LIPOSOMES: FROM PHYSICS TO APPLICATIONS

PT.2

Chapter 11

Liposomes as a drug delivery system

Liposomal drug delivery is a complex field because it incorporates physical, organic and analytical chemistry, biology, pharmacology, toxicology, medicine and some other disciplines. Often these factors are not coherent and synergistic, sometimes even excluding one another, and optimization of the formulation requires skillful concepts and experiments.

As already mentioned, liposomes are normally made from naturally occurring substances and are, therefore, biocompatible, i.e., biodegradable, nontoxic, and do not cause immune response. Not all lipid compositions at all dosage levels are safe, however. Some potential problems will be briefly mentioned in the next section. In general, however, most of the lipids, even at very high doses, do not cause relevant toxicity or other unwanted side effects. A good example are various phospholipid (micro) emulsions, used in parenteral nutrition, which can be administered at doses > 100 g lipid per day.

Toxicology and adverse effects of liposomes

In contrast to other pharmacological studies of liposomes, not much work has been done in the study of potential adverse effects of liposomes [4]. Often it is automatically assumed that liposomes are safe, and researchers back up their assumption by comparing their small concentrations of administered liposomes as mentioned above, with large doses of intravenous lipid nutrition emulsions.

In general, liposomes can cause adverse effects due to their physical or chemical character. In the first case they may be simply too large and can block capillaries, mostly in the lungs, causing pulmonary embolism. Complete saturation of the immune system which is responsible for the clearance of foreign particles from the blood, may be another one. They can also, when in serum, change and influence the functioning of lipoproteins because of the phospholipid exchange. Most of the potential adverse effects will be mentioned in this section and some of them will be further described later. Roughly, these effects can be classified into the following

groups: (i) potential toxicity to RES, especially when liposomes are loaded with drugs, or, an increased susceptibility to various infections in the case of saturated RES; (ii) interactions with plasma proteins and macromolecules, which may induce some changes in blood chemistry; (iii) interactions with circulating cells may cause depletion of lipid and cholesterol, as well as some membrane proteins, such as acetyl choline esterase, from cells, possibly leading to changes in their function. For example, by increasing cholesterol/lipid ratios in the membrane of red blood cells, the activity of Na^+-K^+-ATPase is reduced. Liposomes may also interact with platelets and other molecules and cause alterations in blood clotting. Most of the studies have shown reversible adhesion and platelet aggregation and no relevant significance in blood clotting. Some liposome formulations can cause hemolysis; (iv) capillary blocking of large liposomes and other possible physical and chemical damage, when administered by other routes, especially intracerebrally; (v) possible immunogenicity; (vi) pharmacological effect of certain lipids. Some exogenous lipids may influence cerebral metabolism while polyunsaturated acyl chains from liposomes may be a source of a variety of biologically active compounds. An example is arachidonic acid, whose metabolites include prostoglandins, thromboxanes, leukotrienes and lipoxins; (vii) damage due to lipid or cholesterol degradation products which may be toxic.

In addition to these potential side effects, we should keep in mind that when loaded with potent drugs, the changed pharmacokinetics and biodistribution may, in some cases, increase the toxicity. Typical examples are some liposome-dependent drugs which can increase their toxicity by several hundred times due to their increased activity or bioavailability. Another example is the anticancer drug cytosine arabinose. Encapsulation in liposomes greatly reduces the chemical decomposition of the fragile drug, and increases its toxicity. For instance, one half of the lethal dose in mice (LD_{50}), can decrease from 2.5 g/kg to 0.12 g/kg, i.e., a twenty-fold increase in toxicity upon encapsulation into the liposomes. In this case, toxicity depends upon liposome composition which determines the stability and permeability in a biological environment.

These adverse effects are well known and are also easy to avoid. Most of those encountered in practice originate in the presence of particular lipids in the bilayer. Typical examples are stearylamine and dicetyl phosphate. The first one is thought to damage the lysosome membranes and cause the release of lysosomal enzymes, while the mechanism of the second one is not known. Probably it also damages some membrane structures. The adverse effect of pure phosphatidylserine (PS) vesicles are also known. They cause raised glucose levels in brain and blood and, consequently, brain damage and death in laboratory animals. The metabolic product lysophosphatidylserine produces, via reactions with enzymes, the imbalance between carbohydrate supply and the energy requirement of the brain tissue. Surprisingly, other lysophospholipids did not show this effect which is thought to originate in the serine polar head. 50% lethal doses of PS liposomes in mice were measured at about 5 g/kg, while nonextruded, nonfiltered liposomes showed an

approximately five times lower LD_{50} and mechanical unavailability of the molecules, probably because of clogging capillaries. The LD_{50} of PS is reduced sharply upon diluting PS in the bilayer with other lipids.

Sphingomyelin causes the reversible depression of phagocytic function of liver. These liposomes have also shown, after prolonged injections, enlargement of liver and spleen. The granulomatous inflammatory response is probably a consequence of the overloading of the system with lipids. In contrast to these results liposomes made from PC, Cholesterol, PE and PA seem to be well tolerated. Generally, fluid liposomes show much smaller depression of phagocytic index.

Although in general, liposomes do not cause lysis of red blood cells, a possible hemolysis of liposomes or their constituents should be checked. Later, it will be shown that even liposomes containing detergentlike molecules do not cause observable disintegration of erythrocytes. In addition to adverse effects due to hydrolytic and detergentlike products of lipid hydrolysis, the peroxidation of lipids also causes side effects. The peroxidized lipids can impair reticuloendothelial system and cause hemolysis of erythrocytes. These effects can be greatly reduced or eliminated by the incorporation of radical scavengers and other antioxidants into the liposomal membrane.

