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EXAMINATION OF THE STRUCTURAL AND PERMEABILITY PROPERTIES OF LIPOSOMES STABILIZED BY NEUTRAL POLYMERS

Ph. D. Thesis

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Publications related to the thesis:

I. **Gy. Dékány,** I. Csóka, I. Erős: Adsorption of neutral polymers on negatively charged liposomes. A novel quantitative method to measure the rate of polymer adsorption on the liposomal surface. Colloid and Polymer Science, 279: 966-975 (2001)

II. **Gy. Dékány,** I. Csóka, I. Erős: Interaction between liposomes and neutral polymers: effect of adsorption on drug release. Journal of Dispersion Science and Technology, 22 (5), 461-472 (2001)

III. **Dékány Gy.,** Csóka I., Erős I.: Liposzómák és neutrális polimerek kölcsönhatásának vizsgálata; a stabilizálás hatása a hatóanyag-leadásra. Magyar Kémiai Folyóirat, 107 (11), 491-501 (2001)

Contents

1. Introduction

1.1. The place of liposomal systems in pharmaceutical research

Considerable efforts have been made in pharmaceutical research to develop new drug delivery systems that enhance the efficacy and safety of existing drugs and that underlie optimized drug therapy. Liposomes find perhaps the most extensive use as drug carriers, and many previous studies have demonstrated the enhanced efficacy of encapsulated drugs and the reduction of the side-effects of drugs so entrapped in this manner[1-4]. These vesicles consisted of phospholipid bilayers were discovered by Bangham et al. in 1964 when rational drug delivery research was at a very beginning stage. The few systems then under investigation, now fallen out from the forefront of the research, had no identified targets in humans (i.e. polyclonal antibodies), were too limited in their tasks (i.e. albumin microspheres) whilst other advanced systems (i.e. nylon microcapsules), could not degrade in the body[5]. In contrast, the cell like, organized structure of liposomes together with their unique versatility in terms of composition, physical characteristics and function in vivo, ensured an intellectually multifaceted challenge in the liposomology to apply the system successfully. Structural versatility and its control provided the inclusion of almost any type of drug in the medical application of liposome-based therapeutics and facilitation of related technology. It also enabled us to evade physiological ambushes or to circumvent anatomical barriers. Today, liposome based therapeutics are used routinely in the clinic to prolong or save lives and protect from disease, while others are still being tested in the clinic. Novel ideas and concepts are fermenting in laboratories worldwide, potentially to lead to effective constructs[6].

1.2. Physicochemical and structural properties of lipid membranes and liposomes

Liposomes are simply vesicles which an aqueous volume is entirely enclosed by a membrane composed of lipid molecules (usually phospholipids). They form spontaneously when these lipids are dispersed in aqueous media, giving rise to a population of vesicles which may range in size from tens of nanometers to tens of microns in diameter. Alternatively, liposomes can be composed of entirely artificial components[7-8], chosen for their improved chemical properties. Bilayer membrane vesicles can also be constructed of a wide range of other types of lipid: for example, fatty acids, double chain secondary amines, or cholesterol derivatives[9].

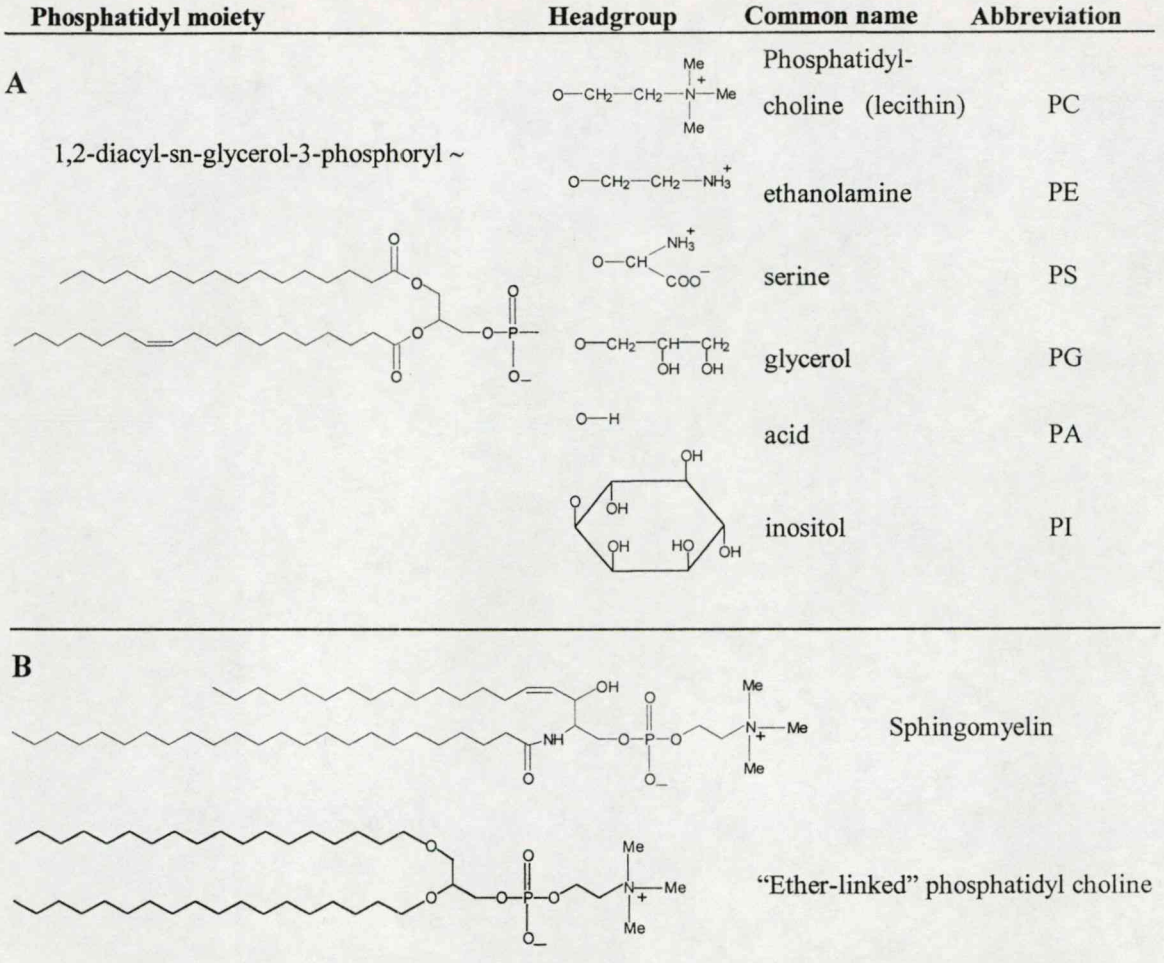


Fig. 1.: Some common naturally-occuring phosphatidyl phospholipids. A: the most frequent phosphoglycero-esters, B: sphingomyelin and ether-linked phosphatidyl choline (from Ref. 10)

Two sorts of phospholipids exist: phosphoglycerides and sphingolipids, together with their corresponding hydrolysis products (Figure 1A and B). The most common phospholipids are phosphatidyl cholines (PC). Molecules of PC are not soluble in water in the accepted sense, and in aqueous media they align themselves closely in planar sheets in order to minimize the unfavourable interactions between the bulk aqueous phase and the long hydrocarbon fatty acid chains. Such interactions are completely eliminated when the sheets themselves to form sealed vesicles. Phosphatidyl cholines contrast markedly with other amphipathic molecules (detergents, lysolecithin) in that bilayer sheets are formed in preference to micellar structures. This is thought to be because the double fatty acid chain gives the molecule an overall tubular shape, more suitable for aggregation in planar sheets

compared with detergents with a polar head and single chain, whose conical shape fits nicely into a spherical micellar structure[10].

1.2.1. Phosphatidyl choline membranes

Phase transitions. At different temperatures, lecithin membranes can exist in different phases, and transitions form one phase to another can be detected by physical techniques as the temperature is increased. The most consistently observed of these phase transitions is the occurring at the highest temperature, in which the membrane passes from a tightly ordered 'gel' or 'solid' phase, to a liquid –crystal phase at raised temperatures where the freedom of movement of individual molecules is higher. The most widely used method for determining the phase transition temperature (T_m) is microcalorimetry[11-13]. In general increasing the chain length, or increasing the saturation of the chains increases the transition temperature. Membranes made from egg yolk lecithin have a transition temperature from –15 °C to –7 °C, compared from mammalian sources which are usually in the range zero to 40 °C.

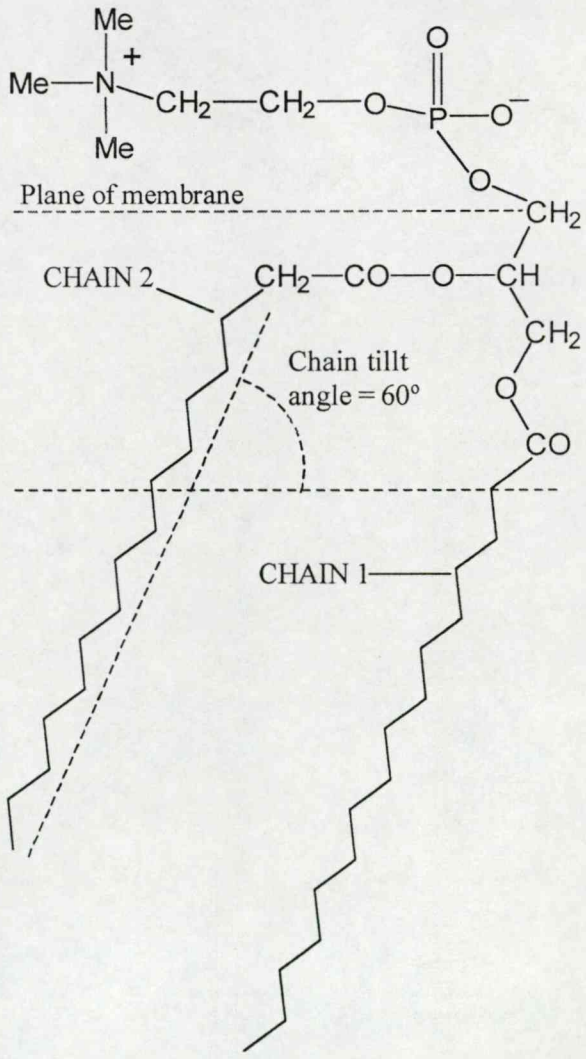


Fig. 2.: Schematic conformation of phosphatidyl choline in biological membranes (from Ref. 10.)

An understanding of phase transitions and fluidity of phospholipid membranes is important both in the manufacture and exploitation of liposomes, since the phase behavior of a liposome membrane determines such propreties as *permeability, fusion, aggregation*, and *protein binding*, all of markedly affect the stability of liposomes, and their behavior in biological systems.

In bilayer membranes, the molecules

appear to be aligned with the glycerol backbone approximately perpendicular to the plane of the membrane, and the phosphocholine head group in a straight line roughly parallel with the membrane surface. This conformation would be expected to reduce the distance between positive and negative charges within the phospholipid molecule. Because of the three methyl groups attached to the quaternary nitrogen, the head group of phosphatidyl choline in this conformation is very bulky, and occupies an area of the membrane (42 $\mathring{A}^2$) greater than that taken up by the two fatty acids in their straight chain configuration (~ 39 $\mathring{A}^2$). In order to overcome this, the hydrocarbon chains are thought to tilt relative to the plane of the membrane at an angle of 58°, in such a way as to fill up the extra space created by the head groups, and to bring the chains of adjacent molecules into closer proximity, to maximze Van der Waals and other non-covalent interactions (Figure 2.).

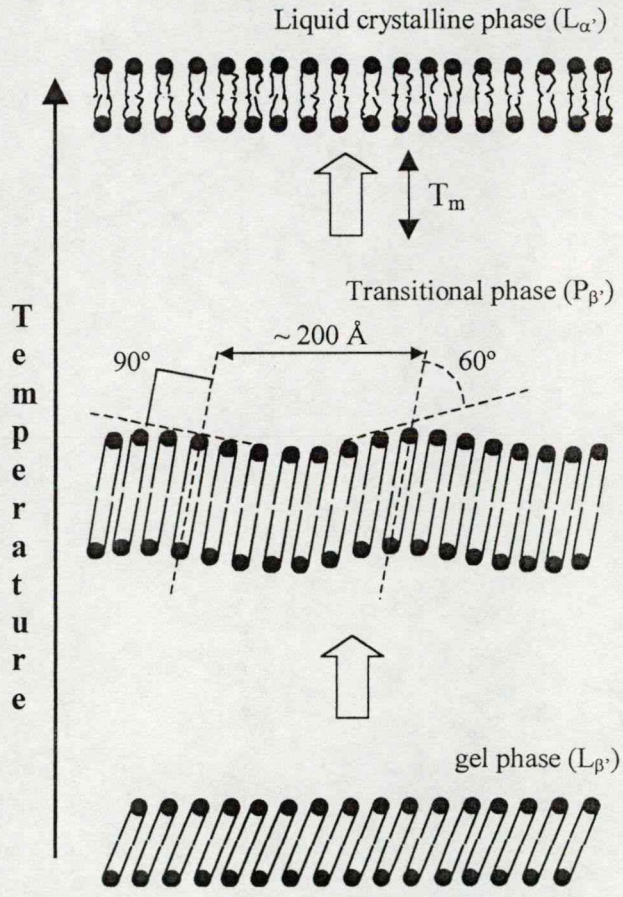


Fig. 3.: Phase transitions of phospholipid membranes during the elevation of temperature (from Ref.10.)

Phase separation. As the temperature increases, the fatty acid chains tend to adopt conformations other than the straight *all-trans* chain configuration, such as the *gauche* conformation state, and this tends to expand the area occupied by the chains, giving rise to a decrease in bilayer thickness upon transition from gel to liquid-crystalline phase (Figure 3.). In fact, the transition from gel to liquid crystal does not occur in a single step for lecithin, but involves two transitions: the main transition already described, and the pre transition, about five degrees below the main transition, at which a change in head group orientation may occur; the heat of this transition is very low compared with the main transition. In the temperature range between the two transitions, the membrane adopts a ruffled appearance, in which it is transformed from a planar to an undulating surface

with a fairly long, regular periodicity. The orientation adopted by the hydro-carbon chains to the plane of the membrane is not certain.

Binary mixtures of synthetic lecithins of different chain lengths give a main transition intermediate in temperature between those of the components, unless the chain lengths are very different, in which case two separate transitions are observed; in the temperature region between the transitions solid and fluid phases may co-exist, each enriched in one or the other of the components[14-16].

1.2.2. Lipids influencing the properties of the biological membranes

1.2.2.1. Other neutral phospholipids

In addition to PC, neutral lipid bilayers may be composed of sphingomyelin (Figure 1B), or alkyl ether lecithin analogues. Replacement of ester groupings by ether linkages increases thev resistance of such lipids to hydrolysis, while not apparently greatly affecting the physical properties of the membranes. The presence in sphingomyelin of the amide linkage and hydroxyl groups, in the region corresponding to the glycerol backbone of lecithin, gives rise to hydrogen bond interactions which may explain the more highly ordered gel phase relative to phosphatidyl choline. Natural mammalian sphingomyelin extracts show phase transitions in the region of 37 °C, in accord with the paucity of unsaturated fatty acid chains found in these mixtures. In contrast, natural phosphatidyl cholines have a much higher content of unsaturations, and give phase transitions well below body temperature. Generally sphingomyelin membranes are considered more stable and tightly packed than lecithin bilayers, as shown by decreased permeability to solutes, greater resistance to lysis by bile salts, and lower membrane fluidity.

The other neutral phospholipid found commonly in natural membranes is phosphatidyl ethanolamine (PE) (Figure 1A)[17-18]. Possessing an unsubstituted quaternary ammonium group, which is protonated at neutral pH. This lipid differs from PC in two respects: first in that its head group is smaller than the bulky phosphocholine of lecithin, and secondly, that it is able to take part in hydrogen bonding interactions with its neighbours in the membrane. In consequence of the small head groups it is not necessary for the chains to tilt in the gel phase; molecules are are oriented perpendicular to the plane of the membrane below the gel phase, and no pre-transition is observed. Saturated PEs have have transition temperatures approx. 20

°C higher than their PC analogues: this is attributed to the formation of intramolecular hydrogen bonds which have to be broken before PE can undergo transition to the more expanded liquid-crystalline phase. At low pH PEs also display PC-like transition temperatures, as the nitrogen becomes protonated, and hydrogen bonding reduced.

