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APPLICATIONS OF LIPOSOMES IN MEDICINE- A REVIEW

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ABSTRACT

Liposomes are structurally and functionally some of the most versatile supramolecular assemblies in existence. Since the beginning of active research on lipid vesicles in 1965, the field has progressed enormously and applications are well established in several areas, such as drug and gene delivery. The medical application of liposomes loaded with small molecular weight drugs is discussed and the use of sterically stabilized liposomes containing doxorubicin in cancer therapy is presented. The review also shows that liposomes have a lot of biomedical applications and uses. They have been used in drug targeting, oral delivery of vaccines, insulins, peptides and some compounds, which are usually degraded in the gastrointestinal tract. It has also found application in topical therapy especially in the eye and lungs. Other areas of application are in cancer chemotherapy and treatment of human immunovirus (HIV) infection. The control of the stability of liposomes is an essential prerequisite for effective use as drug carriers. Liposome immunoassays, for example, benefit greatly from the amplification provided by encapsulated markers, and nanotube-interconnected liposome networks have emerged as ultra small-scale analytical devices. This review provides information about new developments in some of the most actively researched liposome-related topics.

Key words: Liposomes, Conventional Liposomes, Anti-Cancer therapy.

INTRODUCTION

Liposomes are tiny spheres ranging in diameters from 50 nm to several microns^[1]. Liposomes are microscopic vesicles composed of a bilayer of phospholipids or any similar amphipathic lipids. 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]. The

scope of this review is to introduce the application of liposomes and to describe some aspects and mechanisms of stimulating topical and injectable products with liposomes. 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 [3-7]. 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 [8-12]. The benefits and limitations of liposome drug carriers critically depend on the interaction of liposomes with cells and their fate *in vivo* after administration [13,14]. *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 rarer. The fourth possible interaction is exchange of bilayer constituents, such as lipids, cholesterol, and membrane bound molecules with components of cell membranes. 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. 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.

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 ^[15,16]. The benefits of drug loaded 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 encapsulated 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) suppressors;
- (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, ant infection and anti-inflammatory 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.

CONVENTIONAL LIPOSOMES

For historical reasons we shall 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 sensitized liposomes (immunoliposomes as 'guided missiles'), which gave often very encouraging results *in vitro* studies – which are in general performed in the absence of immuno globulins, complement components, and macrophages – failed *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.

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 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 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 targeting. These

benefits, however, can be associated with any colloidal drug carrier. Indeed, similar improvements in therapy were observed with micro emulsions and stable mixed micellar formulations ^[21]. Furthermore, it seems that many of the early liposomal preparations were in fact liquid crystalline colloidal particles rather than self closed 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, is 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, ribavirin, or azido thymidine (AZT) have also shown reduced toxicity and currently more detailed experiments are being performed with respect to their efficacy.

MACROPHAGE ACTIVATION AND VACCINATION

The automatic targeting 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 granules 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 does 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].

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. This cell is 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].

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 anti-inflammatory 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 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. 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. Intra-gastric 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.

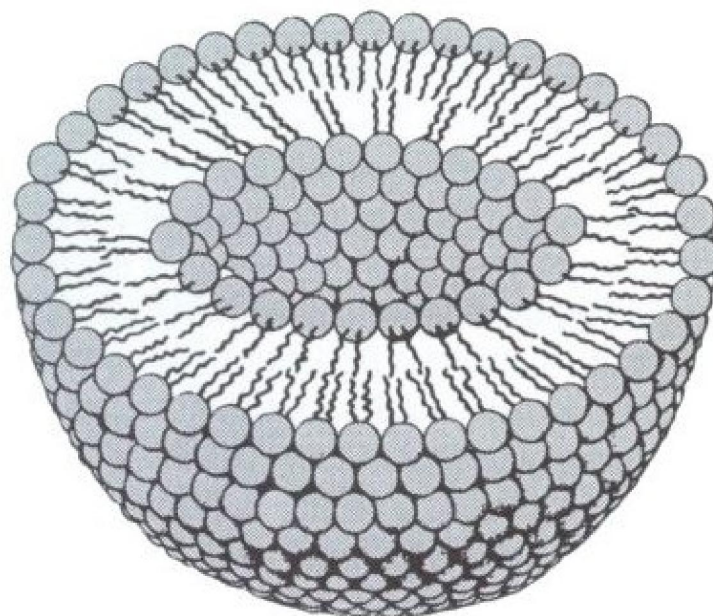
LIPOSOMES WITH ALTERED SURFACE PROPERTIES

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 localized 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.