Neurotoxicity of liposomes was investigated for the chemotherapy of viral infections and tumors in the central nervous system. Many drugs, when injected intracerebrally, are neuroirritant and neurotoxic, because they cannot pass through the blood–brain barrier. Negative dicetylphosphate liposomes were shown to be epileptogenic and caused extensive tissue necrosis. Positively charged stearylamine liposomes caused respiratory failure. Both toxicities are probably due to specific properties of both charged lipids because negative liposomes with phosphatidic acid and neutral DPPC were well tolerated. In these experiments rather large volumes were injected (~ 60 μl), and this could have produced some adverse effects as well.

For negatively charged drugs, including some proteins, positively charged liposomes can be used, and several in vivo applications have shown beneficial effects. The improvements in therapy were observed despite the known fact that positively charged lipids or liposomes can be toxic. Several studies showed brain toxicity and toxic effects to erythrocytes and cells in culture. Recent study compared the interaction of liposomes containing three cationic lipids, single chained stearylamine (SA) and double chained DOTMA and BISHOP [5] with blood. At liposomal positive charge concentrations above 5 μmol/ml SA provoked a strong increase in plasma turbidity, while liposomes containing DOTMA and BISHOP caused a strong clotting response. The BISHOP containing liposomes were the only ones which did not cause hemolysis in vitro. The extent of plasma interactions was dependent on the nature of the positive change, its lipid anchor, charge density and bilayer fluidity. While the detailed origin of these interactions is still not understood, the lytic activity can be correlated with aqueous solubility of the lipid while agglomerate and clot formation can be attributed to electrostatic

interactions with mostly negatively charged plasma components and polyelectrolytes.

Another important characteristic of liposomes is their low or nonexisting immunogenicity. Even repeated injections of liposomes normally do not produce antiliposome antibodies. Some glycolipids, such as lipid A, and several anionic phospholipids such as PG, PA, and especially PS and CL, as well as SM, were shown to possess antigenic properties and can induce the production of antibodies for other, non-immunogenic lipids. Whether the same is true of immunoliposomes has yet to be tested.

In general, most of these adverse effects have not been thoroughly studied yet. But in most cases, it was shown that application of liposomes is safe, and that the motto: 'biocompatible, biodegradable and non-immunogenic' is rather universal.

In order to understand the interaction of liposomes with cells and, especially, their fate in vivo, as well as some of their special potentials as drug delivery system, a brief introduction of immunology will be presented in the next paragraph. A reader familiar with the subject can forward to the next paragraph, however.

An introduction to immunology

Immunology is one of the most rapidly developing areas of scientific research in the last decades. In the past it had great successes in the prevention and virtual elimination of various infectious diseases and, more recently, in organ transplantation. With the advances in genetic engineering, and coupling various polyclonal and monoclonal antibodies to different drugs either directly or indirectly via drug carrier systems, the combination of immunology and drug delivery has become a very promising approach in the treatment of cancer and other diseases. The emergence of the AIDS epidemic also had significant impact on the development of immunology. (For more information on immunology, see refs. 6 and 7.)

By definition, immunity is the ability of the body to protect itself against all foreign matter which enters or comes into contact with the body. The various mechanisms of its action can be classified into two groups. Innate immunity is a general, nonspecific and inherent mechanism of protection. It includes physical barriers, mucous membranes, cough reflex, fever, chemical influences such as pH, secreted fatty acids, digestive enzymes, interferon, and other substances released by leukocytes, serum proteins, and more. Several specialized cells, such as macrophages and granulocytes also participate in the destruction, elimination, or neutralization of foreign material which penetrates into the body.

The innate immunity is supplemented and complemented with acquired immunity. In contrast to the innate immunity, this very delicate protection system is present only in vertebrates. This form of immunity lies dormant until the first contact with a foreign agent. Foreign agents, which can trigger immune response, are immunogenic while the compound responsible is defined as immunogen. The

part of the immunogen which actually reacts with elements of acquired immunity is called antigen. This reaction is called immunization and it triggers a chain of events. Special cells – lymphocytes become activated and a synthesis of antibodies commences. Antibodies are proteins which are specific to the foreign antigen and can neutralize them by binding, which is followed either by precipitation or subsequent digestion and secretion.

This effect was used, without proper understanding, in vaccination for more than one hundred years, because people noticed that few individuals who did not die from some deadly disease were later much more resistant to the same infections than other people.

Immunogens are compounds which can trigger immune response. Normally they are substances with a high molecular weight. Typical examples are proteins. Several relatively simple and low molecular weight compounds, such as penicillin or dinitrophenol, are not immunogenic when they are dissolved in body fluids. However, if they are chemically or physically bound to a carrier or adjuvant, they exhibit immunogenicity. These low molecular weight substances are called haptens. Later, the great potential of liposomes in these aspects (vaccination) will be described.

Macrophages and B and T lymphocytes are the most important cells of acquired immunity. B lymphocytes proliferate in response to particular antigens and secrete antibodies. T lymphocytes do not synthesize antibodies but secrete lymphokines. Macrophages, in contrast to lymphocytes, are not antigen-specific. They phagocytose all foreign substances and cells and, after digestion of antigens, they express and present them to T cells which become activated.

In the body one distinguishes humoral and cellular immunity. The former is mediated by serum antibodies and involves B cells, while the latter is mediated by T lymphocytes.

