Liposomes and Immuno-liposomes as Carriers for Cytostatic Drugs, Magnetic
Resonance Contrast Agents, and Fluorescent Chelates

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Abstract. Biological and medical applications of liposomes as carriers for cytostatic
drugs, magnetic-resonance contrast agents, and fluorescent markers are presented. The
cytostatic effects of liposomal preparations of lipophilic derivatives of cytosine
arabinoside (ara-C), namely N4-oleyl-ara-C (NOAC) and N4-alkyl-ara-C are demon-
strated in the mouse L1210 tumor model. Liposomal drugs were more effective by
factors of 2–10 as compared to ara-C administered in aqueous preparations. Synergistic
effects of the combination of ara-C and mitoxantrone could be shown, again with
significantly increased effects, when the drugs were administered in liposomes. A
practical detergent-dialysis method for the preparation for large volumes of sterile
liposomes for clinical use is presented. Clinical phase II/III studies of liposomal NOAC
and mitoxantrone are currently in progress. Methods of preparation of immunolipo-
somes, i.e. the coupling of antibodies or antibody subunits to liposome membranes, are
discussed and results of specific immunoliposome – cell binding are presented.
Possibilities of increasing the blood circulation times of liposomes by incorporation
of lipophilic derivatives of poly(ethylene glycol) into the liposome membranes are shown.
The successful application of liposomes as carriers of paramagnetic metal complexes
as contrast agents in magnetic-resonance imaging for liver and spleen is documented.
Finally, the concept of liposomes as carriers for fluorescent Europium chelates as
markers for time-resolved flow cytometric applications are presented.

1. General Properties of Liposomes

Liposomes are spherical vesicles pre-
pared from natural or synthetic phosphol-
 lipids and cholesterol. Their mean diam-
ters range from 0.02 to 2 μm depending on
the method of preparation, lipid composi-
tion, and concentration and the properties
of added molecules. Liposomes are com-
posed of either one (unilamellar liposomes)
or several (oligo- or multilamellar lipo-
somes) lipid bilayers surrounding internal
aqueous volumes. According to their mean
size, the liposomes are classified into ei-
ther 'small unilamellar vesicles', (SUV,
20–200 nm) and 'large unilamellar ves-
cles' (LUV, >200 nm), whereas the 'mul-
tilamellar vesicles' (MLV) are of hetero-
genous size distribution (0.1 – 2 μm) [1–
4]. For most of the biological applications,
homogeneous SUV's with average diam-
eters ranging from 20 to 250 nm are better
suited than heterogenous MLV prepara-
tions. Depending on the particular physi-
co-chemical properties, hydrophilic mol-
ecules (drugs, contrast agents, fluorescent
probes, proteins, nucleotides, etc.) can be
entrapped within the internal aqueous space
of a liposome, whereas lipophilic mole-
cules are incorporated into the phospholi-
 pid membrane. Additionally, antibodies,
proteins, and hapten can be covalently
attached to the outer surface of the lipo-
somes either to enable specific binding to
defined targets or to serve as antigens for
immunization. Liposomes allow the par enteral administration of insoluble or
poorly soluble drugs. Toxic side effects on
organs and cells can be reduced or elimi-
nated by liposomal drug delivery. Fast
drug elimination and metabolism can be
retarded with liposomal formulations.

The incorporation of molecules into
the lipid membrane as shown schemati-
cally in Fig. 1 offers several advantages
over the encapsulation of small hydrophilic molecules into the entrapped aqueous
volume of a liposome. Firstly, the stability
of incorporation is higher, because diffusion
and leakage of lipophilic molecules into
the aqueous medium surrounding the lipo-
somes is negligible. Secondly, the rate of
incorporation during liposome formation
is virtually quantitative and procedures
to separate unincorporated molecules from
the liposomes are not necessary (cf. Table
1), and thirdly, the drug-carrying lipo-
2.1. Development of Liposomes with Prolonged Blood Circulation

Liposomes can be prepared by the detergent-dialysis method which offers several advantages mainly for the preparation of large quantities of sterile liposomes (see 2.3). A disadvantage encountered in the use of lipophilic molecules is that chemical modifications of the originally hydrophilic drug are often necessary, e.g. the modification of cytosine arabinoside (ara-C) into N\textsuperscript{4}-oletyl- or N\textsuperscript{4}-alkyl-cytosine arabinoside (NOAC, N\textsuperscript{4}-hexadecyl-ara-C).

