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Introduction

Techniques & Applications

Methods

Further Reading

- eLS
- WIREs
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John Wiley & Sons, Ltd.
ELS subject area: Cell Biology
How to cite: Gregoriadis, Gregory; and Perrie, Yvonne (January 2010) Liposomes.
In: Encyclopedia of Life Sciences (ELS). John Wiley & Sons, Ltd: Chichester.

DOI: 10.1002/9780470015902.a0002656.pub2

Advanced article

Liposomes

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Based in part on the previous version of this Encyclopedia of Life Sciences (ELS) article, Liposomes by Gregory Gregoriadis.

Liposomes are synthetic vesicles consisting of one or more phospholipid bilayers, able to accommodate water- and lipid-soluble molecules. They are used as a delivery system for drugs, genes and vaccines in therapeutics. Liposomes may be formulated with a range of characteristics including different size, charge and drug retention, which can be tailored for a given drug and target site. There is a range of clinical products approved for use which exploit liposomes to passively target drugs or vaccines to the appropriate site of action thereby improving specificity and reducing toxicity. Liposomes can also be actively targeted to specific cells or subcellular regions using targeting

ligands attached to their surface, or by modification of the bilayer to give triggered release under appropriate conditions.

Key concepts:

- Liposomes are bilayer constructs formed when amphiphiles are exposed to water.
- Liposomes can be formulated in different sizes, with different bilayer melting points and with different surface charges allowing for optimization to suit a given drug and target site.
- The lipid head-group dictates the surface charge of the liposomes and the acyl tail influences the melting point of the lipid bilayer and its permeability and therefore influences the drug release rates from liposomes.
- The presence of cholesterol within the bilayers can reduce their permeability and drug leakage.
- In commercial products, phosphatidylcholines in combination with cholesterol are commonly used to formulate the liposomes.
- Drugs can be loaded within the aqueous compartment of the liposomes or within the lipophilic region of the bilayers depending on the lipophilicity of the drug. Through electrostatic interactions, molecules can also be adsorbed on the surface of liposomes.
- Currently, liposomes are used for the delivery of drugs, for example cancer chemotherapy, systemic fungal infections and vaccines.
- Cationic liposomes are being investigated as potential delivery systems for a range of nucleic acid therapies and for subunit vaccines.

Introduction

Liposomes (or synthetic lipid vesicles) form when amphiphiles (surface active molecules with a hydrophilic

group and a hydrophobic chain at opposing ends) are exposed to water. Under appropriate conditions of amphiphile to water mass ratio and temperature, amphiphiles are arranged as one or more concentric bilayers (lamellae) alternating with aqueous compartments (**Figure 1**). Unlike micellar systems, liposomes do not form spontaneously and energy must be added to the system to drive the formation of the lipid bilayers. In the process of their formation, liposomes entrap solute molecules (e.g. drugs) present in the aqueous medium. Alternatively, lipid-soluble drugs mixed with the amphiphile can be accommodated in the lipid phase to become components of the bilayers. In addition, molecules (e.g. nucleic acids and proteins) may be adsorbed onto the surface of liposome membranes through electrostatic interactions. The bilayer structures vary in size (diameter) from approximately 30 nm (and therefore many liposome systems may also be defined as nanotechnology) to many micrometres. They are usually known as small unilamellar vesicles (SUVs), multilamellar vesicles (MLVs) and large unilamellar vesicles (LUVs). Depending on the gel to liquid-crystalline phase transition temperature (T_C) of the amphiphile used (i.e. the temperature at which the hydrophobic chains melt), amphiphiles in liposomal bilayers can be in a gel or fluid state at ambient temperature. Moreover, the composition of liposomes can be tailored to include a sterol (e.g. cholesterol), which contributes to bilayer stability and fluidity, and charged amphiphiles, which render bilayers negatively or positively charged. Physical characteristics of liposomes, such as vesicle size and surface charge, lipid composition and bilayer fluidity, play important roles in determining vesicle behaviour within the biological milieu, and pharmacological activity of the drugs they carry. The structural versatility of liposomes, their innocuous nature and ability to incorporate a plethora of different pharmacologically active molecules have all contributed to

their clinical use in the delivery and targeting of drugs in therapeutics. **See also** Lipids