1.2.2.2. Negatively charged phospholipids

In negatively-charged (acidic) phospholipids all three possible forces regulating head group interactions of bilayer membranes can come into play namely: steric hindrance, hydrogen bonding and electrostatic charge. Thus for dipalmitoyl phosphatidyl glycerol (DPPG), the bulky glycerol group, in conjunction with electrostatic repulsion of the unprotonated phosphate at pH 7, gives it a main transition temperature of almost 10 °C below that of dipalmitoyl phosphatidyl choline (DPPC). In contrast, DPPA (dipalmitoyl phosphatidic acid) has a small head group and single proton at neutral pH which, like PE, can undergo hydrogen bonding, resulting in an elevated main transition temperature for pure DPPA bilayers. At high and low pH, the T_m is brought down, particularly at high pH, where electrostatic repulsion can push the head groups apart.

1.2.2.3. Cholesterol

Cholesterol (Chol) does not by itself form bilayer structures, but it can be incorporated into phospholipid membranes in very high concentrations, even 2 :1 molar ratios of cholesterol to PC. In natural membranes, the molar ratio varies from 0.1-1.0, depending upon the anatomical and cellular location. Being an amphipathic molecule Chol inserts into 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 presence of the rigid steroid nucleus alongside the first ten or so carbons of the phospholipid chain has the effect of reducing the freedom of motion of these carbons, while at the same time creating space for a wide range of movement for the remaining carbons towards the terminal end of the chain. Above a certain concentration of Chol, the membrane area occupied by the acyl chains and sterol combined is greater than or equal to that taken up by the phosphocholine head group, so that PC membranes with high levels of Chol do not show the chain tilt that is observed in the gel phase of liposomes composed of pure PC. Addition of Chol to PC membranes has a marginal

effect on the position of the T_m: in DPPC the T_m changes from 41 °C to 44 °C with 33 mol % Chol. With increasing concentration, however, cholesterol is able to eliminate evidence of a phase transition altogether, reducing the enthalpy of phase change to zero at 50 mol %, and in so doing altering the fluidity of the membrane both below and above the phase transition temperature[19]..

1.2.2.4. Non-structural components

For structural purposes, no components other than phospholipids and sterols need to be incorporated into the membrane. Because the membrane interior is a very fluid aliphatic medium composed of molecules associated by non-covalent interactions it will readily accept and retain a wide range of lipophilic compounds without the need for any fixed chemical structural specificity. Under normal circumstances these compounds can probably be accommodated in the membrane to a concentration of about 1-10 % by weight without the serious disruption[20] of the basic bilayer structure, although the membrane integrity as determined by fluidity or permeability may well be altered. In particular cases, where a specific interaction is known to occur between the compound and other membrane components (i.e. fatty acids, α-tocopherol), concentrations higher than 10 % may be achieved. Conversely, relatively low concentrations of certain substances, such as some polyene antibiotics, will completely disrupt the membrane, as a result of specific interactions.

1.3. Technology of liposomes: from preparation to application

1.3.1. Methods of preparation

The preparation process of liposomes for medical / pharmaceutical purposes mostly consists of three main stages. During the first, hydration step phospholipid molecules associate into lipid bilayer membranes which form liposomes finally. Both the hydration and the sizing stages are recommended to carry out above the gel-liquid crystal phase transition temperature[21]. Several of the preparation approaches listed in Figure 4. have been scaled up to industrial scale. If possible, the use of a high-shear homogenizer for the production of small vesicles is a first choice. No organic solvents are required to dissolve the lipids first, nor are detergents necessary to hydrate the lipids, and there is easy access to the appropriate

(commercially available) equipment. Problems with this method may arise if more than one lipid bilayer component is present. Further upscalable technologies:

- the detergent removal method, where the detergent is removed from the mixed micelles containing lipid bilayer components[22-24].
- the ethanol injection method[25], where the lipids are dissolved in ethanol and then mixed with the aqueous phase; and finally,
- the bilayer forming lipids can be lyophilized in the presence of the (lipophilic) drug. Upon hydration stage the highly porous lipid cake forms liposomes (MLV > 1 μm) and the lipophilic drug is liposome associated[26]. Liposomes are classified in the Table 1. based on their structural parameters (size, lamellarity) which is in close connection with the method of preparation.

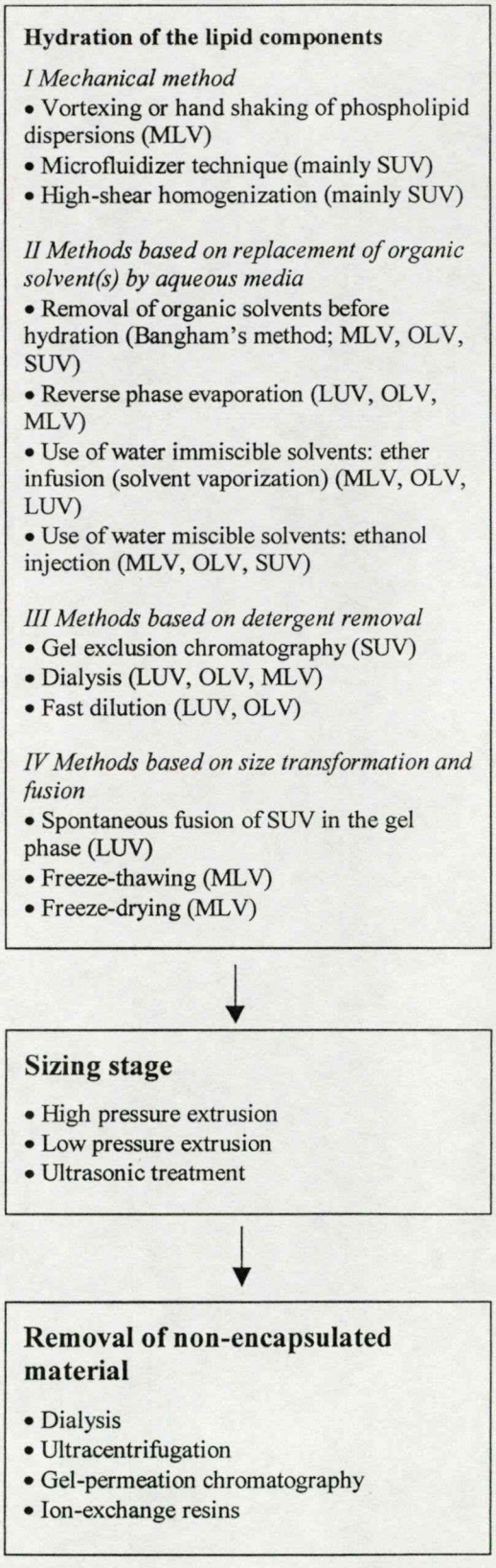


Figure 4.: Methods for the preparation of pharmaceutical liposomes (from Ref. 21.)

1.3.2. Loading of drugs into liposomes

Selection of an encapsulation protocol is largely dictated by concerns such as encapsulation efficiency, drug to lipid ratio, drug retention, ease of preparation, compatibility with regulatory agencies, sterility, ease of scale up, cost efficiency, as well as liposome and drug stability[27,28].

Passive entrapment techniques rely on the ability of liposomes to capture a certain aqueous volume (including dissolved solutes) during vesicle formation. Trapping efficiencies vary dramatically, ranging 1% or less for SUVs to as high as 88% for

Based on structural parameters	Based on method of liposome preparation
• MLV, multilamellar large vesicles- >0.5μm	• REV, single or oligolamellar vesicles made by the reverse-phase evaporation method
• OLV, oligolamellar vesicles- 0.1-1μm	
• SUV, small unilamellar vesicles- 20-100 nm	• MLV-REV, multilamellar vesicles made by the reverse-phase evaporation method
• MUV, medium sized unilamellar vesicles	• SPLV, stable plurilamellar vesicles
• LUV, large unilamellar vesicles- >100 nm	• FATMLV, frozen and thawed MLV
• GUV, giant unilamellar vesicles- >1μm	• VET, vesicles prepared by extrusion methods
• MVV, multivesicular vesicles (usually large >1μm)	• DRV, dehydration-rehydration vesicles

Table 1.: Liposome classification on the basis of pharmaceutical aspects (Ref. 21.)some MLVs[29].

Although maximum trapping efficiencies are obviously desirable, this property is often limited by the type of vesicles (SUV, LUV or MLV) required for in vivo applications. The low trapping efficiencies of SUV systems largely stem from their low trapped volume (0.2-0.8 μl / μmol lipid). LUVs and MLVs can exhibit higher values (1-30 μl / μmol lipid) and also can be prepared at higher lipid concentrations, leading to improved trapping efficiencies. Significant differences in trapping efficiencies are also experienced within each vesicle type depending on lipid concentration limitations and aqueous trapped volumes inherent in the specific liposome preparation procedures.

Active trapping techniques. At these procedures drug / lipid ratios are far greater than predicted on the basis of theoretical aqueous trapped volumes. At the one group of agents which are insoluble in water and can be incorporated into the lipid bilayer during vesicle formation. These materials are generally treated as lipids themselves, being mixed homogenously with the lipid component prior to vesicle hydration step. The second group of the agents are water-soluble materials which interact with the polar head group of phospholipids and are sequestered by the liposomes[30,31].

The most important approach to the active trapping is the pH gradient or remote loading method which was developed for the high performance entrapment of amphiphilic weak bases (such as doxorubicin and adriamycin)[32-37]. The entrapment efficiency can be increased up to 100 % in liposomes by using an ammonium sulfate gradient. This loading strategy, in principle, allow empty liposomes[38] to be loaded with a drug 'at the patients bedside'. This is an interesting option if a labile drug or labile liposomes need to be used.

Assay	Methodology / analytical target
Characterization	
pH	pH meter
Osmolarity	Osmometer
Phospholipid concentration	Lipid phosphorous content / HPLC
Phospholipid composition	TLC and HPLC
Cholesterol concentration	Cholesterol oxidase assay and HPLC
Drug concentration	Appropriate method
Chemical stability	
pH	pH meter
Phospholipid peroxidation	Conjugated dienes, lipid peroxides and FA composition (GLC)
Phospholipid hydrolysis	HPLC, TLC, and FA concentration
Cholesterol autooxidation	HPLC, TLC
Antioxidant degradation	HPLC, TLC
Physical stability	
Vesicle size distribution: submicron range	DLS
micron range	Coulter Counter, light microscopy, laser diffraction and GEC
Electrical surface potential and surface pH	Zeta-potential measurements
Numbers of bilayers	SAXS, NMR
Percentage of entrapped drug	GPC, IEC, and protamine precipitation
Dilution dependent drug release	Retention loss on dilution
Relevant body fluid induced leakage	GPC, IEC and protamine precipitation
Biological characterization	
Sterility	Aerobic and anaerobic cultures
Pyrogenicity	Rabbit or LAL test
Animal toxicity	Monitor survival, histology and pathology

Table 2.: Quality control assays of liposomal formulations; DLS, dynamic light scattering; FA, fatty acid; GPC, gel permeation chromatography; LAL, Limulus Amoebocyte Lysate; NMR, nuclear magnetic resonance; SAXS, small angle X-ray scattering; TLC, thin layer chromatography. Based on Ref. 39.

1.3.3. Sterilization, storage and quality control

Issues that are related to the parenteral administration of liposomes are the product sterility and the absence of pyrogens. The preferred way of sterilizing liposomes is by autoclaving. This is a realistic option; if the pH conditions are optimal, the drug is heat stable and lipophilic. Otherwise, reliance on filtration through membranes with 0.2 μm pores and / or aseptic production is necessary[39].

During the storage of liposomal formulations oxidation can be prevented by excluding oxygen from the injection vial, by addition of an anti-oxidant (i.e. vitamin E) or by selection of saturated acyl-chains in the phospholipid[40]. If those conditions cannot met, freeze drying

may be considered. Liposomes can be successfully freeze dried if the proper lyoprotectant is used and proper freeze-drying conditions are chosen[41]. They prevent aggregation and fusion upon reconstituting the cake. It is not always possible to avoid drug leakage from liposomes after freeze-drying-rehydration cycle, but recent insights into the mechanism of lyoprotection improve the chances for success[35].

The methods of quality control for liposomal formulations are summarized in Table 2. Regarding to the quality there are several special investigating procedures (i.e. drug release[42]) compared to other dosage forms.

1.4. Liposomes for medical applications

1.4.1. Reasons of using liposomes for pharmaceutical purposes

1. *Direction.* Liposomes in one hand can target a drug to the intended site of action in the body, thus enhancing its therapeutic efficacy (drug targeting, site specific delivery), in the other hand they may also direct a drug away from those body sites that are particularly sensitive to the toxic action of it (site avoidance-delivery).

2. *Duration.* Liposomes can act as a depot from which the entrapped compound is slowly released over a time. Such a sustained release process can be exploited to human therapeutic (but nontoxic) drug levels in the bloodstream or at local administration site for prolonged periods of time. Thus, an increased duration of action and decreased frequency of administration are beneficial consequences.

3. *Protection.* Drugs incorporated in liposomes, in particular those entrapped in the aqueous interior, are protected against the action of detrimental factors (i.e. degradative enzymes) present in the host. Conversely, the patient can be protected against detrimental toxic effects of drugs (Duration).

4. *Internalization.* Liposomes can interact with target cells in various ways and therefore able to promote the intracellular delivery of drug molecules that in their free (non-encapsulated) form would not be able to enter the cellular interior due to unfavorable physicochemical characteristics (i.e. DNA molecules).

5. *Amplification.* If the drug is an antigen, liposomes can act as immunological adjuvant in vaccine formulations[43,44].

These objectives for using liposomes are not mutually exclusive; often successful applications of liposomes are based on a combination of two or even more[21].

1.4.2 Classification of liposomes based on their composition and in vivo application

The liposome system has a major advantage over competing other colloidal drug carrier systems: it allows almost infinite possibilities to alter structural and physicochemical characteristics in order to tailor liposome formulations to specific therapeutic needs. The simplest way to classify the plenty of possible liposome versions, four major liposome types can be broadly distinguished on the basis of *composition and in vivo application* (Figure 5.).

Conventional liposomes. These can be defined as liposomes that are typically composed of only phospholipids (neutral and / or negatively charged) and / or cholesterol. They can vary widely in their physicochemical properties such as size, lipid composition, surface charge and fluidity of the phospholipid bilayers. Conventional liposomes are characterized by a relatively short blood circulation time. When administered in vivo by a variety of parenteral routes (often by intravenous administration), they show a strong tendency to accumulate rapidly in the phagocytic cells of the mononuclear phagocyte system (MPS), also often referred as reticuloendothelial system (RES). The major organs of accumulation are the liver and spleen, both in terms of total uptake and uptake per gram of tissue. An abundance of MPS macrophages and a rich blood supply are the primary reasons for the preponderance of particles in the liver and the spleen. A logical therapeutic translation of this MPS-directed distribution behavior is that conventional liposomes may be attractive for drug delivery to MPS macrophages. Indeed, the literature contains many examples of successful applications of conventional liposomes for the delivery of antimicrobial agents to infected macrophages[45]. Another interesting application of macrophage targeting involves the delivery of immunomodulators to

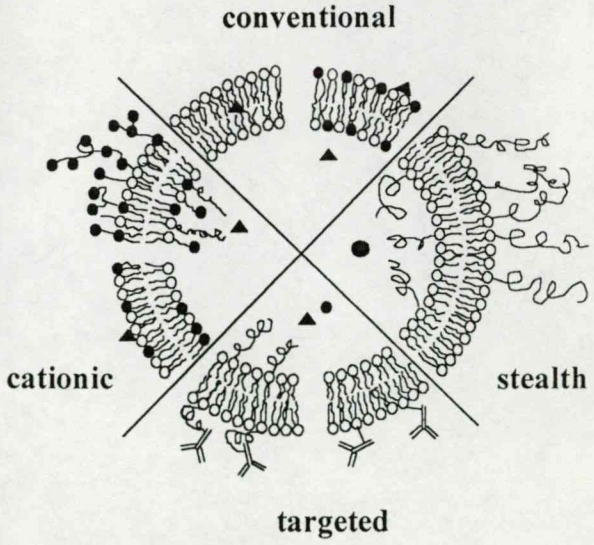


Figure 5.: The four major liposome types applyed for medical purposes. From Ref. 21.

increase the capacity of macrophages to kill neoplastic cells[46,47] and to increase resistance against infectious microorganisms[48]. Liposome-based vaccines have proved effective in experimental models against viral, bacterial and parasitic infections[49-52], as well as tumors[53]. Several liposomal vaccines have been tested in humans, and one of these, a liposomal hepatitis-A vaccine, has received marketing approval in Switzerland[54].

