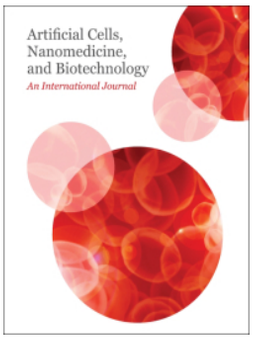

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Application of liposomes in medicine and drug delivery

Hadis Daraee[2], Ali Etemadi[2], Mohammad Kouhi[5], Samira Alimirzalu[4] & Abolfazl Akbarzadeh[1,3]

[1]*Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran,* [2]*Department of Medical Biotechnology, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran,* [3]*Department of Medical Nanotechnology, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran,* [4]*Laboratory of Polymer, Faculty of Chemistry, Payame Noor University, Tabriz, Iran, and* [5]*Department of Physics, College of Science, Tabriz Branch, Islamic Azad University, Tabriz, Iran*

Abstract

Liposomes provide an established basis for the sustainable development of different commercial products for treatment of medical diseases by the smart delivery of drugs. The industrial applications include the use of 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 matrices for various ingredients and penetration enhancers in cosmetics.

In this review, we summarize the main applications and liposome-based commercial products that are currently used in the medical field.

Keywords: commercial products, liposome, medical field, smart delivery

Introduction

The name liposome is derived from two Greek words: 'Lipos' meaning fat and 'Soma' meaning body. A liposome can be formed in a variety of sizes with unilamellar or multilamellar construction, and its name relates to its structural building blocks – the phospholipids, and not to its size. Liposomes were first described by the British hematologist Dr. Alec D Bangham in 1964 (Published 1964), at the Babraham Institute in Cambridge. They were discovered when Bangham and R. W. Horne were testing the institute's new electron microscope by adding negative stain to dry phospholipids (Kumar et al. 2010, Bangham 1993). A liposome is a tiny bubble (vesicle), made out of the same material as a cell membrane. Liposomes can be filled with drugs, and used to deliver drugs for cancer and other diseases. Membranes are usually made of phospholipids, which are molecules that have a head group and a tail group. The head is attracted to water, and the tail, which is made of a long hydrocarbon chain, is repelled by water. In nature, phospholipids are found in stable membranes composed of two layers (a bilayer). In the presence of water, the heads are attracted to water and line up to form a surface facing the water. The tails are repelled by water, and line up to form a surface away from the water. In a cell, one layer of heads faces the outside of the cell, attracted to the water in the environment. Another layer of heads faces the inside of the cell, attracted by the water inside the cell. 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 (Papahadjopoulos 1978). The bilayer structures are called liposomes and the monolayer structures are called micelles. Liposomes are used for drug delivery due to their unique properties. In fact, they can contain a wide variety of hydrophilic and hydrophobic diagnostic or therapeutic agents, providing a larger drug payload per particle and protecting the encapsulated agents from metabolic processes. One of the major drawbacks of conventional liposomes has been their rapid clearance from the blood, due to the adsorption of plasma proteins in their "second generation form", which are called sterically stabilized liposomes. They have been proved to have favorable *in vivo* pharmacokinetic properties due to a surface coating of a hydrophilic carbohydrate or polymer, usually a lipid derivative of polyethylene glycol (PEG), making them attractive vehicles for anticancer drug delivery. Also, the composition of the lipid bilayer can be modulated to obtain other desirable properties, including a prolonged circulatory half- life (stealth liposomes), the ability to form a complex with nucleic acids to mediate gene delivery or genetic regulation, and the capacity to deliver encapsulated contents to the cytosol through the endosomal/ lysosomal pathway (Bangham 1993). Liposomes exhibit several properties which may be useful in various applications. Several practical applications, most notably in drug delivery, emerged in the 1970s (Bangham 1993). Liposomes were first described in 1965 as a model of cellular membranes and quickly were applied to the delivery of substances to

Correspondence: Abolfazl Akbarzadeh and Samira Alimirzalu, Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. Tel/Fax: +984113341933. E-mail: akbarzadehab@tbzmed.ac.ir; Mohammad Kouhi, Department of Physics, College of Science, Tabriz Branch, Islamic Azad University, Tabriz, Iran. E-mail:kouhi@iaut.ac.ir

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cells. Liposomes entrap DNA by one of two mechanisms which have resulted in their classification as either cationic liposomes or pH-sensitive liposomes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex (Bangham 1993).

Today, they are a very useful model, reagent and tool in various scientific disciplines, including mathematics and theoretical physics, biophysics, chemistry, colloid science, biochemistry, and biology. Another interesting property of both conventional and stealth liposomes is their natural ability to increase, by 'passive' targeting, the localization of anticancer drugs to solid tumors. This differential accumulation of liposomal drugs in tumor tissues relative to normal ones is the basis for the increased tumor specificity of liposomal drugs, relative to free drugs. Liposomes have been considered to be excellent models of cell membranes and have been employed as potent drug carriers in which various materials such as drugs, toxins, proteins (peptides), enzymes, antigens (antibodies), and nucleotides are encapsulated (Bangham 1993). In order to achieve direct, effective and selective delivery of the liposome itself or liposome-added materials into a target cell, a deeper understanding of the mechanisms for interaction between the intact cell and the liposome is required. For this purpose, various methodologies, such as chemical modification of liposomal membranes by a cell-specific device (antibodies, saccharides, or other cell specific ligands), fusogens, or boundary lipids, have been proposed. '*Targeting'* must be classified into two categories based on the difference in mechanism *in vivo* after administration. One is *'passive targeting'* (Machy and Leserman 1987, Poznansky and Juliano 1984), with a bulk recognition mechanism, in which the targeting (recognition of a specific cell or tissue) is attained by altering the bulk structural characteristics of the carrier such as its hydrophobicity or hydrophilicity, the charge density, the fluidity or softness, and/or the size of the carrier. Another mechanism is *'active targeting'* which is attained by a molecular recognition mechanism. In this mechanism, the targeting is attained by recognition at the molecular level, through direct and specific interaction between a specific recognition site on the liposomal surface and a receptor on the cytoplasmic membrane of the target cell. When liposomes are administered to the body, primary tissue distribution depends mostly on a passive targeting mechanism. However, when the liposome and target cell approach each other with a very short distance between them, i.e., within a van der Waals radius, an active targeting mechanism becomes dominant. Therefore, active targeting is more eminent and promising than passive targeting.

