CHAPTER 10

Applications of Liposomes

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1. Introduction

Liposomes are spherical, selfclosed vesicles of colloidal dimensions, in which (phospho)lipid bilayer sequesters part of the solvent, in which they freely float, into their interior [1]. In the case of one bilayer encapsulating the aqueous core one speaks either of small or large unilamellar vesicles while in the case of many concentric bilayers one defines large multilamellar vesicles [2].

Due to their structure, chemical composition and colloidal size, all of which can be well controlled by preparation methods, liposomes exhibit several properties which may be useful in various applications. The most important properties are colloidal size, i.e. rather uniform particle size distributions in the range from 20 nm to 10 μm, and special membrane and surface characteristics. They include bilayer phase behavior, its mechanical properties and permeability, charge density, presence of surface bound or grafted polymers, or attachment of special ligands, respectively. Additionally, due to their amphiphilic character, liposomes are a powerful solubilizing system for a wide range of compounds. In addition to these physico-chemical properties, liposomes exhibit many special biological characteristics, including (specific) interactions with biological membranes and various cells [3].

These properties point to several possible applications with liposomes as the solubilizers for difficult-to-dissolve substances, dispersants, sustained release systems, delivery systems for the encapsulated substances, stabilizers, protective agents, microencapsulation systems and microreactors being the most obvious ones. Liposomes can be made entirely from naturally occurring substances and are therefore nontoxic, biodegradable and non immunogenic. In addition to these applications which had significant impact in several industries, the properties of liposomes offer a very useful model system in many fundamental studies from topology, membrane biophysics, photophysics and photochemistry, colloid interactions, cell function, signal transduction, and many others [3–5].

The industrial applications include liposomes as drug delivery vehicles in medicine, adjuvants in vaccination, signal enhancers/carriers in medical diagnostics and analytical biochemistry, solubilizers for various ingredients as well as support matrix for various ingredients and penetration enhancer in cosmetics.

2. Applications of liposomes in basic sciences

Lipid membranes are two dimensional surfaces floating in three dimensional space. In the simplest models, they can be characterised only by their flexibility which is related to their bending elasticity. A number of new theoretical concepts were developed to understand their conformational behaviour [4]. On the other hand they

Table 1

Applications of liposomes in the sciences.

Discipline	Application
Mathematics	Topology of two-dimensional surfaces in three-dimensional space governed only by bilayer elasticity
Physics	Aggregation behaviour, fractals, soft and high-strength materials
Biophysics	Permeability, phase transitions in two-dimensions, photophysics
Physical Chemistry	Colloid behaviour in a system of well-defined physical characteristics, inter- and intra-aggregate forces, DLVO
Chemistry	Photochemistry, artificial photosynthesis, catalysis, microcompartmentalization
Biochemistry	Reconstitution of membrane proteins into artificial membranes
Biology	Model biological membranes, cell function, fusion, recognition
Pharmaceutics	Studies of drug action
Medicine	Drug-delivery and medical diagnostics, gene therapy

Table 2

Liposomes in the pharmaceutical industry.

Liposome Utility	Current Applications	Disease States Treated
Solubilization	Amphotericin B, minoxidil	Fungal infections
Site-Avoidance	Amphotericin B – reduced nephrotoxicity, doxorubicin – decreased cardiotoxicity	Fungal infections, cancer
Sustained-Release	Systemic antineoplastic drugs, hormones, corticosteroids, drug depot in the lungs	Cancer, biotherapeutics
Drug protection	Cytosine arabinoside, interleukins	Cancer, etc.
RES Targeting	Immunomodulators, vaccines, antimalarials, macophage-located diseases	Cancer, MAI, tropical parasites
Specific Targeting	Cells bearing specific antigens	Wide therapeutic applicability
Extravasation	Leaky vasculature of tumours, inflammations, infections	Cancer, bacterial infections
Accumulation	Prostaglandins	Cardiovascular diseases
Enhanced Penetration	Topical vehicles	Dermatology
Drug Depot	Lungs, sub-cutaneous, intra-muscular, ocular	Wide therapeutic applicability

can be used as a model in order to understand the topology, shape fluctuations, phase behaviour, permeability, fission and fusion of biological membranes. Their aggregation leads to fractal clusters. In addition they can serve as a model to study vesiculation, including vesicle shedding and endo- and exo-cytosis, of living cells (table 1).

Despite their widespread application, the mechanism of liposome formation is not yet well understood. The equilibrium calculations of the shapes of giant unilamellar vesicles [7, 8] and their observations (fig. 1A) [9, 10], however, offer a qualitative guidance in the modeling of structural transformations in the various vesiculation processes. Figure 1B shows that similar shapes occur also in multilamellar aggregates and it is reasonable to assume that the gradient of hydration across the stack of concentric lamellae causes also the gradient of surface areas of polar heads in the consecutive monolayers because the area of polar head is proportional to hydration. As a result of this imbalance the curvature is induced. This indicates that a similar

Fig. 1. Phase contrast optical micrographs of various liposomes. (A): Several shapes of giant unilamellar vesicles (from ref. [9], with permission). (B): In a dispersion of multilamellar egg lecithin vesicles (10 mg/ml) several liposomes show similar shapes as giant unilamellar vesicles, as indicated by arrows.

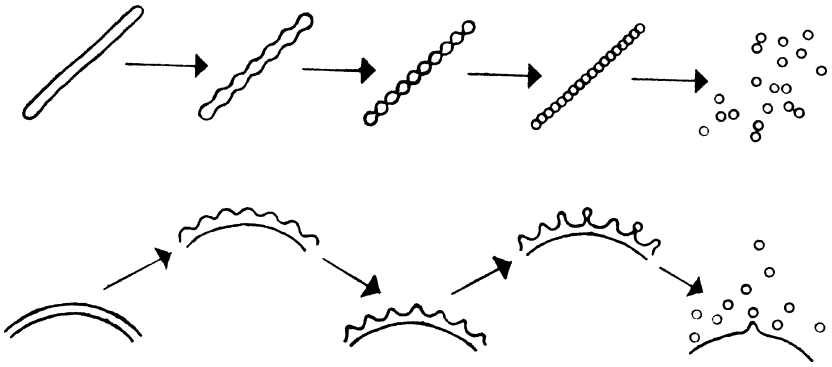

Fig. 2. Schematic presentation of the formation of unilamellar vesicles by pinching off and by budding off mechanisms. Optical micrographs of the transition structures are also shown. (A): Membrane destabilisation: a sinusoidal undulation of the membrane can be seen (arrows). Such membranes often shed off daughter vesicles as observed by Helfrich and collaborators [67] (B): Transition of cylindrical liposome made from polymerizable synthetic lipid distearoyl-dimethyl-ammonium chloride (DODAC) into a 'string of beads' structure. (Courtesy of M. Dvolaitzky and P.G. de Gennes.)

mechanism of curving, bending, and eventually, budding off, as observed in giant unilamellar vesicles can qualitatively explain the formation of large multilamellar vesicles from dry lipid films. Moreover, vesiculation is an essential process in cell

function and communication. Figure 2 shows how smaller unilamellar vesicles can be formed from large membranes or cylindrical liposomes by budding and pinching off, respectively. These processes are contrasted by laboratory preparations in which high energy fragmentation of the bilayers causes the formation of smaller vesicles [11, 12]. It is not unreasonable to assume that similar budding and pinching off mechanisms, as shown in fig. 2, operate in vivo in exocytosis and formation of synaptic vesicles, and in Golgi apparatus, respectively [13]. Of course, there are several enzymatic (i.e. nonspontaneous) processes which provide the driving force for these transformations (fusion and fission) but nevertheless it is important to establish the role of the lipid bilayer itself in these processes.

