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LIPOSOMES AS CARRIERS FOR DRUGS AND ANTIGENS: APPROACHES TO PRESERVE THEIR LONG TERM STABILITY

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ABSTRACT

Liposomes can be used for several different reasons in therapy. In this brief review the sources of chemical and physical instability of liposomes and the approaches for long term stabilization will be discussed.

INTRODUCTION

Recently, the first drug carrying, parenterally administered, liposomal formulation has been approved by several European regulatory authorities. It contains the antifungal drug amphotericin B. More parenteral liposomal formulations carrying cytostatic or antibiotic drugs are expected to reach the stage of registration soon. Apart from liposomes being used as drug carrier, there is a growing interest in the potential utilization of liposomes for the proper presentation of antigens in newly developed, well characterized vaccines.

The reasons why liposomes are preferred as drug carriers or antigen presenting vehicles over alternative systems are: the possibility to manipulate (within limits) their behaviour in vivo, their relative safety (1) and their aqueous core, providing a 'friendly' environment for encapsulated proteins. Liposomes can be

used for several purposes: (i) as solubilizers of lipophilic drugs (fast disintegration in blood upon injection) (2), (ii) as carriers of immunomodulating agents ('passive targeting' to macrophages) (3), (iii) as carriers of cytostatic and antifungal agents ('site avoidance effects' or slow release systems) (4) or (iv) as carriers homed (e.g. immunoliposomes) to pathological tissue or cells (thrombi, tumors, infections): 'active targeting' (5, 6) and (v) as antigen carriers, preferably in conjunction with an adjuvant (7).

Apart from the pathological, physiological and anatomical restrictions (6), liposome use can also be restricted by pharmaceutical limitations. Both chemical (hydrolysis, oxidation) and physical instability (aggregation, phase separation in the bilayer and fusion, loss of encapsulated drug) has been described. This instability on the shelf can jeopardise their performance in vivo. Several approaches to monitor the physical and chemical stability of liposomes (8 - 10) and to stabilise liposomes on long term storage have been developed and evaluated. The problems around the chemical and physical stability of liposomes and the approaches to stabilise liposomes will be outlined in this manuscript. Detailed information can be found in the cited literature references.

CHEMICAL STABILITY OF LIPOSOMES

Phospholipids in liposomes are sensitive to chemical degradation via two pathways: (i) hydrolysis of the ester groups, and (ii) oxidation of the unsaturated acyl chains. The sequence of hydrolysis reactions is shown in Figure 1 (11). Different analytical techniques to monitor hydrolysis and oxidation reactions were reviewed before (11 - 13).

Grit et al. (14 - 17) described in a number of articles different variables that influence the hydrolysis reactions of phosphatidylcholine (PC), the major phospholipid in most liposome preparations, and the charge inducing phospholipid: phosphatidylglycerol (PG). pH and temperature, but also, unexpectedly, buffer species, affect hydrolysis kinetics. An example of the pH dependency is shown in Figure 2 (15).

It is clear that for PC containing vesicles the pH for the lowest hydrolysis rate is around pH 6.5. The rigidity of the PC bilayer (degree of unsaturation) did not influence this optimal pH, but at ambient temperatures degradation rate constants for liposomal dispersions with saturated acyl chains (hydrogenated soy bean PC) tended to be lower than for natural soy bean PC. Grit and Crommelin (17) were able to demonstrate that the surface pH and not the bulk pH was the rel-