Antibodies are protein molecules known as serum globulins. They can bind to the target via hydrogen bonds, van der Waals forces, or due to electrostatic, and hydrophobic interaction with antigens. All the immunoglobins are shaped like the letter 'Y', with two light amino acid chains attached to a heavier stem. The light chains, normally around 25 kDa, are attached to the areas of the heavier stem part (50 kDa) by a disulfide bridge which also binds together the heavier chains. Within the species the stem has, in general, a constant chemical composition while the arms vary, having a different sequence of amino acids for each antigen. Different animals have slightly different antibodies for some antigens – they differ predominantly in the heavier chains. The light arms can be used for immunization in other species while the whole antibody may occasionally produce allergic reactions.

When antibodies are split by proteolytic treatment, such as incubation with the enzyme papain, they disintegrate into three fragments. Two of these fragments retain the ability to bind antigens specifically but lose the ability to precipitate it. The two fragments are called Fab (fragment antigen binding) while the third fragment can be crystallized out of solution and is therefore referred to as Fc

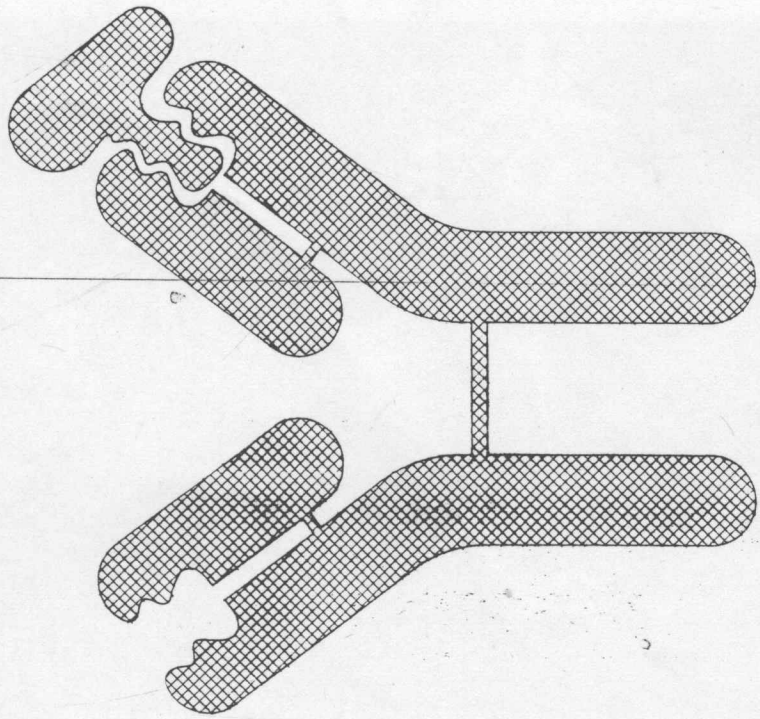

Fig. 11.1. A schematic presentation of an antibody. The upper arm interacted with an antigen.

(fragment crystallizable).

There are five different classes of heavy chains and immunoglobulins: Ig G, Ig M, Ig A, Ig E, and Ig D. They differ in their function. Ig M, for instance, is a powerful antibody which can activate blood components to attach and kill parasites.

Upon binding of antibody to the antigen, the antigen–antibody complex is formed. This results in a precipitate when antigen is soluble, and in clumping or agglutination if the antigen is on colloidal particles, including parasitic microorganisms.

Some antibodies may also activate the humoral system – the complement system which will increase phagocytosis. In such cases, cells from innate immune system also become activated and help to destroy the invader.

Another kind of antibody–antigen reaction may cause the release of pharmacologically active compounds, such as histamine, via the degranulation of mast cells. Later we shall see that this is an extremely important fact for liposome application because histamine produces an increase in vascular permeability. This results in higher accumulation of antibodies, serum and small liposomes, if present, at the site of immune reaction.

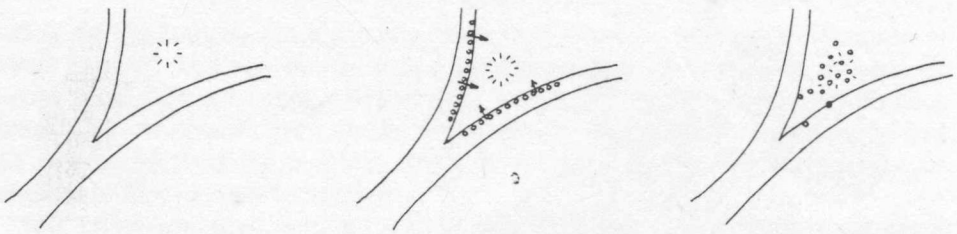

Fig. 11.2. Inflammation. After an inflammatory stimulus, such as a microbial infection, is detected the blood vessels and cells respond by vaso dilatation, increased vascular permeability and extravasation of cells which phagocytose microbes. Adapted from G.P. Lewis: Mediators of Inflammations, Wright, Bristol, 1986.

Figure 11.2 shows a simplified scheme of the inflammation. After the inflammatory stimulus, such as a microbial infection, the presence of various toxins is noted. The response is in vascular vasodilatation (cellular basis of inflammation) followed by migration of cells (in humoral basis of inflammation the sequence is interchanged). These responses increase vascular permeability and new cells, predominantly granulocytes (polymorphonuclear leukocytes), arrive at the site of inflammation and phagocytose invaders.

In the cell-mediated immunity T lymphocytes have, in contrast to antibodies, many different receptors. T cells become activated by antigen presenting cells, such as macrophages. They also release soluble substances such as lymphokines which affect other T cells, and recruit, i.e., attract and activate macrophages, at the area of immune response.