Many methods of liposome preparation and characterization are in use. Together with many applications in biochemistry and biology, they have been described and discussed in several reviews [1-4].

Fate and behaviour of liposomes in vivo are very complex, and mainly depending on liposome size and population homogeneity, lipid composition and site of administration [5]. Two major events can take place when liposomes are introduced into a body compartment: a) liposomal contents may be released under the influence of the biological environment (e.g. interactions with serum proteins) and/or b) liposomes can be transported to certain compartments where liposomes or some or all of the liposomal drug may be taken up by cells. The kinetic rates of a determine the extent of drug uptake in b. Thus, factors that control the physical stability of the liposomes in vivo are of paramount importance. Due to their particular properties, liposomes and most of other drug carriers are taken up by the cells of the mononuclear phagocyte system (MPS), i.e. mainly by liver, spleen, bone marrow, and lymph nodes.

This property may either be advantageous, when the molecule of interest is to be targeted to the MPS, or disadvantageous, when the sites of delivery are elsewhere in the body. Thus, the development of liposome preparations that reduce uptake of the MPS and prolong blood circulation of liposomal drugs are a major task in liposome research.

On the cellular level, liposomes interact with cell membranes by adsorption, endocytose, membrane fusion, and lipid exchange, or by a combination of these mechanisms [9]. It is conceivable that mechanisms of cancer-drug resistance may be circumvented by liposomal drug delivery in a way that cellular drug uptake and intracellular transport mechanisms can be altered [10].

The main purpose of this publication is to present the author's recent work in the field of liposome applications in biology and medicine. For more profound informations on liposome preparation methods, physicochemical properties, in vivo behaviour, and other applications of liposomes, the reader is referred to reviews and publications where these aspects are dealt with [1-5][9-15].

2. Application of Liposomes in Biology and Medicine

2.1. Development of Liposomes with Prolonged Blood Circulation

With synthetic phospholipids of high liquid-crystal to solid-phase transition temperature, of poly(ethylene glycol)-linked lipids or of glycolipids [6][7], liposomes can be made more resistant to degradation in circulation and to fast absorption in the organs of the MPS.

We have used the two lipophilic poly(ethylene glycol) derivatives cholesterol-poly(ethylene glycol) 900 sebacat (cholester-Peg(900)) and poly(ethylene glycol) (5000)-phosphatidylethanolamide (PEG(5000)-PE) [6][8] for the preparation of hydrophilic liposomes. As shown in Fig. 2, the blood kinetics of such liposomes can be influenced by the addition of PEG derivatives. Chol-Peg(900) has little effect upon prolongation of the circulation of the liposomes, whereas with the derivative of higher molecular weight higher and longer lasting levels of the I\textsuperscript{125}, labelled (cf. Methods) liposomes were found in the blood. The degree of ethylene glycol polymerization influences the interactions of the liposomes with blood components. Short PEG chains (e.g. cholest-Peg(900)) provide no increased blood stability of the liposomes. The elimination half-time (t\textsubscript{1/2}) from the circulation of liposomes without cholest-Peg(900) was 1.2 h and that of liposomes containing 40 mol% of cholest-Peg(900) 1.1 h. In contrast, a blood elimination half-time of 3.4 h was determined with the more hydrophilic PE-Peg(5000) derivative.

The increased stability of such liposomes in circulation can for instance be

| Drug | Incorporation | Liposome diameter (mm) after 300 d |
|------|--------------|----------------------------------|
| ara-C | 19 | 3.8 | 110 | 102 |
| N\textsuperscript{4}-oleyl-ara-C | 581 | 88 | 108 | 109 |
| N\textsuperscript{4}-hexadecyl-ara-C | 555 | 91 | 117 | 124 |

- Incorporation of drugs was determined by UV spectroscopy or with radiolabeled ara-C derivatives. The initial drug concentration in the micelles was taken as 100%.
- Hydrodynamic liposome diameters were measured by laser light scattering [62].

