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REVIEW ARTICLE

# CONTROLLING THE DRUG DELIVERY ATTRIBUTES OF LIPID-BASED DRUG FORMULATIONS

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## INTRODUCTION TO THE PROBLEM

Research on liposomes as model membrane systems and as drug carriers facilitated the design of pharmaceutically viable lipid-based drugs. In fact much of the research and technology required to prepare liposomal carriers for testing in clinical trials was well established by 1987 (1–3). By that time, four pivotal hurdles were overcome. First, the importance of carefully assessing structure activity relationships through analysis of physiochemical characteristics was proven to be essential in product development. This is best exemplified by studies contributing to the characterization of the amphotericin-B lipid complex (4,5). Second, biological barriers previously believed to limit the distribution properties of systemically administered macromolecular drug carriers, such as liposomes, proved to be penetrable. In 1979 John Balderswieler and co-workers recognized that liposomal drugs could effectively deliver contents to tumors (6), a phenomena that

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continues to be a fundamental rationale for development of systemically administered liposomal anticancer drugs (7). Third, manufacturing issues for preparing pharmaceutically acceptable formulations were resolved (8–10). This included identification of sources for inexpensive raw materials, the elucidation of procedures for storing lipid-based carriers for extended time periods (11) and the development of methods for reproducibly preparing large batches of liposomes with attributes that could be characterized according to the rigorous guidelines of health boards such as the FDA. Fourth, procedures for loading liposomes with pharmaceutically active agents that relied on the chemical attributes of the lipids prior to liposome formation (e.g. doxorubicin/cardiolipin complex) and/or involved loading of pre-formed liposomes were developed (12–16). The latter involves the use of ion gradients to effect drug loading, a procedure that has proven to be particularly useful and versatile.

At the end of the 1980's investigators confidently suggested that liposomes could be rationally designed to achieve specific therapeutic benefits for a broad range of disease targets. It is perhaps disappointing, therefore, that improvements in the therapeutic properties of liposomal drugs have been relatively incremental since 1990. The most significant revisions of lipid-based carrier technology that have guided research efforts during the 1990's involved three breakthroughs made in the late 1980's: 1) the observation that surface associated polymers (i.e. polyethylene glycol or the ganglioside GM1) cause changes in the liposome surface properties that contribute to increased circulation lifetimes (17,18); 2) the discovery that positively charged liposomes can be used to transfer polynucleotides into cells (19, 20); and 3) the identification of certain lipids that can act as therapeutic molecules (21).

Given this perspective, it is useful to consider how this technology may emerge in the next millenium. Other than the many entrepreneurial interests, we believe that the primary objective that has driven research focused on development of liposomal drug carriers concerns improving drug specificity. This goal is clearly a reflection of any drug discovery program, which under ideal conditions would be able to select for pharmaceutical agents that only affect diseased tissues or cells. Such specificity has not been achieved to date.

For anticancer drugs a drug dose required to obtain therapeutic benefits is often not dissimilar to that dose where toxicity is observed. By definition, these drugs exhibit a low therapeutic index and it is not surprising that much of the research developing lipid-based drugs has focused on cancer applications. This research has identified drug formulations that exhibit an improved therapeutic index in comparison to free drug. It is believe that improvements are a consequence of liposome mediated changes in drug pharmacokinetic and biodistribution characteristics. For the anticancer drug doxorubicin, it is known that liposome encapsulation results in reduced drug levels in tissues where toxicity is a concern (e.g. heart) and increased drug levels in tumors (22–24).

These results are satisfying in terms of obtaining desired improvements in a drug's selectivity and therapeutic index, however, there are some significant

conceptual problems that have largely been ignored. First, all tissues can potentially be exposed to higher levels of drug as a consequence of liposome encapsulation. This potential exists because therapeutically optimized liposomes are retained in the blood compartment for extended time periods, where the circulating drug concentrations (free plus encapsulated drug) can be 2 to 3 orders of magnitude greater than can be achieved with free drug. This is illustrated in Figure 1 for two liposomal formulations of mitoxantrone, where the differences in circulating blood levels measured after i.v. injection of free and liposomal drug is shown. Similar data has been obtained for formulations of vincristine, doxorubicin and cisplatin. The significant increase in circulation lifetime and blood levels obtained is a distinguishing characteristic of liposomal formulations that retain drug well and are designed to exhibit slow elimination rates. On the basis of data showing increased drug levels for an extended time period, it is curious why liposomal formulations of an anticancer drug are not more toxic than free drug.