Physico-chemical studies of cholesterol and other sterols containing membranes may offer also some clues on the evolution of life. It seems that the presence of cholesterol, which could have been synthetised only in certain geological time when the atmosphere became rich with oxygen and renders membrane more cohesive at increased bilayer fluidity, was the cause which enabled endo- and exo-cytosis and therefore the development of multi-organelle prokaryotic cells from the eucaryotic ones, which contain no cholesterol and also no internal organelles [10].

Living species contain also a vast number of different polar and bipolar lipids and sterols, and their occurrence and function in different species may significantly improve our understanding of evolution of plant and animal life.

One of the major research goals is to duplicate photosynthesis and harvest the abundant light energy either by artificial photosynthesis or by splitting water into its reactive elements. These systems are rather complex involving precisely tuned photosensitisers, electron relay mechanisms and catalysts embedded in lipid bilayers. Artificial systems use liposomes to simulate the organization, orientation, and activity of the embedded compounds and their reactivity. Although these are very complex problems, several artificial systems have already photolysed water, even though the yields are still very low [14].

The structure of liposomes offers also a system to compartmentalise chemical reactions. This can be used in catalysis, the studies of biomineralisation or in the synthesis of colloid particles.

One of the most prolific areas of liposome applications is in biochemical investigations of conformation and function of membrane proteins. These are the so-called reconstitution studies and purified membrane proteins, such as ion pumps (sodium-potassium-, or calcium-ATPases), or glucose transport proteins are reconstituted in their active form into liposomes and then studied. This research has important consequences on our understanding of proteins and cell function [15, 16]. Furthermore, cell communication largely depends on the traffic of vesicles. Nerve impulses travel between synapses and neurons in synaptic vesicles carrying neurotransmitters. Various proteins regulate directions, addresses, docking, internalization or fusion of these vesicles with a great efficacy. Cells secrete and ingest macromolecules via exo- and endocytosis, respectively as well as transport molecules to and within Golgi apparatus by the use of vesicles. Obviously, our increasing knowledge of these processes will shed more light on the function of living cells as well as offer some solutions in the case of its disfunction.

3. Applications of liposomes in medicine

Applications of liposomes in pharmacology and medicine can be divided into therapeutic and diagnostic applications of liposomes containing drugs or various markers, and their use as a model, tool, or reagent in the basic studies of cell interactions, recognition processes, and of the mode of action of certain substances [3].

Unfortunately many drugs have a very narrow therapeutic window, meaning that the therapeutic concentration is not much lower than the toxic one. In several cases the toxicity can be reduced or the efficacy enhanced by the use of an appropriate drug carrier which changes the temporal and spatial distribution of the drug, i.e. its pharmacokinetics and biodistribution.

The benefits and limitations of liposome drug carriers critically depend on the interaction of liposomes with cells and their fate in vivo after administration. In vitro and in vivo studies of the interactions with cells have shown that the predominant interaction of liposomes with cells is either simple adsorption or subsequent endocytosis. Fusion with cell membranes is much more rare. The fourth possible interaction is exchange of bilayer constituents, such as lipids, cholesterol, and membrane bound molecules with components of cell membranes. These interactions, schematically shown in fig. 3, determine also the fate of liposomes in vivo.

The body protects itself with a complex defense system. Upon entering into the body, larger objects cause thrombus formation and eventually their surface is passivated by coating with biomacromolecules while smaller particles, including microbes, bacteria, and colloids are eaten up by the cells of the immune system. This response of the immune system has triggered substantial efforts in the development of biocompatible and nonrecognizable surfaces and has also, on the other hand, narrowed the spectrum of applications of microparticulate drug carriers only to targeting of the very same cells of the immune system.

Fig. 3. Schematic presentation of liposome interactions with cells. Endocytosis is shown in the upper left part of the cell. In addition, clockwise fusion, lipid exchange and adsorption (of leaky vesicle) are shown. From ref. [3], with permission.

Although they are composed from natural substances liposomes are no exception. They are rapidly cleared from the circulation by the macrophages which are located mainly in the liver, spleen, and bone marrow.

3.1. Modes of liposome action

Liposomes as drug delivery systems can offer several advantages over conventional dosage forms especially for parenteral (i.e. local or systemic injection or infusion), topical, and pulmonary route of administration. The preceding discussion shows that liposomes exhibit different biodistribution and pharmacokinetics than free drug molecules. In several cases this can be used to improve the therapeutic efficacy of the encapsulated drug molecules. The limitations can be reduced bioavailability of the drug, saturation of the cells of the immune system with lipids and potentially increased toxicity of some drugs due to their increased interactions with particular cells. The benefits of drug loaden liposomes, which can be applied as (colloidal) solution, aerosol, or in (semi) solid forms, such as creams and gels, can be summarized into seven categories:

(i) Improved solubility of lipophilic and amphiphilic drugs. Examples include Porphyrins, Amphotericin B, Minoxidil, some peptides, and anthracyclines, respectively; furthermore, in some cases hydrophilic drugs, such as anticancer agent Doxorubicin or Acyclovir can be encapsulateded in the liposome interior at concentrations several fold above their aqueous solubility. This is possible due to precipitation of the drug or gel formation inside the liposome with appropriate substances encapsulated [17];

(ii) Passive targeting to the cells of the immune system, especially cells of the mononuclear phagocytic system (in older literature reticuloendothelial system). Examples are antimonials, Amphotericin B, porphyrins and also vaccines, immunomodulators or (immuno)supressors;

(iii) Sustained release system of systemically or locally administered liposomes. Examples are doxorubicin, cytosine arabinose, cortisones, biological proteins or peptides such as vasopressin;

(iv) Site-avoidance mechanism: liposomes do not dispose in certain organs, such as heart, kidneys, brain, and nervous system and this reduces cardio-, nephro-, and neuro-toxicity. Typical examples are reduced nephrotoxicity of Amphotericin B, and reduced cardiotoxicity of Doxorubicin liposomes;

(v) Site specific targeting: in certain cases liposomes with surface attached ligands can bind to target cells (‘key and lock’ mechanism), or can be delivered into the target tissue by local anatomical conditions such as leaky and badly formed blood vessels, their basal lamina, and capillaries. Examples include anticancer, antiinfection and antiinflammatory drugs;

(vi) Improved transfer of hydrophilic, charged molecules such as chelators, antibiotics, plasmids, and genes into cells; and

(vii) Improved penetration into tissues, especially in the case of dermally applied liposomal dosage forms. Examples include anaesthetics, corticosteroids, and insulin.

Among numerous studies which showed improved therapeutic index we shall mention only those which had significant impact and are also in various phases of preclinical and clinical studies in humans.

In general, liposome encapsulation is considered when drugs are very potent, toxic and have very short life times in the blood circulation or at the sites of local (subcutaneous, intramuscular or intrapulmonary) administration.