The immune response is, in general, directed against foreign invaders. In some cases, however, it may also be directed against tumor cells which have different surface characteristics. This area is under intensive investigation because it offers promising new anticancer treatments in which liposomes already play a significant role.

In the preceding paragraphs, the well-orchestrated action and complexity of the immune system was briefly presented. Unfortunately, this system may also overreact or malfunction, such as through an inability to recognize what is 'self' and 'nonself'.

In the first case, the immune response is triggered by foreign but harmless substances, such as pollen, food proteins, environmental chemicals, animal hair, dander, dust and similar. This hypersensitivity may result in allergic reactions which may be, depending on the mediators, immediate or delayed. Not recognizing self from nonself, a consequence of defective regulation, is termed autoimmunity and includes diseases such as multiple sclerosis, rheumatoid arthritis, and anemias. It presents one of the toughest challenges to modern medicine.

Phagocytosis is the main mechanism of clearing the body of foreign invaders through engulfment, ingestion and consecutive destruction. Several factors may enhance its rate. Commonly they are referred to as opsonins (from Greek opsonin

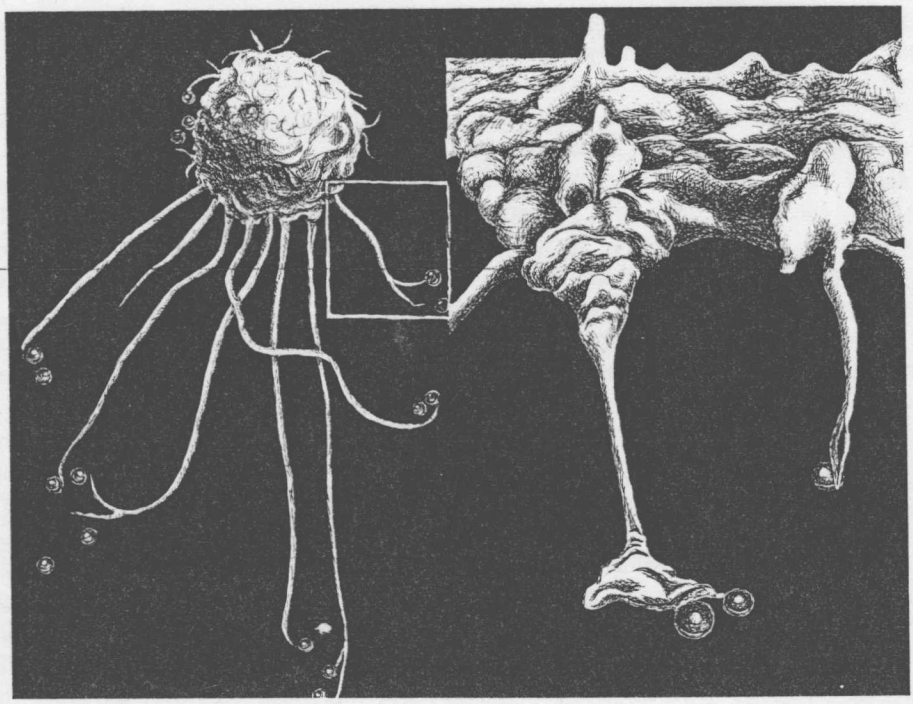

Fig. 11.3. Phagocytosis. Pseudopods of phagocyte surround the foreign particle (liposomes, colloidal particles, microbes, etc.) and engulf it into vacuole. This vacuole fuses with lysosomes which deliver its toxic load, involving digestive enzymes, catalase, lysozyme, hydrogen peroxide, low pH, into phagosome. Upon digestion, the substances are delivered to the ribosomes or cleared out of phagosomes (adapted from Lennart Nilsson's SEM micrographs. See, for instance, Am. Sci. 78, p. 409 and 418, Sept. 1990 or Natl. Geographic 169, 702, June 1986). Drawings: A.D. Lasic.

– prepare for eating). A typical example is IgG which reacts with epitopes, i.e., the smallest immune response triggering parts of the invaders via its Fab portions. After binding, they expose their Fc segments and the antibody coated invaders are engulfed by macrophages or polymorphonuclear phagocytes which bear receptors for the Fc portion of the IgG molecule.

Phagocytosis is commonly divided into endocytosis and pinocytosis. Endocytosis is primarily responsible for the uptake of liposomes, schematically shown in fig. 11.3.

Phagocytic cells include granulocytes, circulating (phagocytic monocytes) and fixed macrophages. Fixed macrophages are the most important in the clearance of liposomes. They are located in liver (Kupffer cells), lymphoid tissue (spleen, lymph nodes), lungs (alveolar macrophages), connective tissue and along the lining of the blood and lymph tissues.

These cells bind and digest, engulf particulate matter and antigens including old red blood cells. Enzymes in macrophages (in Greek 'large eater') break down the

trapped material into simple sugars or amino acids which are reused or secreted. They also take up antigens and denaturate and present them on their surface to specific T cells. We should add that some of the released products, such as endotoxins of gram-negative bacteria, including interleukins and interferons, can be endogenous pyrogens and can cause fever.

Humoral and cellular immunity are highly correlated, especially when they cross activate. Helper T cells are activated by binding the antigen complex and by lymphokine interleukin-1, a small protein (15 kDa) secreted by macrophages. Activated cells then release many lymphokines, such as interleukin-2 (IL-2), which cause the proliferation of T cells, or activation of macrophages by interferons or macrophage activation factor (MAF). Lymphokines also help B cells to synthesize antibodies. A tumor necrosis factor (TNF) is another lymphokine which can cause tumor regression.