Second, it is also clear that drug delivery to sites of tumor growth can be increased substantially when the drug is administered in a liposomal form. Our own data suggests drug exposure within regions of tumor growth can be increased

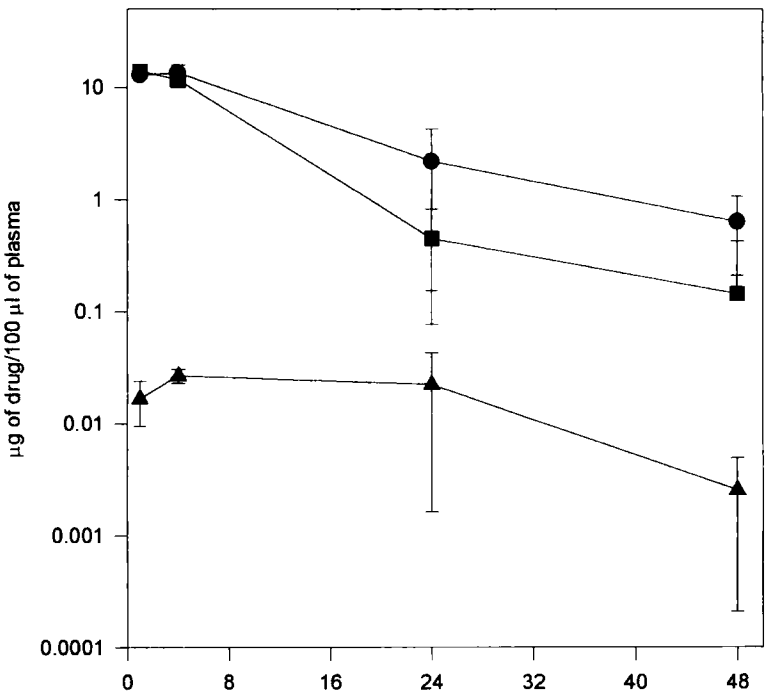


***Figure 1.*** Elimination of mitoxantrone from plasma over 48 hours using DSPC/Chol (filled circles), DMPC/Chol (filled squares) liposomes, and free mitoxantrone (filled triangles). Liposomes were loaded with mitoxantrone at a drug to lipid weight ratio of 0.1 (wt:wt). Female CD1 mice were injected at a 10 mg/kg drug dose i.v. via lateral tail vein. Data represents the mean and standard deviation obtained from at least 4 animals.

3- to 100-fold when the drug is given encapsulated in liposomes. For example, the propensity of liposomal doxorubicin formulations to accumulate in Lewis Lung tumors over a 7 day time period after i.v. administration is shown in Figure 2. Using the mean AUC (μmol doxorubicin/g tissue–time curve, calculated from data integrated from 0 time through to day 7) as an estimate of tumor drug exposure, DSPC/Chol liposomes ($AUC_T$ of 38 μmol·g-1·h) delivered slightly more doxorubicin to tumors than DSPC/Chol/PEG-PE liposomes ($AUC_T$ of 31 μmol·g-

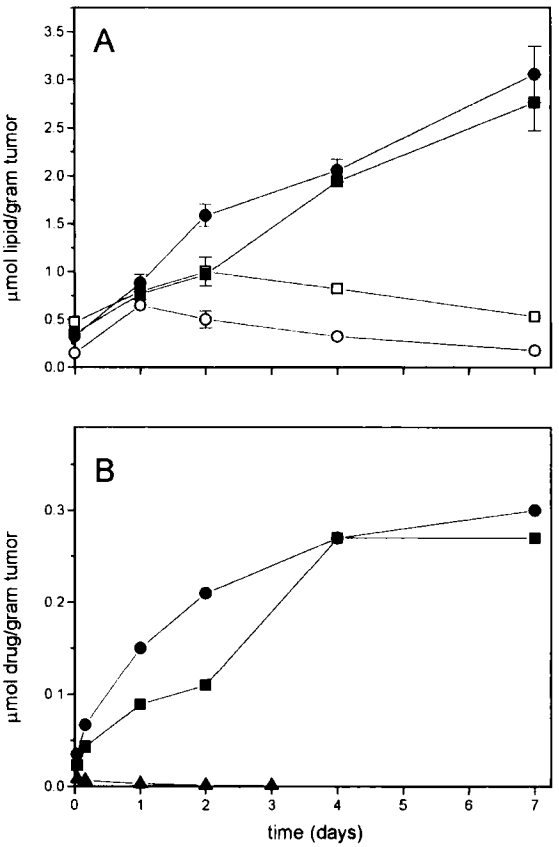


*Figure 2.* Tumor loading of liposomal lipid and doxorubicin following iv administration of either DSPC/Chol or DSPC/Chol/PEG-PE liposomes with or without entrapped doxorubicin (2 μmol drug per injection). The lipid dose was 10 μmol total lipid per mouse. When free drug was given iv at the MTD a dose of 0.66 μmol per mouse was administered. Mice were sacrificed at 1, 4, 24 h, 2, 4, and 7 d, and lipid and drug plasma concentrations determined. Results shown represent the mean of four animals ±S.E.M. per group. If the error bars are not visible they are contained within the space of the symbol. A. Liposome accumulation in the Lewis Lung solid tumor: DSPC/Chol (open circles); DSPC/Chol + doxorubicin (filled circles); DSPC/Chol/PEG-PE (open squares); DSPC/Chol/PEG-PE + doxorubicin (filled squares). B. Drug accumulation. free doxorubicin (filled triangles); doxorubicin in DSPC/Chol; (filled circles), doxorubicin in DSPC/Chol/PEG-PE (filled squares).