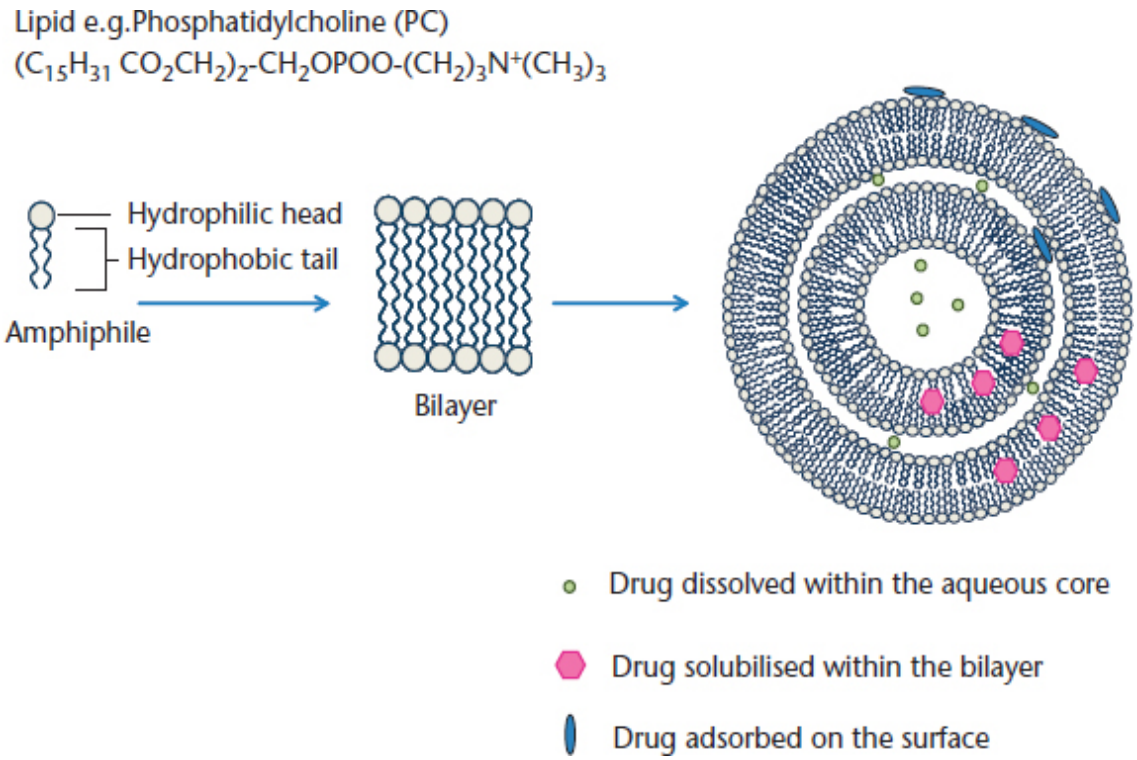


Figure 1 Schematic representation of a lipid, which can form bilayer vesicles with drug entrapped in the aqueous phase or within the bilayer. With appropriate electrostatic interactions, drugs can also be adsorbed onto the surface.

In addition to liposomes, there is a range of surfactant-based vesicle systems, which have similar bilayer structure and include nonionic surfactant vesicles or niosomes which are prepared using nonionic surfactants such as monopalmitoyl glycerol (e.g. Brewer and Alexander, 1992) or which incorporate bile salts (bilosomes) (e.g. Mann *et al.*, 2006) or viral envelop material (virosomes) (e.g. Wilschut, 2009). Cationic liposomes complexed with nucleic acids such as deoxyribonucleic acid (DNA) have also been referred to as lipoplexes.

Methodology

Choice of preparation method

The numerous methods developed so far to meet widely different requirements in liposome-based therapeutics are broadly divided into two categories: those involving physical modification of existing bilayers and those that depend on the generation of new bilayers by the removal of the lipid-solubilizing agents. Examples of the first category include: the preparation of drug-containing SUVs from drug-containing MLVs by sonication, where, however, drug entrapment yield is very low; and the preparation of drug-containing liposomes by the dehydration–rehydration method, where preformed ‘empty’ (water-containing) SUVs are freeze-dried in the presence of drug destined for entrapment. Vesicles produced by this method (dehydration–rehydration vesicles; DRVs) are multilamellar and exhibit a high yield of drug entrapment. Another approach to high-yield drug entrapment is based on the remote loading of preformed liposomes. According to the method, neutral molecules shuttle by diffusion through the bilayers into the liposomes, where they not only become charged as a result of the local pH but also become entrapped because the outward diffusion rates of charged molecules are very low. Alternatively, drugs remain entrapped because of changes in their solubility affected by ions already present within the preformed liposomes.