At the first stage of liposome-cell interaction, liposomes nonspecifically or specifically adhere to the cell surface. Nonspecific adsorption is simply an electrostatic and/or a hydrophobic interaction between the two, while specific adsorption is a receptor-ligand or an antigen-antibody interaction between the two surfaces of the cell and the liposome. Following such binding (irrespective of whether it is specific or nonspecific), the liposome is internalized into the cell by the mechanism of endocytosis (or phagocytosis). This is followed by the enzymatic digestion of the liposome in the intracellular compartment (endosome, phagosome or acidosome), accompanied by the intracellular distribution of liposomal components to cytosol.

Structural components of liposome

Liposomes are globular lipid bilayers of 50–1000 nm in diameter that serve as convenient delivery vehicles for biologically active compounds. Topical application of liposomes has large possibilities in dermatology and in the delivery of anticancer agents in order to reduce the toxic effects of the drugs when given alone, or to increase the circulation time and effectiveness of the drugs. Liposomes might be used to target precise cells by attaching amino acid fragments such as antibodies or proteins or appropriate fragments that target specific receptor sites. DNA vaccination and improved efficiency of gene therapy are just a few of the upcoming applications of liposomes. Liposomes are especially effective in treating diseases that affect the phagocytes of the immune system because they tend to accumulate in the phagocytes, which know them as strange attackers. There are a number of structural and nonstructural components of liposomes. The main structural parts of liposomes are:

Phospholipids

Phospholipids are the major structural component of biological coverings, and two sorts of phospholipids exist - PHOSPHODIGLYCERIDES AND SPHINGOLIPIDS. The most common phospholipid is the phosphatidylcholine (PC) molecule. Particles of phosphatidylcholine are not soluble in water and in aqueous media, they align themselves closely in planar bilayer sheets in order to minimize the unfavorable action between the bulk aqueous phase and the long hydrocarbon fatty series. The glycerols, including phospholipids, are the most commonly used component of liposome formulation and represent greater than 50% of the weight of lipid in biological membranes. These are derivatives of phosphatidic acid. Examples of phospholipids are:

1. Phosphatidyl choline (Lecithin) – PC
2. Phosphatidyl ethanolamine (cephalin) – PE
3. Phosphatidyl serine (PS)
4. Phosphatidyl inositol (PI)
5. Phosphatidyl Glycerol (PG)

Cholesterol

Cholesterol does not form a bilayer construction by itself, but is able to be included into phospholipid membranes in very high concentrations of up to 1:1 or even 2:1 molar ratio of cholesterol to phosphatidylcholine. Cholesterol positions itself in the membrane with its hydroxyl group oriented towards the aqueous surface and the aliphatic chain aligned parallel to the acyl chains in the center of the bilayer. The high solubility of cholesterol in the phospholipid liposome has been attributed to both hydrophobic and definite head group interaction, but there is no clear indication for the arrangement of cholesterol in the bilayer.

Liposomes can be classified in terms of work and mechanism of intracellular delivery into five types as:

1. Conventional liposomes
2. pH sensitive liposomes
3. Cationic liposomes
4. Immune liposomes
5. Long circulating liposomes

Conventional liposomes

The conventional liposome-based mechanism is the first creation of liposomes to be used in *pharmaceutical* applications (Immordino et al. 2006). Conventional liposome formulations are mainly comprised of natural phospholipids or lipids such as 1, 2-distearoryl-sn-glycero- 3-phosphatidyl choline (DSPC), sphingomyelin, egg phosphatidylcholine and monosialoganglioside.

pH-sensitive liposomes

Liposomes of various compositions can extensively bind to cell shells. For gene transport, it was recognized that dioleoylphosphatidylethanolamine (DOPE) is by far the most efficient lipid for in vitro gene transfection for pH-sensitive liposomes or as a lipid helper in cationic liposomes (Wang and Huang 1987). It has been assumed that the function of phosphatidylethanolamine (PE) is that of a membrane synthesis advocate, because in reality this lipid undergoes changes ahead of acidification (Litzinger and Huang 1992). After fastening to the cell surface, liposomes are internalized into endosomes where they encounter a more acidic pH than in the external intermediate. Usually, endosomes normally have an inner pH of 6.50 (Mellman et al. 1986). Conventional, pH-insensitive liposomes and their content are delivered to lysosomes and stained. The last condition for plasmid liposomes after cell penetration is to avoid accumulation in particular cell compartments such as lysosomes. In order to avert this deprivation, pH-sensitive liposomes have been proposed.

pH-sensitive liposomes were designed based on the concept of viruses that fuse with the endosomal membrane, delivering their genetic material to the cytosol before reaching the lysosomes (Chu et al. 1990).