3.2. Conventional liposomes

For historical reasons we shallconventional liposomes distinguish between conventional liposomes and liposomes with altered surface properties. The first generation of liposomes includes various lipid compositions which changed the physicochemical properties of liposomes in a variety of different ways, but could not significantly alter their biological properties upon intravenous administration which is the most widely used route in medical applications. Therefore, the optimistic goals of antibody sensitised liposomes (immunoliposomes as 'guided missiles'), which gave often very encouraging results in in vitro studies – which are in general performed in the absence of immunoglobulins, complement components, and macrophages – failed in in vivo applications.

The first condition for the immunoliposome concept to work is therefore that they escape the clearance by the mononuclear phagocytic system. This was made possible by the introduction of sterically stabilized liposomes in which the presence of surface grafted hydrophilic polymers substantially prolongs the liposome blood circulation times, probably due to reduced interactions with the components of the immune system. This reduction arises from the presence of a steric barrier which prevents adsorption or hydrophobic binding of immune system components onto the foreign body. The liposomes with altered surfaces therefore include sterically stabilized liposomes and immunoliposomes. With respect to sterically stabilized immunoliposomes one should add a note of caution. Even liposomes with prolonged circulation in blood are not likely to be as widely applicable as many researchers envision(ed). The main limitations are extravasation (escaping from the blood circulation) and poor blood circulation in solid tumours. In the latter case, some of the particulates suspended in blood which come in the area extravasate due to leaky capillaries and stay or get stuck in the region of the extravasation and actually the presence of surface attached homing ligand, i.e. active targeting, does not really have much influence. In addition to some other targeting possibilities, such as injections in different body cavities [18], immunoliposomes present a viable option in immunoassays and diagnostic tests.

3.2.1. Liposomes in parasitic diseases and infections

Since conventional liposomes are digested by phagocytic cells in the body after intravenous administration, they are ideal vehicles for the targetting of drug molecules into these macrophages. The best known examples of this 'Trojan horse-like' mechanism are several parasitic diseases which normally reside in the cell of mononuclear phagocytic system. They include leishmaniasis and several fungal infections.

Leishmaniasis is a parasitic infection of macrophages which affects over 100 million people in tropical regions and is often fatal. The efficacious dose of drugs, mostly different antimonials, is not much lower than the toxic one. Liposomes accumulate in the very same cell population which is infected and therefore offer an ideal drug delivery vehicle [19]. Indeed, the therapeutic index was increased in rodents as much as several hundred times upon administration of the drug in various liposomes. Surprisingly, and unfortunately, there was not much interest to scale up the formulations and clinically approve them after several very encouraging studies dating back to 1978. Only now, there are several ongoing studies with various antiparasitic liposome formulations in humans. These formulations use mostly ionophore Amphotericin B and are transplanted from very successful and prolific area of liposome formulations in antifungal therapy.

The best results reported so far in human therapy are probably liposomes as carriers for Amphotericin B in antifungal therapies. This drug is the drug of choice in disseminated fungal infections which often parallel compromised immune system, chemotherapy, or AIDS and are frequently fatal. Unfortunately, the drug itself is very toxic and its dosage is limited due to its nephro- and neuro-toxicity. These toxicities are normally correlated with the size of the drug molecule or its complex and obviously liposome encapsulation prevents accumulation of drug in these organs and drastically reduces toxicity [20]. In addition, often the fungus resides in the cells of the mononuclear phagocytic system and therefore the encapsulation results in reduced toxicity and passive targetting. These benefits, however, can be associated with any colloidal drug carrier. Indeed, similar improvements in therapy were observed with microemulsions and stable mixed micellar formulations [21]. Furthermore, it seems that many of the early liposomal preparations were in fact liquid crystalline colloidal particles rather then selfclosed multilamellar liposomes. Since the lives of the first terminally ill patients, which did not respond to all the conventional therapies, were saved [20], many patients were very successfully treated with a variety of Amphotericin B formulations.

Similar approaches can be implemented in antibacterial, and antiviral therapy [22]. Most of the antibiotics, however, are orally available and liposome encapsulation can be considered only in the case of very potent and toxic ones which are administered parenterally. The preparation of antibiotics loaded liposomes at reasonably high drug to lipid ratios may not be easy because of the interactions of these molecules with bilayers and high densities of their aqueous solutions which often force liposomes to float as a creamy layer on the top of the tube. Several other routes, such as topical or pulmonary (by inhalation) are being considered also.

Liposome encapsulated antivirals such as acyclovir, ribavarin, or azide thymidine (AZT) have also shown reduced toxicity and currently more detailed experiments are being performed with respect to their efficacy.

3.2.2. Macrophage activation and vaccination

The automatic targetting of liposomes to macrophages can be exploited in several other ways, including the macrophage activation and in vaccination.

Some natural toxins induce strong macrophage response which results in macrophage activation. This can be duplicated and improved by the use of liposomes because small molecules with immunogenic properties (haptens) cannot induce immune response without being attached to a larger particle. For instance, liposomes containing muramyl tripeptide, the smallest bacterial cell wall subunit with immunogenic properties cause macrophage activation. Activated macrophages are larger and contain more granulomae and lysosome material. Their state lasts for a few days during which they show enhanced tumouricidal, virocidal, and microbicidal activity. Early expectations in antitumour activity turned out to be too optimistic due to the simple fact that the number of free circulating macrophages is too small for an effective therapy. In cancer therapy, however, surgery or radiotherapy often do(es) not remove all the tumour cells and in these cases, when tumour burden is low, this therapy is very promising for complete eradication of malignant cells. Activation of macrophages was proven useful in the treatment of viral, bacterial, and fungal infections as well. Synergy between encapsulated immunomodulators and other activating factors such as cytokines and lymphokines, including interferon, was shown [23].

Macrophages are involved also in the process of immunisation. Many molecules, however, do not induce an immune response because they are too small. In order to do so, they must be attached to larger particles. Normally this is done by administration of alum or killed bacteria and obviously liposomes offer an elegant alternative [24]. Indeed, liposomes are used in animal vaccination already since 1988, while human vaccinations against malaria are now in clinical trials [25].

3.2.3. Liposomes in anticancer therapy

Many different liposome formulations of various anticancer agents were shown to be less toxic than the free drug [26]. Anthracyclines are drugs which stop the growth of dividing cells by intercalating into the DNA and therefore kill predominantly quickly dividing cells. These cells are in tumours, but also in gastrointestinal mucosa, hair, and blood cells and therefore this class of drugs is very toxic. The most used and studied is Adriamycin (commercial name for Doxorubicin HCl). In addition to the above mentioned acute toxicities its dosage is limited by its cumulative cardiotoxicity. Many different formulations were tried. In most cases the toxicity was reduced about 50%. This includes both, short term and chronic toxicities because liposome encapsulation reduces the distribution of the drug molecules towards those tissues. For the same reason, on the other hand, the efficacy was in many cases compromised due to the reduced bioavailability of the drug, especially if the tumour was not phagocytic, or located in the organs of mononuclear phagocytic system. In some cases, such as systemic lymphoma, the effect of liposome encapsulation showed enhanced efficacy due to the sustained release effect, i.e. longer presence of therapeutic concentrations in the circulation [27] while in several other cases the sequestration of the drug into tissues of mononuclear phagocytic system actually reduced its efficacy.