With increasing knowledge of immunology and immunomodulators, new therapies for cancer are emerging. Several other methods are being tested to stimulate the body's own defense system and induce tumor regression by interferons, IL-2, TNF, MAF and others. Because many of these molecules have rather short circulation times in plasma and because liposomes end up in the cells of the immune system, liposomes are becoming increasingly important in anticancer immunotherapies. With the recent advance of monoclonal antibodies also new chemo- or radio-therapies are being tried and again liposomes with a specific antibody attached on their surface may become very important because they may significantly increase the load of drugs (antineoplastic agents), toxins (ricin, diphtheria), radionuclides (^{99}Tc, ^{111}In, or inactive and activable B) or photosensitive drugs, per target molecule.

For the application of liposomes as a drug delivery system, several aspects of their interactions with various cells and stability in biological fluids will have to be discussed. One should be aware that good stability in vitro is not necessarily correlated to good stability in vivo, and vice versa. This is due to the different mechanisms of interactions in both environments: membrane stability and permeability in vitro, and phagocytosis in vivo. For instance, opsonization of liposomes in in vitro experiments, in the absence of phagocytic cells may not change liposome stability, while in vivo it leads to a rapid uptake. Therefore, very compact polymerized liposomes can be very stable in blood or plasma in in vitro incubation, but exhibit poor stability in blood circulation.

The in vitro and in vivo behavior of liposomes will be discussed next.

In vivo behavior of liposomes

Stability of liposomes in biological fluids

Upon parenteral administration, liposomes are diluted into body fluids such as blood, lymphatic, and extracellular fluid. Because most currently considered lipo-

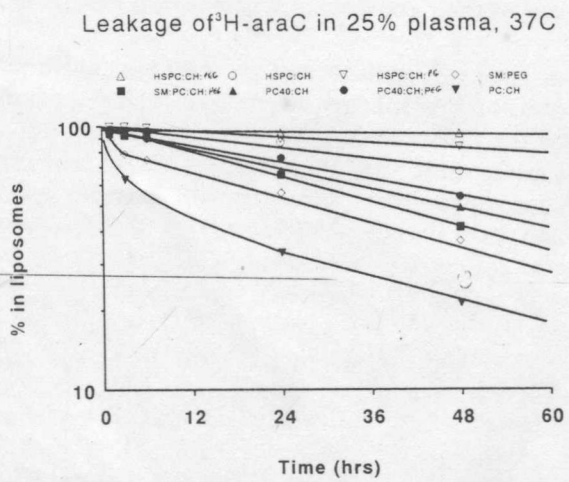

Fig. 11.4. Plasma-induced leakage of tritiated cytosine arabinose in 25% plasma at 37°C. Half-lives for drug release are, from top to bottom, 786, 187, 91, 54, 47, 41, 40, and 18 hours, respectively. These are relatively very stable liposomes. On the other end leakage rates from seconds to minutes can be also achieved. (Courtesy of T.M. Allen, University of Alberta, Canada.)

some-based therapeutic applications would require intravenous injections, and because blood may be the most detrimental in vivo environment, the majority of in vitro evaluations of liposome stability and release of the encapsulated material in vivo, rely on incubation in plasma [8–13]. These are the so-called plasma-induced leakage assays. Normally, liposomes at a given concentration are incubated in physiological conditions (37°C) with an appropriate amount of biological fluid. At various time points, the mixture is assayed with respect to the loss of encapsulated material, liposome properties, or concentration. Figure 11.4 shows an example of plasma-induced leakage of several different liposomal samples.

Plasma, which normally accounts for 55% of the blood volume is an aqueous solution containing several hundred different ions, molecules, and macromolecules. In terms of interactions with liposomes, plasma lipoproteins, and phospholipases are by far the most important. Presence of Ca^{2+} ions, pH around 7, ionic strength, and toxicity (physiological $\sim$ 290 mOsm) also have to be considered. Normally human or murine plasma are used. It should be added that plasma represents a more liposomicidal environment than blood because of higher activity of proteins in cell-free solutions.

As can be seen from fig. 11.4, the stability, as measured by the leakage rate, strongly depends upon the lipid composition. The stability values, i.e., percent of nonreleased drug with time, reported in the table are rather high due to relatively low plasma concentration and to the fact that all the liposome formulations studied are mechanically rather stable and impermeable. For instance, EPC

liposomes can leak half of their contents in whole serum plasma in approximately ten seconds [14].

The interactions of liposomes with various proteins may involve simple charge–charge interactions and/or hydrophobic interactions which lead to protein adhesion or adsorption on the liposome surface or its penetration into the bilayer. In the absence of RES cells, these two types of interactions normally do not have much influence on liposome integrity. The latter one, however, and lipid exchange between liposomes and other particles, especially high-density lipoproteins (HDL), can result in the loss of encapsulated or bound molecules, liposome disintegration, or both. Although most, if not all, lipoproteins may be involved in interactions with liposomes, the major interaction occurs with HDL which is probably the most important for liposome disintegration in vitro. In vivo behavior, as suggested above, can be very different because the opsonized proteins which do not necessarily induce major changes in vitro, can result in enhanced cellular uptake by the RES. The transfer of phospholipid molecules to HDL is greatly enhanced by plasma phospholipid transfer proteins. After incubation with liposomes, liposomal lipid is found associated with the HDL. This exchange also depends on the vesicle to HDL ration showing a saturation at about 0.25 mg PC/mg protein. Such exchange increases the HDL molecular weight but not the size.

