Liposomes

From Biophysics to Therapeutics

edited by

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To my two sons,

David and Benjamin

Preface

The first book in this series, *Liposomes*, was designed to address progress in the basic research on liposomes. Subjects such as vesicle formation methods and mechanisms of liposome-cell and liposome-serum interactions were presented in detail; however, the therapeutic applications of liposomes were presented in only one chapter, written by Eric Mayhew and Demetrios Papahadjopoulos. Since *Liposomes* was published in 1983, considerable progress has been made in the use of liposomes in virtually every field of medicine. It is the purpose of this volume, *From Biophysics to Therapeutics*, to make the transition from the laboratory to the clinic. Although the major thrust of this work is the evaluation of the pharmaceutical potential of liposomes, this subject cannot be presented coherently without first discussing the structural properties of liposomes and their potential as pharmaceutical agents. These subjects are covered by Sol M. Gruner in a unique and thought-provoking chapter, examining liposomes as materials, and by Pieter R. Cullis and co-workers, who discuss recent progress in solving the commercial problems of production, stability, sterility, and reproducibility of liposomes. The biodistribution of liposomes in vivo as well as their potential in drug targeting are thoroughly covered in Chapters 4 and 5, respectively. An understanding of these subjects is essential if one is to design liposome-based therapeutics. It is probably accurate to say that liposome biodistribution dictates how they are used. Certainly much of the applied work in the areas of vaccines, infectious disease, and cancer presented in Chapters 6 through 9 has evolved from an initial understanding of pharmacokinetics. In Chapters 3 and 10, the authors present subjects not often considered in the context of liposome research. Chapter 3 by Steven L. Regan covers the use of polymerizable lipids to make liposomes. These vesicles have the potential to provide controllable time-release systems as well as having possible uses in oral drug delivery. In Chapter 10, Alan L. Weiner investigates the use of lamellar systems as drug-solubilizing agents—an often overlooked application of liposome technology.

Marc J. Ostro

Introduction

In the last century, new drug development progressed at a rapid pace, but the mode of drug administration for the treatment of disease did not improve significantly. Medication is still given parenterally and is still systemically diluted so that only a small fraction of the administered drug can actually be found at the desired site. This archaic manner of introducing drugs to patients is clearly inefficient and often leads to toxic side effects since there is no mechanism for routing drugs away from cells or organs where drug-associated toxicity is elicited.

Almost 75 years ago, Paul Ehrlich envisioned a drug delivery mechanism that would target drugs directly to diseased cells: This was the famous "magic bullet" concept. The pursuit of this dream has been an obsession for many working in the liposome field. It was not long after liposomes were first made by A. D. Bangham in the early 1960s that it was demonstrated that a wide variety of molecules, large and small, could be encapsulated within the aqueous spaces of liposomes or actually inserted into their membranes. It was generally assumed at the time that liposomes, since they were primarily made out of phosphatidylcholine, would not be recognized as particulate antigens and would thus avoid reticuloendothelial system (RES) clearance. Therefore, if one could attach a targeting molecule such as an antibody to the surface of the liposome, the potential for targeted drug delivery could become a reality. Certainly, there are now numerous methods for accomplishing this attachment, thereby allowing one to produce vesicles loaded with drug and coated with a variety of site-specific molecules. Unfortunately, the attempt to use these liposomes for targeted drug delivery has been inhibited by the fact that the initial assumption—RES avoidance by phosphatidylcholine vesicles—is simply not true. Regardless of the composition, size, or charge of the vesicle and regardless of what the vesicle is coated with, eventually liposomes will be deposited in high concentrations in RES organs. An additional problem involves the ability, or lack thereof, of liposomes to leave the circulatory system and enter the extravascular space. This makes targeting to most sites quite difficult.

I am often asked why it has taken 22 years for people to start to realize the commercial potential of liposomes. There are several answers to this question but, in my view, one major factor has been the dogged pursuit of the "magic bullet" concept. For years more practical applications of liposomes

were ignored. There are of course other reasons why the emergence of liposomes as a viable commercial technology has been delayed. For example, until the last five years, most liposome research was done in university settings. In this environment, the endpoint of a successful project is not the development of a product but the publication of a paper. This is not meant as a criticism since it is not the function of universities to produce articles of commerce; however, typical commercial problems have not normally been addressed. For example, most liposome research requires the production of only small amounts of entrapped material. The production of 100 ml of liposomes would be considered large-scale manufacturing by most basic researchers. It is certainly not uncommon for liposomes to be made in the morning and used in the afternoon. Week-old liposomes are usually disposed of. If sterile liposomes are needed, they are usually produced under a sterile hood. Reproducibility of the preparation, pyrogen content, integrity of the lipid, patentability, cost, quality-control methods, and regulatory issues, as well as acute, subacute, and chronic toxicity are seldom if ever addressed.