Applications in man showed in general reduced toxicity, better tolerability of administration with not too encouraging efficacy. Several different formulations are in different phases of clinical studies and show mixed results [28].

3.2.4. Other applications

Small liposomes composed of lipids with long and saturated hydrocarbon chains in mixtures with cholesterol were shown to accumulate at the sites of inflammations. Such liposomes were used for diagnostic purposes [29]. They can also deliver antiinflammatory drugs. Liposomes containing corticosteroids were injected also directly into the sites of inflammations, especially into arthritic joints where they acted as a sustained release system. Additionally, the contamination of healthy tissues with drug molecules was reduced.

Liposomes can be used also to deliver drugs into the lung [30]. This is most often done by inhalation of liposome aerosol. This can be used either for the treatment of various lung disorders, infections, asthma, or using lungs as a drug depot for the systemic delivery. By tailoring lipid composition a variety of release kinetics can be obtained (fig. 4). One of the possible applications of these aerosols is in the asthma relief in which the dosing frequency can be substantially reduced and single inhalation can last overnight [31].

The natural fate of liposomes to accumulate in liver and spleen was exploited in the treatment of neonatal jaundice in an animal model [32]. The application of free and liposomal metalloporphyrins which inhibit enzyme which breaks down hemoglobin into toxic bilirubin, however, did not result in statistically significant reduction of the enzyme activity. This is probably due to the fact that uptake by liver greatly exceeds the uptake into the spleen in which the degradation takes place. However, when the liver uptake was presaturated with a dose of empty liposomes, the enzymatic activity was significantly reduced due to targetting of liposomes to the spleen [32].

Liposomes can be applied also as a thick cream, gel, or tincture. In addition to subcutaneous or intramuscular drug depot these formulations can be applied topically. Several researchers claim increased penetration of lipid and drug molecules into the skin. These data, as well as possible mechanisms, are, however, still a matter of controversy.

Oral applications of liposomes are at present rather limited due to the very liposomicidal environment in stomach and duodenum and normally the administration of free or liposome encapsulated drug exhibits usually no differences. Intragastrical administration, however, shows that liposomes enhance the systemic bioavailability of certain water insoluble drugs and vitamins. Several designs to stabilize liposomes in low pH, degradative enzyme, and bile salts containing environments are being studied. They include liposomes composed from many bilayers with different chemical stability and with programmable degradation kinetics, liposome encapsulated in biodegradable gels or capsules, polymer coated liposomes, and similar. More research is needed, however, to find out if some of these approaches are commercially viable.

3.3. Liposomes with altered surface properties

All these applications have made use of the so-called conventional liposomes. New strategies, including selective targetting of cancer and other diseased cells, however, rely on liposomes with altered surface properties. For selective interactions with

particular cells, liposomes have to bear surface attached antigens. The application of these immunoliposomes, however, suffers from their quick clearance from the blood by the immune system and their inability to extravasate, i.e. leave the blood stream. These two limitations can be bypassed in certain applications, such as in treatments of intraperitoneal tumours or other disorders, in the use of liposomes as localised drug reservoir, in some topical applications, or in pulmonary applications of liposome aerosols, but for the majority of other applications the fast clearance represents the major obstacle. For this reason sterically stabilised liposomes were introduced which can largely avoid detection by the immune system and were shown to have blood circulation times for several days (half lives in humans $>$ 2 days as compared to minutes rather than hours of conventional liposomes). For this reason they are often called Stealth liposomes. Of course only with stealth immunoliposomes, systemic active targetting became a possibility.

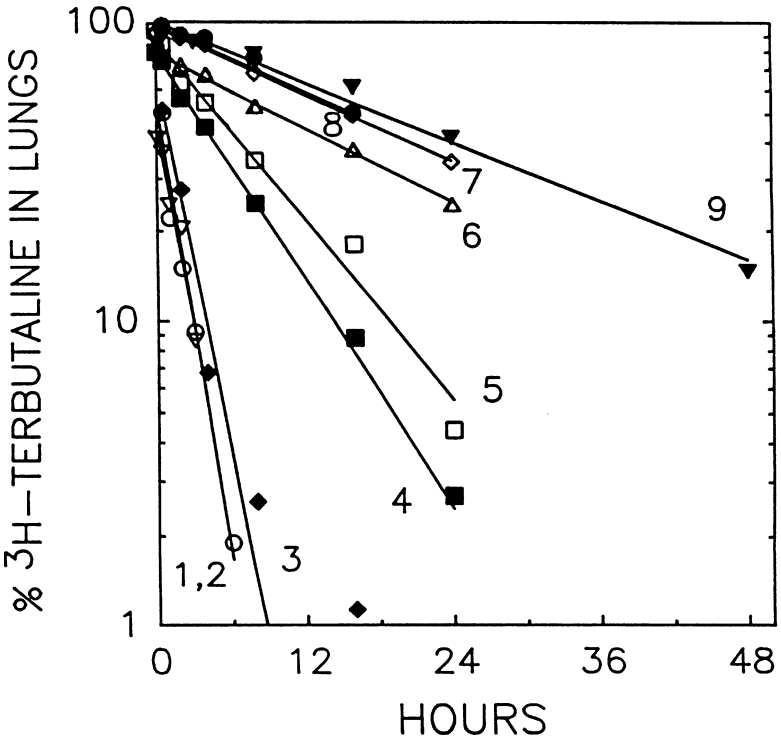

Fig. 4. Disappearance of radioactivity from the lungs after intratracheal instillation of unencapsulated tritium labelled bronchodilator terbutaline (1,2; ○ and ▽, respectively) and drug encapsulated in DPPC/DPPG, size 0.2 μm (3, ♦), EPC/EPG/CHOL, 0.24 μm (4, ■), EPC(IV40)/EPG/CHOL, 0.27 μm (5, □), same at 3.9 μm (6, △), HEPC/EPG/CHOL,0.27 μm (7, ◇), DPPC/DPPG/CHOL 0.24 μm (8, ●), and DSPC/DSPG/CHOL, 0.23 μm (9, ▼). All molar ratios are 55/5/40, only #3 has 95/5. Measured values (symbols) were fitted and numbers on the plots represent various formulations. Abbreviations: E egg, PC phosphatidylcholine, D di, P palmitoyl, G glycerol, H hydrogenated, S stearoyl, CHOL cholesterol, IV iodine value (i.e. degree of acyl chains saturation: EPC IV = app. 65–70, HEPC IV = 1).