Long-circulating (or 'stealth') liposomes. In spite of the above-mentioned valuable applications for conventional liposomes, the fast and efficient elimination of conventional liposomes from the circulation by liver and spleen macrophages has seriously compromised their application for the treatment of the wide range of diseases involving other tissues. Long-circulating liposomes opened a realm of new therapeutic opportunities that were up to then unrealistic because of efficient MPS uptake of conventional liposomes[21]. Perhaps the most important key feature of long circulating is that they are able to extravasate at body sites where the permeability of the vascular wall is increased. Fortunately, regions of increased capillary permeability include pathological areas such as solid tumors and sites of infection and inflammation. It is illustrative for the importance of the long-circulation concept that the only two liposomal anticancer products that are approved for human use are based on the use of long-circulating liposomes for the tumor selective delivery of antitumor drugs (Doxil® or Caelyx® and DaunoXome®). At present the most popular way to produce long-circulating liposomes is to attach hydrophilic polymer polyethylene glycol (PEG) covalently to the outer surface[55-59]. Steric stabilization results from the local surface concentration of highly hydrated PEG groups that create a steric barrier against interaction with molecular and cellular components in the biological environment[60,61]. The ability of these type of liposomes to persist in the blood and to localize in sites of pathology also provides applications for drug delivery outside the oncology. Other examples can be found in recent evaluations of their usefulness for delivery of scintigraphic agents[62,63], antibiotics[64], and cytokines[65,66]. Their persistence in blood suggests that they may also prove effective to provide slow release of therapeutics such as biomacromolecules into the systemic circulation[67]. One study of this approach has been reported using the peptide hormone vasopressin[68].

Targeted liposomes: These vesicles have specific 'homing device' on their surface to enhance target site binding[69]. Till this time among them the immunoliposomes attached with antibodies or antibody fragments were investigated the most extensively[70-73]. Aldough

immunoliposome systems have been examined for various therapeutic applications, the primary focus has been the targeted delivery of anticancer agents[74]. Successful attempts have been made to prolong the half-life of immunoliposomes after intravenous administration by coating with PEG, thus giving them a greater chance to reach target sites other than MPS macrophages. Not only antibodies can be attached to liposomes as homing devices to the surface of liposomes in order to obtain a targeted drug release system but also hyaluronic acid derivatives and folic acid are capable to direct liposomes to different tumor cells. One of these cell types (mostly in lung cancers) over-expresses the CD44 cell surface receptor which binds hyaluronic acid whilst at the other type of these cells folate receptors are presented in enormously high number[75-77].

Cationic liposomes. These are the youngest members of the liposomal systems and they are in spotlight among the delivery systems under development for improving the delivery of genetic material[78-80]. Their cationic lipid components interact with, and neutralize, the negatively charged DNA, thereby condensing the DNA into a more compact structure. The resulting lipid-DNA complexes, rather than DNA encapsulated within liposomes, provide protection and promote cellular internalization and expression of the condensed plasmid[81-84].

1.5. Importance of the interactions between liposomes and polymers

Many attempts have been made to avoid the MPS-trapping of liposomes and to achieve longer half-lives in the bloodstream by modification of the liposomal surface. As we mentioned previously successful results have been obtained by the modification of liposomes with poly(ethylene glycol) (PEG) derivatives. In addition to this approach until recently only a few studies have dealt with other polymers physically adsorbed on the surface of liposomes (Figure 6.). It can be explained by two major reasons why we need for such researches. Primarily; however PEG protects liposomes from MPS it may also inhibit their interaction with the biological milieu at the site of action. For example: if we covered liposomes with biodegradable polymers adsorbed physically it might be easier to transport active agents into cells in pathological tissues. Secondly, this is a more simple and cheap way to prepare steric stabilized vesicles compared to the production of peggylated phospholipids.

Poly (vinyl alcohol) (PVA) and PVA modified with a hydrophobic moiety (PVA-R), were examined as a coating material for liposomes to be loaded with an anticancer drug,

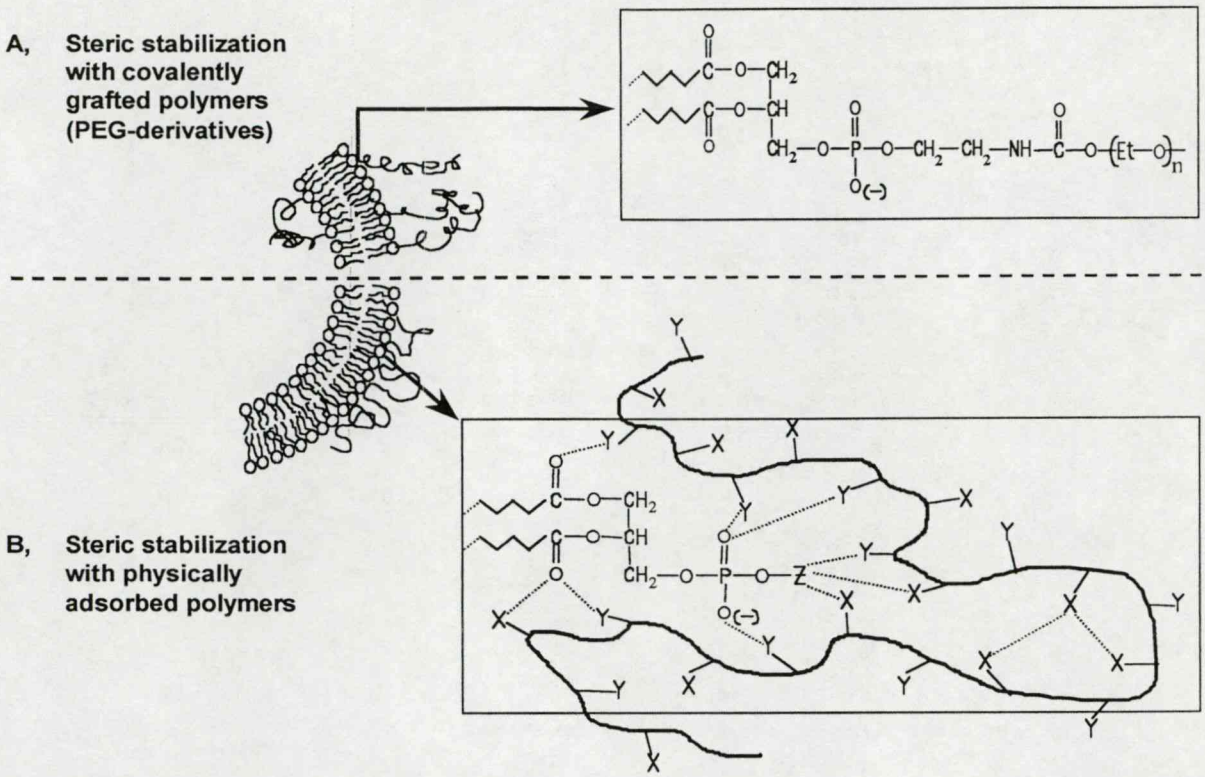


Figure 6.: Section A,: liposome membrane with peggylated phosphatidyl ethanolamine lipid component; section B,: liposome surface with polymers attached by physical forces ie. hydrogen bonds.

doxorubicin by **Takeuchi et al.**[85,86]. Besides the animal experiments (pharmacokinetic and distribution studies) they performed dynamic laser light scattering (DLS) measurements and determined the coating amounts of polymers. It was shown that PVA-R binds to liposomes with higher affinity than PVA due to the anchoring of the 'R' alkyl chains.

The kinetic (long-term) stability of dimyristoyl phosphatidyl choline (DMPC) liposomes was examined by **Grohmann et al.** who incorporated PVA and poly(vinyl pyrrolidone)(PVP) derivatives into the bilayer membrane of the vesicles[87]. These experiments were carried out at different pH values, and the enhancement of the average vesicle diameter was checked by DLS after certain time intervals for 3 months. It was concluded that the rate of diameter increase can be significantly diminished by stabilizing the dispersions with PVP or PVA and its co-vinyl acetal, propional or butyral copolymers. In another approach differential scanning calorimetry was used to examine the polymer-liposome interactions[88]. It is also very noteworthy the work of **de Rubalcava et al.**[89] who made a detailed examination on the interactions between liposomes and hydoxypropylmethylcellulose.

Enteral absorption of insulin was examined from liposomes coated with a mucoadhesive polymer: chitosan (CS). In this study the change in the zeta potential afforded evidence of the polymer layer on the liposome surface[86] and the ability of mucoadhesion was compared with other polymers (PVA and poly(acrylic acid)) attached to cholesterol and 'R'. In the case of insulin loaded liposomes coated with CS, added orally, the rate of diminishing of the blood glucose level was almost the same as it was experienced when insulin was administered itself, subcutaneously.

In another interesting study published by **A. de la Maza et al.**[90] protection of PC liposomes against the solubilizing effect of a nonionic surfactant, Triton X-100 was investigated after a bacterial glycoprotein (GP) exopolymer had been adsorbed onto the surface. They concluded that the higher the proportion of the GP in the system the lower the surfactant ability to saturate and solubilize liposomes. The fact that surfactant concentration at sublytic and lytic level was lower and similar than the surfactant critical micelle concentration indicates that the interaction at these two levels was mainly ruled by the action of surfactant monomers and by the formation of complex mixed micelles: surfactant/PC/GP.

Polymerized surfactant vesicles entrapping colloidal platinum were prepared by **J. H. Fendler et al.** for catalytic and artificial photosynthetic purposes. They built in double bonded functional groups either to the head group or the hydrocarbon chain of the surfactant molecules of which the vesicular systems were prepared. The polymer network was produced by photopolymerization (UV light)[91].

Poly[N-(2-hydroxypropyl)methacrylamide] (polyHPMA)[92] which is a popular biomedical polymer and xanthan gum[93] were also used for steric stabilization of liposomes.

1.6. Research aims

In the knowledge of the preliminaries discussed before (1.5.) my aim was to develop methods for the detailed investigation of the liposome-polymer interactions in model systems composed of three liposome types: **1.** soy lecithin phospholipid (SLPL) / Chol / dicetyl phosphate (DCP) = 25 : 3 : 2, **2.** DPPC / Chol / DCP = 4 : 1 : 1, **3.** DPPC / Chol = 2 : 1, and neutral polymers: PVA, PVP, dextrane. The methods of examinations are summarized in the Figure 7.

Direct methods:

1. Quantitative measurement of the amount of polymers adsorbed on the surface of liposomes with different analytic methods,
2. Dynamic laser light scattering measurements in order to prove the existence of polymer-adsorption on the basis of increasing vesicle diameters,
3. Titration microcalorimetry for the detection of the heat effects caused by the interaction of polymers and the liposome surface,
4. X-ray diffraction examinations to investigate the structural changes of the lipid membrane brought by the polymer-adsorption
5. High precision densimetric measurements to examine the expansion / compression of the liposome membrane on the basis of molar excess volume (V_m^e) values calculated from the densimetric data.

I also used an *indirect*, test dye release method which detects the changes in the membrane permeability caused by the polymer adsorption. This process consisted of two main stages:

1. Optimization of the active entrapment (remote loading) of a weakly basic test dye, acridine orange into the liposomes
2. Test dye release with self-developed membrane diffusion cells.

On the basis of the results obtained I tried to explain the reasons of the differences in the rate of adsorption observed considering the physical and chemical properties of the liposome types and polymers.

2. Experimental

2.1. Materials

Soy lecithin (SL): Lucas Mayer, Germany. Dipalmitoyl phosphatidyl choline (DPPC), dicetyl phosphate (DCP) cholesterol (Chol) and N-[2-Hydroxyethyl] piperazine -N'-[2-ethanesulfonic acid] (Hepes): Sigma, St. Louis, Mo., USA. Poly (vinyl pyrrolidone) K30 (PVP) $M_r \approx 40000$, acridine orange hydrochloride (AO), Tris-(hydroxymethyl)-aminomethane (Tris), and hexadecylpyridinium chloride (HDPCl): Fluka Chemie, Switzerland. Protamine hydrochloride (Prot): F. Hoffmann-La Roche, Switzerland. Sephadex G50f xerogel: Pharmacia, Sweden. Poly (vinyl alcohol) PVA $M_r \approx 72000$, dextrane (DEX) $M_r \approx 40000$ and Triton X-100: Reanal, Budapest, Hungary. All other substances like, sodium chloride, sodium hydroxide, disodium hydrogenphosphate, potassium dihydrogenphosphate, potassium iodide, iodine, ammonium sulphate, ethanol, methanol, chloroform are the products of Reanal and have the purity of analytical grade.

2.2. Methods

2.2.1. Analytics of phospholipids

There were two important reasons to use phospholipid analytics during my works: in one hand it had been important to know the amount of phospholipids in the SL before I started to prepare liposomes made of that, in the other hand it had to be known the loss of liposomes caused by gel permeation in case I separated the liposome entrapped AO from the non-entrapped one. It is also advised to measure the liposome concentration before the gel permeation because at downsizing with the LiposoFast® (Avestin Inc. ON, Canada) the membrane filter may bound a certain amount of liposome. Accordingly I determined the amount of liposomes as phospholipid concentration with the Rouser's assay[94,95]. In this method, phospholipid phosphorus is first acid-hydrolysed to inorganic phosphate with perchloric acid 70 % at 200 °C. This is converted to phospho-molybdic acid by the addition of ammonium molybdate, and the phospho-molybdic acid is quantitatively reduced to a blue coloured compound by ascorbic acid at 100 °C. The intensity of the blue colour is measured by spectrophotometer (UVIKON 930, $\lambda = 810$ nm).

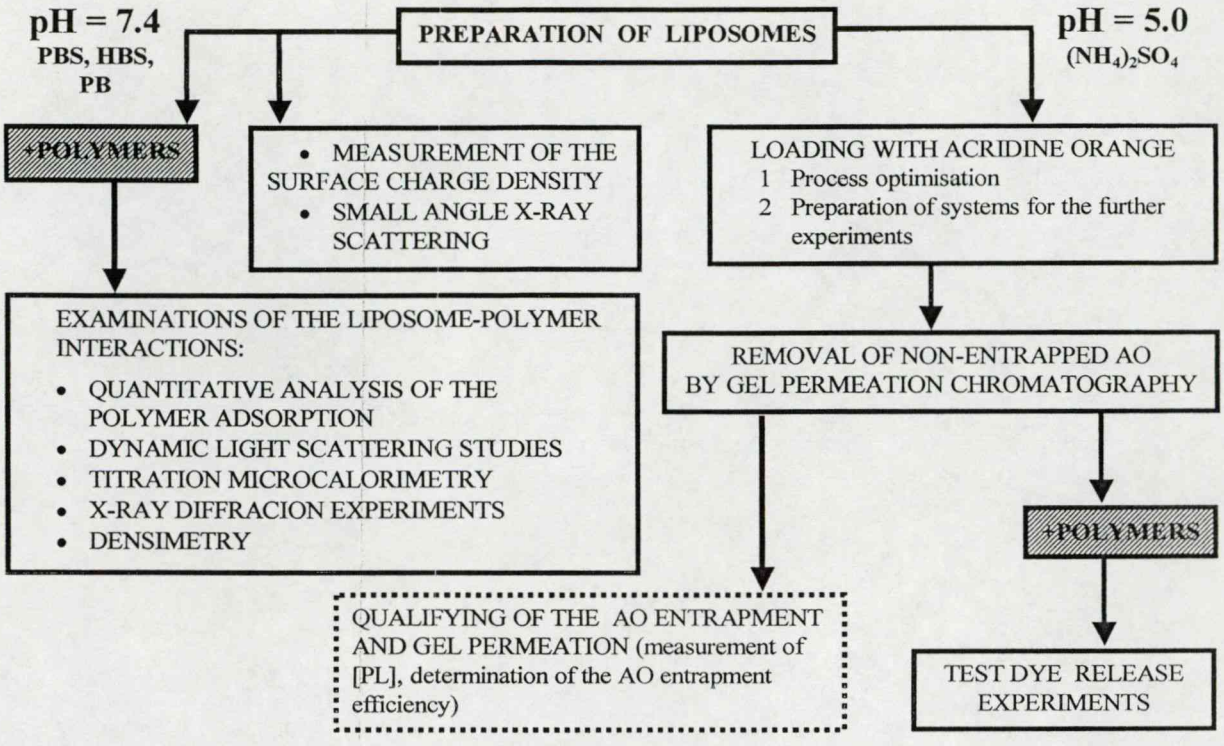


Fig. 7.: Survey figure of the methods used. After preparation of the liposomal systems there are two main approaches to investigate the polymer-liposome interactions: 1. direct examinations with analytic and structure-scanning methods (LEFT), 2. indirect examination on the basis of the change in the AO permeability (RIGHT).

