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Good manufacturing processes require that lipid vesicles be reproducibly uniform in size, carry the drug in sufficient concentration, and remain stable for an acceptable shelf-life under varying temperatures. Following these guidelines improves the analytical processes that support biopharmaceutical drug development.

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Liposomes (a Review)

Part Two: Drug Delivery Systems

FDA considers liposomal drugs to be products in which an active drug is encapsulated in or complexed with lipids. The agency also considers liposomal drug products containing already marketed drug substances as new drug formulations. Some unique requirements for the phospholipids used in liposomal formulations depend on the source of the lipids. As discussed in Part 1, stability of the phospholipids, which are considered critical excipients, plays a key role in the characterization of product performance.

Reduced Toxicity, Entrapped Drugs

Liposomes have the distinct advantages of being both nontoxic and biodegradable because they are composed of naturally occurring substances. Biologically active materials encapsulated within liposomes are protected to varying extent from immediate dilution or degradation, suggesting drug carrier systems for the transport of drugs and other bioactive capsules to disease-affected organs. The unique ability of liposomes to entrap drugs both in an aqueous and a lipid phase make such delivery systems attractive for hydrophilic and hydrophobic drugs.

Because of advancements in the methods of preparing and formulating liposomes, high-entrapment efficiencies are possible for incorporating drugs into liposomes, creating a tremendous pharmaceutical impact. Furthermore, such encapsulation has been shown to reduce drug toxicity while retaining or improving the therapeutic efficacy. Several laboratories have reported the use of liposomes as drug carriers in the treatment of cancer (1,2), leishmaniasis (3), metabolic disorders, and fungal diseases (4). Innovative research in liposomal drugs has led to commercialization of several anticancer therapeutics such as Doxil, Myocet, two liposome-based anticancer drugs; doxorubicin; and an antifungal drug formulation, AmBisome, which is a liposomal formulation of amphotericin B used for systemic therapy.

Liposomes may have a use in gene delivery to correct gene-associated disorders or for vaccine therapy (5). A quantitative entrapment of DNA can be achieved using the preparation of empty liposomes with cationic lipids followed by mixing with DNA or a plasmid of interest. Because of its convenience and efficacy, cationic lipid-mediated gene delivery technology is a promising system for in vivo gene therapy. Clinical trials of large-size lipid-DNA complexes have mostly shown a lack of adverse effects and moderate expression in a relatively low fraction of cells, but no decisive clinical disadvantages.

Sustained Delivery Systems

As systems for the delivery of drugs, liposomal dispersions may be beneficial through several mechanisms. They can solubilize water-insoluble drugs (such as Minoxidil or amphotericin B), provide site avoidance and reduced toxicity through minimized uptake in sensitive tissues (such

Abbreviations Used

DRV	dehydration-rehydration vesicles
HDL	high density lipoproteins
LUV	large unilamellar vesicles
MAB	monoclonal antibodies
MLV	multilamellar vesicles
PC	phosphatidylcholine
PS	phosphatidylserine
RES	reticuloendothelial system
SUV	small unilamellar vesicles

as the heart, kidneys, brain or gut for amphotericin B or doxorubicin), and facilitate sustained release. In theory a drug in a liposomal system may be released through three possible mechanisms: passive diffusion, vesicle erosion, and vesicle retention.

In **passive diffusion**, the drug molecule penetrates a series of bilayers in a gradient to reach the extravascular layer, the circulating blood plasma. This mode of release has been investigated using diffusion models. In its simplest case, the model would correspond to that of diffusion through a membrane (Fick's second law). When multiple layers are present, the model would resemble a heat flux through concentric spheres or diffusion through a series of barriers. The regulation of permeability (the relative leakiness of the vesicles) is affected by certain materials such as cholesterol.

Vesicle erosion. Phospholipases and lipoproteins present in blood plasma can attack and compromise vesicle integrity, gradually exposing the drug entrapped in the interbilayer spaces. Vesicle erosion is manipulated through the compositional variations of the lipid bilayers.