Cationic liposomes

Generally, this is a simple procedure requiring the mixing of cationic lipids with the DNA and adding them to the cells. This results in the configuration of collectives composed of DNA and cationic lipids. The cationic lipid DOTMA was first synthesized and described by Feigner et al. (1987). It is presumed that complex formation simply results from ionic interactions between the positively charged head group of DOTMA and the negatively charged phosphate groups of DNA. In an effort to determine the physicochemical properties of the composite, cationic lipids connected with DOPE and with various amounts of three different cationic surfactants have been examined by cryo transmission electron microscopy (TEM). The results of the cryo TEM analysis suggest that an excess of lipids in terms of charge leads to the entrapment of the DNA molecules between the lamellae in clusters of aggregated multilamellar structures. The option of surfactant use does not seem to influence the morphology of the DNA-lipid-complexes (Gustafsson et al. 1995).

Immune liposomes

Another possible function of liposomes in medicine is the potentiation of the immune response by acting as an immunological adjuvant. The reconstitution of antigens into liposomal membranes or their incorporation into the interior water core of the liposome would cause the enhancement of immune response such as macrophage activation, antibody production (Alving 1991), effective induction of cytotoxic cell (Raphael and Tom 1984) and subsequent antitumor activity (LeGrue 1984). The advantages of liposomes as immunological adjuvants are their biodegradability, low toxicity, low antigenicity and their potential to target specific cells *in vivo*. Indeed, multiple data indicate that liposomes are excellent adjuvants for the enhancement of immunogenicity to a given antigen involving glycolipids (gangliosides), proteins, antigens to pathogenic viruses etc. (Engelhard et al. 1984, Gregoriadis et al. 1987, Steele et al. 1984). The optimistic goals of antibody-sensitized liposomes (immune liposomes as 'guided missiles'), which has often given 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. Considering a number of additional potential targeting applications, such as injection in different body cavities, immune liposomes present a viable option in immunoassays and diagnostic tests.

Long circulating liposomes

The experimental results show that liposomal disposition is capable of being changed, but predominantly inside the mononuclear phagocytic system including the intrahepatic uptake itself. Blood circulation times were extended, but the first considerable improvements were achieved by the incorporation of ganglioside GM1 or phosphatidylinositol at 5–10 mol% into the bilayer (Allen and Chonn 1987, Gabizon and Papahadjopoulos 1988). The best results were obtained by substituting these two lipids with synthetic polymer enclosing lipids. The maximum circulation times were achieved when using polyethylene glycol covalently bound to the phospholipid. It seems that the median molecular mass, between 1500 to 5000 Da is the optimal (Lasic 1994). It was suggested 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, complementary proteins, fibronectin and similar molecules which spot strange particles for following macrophage uptake, since the origin of steric stabilization is schematically well documented although not well understood. It was recently exposed that the Alexander-de-Gennes model of polymers at interfaces (De Gennes 1987) can qualitatively explain the stability of liposomes in biological systems (Lasic 1994). The different types of liposomes are abbreviated below.

There are three types of liposomes:

1. MLV (Multilamellar vesicles)
2. SUV (Small unilamellar vesicles)
3. LUV (Large unilamellar vesicles)

Five groups of phospholipids that can be used for the liposomal preparation can be discerned:

1. Phospholipids from natural sources
2. Modified natural phospholipids
3. Semisynthetic phospholipids
4. Fully synthetic phospholipids
5. Phospholipids with non-natural head groups

Advantages of liposomes

Liposomes offer several advantages in delivering genes to cells.

1. Liposomes can complex both with negatively and positively charged molecules.
2. Liposomes offer a degree of protection to the DNA from degradative processes.
3. Liposomes can carry large pieces of DNA, possibly as big as a chromosome.
4. Liposomes can be targeted to specific cells or tissues.

Disadvantages of liposomes

1. Production cost is high.
2. Leakage and fusion of encapsulated drug / molecules.
3. Sometimes phospholipid undergoes oxidation and hydrolysis-like reactions.
4. Short half-life.
5. Low solubility.
6. Fewer stables.

Properties of liposomes

The system is composed of structures of bimolecular sheets intercalated by aqueous space.

- They are permeable to water.
- They are osmotically sensitive.
- Positively charged membranes are impermeable to cations and negatively charged ones are relatively permeable to anions.

Applications of liposomes in medicine

Liposome encapsulation can alter the spatial and temporal distribution of the encapsulated drug molecules in the body, which may significantly reduce unwanted toxic side effects and increase the efficacy of the treatment. Applications of liposomes in pharmacology and medicine can be divided into therapeutic and diagnostic applications of liposomes containing drugs or a variety of elements, and their utilization as a form, tool, or reagent in the fundamental studies of cell interfaces, recognition procedures, and of the mechanism of action of certain materials. The benefits and limitations of liposomal 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 their 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 rarer. The fourth possible interaction is the exchange of bilayer ingredients, such as cholesterol and lipid: membrane-bound molecules with components of cell membranes. These interactions also determine the fate of liposomes *in vivo*. The body protects itself with a complex defense system. Upon entering the body, larger objects cause thrombus formation and their surface is eventually passivated by coating with bio macromolecules, while minor particles, as well as 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 non-recognizable surfaces and has also, on the other hand, narrowed the spectrum of applications of micro particulate drug carriers only to targeting of the very same cells of the immune system. Although they are composed of natural substances, liposomes are no exception. They are quickly removed from the circulation by the macrophages which are located mainly in the spleen, liver and bone marrow.