2.2.2. Preparation of liposomes

Determination of the phospholipid (PL) content of SL was made according to the PL-assay described previously. This value was found to be 85 ± 1.2 %. The SL was the major component of the one liposome type of which lipid composition was set to soy lecithin phospholipid (SLPL) / Chol / DCP = 25 : 3 : 2 (mol / mol). The other two systems had the composition of DPPC / Chol / DCP = 4 : 1 : 1 and DPPC / Chol = 2 : 1. Liposomes were prepared by the classic lipid film hydration (or Bangham) method[96,97]. The lipids were dissolved in a mixture of 9:1 (v/v) chloroform / methanol. Lipid films were prepared on the wall of a round-bottomed flask with a Rotadest 2118 vacuum evaporator.

After the removal of all traces of organic solvent films were hydrated at 50 °C either in 0.01 M phosphate buffered saline (PBS) or in Hepes buffered saline (HBS) both with the pH value of 7.4. 0.1 M or 0.06 M ammonium sulphate (pH = 5.0) was used as hydration medium if I wanted to prepare AO loaded liposomes afterwards. Lipid films of liposomes intended for

the surface charge density measurements were hydrated in phosphate buffer (PB) which was free of sodium chloride and had the concentration of 0.01 M.

The average vesicle diameter of the coarse multilamellar vesicle (MLV) dispersions formed by powerful hand shaking of the round bottomed flasks was reduced either by sonication with a Realsonic RS-16F ultrasonic bath or extrusion through a 100 nm pore sized polycarbonate filter (device: LiposoFast™) depending on the composition of liposomes. Both the hydration and downsizing processes has to be done above the main phase transition temperature (T_m) of the phospholipids used[98-100]. The size of liposomes was checked by dynamic laser light scattering (DLS) measurements finally. The survey of the experiments made on the freshly prepared liposomes is depicted in the Figure 7.

2.2.3. DLS measurements

DLS measurements were done in order to follow the change of the liposome diameter during the different work stages. I used a SEMA-Tech SEM-633 with He-Ne laser, 5 mW (λ = 633 nm) instrument at a measuring angle of 90 ° for 100 seconds. The samples to be examined were diluted approx. 50-fold with the suitable media. The other important role of DLS exams was to prove the existence of polymer-adsorption on the basis of increasing vesicle diameters. In these cases samples with the polymer / liposome ratios of 0.04-0.80 (w/w) were examined immediately after their dilution.

2.2.4. Determination of surface charge density

The surface charge density of liposomes dispersed in 0.01 M phosphate buffer (pH = 7.4) was determined with a Mütek PCD 02 particle charge detector (PCD). The liposome concentration was diluted to 7.5 mg / ml in each original dispersion, then these systems were titrated with 0.1 M HDPCl solution in the measuring cell (10 ml) of the PCD. The streaming potential was measured simultaneously and the endpoint of this process was indicated by a PCD signal of 0 mV. Based on the consumed volume of HDPCl the surface charge density is calculated by:

$$Z = V^0_{charge} \cdot C_{HDP}{}^{+} / m \qquad (1)$$

where: Z = surface charge density in milliequivalents per gram (mEq / g),

V^0_{charge} = consumed volume of 0.1 M HDPCl (ml) at zero charge,

m = mass of the liposomes in the measuring cell (g),

C_{HDP}^{+} = concentration of HDPCl (0.1 M).

2.2.5. Determination of the amounts of polymer adsorbed

I added PVA or PVP or DEX at the polymer / liposome ratios of 0.04-0.80 (w/w) to the liposomes prepared in PBS and after mixing by vortexing the systems they were incubated at 25 ± 0.1 °C. In the samples got into adsorption equilibrium, liposomes with adsorbed polymers on their surface were separated either by ultracentrifugation with a Beckmann TL 100 ultracentrifuge or after aggregating with protamine by centrifugation from the dispersion media[97,101]. The equilibrium concentrations (C_e) of the non-adsorbed polymers were determined form the supernatants by the following analytic methods (Figure 8.).

A, Analysis of the total organic carbon (TOC) content with Euroglas TOC-1200 Total Organic Carbon Analyzer. This method is suitable for the quantitative determination of all the polymers (PVA, PVP, DEX) we used. The concentration-TOC signal (ppm) calibration lines were recorded between the concentration range of 25-800 μg / ml for each polymer.

B, Spectrophotometric measurement (UV-VIS) with Kontron Uvikon 930 spectrophotometer for the determination of PVA and PVP. In the case of PVA I added specific reagent solution containing iodine, boric acid and potassium iodide[102] to the samples taken from the supernatants. The reagent forms a green coloured complex from PVA and the optical density of this colour is measurable at λ = 670 nm. The concentration-absorbance calibration line was

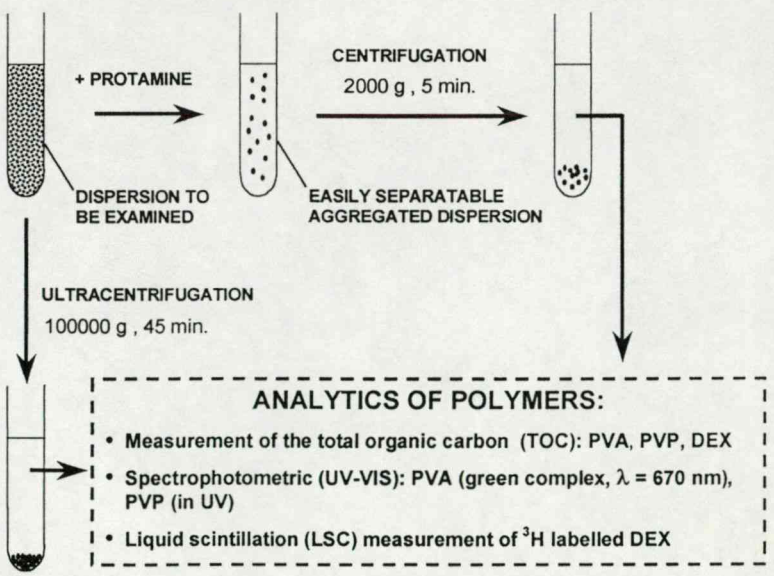


Fig. 8.: Quantitative analysis of the polymers adsorbed. Liposomes are separated from their dispersion media by centrifugation first, then polymers are determined from the supernatants.

recorded between the concentration range of 6.25-40 μg / ml. The measurement of PVP is more complicated: in the absence of a specific chromophore - producing reagent I was forced to measure in the UV. In order to circumvent this problem we minimized the disturbing effect of unbound liposomes and Prot. I added increasing amounts of Prot to dispersions containing only liposomes and after centrifuging them I searched for the absorbance minimum at 210 nm. Figure 9. reveals the optimal ratio of Prot / phospholipid at where PVP displays an absorbance maximum in ethanol : water = 9 : 1 (v/v). I added Prot in this Prot / phospholipid ratio to the liposome-PVP systems to be examined. This was followed by centrifugation, dilution of an aliquot of the supernatant with 96 % ethanol and measurement of the concentration of unadsorbed (equilibrium) PVP at 210 nm.

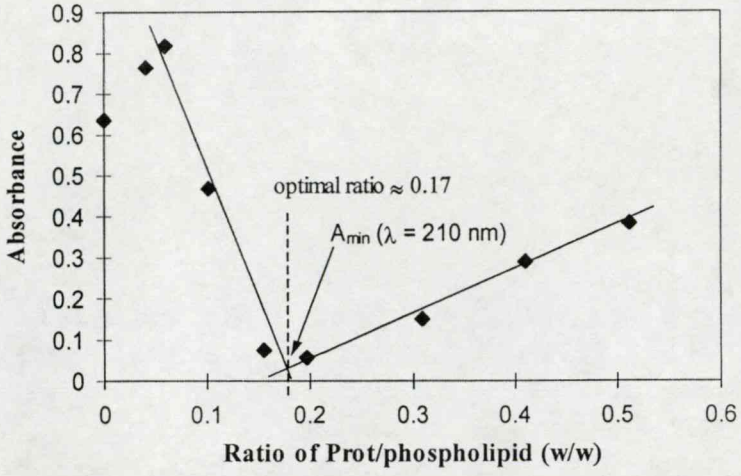


Fig. 9.: Determination of the optimal Prot/phospholipid ratio as the minimum of absorbance in order to minimize the disturbing effect of nonprecipitated liposomes and Prot in the measurement of non-adsorbed PVP.

C, Measurement of the radioactivity with liquid scintillation (LSC) for the determination of DEX. There was certain amount of DEX labelled with tritium by the isotope exchange reaction[103] in a device built specially for this purpose[104]. The non-labelled (cold) DEX and the tritiated DEX (^{3}HDEX) were added to the liposome dispersions at the rate of DEX / ^{3}HDEX = 9 : 1 in order to obtain samples with different DEX / liposome (w/w) ratios. After ultracentrifugation of the samples 10 μl of the supernatants were shaken with 5 ml of toluene-Triton-X scintillation cocktail then the radioactivities were determined with a Searle Delta 300 liquid scintillation counter. The 5th, liposome-free member of the SLPL / Chol / DCP system was chosen as standard with the DEX concentration of 0.181 % (w/v) and the activity of 353278 cpm.

The adsorption was expressed via the adsorption isotherms at 25 ± 1 °C by the following equation:

$$n^s_{polymer} = V \cdot (C_0 - C_e) / 100 \cdot m \qquad (2)$$

where: $n^s_{polymer}$ = the amount of polymer adsorbed on 1 g of liposome (mg / g)

C_0 = the initial concentration of the polymer (mg / 100 ml)

C_e = the measured equilibrium concentration (mg / 100 ml)

V = the volume of the sample (ml)

m = the mass of liposomes in the sample (g).

Examinations are classified by Table 3. regarded to the type of liposomes and polymers.

Type of liposomes	Polymer	Method of analysis after UCF	Method of analysis after PROT
SLPL / Chol / DCP = 25 : 3 : 2	*PVA*	VIS	VIS
	PVP	-	UV
	DEX	LSC	-
DPPC / Chol / DCP = 4 : 1 : 1	*PVA*	VIS, TOC	-
	PVP	TOC	-
	DEX	TOC, LSC	-
DPPC / Chol = 2 : 1	*PVA*	VIS, TOC	-
	PVP	TOC	-
	DEX	TOC, LSC	-

Table 3.: Classification of the analytic methods used for determination of the amount of polymers adsorbed. UCF and PROT mean the way of separation of liposomes either by ultracentrifugation or centrifugation after protamine-aggregation.

2.2.6. Densimetry and molar excess volumes

The molar excess volumes (V_m^E) of the systems can be calculated on the basis of the density and compositional data in cm^3 / mol:

$$V_m^E = [(x_1 \cdot M_1 + x_2 \cdot M_2) / \rho_{1,2}] - [x_1 \cdot M_1 / \rho_1] - [x_2 \cdot M_2 / \rho_2] \qquad (3)$$

where: M(i = 1,2) are the average molar weights of liposomes and polymers, x(i = 1,2) are the molar fraction, ρ_1 and ρ_2 are the densities (g / cm^3) of the liposome dispersions in the absence of polymers and polymer solutions with C_0 concentration. $\rho_{1,2}$ is the measurable density of the polymer / liposome = 0.04-0.50 (w/w) systems.

Measurements were made with an Anton Paar DMA 58 densimeter. Densimetry is based on high precision frequency measurement and the density of the complex liquid examined is calculated from the change of frequency[105].

2.2.7. Calorimetric measurements

These exams were made on the SLPL / Chol / DCP liposomes and PVA. The data were obtained with a thermometric thermal activity monitor (TAM Type 2277) titration microcalorimeter. A portion of the original liposome dispersion (0.333 cm^3 in HBS) was diluted to 2.00 cm^3 with HBS, and the total enthalpies (Table 2) were determined when 800 µl of 0.5 % PVA were added in ten steps. The PVA / liposome ratio (w/w) was set from 0.04 to 0.4. The total duration of this measurement was 17 hours (2 hours for the baseline and 1.5 hours for each step). I also measured the dilution enthalpies of both liposomes and PVA under the same conditions as mentioned above.

2.2.8. XRD studies

The aim of XRD measurements was to detect the structural changes brought about by polymer adsorption in the liposome membrane. The samples containing SLPL / Chol / DCP liposomes in HBS and PVA or PVP were first dried in a heat box and then exposed to water vapour at 60 °C for 12 hours. They were measured in both dry and water-vapour-exposed conditions over angles from 1° to 8° (Philips PW1820 diffractometer Cu Kα, 40 kV, 35 mA). The accuracy of identity period (d_L) was ± 0.01 nm.

2.2.9. Small angle X-ray scattering (SAXS experiments)

These exams were used to justify the transition of the DPPC-liposomes from the gel to liquid crystalline phase. Liposomes were composed of DPPC (100 %) or DPPC : Chol = 2 :1. Samples were heated from 25 °C to 50 °C in eight steps while small angle X-ray scattering was measured using a Kratky camera (KCEC/3 Anton-Paar, Austria) with a position sensitive detector (PSD 50M, Hecus M. Braun-Graz X-Ray Systems GmbH, Austria). The X-ray radiation was generated by a Philips PW 1830 generator, and filtered trough a 20-micron thick Ni plate to obtain a CuKα radiation (λ = 0.1542 nm). The width of the beam was 14 mm and

its thickness, c.a. 80 mm at the sample. The camera was evacuated to about 10-2 torr vacuum. The samples were filled into a 1 mm diameter quartz capillary cell. Data were collected for 1000 seconds in the angle range of 2Θ = 0.1-7.9 degrees.

2.2.10. Entrapment of acridine orange into liposomes; membrane diffusion experiments

2.2.10.1. Preliminary experiments for the optimisation of the loading procedure

The choice of acridine orange as drug modeling test dye can be explained by several reasons. First of all it can easily be detected spectrophotometrically because of its high molar extinction coefficient. The weak basic property (pK_a = 9.25) and the hydrophobic character of the base form are important factors which promote the loading of AO to the internal space of liposomes. Several antineoplastic agents having similar chemical structure can be transported through lipid membranes by this so called remote loading method.