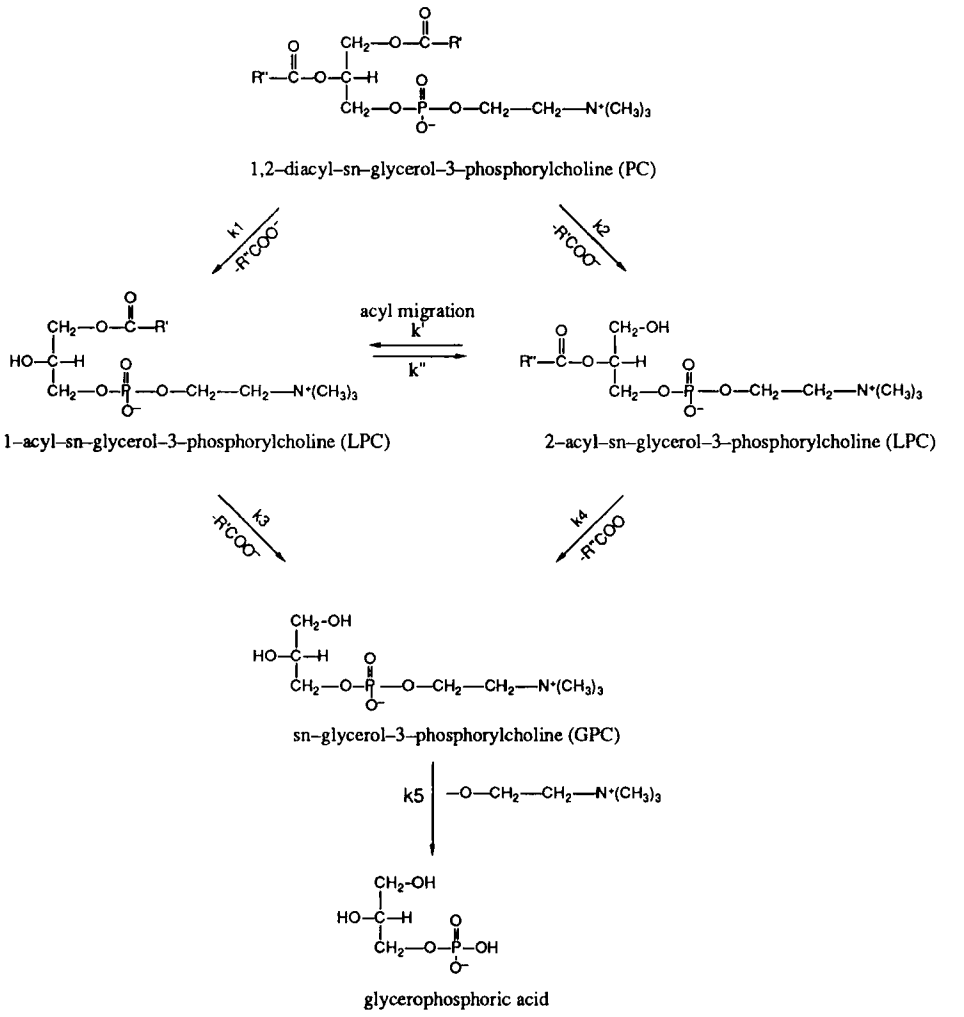

FIGURE 1

Hydrolysis reactions of phosphatidylcholine (PC) in aqueous liposome dispersions. R' and R" are acyl chains. Taken from ref. 11.

evant parameter controlling hydrolysis kinetics. The optimum pH value shifted to higher pH values when high negative surface charge densities (and low ionic strength conditions) prevailed.

Interestingly, the temperature dependence of the hydrolysis rate constants could be described by Arrhenius kinetics, if no bilayer transition temperature occurred in the experimental temperature range (14, 16).

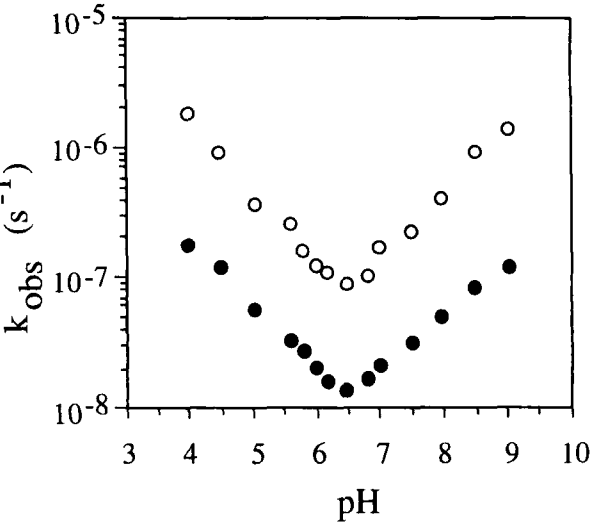

FIGURE 2

The effect of pH on the hydrolysis of saturated soybean phosphatidylcholine (PC). ●: 40°C; o: 70°C. Taken from ref. 15.

