TECHNIQUES FOR ENCAPSULATING BIOACTIVE AGENTS INTO LIPOSOMES

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Received April 29th, 1986

As a prerequisite for the use of liposomes for delivery of biologically active agents, techniques are required for the efficient and rapid entrapment of such agents in liposomes. Here we review the variety of procedures available for trapping hydrophilic and hydrophobic compounds. Considerations which are addressed include factors influencing the choice of a particular liposomal system and techniques for the passive entrapment of drugs in multilamellar vesicles and unilamellar vesicles. Attention is also paid to active trapping procedures relying on the presence of (negatively) charged lipid or transmembrane ion gradients. Such gradients are particularly useful for concentrating lipophilic cationic drugs inside liposomes, allowing trapping efficiencies approaching 100%.

Keywords: liposome; drug encapsulation; targeting; large unilamellar vesicles; trapping efficiency

I. Introduction and Overview

Liposomes have increasing applications as models of biological membranes and as drug delivery vehicles for in vivo application. The generation and use of liposomes as model systems has been reviewed in other papers in this volume and elsewhere [1]. Here we address certain practical problems inherent in the use of liposomal systems for drug delivery. These include the type of liposome to be employed, the techniques for efficiently loading the liposome with a drug of choice, and the stability of the resulting preparation.

Before dealing with these points, however, the question as to why it is of interest to encapsulate a drug in a liposomal carrier and utilize this system as a delivery vehicle (in the absence of any specific targeting factors) should be addressed. The major reason is that whereas the efficacy of targeted liposomes has yet to be demonstrated [2,3], the administration of simple liposomal drug complexes has immediate therapeutic potential [4-6]. This arises due to a lower toxicity and/or enhanced

Abbreviations: MLV, multilamellar vesicle; LW, large unilamellar vesicle; RES, reticuloendothelial system; SUV, small unilamellar vesicle.
efficacy of the drug when encapsulated in a liposome system. The reasons why liposomes buffer drug toxicity are not understood, but are presumably related to altered drug pharmacokinetics or biodistributions. Particular areas in which liposomes display therapeutic promise are as carriers for anticancer agents [7-9], antifungal drugs [10-13], antibacterials [14-20], antivirals [21] and certain antiparasitics [22,23]. Other applications include liposomes as carriers of contrast agents for use in diagnostic X-ray and NMR imaging [24-26].

Questions regarding the type of liposome to employ and the drug trapping procedure used are pertinent due to the large variety of techniques available to prepare liposomes (see Hope et al. [27]), the particular demands arising from the type of drug to be entrapped and the biological effects desired. For example, the technique of drug entrapment must satisfy demands such as high trapping efficiency and reasonably long retention times. In addition, some important drugs exhibit significant lipophilic character which can result in rapid leakage from liposomal systems. Another important consideration is the behaviour of liposomes in vivo which is very sensitive to vesicle size and composition. Approaches to resolve these and other problems are presented in the following sections.

II. Biological Factors which Influence the Choice of a Liposome System

The choice of liposome preparations with regard to lipid composition and size for in vivo drug delivery is dependent on a variety of factors. Work in several laboratories has demonstrated that vesicles composed solely of phospholipid are rendered leaky in serum or plasma [28-31] due to the interaction with high-density lipoproteins [31-34]. This effect is remedied, to a large extent, by the incorporation of cholesterol into the liposomal bilayer [28,30,34]. Although such alterations in lipid composition improve vesicle stability in the circulation, the majority of liposomes administered i.v. are eventually cleared by phagocytic cells of the reticuloendothelial system (RES) [35]. The clearance rate and biodistribution of liposomes, however, are sensitive to vesicle composition, size, dose and mode of administration. For instance, negatively charged vesicles given i.v. are cleared much more rapidly by the RES than are positively charged or neutral vesicles [36]. With respect to vesicle size, it has been shown that i.v. injection of large MLVs ($d = 3 \mu m$) enhances delivery to the lung [37] whereas small unilamellar vesicles (SUVs) appear to exhibit increased partitioning to the bone marrow [38]. Also, increasing the lipid dose or decreasing vesicle size increases the longevity of liposomes in the circulation when given i.v. [36,39-42]. The way in which liposomes are administered is also an important consideration in the development of therapeutic liposomes. Liposomes injected i.m. or s.c. slowly disperse from the injection site, are adsorbed into the lymphatic system and then return to the bloodstream where they are sequestered by the RES. Administration of liposomes i.m. is generally employed where the slow-release property of liposome-encapsulated drugs is desired. Under these conditions retention of the vesicles at the site of injection is preferred. Large
most suitable for this purpose [43-46] since they remain localized at the site of injection much longer than do small vesicles ($d < 0.5 \mu m$) has been shown to be effective in delivering drugs to lymph nodes [47-49] and may be of use in the treatment of lymph node metastases [50].