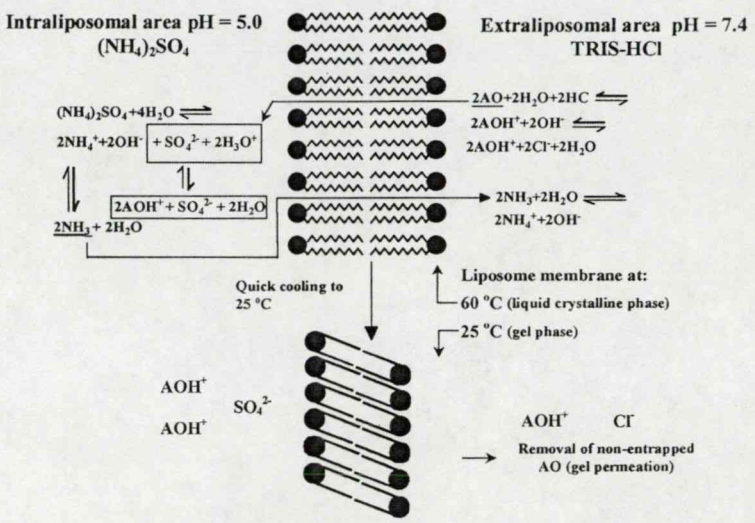


Fig. 10.: Mechanism of the remote loading process. The uncharged form of the weak base passes through the membrane and its sulphate salt forms in the intraliposomal space.

During the loading optimisation experiments we examined the effect of AO / PL ratio and the quality of the buffer in the extraliposomal area on the entrapment efficiency. Since liposomes may undesirablely aggregate during loading[34] it is also necessary to check the average vesicle size by DLS simultaneously. I searched for the optimal AO / PL ratio and buffer quality by which the highest entrapment efficiency with the smallest aggregation rate can be resulted.

The pH of the SLPL / Chol / DCP liposome dispersion hydrated in 0.06 M ammonium sulphate was set from 5.0 to 7.4 with 0.1 M sodium hydroxyde and after it had been divided into three equal volumes, three buffer solutions with pH 7.4 (Hepes-sodium hydroxide, Tris-hydrochloric acid, and phosphate buffer) were added so that the intra- and extraliposomal

spaces remain isoosmotic. This fact was achieved by addition of sodium chloride. The [PL] was cca. 0.027 M and the concentration of the appropriate buffer was 0.01 M in each dispersions at the end of this step. AO was added to the 2 ml of aliquots of these dispersions so that the molar ratio of AO / PL change between 0.02-0.30 and [AO] (C_1) be in the range of 0.30-4.50 mM. Due to the pH-gradient ($\Delta pH \approx 2.4$) set up between the intra- and extraliposomal areas and the incubation of the samples at 50 °C for 1 hour AO can be concentrated into the intraliposomal aqueous phase (Fig. 10.).

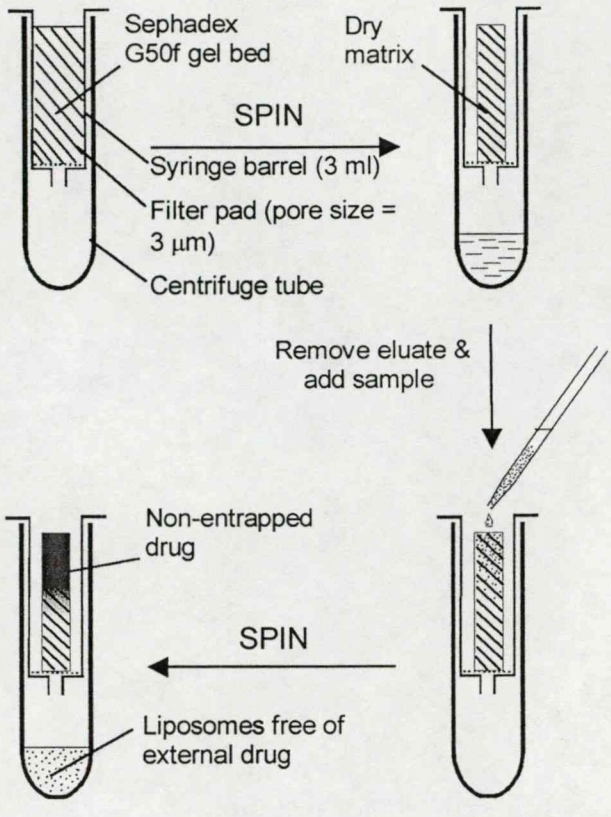


Fig. 11.: Removal of the non-entrapped material by minicolumn-centrifugation. The method is satisfactory for solutes less than 7000 molecular weight

The entrapment efficiency (E^{opt} %) of AO was determined with a similar manner as it was described before, at the quantitative measurement of the polymer adsorption. 200 μl of volumes were taken from the samples and equal volume of Prot solution was mixed with them so that we set the Prot / PL (w/w ratio) to approx. 0.3. The aggregated liposomes were centrifuged at 2000 g for 5 minutes and the concentration of the non-entrapped AO (C_2) was measured from the supernatants spectrophotometrically at 492 nm. Based on these measurements:

$$E^{opt}\ \% = (1-C_2/C_1) \cdot 100. \qquad (4).$$

2.2.10.2. Gel permeation chromatography

I used gel permeation chromatography combined with centrifugation in order to separate the non-entrapped AO from the loaded liposomes. According to Ref. 106. the Sephadex G 50 (fine) xerogel was hydrated in HBS at room temperature for 24 hours. The hydrated gel was filled into plastic syringe barrels (3 ml) which were placed into centrifuge tubes with suitable

size. The syringe barrels were plugged with Marcherey-Nagel (type: MV) filter pads with the pore size of 3 μm. The processing of this method is depicted in the Figure 11.

2.2.10.3. Measurement of AO release from liposomes

According to the optimisation of the AO loading process –the maximum loading effect with the minimum aggregation rate- we added Tris-HCl buffer (pH = 7.4, 0.1 M) to the dispersions in order to provide the permanence of pH gradient between the intra- and extraliposomal spaces during loading. In the case of SLPL / Chol / DCP liposomes the AO / PL, at the DPPC / Chol systems the AO / total lipid (DPPC + Chol) molar ratio was set to 0.1. The extraliposomal pH of the 2 ml of liposome dispersions hydrated with 0.1 M ammonium sulphate solution was set from 5.0 to 7.4 by the addition of 0.1 ml of sodium hydroxide solution (0.047 M). The osmotic equivalence of the inner- and outer liposomal spaces was reached by adding calculated amount of sodium chloride to the Tris-HCl buffer solution. Samples with the final volume of 3 ml were incubated for 1 hour at 50 °C and then cooled to room temperature in icy bath suddenly. The non-entrapped AO was removed by gel permeation combined with centrifugation from the extraliposomal media (Figure 11.). While the loss of liposomes caused by gel permeation can not be predicted I determined the amount of liposomes as PL concentration in the eluted dispersion with the Rouser's assay.

PVA or DEX had been added to the samples with known PL content in the polymer / liposome ratio of 0.1, 0.3 and 0.8 (w/w) before 0.3 ml of them were put into the donor phase of

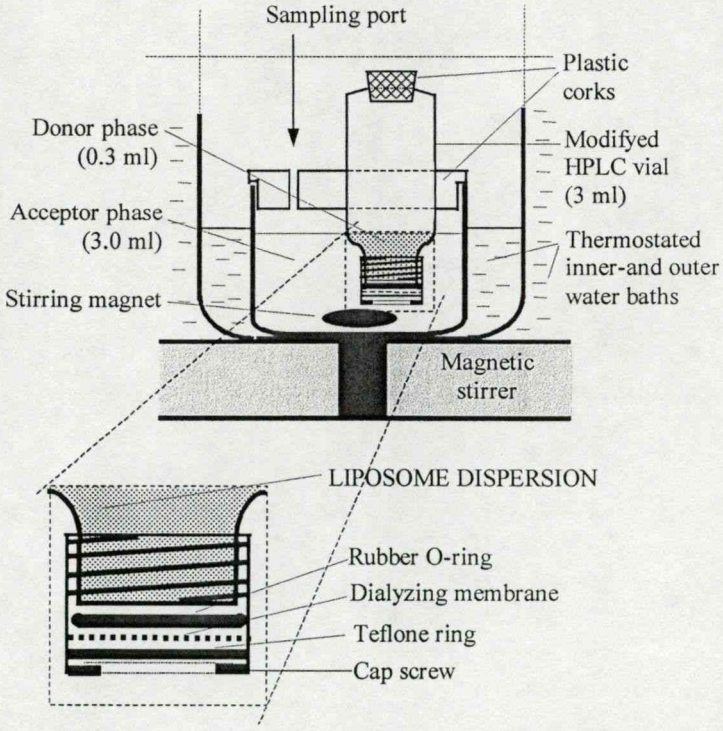


Fig. 12.: Self-developed membrane diffusion cell for the tests of liposomal acridine orange release.

the membrane diffusion cell (Figure 12.) thermostated to 37 °C. 0.5 ml of samples for the test dye release experiments were taken from the receptor phase at the following sampling times: 2, 4, 8, 12 and 24 hours and their absorbance was measured spectrophotometrically (λ = 492 nm). The volume of each sample was replaced with PBS to the receptor phase.

For the calculation of AO released from liposomes till certain sampling times I need to know the amount of AO in the donor phase at the zero time point. This value can be calculated from the result of the concentration measurement (C_Y) made after the disruption of eluted liposomes with Triton-X 100 [107]. This result gives the entrapment efficiency (E %) of AO according to the Eq. 5.:

$$E\% = (C_Y \cdot [PL]_X / C_X \cdot [PL]_Y) \cdot 100 \qquad (5)$$

where: C_X is the measured-in concentration at the beginning of loading and C_Y is the entrapment concentration of AO. $[PL]_X$ and $[PL]_Y$ are the phospholipid concentrations before and after the gel permeation chromatography.

If E % is substituted for the Eq. 6. the N_0 amount of AO in the donor phase (V_2 = 0.3 ml) is obtained:

$$N_0 = N_X \cdot E\% / 100 \cdot V_2 / V_1 \cdot [PL]_Y / [PL]_X \qquad (6)$$

where N_X (mol) is the amount of drug in the V_1 volume (ml) applicated to the gel column. N_0 is the base value for the calculation of the drug release results as follows:

$$N_1 = C_1 \cdot V^c \qquad (t_1)$$

$$N_2 = C_2 \cdot V^c + V^s \cdot C_1 \qquad (t_2)$$

$$N_3 = C_3 \cdot V^c + V^s \cdot (C_1 + C_2) \qquad (t_3)$$

$$N_n = C_n \cdot V^c + V^s \cdot (C_1 + C_2 + ... + C_{n-1}) \qquad (t_n) \qquad (7)$$

where $C_{1\text{-}n}$ values are the drug concentrations (M) of the samples taken from the acceptor phase and $N_{1\text{-}n}$ are the total amounts of drugs released from the liposomes till predicted sampling time points (t_1-t_n). V^c and V^s are the volumes of the whole diffusion cell (V^c = 0.0033 l) and samples (V^s = 0.0005 l). The rate of the cumulative drug release (Rel% as the percentage of N_0) at the n^{th} sampling is:

$$Rel\ \% = N_n / N_0 \cdot 100. \qquad (8).$$

3. Results and Discussion

3.1. Preparation of liposomes and downsizing

The final average diameters of liposomes used for different examinations are summarized in the Table 4. Downsizing was made in order to uniform the size of the liposomes. On the basis of DLS data we have concluded that downsizing is easier at physiologic pH value (7.4) with both ultrasonic bath and extrusion in the case of all the three liposome type than in the 0.1 M ammonium sulphate. While the size of SLPL / Chol / DCP liposomes could only be diminished by sonication, at the other two liposome types containing DPPC as major component the membrane-extrusion method was employed which is more effective than sonication considered to the favouring of downsizing effect and monodispersity. This method resulted average size of approx. 110 nm for the DPPC / Chol / DCP and DPPC / Chol liposomes. We suppose that the reason of the more troubled downsizing at pH = 5.0 is due to weakly acidic functional groups on the polar end of phospholipids of which has decreasing dissociation ability as the pH of the dispersion media grows more acidic. This phenomena makes the surfaces of both the lipid films to be hydrated and the liposomes more hydrophobic therefore it is more difficult to hydrate the films and to downsize the coarse liposome

Type of liposome	Method of downsizing	pH = 7.4 0.01 M PB	pH = 7.4 0.01 M PBS	pH = 5.0 0.1 M $(NH_4)_2SO_4$
SLPL / Chol / DCP = 25 : 3 : 2	Bath sonication	148.7 ± 13.8 nm (±sd) *≈ 40 mM*	150.3 ± 14.2 nm (±sd) *≈ 40 mM*	164.3 ± 13.4 nm (±sd) *≈ 40 mM*
DPPC / Chol / DCP = 4 : 1 : 1	Extrusion	110.4 ± 5.3 nm (±sd) *20.26 mM*	111.7 ± 5.4 nm (±sd) *20.26 mM*	1642 ± 152 nm (±sd) *60 mM*
DPPC / Chol = 2 : 1	Extrusion	114.5 ± 6.2 nm (±sd) *16.16 mM*	116.2 ± 7.3 nm (±sd) *16.16 mM*	151.2 ± 8.1 nm (±sd) *60 mM*

Table 4.: Average sizes of the liposomes used for different experiments. Measurements were done with DLS and data written by italic letters show the PL concentrations of the dispersions.

dispersion formed because liposomes with higher surface hydrophobicity have tendency to fuse or aggregate in aqueous media. The effectiveness of downsizing is influenced in one hand by the saturation grade of the fatty acid chains of phospholipids which has effect on the

fluidity of the liposome membrane, and in the other hand by the lipid concentration of the dispersions. This latter fact was set to higher values (40 and 60 mM) in the dispersions prepared at pH = 5.0 because the dilution resulted by the entrapment and separation of AO would make the final AO diffusion examinations impossible. Probably the combined effect of the saturated lipids composing the lipid membrane, the presence of DCP in 16.7 % (mol/mol) which gives a weakly acidic characteristic to the membrane and the relatively high PL concentration (60 mM) might cause the fact that downsizing of the DPPC / Chol / DCP liposomes in ammonium sulphate was not successful either we used extrusion or bath sonication. The average liposome diameter in this easily sedimenting dispersion was approx. 1642 nm.

3.2. Results of the surface charge density measurements

At the streaming potential of 0 mV which indicates the neutralisation of the liposomal surface, the V^0_{charge} values were calculated from the equations of the regression lines fitted to the titration curves depicted in Figure 13. If these data are substituted for Eq. 1. the Z surface charge density values are given. In the case of DPPC / Chol / DCP liposomes the regression line was fitted to the last two data points. Regarding to the Z values it is ascertainable that the surface of DPPC / Chol liposomes is neutral (Z = 0.013 mEq / g) because of the equal amount of positive and negative charges on the head group of the phosphatidyl choline molecules. The Z value of 0.136 mEq / g is given by negative charge represented by the known amount of DCP (16.7 mol/mol %) at the DPPC / Chol / DCP liposomes. The surface charge density of the SLPL / Chol / DCP system is more than three times higher (Z = 0.42 mEq / g) compared to the

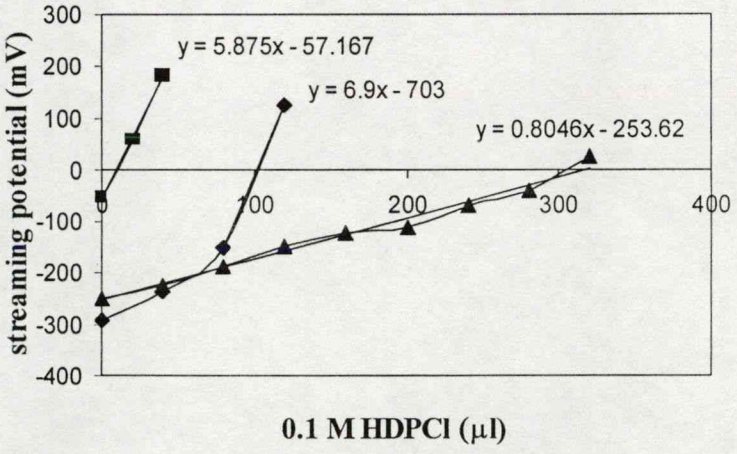


Fig. 13.: Results of the streaming potentiometric titration of DPPC / Chol = 2 : 1 —■—, DPPC / Chol / DCP = 4 : 1 : 1 —♦—, and SLPL / Chol / DCP = 25 : 3 : 2 —▲— liposomes with HDPCl. The surface charge density of 0.42 mEq / g of the SLPL / Chol / DCP liposomes derives from the negatively charged lipids occurred with high amount in SL.

previous one. This relatively big negative charge based on the composition derives partly from the DCP (6.7 mol/mol %) and partly from the components of SL like phospholipids (85 w/w %), glycolipids, sterols, tocopherols, and carbohydrates[108] which wear negative charge in unknown rate. Dispersions have titrated with HDPCl became aggregated systems after the negative charge of liposomes had been neutralized.