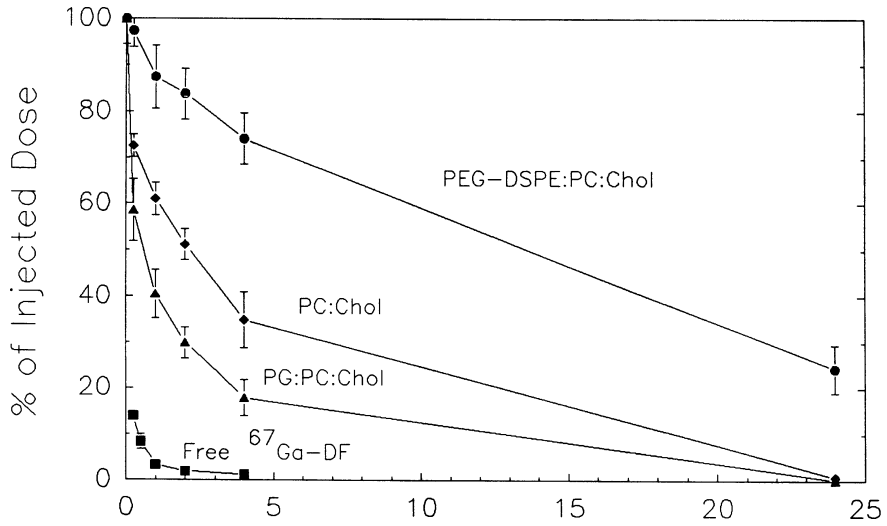

Fig. 5. Comparison of blood clearance of various liposome formulations in rats following intravenous administration of 5 μM lipid/kg. Liposomes contained 39.5 mol% of egg phosphatidylcholine (PC), 33 mol% cholesterol (Chol) and 7.5 mol% of either egg phosphatidylglycerol (PG) or polyethylene (1900 Da) coupled to distearoyl phosphatidylethanolamine (^{1900}PEG-DSPE). Clearance of the free label, Galium desferal is also shown. Courtesy M.C. Woodle and M. Newman.

3.3.1. Sterically stabilised liposomes

The fate of liposomes, i.e. their rapid clearance from the body, was realized rather early. First attempts to alter their biodistribution by either surface ligands or membrane composition were undertaken in the late 70's. The results showed that liposome disposition can be altered, but predominantly within the mononuclear phagocytic system including the intrahepatic uptake itself. Blood circulation times were prolonged but the first substantial improvements were achieved by the incorporation of ganglioside G_{M1} or phosphatidylinositol at 5–10 mol% into the bilayer [33, 34]. The best results were obtained by substituting these two lipids with synthetic polymer containing lipids. The longest circulation times were achieved when polyethylene glycol covalently bound to the phospholipid was used. It seems that intermediate molecular weights, from 1500 to 5000 Da are the optimum [35]. Figure 5 shows blood clearance profiles of several different formulations.

It was suggested [36] that the presence of a steric barrier reduces adhesion and adsorption (or at least adsorption with a conformational change) of blood components, such as immunoglobulins, complement proteins, fibronection and similar molecules, which mark foreign particles for subsequent macrophage uptake as schematically shown in fig. 6.

The origin of steric stabilisation is well documented although not well understood. Recently it was shown that the Alexander-de-Gennes model of polymers at interfaces [37] can qualitatively explain the stability of liposomes in biological systems [35]. The model can explain minimal polymer concentration above the surface of the bilayer at which polymer forms the so-called brush conformation and which acts as

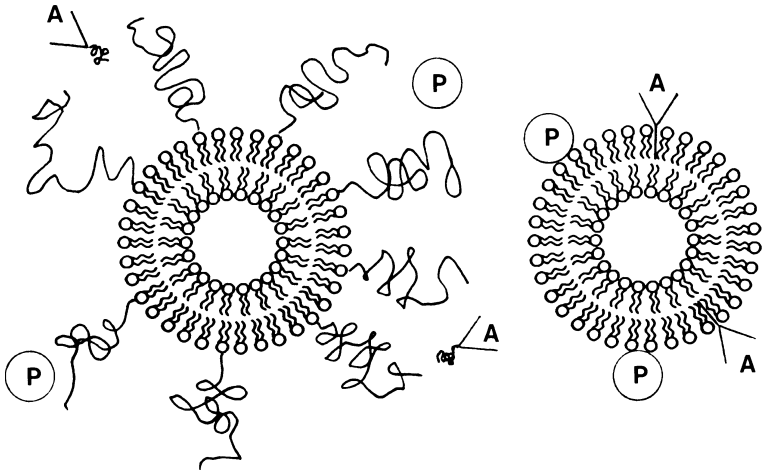

Fig. 6. A schematic presentation of the proposed mechanism of the stabilisation of liposomes in biological environments. The presence of the polymer mushroom or brush [68] reduces the adsorption and adhesion rate of immunoglobulins and other antibodies (A) and plasma (lipo) proteins (P) which either mark liposomes for the subsequent macrophage uptake or deplete lipids which can cause liposome disintegration.

a steric shield. Small angle X-ray scattering measurements of force-distance profiles of polyethylene glycol grafted liposomes have shown enhanced bilayer repulsion [38, 39] in agreement with the hypothesis that reduced surface adhesion and adsorption stabilises liposomes. Recent theoretical work also explained the experimentally well-known fact that increased concentrations of longer chains start to reverse the effect at particular polymer density. This is due to the so-called collapse of the brush which occurs at certain polymer density and results in polymer selfaggregation [40], a well-known fact from the experimental polymer science. Longer chains can also exhibit increased attractive and bridging forces with macromolecules [41]. Of course, the in vivo and in vitro stability are not necessarily correlated and, for example, in vitro very stable formulations, such as highly charged ones, or the ones with charged brush, are cleared in vivo very rapidly. Another factor which may differ between the two tests is the role of chain flexibility on the interactions with particles and proteins. It is possible that the decreased mobility of chains in the denser brush regimes, when the chain motion correlation times may approach times required for protein binding, can account for the weak physisorption of proteins.

3.3.2. Medical applications of stealth liposomes

Sterically stabilised vesicles can act either as long circulating microreservoirs or tumour (or site of inflammation and infection) targetting vehicles. The former applications requires larger liposomes ($\sim$ 0.2 μm) while the latter one is due to the ability of small vesicles to leave the blood circulation. The prolonged presence of small particulates in blood results in effective extravasation in regions with porous, damaged, or badly formed blood vessels which often characterise tumours or their

Fig. 7 Blood vessels (vasculature) of (A) normal and (B) tumour tissue as viewed by rhodamine-phosphatidylethanolamine labelled long circulating liposomes approximately one hour after injection. The difference between the healthy and tumour tissue, which shows accumulation of fluorescent liposomes in extravascular sites can be clearly observed. Plates C and D show accumulation of Doxorubicin loaded Stealth liposomes in mammary adenocarcinoma tumour one hour and one day, respectively, after tail vein bolus injection in female Fischer rat skin flap window chamber model. In this study fluorescence of the encapsulated drug Doxorubicin was used as a marker. The intensity is much lower, however, due to the self quenching effect of highly concentrated drug inside the liposomes. Due to this high concentration, which is 3–5 fold above its aqueous solubility the drug is precipitated or gelled with counterions in the vesicle interior. Because these liposomes practically do not leak their contents the blood vessels can be barely seen either immediately after injection or one hour later (C). Few focal points of accumulation which are outside blood circulation (as can be easily verified by the bright field light microscopy of the same area) show that after one hour there is already some extravasation (C). The increase of fluorescence intensity at 24 hours, however, indicates not only high accumulation of the drug but also the fact that it is being released from the liposomes which reduces or eliminates selfquenching (D). (N. Wu, M. Dewhirst, D.D. Lasic, D. Needham, unpublished data. For details see ref. [39].)

vicinity. While normal molecules and macromolecules quickly come to equilibrium large doses of liposomes can accumulate due to their adhesion or immobilization. (In analogy with biocompatible surfaces we can speculate that PEG chains effectively reduce the adsorption of proteins while for the prevention of cell adhesion much longer chains would be required [42]. At present, it is still not known if such long chains can be effectively incorporated into liposomes.) This allows larger doses of liposome loaden drugs to be delivered to malignant tissues. For instance more then 10% of the injected dose of stealth liposome encapsulated Doxorubicin was found in tumours [43] as opposed to around 1% when free drug was administered.