Modes of liposome action

The preceding discussion shows that liposomes exhibit different biodistribution and pharmacokinetics when compared to free drug particles. In some cases, this can be used to improve the therapeutic effectiveness of the encapsulated drug molecules. The benefits of drug-loaded liposomes, which can be applied as (colloidal) solution, aerosol, or in (semi) solid structures, such as creams and gels, can be classified into seven categories:

(i) Enhanced solubility of amphiphilic and lipophilic drugs:
Furthermore, in some cases, hydrophilic drugs, such as the anticancer agent Doxorubicin or Acyclovir, can be encapsulated in the liposomal interior at concentrations several fold above their aqueous solubility. This is possible because of the precipitation of the drug or gel configuration inside the liposome with appropriate substances encapsulated (Lasic et al. 1992).

(ii) Inactive objective to the cells of the immune system:
Instances are antimonials, porphyrins, Amphotericin B, and also vaccines, immune modulators or (immune) suppressors.

(iii) Maintained free system of systemically or locally administered liposomes.
Cases in points are doxorubicin, cortisones cytosine, arabinose, 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 decreases cardio-, nephro-, and neuro-toxicity. Characteristic exam-

ples are reduced nephrotoxicity of Amphotericin B, and reduced cardiotoxicity of Doxorubicin liposomes.

(v) Precise targeting of Location:
In certain cases, liposomes with surface attached ligands can bind to target cells, or can be delivered into the target tissue by local anatomical conditions such as leaky and badly formed blood vessels, their capillaries and basal lamina. Instances include anti-cancer, anti-disease and anti-provocative drugs.

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

(vii) Improved penetration into tissues, particularly in the case of dermally functional liposomal dosage forms:
Usually, liposome encapsulation is done carefully when the 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.

Liposomes in infections and parasitic diseases

While usually liposomes are digested by phagocytic cells in the body after intravenous administration, they are ideal vehicles for the targeting 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 cells of the mononuclear phagocytic system. Liposomes accumulate in the very same cell population which is infected and therefore offer an ideal drug delivery vehicle (New et al. 1978). These formulations mostly use the ionophore amphotericin B and are relocated from very thriving and prolific areas of liposome formulations in antifungal therapy. These toxicities are normally correlated with the size of the drug molecule or its complex, and liposome encapsulation obviously prevents the accumulation of drug in these organs and drastically reduces toxicity (Lopez-Berestein et al. 1985). Similar approaches can be implemented in antibacterial and antiviral therapy (Svenson et al. 1988). The preparation of antibiotic-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 application or pulmonary (by inhalation) administration are also being considered.

The automatic targeting of liposomes to macrophages can be exploited in several other ways, including by macrophage activation and injection. Some usual toxins persuade tough 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. Normally, this is done by administration of alum or killed bacteria, and liposomes evidently offer an elegant alternative (Gregoriadis 1990). Indeed, liposomes are being used in animal vaccination already since 1988, while human vaccinations against malaria are now in clinical trials (Alving 1991).

Liposomes in anticancer therapy

Very early studies mostly showed decreased toxicity of the liposome-encapsulated drug, but in most of the cases, the drug molecules were not bioavailable, resulting not only in reduced toxicity but also in severely compromised efficacy. Unfortunately, this was also found to be true for primary and secondary liver tumors. Many different liposome formulations of various anticancer agents were shown to be less toxic than the free drug (Weiner et al. 1989). This includes both short term and chronic toxicities because liposome encapsulation reduces the distribution of the drug molecules towards those tissues. Applications in humans generally showed reduced toxicity and better tolerability of administration but with not too much hope of usefulness. Numerous different formulations are in diverse stages of clinical studies and show mixed results (Bakker-Woudenberg et al. 1994).

Other applications

Tiny liposomes composed of lipids with long and saturated hydrocarbon chains in mixtures with cholesterol were shown to build up at the sites of inflammations. Such liposomes were used for analytical studies (Williams et al. 1986). Liposomes can also be used to deliver drugs targeted at the lung (McCalden 1990). The usual nature of liposomes to collect in the liver and spleen was used in the treatment of neonatal jaundice in an animal model (Hamori et al. 1993). Liposomes can also be applied as a thick cream, gel, or tincture. Oral applications of liposomes are at present rather limited due to the very liposomicidal surroundings in the stomach and duodenum, and in general, the administration of free or liposome-encapsulated drug usually exhibits no differences.

Liposomes with altered surface properties

New strategies, including selective targeting of cancer and other diseased cells, however, rely on liposomes with changed surface properties which, however, suffer from their quick clearance from the blood by the immune system. Their inability to extravasate, i.e., leave the blood stream, is useful in the use of some liposomes as localized drug reservoirs in some topical applications, or in pulmonary applications of liposomal aerosols, but for the majority of other applications the fast clearance represents a major obstacle.