Since academic researchers have had little reason to focus their efforts and scarce resources on these problems, solutions have been slow in coming. It is therefore not surprising that I have been told many times during the last five years that liposomes can never be made stable enough to last for two years on a pharmacy shelf; that scale-up to multiple-kilogram batches of liposomes cannot be achieved in a manner that would result in a reproducible product; that terminal sterilization of liposomes is impossible; and that the prohibitive cost of certain purified phospholipids precludes the sale of any liposome product. It is now clear that these conclusions have been based upon 20 years of effort devoted to basic research and not to the solution of purely commercial problems.

During the last five years, thanks to the commitment of considerable financial and human resources, most of these problems have been solved, paving the way for the market introduction of liposome-based pharmaceuticals. For instance, stable liposomes can now be made in a number of ways: They can be lyophilized, dried, frozen, and even loaded with drug after they are produced (see Chapter 2). Liposomes can be sterilized by terminal filtration, and sterile batch sizes as large as 40 liters have been produced. Analytical methods have been developed that can assess reproducibility of liposomes, and raw material costs have dropped to the point where liposome formulations will be cost-effective to manufacture.

While there is still a long way to go before an actual pharmaceutical product can be made for sale, current technology will allow for the production of adequate material for the initiation of several clinical trials. In fact, several clinical trials of liposome-encapsulated drugs have already begun, with many more planned to start during the next two years. Specifically, liposome-encapsulated doxorubicin (a commonly used anticancer drug) is now in Phase I clinical trials

Anticipated Clinical Trials During the Next Two Years

Encapsulated drug	Disease treated	Probable benefit rendered by liposomes
Amphotericin B	Systemic fungal infections	Significantly reduced nephrotoxicity; greater efficacy
Doxorubicin	Cancer	Reduced cardiotoxicity, immunosuppression, emesis, alopecia; enhanced efficacy
Cisplatin derivatives	Cancer	Reduced nephrotoxicity and emesis. New indications in leukemias, lymphomas, and liver metastases. Not cross-resistant with cisplatin
Vincristine	Cancer	Reduced neurotoxicity; enhanced efficacy
Muramyltripeptide	Cancer-immunomodulator	Only acceptable carrier, targets to macrophages
Gentamicin	Gram-negative pneumonia and other serious gram-negative infections	Reduced nephrotoxicity; significantly improved efficacy
Streptomycin	Bovine brucellosis	Only cure
Indomethacin	Arthritis	Reduced gastric toxicity
Bovine somatotrophic hormone	Increase milk production	IM dosing once every two weeks
Epithelial growth factor	Aid in wound healing	Infrequent dosing
Pilocarpine	Glaucoma	Once per day drop
Tear components	Dry eye	Increased comfort that lasts longer
^{111}Indium	Tumor imaging	Preferential accumulation in tumor

in the United States at Georgetown University, and in Canada and Israel, and muramyltripeptide liposomes are now being tested in the United States as immune modulators. The most advanced product involves the use of liposomal amphotericin B for the treatment of systemic fungal infections (see Chapter 8). This work is being done under a Compassionate Investigational New Drug permit by Dr. Gabriel Lopez-Berestein's group at M. D. Anderson Hospital and

Tumor Institute in Houston. To date, over 20 patients with terminal disease have been treated and over half have been completely cured. Given the fact that in order to qualify for this study patients must have failed all conventional therapy, including free amphotericin B, these results are quite exciting. The table lists most of the clinical trials currently underway as well as those expected during the next two years.

As the table indicates, the utility of liposomes spans many fields of medicine. Liposomes have been shown to be useful administered intravenously (anticancer drugs, antibiotics), intramuscularly (peptides), and topically (ophthalmics). In fact, liposomes recently have even been used as emollients in cosmetics. The field of liposome research has literally exploded during the last five years. This book is an attempt to keep pace with that explosion.