Figure 7 shows extravasation of Doxorubicin encapsulated in Stealth liposomes in

the dorsal flap window rat model, i.e. an animal model which allows the viewing in the fluorescence microscope of the biodistribution of fluorescent molecules or labelled liposomes in vivo, in this case dorsal tissue of rat clipped between special microscopic slides [39]. Extensive liposome localization in the tumours was observed. Healthy tissue did not accumulate any signal which was due to Doxorubicin fluorescence [39].

Efficacy studies in various mice tumour models, such as implanted solid C26 carcinoma and inoculated mammary carcinoma, have shown dramatic improvements [44–48]. Solid C26 colon tumour is practically resistant to free drug, conventional Epirubicin (a very similar drug to Doxorubucin) liposomes, and mixtures of free drug and empty stealth liposomes. Stealth Epirubicin and Doxorubicin liposomes resulted, however, in complete remissions of tumours in the early treatment schedule and substantial reduction of tumour size in the delayed treatment regime (fig. 8) [45]. Similar improvement in therapy was observed also in mammary carcinoma (fig. 9) [46]. These formulations were substantially more effective not only in curing mice with recent implants from various tumours but also in reducing the incidence of metastases originating from these intra mammary implants. Similarly, several fold increased drug accumulation was observed also in sites of infections which are also characterized by the enhanced vascular permeability. For instance, in mice with infected lungs 10 fold more antibiotic drug accumulated in the infected lung as compared to the noninfected one [49].

Sterically stabilised liposomes may act also as a sustained drug release system either as a long circulating microreservoir or localised drug depot. The first example

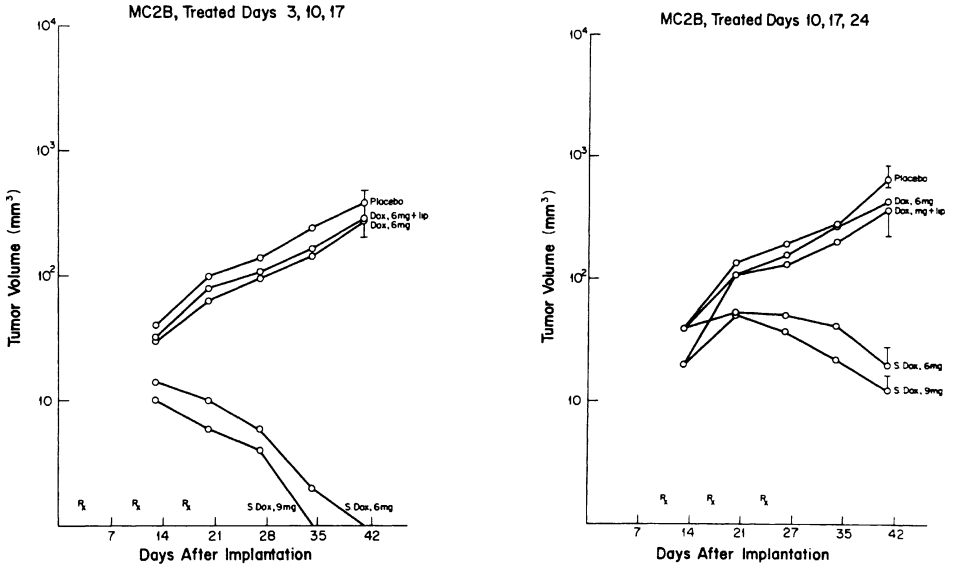

Fig. 9. Tumour size as a function of time for various treatments. Mammary carcinima MC2 were implanted into syngeneic female mice and animals were treated at days 3, 10, 17 (A) or 10, 17, 24 (B) after implantation with saline, free doxorubicin (Dox) and doxorubicin encapsulated in Stealth liposomes (S-Dox) at 2 concentrations. Each point is the average of 20 tumours. (From ref. [46], with permission)

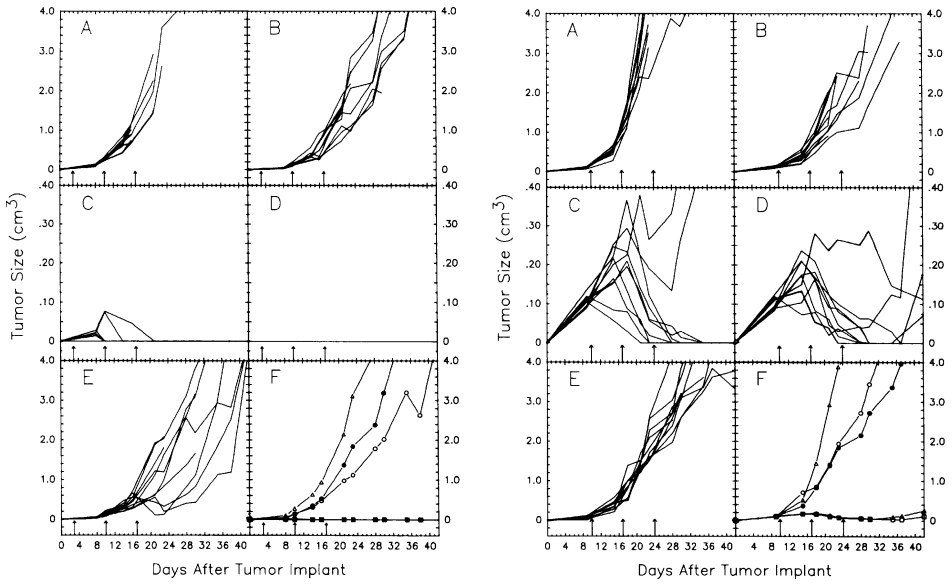

Fig. 8. Effect of various formulations of Epirubicin on the growth of C26 tumour. Ten mice in each group were injected with one million C26 cells and treatments began and continued on days 3, 10, 17 (left) or 10, 17, 24 (right) after inoculation. (A): saline control, (B): free Epi at 6 mg/ml, (C): Epirubicin in stealth liposomes (S-Epi) at 6 mg/ml and, (D): at 9 mg/ml (which resulted in no observable tumour at all). (E): mixture of free drug and empty liposomes. (F): the average values of ten animals in each experiment from A to E. From ref. [45], with permission. Practically identical results were also observed with Doxorubicin (see ref. [47]).

is provided by improved therapeutic efficacy of cytosine arabinose in the treatment of lymphoma [48] while the subcutaneous/intramuscular sustained release system was demonstrated by the action of polypeptide vasopressin [50]. Its action was prolonged up to a month as compared to few days for a free drug and a week for the peptide encapsulated in conventional liposomes. It is important to note that these concepts are becoming more and more important with the introduction of genetically engineered polypeptides and proteins which are hampered by the rapid blood clearance, degradation and/or deactivation in the body.