3.3. Quantitative description of the polymer-adsorption

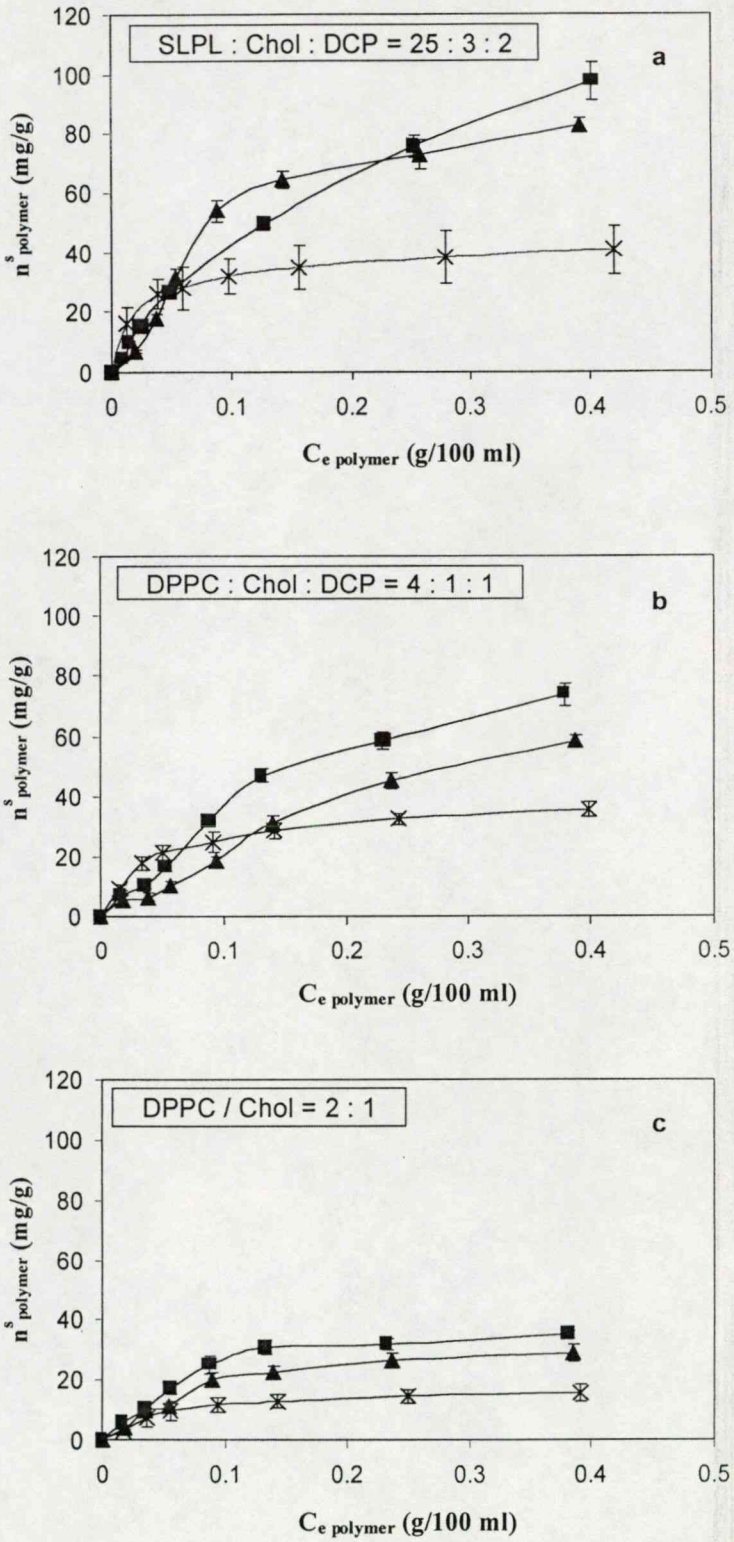
The C_0-C_e value of polymer-concentration change in the dispersions should be known in order to calculate the amount of polymer adsorbed. Determination of the C_e equilibrium concentrations was carried out with the methods described in the experimental section. According to the Table 3. where two methods were used to measure the polymer-adsorption in a certain polymer / liposome = 0.04-0.80 (w/w) system there adsorption isotherms were recorded on the basis of the averages calculated from the results of the two different measurements. Where we worked only with one method there the averages calculated from three parallel measurements (n=3) were used for the same purpose. Two major conclusions can be obtained from the results shown in Figures 14a-c and 15.: in one hand the rate of adsorption for all the polymers is increasing in the row of DPPC / Chol < DPPC / Chol / DCP < SLPL / Chol / DCP liposomes, in the other hand apart from small deviations the amount of polymers adsorbed is decreasing considered to the row of PVA>DEX>PVP. In the following part we try to explain these experiences regarding to the composition of the liposome types and the physical-chemical properties of the polymers.

A, The role of the phospholipid fatty acid-saturation

As the temperature increases biological membranes get to the liquid crystalline phase ($L_{\alpha'}$) from the gel phase ($L_{\beta'}$) through a transitional state ($P_{\beta'}$). In the $L_{\alpha'}$ phase the membrane is more fluid and has looser construction than in the $L_{\beta'}$ phase therefore if the membrane is in the $L_{\alpha'}$ phase the functional groups on the membrane are able to get interacted with other molecules easier. At different membranes composed of chemically homogenous phospholipids the $P_{\beta'}$-$L_{\alpha'}$ transition is well characterised with the main phase transition temperature (T_m). The T_m depends on the quality of the PL head group, the chain length of the PL fatty acid and the saturation of these mostly. While at the membranes containing DPPC as main component the T_m is approx. 41 °C [15], at the bilayer-systems built up from SL

phospholipids which are rich in unsaturated fatty acids, this value is much lower (Figure 3.). An exact T_m value can not be given here because of the chemical heterogeneity. Apart from this it is known that the T_m values of unsaturated phospholipids with different head groups are in the range of –30 to +30 °C.

The measurements of the polymer-adsorption were made at 25 ± 1°C at which temperature DPPC liposomes (Figures 14b and c) are in the orderly gel phase and have more rigid construction than SLPL liposomes. These latter systems are in the fluid, liquid crystalline phase and due to the loose membrane structure are more susceptible to form secondary binding forces (i.e. hydrogen bridges). This may partly explain that SLPL liposomes have high polymer adsorbing ability reaching 100 mg / g facing the smaller values of 35-75 mg / g of the DPPC liposomes.



Figures 14a-c: Adsorption isotherms of PVA —■—, PVP —x—, and dextrane —▲— on liposomes with different membrane compositions.

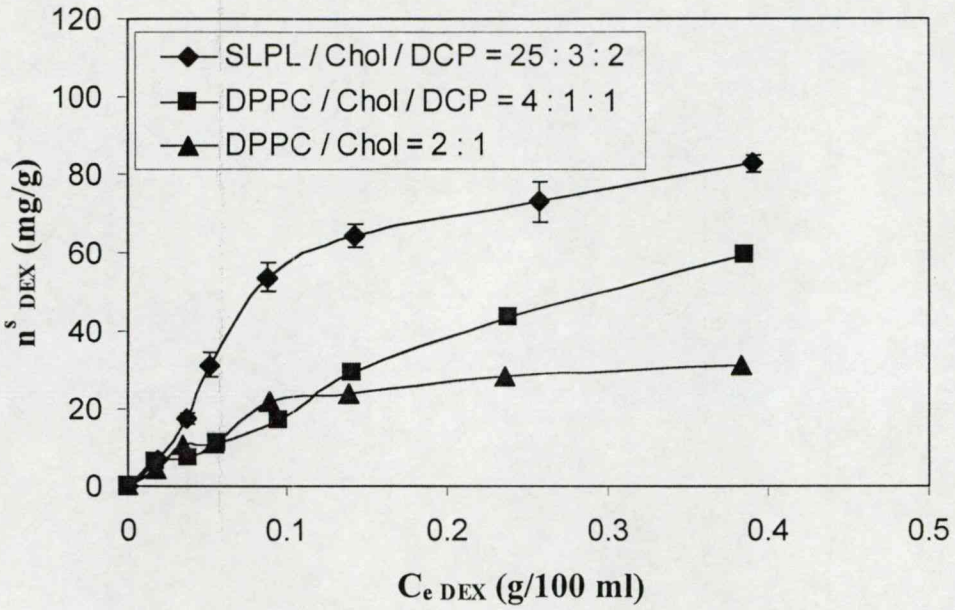


Fig. 15.: Results of dextrane adsorption based on the liquid scintillation measurements

B, The role of cholesterol content, SAXS experiments, temperature dependency

In addition to the PL-quality the liposome-membrane fluidity is influenced by other membrane composing lipids. Among them cholesterol has outstanding importance because it decreases the fluidity and increases the integrity of the biological membranes through filling the "extra spaces" between the PL molecules[109]. The increasing of the membrane composing molecule-integrity involved by the higher cholesterol content inhibits the permeation of the polymer –segments into the membrane by which the amount of the polymer adsorbed may be further decreased.

The comparison of the pure DPPC and DPPC / Chol liposomes can be seen in the Figures 16a and b obtained by SAXS. Intensities of the peaks recorded in the h (nm^{-1}) range of 1.50-2.50 represent the rate of orderliness of the lipid membrane. At the pure DPPC liposomes these peaks disappeared in the temperature range of pre-transition and transition (a). This phenomena is not particular to the DPPC / Chol liposomes (b) and may be explained by the literatural experiences: the presence of Chol decreases or eliminates (from 50 mol/mol %) the differential calorimetric (DSC) heat effect of the gel to liquid crystalline phase transition[19].

According to my conception the diminishing of the polymer adsorbing-ability is explainable with the decreasing of the membrane fluidity and the growing of the membrane

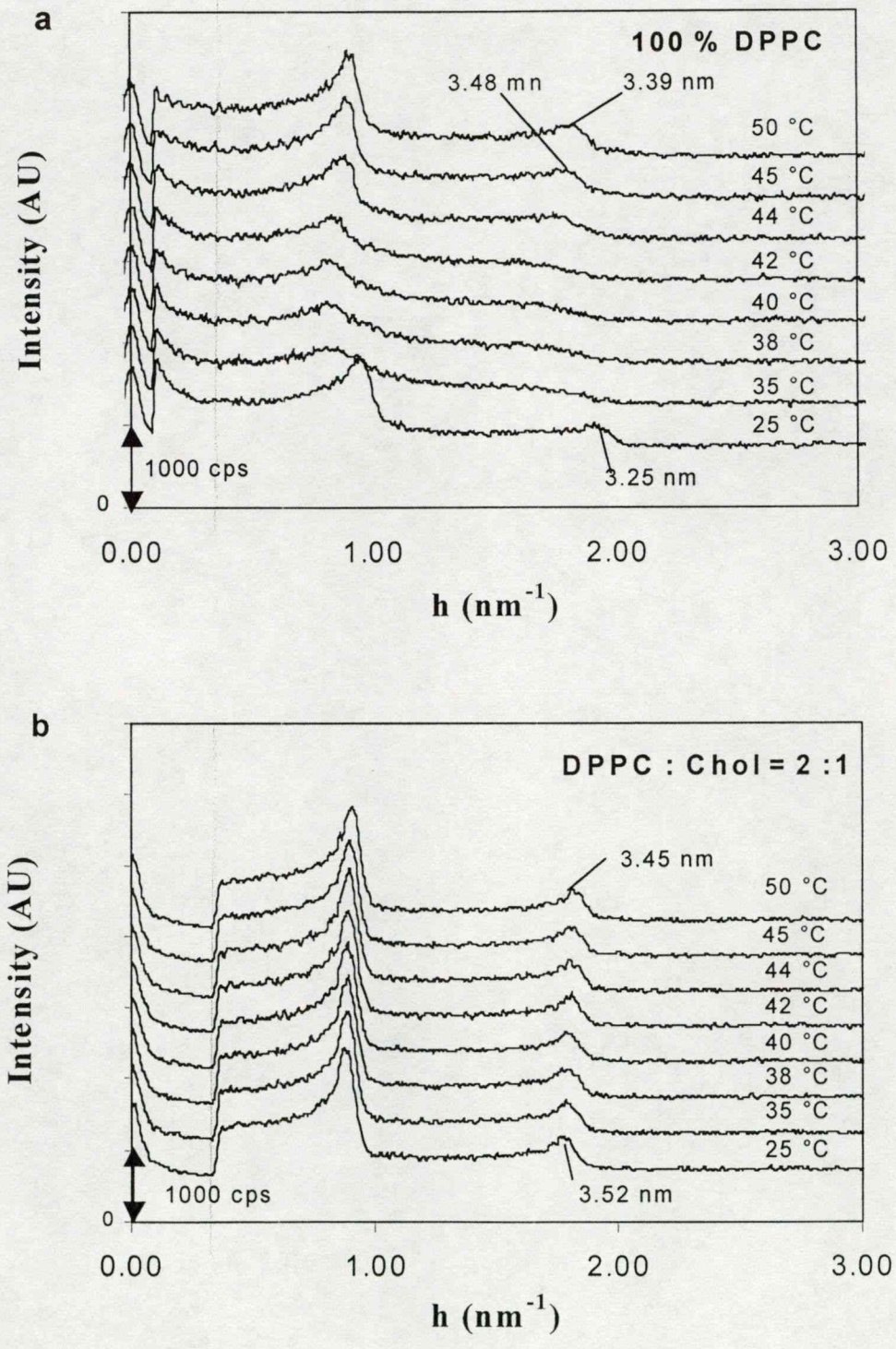


Fig. 16a and b: SAXS results. While the orderliness of the membrane is decreased at the DPPC liposomes (a), the DPPC / Chol liposomes has permanent structure during the temperature range (35-42 °C) of the $L_{\beta'}$-L_{α} phase transition (b). The lamellar repeat distances (d_L) slightly decreased (from 3.52 nm to 3.45 nm) at this latter system which phenomena probably due to the change of the PL-hydrocarbon chain conformation during the phase transition[109].

integrity experienced from the Figures 14a to 14c. These effects are summarized on the Fig. 15. which represents the adsorption of DEX on different liposomes and was prepared on the basis of the quantitative analysis of DEX with the use of ^{3}HDEX.