The clearance of liposomes by the RES has proven to be a major obstacle in achieving delivery to non-RES cells. However, many investigators have utilized this passive targeting to deliver drugs to phagocytic (monocyte, macrophage) cells. Entrapped immunomodulators, for example, can activate macrophages and increase their cytotoxicity to invading cells. This approach may prove useful in enhancing macrophage-mediated host resistance to cancer metastases [51,52], viral infection [21] and bacterial infection [53,54]. In addition, therapeutic agents can be targeted in this manner to parasites which reside in the RES [22,23,55]: For such applications, it appears that negatively charged vesicles of medium size (between 0.1 and 1.0 $\mu m$) most efficiently deliver drugs to these phagocytic cells [52].

III. Techniques for Encapsulating Bioactive Agents

A. Passive trapping techniques

1. General comments

Selection of an encapsulation protocol is largely dictated by concerns such as encapsulation efficiency, drug/lipid ratio, drug retention, ease of preparation, compatibility with regulatory agencies, sterility, ease of scaleup, cost efficiency, as well as liposome and drug stability [56,57]. In the discussions to follow, entrapment procedures will be analyzed with special attention being given to these aspects.

Table I provides an abridged survey on the properties of liposome formulations employed in the encapsulation of various aqueous markers and water-soluble bioactive agents. These ‘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 from 1% or less for SUVs to as high as 88% for some MLVs. 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 $\mu l/\mu mol$ lipid). LUVs and MLVs can exhibit higher values (1-30 $\mu l/\mu mol$ 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 (Table I) depending on lipid concentration limitations and aqueous trapped volumes inherent in the specific liposome preparation procedures. At this point it should be stressed that many agents one may wish to encapsulate often do not act as ideal aqueous markers and therefore theoretical trapped volume determinations may not always reflect the actual encapsulation properties for these materials.
<table>
<thead>
<tr>
<th>Vesicle type</th>
<th>Preparation procedure</th>
<th>Vesicle diameter (μm)</th>
<th>Entrapped agent</th>
<th>% Trapping efficiency</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUV</td>
<td>Sonication</td>
<td>0.020-0.040</td>
<td>Cytosine arabinoside, methotrexate, carboxyfluorescein</td>
<td>1-5</td>
<td>56,61,62</td>
</tr>
<tr>
<td>SUV</td>
<td>French press</td>
<td>0.020-0.050</td>
<td>Carboxyfluorescein, inulin, trypsin, BSA</td>
<td>5-25</td>
<td>63</td>
</tr>
<tr>
<td>SUV</td>
<td>Detergent removal</td>
<td>0.036-0.050</td>
<td>Carboxyfluorescein, inulin</td>
<td>12</td>
<td>66</td>
</tr>
<tr>
<td>LUV</td>
<td>Detergent removal</td>
<td>0.1-10.0</td>
<td>Inulin, cytochrome c, carboxyfluorescein</td>
<td>12-42</td>
<td>66,70, 71</td>
</tr>
<tr>
<td>LUV</td>
<td>Reverse phase evaporation</td>
<td>0.2-1.0</td>
<td>Carboxyfluorescein, cytosine, arabinoside, 25s RNA, DNA, insulin, albumin</td>
<td>28-45</td>
<td>61,75,78</td>
</tr>
<tr>
<td>LUV</td>
<td>Solvent vaporization</td>
<td>0.1-0.5</td>
<td>Pi, chromate, glucose, soy bean trypsin inhibitor, DNA</td>
<td>2-45</td>
<td>72-74,76</td>
</tr>
<tr>
<td>LUV</td>
<td>Extrusion</td>
<td>0.056-0.2</td>
<td>$^{22}$Na, inulin, methotrexate, cytosine arabinoside, DNA</td>
<td>15-60</td>
<td>80,81,83</td>
</tr>
<tr>
<td>MLV</td>
<td>Mechanical mixing</td>
<td>0.4-3.5</td>
<td>$^{22}$Na, carboxyfluorescein, glucose albumin, DNA</td>
<td>1-8.5</td>
<td>56,89</td>
</tr>
<tr>
<td>MLV</td>
<td>Sonicate-freeze-thaw</td>
<td>0.17-0.26</td>
<td>Asparaginase</td>
<td>50-56</td>
<td>91</td>
</tr>
<tr>
<td>MLV</td>
<td>Freeze-thaw</td>
<td>0.5-5.0</td>
<td>$^{22}$Na, inulin</td>
<td>35-88</td>
<td>89</td>
</tr>
<tr>
<td>MLV</td>
<td>Sonicate-dehydrate-rehydrate</td>
<td>0.3-2.0</td>
<td>Carboxyfluorescein, sucrose, albumin, Factor VIII, ATP, vincristine, melphalan</td>
<td>27-54</td>
<td>56</td>
</tr>
<tr>
<td>MLV</td>
<td>Solvent evaporation-sonication</td>
<td>0.3-2.0</td>
<td>Inulin, streptomycin sulfate, chloramphenicol, oxytetracycline sulfamerazine</td>
<td>6.3-38</td>
<td>84</td>
</tr>
</tbody>
</table>
2. Drug entrapment in SUVs