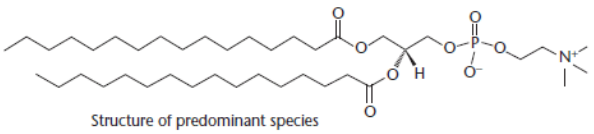
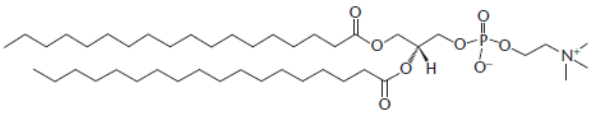
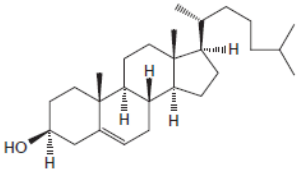
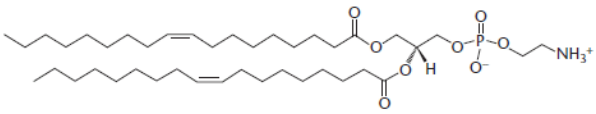
Methods of the second category include hydration of a dry amphiphile film to produce MLVs with low drug entrapment yield, and the more efficient reverse-phase evaporation method, in which an aqueous phase containing the drug is emulsified together with a solution of amphiphile in ether, followed by evaporation of the solvent to produce LUVs. Most of such methods will produce large liposomes of heterogeneous size, which can be a disadvantage. Size and heterogeneity, however, can both be reduced by a variety of techniques, including sonication, microfluidization, high-

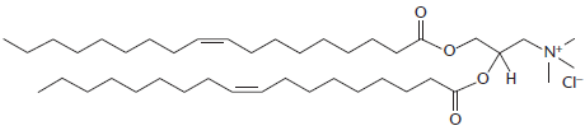
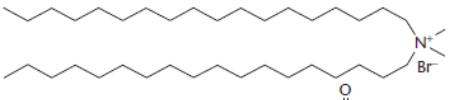
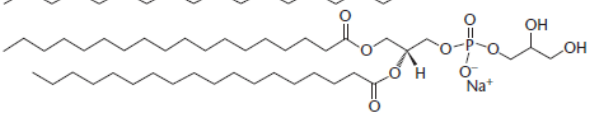
pressure homogenization and extrusion. Many of the procedures available have been scaled up successfully for industrial production. These various methods are explored in more detail in Gregoriadis (2006).

Commonly used lipids and their characteristics

Within the clinically approved liposome preparations the commonly used lipids include the phosphatidylcholines (e.g. egg phosphatidylcholine; dioleoylphosphatidylcholine; dimyristoyl phosphatidylcholine), cholesterol and phosphytidylglycerol. The selection of lipids used within the liposome formulation will influence the characteristics of liposomes including their surface charge, their bilayer fluidity, permeability and drug retention; the choice of lipid head-group will influence the surface charge of the liposomes and the alkyl 'tail' of the lipid (in terms of the number of carbons and its degree of saturation) will influence the transition temperature of lipid and hence dictate whether the liposome is in the gel or fluid phase. Examples of the key characteristics of commonly used lipids are given in **Table 1**. Cholesterol is also a major component in most liposome formulations. Although cholesterol does not form bilayers on its own, it can be incorporated into liposome bilayers at concentrations up to 50% of total lipid. The presence of cholesterol in the bilayer influences the freedom of movement of the phospholipid alkyl chains and at higher concentrations can reduce the bilayer permeability of the liposomes (Demel *et al.*, 1972).