Vesicle retention. The vesicles can be made unrecognizable to the macrophages of the reticuloendothelial system (RES, discussed in detail below) to increase the circulatory lifetime of a liposomal formulation. Similarly, their opsonization (modification by opsonins, which are blood serum constituents that enable phagocyte action) in vivo can be reduced, creating so-called stealth liposomes. Certain plasma proteins (such as opsonins) have an affinity for liposomes. That affinity is enhanced if the liposome is charged. Electrically neutral liposomes exhibit extended circulation half-lives. Tightly packed liposomes are less prone to absorption and penetration by opsonins than are vesicles with loosely packed bilayers. Hence, cholesterol-incorporated liposome formulations exhibit enhanced circulatory times. Smaller liposomes seem less likely to absorb opsonins because of their higher curvature radius compared with larger liposomes.

Some ambiguity is associated with referring to liposomal formulations as "controlled release dosage forms." That arises from an inability to control the number of variables associated with the

manufacture and use of liposomal dispersions, which influences passive diffusion, erosion, and retention in the circulation. Usually some in vitro testing must be carried out on a controlled-release formulation. Such testing is very sensitive to particle size and polydispersity, which can be difficult to control in a liposomal formulation. Even when those parameters are strictly controlled, a correlation to in vivo release is difficult. In addition,

BOTH vesicle size and lipid composition affect the in vivo drug release profile of liposomal formulations.

stability-related aging effects on vesicle integrity would undoubtedly alter permeability over time. The term "sustained-release drug delivery system" is probably more correct here, given the complexity of drug/liposome clearance in the body.

Factors affecting release. The effect of vesicle size on in vivo release of daunorubicin (an anthracycline anticancer drug) from liposomes into the circulation was studied in rats (4). Those liposomes were prepared from hydrogenated egg phosphatidylcholine (HEPC) or egg phosphatidylcholine (EPC), cholesterol, and dicetylphosphate at a molar ratio of 5:4:1, and their mean vesicle sizes were adjusted to about 50 and 100 nm. The drug retained and that which leaked were separated from plasma by gel filtration. On the assumption that lipid content does not change, the drug released from each liposome preparation was estimated by a latency percentage calculated from the drug/lipid concentration ratio of the liposome preparation.

From HEPC-liposomes 50 nm in diameter, the drug was released gradually after intravenous administration, and the cumulative release reached 40% after 240 minutes. However, from EPC-liposomes of the same size, 50% of the drug was

released within five minutes, and more than 90% of the drug was released within 60 minutes. From HEPC-liposomes 100 nm in diameter, no drug release was observed more than 240 minutes after administration. Those findings indicate that both vesicle size and lipid composition affect the in vivo drug release.

In Vivo Fate of Liposomes

The RES is the major site of liposome accumulation after systemic administration (6). Primary organs associated with the RES are the liver, spleen, and lungs. The liver exhibits the largest capacity for uptake, whereas the spleen can accumulate liposomes so that its tissue concentration is 10-fold higher than those of other organs. Assuming that liposomes are designed to minimize systemic protein binding and cell interactions, the extent of liposome accumulation in the lungs is below 1% of the injected dose.

Early studies demonstrated that the liver removed large, charged liposomes rapidly, with spleen clearance half-life of less than one hour. The rate of clearance from circulation could be reduced to some extent by increasing the amount of lipid administered; however, only small (about 100 nm), electrically neutral liposomes containing cholesterol had circulation lifetimes in the range of several hours.

Removal of liposomes from the blood is attributed to phagocytic cells that reside in the RES and is mediated through direct interactions between those cells and the liposomes. In vitro studies have shown that liposome uptake into macrophages can occur in the absence of serum proteins. But recognition, mediated by protein elements that associate with liposome surfaces, is likely to play a dominating effect on interactions with the RES. Clearance of liposomes from blood circulation by components of the RES most likely occurs after opsonization of the vesicles (10). Although the deposition of lipids in the liver is substantial with MLV, liposome size has the same effect on uptake by RES system (7).

A study by Liu et al., using a liver perfusion system, suggested that liposome uptake by the liver is serum and vesicle size dependent (8). Serum-dependent uptake occurs through opsonization of liposomes with serum proteins (9). The extent of

opsonization decreased with a decrease in liposome size from 800 to 200 nm in diameter, and no enhancement of uptake was observed at 200 nm. Small liposomes could not support opsonic activity, whereas the larger ones did so substantially. That size-dependent degradation of liposomes in serum is inconsistent with complement activation. The profound effect of size on complement recognition affects liver uptake, depending on the extent of liposome opsonization.

