [12]. It has been demonstrated that lipids which adopt inverted lipid phases promote fusion of lipid bilayers [7,26] and structural intermediates involved in membrane fusion are similar to those involved in bilayer to H_{II} phase transitions [24,29]. 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 [12]. 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 [30].

The primary route of internalization of liposomes by cells is the endocytic pathway via clathrin-coated pits [31–35]. 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 [36], target-sensitive immunoliposomes [37], cationic lipoplexes [38], stabilized plasmid lipid particles (SPLPs) [39], and programmable fusogenic vesicles (PFVs) [40] 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 [41] and on intracellular delivery [42]. 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 [31]. 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 [36]. 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 [5,9]. Following endocytosis it is proposed that pH-sensitive

Table 1
pH-sensitive liposome formulations

Nonbilayer lipid	Titratable lipid	Ref.
DOPE	Phosphatidylserine	[8]
DOPE	Palmitoylhomocysteine (PHC)	[43]
DOPE	Cholesteryl hemisuccinate (CHEMS)	[44]
DOPE	*N*-Succinyldioleoylphosphatidylethanolamine (*N*-Succ-DOPE)	[45]
DOPE	Oleic acid	[46]
DOPE	Series of double-chain amphiphiles	[47]
DOPE	Diacylsuccinylglycerols (SGs)	[48]
POPE	α-Tocopherol hemisuccinate	[49]
DOPE	Sulfatide	[50]

Table 2
Intracellular delivery using pH-sensitive liposomes

Entrapped molecule	Assay method	Lipid formulation	Ref.
Calcein	Fluorescence microscopy	Oleic acid/DOPE	[36]
Calcein	Fluorescence microscopy	PHC/DOPE	[51]
Arabinoside-C	Cell killing	Oleic acid/DOPE	[52]
Diphtheria Toxin A	Cell killing	Oleic acid/DOPE	[53]
CAT-Plasmid DNA	CAT activity	Oleic acid/Chol/DOPE	[54]
FITC-Dextran (4.2 kDa)	Fluorescence microscopy	CHEMS/DOPE	[55]
Ovalbumin	MHC class-1 presentation	SG/DOPE	[56]
Oligonucleotide	Friend retrovirus inhibition	Oleic acid/Chol/DOPE	[57]
PolyIC RNA	IFN production	Oleic acid/Chol/DOPE	[58]
Superoxide dismutase (SOD)	Cell-associated SOD activity	SG/DOPE	[59]
Listeriolysin O/ovalbumin and HPTS	Fluorescence microscopy/ MHC class-I presentation	CHEMS/DOPE	[60]
Gentamycin	Bacterial killing	*N*-Succ-DOPE/DOPE	[42]

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 nonbilayer 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 dioloeyldimethylammoinum 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 nonbilayer phases such as the hexagonal H_{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_{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 lipo-some formulations can be enhanced by the presence of the H_{II} phase forming lipid DOPE [38,66–70]. We have recently demonstrated that cationic lipids are able to actively induce H_{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_{II} phase [18,73].

A strong correlation is therefore observed between the potentiation of transfection and the inclusion of H_{II} phase lipids in cationic lipoplexes. Cationic lipids, which themselves actively promote the formation of the H_{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.

References

[1] A.D. Bangham, Surrogate cells or Trojan horses. The discovery of liposomes, Bioassays 17 (1995) 1081–1088.

[2] B. de Kruijff, P.R. Cullis, The influence of poly(L-lysine) on phospholipid polymorphism. Evidence that electrostatic polypeptide-phospholipid interactions can modulate bilayer/non-bilayer transitions, Biochim. Biophys. Acta 601 (1) (1980) 235–240.

[3] S.M. Gruner, Intrinsic curvature hypothesis for biomembrane lipid composition: a role for nonbilayer lipids, Proc. Natl. Acad. Sci. USA 82 (1985) 3665–3669.

[4] R.M. Epand, Lipid polymorphism and protein–lipid interactions, Biochim. Biophys. Acta 1376 (1998) 353–368.

[5] D.C. Litzinger, L. Huang, Phosphatidylethanolamine liposomes: drug delivery, gene transfer and immunodiagnostic applications, Biochim. Biophys. Acta 1113 (1992) 201–227.

[6] E. Gorter, F. Grendel, On biomolecular layers of lipoids on the chromocytes of the blood, J. Exp. Med. 41 (1925) 439–443.