Table 1 Examples of commercially available liposomes

Name	Structure	Transition temperature (°C)	Application
Egg phosphatidylcholine (egg PC)	 Structure of predominant species	Below room temperature	PC (also known as lethicin) from natural sources is actually a mixture of phosphatidylcholines, with chains of different lengths and varying degrees of unsaturation. Commonly used as the main lipid in liposome formulations
1,2-distearoyl-phosphatidylcholine (18:0 PC or DSPC)		55	A high transition temperature PC which can enhance stability of liposomes both on storage and in the circulation
Cholesterol		NA	This inclusion of cholesterol within liposomal bilayers has been shown to result in an increased packing densities of phospholipids molecules which is thought to result from the accommodation of cholesterol in the molecular cavities formed by surfactant monomers assembled into vesicles. This space-filling action combined with the ability of cholesterol to complex with phospholipids can reduce bilayer permeability to small hydrophilic solutes and ions
1,2-dioleoyl-phosphatidylethanolamine		−16	Fusogenic helper lipid, which enhances membrane fusion and destabilization, thus facilitating the disruption of endosomes and the release of the incorporated material into the cytoplasm of the cell. High levels of transfection efficiency when in combination with a cationic lipid

Name	Structure	Transition temperature (°C)	Application
1,2-dioeoyl-3-trimethylammonium propane (DOTAP)		<0	Monovalent cationic lipid consisting of a quarternary amine head group linked by ester groups to two unsaturated hydrocarbon chains
Dimethyldioctadecylammonium bromide (DDA or DDAB)		47	A monovalent synthetic cationic lipid with adjuvant properties
1,2-distearoyl-phosphatidylglycerol (DSPG)		55	An anionic lipid which can be used in liposomes to reduce fusion and enhance stability

Note: NA, not applicable.
Source: All structures taken from www.avantilipids.com.

Considerations for drug loading

As mentioned, due to the biphasic nature of liposomes, drugs may be:

1. Entrapped within the aqueous compartments of liposomes.
2. Incorporated into the lipophilic region of the bilayers.

3. Adsorbed onto the surface of liposomes through appropriate electrostatic interactions.

Therefore, consideration of the drug properties should be given when developing liposome formulations strategies. For considerations of drug loading between the aqueous and lipophilic bilayer compartments of the liposomes, the following can be considered as a guide: (**Table 2**).

Table 2 Effect of drug characteristics on loading within liposomes

Hydrophilic drugs (log *P*<1.7)	Drugs with intermediate log *P* values (log *P* 1.7–5)	Lipophilic drugs (log *P*>5):
Entrapped and retained in the aqueous core of liposomes	Such drugs can partition between the bilayer and aqueous phase resulting in low drug incorporation and retention within the liposomes	Incorporated and retained within the liposome bilayers

In terms of surface adsorption onto liposomes, opposing charges between the liposomal membranes and the drug to be adsorbed are required. For example cationic liposomes effectively adsorb a range of negatively charged nucleic acid systems including plasmid DNA (e.g. Felgner and Ringold, 1989), oligonucleotides and ribonucleic acid (RNA) (e.g. Tseng *et al.*, 2009). Proteins can also be adsorbed onto liposome systems and in this case, the isoelectric point (pI; the point at which a molecule carries no net electrical charge) is an easy indication of the type of liposome formulation required: proteins with a high pI value proteins (and therefore cationic at neutral pH) may be adsorbed onto negatively charged liposome whereas proteins with a low pI (and hence anionic at neutral pH) will be adsorbed well onto cationic liposomes (e.g. Vangala *et al.*, 2006).

Interactions with the Biological Milieu and Applications

Liposomes were originally described by Bangham *et al.* (1965). Since then, owing to their similarity with cell

membranes, liposomes have served successfully as models for the study of membrane biophysics. Following their introduction in the early 1970s by Gregoriadis and Ryman (1972) as a drug delivery system, liposomes were investigated extensively, both in terms of their fate *in vivo* and their application in therapeutics. It was established that intravenously injected liposomes interact with blood opsonins, which cause their removal from the blood circulation at rates that depend on vesicle size, lipid composition and surface charge, to end up (via opsonin recognition by appropriate cell receptors) in the tissues of the reticuloendothelial system (RES), mostly fixed macrophages in the liver and spleen. However, intramuscularly or subcutaneously injected liposomes end up in the lymphatic system including the lymph nodes draining the injected site. Therefore, liposomes can be used for passive targeting of such sites and as such can be exploited to deliver drugs to parts of the RES such as the liver or spleen or to lymph nodes which is advantageous for vaccines.