Table 1. Incorporation Characteristics of Cytostatic Drugs and Derivatives in Liposomes Prepared by Detergent Dialysis

Fig. 1. Schematic representation of the incorporation of lipophilic molecules into a liposome bilayer membrane. Hydrophilic molecules are modified with long-chain fatty acids, alkyl chains, or they are attached to phospholipids (e.g. phosphatidyl ethanolamine). Strong complex formation between lipophilic acids (phosphatidic acid) and basic molecules (e.g. mitoxantrone) yield stable drug-containing liposomes.
exploited for the development of liposome preparations containing cytostatic drugs or contrast agents for the vascular system (see 4). Blume and Cevc [8] prepared liposomes with the saturated phospholipid distearyl phosphatidylcholine (DSPC) and 10 mol-% of the DSPE-PEG (5000) derivative. The blood elimination half-time of these very stable liposomes was 8.4 h. These results demonstrate that liposomes of variable kinetic properties can be prepared. According to their particular compositions, their in vivo behaviour can be tailored according to the desired application, i.e. long lasting blood circulation or fast absorption in the organs of the MPS.

2.2. Liposomes as Carriers for Pharmacologically Active Compounds

In the search of new approaches for the chemotherapy of malignant diseases the following strategies are being pursued: a) Exploration of new combinations with different dosing and therapy regimens with existing drugs [16]; b) Search for new drugs with new pharmacological properties, and c) the improvement of the effectiveness of existing drugs using methods to alter drug disposition, kinetics and doseresponse relationships with the aim to achieve a higher therapeutic index [11]. Such improvements may be accomplished by chemical modifications of the active drugs and with the development of drug delivery systems. An optimal drug delivery system must be capable to selectively localize the targets in an organism where disease resides and to release the drugs at suitable rates while producing the minimum of untoward side effects. Among a variety of possible drug delivery systems, liposomes offer several advantages: a) Flexibility in formulation of their composition and size and b) the opportunity to use drugs of known pharmacological properties.

These advantages may, however, also evoke disadvantages. Since the possibilities of liposome preparation and composition are almost infinite and each formulation involves different clinical pharmacology trials, it is necessary to perform time consuming preclinical toxicological and pharmacological studies.

The application of liposome preparations on humans is most advanced in the field of cancer chemotherapy. Phase II/III studies have been reported with liposomal doxorubicin [17][18] and a water-insoluble quinazolone drug [19].

Other clinical applications of liposome preparations comprise the treatment of systemic mycosal infections which are common in leukemia and AIDS. The antifungal drug amphotericin B can be administered at higher doses and with improved effects in a liposomal preparation [20].

The lipophilic muramyl tripeptide-phosphatidyl-ethanolamide (MTP-PE), a macrophage-activating agent, is presently being studied as liposome preparation in patients with advanced cancer [21].

Liposomes loaded with In³¹¹ complexes or other radionuclides can successfully be used for the diagnostic imaging of human primary and metastatic cancer [22].

Synergistic effects between cytostatic drugs have been demonstrated in vivo with different conventional and liposomal drug combinations [23–27]. We investigated the cytotoxic effect of the combination therapy of ara-C and mitoxantrone in the L1210 mouse leukemia model. In Fig. 3 the synergism of the lipophilic ara-C derivative N³-oleyl-cytosine arabinoside (NOAC) and mitoxantrone, both incorporated into liposomes is shown. Female BDFI mice inoculated intraperitoneally

![Fig. 2. Blood clearance of PEG-modified liposomes. The incorporation of lipophilic PEG derivatives into the liposome membranes increases their blood circulation time. With PE-PEG(5000)-liposomes the circulation half-time is increased to 3.4 h vs. 1.1–1.2 h of unmodified liposomes or liposomes containing the less effective chol-PEG(900) derivative.](image1)

![Fig. 3. Synergistic effect of cytostatic drug combinations. Survival curves of the treatment of L1210 leukemia in mice with single drugs and drug combinations either as free drugs or as liposomal drug applications.](image2)
with L1210 leukemia cells were treated by the intravenous route either with the single drugs dissolved in buffer (ara-C, mitoxantrone), or with the single drugs in liposomes (NOAC, mitoxantrone-phosphatidic acid complex, [28]), and finally with the combination of both liposomal drugs. The liposomal drugs were in all cases more effective, compared to the single drug therapy and especially as combination of NOAC and mitoxantrone. We all treated animals survived 60 days [29][30].