Several studies have shown that liposome stability in plasma depends on the relative concentrations, liposome size and lamellarity, lipid composition, and the incubation temperature. The kinetics of liposome leakage depends on the type of label used with(in) the liposome but normally show single exponential release profiles.

The molecular origin of lipid exchange and protein penetration into bilayers depends on the physical state of the liposome. Presence of nonliposomal substances, impurities, single-chain surfactants, rough surface, structural defects, phase boundaries and high radii or curvature make the surface easier to penetrate. A typical example of structural defects occurs in smaller liposomes rapidly cooled to temperatures below their phase transition (T_c) from the liquid crystalline to the gel state. In this case, the surface appears as a faceted polyhedron, composed of jaggedly apposed patches of relatively planar bilayers [15] as can be clearly observed in fig. 11.5. The defects, however, can be annealed, i.e., transformed into a smooth curved membrane with a constant radius of curvature, by heating the system into the liquid crystalline phase. This, in general, reduces the number of defects and yields defect-free polyhedra upon re-cooling of the system, and, in many cases, by inclusion of cholesterol at concentrations above 33 mol%, which gives rise to a liquid ordered phase over a wide temperature range in which the bilayer would otherwise exhibit ordered solid and disordered liquid (fluid) phase at lower and higher temperatures, respectively.

Phase boundaries are another irregularity in lipid bilayers. Normally they occur in liposomes made of synthetic lipids held at the phase transition temperature, T_c. Because this transition is a first-order phase transition, it is characterized by

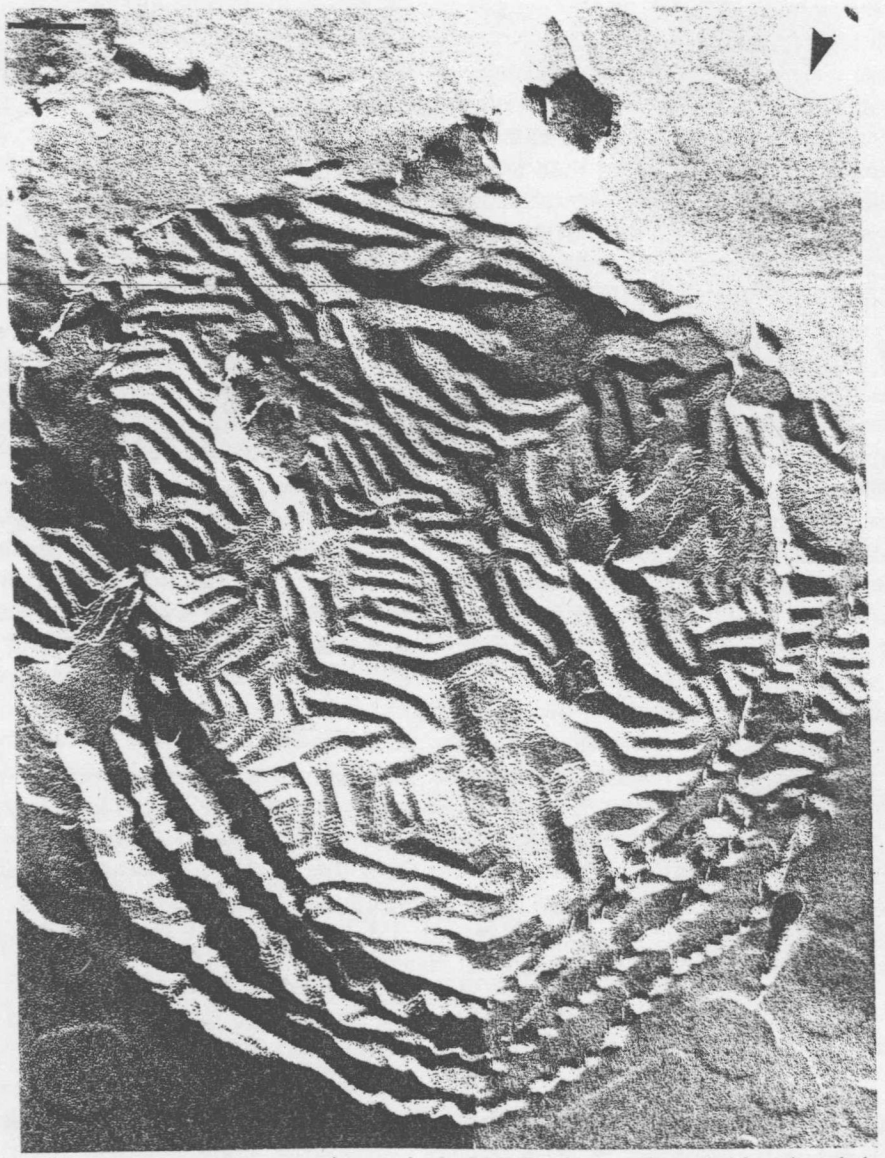

Fig. 11.5. A freeze fracture electron micrograph of a DMPC vesicle rapidly cooled from its gel phase. In addition to nonspherical polyhedral shapes, ripple structure can be clearly observed. Courtesy of D. Papahadjopoulos from the University of California at San Francisco.

coexistence of fluid and solid domains and the leakage is especially severe at the boundaries of the two phases. The addition of cholesterol at 30–50 mol% abolishes the appearance of a phase transition and also drastically reduces the leakage

of entrapped material and susceptibility to penetration into the bilayer. The addition of cholesterol in general transforms the membrane into an ordered fluid phase over a wide temperature range, as determined from the loss of the phase transition and structural-dynamic evidence. This increases the lateral packing in the liquid crystalline phase and eliminates phase boundaries in the 'fluid' gel phase which has lower local order than in a pure phospholipid bilayer but maintaining a smooth surface. Although lateral phase segregation does not occur in most cases, pure lipids with well documented ideal mixing behavior are recommended if long-term stability in either liquid or solid, frozen, or dried forms is required.

