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OVERVIEW

Biophysical Aspects of Using Liposomes as Delivery Vehicles

Anne S. Ulrich[1]

Liposomes are used as biocompatible carriers of drugs, peptides, proteins, plasmic DNA, antisense oligonucleotides or ribozymes, for pharmaceutical, cosmetic, and biochemical purposes. The enormous versatility in particle size and in the physical parameters of the lipids affords an attractive potential for constructing tailor-made vehicles for a wide range of applications. Some of the recent literature will be reviewed here and presented from a biophysical point of view, thus providing a background for the more specialized articles in this special issue on liposome technology. Different properties (size, colloidal behavior, phase transitions, and polymorphism) of diverse lipid formulations (liposomes, lipoplexes, cubic phases, emulsions, and solid lipid nanoparticles) for distinct applications (parenteral, transdermal, pulmonary, and oral administration) will be rationalized in terms of common structural, thermodynamic and kinetic parameters of the lipids. This general biophysical basis helps to understand pharmaceutically relevant aspects such as liposome stability during storage and towards serum, the biodistribution and specific targeting of cargo, and how to trigger drug release and membrane fusion. Methods for the preparation and characterization of liposomal formulations *in vitro* will be outlined, too.

KEY WORDS: Biophysics; drug delivery; lipid; liposome.

ABBREVIATIONS: CHEMS, cholesterylhemisuccinate; CHOL, cholesterol; DC Cholesterol, 3β-(*N*-(*N′*,*N′*,-dimethylaminoethane)-carbimol) cholesterol; DPMC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine; DMRIE, 1,2-dimyristoyloxypropyl-3-dimethyl-hydroxyethyl ammonium; DODAc, dioctadecyldimethylammonium chloride; DOGS, dioctadecylamidoglycyl spermine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; DOSPA, 2,3-dioleoyloxy-*N*-(2(sperminecarboxamide)ethyl)-*N*,*N*-dimethyl-1-propananninium; DOTAP, 1,2-dioleoyloxy-3-(trimethylammonio) propane; DOTMA, *N*-(2,3-(dioleoyloxy)propyl-*N*,*N*,*N*-trimethyl ammonium; DPPS, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylserine; DPSG, *N*-(succinyl)dipalmitoyl phosphatidylethanolamine; DSPG, 1,2-distearoyl-*sn*-glycero-3-phosphatidylglycerol; GMO, glycerol monoleate; *N*-maleyl-DOPE, *N*-(maleyl)dioleoyl phosphatidylethanolamine; OA, oleic acid.

LIPOSOME COMPOSITION AND SIZE

Many reviews and book chapters have focused on the application of liposomes for drug delivery, gene therapy, and immunization. Only a few recent papers shall be pointed out here [1–18], even if this may not pay justice to the pioneers in some areas. When using liposomes as *drug carriers* the main aim is to reduce toxic side effects in sensitive organs such as heart and kidneys and to target specific tissues such as tumors. By optimizing the lipid composition, liposomal size, membrane

[1]Friedrich-Schiller-University of Jena, Institute of Molecular Biology, Winzerlaer Str. 10, 07745 Jena, Germany. E-mail: ulrich@molebio.uni-jena.de

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fluidity, surface charge, and steric stabilization, it is possible to extend the therapeutic index of liposomal carriers over that of the corresponding conventional formulations. Likewise, when designing novel cationic lipids for *gene therapy*, their ability to mediate transfection can be attributed to several physical factors, including the spontaneous condensation of the DNA due to electrostatic interactions, the net positive charge of the system that may promote association with negatively charged cell surfaces, as well as the fusogenic properties of the lipids which can destabilize the plasma membrane or endosomal compartment. Finally, the rationale behind the use of liposomes for *immunization* purposes relies on their ability to deliver an antigen into selected immune cells and to stimulate an immune response. Before examining the pharmaceutical behavior of these different types of liposomal carriers in terms of their physicochemical parameters, some general information and basic definitions will be summarized in the following paragraphs.

Lipid Structure and Assembly

The amphilic molecules used for the preparation of liposomes are derived from or based on the structure of biological membranes lipids, as summarized in Fig. 1 [1, 3, 10–12, 19–21]. Two hydrocarbon chains are usually esterified to a glycerol backbone (''glycerolipids'', or ''plasmalogens'' in the case of an α-β unsaturated ether), or they constitute the hydrophobic ceramide moiety (''sphingolipids''). This hydrophobic part is linked to a hydrophilic headgroup containing either a phosphate (''phospholipids'') or some carbohydrate units (''glycolipids''). Biologically relevant lipid headgroups are either zwitterionic [phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM)], negatively charged [phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylinositol (PI), cardiolipin (CL), substituted glycolipids such as monosiagloganglioside (GM1)], or entirely uncharged [unsubstituted glycolipids]. Saturated acyl chains typically vary in length from 10 carbons (lauryl), 12 (myristoyl), 14 (palmitoyl) to 16 (stearoyl), and the longer 18-carbon chains are usually unsaturated with one (oleoyl), two (linoleyl) or three (linolenyl) *cis*-double bonds. Positively charged lipids are custom-made molecules, based on the same structural principles, which are designed to condense the DNA and to interact with oppositely charged biological membranes. Examples of cationic amphiphiles include DOTAP, DOTMA, DODAC, DC-Chol, DMRIE, DOSPA, DOGS, amongst many others, seen in Fig. 1. Cholesterol is readily incorporated into lipid assemblies up to 50%, whereas purely hydrophobic lipids such as triacylglycerides (TAG) do not disperse in water, unless they are surrounded by a surfactant monolayer (e.g. phospholipid, detergent) to form an emulsion or a solid lipid nanoparticle (SLN).

Amphiphilic lipids are poorly soluble in water as monomers, with a low critical micelle concentration (CMC) typically between 10^{-8} and 10^{-12} M, depending on the hydrocarbon chain length. While single-chain lipids (lysolipids, free unsaturated acyl chains, detergents, etc.) spontaneously assemble into micelles, most membrane-derived lipids tend to be driven into bilayers. The resulting lamellar structures form closed vesicles, i.e. liposomes, as illustrated in Fig. 2. One commonly distinguishes between multilamellar vesicles (MLV, 0.1–10 μm), and unilamellar ones that can be