[7] M.J. Hope, P.R. Cullis, The bilayer stability of inner monolayer lipids from the human erythrocyte, FEBS Lett. 107 (1979) 323–326.

[8] M.J. Hope, D.C. Walker, P.R. Cullis, Ca^{2+} and pH induced fusion of small unilamellar vesicles consisting of phosphatidylethanolamine and negatively charged phospholipids: a freeze fracture study, Biochem. Biophys. Res. Commun. 110 (1983) 15–22.

[9] R.M. Straubinger, pH-sensitive liposomes for delivery of macromolecules into cytoplasm of cultured cells, Methods Enzymol. 221 (1993) 361–376.

[10] S.M. Gruner, P.R. Cullis, M.J. Hope, C.P. Tilcock, Lipid polymorphism: the molecular basis of nonbilayer phases, Annu. Rev. Biophys. Biophys. Chem. 14 (1985) 211–238.

[11] G. Lindblom, L. Rilfors, Nonlamellar phases formed by membrane lipids, Adv. Colloid Interface Sci. 41 (1992) 101–125.

[12] P.R. Cullis, B. de Kruijff, The polymorphic phase behaviour of phosphatidylethanolamines of natural and synthetic origin. A ^{31}P-NMR study, Biochim. Biophys. Acta 513 (1978) 31–42.

[13] R.N. Lewis, D.A. Mannock, R.N. McElhaney, D.C. Turner, S.M. Gruner, Effect of fatty acyl chain length and structure on the lamellar gel to liquid-crystalline and lamellar to reversed hexagonal phase transitions of aqueous phosphatidylethanolamine dispersions, Biochemistry 28 (1989) 541–548.

[14] D. Papahadjopoulos, W.J. Vail, K. Jacobson, G. Poste, Cochleate lipid cylinders: formation by fusion of unilamellar lipid vesicles, Biochim. Biophys. Acta 394 (1975) 483–491.

[15] P. Yager, J. Chappell, D.D. Archibald, When lipid bilayers won't form liposomes: tubules, helices and cochleate cylinders, in: B.P. Gaber, K.R.K. Easwaran (Eds.), Biomembrane Structure and Function – The State of the Art, Adenine Press, Schenectady, NY, 1991, p. 1.

[16] E.M. Wilson-Kubalek, R.E. Brown, H. Celia, R.A. Milligan, Lipid nanotubes as substrates for helical crystallization of macromolecules, Proc. Natl. Acad. Sci. USA 95 (1998) 8040–8045.

[17] T.D. Madden, P.R. Cullis, Stabilization of bilayer structure for unsaturated phosphatidylethanolamines by detergents, Biochim. Biophys. Acta 684 (1982) 149–153.

[18] J.W. Holland, P.R. Cullis, T.D. Madden, Poly(ethylene glycol)–lipid conjugates promote bilayer formation in mixtures of non-bilayer-forming lipids, Biochemistry 35 (1996) 2610–2617.

[19] E.W. Kaler, A.K. Murthy, B.E. Rodriguez, J.A. Zasadzinski, Spontaneous vesicle formation in aqueous mixtures of single-tailed surfactants, Science 245 (1989) 1371–1374.

[20] P.R. Cullis, M.J. Hope, Effects of fusogenic agent on membrane structure of erythrocyte ghosts and the mechanism of membrane fusion, Nature 271 (1978) 672–674.

[21] V.S. Markin, M.M. Kozlov, V.L. Borovjagin, On the theory of membrane fusion. The stalk mechanism, Gen. Physiol. Biophys. 3 (1984) 361–377.

[22] L.V. Chernomordik, J. Zimmerberg, Bending membranes to the task: structural intermediates in bilayer fusion, Curr. Opin. Struct. Biol. 5 (1995) 541–547.

[23] D.P. Siegel, The modified stalk mechanism of lamellar/inverted phase transitions and its implications for membrane fusion, Biophys. J. 76 (1999) 291–313.

[24] D.P. Siegel, R.M. Epand, The mechanism of lamellar-to-inverted hexagonal phase transitions in phosphatidylethanolamine: Implications for membrane fusion mechanisms, Biophys. J. 73 (1997) 3089–3111.