Most intravenously injected liposomes are taken up by the mononuclear phagocytic cells of the reticuloendothelial system (6), and as a result, other tissues have little access. That problem could be overcome by incorporating ganglioside GM1 (10,11) or using polyethyleneglycol conjugated lipids (12) as one component of liposomes. The resulting liposomes are often referred as "sterically stabilized."

Incorporation of ganglioside GM1 or polyethyleneglycol (PEG) conjugation keeps liposomes in circulation longer, thereby reducing uptake by the liver. Although stealth liposomes have more retention time, they may induce adverse skin reactions.

Besides major uptake by the RES, the endothelial barrier could pose another problem for delivery of liposomal drug formulations, especially to undamaged tissues (6). That might be avoided by delayed release using pH-sensitive (13) or temperature-sensitive liposomes (14).

A major concern in using liposomes for therapeutic purposes is their fast removal from blood circulation by components of the RES, most likely after opsonization of the vesicles (1). A study was performed to quantify the reduction of in vitro macrophage uptake of sterically stabilized liposomes. That was determined using liposomes of different defined sizes (125, 250 and 1,000 nm) with and without sterical stabilization. HPTS was used as a fluorescence marker to discriminate between general uptake and the portion of liposomes internalized into the low-pH compartment. The sizes of liposomes used in the study had only a minor influence on uptake, but the highest concentration of lipid was found for large MLV.

Targeting Specific Tissues

Use of liposome-encapsulated enzymes for delivery into cells was first reported in 1971. About the same time, a specific receptor on

hepatocytes was demonstrated to mediate clearance of β -galactose-terminated glycoproteins from circulation. A mannoside-specific receptor was recognized on the cell surface of the RES of rats (including the liver sinusiod and macrophages).

By grafting different glycosides on the surface of liposomes, it is possible to direct the latter to different cell types of rat liver (15). Galactosylated liposomes are mainly taken up by liver hepatocytes, whereas mannosylated liposomes are mainly taken up by nonparenchymal cells (16). Grafting specific ligands to the liposome surface facilitates a fusion of the liposome with target cells by endocytosis, thus releasing material to be delivered.

In cancer chemotherapy, the toxicity of anticancer drugs is of major concern. Liposomes could be used to deliver such drugs and minimize their toxic effects on healthy cells. Targeted delivery to cancer cells could be achieved by coating monoclonal antibodies (MAbs) raised against tumor-cell specific antigens. In vitro and in vivo studies by Ahmad et al. of squamous-cell carcinoma in mouse models provided evidence that antibody-coated polyethyleneglycol liposomes containing doxorubicin were more effective and less toxic than free drugs, drugs incorporated into antibody-free liposomes, and antibody-coated conventional liposomes (1,4).

The major concern in antibody-grafted liposome use is the induction of immune response to the grafted antibodies. Basten et al. suggested a novel approach to overcoming that difficulty (17). They used ^{125}I -labeled antigen to kill the cells responsible for immune induction (the "antigen suicide" technique). Other possible approaches to overcome the immune-system problem include immunosuppressive drugs and humanized antibodies or establishing neutral immune windows for subsequent injection. Liposomes can be designed to release their entrapped contents under certain controlled conditions: pH-sensitive and temperature-dependent liposomal systems (1,13).

Drug targeting using liposomes as carriers holds much promise, especially in reducing toxicity and targeting delivery to disease sites. The future is bright for liposome research, with a large number of clinical trials ongoing in several countries

For Further Reading

Handbook of Nonmedical Applications of Liposomes — Theory and Basic Sciences, D. Lasic and Y. Barenholz, Eds. (CRC, New York, 1996).

Liposomes: From Physics to Applications, D. Lasic, Ed. (Elsevier, The Netherlands, 1993).

Medical Applications of Liposomes, D. Lasic and D. Papahadjopoulos, Eds. (Elsevier, The Netherlands, 1998).

Pharmaceutical Dosage Forms: Disperse Systems, Vol. 3, 2nd Ed., Lieberman, Rieger, and Banker, Eds. (Marcel Dekker, Inc., New York, 1998).

Specialized Drug Delivery Systems: Manufacturing and Production Technology, Praveen Tyle, Ed. (Marcel Dekker, Inc., New York, 1990).