These experiments made clear that liposomes formulated with hydrogenated PC (and PG, as well) or dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) are sufficiently resistant to hydrolytic degradation, if the correct pH and temperature conditions are chosen.

The mechanism of oxidation reactions occurring at unsaturated acyl chains of phospholipids has been investigated by many groups in the past (13, 18). Pharmaceutical preparations containing lipid material that is oxidation sensitive should be filled under nitrogen or a rare gas (argon) and kept in the dark. If necessary, antioxidants such as α-tocopherol or chelators can be added to further reduce the tendency for oxidation. It turns out that if the proper measures have been taken, oxidative degradation does not limit shelf life.

Recently, it was reported that liposomes of different lipid composition (with saturated or unsaturated PC and PG) could be steam sterilized without substantial hydrolitic or oxidative degradation (19).

PHYSICAL STABILITY OF LIPOSOMES

Liposomes can change their physical characteristics in several ways. (1) Particle size (an important parameter for the in vivo disposition of liposomes and,

therefore, the encapsulated drug or antigen) can change because of aggregate formation and fusion, (2) Phase separation of bilayer components upon storage can occur, and, finally, (3) Leakage of encapsulated material from liposomes can occur. First, the option to store liposomes as an aqueous dispersion will be discussed. Then, alternative approaches to solve potential shelf life problems will be dealt with: (freeze) drying and the 'proliposome' concept.

Liposomes Stored as Aqueous Dispersions

Ad 1 Size: Particle size changes upon storage of PC containing liposomes over pharmaceutically relevant time intervals can be, in general, minimised by selection of proper charge inducing agents. Mostly, negatively charged phospholipids (in particular PG) are used to successfully stabilise the liposomes.

Ad 2 Phase Separation: Phase separation can occur when the bilayer composition changes because of chemical degradation reactions, or when the bilayer goes through temperature cycles (8). Proper selection of bilayer components can avoid these problems. Sometimes, phase separation occurs in vivo, when bilayer components are selectively drawn from the bilayer by plasma components (20). If this effect is undesired, components that form more rigid bilayers are to be preferred. In other cases one might wish to deliberately destabilise the liposomes in vivo so that a rapid release of the encapsulated drug is induced. An example of plasma destabilised liposomes are liposomes composed of phosphatidylethanolamine (PE) and oleic acid (21). Oleic acid has a high affinity for plasma albumin and leaves the bilayer. The PE enriched bilayer subsequently converts into a hexagonal phase releasing all encapsulated hydrophilic material.

Ad 3 Leakage of Encapsulated Material: The permeability for bilayers is highly dependent on the physico-chemical properties of the bilayer and drug, and the temperature (21). Three categories of drugs can be discerned (22): (i) highly hydrophilic, non-bilayer interacting drugs, (ii) drugs with 'some' lipophilicity and (iii) strongly lipophilic drugs. An example of the dependence of the bilayer composition on leakage kinetics for a low molecular weight, hydrophilic, non-bilayer interacting compound (carboxyfluorescein, CF, category i) is shown in Figure 3 (23).