One should be aware that phase separation also may occur isothermally for one of many reasons: limited miscibility of particular lipids, changes in ionic strength, pH, or interactions of a bilayer component with a plasma component leading to a lamellar to hexagonal phase transition and concomitant liposome disintegration. This has been well documented by the formation of Ca^{2+}-phosphatidylserine complex or removal of lipid components coupled to antibodies leaving a PE rich nonlamellar phase. From the preceding discussion and from fig. 11.4, it is clear that the liposome composition must have the dominant role in the liposome stability in plasma. These results can be understood by increased intrabilayer van der Waals attraction, which increase the cohesivity of the bilayer. Measurements of membrane bending elasticity and mechanical stability have shown that maximal van der Waals attraction occurs between C_{18} saturated chains and cholesterol [16]. It seems that PC polar heads can pack together better when located on glycerol backbone as compared to the sphingosine and therefore, PC/Chol mixtures exhibit the highest mechanical stability. SM, however, can form an additional hydrogen bond on the backbone which can also stabilize the bilayer and the questions which composition, i.e., DSPC/Chol = 1/1 or SM/Chol = 1/1 mol/mol, is more stable in different conditions still has to be critically evaluated. Definitively, however, results from reference 14 show that in order to increase mechanical strength maximally, bilayer must be saturated, i.e., contain 50 mol%, with cholesterol. Also, this shows that mixing SM and PC lipids in vitro can rather decrease than increase the mechanical stability.

Interactions of liposomes with cells

The interactions of liposomes with cells are of wide biological interest because they are not only important in drug delivery, but also in understanding of cell–cell interactions [17] and recognition processes.

The interactions fall into four categories: (1) exchange of lipids, or proteins, with cell membranes; (2) adsorption, or binding, of liposomes to cells; once bound to the cell surface, (3) liposomes can be internalized such as by endocytosis or phagocytosis, or (4) the bound liposome bilayer may fuse with the cell membrane. In all of these interactions, a strong dependence exists on lipid composition, type

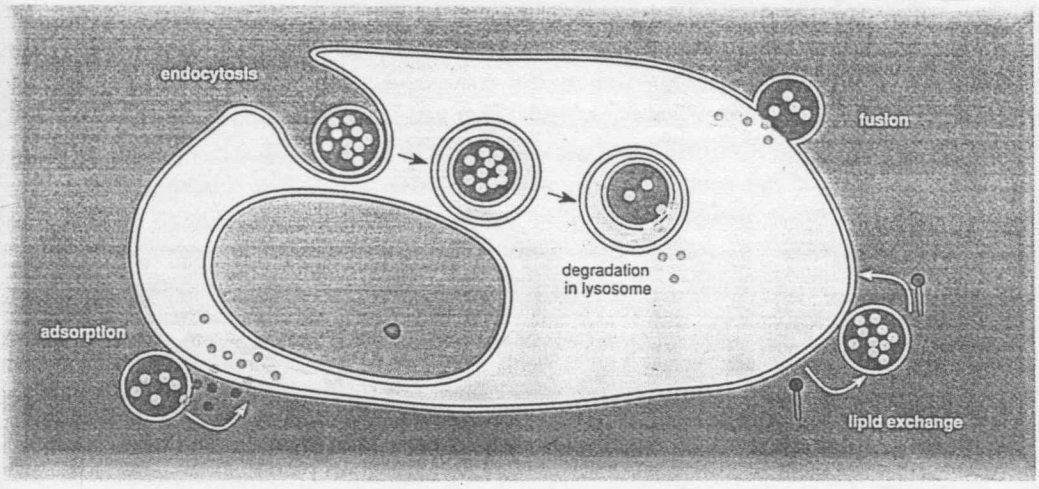

Fig. 11.6. Liposomes and cells interact in four ways. A liposome must first adsorb to the cell membrane (lower left), where it may release some of its contents into the extracellular fluid. In turn, some of the contents may pass through the membrane into the cell. Some liposomes are ingested by the process of endocytosis (upper left), and then degraded in organelles called lysosomes, which release the liposomes's contents into the cytoplasm. The liposome's contents may also enter the cytoplasm directly if the liposome fuses with the cell membrane (upper right). A liposome may also exchange its lipids with those of the cell membrane (lower right). From ref. 18.

of cell, presence of specific receptors, and many other parameters. Each category will be discussed separately and is schematically shown in fig. 11.6.

Lipid transfer occurs by two separate processes; either by associated transfer proteins or via molecular solubility in the aqueous phase or upon events during collisions. A number of lipid transfer proteins have been detected in plasma. Due to the similarity of liposomes with the phospholipid monolayer of lipoproteins, these lipid transfer proteins also recognize liposomes. Lipid exchange, including cholesterol, can occur also in the absence of enzymatic activity. A number of early reports of liposome fusion with cells, based on transfer of lipid markers, were later shown to be more representative of lipid exchange. These results demonstrated the occurrence of lipid exchange in the absence of lipid transfer proteins. The mechanisms of lipid exchange are unresolved. During lipid exchange there is practically no mixing of liposome and cell contents. However, in the case of liposomes carrying lipophilic drugs in plasma some transfer of the drug molecules to plasma lipoproteins may occur.

Liposomes adsorb avidly to the surface of a variety of cells, as revealed by electron microscopic evidence, and this may or may not lead to other interactions. In some cases, bound liposomes remain in that condition for extended periods.