Sterically stabilized liposomes

The results showed that liposome disposition can be changed, but mostly inside the mononuclear phagocytic system as well as the intrahepatic uptake itself. Blood circulation times were extended but the primary and considerable developments were attained by the incorporation of ganglioside GM1 or phosphatidylinositol at 5–10 mol% into the bilayer (Allen and Chonn 1987). 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 the mid-range molecular mass, from 1500 to 5000 Da, is the optimum (Lasic 1994). It was suggested 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, fibronectin and similar molecules, which mark foreign particles for subsequent macrophage uptake. The origin of steric stabilization is well documented although not well understood. Recently, it was shown that the Alexander-de-Gennes model of polymers at interfaces (De Gennes 1987) can qualitatively explain the stability of liposomes in biological systems (Lasic 1994).

Medical applications of stealth liposomes

The former application requires larger liposomes (0.2μm) while the latter one is due to the ability of small vesicles to leave the blood circulation. Sterically stabilized liposomes may also act as a sustained drug release system either as a long circulating micro-reservoir or localized drug depot. The first example is provided by improved therapeutic efficacy of cytosine arabinose in the treatment of lymphoma (Allen et al. 1992), while the subcutaneous/intramuscular sustained release system was demonstrated by the action of the polypeptide vasopressin (Woodle et al. 1992). 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 significant to note that these ideas are becoming more and more important with the introduction of genetically engineered polypeptides and proteins which are hampered by the rapid blood acceptance, degradation and/or deactivation in the body. The altered biodistribution of stealth liposomes, in addition to the accumulation at the sites characterized with porous blood capillaries, such as in tumors, inflammations, and infections, might profit numerous other applications. In the intact vasculature, the distribution of stealth liposomes is shifted from the liver, spleen and bone marrow, to the skin. This eliminates the chance to transport antivirals and dermatological agents to these sites. On the other hand, while it was shown that the administration of empty stealth liposomes is well tolerated (Lasic 1993), it requires careful toxicology and tolerability studies when liposomes loaded with potent drugs are used.

Applications of stealth liposomes in humans

The encouraging results of Doxorubicin encapsulated in stealth liposomes in preclinical studies were also observed in clinical trials in humans. The drug remained encapsulated in circulating liposomes for up to one week after injection, while drug metabolites were found at tumor sites, indicating that they had been released by the liposomes. The absorption of the drug in tumors was 4–10 times superior to that in the control group which was treated with free drug. The high efficacy was due to the approximately 10-fold higher drug concentration in lesions as compared to the administration of free drug. In summary, it seems that stealth liposomes loaded with anticancer drugs will achieve substantial improvements in the treatments of a variety of tumors. In addition, we trust that they will be also successful in the treatments of inflammations, infections, and in antiviral therapy.

Vaccination, gene therapy and diagnostics

As a result of their special properties, liposomes are being studied for the treatment of other diseases and for vaccination applications. Examples are liposomal antibiotics, antivirals, prostaglandins, steroid and non-steroid anti-inflammatory drugs, insulin and many others.

Recombinant-DNA technology and studies of gene function and gene therapy all depend on the delivery of nucleic acids into cells *in vitro* and *in vivo*. Although *in vitro* techniques can rely on a number of physical and chemical methods, *in vivo* delivery is more demanding. DNA-carrier systems include several colloidal particles: cationic liposomes have been shown to form complexes (negatively charged) DNA, and such complexes were able to transfect cells *in vitro*, resulting in the expression of the protein encoded in the DNA plasmid in the target cells. Obviously, for gene therapy (the treatment of diseases at the molecular level by switching genes on or off), *in vivo* delivery is preferred. It was discovered that cationic lipid-based DNA complexes can transfect certain cells *in vivo* upon localized (mostly lung-epithelial cells upon intratracheal instillation) or systemic (through endothelial cells in the lung) administration. The liposomes act as an adjuvant and carrier of co-adjuvants (viral glycolipids and glycoprotein) and potentiate an immune response to the vaccine antigen. A significant barrier to the development of liposomes for oral or mucosal delivery of macromolecules has been the susceptibility of liposomes formed from commonly available lipids to enzymatic degradation and to the low pH in the stomach and the bile-salt dissolution in the intestine. Common liposomes thus fail to protect macromolecules against enzymatic degradation in the gastrointestinal tract. Mechanically and sterically stabilized liposomes can survive these conditions, but are consequently too stable in the intestine to deliver the encapsulated drug via the normal absorption process. Alternatively, liposomes (or their precursors) can be delivered directly into the intestine in various capsules. It is possible that the mere presence of lipids increases absorption in the gut, as it is well known that various micelles, mixed micelles and vesicles help to transport hydrophobic molecules across the epithelial membranes in the intestine. Furthermore, lipids may inactivate some enzymes that would take in or pump out drugs.

Cancer therapy

Cytotoxic drugs can distribute non-specifically all over the body, and cause the death of normal as well as malignant cells, thereby giving rise to a variety of side effects. The entrapment of these drugs into liposomes resulted in increased flow life span, improved deposition in the infected tissues and defense from the drug metabolic deprivation, altered tissue release of the drug, with its improved uptake in organs rich in mononuclear phagocytic cells (liver, spleen and bone marrow) and decreased uptake in the kidney, myocardium and brain. To target tumors, liposomes must be competent in leaving the blood and accessing the tumor. Liposomal entrapment of these drugs showed reduced cardiotoxicity,