In another experiment summarized in Table 2, we could demonstrate the superior or cytostatic action of liposomal lipophilic derivatives of ara-C in comparison to the free drug. Total doses of the liposomal ara-C derivatives which were 2-4 times below those of free ara-C were more effective against the L1210 leukemia. The length of the alkyl chain has a marked influence on the cytostatic action of the derivative. Chain lengths of less than 14 C-atoms showed no effect. This effect of side-chain length was shown before with N^4-acetyl derivatives of ara-C [31].

At the University Hospital in Zurich, we are presently conducting phase I/II studies with liposomal mitoxantrone [28][32][33] for the treatment of advanced breast cancer and indolent non-Hodgkin lymphomas. In another study, patients suffering from refractive, acute myeloid leukemia (AML) are treated with a combination of liposomal NOAC and mitoxantrone liposomes.

### 2.3. Preparation of Liposomes for Clinical Applications

The use of liposome preparations for clinical trials requires that large volumes of stable and sterile liposomes, are made available. For the preparation of the NOAC- and mitoxantrone-liposomes we have developed a convenient method of preparation which is based on the removal of detergent from mixed lipid/drug/detergent micelles by fast dialysis through capillary dialyzers [34][35]. The setup of the dialysis instrument is shown in Fig. 4. The sterile micelle solution is pumped through an in-line filter (F) into the system. Driven by pump PM/l, the micelles/liposomes (M/L) circulate in a closed circuit from the reservoirs (R1,R2) to the serially connected capillary dialyzers (dialyzers 1–3). In a counter current flow, the dialysis buffer is drawn with pump P1 alongside the capillaries, whereby the detergent is effectively removed from the micelle solution. During dialysis, the micelles are transformed into homogeneous bilayer liposomes. The extensive dialysis guarantees the quantitative detergent removal. Depending on the lipid concentration and duration of dialysis, the amount of residual detergent can be reduced to less than 0.2% of the initial amount [34]. An example of liposome preparation for clinical application is given in the experimental section.

### 3. Immunoliposomes

The targeting of drugs, toxins, peptides, and proteins to a specific site is subject of many approaches. In cancer therapy, cytotoxic antibodies, antibody-toxin conjugates, and antibody-drug conjugates are used to obtain increased tumorcell specific cytotoxicity [36–42]. However, the use of heterologous antibodies elicits an immune reaction and the production of human-anti-mouse antibodies (HAMA). Presently, the production of human monoclonal antibodies is still not easy to achieve because of the limited possibilities of immunizing humans. Furthermore, methods for the production of stable cell lines that efficiently secrete...
human monoclonal antibodies have to be developed. Because of the limited possibilities of immunizing humans, it is presently not achievable to prepare human monoclonal antibodies. To circumvent these problems, chimeric human-mouse antibodies are being produced with molecular-biological methods [43]. Attempts are made to reduce the antibody molecules to the size of the so-called 'minimal reactive unit' (mru) which is the smallest possible peptide that recognizes and binds to specific cellular antigens [44]. Such mru's may have significant advantages over the original antibody molecules whereby preparation methods of such units and their linkage to toxins, cytostatic drugs, radionuclides, and macromolecular carriers such as liposomes have to be developed. The possibilities of fragmentation of an IgG antibody molecule into smaller subunits, namely F(ab)2-, Fab-, and Fv-fragments, single chain V, and minimal reactive units, are schematically shown in Fig. 5.

3.1. Preparation and Application of Immunoliposomes

Immunoliposomes are obtained by the linkage of antigen specific molecules to their surface. In Fig. 6, a schematic view of the coupling of antibodies, fragments, and smaller molecules (cf. Fig. 5) to drug-carrying liposomes is given. The covalent linkage of such molecules to the surface of liposomes can be effected by various methods. A variety of bifunctional reagents are available which offer multiple coupling possibilities [45] [46]. We have investigated the coupling of antibodies to liposomes using N-succinimidyl-S-acetylthio- propionate (SPDP) as bifunctional linkers.

In Fig. 7, scanning electron micrographs from in vitro incubations of liposomes (a) and immunoliposomes (b) with EL4 T-cell lymphoma cells are shown. Few liposomes and liposome aggregates are unspecifically adsorbed to the cell surface (a), whereas the antibody mediated binding of the immunoliposomes is demonstrated in (b) where a high number of single liposomes bound to the cell surface and the protruding microvilli can be recognized [47].