Marc J. Ostro

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Contents

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Materials Properties of Liposomal Bilayers

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INTRODUCTION

Liposomes are useful because of the highly structured way bilayers can be organized to sequester solutes. Effective utilization of liposome technology requires an understanding of how liposome structure results from the chemistry of the molecules of which liposomes are made, the materials properties of the bilayer, and the way the bilayers are assembled into liposomes. Although the literature contains excellent reviews covering many aspects of liposomes (1-7), little attention has been specifically given to distinguishing between the chemical, materials, and the morphological properties of liposomes. *Chemical properties* refer to characteristics that may be understood by consideration of one, or at most a few, molecules, by length scales of about a nanometer or less, and by interactions that are usually nearest-neighbor interactions. Examples include the pK of a headgroup, the degree of saturation of a lipid chain, or the specific protein-lipid substrate interactions of the phosphatidylinositol/diacylglycerol cycle (8). *Materials properties*, on the other hand, result from the cooperative interactions of many molecules and are not readily understood by considering only isolated molecules. Examples include the bilayer isothermal compressibility, main transition enthalpy, and tensile elastic limit. Materials properties are notable in that often very different collections of molecules yield similar materials properties. Finally, *morphological organization* refers to the different way similar materials

can be macroscopically organized. For example, large unilamellar vesicles (SUV) and multilamellar vesicles (MLV) are two morphologically distinct organizations that may be made of a single kind of bilayer material.

This article is a review of recent work on the materials and morphological aspects of bilayers and liposomes. Relatively little will be said about the purely chemical aspects of lipids because there is an extensive literature on this subject (see, for instance, Ref. 9).

This review is divided into two parts. The first is a discussion of important properties of lipid bilayers from a materials point of view. The second deals with the way bilayers are organized into liposomes, with special emphasis on the interaction between bilayer properties and morphology. It is well recognized that the basic characteristics of the constituent bilayers affect the morphology of the liposomes. For example, the surface charge of the bilayer affects the spacing between the layers in an MLV. As another example, bilayers of different compositions and hence different bulk properties yield different sized liposomes under a given method of preparation. The reverse aspect of this interaction, namely, the way morphology affects the basic materials properties of bilayers, is less well known. Some examples are that the size of the liposome and hence the surface curvature, changes the main transition enthalpy (10), the main transition temperature (11), as well as the fusogenic behavior and the interactions with divalent cations (12-18).

BILAYERS AS MATERIALS

Liposomes are lyotropic liquid crystals composed preponderantly of ampiphilic bilayers. It is noteworthy that most of the nouns of the preceding sentence refer to materials and materials properties, i.e., properties that arise as a consequence of the cooperative action of many molecules. Although the distinction is not always clear-cut, materials properties are to be contrasted with purely chemical properties, e.g., properties that may be understood by considering one or, at most, a few molecules. Although it is dangerous to draw too fine a distinction between chemical and materials properties, it is important to recognize that many different combinations of chemicals often lead to identical materials properties. Thus for instance, there is a wide latitude of phospholipid substitutions that will yield a bilayer of a given compressibility or tensile strength. The technology of liposomes deals largely with the procedures and variety of chemicals that can be manipulated to form closed structural units of bilayers endowed with general materials properties.

The materials properties of bilayers are of importance to liposome technology for fundamentally the same reason that the materials properties of a metal determine the uses to which the metal will be placed. In both the lipid and the metals examples, the materials properties determine constraints placed

on objects fabricated from the materials. A trivial example is that a soft metal would not be used for a bearing. Likewise, a bilayer with a low tensile elastic limit should not be used in instances where it had to sustain a large osmotic imbalance. Other aspects of the metal-bilayer analogy are also appropriate, although likely to be unfamiliar to many liposome workers. When a metallurgist wishes to find a substitute material from which to fashion an engine part, he first considers the desirable elastic properties the substitute material should have. It is much more efficient to test the general properties of the material than to attempt to fabricate the part from each possible candidate metal and test the resulting part. Likewise, the bulk materials properties of bilayers are likely to be important determinants in the use of liposomes. Knowledge of the role of materials properies, and convenient methods of measuring the properties, will eventually add efficiency to the process of fabricating liposomes for specialized uses, especially when tests of the desired results (e.g., buffering of drug toxicity) are expensive and difficult.