[25] M.J. Hope, P.R. Cullis, Effects of divalent cations and pH on phosphatidylserine model membranes: a ^{31}P-NMR study, Biochem. Biophys. Res. Commun. 92 (1980) 846–852.

[26] H. Ellens, J. Bentz, F.C. Szoka, H^+- and Ca^{2+}-induced fusion and destabilization of liposomes, Biochemistry 24 (1985) 3099–3106.

[27] P.L. Yeagle, F.T. Smith, J.E. Young, T.D. Flanagan, Inhibition of membrane fusion by lysophosphatidylcholine, Biochemistry 33 (1994) 1820–1827.

[28] L.V. Chernomordik, S.S. Vogel, A. Sokoloff, H.O. Onaran, E.A. Leikina, J. Zimmerberg, Lysolipids reversibly inhibit Ca(2 +)-, GTP- and pH-dependent fusion of biological membranes, FEBS Lett. 318 (1993) 71–76.

[29] H. Ellens, D.P. Siegel, D. Alford, P.L. Yeagle, L. Boni, L.J. Lis, P.J. Quinn, J. Bentz, Membrane fusion and inverted phases, Biochemistry 28 (1989) 3692–3703.

[30] I. Wrobel, D. Collins, Fusion of cationic liposomes with mammalian cells occurs after endocytosis, Biochim. Biophys. Acta 1235 (1995) 296–304.
[31] R.M. Straubinger, K. Hong, D.S. Friend, D. Papahadjopoulos, Endocytosis of liposomes and intracellular fate of encapsulated molecules: encounter with a low pH compartment after internalization in coated vesicles, Cell 32 (1983) 1069–1079.
[32] R.M. Straubinger, D. Papahadjopoulos, K.L. Hong, Endocytosis and intracellular fate of liposomes using pyranine as a probe, Biochemistry 29 (1990) 4929–4939.
[33] D.L. Daleke, K. Hong, D. Papahadjopoulos, Endocytosis of liposomes by macrophages: binding, acidification and leakage of liposomes monitored by a new fluorescence assay, Biochim. Biophys. Acta 1024 (1990) 352–366.
[34] K.D. Lee, K. Hong, D. Papahadjopoulos, Recognition of liposomes by cells: in vitro binding and endocytosis mediated by specific lipid headgroups and surface charge density, Biochim. Biophys. Acta 1103 (1992) 185–197.
[35] D.S. Friend, D. Papahadjopoulos, R.J. Debs, Endocytosis and intracellular processing accompanying transfection mediated by cationic liposomes, Biochim. Biophys. Acta 1278 (1996) 41–50.
[36] R.M. Straubinger, N. Duzgunes, D. Papahadjopoulos, pH-sensitive liposomes mediate cytoplasmic delivery of encapsulated macromolecules, FEBS Lett. 179 (1985) 148–154.
[37] R.J. Ho, B.T. Rouse, L. Huang, Target-sensitive immunoliposomes: preparation and characterization, Biochemistry 25 (1986) 5500–5506.
[38] P.L. Felgner, T.R. Gadek, M. Holm, R. Roman, H.W. Chan, M. Wenz, J.P. Northrop, G.M. Ringold, M. Danielsen, Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure, Proc. Natl. Acad. Sci. USA 84 (1987) 7413–7417.
[39] J.J. Wheeler, L. Palmer, M. Ossanlou, I. MacLachlan, R.W. Graham, Y.P. Zhang, M.J. Hope, P. Scherrer, P.R. Cullis, Stabilized plasmid–lipid particles: construction and characterization, Gene Ther. 6 (1999) 271–281.
[40] G. Adlakha-Hutcheon, M.B. Bally, C.R. Shew, T.D. Madden, Controlled destabilization of a liposomal drug delivery system enhances mitoxantrone antitumor activity, Nat. Biotechnol. 17 (1999) 775–779.
[41] J.W. Holland, C. Hui, P.R. Cullis, T.D. Madden, Poly(ethylene glycol)–lipid conjugates regulate the calcium-induced fusion of liposomes composed of phosphatidylethanolamine and phosphatidylserine, Biochemistry 35 (1996) 2618–2624.