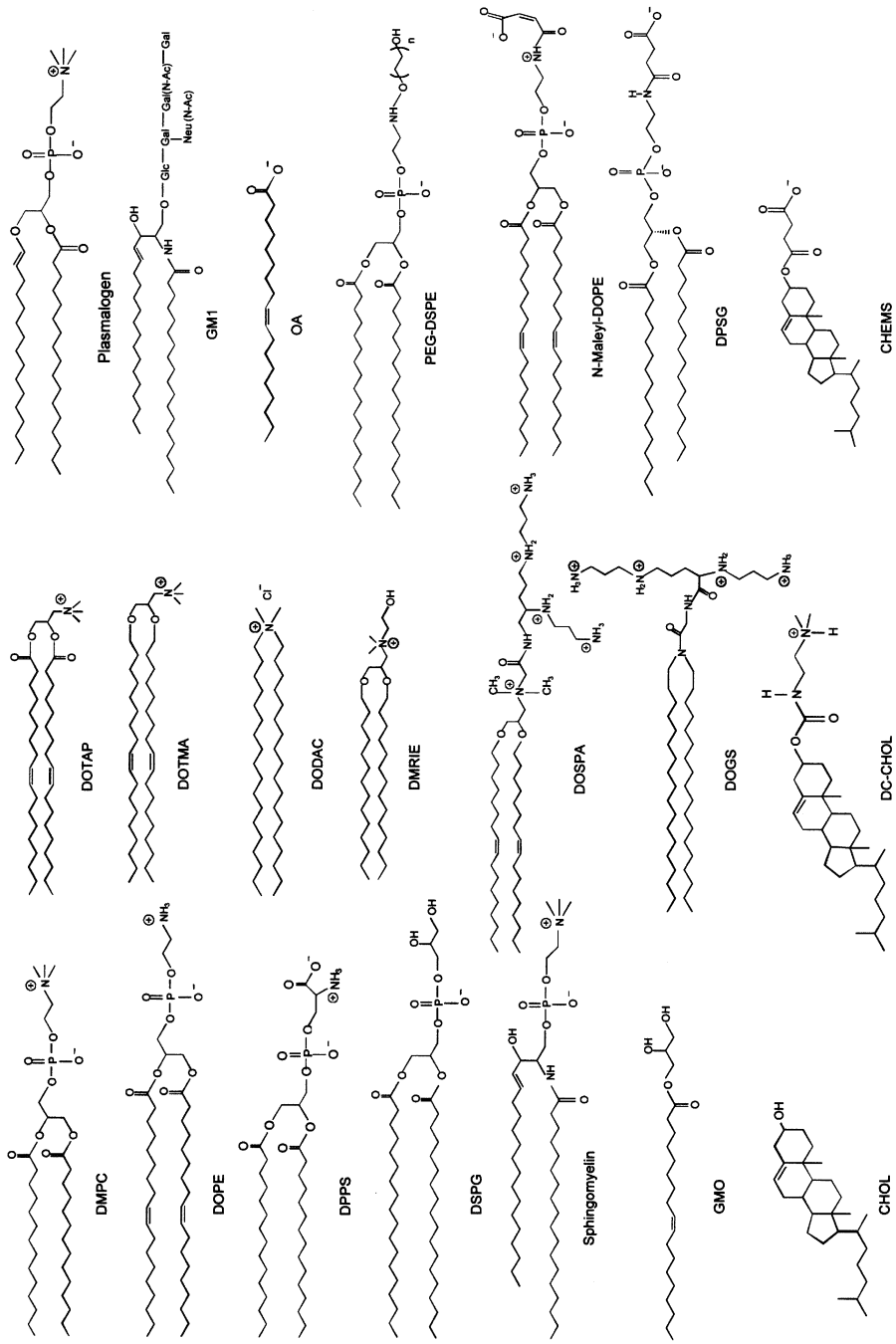

Fig. 1. Overview of representative lipid and amphilphile structures (named are listed in abbreviations).

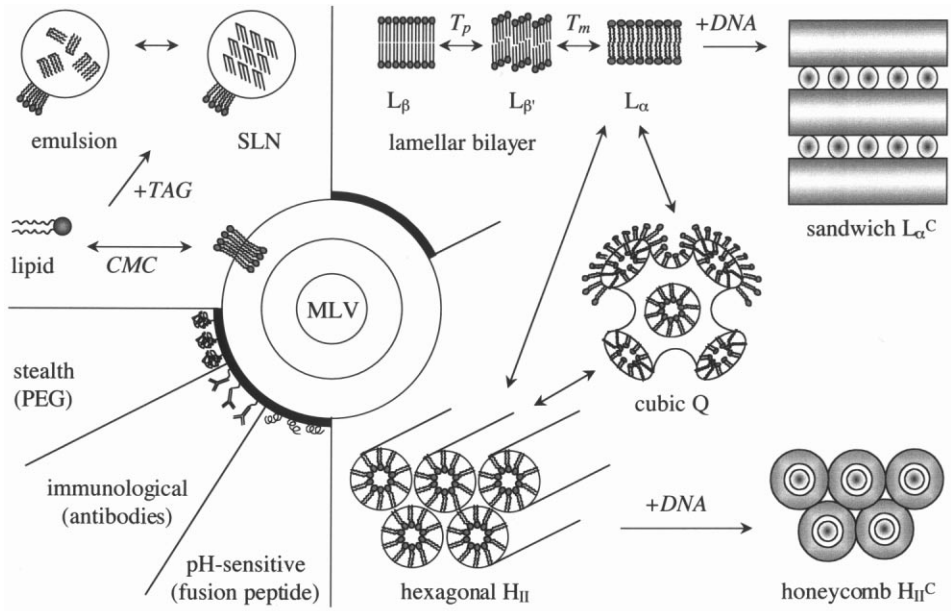

Fig. 2. Overview of lipid phase transitions and polymorphism.

small (SUV, <100 nm), large (LUV, 100–500 nm) or giant (GUV, ≥1 μm). Certain isolated lipids or lipid mixtures may prefer non-bilayer morphologies such as hexagonal (H_{II}) and cubic (Q) phases –*see below*). Mixtures of cationic lipids and DNA, i.e. so-called lipoplexes'', can adopt closely related structures.

Preparation of Liposomes

A suspension of MVL is readily obtained by co-dissolving the desired lipids (including any hydrophobic cargo) in organic solvent, subsequent drying, and finally hydrating under agitation above the lipid phase transition temperature. The resulting size distribution and lamellarity of the MLV is very heterogeneous, but sophisticated procedures have been developed to produce uniformly sized liposomes [1, 20, 22]. Sonication disrupts MLV to produce SUV with radii around 30–60 nm, which carry defects and have a tendency to undergo aggregation and fusion. Repeated extrusion of a MLV suspension through a polycarbonate filter of well-defined pore-size produces LUV with any designated diameter between 80–400 nm. Interestingly, extruded vesicles have been reported to retain significantly elongated elliptical shapes, which has to be taken in to account when evaluating their size and entrapped volume [23]. For the large-scale production of LUV the easiest and most efficient method is by homogenization, where a lipid suspension is repeatedly forced at high pressure through a small orifice to collide with a wall or an oncoming fluid beam. Disadvantages are a relatively high polydispersity, difficulties in fine-tuning the size, and some risk of sample degradation. Another very rapid but cumbersome approach

relies on dissolving a mixture of lipid and hydrophobic carbon in ethanol or ether, which is then injected into an appropriate buffer to form a heterogeneous mixture of SUV, LUV or MLV, depending on concentration.