Another example of the metals analogy pertains to alloying. The properties of mixtures of metals vary systematically with the ratios of the constituents, as do the properties of lipid mixtures. In the former, the metallurgist does not form random mixtures; rather, he mixes judiciously based on an understanding of how a given component affects a desired materials property. Experienced researchers who fabricate liposomes do likewise, although, currently, the process is much more intuitive and less systematic. The effects of mixtures are likely to be especially pertinent to the delivery of high concentrations of lipophilic pharmaceuticals. Such substances are often potent modifiers of bilayer properties. It will become necessary to understand how the drug is modifying the properties of the bilayer, which modifications are deleterious, and how to adjust the lipid composition to compensate for effects of the drug.

Systematic use of materials properties of liposomes is hampered by a lack of appreciation of the differences between chemical and materials properties, by the difficulty of measuring many of the properties and, most of all, by a lack of consensus about which properties are important for a given application. All of these reasons may be taken as a sign of the immaturity of liposome technology. There is every reason to believe that this situation will change as liposomes evolve from a laboratory tool to a commercially important technology.

What bilayer materials properties are important for liposome technology? Recognizing that the answer to this question is biased by one's perspective and by current knowledge, we would certainly include the following:

1. Gel to liquid crystalline ($L_\beta - L_\alpha$) transition
2. Bilayer permeability
3. Partition coefficients
4. Electrical properties
5. Elastic properties

These properties are a direct consequence of the balance of forces that exists in the bilayer; therefore before discussion of the properties, it is appropriate to consider why ampiphiles aggregate to form bilayers and to understand the internal forces that result from the process of aggregation.

Molecular Aggregation into Bilayers

The driving force behind bilayer assembly is, of course, the hydrophobic effect (19) coupled with the ampiphilic nature of the phospholipids of which bilayers are usually composed. Phospholipids typically consist of a water-loving, or hydrophilic, headgroup covalently attached to two very hydrophobic hydrocarbon tails. By *hydrophobic*, we refer to the fact that the chemical potential of a hydrocarbon is lowered substantially [approximately 3.7 kJ mol^{-1} per methylene group (19)] in going from an aqueous to an oillike environment, e.g., one in which the near-neighbor interactions are nonpolar, nonionic, and nonhydrogen bonding.

The drop in chemical potential accomplished by segregating the tails out of contact with water while keeping the headgroups suitably hydrated may be achieved if the lipids aggregate into bilayers (Fig. 1a-c). It is important to recognize however that alternative geometries, such as micelles, and tubular aggregates, such as the hexagonal (H_{II} and H_I) phases shown in Fig. 1d-e, also meet the constraints of the hydrophobic effect. The geometry that actually occurs in equilibrium is the one in which compromises are made among the hydrophobic effect, entropy, and all the intermolecular interactions such that the overall free energy per molecule is minimized. The complex of interactions that has to be considered include van der Waals forces, hard core molecular packing constraints, electrostatic interactions, and hydrogen bonds. The various geometries assumed by lipid-water systems are referred to as *lipid polymorphism* (20). The molecular basis of lipid polymorphism has recently been reviewed (21).

It is erroneous to assume that, because liposomal lipids are in bilayer form, nonbilayer aggregates need not be considered. The reasons for this are twofold. First, although the lipid geometry observed may vary discretely, as in a bilayer to H_{II} phase transition, the complex of underlying forces that determine the geometry with the lowest overall free energy varies continously. Thus the liposomal bilayers may be poised at the edge of a phase transition by a suitable combination of temperature and composition. As discussed later, relevant properties, such as leakage and stability, are dramatically different when bilayers are near phase transitions. In terms of a classic reaction diagram (Fig. 2), the bilayer and nonbilayer states may be at nearly identical energies.