N. J. Zuidam, *Stability of Liposomes: Chemical and Physical Characterization of Liposomes upon Autoclaving, Gamma-Irradiation and Storage* (University Utrecht, The Netherlands, 1994).

with liposomal formulations of various anticancer drugs, antisense, cytokines, peptides and proteins. In the near future, several more liposome-based drugs will find their way into the pharmaceutical market.

References

- (1) I. Ahmad et al., "Antibody-Targeted Delivery of Doxorubicin Entrapped in Sterically Stabilized Liposomes Can Eradicate Lung Cancer in Mice," *Cancer Res.* 53, 1484–1488 (1993).
- (2) F.J. Mayhew, R. Goldrosen, and J. Vaage, "Effects of Liposome-Entrapped Doxorubicin on Liver Metastases of Mouse Colon Carcinomas 26 and 38," *J. Natl. Cancer Inst.* 78, 707–713 (1987).
- (3) C.R. Alving et al., "Therapy of Leishmaniasis: Superior Efficacies of Liposome-Encapsulated Drugs," *Proc. Natl. Acad. Sci. (USA)* 75, 2959–2963 (1978).
- (4) G. Lopez-Berestein et al., "Liposomal Amphotericin B for the treatment of Systemic Fungal Infections in Patients with Cancer: A Preliminary Study," *J. Infect. Dis.* 151, 704–710 (1985).
- (5) G. Gregoriadis, "Genetic Vaccines: Strategies for Optimization," *Pharm. Res.* 15, 661–670 (1998).
- (6) G. Poste and P. Papahadjopoulos, "Lipid Vesicles as Carriers for Introducing Materials

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- into Cultured Cells: Influence of Vesicle Lipid Composition on Mechanism(s) of Vesicle Incorporation into Cells," *Proc. Natl. Acad. Sci. (USA)* 73, 1603–1607 (1976).
- (7) R. Zeisig et al., "Effect of Sterical Stabilization on Macrophage Uptake In Vitro and on Thickness of the Fixed Aqueous Layer of Liposomes Made from Alkylphosphocholines," *Biochim. Biophys. Acta*, 1285, 237–245 (1994).
- (8) D. Liu, F. Liu, and Y.K. Song, "Recognition and Clearance of Liposomes Containing Phosphatidiserine Are Mediated by Serum Opsonin," *Biochim. Biophys. Acta.*, 1235, 140–146 (1995).
- (9) H. Harashima et al., "Enhanced Hepatic Uptake of Liposomes Through Complement Activation Depending on the Size of Liposomes," *Pharm. Res.*, 11, 402–406 (1994).
- (10) T.M. Allen and A. Chonn, "Large Unilemellar Liposomes with Low Uptake into the Reticuloendothelial System," *FEBS Lett.* 223, 42–46 (1987).
- (11) A. Gabizon and D. Papahadjopoulos, "Liposome Formulations with Prolonged Circulation Time in Blood and Enhanced Uptake by Tumors," *Proc. Natl. Acad. Sci. (USA)* 6949–6953 (1988).
- (12) T.M. Allen, "Stealth Liposomes Avoiding Reticuloendothelial Uptake" in *Liposomes in the Therapy of Infectious Diseases and Cancer*, G. Lopez-Berestein and I.J. Fidler, Eds. (Alan R. Liss, Inc., New York, 1989), pp. 405–415.
- (13) J.N. Weinstein et al., "Liposomes and Local Hyperthermia: Selective Delivery of Methotrexate to Heated Tumors," *Science* 204, 188–191 (1979).
- (14) C.M. Cohen et al., "Introduction of Purified Hexosaminidase A into Tay-Sachs Leukocytes by Means of Immunoglobulin-Coated Liposomes," *Biochemistry* 15, 452–460 (1976).
- (15) P. Ghosh, B.K. Bachhawat, and A. Surolia, "Synthetic Glycolipids: Interaction with Galactose-Binding Lectin and Hepatic Cells," *Arch. Biochem. Biophys.* 206, 454–457 (1981).
- (16) P. Ghosh, P.K. Das, and B.K. Bachhawat, *Arch Biochem Biophys* 231, 266–270 (1982).
- (17) A. Basten et al., *Nature New Biol.* 231, 104–106 (1971). **BP**