Hydrophobic and amphiphilic drugs can be readily incorporated into liposomes using the methods above [24, 25], but for water-soluble compounds there are more efficient approaches to achieve a high yield of encapsulation [22]. For example, vesicles can be passively charged using reverse phase evaporation (REV), by injecting an aqueous solution of the drug rapidly into an organic phase containing the lipid. The resulting "water-in-oil" emulsion is sonicated and partially dried down to a semi-solid gel, which is then converted by vigorous shaking into a concentrated suspension of vesicles in water (typically 0.1 to 1.0 μm diameter, with up to 50% entrapment). Alternatively, dehydration–rehydration vesicles (DRV) can be produced at large-scale by mixing an aqueous solution of the drug with a suspension of "empty" LUV. Freeze-drying and rehydration induces fusion between adjacent membranes and the solute is taken up by the resulting MLV (0.1 to 2.0 μm diameter, with up to 80% entrapment). Another, more gentle method for the passive entrapment or reconstitution of sensitive proteins and membrane-anchored ligands (e.g. for targeting purposes or antigen presentation) relies on dissolving the lipids and cargo have in a detergent. Subsequent dilution or detergent removal by dialysis, biobeads, or gel filtration leads to the formation of liposomes, although it is still difficult to control the reconstitution mechanism rationally [26]. As opposed to passive loading, active loading procedures exploit differences in the partition coefficient of a drug on pH and ionic strength [27]. For example, basic compounds carrying amino groups are relatively lipophilic at high pH by hydrophilic at low pH. by setting up a pH gradient across the liposomal membrane (either by adding base to vesicles prepared at low pH, or by loading the vesicles with $(NH_4)_2SO_4$ which will release NH_3 and thus turn acidic inside), the drug will actively diffuse into the liposome and be trapped.

Preparation of Cationic Lipid-DNA Complexes

Early attempts to incorporate large DNA fragments or plasmids into conventional zwitterionic or anionic liposomes were rather inefficient. Only in the presence of Ca^{2+} does negatively charged DOPS accommodate DNA in stable particles of several μm diameter, exhibiting a cochleate structure (a continuous multilayered sheet rolled up in a cylinder) [28]. The use of cationic amphiphiles to condense and deliver DNA, on the other hand, is one of the most popular non-viral approaches for gene therapy [2, 5, 6, 8, 9, 13, 15, 16, 18, 29]. It is well known that the concentrations of lipid and DNA, the ionic strength and temperature of the suspending medium, the order of addition, and the rate of mixing affect the resulting lipoplex size and homogeneity. One of the most critical parameters for the colloidal stability and transfection activity of lipoplexes is the initial charge ratio of cationic lipid:DNA. Highly positively charged complexes, where the DNA is completely sequestered, exhibit a relatively uniform diameter of about 100–450 nm, and a similar sized distribution is observed when complexes are prepared with an excess of DNA. Empirically, the most uniform results are obtained when rapidly adding the

limited component to the one in excess, whereas very slow mixing or the reverse order frequently leads to precipitation [16]. At either charge ratio far from 1:1, the resulting high colloidal stability is attributed to the net surface charge. On the other hand, when neutral complexes are prepared, very heterogeneous particles are obtained (350–1200 nm diameter). Since the lipid-DNA assembly is governed by multivalent electrostatic interactions, macroscopic aggregation tends to be kinetically controlled and irreversible, while thermodynamic equilibrium can only be locally established at the microscopic level [30]. It is not surprising that different transfection activities have been reported for any given liposome composition, depending on the use of MLV, LUV, or SUV. With a carefully controlled mixing procedure, however, uniform complexes with a narrow size distribution could be reproducibly obtained [5], irrespective of the type of liposomes used [31]. The transfection activity of these particles was dependent only on the final size (300–2000 nm diameter) which determines the corresponding mode of cellular uptake (see below). Remarkably, their biological activity was not affected by altering the overall lipid/DNA charge ratio following the initial condensation [30], suggesting that the initial steps of mixing are most critical.

Particle Size Characterization

Spectroscopic methods for describing particle size and lipid assembly have been recently reviewed [14]. Both turbidimetry and light scattering are based on the same physical phenomenon, despite the different instrumentation required. Turbidity is readily measured in conventional spectrophotometers by determining the optical density (OD) typically at 400 nm [2]. Even though it is not possible to estimate the particle size, this method is very useful for quickly checking the reproducibility of preparations or for monitoring the solubilization and reconstitution of vesicles. Light scattering methods, on the other hand, are more sensitive as they detect the 90°-scattering, which can be measured either under steady-state conditions in a fluorimeter, or dynamically in a designated instrument equipped with a laser. The latter method, also called quasielastic light scattering or photon correlation spectroscopy (PCS), analyzes the intensity of the scattered light in the millisecond time regime through autocorrelation analysis. The Brownian motion of the particles induces a broadening of the spectrum that is related to their size and shape [23]. Dynamic light scattering is thus the most popular method to calculate the mean hydrodynamic radius of suspended particles and their polydispersity index, and it coves a size range from a few nanometers to several μm [13, 14]. An alternative approach to determine the distribution profile is afforded by field-flow-fractionation (FFF). The separation principle relies on the differential behavior of particles under laminar flow when they are exposed to a perpendicular field, which may affect their mass (sedimentation FFF), size (cross-flow FFF), or charge (electric-field FFF) [32].

The homogeneity and morphology of liposomes and lipoplexes can also be visualized by electron microscopy (EM) [33–37]. Negative-stain EM gives a straightforward impression of the particle size distribution (provided there are no staining artefacts due to pH, ions, osmolarity), although the lamellarity and morphology of the lipid are difficult to assess. In freeze-fracture EM, the hydrophobic monolayer

faces are exposed and depicted in detail by the shadowed replicas. These images readily reveal the packing geometries of lamellar and hexagonal phases as well as rippled morphologies (*see below*). Cryo-EM, finally, is a powerful approach to visualize the three-dimensional geometry and the DNA-load of vesicular structures trapped within a thin layer of ice, even though the contrast is comparatively low.

Colloidal Stability and ζ-potential

Following the entrapment of a drug, antigen, or DNA, the physical stability of a liposome formulation is determined by its colloidal behavior and its ability to retain the cargo for long periods during storage. The liposomes should ideally remain intact upon dilution or changes in the ionic strength, as typically encountered during administration. From a thermodynamical point of view it is notable that plain liposomes, and lipoplexes in particular, are not at equilibrium but represent kinetically trapped systems. Hence, their structures are relatively stable upon dilution, whereas thermodynamically reversible systems such as micelles and microemulsions would immediately aggregate or disintegrate [38]. According to the basic DLVO (Derjaguin–Landau–Verwey–Overbeek) theory, a system will be stable in simple electrolyte solutions if the electrostatic repulsion between two particles is larger than their van der Waals attraction. Charged liposomes are thus most suitable stored at low ionic strength, but aggregation may occur at high lipid concentrations or in the presence of multivalent ions with high affinities (e.g. PS with Ca^{2+}) [1, 39]. Coating uncharged colloidal particles with non-ionic hydrophilic polymers, such as PEG (*see below*), renders them stable against nonspecific interactions and self-aggregation, because the hydration repulsion and the steric barrier prevent close approach.