SUVs have traditionally been prepared by sonication [58-60], which converts an MLV dispersion to an opalescent solution of vesicles ranging in size from about 0.02 to 0.05 \( \mu \text{m} \). The simplicity of this procedure has attracted widespread use. However, there exist several drawbacks in the technique when applied to drug encapsulation. These include the low trapping efficiencies obtained, which range from 1% to 5% (61,62,63). In addition, this approach appears inappropriate for entrapment of many proteins and DNA due to ultrasonic degradation of these biological materials [63]. Advantages of sonicated SUVs are that small homogeneous populations of vesicles are rapidly formed and virtually any lipid composition can be employed.

Under appropriate conditions, SUVs can also be produced employing detergent removal techniques [64-66]. These vesicles exhibit improved trapping efficiencies over sonicated systems (Table I) and the average diameter of the liposomes can be controlled to some extent by varying the initial detergent/lipid ratio [66]. However, problems arise due to difficulties in removing residual detergent [67].

The French pressure cell produces fairly homogeneous SUVs of a size 0.05 \( \mu \text{m} \) (depending on lipid composition) by passing a lipid suspension under extreme pressures through a small outlet orifice [63,68]. Trapping efficiencies up to 25% can be achieved although this value decreases somewhat with increasing molecular weight of the trapped solute [63]. An important feature of the French press vesicle (FPV) technique is that proteins do not appear to be significantly affected during the process as they are in sonication [63]. An interesting observation is that FPVs appear to retain entrapped solutes significantly longer than do SUVs produced by sonication or detergent removal [56,63,66].

3. Drug entrapment in LUVs

One of the first procedures developed to produce LUVs was removal of detergent from a mixed micellar solution [69]. Applications of this technique have expanded from its original use in reconstituting membrane proteins [69] to include encapsulation of bioactive agents. The large aqueous trapped volumes (1.5-30.0 \( \mu \text{l}/\mu \text{mol} \)) of these vesicles enable the preparation of liposome systems which exhibit drug/lipid ratios and trapping efficiencies which are superior to most SUVs and MLVs. LUVs ranging in size from 0.1 to 10 \( \mu \text{m} \) are obtained by utilizing various detergents or lipid/detergent ratios [65,66,70,71] and yield trapping efficiencies as high as 42% (Table I).