C, The role of surface charge density

The deviance of the surface charge density of liposomes shown in Figure 13. may also influence the characteristics of the polymer-adsorption. The negative surface charge provided by the weakly acidic functional groups may polarize the electron-structure of the polymers to be adsorbed. This effect can only succeed at the PVP more significantly among the polymers examined. The nitrogen in the 2-pyrrolidone is very weakly basic and we suppose that it gets protonated in aqueous solution. Through this it becomes slightly positively charged which favours the attachment of PVP to negatively charged surfaces. This theory might be the reason of the phenomena that PVP bounds better to the negatively charged SLPL / Chol / DCP and DPPC

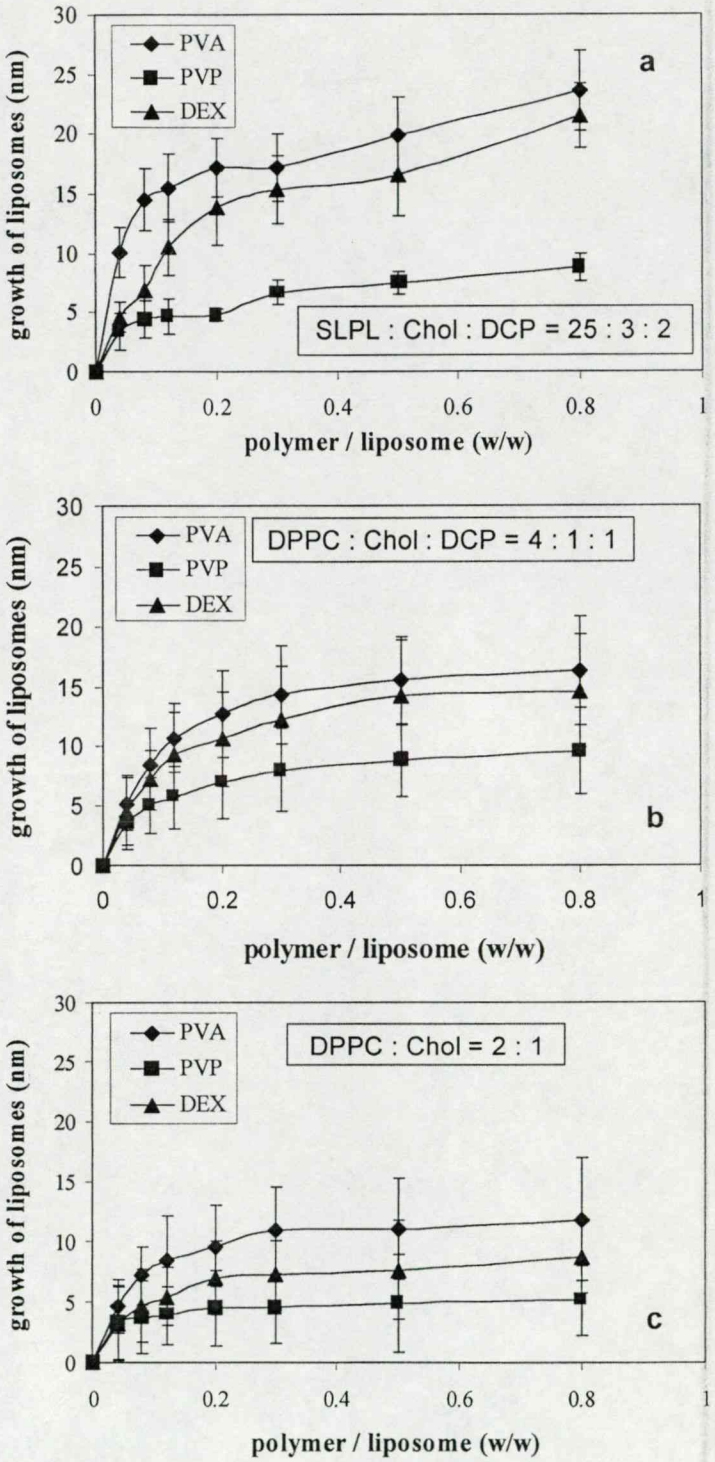


Fig. 17a-c: Detection of the polymer-adsorpion with DLS. The initial vesicle size diameters are in the Table 4., n=3

/ Chol / DCP liposomes at the beginning stage of adsorption than PVA and DEX which interact stronger other cases.

D, The role of the structural properties of the polymers

At the explanation of the change of adsorption ability observed at different polymers it may be remarkable the quality and frequency of the functional groups on the polymers suitable to form H-bridges, and the average molar weight of the polymers. We can see on the figures 14a-c that PVA and DEX adsorb in higher ratio on all the three liposome types than PVP does. The reason of this deviance should probably be looked for in the better H-bond forming ability of the hydroxyl groups on the PVA and DEX compared to the oxo groups on the PVP molecules. We did not find any significant difference between the quantitative adsorption properties of PVA and DEX at the SLPL / Chol / DCP liposomes where the polymer segments have possibility to penetrate into the membrane. On the contrary at the other two DPPC-liposomes with more rigid membrane where polymers can only bound onto the surface we experienced measurable difference between the adsorption of PVA (35-75 mg/g) and DEX (30-55 mg/g). According to our supposition this deviance is due to the more massive adsorption layer of the DEX resulted by the smaller molecular weight, the ramified structure[110] and the three H-bond forming hydroxyl groups per monomer unit. Whereas this compact layer does not allows the adsorption of further DEX molecules on the liposome surface, in the case of PVA which has higher molecular weight and only one hydroxyl group per monomer unit, the structure of the adsorption layer is looser and gives chance for further PVA molecules to bind as the PVA / liposome mass ratio increases (Figures 14b and c).

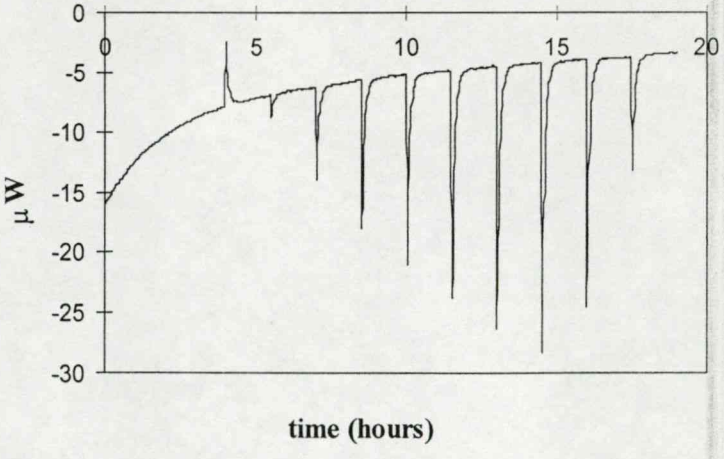


Fig. 18.: Titration microcalorimetric enthalpogram of PVA/liposome 0.04-0.4 (w/w) systems from which the ΔH_{tot} values were calculated by integration of the areas under peaks.

3.4. Examination of polymer adsorption with DLS technique

DLS measurements confirmed the results described before. The actual vesicle sizes were compared to the original sizes (Table 4.) and the growth of liposomes were given this way. On the basis of the Figure 17a-c we can conclude that PVA formed the thickest adsorption layers on the surface of all the three liposome types with the maximum liposome growing values of 11.8 – 23.5 nm.

3.5. Heat evaluation of the liposome-polymer interaction determined by titration microcalorimetry

The heat production / absorption during titration of liposomes with PVA is illustrated in Figure 18. The first peak reflects an exothermic process and the others endothermic effects. The enthalpy values required to obtain the adsorption enthalpy are listed in Table 5. This enthalpy is obtained from the relation:

$$\Delta H_{ads} = \Delta H_{tot} - (\Delta H_{dil(PVA)} + \Delta H_{dil(liposomes)}) \quad (9)$$

where ΔH_{ads} is the adsorption enthalpy,

ΔH_{tot} the total enthalpy and

ΔH_{dil} are the dilution enthalpies of the components (PVA and liposomes).

The adsorption enthalpy is plotted aganist the equilibrium concentration of PVA (Fig.

PVA / liposome (w/w)	ΔH_{tot} (mJ)	ΔH_{dil} (mJ) of liposomes (with buffer)	ΔH_{dil} (mJ) of PVA (added to buffer)	$\Sigma\Delta H_{dil}$ (mJ) of PVA and liposomes	ΔH_{ads} (mJ)
0.04	2.16	4.88	0.56	5.44	-3.28
0.08.	-0.78	3.43	-1.10	2.33	-3.11
0.12	-2.99	1.23	-2.77	-1.54	-1.45
0.16	-4.69	-1.94	-3.69	-5.63	0.95
0.20	-6.16	-4.43	-4.66	-9.09	2.93
0.24	-7.32	-5.90	-5.00	-10.90	3.58
0.28	-8.46	-7.70	-4.87	-12.57	4.11
0.32	-9.46	-9.45	-4.34	-13.79	4.33
0.36	-7.99	-9.73	-2.76	-12.49	4.50
0.40	-3.81	-4.70	-3.44	-8.14	4.33

Table 5.: Summary of TAM results. ΔH_{ads} shows exothermic interaction up to approximately $C_{e\ PVA}$ = 0.04 g/100cm^3. The standard deviation of ΔH_{ads} was ±2.1-6.5 %, n=3.

19a). The interaction is exothermic up to a PVA conentration of 0.04g / 100 cm^3 but is endothermic at higher concentration. An analogous representation may be seen in Fig. 19b, where the adsorption enthalpy is related to 1 g liposome (ΔH_{ads}') as a function of the amount of PVA adsorbed. This relation shows that in the initial stage of the isotherm, up to an adsorbed amount of 40 mg/g, exothermic effects are recorded. The differential adsorption enthalpy in Fig. 19c shows the adsorption enthalpy related to the unit mass of PVA.

These data clearly demonstrate a strong exothermic interaction between the liposome membranes and the segments of the PVA molecules in the initial state of the adsorption. The interaction may primarily involve hydrogen bonding. At higher PVA concentrations endothermic interactions occur which indicates that the polymer is incorporated between the alkyl chains of the bilayer. This

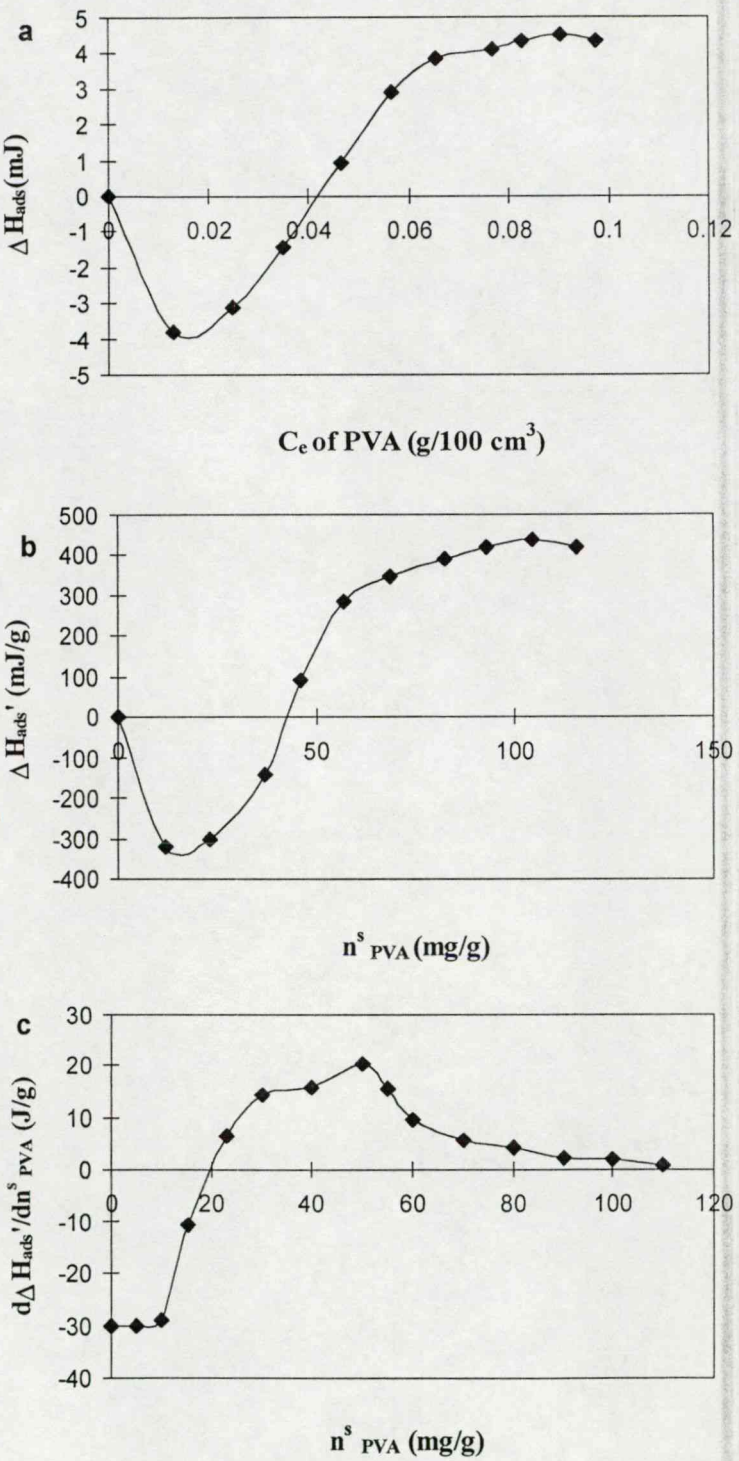


Fig. 19a: ΔH_{ads} shows exothermic interaction up to c_e = 0.04 g / 100 cm^3. The c_e values were calculated from the adsorption isotherm (Fig. 14a, Prot aggregated system). 19b: ΔH_{ads} per gram of liposome as a function of the amount of PVA adsorbed per gram of liposome (n^S_{PVA}). 19c: Derived form of Fig. 18b. Calculation of the amount of energy liberated by the adsorption of 1 g PVA.

process decreases in the structural order of the bilayers, the disorientation of the alkyl chains being controlled by entropy. It must also be considered that there are cholesterol and dicetyl phosphate molecules between the lecithin phospholipids and the hydroxyl groups of cholesterol might favour the penetration of PVA chains into the lipid layer.

3.6. XRD results

In the PVA-liposome systems a lamellar repeat distance (d_L) of 4.71 nm was found. After storage in saturated water vapour a small degree of swelling was observed with d_L= 4.85 nm. The layer thickness was related to the structure of the hydrated lipid membrane (Fig. 20.). At a ratio of 0.305 PVA / liposome d_L in the dry condition was 3.94 nm. This reveals that the adsorbed PVA compresses the bilayer by connecting molecules in the membrane. When this sample was exposed to water vapour, two well-characterized peaks appeared with d_L= 6.13 nm and d_L= 3.57 nm, which are probably the (001) and (002) reflections of a highly expanded bilayer with a spacing of about 7 nm. The nonintegrality (d_{001} is not equal to d_{002}) indicates a certain interstratification of somewhat differently expanded interlayer spaces, i.e. the distances from interlayer to interlayer of the expanded phase is not exactly the same. The XRD records of PVP-liposomes reveal essentially the same effects (Fig. 21.). In dry condition PVP compresses the bilayer, while saturated water vapour expands the membrane to d_L= 4.43

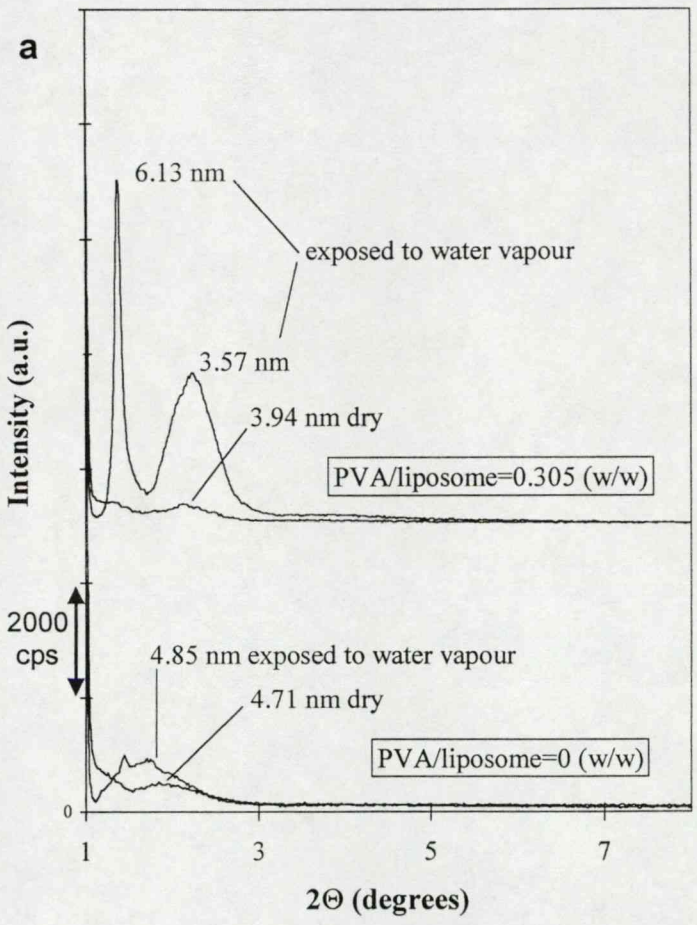


Fig. 20.: XRD diffractograms of PVA-liposomes

nm. This expansion is maintained after further addition of PVP but another peak appears at 7.06 nm which is characteristic of completely expanded bilayers.