The altered biodistribution of stealth liposomes, in addition to the accumulation at the sites characterised with porous blood capillaries, such as in tumours, inflammations, and infections, may benefit several other applications. In the intact vasculature the distribution of stealth liposomes is shifted from the liver, spleen, and bone marrow towards skin. This opens the opportunity to deliver antivirals and dermatological agents to these sites. On the other hand, and while it was shown that the administration of empty stealth liposomes is well tolerated [5], it requires careful toxicology and tolerability studies when liposomes loaden with potent drugs are used.

3.3.3. Applications of Stealth liposomes in man

The encouraging results of Doxorubicin encapsulated in Stealth liposomes in preclinical studies were observed also in clinical trials in humans. Blood circulation times around 45 hours were found [51] and at reduced toxicity very good response in AIDS patients with Kaposi sarcoma was observed [52, 53]. Long circulation times significantly, i.e. 200-fold, increased the area under curve of drug concentration vs time and accumulation in various tumours was proportional to the liposome circulation times [51]. The drug remained encapsulated in circulating liposomes up to one week after injection while at tumour sites drug metabolites were found indicating that it had been released from liposomes. The concentration of the drug in tumours was 4–10 times greater than in control group which was treated with free drug [51]. The same selective targetting was observed also in patients with Kaposi sarcoma. Practically all patients showed considerable decrease in modularity of skin lesions while total flattening was observed in 25% of the cases [52]. The high efficacy was due to the approximately ten fold higher drug concentration in lesions as compared to the administration of free drug (table 3) [53].

In conclusion, it seems that stealth liposomes loaded with anticancer drugs will achieve substantial improvements in the treatments of various tumours. In addition, it

Table 3
Localization of Doxorubicin in Kaposi Sarcoma lesions after intravenous injection of free drug and drug encapsulated in Stealth liposomes (from ref. [53]).

dose [mg/m^2]	Doxorubicin concentration μg/g tissue		Selectivity Index
	free drug	in stealth liposome	
10	0.18	2.06	11.4
20	0.31	1.61	5.2
40	0.72	7.11	9.9

is hoped that they will be effective also in the treatments of inflammations, infections, and in antiviral therapy.

4. Liposomes in bioengineering

Modern genetic engineering and gene recombinant technology is based on the delivery of genetic material, i.e. fragments of DNA, into various cells and microorganisms in order to alter their genetic code and force them to produce particular proteins or polypeptides [54].

Nucleic acids used in gene transfer are large, with molecular weights up to several million Daltons, highly charged and hydrophilic and therefore not easy to transfer across cell membranes. Additionally to classical methods, such as direct injection, phosphate precipitation and others, liposomes were tried as transfection vectors. They can deliver the encapsulated or bound nucleic acid into cells predominantly in two ways: the classical approach is to encapsulate the genetic material into liposomes and liposomes act as an endocytosis enhancer while recently the phosphate or DEAE precipitation was simulated by liposomes. In these cases the nucleic acid forms a complex with several cationic liposomes and the size of the complex and its adsorption on the cell surface catalyses endocytosis or, possibly, fusion. The third, still unexplored way would be to use fusogenic liposomes or cause fusion upon adsorption of the liposome on the cell surface.

The classical approach used predominantly large unilamellar vesicles made from negatively charged phosphatidylserine in order to prevent interaction with DNA molecules which may contain up to several thousand negative charges [55]. In some cases transfection efficiencies were improved several hundred times and plant protoplast which are very difficult to transfect were successfully genetically altered [56]. In the mid 80's, however, electroporation showed better results and the interest for liposomes markedly diminished.

Recently, however, transfection was successfully performed using small unilamellar vesicles made from positively charged lipids. First studies used cationic lipid dioleoyl-propyl-trimethylammonium (DOTMA) [57]. Later studies showed better transfection efficiencies by using some of the commercially available cationic lipids [58]. Better transfection efficiencies at reduced toxicity were found by using liposomes containing positively charged cholesterol [59]. Many novel cationic lipids are being synthetised in order to improve transfection, especially in vivo.

These methods can be used also in gene therapy. The idea is to deliver the nondefective gene into the appropriate cells and hope that they will respond. For instance, patients with cystic fibrosis have a defective gene that encodes the code for a protein critical to a transfer of salts through the cell membrane in the lungs. In recent experiments, upon inhalation of the copies of human gene mixed with liposomes, 70% of the cells lining the lungs of mice incorporated the gene and began using it to make proteins in large amounts [60]. This promises elimination or drastic reduction of the symptoms of the disease if it can be repeated in humans.

5. Application of liposomes in cosmetics

The same properties of liposomes can be utilized also in the delivery of ingredients in cosmetics. In addition, liposomes as a carrier itself offers advantages because lipids are well hydrated and can reduce the dryness of the skin which is a primary cause for its ageing. Also, liposomes can act as a supply which acts to replenish lipids and, importantly, linolenic acid.

In general the rules for topical drug applications and delivery of other compounds are less stringent than the ones for parenteral administration and several hundred cosmetic products are commercially available since Capture (C. Dior) and Niosomes (L'Oréal) were introduced in 1987. They range from simple liposome pastes which are used as a replacement for creams, gels, and ointments for do-it-yourself cosmetical products to formulations containing various extracts, moisturizers, antibiotics, and to complex products containing recombinant proteins for wound or sunburn healing. Most of the products are anti-ageing skin creams. Unrinsable sunscreens, long lasting perfumes, hair conditioners, aftershaves and similar products, are also gaining large fractions of the market. Liposomal skin creams already share more than 10% of the over $10 billion market. Table 4 shows some of these products.

As in the case of topical delivery in medical applications, the workers in the field do not agree on the mechanism of action. While some claim enhanced permeability into the skin the others claim mostly that liposomes are a noninteractive, skin-non-irritating, water based matrix (without alcohols, detergents, oils and other non-natural solubilizers) for the active ingredients.

In addition to the natural lipids, either phospholipids or 'skin lipids', which contain mostly sphingolipids, ceramides, oleic acid, and cholesterol sulphate, liposomes made from synthetic lipids are also being used. They include mostly liposomes made from nonionic surfactant lipids, which can be chemically more stable. Some of these

Table 4

Some liposomal cosmetic formulations currently on the market. According to the manufacturers, liposomes may deliver moisture and a novel supply of lipid molecules to skin tissue in a superior fashion to other formulations. In addition they can entrap a variety of active molecules and can therefore be utilized for skin creams, anti-aging creams, after shave, lipstic, sun screen and make-up.

Product	Manufacturer	Liposomes and key ingredients
Capture	Cristian Dior	Liposomes in gel with ingredients
Efect du Soleil	L'Oréal	Tanning agents in liposomes
Niosomes	Lancome (L'Oréal)	Glyceropolyether with moisturizers
Nactosomes	Lancome (L'Oréal	Vitamins
Formule Liposome Gel	Payot (Ferdinand Muehlens)	Thymoxin, hyaluronic acid
Future Perfect Skin Gel	Estee Lauder	TMF, vitamins E, A palmitate, cerebroside ceramide, phospholipid
Symphatic 2000	Biopharm GmbH	Thymus extract, vitamin A palmitate
Natipide II	Nattermann PL	Liposomal gel for do-it-yourself cosmetics
Flawless finish	Elizabeth Arden	Liquid make-up
Inovita	Pharm/Apotheke	Thymus extract, hyaluronic acid, vitamin E
Eye Perfector	Avon	Soothing cream to reduce eye irritation
Aquasome LA	Nikko Chemical Co.	Liposomes with humectant

liposomes can be made very easily by mixing and homogenizing aqueous solutions with molten surfactants. These liposomes can be more stable than their natural analogues and can be easily produced in large quantities and are very inexpensive.