The electrical properties of liposomal surfaces are conveniently examined by microelectrophoresis, which yields the ζ-potential and the surface charge density as characteristic parameters [39, 40]. Even uncharged'' liposomes prepared from pure PC possess non-zero ζ-potentials over a wide range of ionic strengths. In most electrolyte solutions the ζ-potential of PC tends to be negative due to an anion layer adsorbed to the raised zwitterionic headgroup dipoles. The ζ-potential is sensitive to lipid phase transitions, the adsorption of amphiphiles and proteins, steric stabilization with uncharged PEG, and the presence of surface modifications—hence it is a useful parameter for monitoring liposome stability and for checking the reproducibility of batches.

Chemical stability of the lipids during storage is another point of concern, especially against hydrolysis, and in the case of unsaturated lipid chains also against oxidation. Liposomes can be stored frozen or as lyophilized powders, but it is essential to re-check their size distribution, morphology, and entrapped cargo before use. A cryoprotectant, such as trehalose, is suitably added to avoid phase transitions and membrane fusion [27].

To characterize lipid identity, purity, and quantity, MALDI-TOF offer several advantages over conventional chromatographic methods such as TLC, GC, or LC [13]. Normal-phase HPLC is also suitable for analyzing lipid mixtures, which can

be detected by evaporative light scattering rather than UV absorption. Similar analytical methods apply to the characterization of lipid-protein and lipid-DNA complexes [6].

LIPID PHASE TRANSITIONS AND POLYMORPHISM

Lipid Chain-melting Transition

The "fluidity" of a lipid bilayer, composed of a single species, depends on its lipid chain melting transition, T_m, relative to ambient or physiological temperature. As illustrated in Fig. 2, in the lamellar "solid" gel phase (L_β) the lipid acyl chains are preferentially aligned in an all-*trans* conformation, and lateral diffusion is very slow. When the temperature is raised and reaches T_m, the membrane passes into the "fluid" liquid crystalline phase (L_α), a disordered state, in which the lipids are free to diffuse laterally and the acyl chains undergo rapid *trans-gauche* fluctuations. Bilayers in the gel phase sometimes exhibit regular rippled (L_β) or crumpled (P_{CC}) morphologies, as a result of slightly different cross sections for the headgroups and acyl chains which thereby optimize their lateral packing. An unusual combination of high acyl chain order and high lateral mobility has been reported for sphingomyelin/cholesterol-rich mixtures, and more recently also for other lipids such as DPMC when hydrated below the chain melting temperature, as shown in Fig. 3 [33, 35]. In this so-called "lipid-ordered" state (l_o), the acyl chains are straight (as in the gel

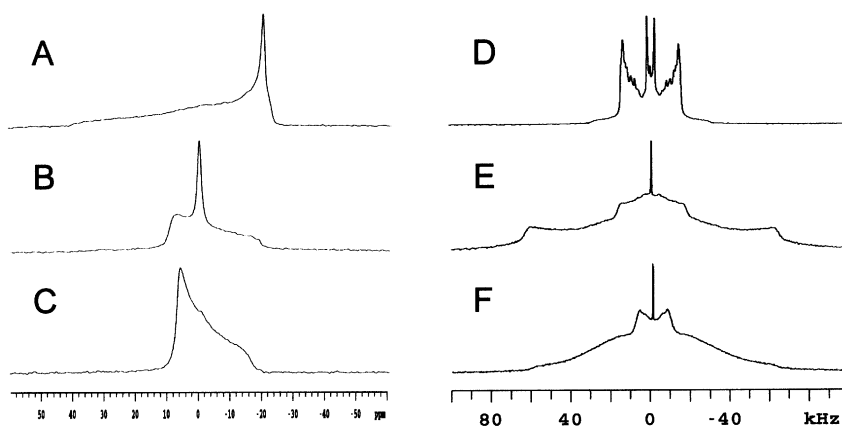

Fig. 3. Solid state NMR characterization of lipoplex morphology (left) and liposome phase transitions (right). *Left:* Typical ^{31}P-NMR lineshapes for a lamellar phase (A), a mixture of hexagonal and cubic phases (B), and a pure hexagonal phase (C). These spectra represent the ^{31}P-signal of the helper lipid DOPE in lipoplexes (cationic lipid/DNA charge ratio = 1.4), illustrating its tendency to promote inverted phases in three DNA-containing samples of 60% DOTAP + 40% DOPE (A), 40% DOTAP + 60% DOPE (B), and 20% DOTAP + 80% DOPE (C) unpublished results by Dürr *et al.*]. *Right:* Characteristic ^{2}H-NMR spectra of chain-deuterated DMPC-d_{27} in the fluid $L\alpha$ (or liquid disordered" l_d) phase at 30°C (D), in the unusual liquid ordered" l_o phase at 3°C that is obtained upon hydration below T_m (E), and in the L_β gel state (or solid ordered" s_o phase) at 3°C that is obtained upon hydrating above T_m (F) [35].

phase) but the lipid molecules as a whole undergo rapid diffusion in the plane of the membrane (as in the fluid phase). It remains to be investigated whether liposomes in the l_o state may exhibit an enhanced toughness and elasticity.

The lipid chain melting transition temperature of membranes increases with aclyl chain length (DMPC: 24°C, DPPC: 42°C, and DPSC: 55°C), with the degree of chain saturation (DOPC: –15°C), and with loss of headgroup hydration (dehydrated DPMC: ~40°C). Many natural membrane lipids (e.g. egg PC) typically possess an unsaturated chain in the *sn*-1 position and a saturated one in *sn*-2, such that they are fluid at physiological temperature. The addition of cholesterol increases disorder amongst the acyl chains in the gel phase, whereas the fluid phase becomes more ordered. At 50% cholesterol the membrane is saturated and the lipid phase transition completely abolished. Cholesterol is thus used as a universal membrane sealer, minimizing the bilayer permeability and providing it with improved mechanical and cohesive strength, but exceptions to this general rule have been reported [3, 11].

Depth Profile of the Bilayer

A lipid membrane may be regarded as a composit of layers with very distinct characteristics. Much information has been derived from computer simulations [3, 11, 41], as discussed by Saiz *et al.* in this issue [42]. Basically, the outermost layer consists of perturbed water, which has a considerably lower dielectric constant than the bulk and is less capable of forming hydrogen bonds with approaching compounds. The second layer contains water, lipid headgroups, and the upper paths of some acyl chains, as the hydrophilic/hydrophobic interface is smeared-out over a significant depth. This layer is available for non-covalent interactions with drugs, and is likely to play a role in the folding of peptides and proteins. The third layer consists of conformationally ordered acyl-chain segment, which impose an anisotropic potential on hydrophobic molecules penetrating the membrane. Finally, the innermost layer consists of the acyl chain termini and is as conformationally disordered as liquid decane.