Figure 3 shows that the presence of cholesterol in the bilayer of the egg PC liposomes dramatically reduces the permeability. For 'gel state' bilayers (distearoylphosphatidylcholine and distearoylphosphatidylglycerol, DSPC and DSPG, respectively) permeability is low, with or without cholesterol. It is clear that if in

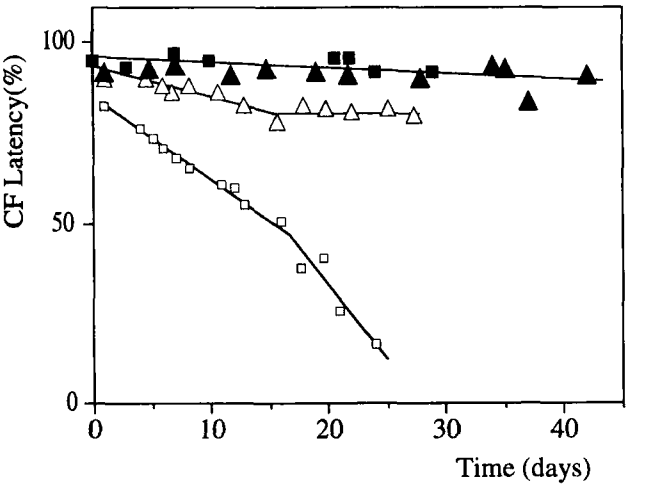

FIGURE 3

The critical importance of the proper selection of bilayer components on the retention of CF. □ : PC/PS (9/1, molar ratio); Δ : PC/PS/CHOL (10/1/4, molar ratio); ● : DSPC/DSPG (10/1, molar ratio); ▲ :DSPC/DSPG/CHOL (10/1/5, molar ratio). Taken from ref. 23.
(PC = egg phosphatidylcholine; PS = phosphatidylserine; CHOL = cholesterol; DSPC = distearoylphosphatidylcholine; DSPG = distearoylphosphatidylglycerol)

vivo performance allows 'gel state' bilayers to be used, shelf life of the liposomes in aqueous media with the proper pH might easily meet industrial demands. The second (ii) category of drugs ('some lipophilicity') tends to be difficult to keep entrapped over periods of months as long as outside 'sink conditions' prevail. The third category of strongly lipophilic drugs (iii) has a high affinity to the bilayer and these drugs stay there over a long period of time, independently of the 'state' of the bilayer.

As a final remark, the presence of hydrolysis (lyso-phosphatidylcholine) or oxidation reaction products can affect bilayer properties. Although lysophosphatidylcholine is known to be a lipid bilayer solubilizer, the solubilizing effect of lyso-phosphatidylcholine in degrading liposomes is 'neutralised' by the simultaneous appearance of fatty acids (the other part of the split PC molecule) in the bilayer (24). The permeability of these bilayers for calcein (low molecular weight, hydrophilic, non-bilayer interacting probe) does not increase until over 10% of the bilayer lipids is hydrolysed.

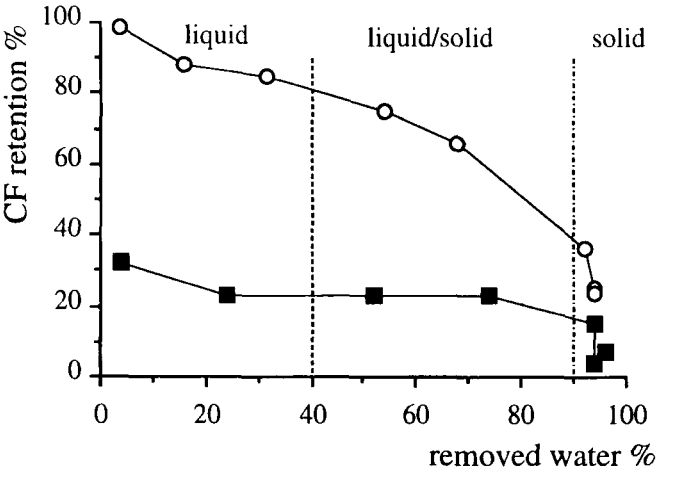

FIGURE 4

Retention of encapsulated CF after thawing or rehydration as a function of the percentage of removed water by freeze drying. Particle size: ■ :0.28 μm; o : 0.13 μm. Taken from ref. 28.