6. Application of liposomes in agro-food industry

The ability of liposomes to solubilize compounds with demanding solubility properties, sequester compounds from potentially harmful milieu, and release incorporated molecules in a sustained and predictable fashion can be used also in the food processing industry.

Lipid molecules, from fats to polar lipids, are one of the fundamental ingredients in almost any food. For instance, lecithin and some other polar lipids are routinely extracted from nutrients, such as egg yolks or soya beans. Traditionally polar lipids were used to stabilize water-in-oil and oil-in-water emulsions and creams, or to improve dispersal of various instant powders in water. With the advent of microencapsulation technology, however, liposomes have become an attractive system because they are composed entirely from food acceptable compounds.

The sustained release system concept can be used in various fermentation processes in which the encapsulated enzymes can greatly shorten fermentation times and improve the quality of the product. This is due to improved spatial and temporal release of the ingredient(s) as well as to their protection in particular phases of the process against chemical degradation. A classical example is cheesemaking. The first serious attempts to decrease the fermentation time using cell-wall-free bacterial extracts were encouraging enough to stimulate efforts to improve enzyme presentation. After preliminary studies in which liposome systems were optimized the cheese ripening times can be shortened by 30–50% [61–63]. This means a substantial economic profit knowing that ripening times of some cheeses, such as Cheddar, say, are about one year during which they require well controlled conditions. In addition, due to the better dispersal of the enzymes the texture of cheeses was even and bitterness and inconsistent flavour due to the proteolysis of enzymes in the early phase of fermentation was much improved [61, 62].

In addition to improved fermentation, liposomes are being tried in the preservation of cheeses. Addition of nitrates to cheese milk to suppress the growth of spore-forming bacteria is now being questioned due to health concerns and natural alternatives are under study. Lysozyme is effective but quickly inactivated due to binding to cassein. Liposome encapsulation can both preserve potency and increase effectivenes because liposomes become localized in the water spaces between the casein matrix and fat globules of curd and cheese. This also happens to be where most of the spoilage organisms are located [63]. These applications of enhancing natural preservatives, including antioxidants such as vitamins E and C, will undoubtedly become very important due to recent dietary trends which tend to reduce the addition of artificial preservatives and everlarger portion of unsaturated fats in the diet.

In other areas of the agro-food industry, liposomes encapsulated biocides have shown superior action due to prolonged presence of the fungicides, herbicides or

pesticides at reduced damage to other life forms [64]. Liposome surface can be made sticky so that they remain on the leafs for longer times and they do not wash into the ground. In these applications inexpensive liposomes produced from synthetic lipids are used.

The same liposomes are being tried in shellfish farms. These animals are susceptible for many parasitic infections. They are filter feeders and they pump large amounts of water through their body. This seems to offset large dilutions of liposomes in the pool and the drug molecules as well as some essential nutrients needed in ppm to ppb quantities can be delivered.

7. Other applications of liposomes

The potential of making large quantities of inexpensive and stable liposomes may put forward several other applications. They range from water based paints, single tube two component glues or resins, self healing paints, and similar products. They are based mostly on the dissolving potential of liposomes and their ability to protect the encapsulated substance until an external stimulus such as the presence of oxygen, light, or change in hydration. Figure 10 presents schematic view for the light induced trigger for the release of liposome encapsulated substances. Critical evaluation of these applications is difficult, however, because the information is mostly concentrated in progress reports, business analyses, or prospectuses of various producers.

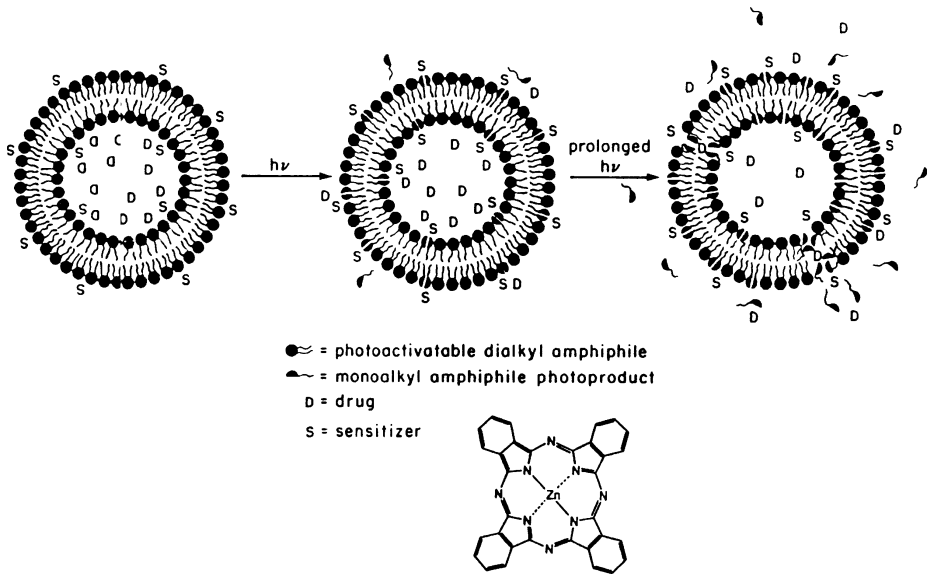

Fig. 10. Schematic presentation of light induced release of encapsulated material. Photocleavable lipid, such as naturally occurring plasmalogens, is cleaved by radicals generated in photosensitiser (zinc porphyrin, S, see chemical formula) under the adsorption of light quanta. (Courtesy D. Thompson).

In ecology, liposomes offer improvements in bioreclamation and various monitoring and analytical-diagnostic applications. For instance, it was shown that in an oil spill, the addition of various bacteria with possible nutrients encapsulated in liposomes improves the degradation rates of carbohydrates, which are otherwise very slow. Due to the surfactant action liposomes also improve the coagulation and sinking of oil spread on the water surface or its cleaning up with floating booms [65]. The Environmental Protection Agency is testing liposomes' ability to deliver nutrients to oil spills to speed up the degradation [66].

Liposomes containing membrane anchored chelators can be used to clean toxic or radioactive metals from solutions. For instance, water contaminated in a nuclear reactor can be purified by addition of such liposomes which could be easily precipitated after binding of the toxic ions.

In addition to the above mentioned liposome applications there are many others which were not mentioned. An interested reader may find more information in ref. [5] and references therein.

In conclusion, it seems that liposomes established themselves as an important model system in several different basic sciences and as a viable alternative in several applications. Despite over a 1 billion dollar cosmetic liposome industry, I dare to say that the real future of liposomes is in anticancer and possibly other chemotherapies, gene therapy as well as some other medical applications such as artificial blood.

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