A second reason why lipid polymorphism must be considered is that liposomes are assemblies that may be far from thermodynamic equilibrium. Although the equilibrium state may be nonbilayer, the approach to equilibrium

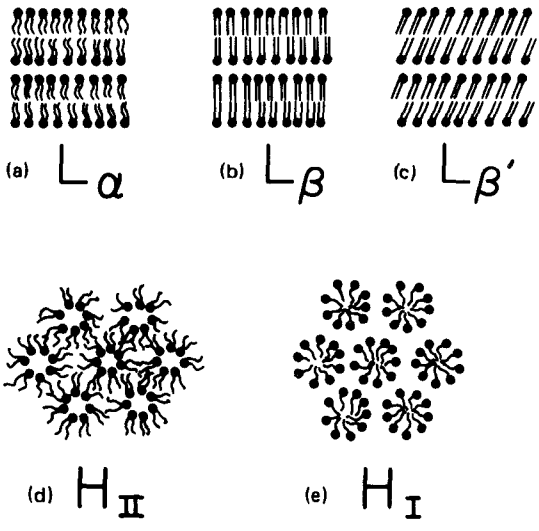

Figure 1 A schematic representation of several lipid polymorphic phases. The L_α phase is a liquid crystalline (melted chain) bilayer phase, whereas in the L_β and $L_{\beta'}$ phases the chains are in a gel (frozen) state. The H_{II} and H_I phases consist of hexagonally packed tubes of liquid crystalline lipids (*Source:* from Ref. 21).

may be kinetically blocked by the liposomal morphology. For example, it may be that there exists a nonbilayer rearrangement of the molecules in a system, which yields a state of lower overall free energy; however movement of the lipids into this state may involve the extensive exposure of hydrocarbon chains to water. Here the liposomes may be unstable and slowly degrade. A relatively minor perturbation of the system (e.g., contamination by small amounts of oil or divalent cations) may precipitate the degradation of the liposomes. In terms of Fig. 2, the nonbilayer state B, may actually be at a lower energy than the bilayer state, A, but the activation barrier that separates the two minima is sufficiently high that movement from A to B proceeds very slowly. (In some sense, the latter state of affairs is the norm when liposomes are used to sequester aqueous solutes. In this instance the state of lowest free energy may be when the solute has leaked and is uniformly dispersed throughout the system. Note that this is not necessarily true for hydrophobic solutes.)

Considerable variability of the materials properties of liposomes may be obtained by altering the vesicle composition; indeed, compositional variation is the subject of an enormous and complex literature (for reviews see Refs. 5,7). Unfortunately, at present, there is only a limited, phenomenological understanding of how composition affects the intermolecular forces and the consequent

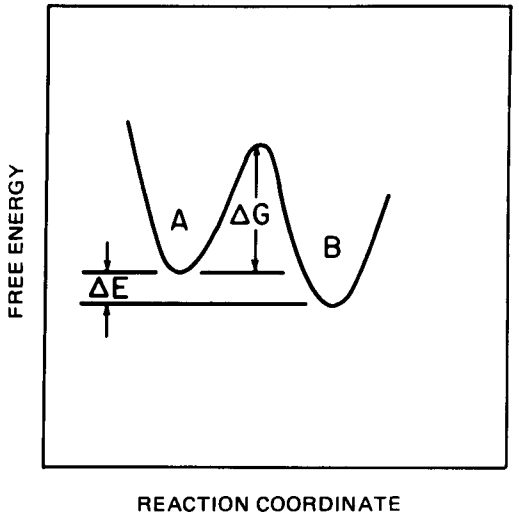

Figure 2 The L_α and H_{II} phases may be thought of as local minima (A and B) in a generalized reaction coordinate diagram. The thermodynamically stable phase is that associated with the deepest minimum under a given set of environmental conditions. In general, the L_α minimum rises relative to the H_{II} minimum as the temperature rises or the water content falls. The free energy difference, ΔE, drives a phase transition between minima. The transition may also be kinetically blocked if the activation barrier, ΔG, is too large.

materials properties of the liposomes. In so far as the liposome is used to deliver dilute drugs, the bilayer properties may be independently analyzed. However in a more typical situation, the liposome may be used to deliver a concentrated drug, which may be charged, may be lipophilic, and may partition strongly into the bilayer. Here, the bilayer must be regarded as a new material of altered composition, often with properties considerably different from those of the pure lipid bilayer. The study of the properties of drug-lipid materials is in its infancy. Because liposomes are one of the few ways to deliver high concentrations of very lipophilic materials, it is safe to predict that the study of lipid-drug materials will experience considerable growth in the near future.

The most important use of liposomes results from the ability to retain solutes for long periods. This ability depends both upon properties intrinsic to the bilayer (e.g., the rate at which a solute diffuses through an intact bilayer) and on morphology (e.g., do the bilayers completely enclose an aqueous volume). In the next five sections we deal exclusively with properties intrinsic to the bilayer.