[42] P. Lutwyche, C. Cordeiro, D.J. Wiseman, M. St.-Louis, M. Uh, M.J. Hope, M.S. Webb, B.B. Finlay, Intracellular delivery and antibacterial activity of gentamicin encapsulated in pH-sensitive liposomes, Antimicrob. Agents Chemother. 42 (1999) 2511–2520.
[43] J. Connor, M.B. Yatvin, L. Huang, pH-sensitive liposomes: acid-induced liposome fusion, Proc. Natl. Acad. Sci. USA 81 (1984) 1715–1718.
[44] H. Ellens, J. Bentz, F.C. Szoka, pH-induced destabilization of phosphatidylethanolamine-containing liposomes: role of bilayer contact, Biochemistry 23 (1984) 1532–1538.
[45] R. Nayar, A.J. Schroit, Generation of pH-sensitive liposomes: use of large unilamellar vesicles containing *N*-succinyldioleoylphosphatidylethanolamine, Biochemistry 24 (1985) 5967–5971.
[46] N. Duzgunes, R.M. Straubinger, P.A. Baldwin, D.S. Friend, D. Papahadjopoulos, Proton-induced fusion of oleic acid–phosphatidylethanolamine liposomes, Biochemistry 24 (1985) 3091–3098.
[47] R. Leventis, T. Diacovo, J.R. Silvius, pH-dependent stability and fusion of liposomes combining protonatable double-chain amphiphiles with phosphatidylethanolamine, Biochemistry 26 (1987) 3267–3276.
[48] D. Collins, D.C. Litzinger, L. Huang, Structural and functional comparisons of pH-sensitive liposomes composed of phosphatidylethanolamine and three different diacylsuccinylglycerols, Biochim. Biophys. Acta 1025 (1990) 234–242.
[49] H. Jizomoto, E. Kanaoka, K. Hirano, pH-sensitive liposomes composed of tocopherol hemisuccinate and of phosphatidylethanolamine including tocopherol hemisuccinate, Biochim. Biophys. Acta 1213 (1994) 343–348.
[50] X. Wu, K.H. Lee, Q.T. Li, Stability and pH sensitivity of sulfatide-containing phosphatidylethanolamine small unilamellar vesicles, Biochim. Biophys. Acta 1284 (1996) 13–19.
[51] J. Connor, L. Huang, Efficient cytoplasmic delivery of a fluorescent dye by pH-sensitive immunoliposomes, J. Cell Biol. 101 (1985) 582–589.
[52] J. Connor, L. Huang, pH-sensitive immunoliposomes as an efficient and target-specific carrier for antitumor drugs, Cancer Res. 46 (1986) 3431–3435.
[53] D. Collins, L. Huang, Cytotoxicity of diphtheria toxin A fragment to toxin-resistant murine cells delivered by pH-sensitive immunoliposomes, Cancer Res. 47 (1987) 735–739.
[54] C.Y. Wang, L. Huang, Highly efficient DNA delivery mediated by pH-sensitive immunoliposomes, Biochemistry 28 (1987) 9508–9514.
[55] C.J. Chu, J. Dijkstra, M.Z. Lai, K. Hong, F.C. Szoka, Efficiency of cytoplasmic delivery by pH-sensitive liposomes to cells in culture, Pharm. Res. 7 (1990) 824–834.
[56] S. Nair, F. Zhou, R. Reddy, L. Huang, B.T. Rouse, Soluble proteins delivered to dendritic cells via pH-sensitive liposomes induce primary cytotoxic T lymphocyte responses in vitro, J. Exp. Med. 175 (1992) 609–612.
[57] C. Ropert, M. Lavignon, C. Dubernet, P. Couvreur, C. Malvy, Oligonucleotides encapsulated in pH sensitive liposomes are efficient toward Friend retrovirus, Biochem. Biophys. Res. Commun. 183 (1992) 879–885.
[58] P.G. Milhaud, B. Compagnon, A. Bienvenue, J.R. Philippot, Interferon production of L929 and HeLa cells enhanced by polyriboinosinic acid–polyribocytidylic acid pH-sensitive liposomes, Bioconjug. Chem. 3 (1992) 402–407.
[59] P. Briscoe, I. Caniggia, A. Graves, B. Benson, L. Huang, A.K. Tanswell, B.A. Freeman, Delivery of superoxide