4. Liposomes as Carriers for Contrast Agents

For the improvement of diagnostic accuracy in computed tomography (CT) and in magnetic-resonance imaging (MRI), liposomal contrast agents are being investigated. In CT, multimellar liposomes have been used as carriers for I125 labeled X-ray contrast agents for the imaging of liver and spleen [12]. In MRI, paramagnetic complexes were encapsulated into liposomes also with the intention to improve contrast in the abdominal organs. Paramagnetic ions (Gd3+, Fe3+, Mn2+) have the property to enhance the spin-lattice relaxation of protons which increases the contrast of the tissue where these ions are located [48]. Because of the toxicity of these ions, stable complexes with ligands like diethylenetriaminepentaacetic acid (DTPA) have to be formed [49]. We have prepared liposomes containing a lipophilic derivative of DTPA, namely DTPA-stearate, for the complexation with Gd3+, Fe3+, and Mn2+ as paramagnetic ions. As shown in Fig. 8, Gd3+-DTPA-stearate liposomes are distributed at very high concentrations in the liver. MR imaging of the liver was performed with rats and dogs, and strong contrast enhancement persisted 1–5 h af-
ter application of the liposomal preparation [50][51]. As shown in Fig. 8, the initially high blood concentration of the liposomal Gd\(^{3+}\)-chelate is decreasing, as liver accumulation is increasing. In MRI, contrast agents for the vascular system are needed, and attempts are being made to increase the blood circulation times of paramagnetic ion chelates by attachment to macromolecules and particulate carriers [52][53]. The modification of chelate-carrying liposomes with PEG derivatives as described above (cf. 2.1) may allow the use of such preparations for the imaging of the vascular system.

5. Fluorescent Liposomes and Their Use in Cytofluorometry and Immunofluorescence

In biological research, fluorescent liposomes are used for many purposes [13–15]. Similar to drugs and contrast agents, fluorescent molecules can either be encapsulated in the trapped aqueous volume or incorporated into the lipid membrane of a liposome. The binding of liposomes and immunoliposomes to target cells can be monitored by flow cytometry and/or fluorescence microscopy. We demonstrated specific liposome binding to tumor cells using the highly lipophilic perylene dye \(N,N'\)-bis(1-hexylheptyl)-3,4,9,10-perylenebis(dicarboximide) (BHPD) [47].

In flow cytometry (FCM), ways are being sought after to increase the sensitivity of the detection methods. The specific fluorescence of a labeled molecule is often overlaid and disturbed by the short lived autofluorescence of the cell. Most of the fluorescent probes presently used in FCM have very short fluorescence emission lifetimes in the ns range. Chelate complexes of Eu display unusual fluorescent properties like a Stoke’s shift of 250–350 nm as compared to 28 nm for fluorescein and very long fluorescence lifetimes in the order of 0.1–1 millisecond [54]. Such Eu\(^{3+}\)-chelates have found various applications in fluoroimmunoassays [55][56]. In collaboration with the Institute of Biomedical Engineering of the ETH-Zürich (Prof. M. Anliker), we are developing a method which allows the use of time-resolved fluorescence (TR-FCM) in flow cytometry [57][58]. To achieve a signal to noise ratio which is high enough for the detection of a low number of antibodies bound to a cell ca. 500 Eu\(^{3+}\)-chelates per antibody are necessary [58]. For this purpose, we are developing liposomes which can carry high numbers (>300–10\(^6\)) of either a \(\text{H}_2\text{O}\)-soluble Eu\(^{3+}\) complex or a lipophilic derivative of this complex. The concept of specific binding of Eu\(^{3+}\)-labeled immunoliposomes to target cells is shown in Fig. 9. A biotinylated anti-CD8 antibody is noncovalently linked via avidin to biotinylated liposomes containing entrapped chelate complexes of Eu\(^{3+}\) with 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (Eu\(^{3+}\)-BCPDA) and BHPD as short-lived fluorescence marker [47][54]. These immunoliposomes contain sufficient Eu molecules to allow the detection of target molecules on autofluorescent cells [57]. For the measurement of time-resolved fluorescence, a conventional cytometer was modified enabling the time delayed detection of the Eu\(^{3+}\)-fluorescence after the extinction of short lived autofluorescence [58]. With both methods, conventional FCM using the liposomal BHPD fluorescence and TR-FCM of the liposomal Eu\(^{3+}\)-chelates, we could demonstrate as preliminary results specific liposome binding via the CD8 antigen on human lymphocytes.