Solvent evaporation/vaporization techniques have gained widespread popularity for entrapping drugs in LUVs. Lipids dissolved in organic solvent are mixed with an aqueous phase (containing the agent to be trapped) and removal of the solvent by heat and/or reduced pressure induces the spontaneous formation of LUVs [61, 72-74]. These procedures have been shown to yield trapping efficiencies between 30 and 45% for a wide range of solutes including proteins, DNA and RNA [61]. The corresponding large trapped volumes and high drug/lipid ratios may
be important in order to keep lipid doses below toxic levels [79]. In addition, several studies have demonstrated that entrapped agents are efficiently delivered to target cells in vitro utilizing these systems [75-78]. A disadvantage of solvent-based liposomes is that some macromolecules are deactivated by this procedure [66].

Recent reports [80,81] have described the production of homogeneously sized LUVs by extrusion of MLVs through polycarbonate filters of defined pore size under moderate pressures (≤800 lb/in²). These pressures allow the preparation of homogeneous LUVs with average diameters ranging from 0.056 to 0.2 μm [81]. Since, at present, LUVs larger than 0.2 μm cannot be produced, the aqueous trapped volumes of LUVETs (LUVs produced by extrusion techniques) are somewhat lower (0.8-2.5 μl/μmol) than for some systems obtained employing solvent evaporation/vapourization techniques (2-30 μl/μmol); [82]. This does not limit trapping efficiencies for LUVET preparations, however, since very high lipid concentrations can be utilized. For instance, extrusion of frozen and thawed MLVs (see next section) [81] prepared at 400 mg phospholipid/ml through 0.1 μm filters yields a very homogeneous LUV preparation (mean diameter = 0.11 μm [81]) in which 56% of the aqueous volume (Table I) is contained within the vesicles [81]. An attractive feature of this technique is that vesicles composed of virtually any lipid composition which adopts liquid crystalline lamellar systems can be obtained within a very short period of time (<30 min). In addition, the vesicles display optimal retention of entrapped solutes [80,83], degradation of proteins and DNA is negligible (M.B. Bally et al., unpublished data), extrusion renders the preparation sterile and scale-up would appear to be straightforward.

4. Drug entrapment in MLVs

Since the efflux of water-soluble drugs from liposomes is often dictated by their membrane permeability, MLVs can exhibit maximal drug retention due to the number of lamellae the agent must cross to reach the vesicle exterior. Major problems typically experienced with MLVs are the low aqueous trapped volumes and trapping efficiencies obtained for traditional MLV dispersions. Numerous techniques, therefore, have been developed to yield multilamellar preparations which entrap higher proportions of the drug. These increased trapping efficiencies are achieved by increasing the aqueous trapped volume of the vesicles and utilizing high lipid concentrations. The resulting preparations tend to be quite heterogeneous in size (Table I), and the extent of multilamellar character appears to vary for different procedures. For the purpose of this discussion, MLV will refer to any liposome system that displays an average number of lamellar per vesicle ≥3.

Under appropriate conditions, MLVs can be produced employing solvent evaporation techniques [84]. These MLVs display trapping efficiencies between 6 and 38% and appear to retain entrapped solutes longer than traditional MLVs [84]. Such observations may be related to the fact that solvent evaporation MLVs exhibit equilibrium transmembrane solute distribution whereas traditional MLVs do not [84]. The osmotic stress thus imposed on the latter MLVs may result in their poorer
retention of entrapped solutes. Antibiotics entrapped in solvent evaporation MLVs have been shown to be effective for the treatment of Brucella infections in mice and guinea pigs [19], indicating that these formulations may be very useful as a delivery system for certain drugs.