Liposome dispersions (hydrogenated soybean phosphatidylcholine/ dicetylphosphate; molar ratio 10:1) contained 10% m/m saccharose in/outside the vesicles. The liposomes were prepared as described by Fransen et al. (Int. J. Pharm. 33, 27, 1986) with slight modifications. 5 mM CF (in 10 mM tris buffer, pH 7.4) was encapsulated. Freezing temperature: boiling liquid nitrogen; primary drying phase: 2-14 hrs, dP (Pa) 10, temperature -40°C; secondary drying phase: temperature was raised from -40 to 20°C at a rate of 10°C/h. The vials were sequentially stoppered during the freeze drying process. The samples with less than 40% water removed -the vials closed in the early stage of the process- still contained liquid material upon thawing after the cycle was finished. When between 40% and 90% water was removed both solid and fluid material was present. When over 90% water was removed the system was solid.

Liposomes Stored in Freeze-Dried Form

If, for the proper in vivo performance of liposomes a bilayer structure is preferred with insufficient long term stability, freeze drying of liposomes might be an option to obtain pharmaceutically stable liposomes. To maintain the same particle size distribution after the freeze drying-rehydration cycle, a cryoprotectant needs to be added. The different types of cryoprotectants and their possible mechanism

of action have been discussed by Crowe et al. and Özer et al. (25, 26). For liposome stabilization usually sugars are used as cryoprotectant, although other types of excipients also have been reported to exert cryoprotective effects (23, 27). A number of effects may contribute to the cryoprotective action: (i) the formation of amorphous glass structures during the freeze drying process might avoid mechanical damage inflicted by ice crystals. It is recommended to store these cakes below the glass transition temperature. And, (ii) the sugars may interact with the polar head groups of the phospholipids and stabilise the membranes when the bilayer stabilizing water is removed by sublimation ('pseudo-hydration layer').

Substantial loss of the encapsulated drug after a freeze drying-rehydration cycle has been reported. This loss depended on the encapsulated drug or probe and the experimental conditions. Apart from the required presence of cryprotectants, parameters as bilayer composition, freezing rate and temperature, primary and secundary freeze drying protocol, and residual water level play a critical role (2). For instance, Talsma and Crommelin (28) reported that small sized liposomes tend to be more resistant to CF loss after a cycle than larger ones (Figure 4). On the other hand, highly lipophilic compounds, such as Sudan Red, do not leave the bilayer throughout the freeze drying-rehydration cycle and successful freeze drying of these liposome dispersions is possible, provided the proper cryoprotectant is used to avoid aggregation and fusion to occur.

The 'Proliposome' Concept

In proliposome formulations liposomes are formed by hydrating lipids 'at the bedside'. The dry lipids (coated as a film on the glass vessel wall, in the form of a freeze dried cake or coated on a water soluble, particulate carrier (sodium chloride crystals) are hydrated by shaking with an aqueous medium just before injection. A prerequisite is that the drug involved has a strong tendency to be taken up into the bilayer (such as palmitoylated drugs and muramyltripeptide (MTP)-PE) and that rather wide particle size distributions are acceptable. Several groups have discussed this approach (29 - 32). The MTP-PE liposomes are presently tested in the clinic, indicating that this preparation is safe in animals and pharmaceutically acceptable.

CONCLUDING REMARKS

Liposome use can be restricted by pharmaceutical limitations. Both chemical (hydrolysis, oxidation) and physical instability (aggregation, phase separation

in the bilayer, fusion and retention loss) have been described. This instability can affect their performance in vivo and cause irreproducible results. Several approaches to solve problems related to the physical and chemical instability of liposomes on long term storage have been developed and evaluated. It can be concluded that today, there is no general solution to produce pharmaceutically stable liposomes. On a case to case basis the formulation protocols for optimum stability should be selected from the above discussed possibilities.

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