The stability of liposomes in blood or interstitial fluids, vesicle clearance rates and tissue distribution can be controlled to satisfy particular needs by tailoring the structural characteristics of the vesicles. This includes coating of the vesicle surface with ligands (e.g. antibodies and glycoproteins) that exhibit selective affinity for receptors on the surface of target cells (promoting active targeting), or with polymers, such as polysialic acids and polyethyleneglycol (PEG), which render the vesicle surface highly hydrophilic. A hydrophilic surface helps to repel opsonins and thus contributes to longer vesicle circulation times and, therefore, to opportunities of interaction with target cells other than those of the reticuloendothelial system. The ability of liposomes to avoid RES uptake offers them the opportunity to accumulate in pathological sites with leaky vasculature including tumour sites and sites of

inflammation where the integrity of the endothelial barrier is disrupted. This modified permeability of the endothelium resulting from pathological conditions can be exploited in drug targeting strategies to allow the escape of the liposomes from the central circulation. This phenomenon is called enhanced permeability and retention (EPR) effect. The EPR effect can be exploited to passively target liposomes (and their incorporated drugs) to a site where the vasculature is leaky and gaps in the endothelium are present. By modifying the liposome surface to avoid their recognition and clearance by the RES, vesicle circulation time in the blood compartment can be increased to high enough values so as to allow the carrier to accumulate at other sites (e.g. tumours) and thus release the drug *in situ*. Targeting via the EPR effect is driven by the plasma concentration of liposomes.

This accumulated knowledge of the fate of liposomes *in vivo* and its control have resulted in the successful use of the system in a wide range of therapeutic applications where the pharmacokinetics of drugs is optimized by their liposomal carrier, leading, in turn, to improved pharmacological activity.

Clinical applications of liposomes

Liposome-based products approved for clinical use include those employed in the treatment of systemic fungal infections (e.g. AmBisome), the treatment of certain cancers, for instance Kaposi sarcoma (Caelyx/Doxil, DaunoXome), and vaccination against hepatitis A (Epaxol-Berna) and influenza (Infexal Berna V; **Table 3**). Most of these applications have relied either on the ability of liposomes to transport drugs into macrophages where target pathogens reside (passive targeting of the RES), or to their ability to bypass tissues (e.g. heart or kidneys) that are sensitive to certain drugs and to target tumours with increased blood vessel permeability (passive targeting via EPR). **See also** Membrane Dynamics

Table 3 Examples of commercially available liposomes

Trade name	Drug	Description	Indications
Caelyx/Doxil	Doxorubicin	80–100 nm sterically stabilized liposomes composed of pegylated distearoyl phosphatidylethanolamine, hydrogenated soy phosphatidylcholine and cholesterol	Advanced ovarian cancer Advanced breast cancer Aids-related Kaposi sarcoma.
DaunoXome	Daunorubicin	~45 nm liposomes composed of distearoyl phosphatidylcholine and cholesterol	Kaposi sarcoma
Myocet	Doxorubicin	150–190 nm size liposomes composed of egg phosphatidylcholine and cholesterol	First-line treatment of metastatic breast cancer in women
Depocyt	Cytarabine	Multivesicular liposomes composed of dioleoylphosphatidylcholine (DOPC), cholesterol, triolein, and dipalmitoylphosphatidylglycerol (DPPG). The vesicles range in size from 3 to 30 microns	Intrathecal treatment of lymphomatous meningitis
AmBisome	Amphotericin B	Vesicles are < 100 nm in size and composed of soy phosphatidylcholine, cholesterol, distearoylphosphatidylglycerol	Systemic fungal infections
Epaxal Berna	Formalin inactivated hepatitis A virus	150 nm virosomes composed of purified influenza virus surface antigens phosphatidycholine and phosphatidylethanolamine	Hepatitis A vaccine
Inflexal V Berna	Purified influenza haemagglutinin glycoprotein and neuraminidase	Virosome system similar to Epaxal	Influenza vaccine
Visudyne	Vereporfin	Vesicles composed of dimyristoyl phosphatidylcholine (DMPC) and egg phosphatidylglycerol (PG)	Photodynamic therapy for macular degradation