Lipid bilayers in the fluid state readily accommodate hydrophobic drugs and anesthetics, whose solubility correlates with their octanol-water partition coefficients. In the gel state, on the other hand, hydrophobic compounds are less soluble in membranes and tend to be expelled, especially in the case of saturated acyl chains that will minimize their packing defects. The temperature-dependent solubility of drugs plays an important role in the production of solid lipid nanoparticles, which consist of a solid lipid core (usually triacylglyceride) that is emulsified by a phospholipid monolayer or amphilphilic polymer, as illustrated in Fig. 2. Loading is achieved by melting the neutral matrix lipid and dissolving the drug in the emulsion. Upon cooling, the differential solubility of the drug in the two lipid phases (fluid and gel) and in the aqueous buffer can lead to a relative enrichment in either the core of the SLN (useful for sustained release) or in its periphery (useful for a burst phase) [43]. To enhance the loading capacity of a SLN, more complex lipid mixtures need to be employed, containing mono-, di- and tricylglycerides with fatty acids of different chain lengths, which form less perfect crystals with many imperfections to accommodate the drug.

Lateral Phase Separation

The lateral organization of a lipid bilayer can be rather inhomogenous and plays a major role in drug penetration and membrane permeability [3]. Static lateral phase separation is frequently encountered in lipid mixtures with very different acyl chain lengths, or when certain headgroups couple to extramembraneous surfaces. Dynamic structural heterogeneity is much harder to characterize, but is manifest as density fluctuations close to the chain-melting phase transition, or as compositional fluctuations in moderately-matched lipid mixtures. Since the molecular packing is laterally disturbed at the interfaces between the domains, these boundaries will enhance the penetration of drugs and peptides into the membrane and greatly increase bilayer permeability. A dynamic heterogeneity also implies a softening of the membrane and a decrease in its bending rigidity, which in turn affects its capacity to undergo fusion, vesiculation, or interaction with other surfaces. these effects have been exploited to design permeability enhances and fusogenic systems that can be actively triggered by a change in temperature (*see below*).

A related phase separation phenomenon can be triggered by the strong interaction between Ca^{2+} ions and negatively charged PS headgroups. A fluid-fluid phase coexistence has been reported after addition of Ca^{2+} to a mixture of PC + PS due to the formation of *cis*-complexes in the bilayer phase. Vesicles consisting of pure PS are driven to fuse by the addition of Ca^{2+}, which is attributed to the formation of *trans*-complexes in apposing bilayers, being accompanied by the formation of local gel-phase domains. Likewise, the interaction of cationic amphiphiles with DNA may affect their mixing behavior with uncharged helper lipids and possibly promote lateral phase-separation [3]. A dehydration-induced lipid phase-transition upon lipoplex assembly has nevertheless been ruled out, even though a significant release of water and counterions occurs upon intimate DNA-lipid contact [44].

Lipid Polymorphism

Lipid polymorphism is an essential aspect to consider in the rational design of liposomes for drug delivery and gene therapy, and has been well reviewed [7, 9, 19]. Besides the lamellar phase many lipids and surfactants can also adopt other morhpologies, such as the inverted hexagonal phase (H_{II}) or various cubic phases (Q), summarized in Fig. 2. The hexagonal phase consists of an array of cyclindrical rods, wherein the lipids are oriented with their headgroups towards the aqueous core. Cubic phases are made up of bicontinuous surfaces, such that every point of the internal surface of this macroscopic assembly has access to the external aqueous space via aqueous channels (about 5 nm diameter), and any lipid molecule may exchange places with another in the same monolayer simply by diffusion [45].

The preference of lipids for certain geometries is conveniently rationalized by ''molecular shape'' arguments. Lipids with a large headgroup and a small hydrocarbon cross-section have a cone-like geometry, self-assemble into micelles, and are said to exhibit positive membrane curvature. Lipids that are cylindrical in shape, having nearly equal headgroup to hydrocarbon area, self-assemble into bilayers. Finally, lipids with small headgroups adopt ''inverted'' phases such as H_{II} and Q,

are said to exhibit negative membrane curvature. Examples of cone-shape lipids are detergents, lysolipids, and ganglioside GM1, whereas most of the natural membrane lipids are cylindrical. The tendency to form inverted phases is pronounced for pure PE, diaclyglycerol (DAG), PA (with Ca^{2+}, or at low pH), PS (with Ca^{2+}), and CL (with Ca^{2+}). Long and unsaturated acyl chains have particularly large cross-sections, especially at high temperature, whereas the removal of water will reduce the effective headgroup volume.

Unsaturated PE lipids in isolation prefer the inverted H_{II} phase, e.g. DOPE undergoes an $L_\alpha \rightarrow H_{II}$ transition at 10°C. This tendency is supposed to play a key role in the delivery of drugs and DNA across cellular membranes, as DOPE facilitates the formation of highly curved intermediates that are necessary for membrane fusion [12, 16, 19]. Unsaturated PEs will adopt a lamellar bilayer structure in the presence of stabilizing lipids such as PCs and PEG-lipid constructs. However, once the stabilizing function is removed, for example by pH-triggering (*see below*), a lamellar-to-hexagonal phase transition can be triggered that is accompanied by release of contents and lipid mixing, thus being reminiscent of membrane fusion events. On the other hand, by inducing a positive curvature in the outer monolayer of vesicles, for example by the addition of lysolipids or detergents, it is possible to suppress their ability to participate in fusion. Cationic amphiphiles, in particular, display a very interesting phase behavior with regard to the delivery of DNA, as they are usually stable as bilayers in isolation or when supplemented with neutral helper lipids. However, when they are combined with negatively charged lamellar lipids (e.g. DODAC plus CHEMS), the mixture will adopt an inverted H_{II} phase. This transition is attributed to the formation of ion pairs and an effective reduction in headgroup volumes.

The formation of cubic phases, finally, is attributed to a moderately "inverted" molecular shape, as exemplified by glycerol monooleate (GMO) [45]. At a reduced water content of 20% (w/w), on raising the temperature this lipid goes through the sequence $L_\alpha \rightarrow Q \rightarrow H_{II} \rightarrow$inverted micelles (see Fig. 2). Likewise, the addition of water to its L_α phase at room temperature triggers conversion into the very viscous cubic phase. This transition is suitably exploited in using GMO as a matrix for drug delivery, since the fluid lamellar phase has a sufficiently low viscosity to be readily injected into a cavity, where it will take up water and transform into a stiff gel.

It must be generally noted that any hydrophobic drug, amphiphilic compound or proteinaceous cargo is likely to affect the phase diagram of its liposomal carrier, hence great care has to be taken to characterize the corresponding behavior of the loaded system [24, 25, 46–48].