Previous work has demonstrated that freezing and thawing SUVs results in a dramatic increase in vesicle size and trapped volume [85]. Although this technique was used initially to produce LUVs, under proper conditions MLVs can be prepared in this manner to efficiently encapsulated numerous solutes (Table I) without significantly affecting the biological activity of more sensitive agents such as DNA and proteins [86-88]. More recently, studies have shown the multiple freeze-thaw steps completed directly on MLV dispersions induce a remarkable morphological change in the liposomes and results in a 10-fold or greater increase in the aqueous trapped volumes [89]. Trapping efficiencies as high as 88% for these systems can be achieved by utilizing high lipid concentrations, although entrapped solute/lipid ratios appear to decrease above 100 mg lipid/ml, presumably due to a lack of available aqueous media. These elevated trapping efficiencies are clearly desirable and such preparations may be very useful under conditions where lipid dose is not a limiting factor.

Encapsulation efficiencies approaching 50% for MLVs can also be obtained by dehydration-rehydration procedures [55,90,91] where SUVs are dried in the presence of the desired agent by lyophilization or other methods. Upon addition of water to the dehydrated material, the multilayers swell, forming MLVs which entrap a significant amount of the original solutes. Important features of the freeze-thaw and dehydrate-rehydrate processes, in addition to the large trapping efficiencies are that they appear applicable to a broad spectrum of biologically active agents, they produce MLVs which exhibit stable solute retention characteristics, they are relatively simple, they do not require organic solvents or detergents and they display extended stabilities by storage in the frozen or dried state.

B. Active trapping techniques

The previous section addressed the techniques used to liposomally entrap materials which behave as true aqueous markers. Here we deal with procedures where drug/lipid ratios of the final preparations are far greater than predicted on the basis of theoretical aqueous trapped volumes. At one extreme are agents which are virtually insoluble in water and can be incorporated into the lipid bilayer during vesicle formation. These materials are generally treated as lipids themselves, being mixed homogeneously with the lipid component prior to vesicle hydration step. The amount of hydrophobic drug that can be introduced in a liposome is therefore highly dependent on packing restrictions in the lipid bilayer and, as a result, liposome formulations for this class of drugs vary dramatically from one agent to the next. At the other extreme are water-soluble materials which interact with the polar headgroup of phospholipids and are sequestered- by the liposomes. Between these
two extremes are amphiphilic agents which are often the most difficult to retain inside liposomes as they can rapidly permeate through lipid bilayers. Since a large number of commonly used drugs are amphiphilic molecules, this property poses a major difficulty. Alterations in the lipid composition of liposomes have been used to enhance the encapsulation efficiency and decrease the release rates of these agents through ionic interactions between the drug and charged lipid components. A good example is the amphiphilic anticancer drug adriamycin. This positively charged anthracycline derivative interacts with negatively charged phospholipids, particularly with cardiolipin, and several investigators have utilized this association to efficiently entrap adriamycin into liposomes and increase drug-retention [92,93].

Drug loading in response to ion gradients offers a more general means for encapsulating agents which are amphiphilic cations (such as adriamycin). This process is analogous to the ability of various probes of membrane potentials and transmembrane proton gradients to redistribute across lipid bilayers in response to $\Delta\psi$ or $\Delta p\text{H}$. This can result in very large transmembrane concentration gradients of the probe molecule. Table II lists several amphiphilic drugs in various classes which are sequestered into the vesicles in response to $\Delta\psi$ and/or $\Delta p\text{H}$. Bulk accumulation of antineoplastic agents [83,94,95], local anaesthetics [95,96] and biogenic amines [97,98] inside liposomes~ can be obtained by incubating the drug in the presence of vesicles exhibiting transmembrane $K^+ (\Delta\psi)$ or $H^+ (\Delta p\text{H})$ gradients. Although both $\Delta\psi$ and $\Delta p\text{H}$ systems induce efficient entrapment of lipophilic cations, drug uptake in response to $\Delta p\text{H}$ most likely will be of more practical use. This is due to the fact that $\Delta\psi$-dependent encapsulation requires the use of exogenous ionophores whereas $\Delta p\text{H}$-dependent uptake does not. The mechanism of drug uptake induced by pH gradients is probably similar to the