Cancer chemotherapy

There are three commercial liposome formulations of anthracyclines all with different formulations: Myocet® liposomes which are relatively large in size (~180 nm) and are composed of egg phosphatidylcholine and cholesterol, and therefore do not have a modified hydrophilic surface coating. Owing to their larger size and surface properties, they are rapidly taken up by the RES and thereafter act as an 'RES depot' from which drugs re-enter the blood stream similarly to a slow influsion, resulting in lower toxicity profiles. DaunoXome® liposomes are prepared from distearoylphoshatidylcholine and cholesterol but are much smaller in size (45 nm). Although these liposomes have no hydrophilic coating, the use of the high transition temperature lipid in combination with cholesterol is

considered to support prolonged blood residence time (Senior and Gregoriadis, 1982). In contrast, Caelyx® (also known as Doxil®) are liposomes approximately 80–100 nm in size. In addition to phosphatidylcholine and cholesterol they incorporate PEG_{2000} – distearoyl phosphatidyl ethanolamine and α-tocopherol (56:38:5:0.2 mol% respectively) in the bilayers. The PEG_{2000} coating renders the liposomal surface hydrophilic, leading to liposomes that are less prone to opsonin adsorption and therefore to their reduced uptake by the RES. The α-tocopherol is used as an anti-oxidant in the formulation.

Systemic fungal infections

Amphotericin B is generally the first line drug for the treatment of life-threatening systemic fungal and protozoal infections. However, its application is limited by its associated adverse side effects. There are three lipid-based formulations with AmBisome often being described as the only ‘true’ liposome due to its aqueous core within a bilayer system. The AmBisome is composed of hydrogenated soy phosphatidylcholine, cholesterol, distearoylphosphatidylglycerol and α-tocopherol. In these liposomes the amphotericin B is intercalated within the liposomal membrane due to its low solubility.

Vaccines

Liposomes were first identified as being immunologically effective by Allison and Gregoriadis (1974). Currently licensed vaccines based on vesicular systems are those based on virosome technology. Epaxal® is a hepatitis A vaccine where the inactivated virus has been adsorbed onto virosomes, which are small vesicle structures, ~150 nm in size, prepared from the outer coat of influenza virus and additional phospholipids. Similarly, Inflexal® V is a virosomal

influenza vaccine. Haemagglutinins, isolated from the influenza virus envelope, are purified and combined with lipids to form virosomes.

A further development in the use of liposomes as carriers of vaccines is the finding that plasmid DNA encoding for a vaccine antigen entrapped in cationic liposomes leads to a much higher immune response in injected mice than when a plasmid DNA vaccine is administered as such (Perrie *et al.*, 2001). Recent work (Gregoriadis *et al.*, 2007) has now shown that when both the plasmid DNA encoding the vaccine antigen and the antigen itself are co-entrapped in the same liposomes which are then used in immunization studies (one injection only), immune responses are augmented to levels that are considerably higher than those seen with liposomes containing either the plasmid DNA or the protein vaccine alone. This co-delivery approach (Gregoriadis *et al.*, 2007) to DNA immunization could lead to single-shot vaccines.

Photodynamic therapy

Visudyne® is a Food and Drug Administration (FDA)-approved light sensitive delivery system. It is a liposomal formulation used to treat age-related macular degeneration which can result in vision loss. The liposomes are composed of dimyristoyl phosphatidylcholine and phosphatidylglycerol and entrap the photosensitizer verteporfin. After intravenous infusion, the liposomes are retained in the neovascular spots of the eye due to the enhanced permeability and slow clearance of liposomes from these areas. Thus, these liposomes act as a delivery system to localize the photosensitizing compound selectively to the target tissue. The localized liposomes are subsequently activated via a nonthermal laser light which promotes release of the photosensitizing agent (verteporfin). This promotes light-induced toxicity, thus reducing undesirable cell proliferation (Christie and Kompella, 2008).