DNA-lipid Structures

The structures of cationic liposome-DNA complexes have been comprehensively characterized in homogeneous bulk samples by X-ray diffraction and by theoretical means [49–51]. There exist two basic architectures, L_α^C and H_{II}^C, which resemble the pure lipid phases as shown in Fig. 2. The lamellar architecture comprises of parallel strands of DNA sandwiched between fluid lipid bilayers. Replacement of DOPC by DOPE in DOTAP-containing liposomes was shown to lead to a

transition from the multilamellar "sandwich" structure to an inverted "honeycomb" phase, where single DNA strands are coated by a lipid monolayers and arranged in a hexagonal array [9, 16, 18]. Another, so-called "spaghetti and meatball" morphology has been described by EM in terms of a single DNA strand that is covered by a lipid bilayer and connects larger lipid/DNA aggregates.

The small suspended lipoplex particles used in transfection are less likely to possess a homogeneous structure. Invaginated bilamellar liposomes, as described by Smyth Templeton in this issue [52], represent one of the few well-characterized morphologies that have been observed by cryo-EM for several cationic lipids after extrusion [1, 2]. It has been suggested that the initial interaction of DNA with the outer surface of a unilamellar vesicle induces a contraction of its outer lipid layer due to charge neutralization. This in turn leads to an ivnersion of the vesicle around the DNA, such that a bilamellar vesicular structure is formed. The DNA is engulfed in the innermost compartment, where it appears to be well protected and highly effective in transfection.

Methods for Characterizing Lipid Phases and Transitions

Differential scanning calorimetry (DSC) is used to determine the onset temperature of the lipid chain-melting and other phase transitions, and the area under the curve (i.e. the enthalpy) is representative of the cooperativity [44]. Since the transition temperature is sensitive to additives in the bilayer, it is a suitable parameter to monitor drug-lipid interactions and to check for break-down products or impurities. To determine the total amount of water in concentrated lipid dispersions, it is possible to analyze the area under the ice-melting peak [53]. The related technique of high sensitivity titration calorimetry finally can provide accurate thermodynamic parameters of a drug partitioning into a membrane [47].

Both nuclear magnetic resonance (NMR) and electron spin resonance (ESR) spectroscopy are powerful tools to examine the local structural and motional features of designated lipid segments, thus providing an overall picture of lipid morphology as well as detailed insights into the local molecular architecture. Technical details and applications have been extensively reviewed [14, 20, 21, 24, 47, 54–56]. For example, the solid state ^{31}P-NMR signal of phospholipids is a straightforward indicator of lamellar, hexagonal and micellar/cubic phases. Not only pure liposomes but also lipoplexes have been characterized that way, as illustrated in Fig. 3. [Dürr *et al.*, *in preparation*]. Additionally, the local order and mobility of individual molecular segments can be studied either by selectively deuterating them for ^{2}H-NMR or by labelling them with a nitroxide radical for ESR. Characteristic ^{2}H-NMR spectra of the lipid acyl chains are useful for distinguishing the gel phase, the fluid phase, and the unusual liquid ordered" state, as depicted in Fig. 3. [35]. Analysis of deuterated lipid headgroups has shown that the alignment of their dipole moments sensitively reflects the association of charged molecules as well as water with the membrane surface [57, 58]. Likewise, the penetration and localization of hydrophobic drugs and anesthetics (see Xu *et al.*, in this issue [59]), as well as the local conformation, orientation and lipid-perturbation of an amphiphilic peptide or transmembrane protein can be described in detail by solid state NMR (55, 60–62].

Fluorescence spectroscopy provides many complementary approaches for studying bilayer re-arrangements such as vesicle leakage, membrane fusion, and hydrophobic exposure [12, 15, 18, 63]. An extensive arsenal of highly sensitive water-soluble or lipid-anchored dyes is available for monitoring the escape of aqueous contents by de-quenching of ANTS and DPX, the mixing of lipids by resonance-energy transfer between rhodamine-PE and NBD-PE, and the dehydration-induced spectral shifts of ANS or laurdan [44].

SURFACE MODIFICATIONS OF LIPOSOMES

Steric Stabilization

One of the most critical aspects in drug delivery and gene therapy is to improve liposome stability and enhance their circulation times in the blood. It has been demonstrated that coating the lipoplexes with neutral or negatively charged polymers can enhance their stability. For example, including ganglioside GM1 into liposomes leads to significantly enhanced stability in serum. A major improvement was achieved by coating with polyethyleneglycol (PEG), as illustrated in Fig. 2. This hydrophilic polymer prevents liposomal aggregation during storage and particularly in serum after administration, as discussed in more detail by Allen *et al.* in this issue [64] (see also [7, 8, 12]). The incorporation of PEG-PE into so-called ''stealth'' liposomes establishes a steric barrier that delays their recognition and clearance from the blood stream by the mononuclear phagocyte system. Circulation times of conventional liposomes (typically several minutes) have been increased this way up to many hours. It has even been possible to insert PEG-coupled lipids into the outer leaflet of preformed liposomes and lipoplexes, causing little loss of the entrapped contents.

Optimum stabilization is typically achieved with 5–10% PEG-PE with a molecular mass in the range 1000–2000 Da. At lower concentrations the polymer chain configuration changes from a so-called brush-structure to a mushroom-structure, hence the surface tends to remain fully covered. A general drawback of PEGylated liposomes is their reduced ability to approach the target membrane and undergo fusion. To circumvent this limitation, various liposome formulations have been designed to shed their PEG coat during circulation. For example, PEG can be attached to short acyl chains which readily escape from the vesicle, or an acceptor population of uncoated vesicles can be co-administered as a sink''. alternatively, PEG can be actively unmasked upon acidification in the endosome, using acid-labile chemical linkages (*see below*).

Use of Fusogenic Peptides and pH-titrable Polymers

Enveloped viruses, such as influenza or HIV, carry fusogenic proteins on their surface by which they enter the host cell [12]. Membrane destabilization and fusion is accomplished by a short amphiphilic sequence, the fusion peptide, located either at the tip or in an internal region of the protein, which gets exposed upon receptor recognition or at acid pH upon endocytosis. Such synthetic peptides can trigger

fusion of liposomes *in vitro*, in many cases in a pH-dependent manner [63]. Their activity is attributed to a conformational change and possibly oligomerization of the peptide [37]. In an attempt to mimick nature, liposomes have been equipped with fusion peptides, as for example discussed by Bungener e*t al*. in this issue [65] and others [7, 12, 15]. Anchoring the peptide to the lipid bilayer via a myristic chain was shown to greatly enhance the fusogenic action and may provide a convenient way of incorporating the peptides into vesicular carriers, as illustrated in Fig. 3. Lipoplexes, too, have exhibited improved transfection activities when mixed with charged fusion peptides. A drawback may concern the risk of an immunogenic response *in vivo*, besides the relatively high cost of synthetic peptides.