6. Experimental

Materials. Soy phosphatidylcholine (SPC, Epikuron 200) was from L. Meyer, Hamburg. FRG, egg phosphatidyl choline and phosphatidic acid were from Lipid Products, S. Nutfield, U.K. Cholesterol (recrystallized from MeOH), choles-
teryl-poly(ethylene glycol) 900 sebacat (cholesterol, stearylamine, and thiglycerol) were from *Fluka*. Sodium cholate, DL-α-tocopherol, org. solvents and buffer salts were from *Merck*, Darmstadt, FRG. The ara-C derivatives were synthesized as described in [29]. The fluorescent probe BHPD was synthesized by Dennig and Langhals [59]. Mitoxantrone dihydrochloride was from Lederle Arzneimittel GmbH, Wolfhagen, FRG. All other reagents used were of anal. grade and from *Fluka* or *Merck*. The radioactive salts Na$^{25}$, Gd$^{153}$, Fe$^{59}$, and Mn$^{54}$Cl were obtained from Amersham Int., Amersham, U.K.

**Methods.** Liposomes containing the ara-C derivatives $N^4$-oleyl-ara-C and the $N^4$-alkyl-ara-C compounds (cf. Table 2) were prepared in vol. of 20–50 ml by detergent dialysis as described in [29]. Briefly, the org. (CHCl$_3$/MeOH, 1:1 v/v) lipid/drug/detergent mixtures (egg- or soy phosphatidylycholine, cholesterol, lipophilic drug, charged lipid (phosphatidic acid or stearylamine), nα tocopherol) were dried by rotary evaporation (Büchi rotavapor 01) and solubilized with 20–50 ml of phosphate buffered saline (PBS, pH 7.4). The micellar soln. were dialyzed against 10–20 l PBS during 15–20 h in a Lipoprep GD-1 instrument (Biechi, Munich, FRG). After dialysis the liposomes were removed from the instrument and filtered through 0.45 μm sterile filters (Van Leer Medical, Irigny, France).

Large Volume Liposome Preparation for Clinical Trials. The cancer drug mitoxantrone (Novantrone$^*$) forms a strong complex with negatively charged lipids [28]. The drug can be bound to liposomes containing phosphatidic acid by the formation of a 1:2 molar complex (cf. Fig. 1). Homogeneous and stable liposomes can be prepared by detergent dialysis in spite of the fact that mitoxantrone is a small H$_2$O-soluble molecule. The preparation of 3 l of mitoxantrone liposomes with a drug concentration of 0.1 mg/ml is given below.

20 g of soy phosphatidylycholine (SPC, Epikuron 200), 2 g of cholesterol, 0.355 g of mitoxantrone dihydrochloride, 0.95 g of phosphatidic acid, and 0.12 g of nα-tocopherol plus 20.6 g of sodium cholate as detergent are dissolved in 500 ml of CHCl$_3$/MeOH (1:1 v/v). After filtration and exhaustive removal of the org. solvents by rotary evaporation (3–4 h, 40$^\circ$C), the lipid/drug/detergent mixture is dissolved in 500 ml of sterile phosphate buffer (PB, 67 mM, pH 7.4). After sterile filtration (0.2 μm), the micelle soln. is pumped into the dialysis instrument (cf. Fig. 4) which is set up with two capillary dialysis cartridges (Baxter-Travenol, Type ST-25). After dialysis of 20 l of PB, the mitoxantrone liposomes are collected and diluted to 3000 ml with sterile PB. Thiglycerol (0.5 g) is added as an antioxidant and the liposomes are filled under sterile conditions and nitrogen purging into 100 ml ampoules. The liposomes have mean diameters of 60–80 nm and can be stored up to 12 months at 4–7$^\circ$C. The cap. dialysis method can be used for the preparation of liposome vols. of 0.1–1 ml. The upscaling of the capillary dialysis method for the preparation of larger batches should be feasible.