dermal toxicity and better survival of experimental animals compared to the controls receiving free drugs. Furthermore, a significant increase of the liposomal drug in tumor tissue was observed when TNF-alpha was coadministered. The antitumor effects of liposomes against solid tumors were superior to those of TNF solution. The ability to selectively target liposomal anticancer drugs via specific ligands against antigens expressed on malignant cells could improve the therapeutic effectiveness of liposomal preparations as well as reduce the adverse side effects associated with chemotherapy. A new type of long-circulating immune liposomes, *i.e.,* polyethylene glycol (PEG) immune liposome-attached antibodies at the distal end of PEG series, the so-called accessory form of immune liposomes, showed much superior target ability than the usual immune liposomes on collectively targeting sites of lung endothelial cells and solid tumor tissue. The pendant type immune liposomes can flee from the gaps between adjacent endothelial cells and openings at the vessel termini during tumor angiogenesis by passive convective transport, much higher than ligand-directed targeting. Active targeting of tumor tissue with the pendant type immune liposomes is particularly important for many highly toxic anticancer drugs in cancer chemotherapy. The ultimate goal of the pendant type immune liposome is the incorporation of a fusogenic molecule, which would persuade liposomal combinations subsequent to their binding to the target cells or their internalization by endocytosis. Photodynamic therapy (PDT) as a cancer treatment is notable for its relatively low side effects in comparison with those of chemotherapy and radiotherapy. These results suggest that a long-circulating liposomal formulation of photosensitive agents is useful for PDT. Enhanced *in vitro* cytotoxic activity against leukemic cells was found for combinations of the ether lipids, octadecylphosphocholine and ET-18-OCH_3, with both teniposide and paclitaxel. The benefit of the liposomal formulation form for ether lipids was supported by the fact that their hemolytic activity was much reduced when they were incorporated into liposomes.

Antimicrobial therapy

Treatment of mycobacterial infections differs from that of other bacterial diseases because of several properties possessed by the mycobacteria and the host. In a simple *in vitro* culture, liposomal neomycin and penicillin were found to be active beside bacteria, while the liposome trap clearly restricted the antimicrobial motion of chloramphenicol. Liposome encapsulation changes the tissue distribution of gentamicin when given by the intravenous route to rabbits. The incorporation of rifabutin in liposomes resulted in a significant enhancement of activity against *Mycobacterium avium* infection compared to free rifabutin. Furthermore, the antitubercular action of rifampin was significantly increased when encapsulated in egg phosphatidylcholine liposomes.

Liposome for respiratory drug delivery system

Liposomes are widely used in several types of respiratory disorders. Liposomal aerosol has several advantages over ordinary aerosol, as follows (Fendler and Romero 1977):

1. Sustained release
2. Prevention of local irritation
3. Reduced toxicity
4. Improved stability in the large aqueous core

Several injectable liposome-based products are now in the market including AmBisome, fungisome and mycoses. To be efficient, liposomal drug delivery structure for the lung is dependent on the following parameters:

1. Lipid composition
2. Size
3. Charge
4. Drug and Lipid ratio
5. Method of delivery

Liposomes for brain-targeted drug delivery

Yagi et al. have developed a liposome that can pass through the blood-brain barrier (BBB) and reach human glioma. They employed sulfatide and a monoclonal antibody as the sensory device in order to increase the target ability of the liposome (Lasic and Papahadjopoulos 1998). Egg PC liposomes coated with CHP also were significantly accumulated in brain tumors of the rat (Ochi et al. 1990). CHP-coated liposomes labeled with [14C]-DPPC were injected by the carotid route into Fisher-344 rats implanted with 9L-gliosarcoma. Each tissue (tumor, ipsilateral and contralateral brain, liver, spleen, kidney and blood) was collected 30 min after injection of the liposome. Tissue distribution of the liposome with or without CHP-coating was investigated. Distribution of the CHP-coated liposome increased by 4.5 times in the tumor and by 2.1 times in the ipsilateral brain, and decreased by 4 times in the spleen compared with that of control liposome. The survival of 9L-glioma-implanted rats was investigated by the use of liposomes in which an antitumor drug (CDDP, c/s-platinum diamino dichloride) was loaded. In all runs, 7.5/~g/kg of CDDP was administered via the carotid route into rats, 5 days after tumor inoculation. Average survival was 35.3 days for the group treated with the CHP-coated liposomes. This was statistically significant ($P < 0.05$) compared with the case of the untreated group (20.3 days) (Ochi et al. 1990).

Procedures for the formation of targeted long-circulating liposomes

Targeted liposomes must survive in the systemic circulation long enough to reach and bind to their target, and a critical step to achieving this was the development of liposomes which remained long-circulating following the coupling of ligands at the liposomal surface. Strategies to increase the circulation time of liposomes, like a reduction in liposomal size (Juliano and Stamp 1975) or the inclusion of cholesterol and/or high phase transition lipids (Senior 1986) provided a modest decrease in the clearance rate of liposomes. More success came from the tactic of including a hydrophilic molecule at the liposomal surface, e.g., GMi, and phosphatidylinositol (Allen 1994) or lipid derivatives of polymers like PEG (Klibanov et al. 1990), poly(acrylamide), poly(vinyl

pyrrolidone) (Torchilin et al. 1994) or poly(methyl or ethyl oxazoline). While not all of the above polymers have been investigated for their ability to prolong the circulation time of antibody- or ligand-containing liposomes, it has been shown that both GMi and PEG have been successful in this regard.