An alternative, more cost-effective and less immunogenic approach relies on the adsorption of pH-titrable polymers to the liposomal surface [12]. Poly-histidine and poly-lysine, for example, become positively charged upon lowering the pH and have been shown to destabilize and fuse negatively charged membranes *in vitro*. Poly-(amidoamine)s undergo a marked conformational change from a relatively coiled hydrophobic structure at neutral pH to a relaxed hydrophilic structure at acidic pH, and may be thus exploited as endosomolytic agents. Catinoic poly)ethyleneimine) can induce non-leaky fusion at pH <7, and is even being investigated as a gene delivery vesicle on its own. Interestingly, its activity as a transfection enhancer has been attributed to its high buffering capacity, which may raise the endlysosomal pH and inactivate the lytic enzymes, before ultimately the osmolarity increases and the lysosome bursts. Polyanions, bearing carboxyl groups, are useful for destabilizing and fusing both negatively charged as well as uncharged membranes, due to their hydrophobic character at low pH. Promising pH-sensitive polyanions are, for example, poly(acrylic acid) derivatives and succinylated poly(glycidol)s. Further triggering approaches using synthetic polymers are based on the temperature-dependent lipid affinity of *N*-isoproplylacrylamide, which undergoes a solubility change at ambient temperature. Altogether, there remains much potential to be explored with synthetic polymers and many open questions concerning their behavior in combination with liposomal carriers *in vivo*.

Ligand-targeted Liposomes

Ligand-targeted liposomes and lipoplexes offer a vast potential for directing the cargo to designated cell types *in vivo*, using surface-bound site-specific ligands [4, 8, 66]. Many different kinds of ligands are available, such as antibodies (see Fig. 2), receptors, peptides, vitamins, oligonucleotides (aptamers), or carbohydrates as described by Park in this issue [67]. Large numbers of ligands can be positioned onto the liposome surface, and the resulting multivalency strongly enhances the binding affinity. Ligands of different types can be combined onto the same carrier, providing a more precise target selection. Since lipid assemblies are usually dynamic structures, surface-coupled ligands have a high motional freedom to position themselves for optimal substrate-interactions. In stealth liposomes, however, the extended PEG molecules (~50–75 Å for PEG_{2000}) would prevent the recognition of cell surface receptors, but ligands have been successfully attached to the distal ends of the PEG chains to space them far from the vesicle surface, as described by Maruyama in this

issue [68]. Besides reaching selected cellular targets, surface displayed proteins are also particularly useful for antigen presentation and immunization purposes.

LIPOSOMAL DELIVERY, DRUG RELEASE AND TRANSFECTION

Liposome Stability

The capacity of liposomes to retain their cargo *in vitro* depends mainly on the lipid composition and temperature [10]. A general sequence of hydrophilic solute permeability is: water > small non-electrolytes > anions > cations≡large non-electrolytes > large poly-electrolytes. Membrane permeability is highest at the lipid phase transition, and is generally lower in the gel phase than in the fluid phase [3]. Stability against leakage has thus been promoted using phospholipids that remain in the solid phase at physiological temperatures, such as TPPC and DSPC. Incorporation of cholesterol generally enhances bilayer stability against leakage, whereas lipid impurities (e.g. lysolipids, unsaturated fatty acids, uneven chain lengths, etc.) can weaken the bilayers and cause loss of contents. The use of multilamellar carriers retards leakage to some extent, but polymerized liposomes (e.g. made of 2,4-octadecadienoyl chains) are ultimately the most resistant [69].

Under physiological conditions, solute leakage depends not only on the intrinsic membrane permeability but also in its interactions with components of the biological fluids, which strongly affect liposome clearance as discussed by Ishida *et al.* in this issue [70]. For example, in blood the lipid molecules can be transferred from the liposomal membrane to plasma lipoproteins (HDL, LDL, etc.). This escape is particularly pronounced for fluid liposomes (e.g. DOPC) or those containing single- or short-chain lipids, which distintegrate rapidly and release their contents within a few minutes after intravenous injection. The longest lifetimes have been generally observed for gel-phase vesicles that are small (~50 nm) and carry no net charge. The incorporation of cholesterol minimizes lipid exchange, and circulation periods are significantly enhanced by incorporating PEG-DSPE in liposomes and lipoplexes.

Since hydrophobic compounds are readily accommodated in the fluid hydrocarbon region of a bilayer, liposomes might be intuitively considered to be excellent carriers for lipophilic cargo. However, it has been realized that hydrophobic solutes are often rapidly (within minutes) depleted from their carriers by exchange mechanisms, leading to their equilibration amongst all other lipidic structures in the circulation (lipoproteins, erythrocyte membranes, etc.) [25, 47, 48]. If solubility permits, hydrophobic drugs may be preferably accommodated in gel state MLV or in multilayered solid cochlates [28]. Alternatively, solid lipid nanoparticles offer a stable hydrophobic environment with a high-loading capacity [32, 43].

Delivery of Drugs and Antigens from Liposomes

The exact mechanism of liposome breakdown remains speculative, once it has been trapped on the cell surface. A drug may simply leak out and traverse the plasma membrane by diffusion or pore formation. Alternatively, the liposome may either fuse directly with the plasma membrane, or it may be taken up by endocytosis or

phagocytosis. In receptor-mediated endocytosis, small particles (<150 nm diameter) bind to cell surface receptors and are taken up by clathrin-coated pits to form coated vesicles. After internalization the clathrin coat is removed and the vesicle fuses with lysosomes, which induces the breakdown of the lipids and release of their contents. Large particles (>150 nm), on the other hand, are taken up principally by phagocytosis, which is usually limited to specific cells such as macrophages but can be induced in many other cell types with appropriate ligands. In both cases, the liposome could either be degraded in the low pH environment, or it could fuse directly with the endosomal or lysosomal membrane. The actual route of intracellular processing is highly relevant for antigen presentation purposes. *In vitro*, conventional liposomes have been found to deliver their proteinaceous contents to the lysosome, such that antigens are processed for presentation via the major histocompatibility complex (MHC) II pathway [71]. On the other hand, pH-sensitive liposomes have been observed to deliver antigens to antigen-presenting cells via the cytosolic MHC I pathway [72]. *In vitro*, however, the situation is significantly more complex, and further details about using liposomes for immunization are covered in this issue by Bungener *et al.* [65], by Chikh *et al.* [73], by Zhou and Neutra [74], and by Wilson *et al.* [75].

One of the most important tools for triggering liposomal delivery is to exploit the pH-sensitivity of the endocytotic pathway, and excellent reviews have been presented [7, 12, 19]. Endosomes and lysosomes can reach pH values below 5.0, and liposomes can be in principle by internalized by any kind of cell when suitably targeted. There are four different classes of pH-sensitive liposomes, two of which have already been discussed above, namely those containing fusogenic peptides and those covered with acid-titratable synthetic polymers. An alternative, lipid-based approach relies on the combination of polymorphic lipids such as DOPE with mildly acidic amphiphiles that act as stabilizers at neutral pH [12]. For example, oleic acid (OA) or CHEMS have a cylindrical shape in their charged state, which keeps DOPE in a lamellar phase and prevents liposome aggregation due to electrostatic repulsion. Protonation of the weakly acidic lipid, however, renders its headgroup less hydrophilic. As a result, its shape becomes conical and reinforces the H_{II} preference of DOPE, and moreover the colloidal suspension will aggregate due to the resultant loss of electrostatic and hydration repulsion. Yet another kind of pH-triggering mechanism involves "caged" lipid-derivatives with specifically engineered chemical bonds that are hydrolyzed by acid-catalysis (see Fig. 1). For example, the lipid headgroup of *N*-maleyl-DOPE has been covalently modified such that cleavage leaves behind plain DOPE which favors membrane fusion. Likewise, the lipid chains of acid-sensitive plasmalogens are hydrolyzable to produce membrane destabilizing fatty aldehydes and lysolipids [7].