**Filter Extrusion.** The liposomes containing Eu$^{3+}$ chelates were prepared by extrusion of multilamellar liposomes through Nucleapore$^*$ (Sterico AG, Dietikon) membranes by calculation of the mean survival time in days

![Fig. 8. Pharmacokinetics of Gd-DTPA-seeurate liposomes as MRI contrast agent. The liposomes are accumulated in the liver tissue yielding high liver contrast over 1–5 h after injection.](image)

**Fig. 8.** Pharmacokinetics of Gd-DTPA-seeurate liposomes as MRI contrast agent. The liposomes are accumulated in the liver tissue yielding high liver contrast over 1–5 h after injection.

![Fig. 9. Concept of the use of immunoliposomes as carriers for Eu chelates for the time-resolved detection of fluorescence on target cells.](image)

**Fig. 9.** Concept of the use of immunoliposomes as carriers for Eu chelates for the time-resolved detection of fluorescence on target cells
of the treated (T) animals as compared to that of the control (C) animals, expressed as TIC in %.

The animals were observed daily until death or gamma counter described in [50].

The liposomes containing the paramagnetic chelate Gd\(^{3+}\), and Mn\(^{2+}\)-DTPA liposome were composed of SPC, cholesterol, \(\mathrm{Na}_{2}\)EDTA and Mn\(^{2+}\)DTPA liposome, DTPA-stearate (10:2:0.1:0.4 mol parts) and were prepared in vols. of 5–10 ml by dialysis as described above.\(^{125}\)labeled SPC was obtained by the photoreaction with 3-(trifluoromethyl)-3-(iodophenyl)diazirine (TID) as described by Brunner and Sennetti.\(^{125}\)labeled SPC was added in trace amounts to the lipid mixtures and liposomes (5–10 ml) were prepared by sodium-cholate dialysis (see above).

The liposomes containing the paramagnetic chelate Gd\(^{3+}\), and Mn\(^{2+}\)-DTPA liposome were composed of SPC, cholesterol, \(\mathrm{Na}_{2}\)EDTA and Mn\(^{2+}\)DTPA were added during complex formation to the DTPA-stearate liposomes as described before [50][51].

The pharmacokinetic parameters and organ distribution data were obtained as follows: male or female rats (ZUR SIV, Institut für Zuchthygiene, University of Zürich) of 160–240 g b. wt. were used. Liposomes (0.5–1.5 ml) containing 30 \(\mu\)g of the metal chelate were injected i. v. into the tail vein or the exposed jugular vein. Three animals per time point were used and killed after 0.5, 1, 1.5, 2, 3, 4, 24, 72, 192 h. Blood and the main organs were removed and the concentration of the radioactively labeled chelates determined in a gamma counter (Packard Auto-gamma 5000).

Pharmacokinetic parameters were calculated as described in [50][51]. The distribution of Gd\(^{3+}\)DTPA in liposomes in blood and liver are shown in Fig. 8.

Immunoliposomes. Antibody Modification and Liposome Coupling. The detailed methods of antibody modification and coupling to liposomes are given in [47]. Summarized, the IgG monoclonal antibodies (anti-B8.24.3, anti-B16.6-6) were modified with the bifunctional coupling reagents SPDP (Pharmacia) and SADA. SADA was synthesized according to Duncan et al. [63]. Optimal coupling conditions (molar ratios of SADA: antibody, incubation time, temperature, pH) were determined. The modified antibodies were linked to liposomes of the usual composition (SPC, cholesterol, \(\mathrm{Na}_{2}\)EDTA) and DTPA-stearate (10:2:0.01:0.4 mol parts). Additionally, the liposomes contained NOAC as cytostatic drug and a lipophilic maleimide derivative (N-(3-maleimidopropionyl)-N'-(3-aminopropyl)-1-lysine methyl ester, (MP-PL)) to which the SADA modified antibodies could be attached via a thioether linkage. To be able to determine liposomes – cell binding by flow cytometry the lipophilic fluorescent marker BHPD was incorporated into the liposome membranes.