Coupling strategies

A ligand is coupled, often through a spacer molecule, to a hydrophobic anchor via cross-linking molecules. The hydrophobic anchor is required for stable insertion of the conjugate into the lipid bilayer of a liposome. The hydrophobic anchor must be sufficiently strong to bind a ligand (e.g., an antibody) and a spacer molecule (e.g., a large hydrophilic polymer like PEG) securely to the liposomal surface. The choice of anchor depends on the general coupling strategies available, i.e., which reactive groups are presented on the anchor and the ligand, the availability of heterofunctional cross linking molecules, and the type of chemical bond desired (e.g., stable versus unstable, covalent versus non-covalent). Generally, the anchor of choice has been phosphatidylethanolamine (PE) because of the reactive amine in its head group and the availability of various acyl chain lengths of different degrees of unsaturation, e.g., dimyristoyl PE (DMPE), dipalmitoyl PE (DPPE), distearoyl PE (DSPE), dioleoyl PE (DOPE) or 1-palmitoyl- 2-oleoyl PE (POPE). To confer long circulation times, PEG-lipids are normally incorporated into liposomes at 4–10% of total lipid. Also, the optimal weight range of the grafted PEG is between 2000–5000 Da (PEG-2000 to PEG-5000, 45–115 repeat units). This amount of polymer, with long hydrophilic chains, when grafted onto the liposomes, hinders the interaction of proteins, including antibodies, with liposomal surfaces. This will reduce the efficiency of both the ligands and antibodies in coupling to the liposomes and their ability to bind to their target.

Coupling ligands to the liposomal surface

Attaching ligands to the surface of pre-formed, PEG-containing liposomes

Some of the chemistry for coupling ligands to long-circulating liposomes was adapted from procedures for conjugating antibodies and/or antibody fragments (Fab' and F(ab')2) to the liposomal surface. The first major class of linkage chemistry involves sulfhydryl reactions. Two thiol-reactive PE derivatives which can be incorporated into liposomes are N-pyridyldithiopropionyl-PE (PDP pE)io9-iii maleimidophenyl butyrate-PE (MPB-PE). PDP-PE is easily reduced to form free thiol groups at the liposomal surface. The free thiol groups can then be coupled through thioether bonds to maleimide groups on proteins (introduced onto exposed amino groups) through the use of a hetero bifunctional cross-linking reagent like SMPB (H-succinimidyl-4-)p-maleimidophenyl)butyrate). Attempts to couple antibodies to the surface of PEG-2000-grafted liposomes with PDP-DOPE or a maleimidobenzoyl-derivative of DPPE (MB-PE) incorporated in the bilayer resulted in low antibody densities and low coupling efficiencies. PEG-lipids, at concentrations of approximately 5 mol% or higher in the bilayer, probably sterically interfere with the accessibility of the antibody to the liposomal surf ace.

Addition of PEG after ligand coupling

To overcome the interference of PEG in the conjugation of antibodies onto the liposomal surface, PEG could be incorporated into liposomes after ligand coupling occurs. PEG-2000-DSPE was effectively transferred from micelles and inserted into preformed liposomes; however, high temperatures were needed for efficient transfer, which may destroy protein ligands like antibodies. In another method, PEG was covalently coupled onto the liposomal surface after antibody conjugation. Liposomes containing maleimidobenzoyl-DPPE (MB-PE) were conjugated first with thiolated antibodies, then PEG was grafted to the surface of the immune liposomes through the use of PEG-succinyl cysteine (PEG-SC) of various polymer chain lengths. This post-coating method resulted in both efficient antibody conjugation and efficient grafting of PEG-750-SC, PEG-2000-SC and PEG-5000-SC onto the liposomal surface. However, only PEG-2000-SC-immunoliposomes retained extended circulation times and *in vitro* target binding, compared to the control, non-PEG liposomes. The advantage of this post-coating method of immune liposome preparation is that both the ligand and the grafted polymer occur on the outside leaflet only, leaving the maximal interior space for drug loading.

Formation of ligand-anchor conjugates prior to liposome formation

The liposomes can then be formed by either co-solubilizing the conjugates in detergent followed by dialysis or by hydrating a dry lipid film containing the lipid ligand conjugate plus other lipids. The first method uses carbodiimide activation of the carboxyl groups of N-glutaryl-PE (NGPE) followed by coupling, to free amine groups on antibodies in an octyl glucoside solution. Additional lipids are added to the detergent solution and liposomes are formed following detergent removal through dialysis. A portion of the incorporated antibody will be oriented to the interior aqueous space of the liposome, making it unavailable for target binding. Also, the internal antibody will also occupy internal space which will reduce the available volume for drug loading. Immuno liposomes formed by this method, in liposomes containing PEG-5000-PE, showed poor target binding, due to steric hindrance of antibody-antigen interactions. However, the amount of antibody-lipid conjugate incorporated into PEG-grafted liposomes was reported to be independent of the polymer size and surface density.

Non-covalent coupling methods

The non-covalent, but high affinity interaction of avidin or streptavidin with biotin has been adapted for coupling ligands to the liposomal surface. The cross linking of a ligand and liposomes can proceed via an avidin bridge either before or after target binding. In one variation, ligands are non-covalently bound to preformed liposomes by first binding avidin or streptavidin to liposomes containing

a biotinylated lipid (usually a derivative of PE) and then incubating with a biotinylated ligand. In another variation, biotinylated-ligands (or an avidin-ligand conjugate) are first bound to the target epitope. A chase step follows, using either streptavidin- or avidin-liposomes (or biotinylated liposomes). The advantage of this two-step protocol is that many different ligand conjugates can be synthesized and bound to their intended targets, independent of the drug carrier. We can hypothesize at least two reasons for this success: at low antibody coupling densities the circulation times of immune liposomes are long, and these preparations have good target binding abilities.