Light-activation, too, can be used to trigger a chemical reaction between plasmologens and free radical species that are generated from a burst of singlet oxygen, following illumination in the presence of a suitable sensitizer. Other, enzymatic triggering mechanisms have been based on the simultaneous administration of two kinds of liposomes. For example, Ca^{2+} has been encapsulated in a photo-oxidatively triggered subpopulation of vesicles, which upon release stimulates the hydrolysis of the second liposome population by an exogenous Ca^{2+}-dependent phospholipase A_2 [12].

Thermosensitive liposomes, finally, rely on a relatively sharp phase transition in the lipid phase at temperatures only a few degrees above 37°C. Packing defects observed at the gel-to-liquid crystalline phase transition result in increased permeability to entrapped solutes or drugs [3]. Such ''thermosomes'' require an external stimulation by heat in a spatially well-defined region, e.g. around a tumor, but whole-body hypothermia is also being pursued in order to trap distant metastases.

Transfection Mechanisms of Lipoplexes

The mechanisms of DNA transfer from the lipoplex to the nucleus remain rather elusive and are difficult to control. The initial release of DNA into the cytoplasm is generally attributed to an intrinsic ability of the cationic lipids to destabalize the plasma membrane or the endosomal compartment (depending on the mode of uptake). Specifically, a flip-flop of anionic lipids from the cytoplasmic leaflet to the lumenal leaflet of the endosome appears to result in the formation of charge-neutral ion pairs, thereby releasing the DNA from the complex [9, 16]. An additional membrane-destabilizing role has been attributed to the resulting mixture of cationic with anionic lipids, which exhibits a high tendency to undergo fusion due to the effective decrease in headgroup size and hydration as an ion-pair [8, 19].

When DOPE is used as a helper lipid, cationic liposomes generally mediate higher levels of transfection than those containing DOPC. This observation is attributed to the tendency of DOPE to undergo a lamellar-to-hexagonal phase transition in a pH-, composition- or temperature-dependent manner, which is likely to facilitate fusion with or destabilization of the target membrane. Additionally, it has been proposed that the amine group of PE may compete with the cationic lipids for the DNA phosphate backbone, thus assisting disassembly of the complex and allowing the DNA to escape from the endosome [15, 16, 76]. Cholesterol has been used as an alternative helper lipid, resulting in the formation of more stable complexes *in vitro* than those containing DOPE. Since the beneficial effects of different helper lipids vary with the cell lines and depend on the exact conditions *in vivo*, it is not straightforward to draw general conclusions from biophysical parameters *in vitro*. As illustrated by Hirko *et al*. in this issue [77], there are many further biological barriers to be overcome and different environments to be encountered *in vivo* [66]. For example, it has been shown that DNA which is coated by liposomes remains protected against nucleuses while traversing the cytosol, whereas the concomitant condensation/compaction of the DNA appears to be unfavorable for gene expression once it reaches the nucleus.

Administration of Liposome Formulations

Different applications may require distinct release kinetics and different particles sizes [4, 22]. To deliver tomographic imaging agents, for example, a rapid burst will achieve maximum contrast between the circulation system and the target tissues. For the sustained delivery of therapeutic agents, on the other hand, liposomes should provide a continuous source of material to be taken up by the target site over extended periods of time. Following an intravenous injection, the first capillaries to

be encountered by a delivery vehicle are in the lung tissue, which tends to mechanically entrap large particles such as MLV or serum-induced aggregates. The lung thus represents a unique site for drug delivery if large liposomes are employed, but care has to be taken to avoid pulmonary emboli. Administration of liposomal formulations to the epithelial cells in the lower airways of the lung by nebulization, on the other hand, requires smaller particles (~300 nm) that are in the respirable range [78].

The other "first pass" organ to be encountered after systemic administration is the liver, which can thus be targeted with fast-release liposomes. Large vesicles are generally more rapidly eliminated from the bloodstream, whereas small ones circulate for longer, especially those with neutral or positively charged, rigid bilayers. Liver, lung and spleen may be circumvented using stealth liposomes. Although liposomes do not usually pass through the endothelial barrier of the vascular system, under pathological conditions small particles (<100 nm) are able to extravasate at sites of inflammation and through the poorly formed neovasculatore of some solid tumors [8]. Regardless of the circulation time of an intravenously injected liposomal dose, however, the majority will eventually be taken up by the mononuclear phagocyte system. Using alternate parenteral routes, such as intraperitoneal, subcutaneous or intramuscular, large liposomes generally tend to be retained longer at the site of injection, while small ones are mainly taken up by the draining lymph system.

Transdermal drug delivery requires small and highly fluid liposomes (100–200 nm) that are able to squeeze through the intercellular regions of the *stratum corneum*. Surfactants can be included to increase the elasticity of the liposomes, together with PEGylated lipids or charged amphiphiles that improve colloidal stability [79]. Rapid disintegration of the liposomes into mixed micelles may further enhance the skin permeation [46]. The epidermal lipid barrier itself can be softened by adding permeability enhances such as oleic acid or azone, which decrease the lipid acyl chain order presumably by increasing the lateral phase inhomogeneity [3].

Oral administration of liposomes is limited to very stable formulations that survive the low pH of the stomach and the bile salts and hydrolytic enzymes of the intestine, as discussed by Zhou and Neutra in this issue [74]. In the gut, comparatively large particles of several μm size can be taken up by Peyer's patches, which are part of the lymphoid tissue. Polymerized MVL have been sown to retain their hydrophilic cargo for extended periods over 24 hr [69], and solid cochleates are also sufficiently stable to deliver oligonucleotides and proteins orally [28]. Solid lipid nanoparticles, too, are promising vesicles for hydrophobic compounds, offering a high loading capacity and slow release kinetics of up to a week [32, 43].

A novel lipid-based material for intramuscular, subcutaneous, periodontal, vaginal, and post-surgical implants is provided by cubic phases, which serve as a macroscopic matrix for the local delivery of hydrophobic as well as hydrophilic drugs and enzymes [45]. The release is typically controlled by diffusion within the gel, but is limited by biodegradation and by an initial burst phase during injection of the lipid in the lamellar state.

Besides liposomes, emulsions, and solid lipid particles, other carrier systems are being developed, based on polymeric or ceramic particles, offering complementary modes of delivery according to their respective physical properties.

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