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 GM1 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]. 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 immunoglobulin, complement proteins, fibronectin and similar molecules, which mark foreign particles for subsequent macrophage uptake as schematically shown in (fig. 1).



Liposome structure formed by phospholipids

Figure 1

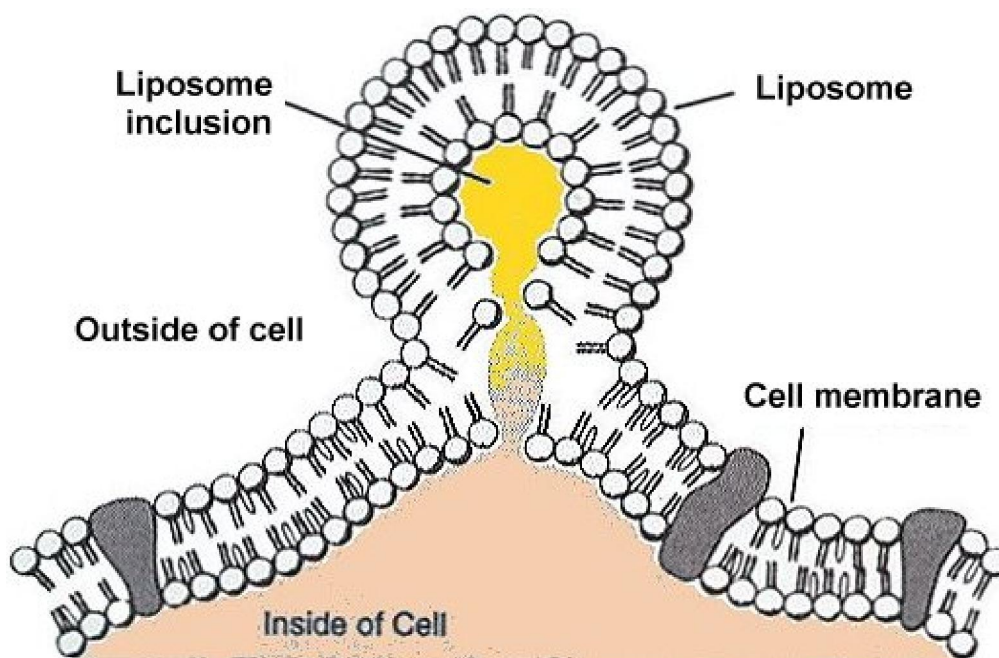
The origin of steric stabilization is well documented although not well understood. Recently it was shown that the Alexander-de-Gennes model of polymers at interfaces ^[37] can qualitatively explain the stability of liposomes in biological systems ^[35]. The model can explain minimal polymer concentration above the surface of the bilayer at which polymer forms the so-called brush conformation and which acts as 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 stabilizes liposomes. Recent theoretical work also explained the experimentally wellknown 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 self aggregation^[40], a wellknown 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.

MEDICAL APPLICATIONS OF STEALTH LIPOSOMES

Sterically stabilised vesicles can act either as long circulating micro reservoirs or tumour (or site of inflammation and infection) targeting vehicles. The former applications require larger liposomes 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 characterize tumours or their 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 laden drugs to be delivered to malignant tissues. For instance more than 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. 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 Doxorubicin) 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. 2) ^[45]^[46].



Acceptance of liposome into cell

Figure 2

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 micro reservoir or localised drug depot. The first example is provided by improved therapeutic efficacy of cytosine arabinose in the treatment of lymphoma ^[48] while the subcutaneous 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 characterized 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, it requires careful toxicology and tolerability studies when liposomes loaded with potent drugs are used.

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. 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. In conclusion, it seems that stealth liposomes loaded with anticancer drugs will achieve substantial improvements in the treatments of various tumours. In addition, it is hoped that they will be effective also in the treatments of inflammations, infections, and in antiviral therapy.

CONCLUSION

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. 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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