dismutase to pulmonary epithelium via pH-sensitive liposomes, Am. J. Physiol. 268 (3, Pt. 1) (1995) L374–380.
[60] K.D. Lee, Y.K. Oh, D.A. Portnoy, J.A. Swanson, Delivery of macromolecules into cytosol using liposomes containing hemolysin from *Listeria monocytogenes*, J. Biol. Chem. 271 (1996) 7249–7252.
[61] I.M. Hafez, S. Ansell, P.R. Cullis, Tunable pH-sensitive liposomes composed of mixtures of cationic and anionic lipids, Biophys. J. 79 (2000) 1438–1446.
[62] R.N. Lewis, R.N. McElhaney, Surface charge markedly attenuates the nonlamellar phase-forming propensities of lipid bilayer membranes: calorimetric and ^{31}P-nuclear magnetic resonance studies of mixtures of cationic, anionic, and zwitterionic lipids, Biophys. J. 79 (2000) 1455–1464.
[63] Y. Xu, F.C. Szoka Jr., Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection, Biochemistry 35 (1996) 5616–5623.
[64] O. Zelphati, F.C. Szoka Jr., Mechanism of oligonucleotide release from cationic liposomes, Proc. Natl. Acad. Sci. USA 93 (1996) 11493–11498.
[65] S. Bhattacharya, S.S. Mandal, Evidence of interlipidic ion pairing in anion-induced DNA release from cationic amphiphile–DNA complexes. Mechanistic implications in transfection, Biochemistry 37 (1998) 7764–7777.
[66] J.H. Felgner, R. Kumar, C.N. Sridhar, C.J. Wheeler, Y.J. Tsai, R. Border, P. Ramsey, M. Martin, P.L. Felgner, Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations, J. Biol. Chem. 269 (1994) 2550–2561.
[67] X. Zhou, L. Huang, DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action, Biochim. Biophys. Acta 1189 (1994) 195–203.
[68] H. Farhood, N. Serbina, L. Huang, The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer, Biochim. Biophys. Acta 1235 (1995) 289–295.
[69] K.W. Mok, P.R. Cullis, Structural and fusogenic properties of cationic liposomes in the presence of plasmid DNA, Biophys. J. 73 (1997) 2534–2545.
[70] M.J. Hope, B. Mui, S. Ansell, Q.F. Ahkong, Cationic lipids, phosphatidylethanolamine and the intracellular delivery of polymeric, nucleic acid-based drugs, Mol. Membr. Biol. 15 (1998) 1–14.
[71] A.M. Lam, P.R. Cullis, Calcium enhances the transfection potency of plasmid DNA–cationic liposome complexes, Biochim. Biophys. Acta 1463 (2000) 279–290.
[72] P. Harvie, F.M. Wong, M.B. Bally, Use of poly(ethylene glycol)–lipid conjugates to regulate the surface attributes and transfection activity of lipid–DNA particles, J. Pharm. Sci. 89 (2000) 652–663.
[73] P.R. Cullis, B. de Kruijff, Polymorphic phase behaviour of lipid mixtures as detected by ^{31}P-NMR. Evidence that cholesterol may destabilize bilayer structure in membrane systems containing phosphatidylethanolamine, Biochim. Biophys. Acta 507 (1978) 207–218.
[74] J.S. Remy, C. Sirlin, P. Vierling, J.P. Behr, Gene transfer with a series of lipophilic DNA-binding molecules, Bioconjug. Chem. 5 (1994) 647–654.
[75] C.J. Wheeler, L. Sukhu, G. Yang, Y. Tsai, C. Bustamente, P. Felgner, J. Norman, M. Manthorpe, Converting an alcohol to an amine in a cationic lipid dramatically alters the co-lipid requirement, cellular transfection activity and the ultrastructure of DNA–cytofectin complexes, Biochim. Biophys. Acta 1280 (1996) 1–11.
[76] Y. Xu, S.W. Hui, P. Frederik, F.C. Szoka, Physicochemical characterization and purification of cationic lipoplexes, Biophys. J. 77 (1999) 341–353.
[77] O.V. Gerasimov, Y. Rui, D.H. Thompson, Triggered release from liposomes mediated by physically and chemically induced phase transitions, in: M. Rosoff (Ed.), Vesicles, Marcel Dekker, New York, 1996, p. 679.
[78] S. Simoes, V. Slepushkin, P. Pires, R. Gaspar, M.P. de Lima, N. Duzgunes, Mechanisms of gene transfer mediated by lipoplexes associated with targeting ligands or pH-sensitive peptides, Gene Ther. 6 (1999) 1798–1807.