Binding experiments of liposomes and target cell specific immunoliposomes were made as follows: 10\(^{-5}\) or 10\(^{-6}\) ELA B-cells (or 10\(^{-6}\) ELA B-cells) suspended in PB were mixed with liposomes (1–130 nm, corresponding to 0.02–2.5·10\(^5\) liposomes/cell) in a vol. of 0.4 ml. After incubation during 60 min at 4\(^\circ\) and several washing cycles, the cell-bound liposomal BHPD fluorescence intensity was measured on an Epics Profile Fluoro Cytometer (Coulter Corp.) at 470-nm excitation and 530-nm emission wavelength. For the scanning electron microscopy experiments (cf. Fig. 7 a, b) the binding of the liposomes to EL4 cells was performed as described above. After incubation, the cells were fixed with 2% glutaraldehyde and seeded on glass cover slips precoated with poly-L-lysine (Sigma, \(\mu\)l: 5,000). The dehydrated and dried samples were coated with gold (10 nm) and examined in a SEM 505 (Philips).

7. Concluding Remarks

In this review of the author's recent work, it is demonstrated that liposomes can be used as very versatile macromolecular carriers for a large diversity of hydrophilic or lipophilic molecules. The use of liposomes as drug carriers for in vivo applications offers several advantages over other carrier systems. Liposomes can be prepared with natural, toxic and degradable, and the lipidic membrane can be composed according to particular requirements. Size and homogeneity which contribute to the in vivo properties of liposomes can also be influenced by the use of appropriate preparation methods and by selection of the lipid composition. Compared to other carrier systems like albumins, dextrans, antibodies, nanocapsules, etc., liposomes offer the advantage that significantly higher numbers of small molecules can be loaded into one vesicle. Generally, an antibody can carry 10–50 small molecules without impairment of its specific binding activity. The loading capacity of albumins and dextrans varies between 100 to a few thousands of molecules, whereas liposomes can be loaded with very high numbers of small molecules. For example, a 1 mm solution of ara-C entrapped in 1 ml of liposome suspension with an entrapment efficiency of 1% yields 6·10\(^{15}\) trapped ara-C molecules per liposome. The entrapment of high numbers of small hydrophilic molecules into the inner aqueous space of a liposome has the disadvantage that these molecules leak through the lipid bilayer and that, therefore, stable preparations are not obtained. Lipophilic molecules like, e.g. the ara-C derivatives N\(^4\)-oleyl-ara-C and N\(^4\)-hexadecyl-ara-C can be incorporated at numbers of 10\(^5\)–10\(^6\) molecules per liposome, depending on the drug properties, and on size and concentration of the liposomes. Here, the incorporation efficiency is virtually quantitative and lipidosome preparations of high stability are obtained.

With the possibility to vary these parameters, optimized liposomal preparations can be obtained which meet the requirements of their intended application. Liposomes loaded with an antitumor drug can either be made to stay in circulation, when a circulating leukemia cell is the target, or the liposomes are composed and prepared in such a fashion that they accumulate in the liver, when a liver tumor or tumor metastases have to be treated. The natural distribution and elimination kinetics of liposomes can further be altered by the attachment of cell-specific antibodies or antibody subunits to the liposome surface. With the concept of cell-specific immunoliposome binding, higher uptake of cytostatic drug molecules in the tumor cell and less unwanted toxic effects on healthy cells are expected. The circumvention of multidrug resistance of tumor cells by their treatment with liposomal cytostatic drugs may be possible. Uptake mechanisms and intracellular drug distribution of drugs delivered by liposomes to resistant cells are presently under investigation [61].

The clinical utility of liposomal drug preparations depends on the availability of large batch preparation methods. Most of the presently used methods allow only the preparation of small batch volumes for laboratory use. Methods which allow the preparation of larger liposome batches are under development. The filter extrusion and detergent-dialysis methods can readily be enlarged in scale. The detergent-dialysis instrument described in this publication allows the preparation of intermediate batches of 0.1–10 l of liposomes. It can easily be set up and operated in a sterile facility, e.g. in a hospital pharmacy. The utilization of this method could allow the performance of important clinical trials with liposomal drug preparations.

The lyophilization of liposomes would greatly encourage the use of clinical liposome applications. The method is under investigation, and lyophilized MLV's containing muramyltripeptides are in use [21]. The future developments in this field may contribute to new approaches of the use of known drugs, of insoluble lipophilic molecules as intravenous application forms, of more effective and less toxic preparations, of new vaccination methods and of general aspects of drug-membrane interactions.

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