Future Developments

Future challenges that could potentially be met by the use of liposomes include manipulation of the immune system by the use of tailored liposomes, leading to the production of vaccines (both conventional and genetic) with much higher potency and selectivity, and *in vivo* targeting to tumours and gene therapy. In all such cases, designer liposomes carrying drugs, vaccines or genes should be able to recognize their cell target, interact with it specifically and deliver their therapeutic content intracellularly, directly into the appropriate target organelle (e.g. the nucleus). The posttranscriptional gene silencing of RNA using small interfering RNA (siRNA) also offers potential in gene therapy. The ability of short stretches of double-stranded RNA to promote the degradation of complementary messenger RNA (mRNA) sequences allow for the specific suppression of gene expression. However, like other nucleic acid-based therapies, naked siRNA is prone to degradation and has a very short half-life. Therefore, effective delivery systems are required and various cationic lipid-based systems are being investigated (Tseng *et al.*, 2009). **See also** Human Gene Therapy

Interestingly, cationic liposomes have also been extensively investigated as vaccine adjuvants. The use of cationic liposomes as drug delivery systems has been limited due to problems of cationic moieties interacting with serum proteins. As a result, much work has been focused on avoiding such interactions which lead to the destablization and rapid clearance of the cationic liposomes after intravenous injection. However, the issues with biological recognition of cationic lipids may actually be advantageous in the design of vaccine adjuvants for protein-based vaccines. Additionally, cationic liposomes as delivery systems for vaccines antigens offer the possibility of adsorption of antigens onto the liposomal surface and their

delivery to antigen-presenting cells (APC) of the immune system. A range of cationic lipids have been investigated including 3β-(N-N′N′-dimethylaminoethane-carbamoyl) cholesterol (DC-Chol), 1,2-dioleoyl-3-trimethylammonium-proprane (DOTAP) and N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium choloride (DOTMA) and for example the use of DC-Chol liposomes has been shown to overcome the observed nonresponse to hepatitis B vaccine. The combination of a cationic lipid (particularly dimethyldioctadecylammonium bromide, DDA) with an immunostimulatory agent (trehalose 6,6′ dibehenate, TDB) has been shown to be a versatile adjuvant system which mediates protection against tuberculosis and has shown promising protective efficacy against other infectious diseases requiring different immunological profiles. These cationic liposomes systems are currently undergoing clinical trials (Christensen *et al.*, 2009).

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John Wiley & Sons, Inc.
Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology
Volume 3, July/August 2011

DOI: 10.1002/wnan.131

Advanced Review

Topical and mucosal liposomes for vaccine delivery

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Mucosal (and in minor extent transcutanous) stimulation can induce local or distant mucosa secretory IgA. Liposomes and other vesicles as mucosal and transcutaneous adjuvants are attractive alternatives to parenteral vaccination. Liposomes can be massively produced under good manufacturing practices and stored for long periods, at high antigen/vesicle mass ratios. However, their uptake by antigen-presenting cells (APC) at the inductive sites remains as a major challenge. As neurotoxicity is a major concern in intranasal delivery, complexes between archaeosomes and calcium as well as cationic liposomes complexed with plasmids encoding for antigenic proteins could safely elicit secretory and

systemic antigen-specific immune responses. Oral bilosomes generate intense immune responses that remain to be tested against challenge, but the admixing with toxins or derivatives is mandatory to reduce the amount of antigen. Most of the current experimental designs, however, underestimate the mucus blanket 100- to 1000-fold thicker than a 100-nm diameter liposome, which has first to be penetrated to access the underlying M cells. Overall, designing mucoadhesive chemoenzymatic resistant liposomes, or selectively targeted to M cells, has produced less relevant results than tailoring the liposomes to make them mucus penetrating. Opposing, the nearly 10 μm thickness stratum corneum interposed between liposomes and underlying APC can be surpassed by ultradeformable liposomes (UDL), with lipid matrices that penetrate up to the limit with the viable epidermis. UDL made of phospholipids and detergents, proved to be better transfection agents than conventional liposomes and niosomes, without the toxicity of ethosomes, in the absence of classical immunomodulators. *WIREs Nanomed Nanobiotechnol* 2011 3 356–375 DOI: 10.1002/wnan.131

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INTRODUCTION

In first place, a brief overview on the anatomical and phenomenological constraints for mucosal and transdermal delivery of particulate material to antigen-presenting cells (APC) will be presented. Later selected results upon administration of intranasal, oral, and transdermal liposomes and other vesicles as adjuvants will be critically discussed. On those basis, a relationship between structure and function of liposomes and immune response will be elaborated.