<table>
<thead>
<tr>
<th>TABLE II</th>
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<tbody>
<tr>
<td>DRUGS WHICH RESPOND TO $K^+ (\Delta\psi)$ AND $H^+(\Delta p\text{H})$ TRANSMEMBRANE GRADIENTS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug</th>
<th>Trapping efficiency (%)</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta\psi$</td>
<td>$\Delta p\text{H}$</td>
</tr>
<tr>
<td>Antineoplastic agents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adriamycin</td>
<td>95-98</td>
<td>80-98</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>90</td>
<td>N.D.</td>
</tr>
<tr>
<td>Local anaesthetics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dibucaine</td>
<td>60-98</td>
<td>60-95</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>80</td>
<td>N.D.</td>
</tr>
<tr>
<td>Biogenic amines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dopamine</td>
<td>50-90</td>
<td>60</td>
</tr>
<tr>
<td>Seratonin</td>
<td>20-40</td>
<td>20-40</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>20-40</td>
<td>20-40</td>
</tr>
</tbody>
</table>

N.D., not determined.
ApH-dependent transmembrane redistribution of other weak bases, where the unprotonated (neutral) species crosses the membrane and accumulates in the vesicle interior until 
$\frac{[\text{AI-I}^+]}{[\text{AH}^+]}_{\text{out}} = \frac{[\text{H}^+]}{[\text{H}^+]_{\text{out}}}$ according to classical Henderson-Hasselbach relationships, where AI-I? indicates the protonated form of the agent in question. For example, under appropriate conditions adriamycin can be liposomally encapsulated by this procedure yielding transmembrane drug concentration gradients of $10^3$ and trapping efficiencies approaching 100% [83,94]. In addition, this results in drug-lipid ratios which are significantly greater than possible by other methods. Clearly, this is an extremely cost-efficient method for entrapment. The transmembrane ion gradients not only accomplish efficient drug encapsulation but also decrease the rate of drug efflux from the vesicles by as much as 30-fold [83,94]. Finally, this procedure can be employed to entrap drugs after the liposomal carrier system itself has been generated. Such ‘remote loading’ could be of utility for labile drugs where it may be important to encapsulate the agent immediately prior to use.

IV. Stability of Liposome-Drug Systems

An important consideration for liposomes as pharmaceuticals concerns is the stability of the sample from the time of drug encapsulation until its use in vivo. While the chemical stability of lipids and drugs often can be increased with the inclusion of antioxidants and the like, the physical stability of liposomes poses a more difficult problem. In particular, pharmaceutically acceptable liposome-encapsulated agents must be maintained as such during storage times of 1 year or more. The release of entrapped solutes from vesicle carrier systems over this period of time may render many liposome-drug preparations inappropriate for therapeutic use. The ability to efficiently load lipophilic, cationic drugs into liposomes employing $\Delta \psi$ or $\Delta \text{pH}$ [83,94-98] just prior to use provides a means to circumvent such difficulties. Another approach to solve this problem has been the use of dehydrated liposome systems. Freeze-drying or drying vesicles in the presence of appropriate cryoprotectants (e.g. trehalose) yields dehydrated liposome preparations which can easily be stored and upon rehydration with water retain >90% of the entrapped agents [99-101]. Vesicles generated in the presence of specific saccharides also maintain their original chemical and physical (size) properties[101]. These results are especially pertinent for SUV and LUV systems where vesicle size is of particular importance. In addition, dehydrated liposomes can be prepared such that addition of water results in vesicle systems exhibiting transmembrane pH gradients which can be utilized for ‘remote loading’ of lipophilic, cationic drugs [101]. Our laboratory has found that vesicles can be used in this manner after storage at 4°C for over 18 months (L.D. Mayer, unpublished data).

V. Final Remarks

The use of liposomes either as model systems or as drug delivery vehicles usually requires the entrapment of material either inside the vesicle or in the liposome
membrane itself. It should be clear from the results presented here that techniques for the efficient entrapment of a large variety of lipophilic and hydrophilic agents inside vesicles of differing size and lipid compositions are now available. Further, the use of appropriate cryoprotectants promises that such formulations can be stable for considerable lengths of time when stored in the dry form. Thus, solutions for many of the technical difficulties associated with liposomal drug delivery appear possible.

References