A comparison of the XRD results for the the two neutral polymers with liposomes suggests that, as already proved in connection with adsorption isotherms, PVP binds more weakly on the surface of the bilayer membrane than PVA: at PVP/liposome (w/w) ratios of 0.025 and 0.61 the expanded structure show d_L= 4.43 nm and 4.56 nm. The amount of the highly expanded phase with d_L = 7.06 nm seems to be distinctly smaller than for PVA.

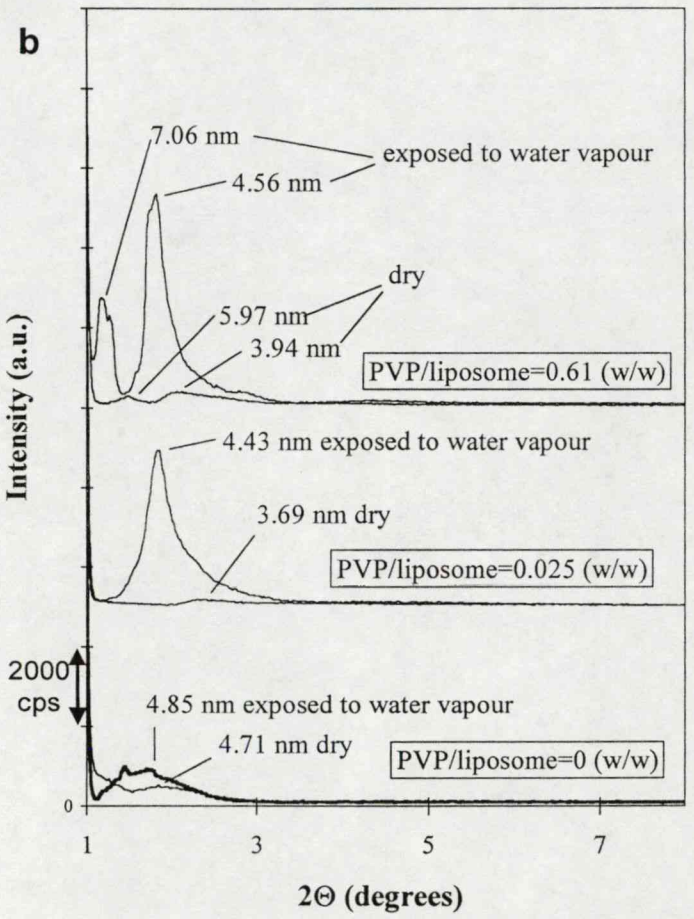


Fig. 21.: XRD diffractograms PVP-liposomes

3.7. Results of densimetry

The high precision densimertic measurements were done on the SLPL / Chol / DCP systems. The molar excess volume (V_m^e) values which were calculated with the Eq. 3. are plotted in the function of polymer / liposome mass ratio in the Figure 22. At all the polymers decreasing of the V_m^e was observed which refers to the compression of the liposome membrane structure caused by polymer adsorption. The rate of compression is the most expressed in the case of DEX: 5.71 cm^3 / mol at DEX / liposome = 0.5 (w/w). The membrane-compressing effect of the polymers is smaller rated in the PVA-liposome systems and the least expressed at the PVP where the deviance compared to the PVA and DEX is already measurable at PVP / liposome = 0.04-012 (w/w). Densimetric results support the theory described in 3/D where we searched for explanation on the different adsorption character-istics of polymers examined.

3.8. Description of the acridine orange entrapment and release

3.8.1. Optimisation of the remote loading

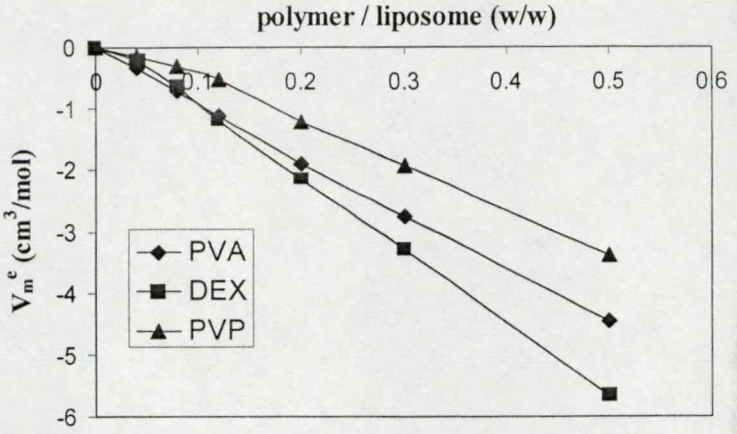


Fig. 22. : Molar excess volumes calculated from the results of high precision densimetry reveal the compression of the membranes of SLPL / Chol / DCP liposomes.

The results obtained from the experiments made on the remote loading process with three buffers having different quality can be seen in the Table 6. It is obvious that the best performance was given by the systems buffered with Tris-HCl. Among them the sample AO / PL = 0.1 (molar ratio) was found to be the most suitable for the test dye release examinations. The biggest rate of entrapment efficiency (47.8 %) with the smallest increasement of the liposome diameter (195.2 nm) was observed at this sample. The phenomena of vesicle enlargement is probably explained with the neutralizing effect of AO cations on the negatively charged phospholipids packed in the liposomal membrane. The negative surface charge of vesicles which maintains the stability of the real colloid system is neutralized after the addition of a certain amount of AO. This interaction is followed by aggregation.

It might be connection between the quality of the buffers used and the entrapment efficiency too. Anions in the buffer systems may form ionic pairs with AO cations inhibiting the formation and the transport of the AO base into the liposomes. Hepes molecules with acidic character may form salt with AO cations in the extraliposomal area while Tris having basic property does not keeps them back from participating in the membrane transport mechanism. Between these two extremities can be discussed the phosphate buffered system in which the hydrogen- and dihydrogenphosphate anions inhibit the AO transport into liposomes in lesser extent than it was experienced at Hepes anions due to the absence of non-polar interactions and the smaller molecular size.

AO/PL (mol/mol)	Tris-HCl buffer		phosphate buffer		Hepes-NaOH buffer	
	E^{opt} % ± sd	d (nm) ± sd	E^{opt} % ± sd	d (nm) ± sd	E^{opt} % ± sd	d (nm) ± sd
0	0	150.8±15.0	0	153.1±15.6	0	153.9±15.9
0.02	36.61±3.11	160.2±17.7	25.51±3.12	162.7±16.2	13.02±2.13	162.4±17.1
0.05	50.00±4.22	164.6±18.9	38.72±4.21	178.4±25.5	39.21±3.11	174.9±24.6
0.10	***47.86±4.61***	***195.2±27.1***	42.93±3.68	314.3±30.3	33.31±3.75	241.1±32.8
0.15	51.10±4.82	296.8±25.8	46.02±5.01	594.1±40.8	30.53±4.73	326.0±30.9
0.20	50.80±3.73	364.2±36.3	48.35±4.12	1588.1±51.6	33.62±4.36	658.8±45.6
0.25	46.54±3.92	643.4±42.9	43.12±3.56	2220.0±52.5	27.91±5.12	1304.9±48.6
0.30	45.45±4.31	1011.9±63.0	42.71±4.26	2256.0±96.6	26.92±4.03	1905.1±90.0

Table 6.: The optimal entrapment efficiency (E^{opt} %) and average vesicle diameter (d) results of the remote loading process of AO into liposomes. The data used for AO release experiments are shown by italicised fonts, n=3.

3.8.2. Results of the membrane diffusion measurements

These experiments were made on the SLPL / Chol / DCP and DPPC / Chol liposomes only because the average vesicle size (≈ 1642 nm) of the DPPC / Chol / DCP systems would not have rendered the entrapment of AO followed by separation with gel permeation possible. Results of the operations of AO entrapment made by the conditions described in the experimental section and the gel permeation combined with centrifugation are summarized in the Table 7. E % values were calculated by the Eq. 4. and the m_Y mass of AO in the gel

Type of liposomes	$[PL]_x$ (mM)	$[PL]_y$ (mM ± sd)	E % (% ± sd)	V_2 / V_1	N_0 (mol)	DLS_2 (nm ± sd)
SLPL / Chol / DCP = 25 : 3 : 2	26.66	17.89 ± 2.01	54.90 ± 6.40	0.954	$2.12 \cdot 10^{-6}$	211 ± 12
DPPC / Chol = 2 : 1	40	24.75 ± 1.93	72.05 ± 7.93	0.943	$3.36 \cdot 10^{-6}$	304 ± 21

Table 7.: Results related to the loading of AO into liposomes and the removal of unentrapped AO by gel prmeation. The DLS_2 values mean the increased average diameters of liposomes after the remote loading step. Standard deviations were calculated on the basis of three parallel measurements (n=3).

permeated liposomes with V_2 volume by the Eq. 5. During the processes of AO entrapment the growth of the neutral DPPC / Chol liposomes was more expressed ($DLS_2 = 304 \pm 21$ nm) than the SLPL / Chol / DCP liposomes which have negative surface charge providing increased kinetic stability for these dispersions.

The final results of the AO release exams are calculated by Eq.8. and depicted in the Figures 23a and b. The polymer –free SLPL / Chol / DCP dispersions released 7.51 % of the originally entrapped test dye. This release-rate is similar by the order of magnitude to the liposomes containing SL-phospholipids with the purity of 99 % as major component[111,112]. At the samples containing PVA and DEX it was obviously revealed the inhibition of the AO diffusion which is due to the membrane structure compression shown at densimetry and the polymer layer formed on the surface of the vesicles. Whereas at the DEX / liposome systems of the Figure 23a mostly the membrane compression of DEX, at the systems containing PVA rather the thickening of the polymer layer has influence on the efflux of AO (Figure 23b). On the basis of the measurements the amount of AO released during 24 hours decreased more determined to 5.2 % at the DEX / liposome = 0.1 (w/w) systems than at the systems containing PVA in the same mass ratio.

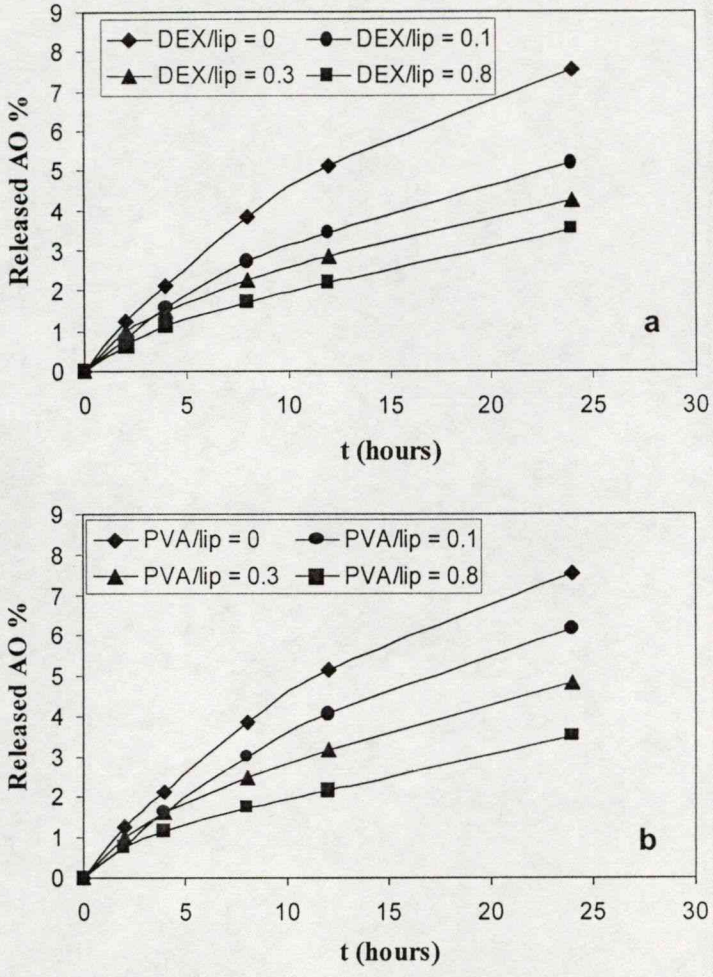


Fig. 23a and b: Efflux of acridine orange from the SLPL / Chol / DCP liposomal systems containing dextrane (a) and PVA (b)

In the case of DPPC / Chol liposomes AO release was not experienced either at the polymer free or the polymer contained samples. The explanation of this phenomena can

obviously be traced back to the 3 A and B points. Thus the rigid membrane of the liposomes containing cholesterol in 33.3 % (mol/mol) is in the $P_{\beta'}$ (rippled) transitional phase at 37 °C which is still very orderly (Figure 4.). Through these facts and the pH gradient exists between the inner and outer sides of liposomes AO molecules are unable to leak out from liposomes having such lipid composition.

4. Summary

Interactions between liposomal systems with three different lipid compositions and PVA, PVP and dextrane macromolecules were examined.

1. Considerable deviations were found in the behaviour of the systems having different lipid compositions at the preparation. In addition to the pH of the dispersing media important role have the grade of phospholipid fatty acid saturation, the surface charge, and the lipid concentration. In general I can say that physiologic pH (7.4) provides the best conditions to prepare any kind of liposome easily. SLPL / Chol / DCP liposomes, pH = 7.4: d = 148 ± 10 in phosphate buffer (PB) and d = 150 ± 14 nm in phosphate buffered saline (PBS), pH = 5.0: d = 164 ± 13 nm. DPPC / Chol / DCP liposomes, pH = 7.4: d = 110.4 ± 5.3 (PB) and d = 111.7 ± 5.4 (PBS), pH = 5.0: 1642 ± 132 nm. DPPC / Chol liposomes, pH = 7.4: d = 114 ± 6.2 nm (PB) and 116 ± 7.3 nm (PBS), pH = 5.0: d = 151 ± 8.1 nm.

2. The composition of liposomes influences not only the preparation process but also the polymer adsorption on the liposomal membrane. At the experimental conditions employed the fluid, liquid crystal phased ($L_{\alpha'}$) SLPL / Chol / DCP liposomes bind all the three polymers in higher ratio than the other two systems which contain DPPC as major component and are in the orderly gel ($L_{\beta'}$) phase. Polymers are not only able to adsorb onto the surface of the ($L_{\alpha'}$) phased membranes but also penetrate into their structure.

3. The surface charge density of liposomes and physical chemical properties of the polymers have also effect on the adsorption characteristics. In general PVA and dextrane which are rich in hydroxyl groups adsorb better than PVP at which adsorption properties has determining role the negative surface charge of the lipid membranes.

4. Results experienced at the quantitative polymer adsorption measurements were also confirmed by DLS examinations where I found the biggest adsorption layer thicknesses -

which as value (t^s) is half of the growth of liposomes- at the SLPL / Chol / DCP systems containing PVA ($t^s_{max} \approx 11.8$ nm) and dextrane ($t^s_{max} \approx 10.8$ nm).

5. Enthalpy values of the titration microcalorimetric exams are exothermic at the beginning stage of the adsorption which fact reveals that these interactions are hydrogen bonding. If we increased the PVA concentration, the heat effects grew endothermic which probably the consequence of the polymer-penetration into the lipid membrane.

6. XRD diffractograms show that PVA allows smaller expansion for the lipid bilayer than PVP if I exposed the lipid films prepared from liposome dispersions containing different amount of polymers to water vapour. The diminishment of the molar excess volume values (V_m^e) obtained by further calculation from the results of high precision densimetry revealed the compression of the lipid membrane structure. This phenomena was the most expressed at the systems containing dextrane.

7. At the preliminary experiments of the acridine orange (AO) release tests I have concluded that the active- or remote loading of AO is optimal at the AO / PL rate of 0.1 in Tris buffered saline considered to the entrapment efficiency and the rate of aggregation.

8. The polymer barrier and the membrane compression formed through the adsorption process decreased the AO release of SLPL / Chol / DCP liposomes. There was no AO release experienced at the DPPC / Chol liposomes which is an obvious evidence that the efflux of the test dye is influenced by the composition of the liposome membrane primarily.

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Annex

Papers related to the thesis.