Despite extensive studies, the mechanisms of liposome uptake leading to their localization in endosomal and lysosomal compartments are still poorly understood. Perhaps best characterized is phagocytosis by freshly isolated monocytes,

macrophages, and Kupffer cells. The results have shown that binding of the liposomes occurs as a result of specific opsonization followed by uptake. The liposomes and their contents are degraded in the endosomes or subsequently by lysosomal digestion. Targeted liposomes, containing antibodies (Ab) covalently attached to the surface, can be endocytosed after binding of the Ab with its cellular receptor, but only if the receptor falls within a class exhibiting internalization. Otherwise, as mentioned above, the liposomes may remain bound to the cell surface until they are degraded by one of several possible mechanisms: macrophage recognition and uptake, degradation by lipases and other enzymes, physical disintegration, or they are washed away.

The most common mechanism of the delivery of liposome contents into cells is endocytosis. There are, however, only a few cell types, in general derived from bone marrow, which can effectively phagocytose, especially larger, liposomes. After adsorbing on the cell surface the liposomes are engulfed into phagosomes which transport them to the lysosomes. After fusion the lipids are digested and the encapsulated molecules are released into surroundings. In these cases most of the liposomes end up in lysosomes and if their content is not affeceted by lower values of pH, binding or intercalating with lysosome molecules, and enzymatic activity in lysosomes where liposomal lipids are digested, the drug can leak out into cytoplasm.

Some cells, however, are rather phagocytically inactive and in such cases liposomes can be adsorbed, especially if labelled with antibodies for the antigens expressed on the cell surface for prolonged periods of time; up to days if they are composed of rigid and cholesterol saturated lipid mixtures or polymerized lipids. Such liposomes and also sterically stabilized liposomes which can inhibit activity of phospholipases are rather refractory against disintegration and digestion. In these cases the drug can still leak out and enter the cell via some transport proteins or passively by diffusion if the drug has also appreciable lipid solubility. If this mechanism cannot operate, targeted liposomes can still be used to deliver radionuclides for radiotherapy, photosensitizers for phototherapy, or they can be tried to be physically or chemically disintegrated after adsorption. This or the induction of leakage can be done either by ultrasound, microwaves, local temperature or acidity changes, or by trying to home special ionophores or other disruptive agents to liposomes. Liposomes which are unstable due to their composition, the so-called 'time bombs' or 'suicidal' liposomes were also tried to be designed. Most of the work in the triggered release of liposome contents is still in its infancy and will be briefly discussed after a chapter dealing with loading of liposomes with drugs.

The original idealized model of drug delivery with liposomes envisioned fusion of liposomes with cells delivering their contents directly to the cytoplasm. While fusion is an essential cellular process, for example in exocytosis and in reverse in endocytosis, it is highly regulated and controlled. As discussed above, it appears that liposome fusion with cells occurs very rarely. Apparently, this process is largely controlled by membrane proteins, either those of a cell or of a virus.

Enhanced fusion by reconstitution of viral surface proteins in liposomes (or virosomes) gives support for this vision. Consequently, it is unrealistic to consider that simple lipid bilayers will fuse with cells without the action of a fusogen in vivo. This can be done either by incorporating fusogenic proteins or, in vitro, addition of fusogens.

By using the fusion mechanism, some biologically active molecules, such as mRNA or DNA which are normally deactivated by lysosomal enzymes in the endocytic pathway, were transferred into cells in their active form. In in vitro experiments fusion is normally enhanced by adding substances which can induce fusion. These compounds include polyethylene glycols, with molecular weights normally about 5000–20 000 Da up to 40–60 wt.%, fragments of some viruses or synthetic peptides. Some liposome compositions are also more susceptible for vesicle fusion because they can contain fusogenic lipids such as phosphatidylethanolamines, short saturated or long, possibly unsaturated, fatty acids or they have asymmetric membrane composition. Some of the fusogenic molecules, such as fragments of Sendai virus can also be incorporated into liposomes and the resulting virosomes have normally high specific fusogenic activity and can be used to incorporate some reconstituted membrane proteins into bilayers.

Fusogenic virosomes can be prepared by a detergent depletion method. For instance, Sendai virus protein containing liposomes can be prepared by dissolving viral envelopes with non-ionic detergents, such as Triton X-100. This detergent does not dissolve nucleocapsid nor inactivate the fusogenic activity of HN and F glycoproteins of Sendai virus. Briefly, virus particles, normally grown in fertilized hen eggs, are dissolved with the detergent. The nondissolved material is pelleted away. Supernatant is normally shaken with detergent adsorbing beads such as BioBeads SM-2. Fusogenic reconstituted Sendai virus virosomes with diameters between 100 and 300 nm are obtained. Depending on the experiment, other lipids can be added into the mixed micellar solution to improve the yield. Normally one milligram of intact virus yields 0.4 mg of reconstituted envelopes.

Virosomes bind to cells which bear receptor for the HN glycoprotein and the action of the F protein results in fusion. Otherwise, specific antibodies for particular cells have to be incorporated into virosomes.

Temperature dependence of these processes is related to the liposome phase transition temperature, as well as that of the biological membranes. In general, endocytosis and adsorption are unaffected while rigid liposomes show reduced fusion with cells. They also show no measurable lipid or protein exchange despite retaining the ability to adsorb onto cells.

Fate of liposomes in vivo

The living bodies protect themselves with a complicated defense systems and macrophages are their crucial components. Macrophages are specialized cells which work either by processing and presenting antigens, secreting factors which