Antibacterial agents in long circulating liposomes

Sterically stabilized liposomes containing gentamicin or ceftazidime

In a rat model of left-sided pneumonia caused by *Klebsiella pneumoniae* (an infection fatal within 5 days) the behavior of sterically stabilized liposomes composed of PEG-DSPE: PHEPC: Choi (molar ratio, 0.15:1:1.85) with a mean particle size of 80 nm was investigated. The circulation half-life for these liposomes in blood was about 20 h. The liposomes showed relatively low hepatosplenic uptake. After intravenous administration these liposomes are passively targeted towards the infected lung tissue. The efficacy of gentamicin or ceftazidime encapsulated in these liposomes was investigated in this experimental pneumonia model. At a single-dose treatment schedule started at 24 h after bacterial inoculation, a superior therapeutic efficacy of the liposome-encapsulated antibiotic was observed compared to the effects of free antibiotic, in terms of increased survival of the infected rats, as well as increased bacterial killing in the infected lung tissue. During the long circulation in blood the antibiotic-containing liposomes are relatively stable (Senior et al. 1991; Papahadjopoulos et al. 1991).

MiKasome®, amikacin-containing liposomes

In more recent animal studies, the efficacy of MiKasome® was investigated in a model of intra peritoneal infection caused by *Klebsiella pneumoniae* in immune suppressed mice, resulting in sepsis. In prophylactic treatment MiKasome® showed improved efficacy in terms of survival of animals and bacterial killing in blood, liver and spleen. *A*55 nm unilamellar liposomal preparation of amikacin, MiKasome® consisting of HSPC: Choi: DSPG (molar ratio, 2:1:0.1) was developed (NeXstar Pharmaceuticals Inc., San Dimas, CA), and the pharmacokinetics and toxicity were investigated in animal models. Data on biodistribution in rats at relatively high dosage show that MiKasome® can more effectively deliver amikacin to liver, spleen, lung and kidney than free drug treatment.

Antifungal agents in long circulating liposomes

AmBisome®, amphotericin B-containing liposomes

AmBisome® shows prolonged blood residence time at therapeutically effective doses, the elimination half-life being about 32 h in humans. However, using AmBisome® at much higher dosages is standard, and these elevated doses result in enhanced antifungal effectiveness. Yet, in rigorous infection, basic studies in immune cooperation on the mechanism of action of AmBisome® in animals show that intact AmBisome® can reach the site of infection; at the site of infection direct interaction between AmBisome® and the fungal cell may occur or AMB may be released from AmBisome® in the close vicinity of the fungus. To achieve prolonged circulation of AMB liposomes without the constraint of high lipid dose, sterically stabilized AMB-containing liposomes were prepared at our laboratory.

Sterically stabilized liposomes containing amphotericin B

Two different formulations of AMB in PEG-grafted liposomes have been studied. Liposome preparations consisted of PEG-DSPE: HSPC: Choi: DSPG: AMB (molar ratio 0.29:2:1:0.8:0.4), further referred to as PEG/DSPG-AMB, and PEG-DSPE: HSPC: Choi: AMB (molar ratio, 0.21:1.79:1:0.32), further referred to as PEG-AMB. The two different preparations showed a large difference in toxicity in uninfected mice. PEG/DSPG-AMB was as toxic as conventional AMB, whereas the liposomal formulation PEG-AMB greatly reduced the toxicity of AMB. In conclusion, the PEG-AMB formulation shows three characteristics that are expected to be important for improved antifungal efficacy: low toxicity, elevated fundamental antifungal action, and extended circulation time of integral AMB-containing liposomes in blood.

Design of liposome-based drug carriers

Liposomes are used for drug delivery due to their unique properties. A liposome encapsulates a region on aqueous solution inside a hydrophobic membrane; dissolved hydrophilic solutes cannot readily increase the lipids. Hydrophobic chemicals are able to be dissolved into the membrane, and in this method liposomes are capable of taking both hydrophobic molecules and hydrophilic molecules. The use of liposomes for transformation or transfection of DNA into a host cell is known as lipofection. In addition to gene and drug delivery applications, liposomes can be used as carriers. The design of liposomes as a drug carrier is much more complicated and "full of mines" than it seemed at the birth of the field 30 years ago. Major scientific input in the fields of physical chemistry, chemistry, 1ife and medical sciences, including pharmacy, should be used in a cross-talk with technology. This field is an excellent example of the need for science and technology to move together hand-in-hand in order to achieve a product.

Conclusion and future outlook

The synergistic input from colloid science, physics, chemistry, biology, pharmacology and medicine has resulted in the successful development of liposomal drug delivery in less than 30 years, and the solid theoretical and experimental bases that have been developed promise new improvements

and products. Only time will tell which of the above applications and speculations will prove to be successful. However, based on the already available products, we can say that liposomes have definitely established their position in modern technology.

One of these successful technologies is the commercial formulation of topically applied liposomal formulations, particularly those prepared from lipid mixtures of a composition similar to the *stratum corneum,* which would be an effective delivery system for the treatment of skin diseases. With the advancement of other technologies in medicine, the field of liposomes will be a more advanced and reliable platform for the development of more useful bioproducts, especially in terms of medical diagnostics and public health areas.

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Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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