1-h) in this study. The peak level of drug obtained in tumors was approximately 250 nmol per g and this represents approximately 140 μg equivalents of doxorubicin per g tumor. In contrast, after administration of free doxorubicin peak drug levels were achieved within 15 min. and these levels (10 nmol per g) were 25-fold lower than those obtained following administration of the liposomal formulations. Although the liposomal drug is typically more active then free drug (see Figure 3 for efficacy data obtained following treatment of mice bearing Lewis Lung carcinoma with free and liposomal drug at the maximum tolerated dose),

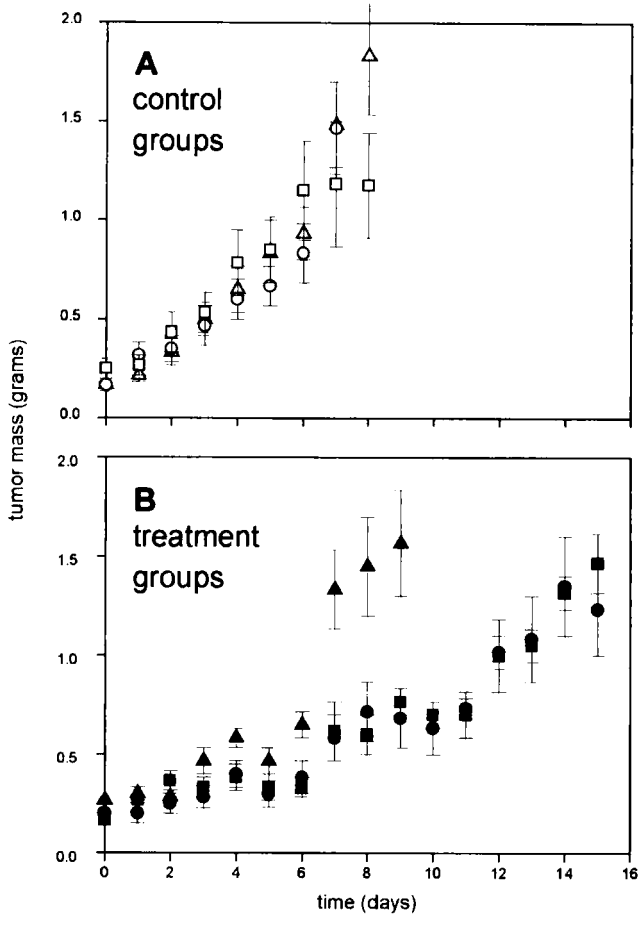


*Figure 3.* Doxorubicin mediated Lewis Lung solid tumor growth inhibition. Tumor bearing mice were given various treatments and tumor mass was estimated daily using caliper measurements. Control groups: saline treated control (open triangle); 10 (μmol empty DSPC/Chol (open circle); 10 μmol empty DSPC/Chol/PEG-PE (open square). Treatment groups: 0.66 mmol free doxorubicin (filled triangle); 2 μmol doxorubicin in 10 (μmol DSPC/Chol (filled circle); 2 (μmol doxorubicin in 10 (μmol DSPC/Chol/PEG-PE (filled square). Results shown represent the mean of four animals ± S.E. per group.

the improvements can be disappointing when one considers the relative increases in drug exposure obtained through use of a liposomal drug. Such data raise the question as to why liposomal formulations of anticancer drugs are not more efficacious.

Third, it is well established that liposomal formulations, even those with surface attributes that result in reduced elimination rates, are removed by organs such as the liver and spleen. Logically one would assume that diseases that are localized in these organs would be effectively treated with liposomal anticancer drugs. Using a model where drug sensitive tumor cells seed and grow in the liver, we have measured the therapeutic activity of a broad variety of liposomal anticancer drugs. These drugs are known to be very active against tumor progression when given i.v. to animals bearing the tumor cells grown in the peritoneal cavity. As shown in Table 1, only one of the liposomal drugs proved to be effective in treating animals effected by liver localized tumors. These data force us to consider why some liposomal drugs are more effective than other liposomal drugs at treating tumors derived from cell lines that are equally sensitive to the drugs given in free form. This question cannot be resolved simply on the basis of differences in tumor cell division rates or regional localization of the drug loaded carrier systems.

***Table 1.*** Therapeutic Activity of Free and Liposomal Anti-Cancer Drugs Given at the *Maximum Therapeutic Dose* to Mice Bearing the L1210 i.v. Tumor Model

| TREATMENT | DRUG DOSE (mg/kg) | MEAN OF THE MEDIAN SURVIVAL TIME (Days) | % ILS[e] | % SURVIVAL |
|---|---|---|---|---|
| Control (saline) | | 9.8[a] | | N/A |
| Control (EPC/Chol) | | 11.5[b] | 17 | 0 |
| Control (DSPC/Chol) | | 10.5[b] | 7 | 0 |
| Free Mitoxantrone | 10 | 17.2[c] | 76 | 0 |
| DSPC/Chol Mitoxantrone | 20 | 25.1[c] | 156 | 0 |
| DMPC/Chol Mitoxantrone | 10 | >60[c] | ND[f] | 100 |
| Free Doxorubicin | 10 | 13.5[b] | 38 | 0 |
| EPC/Chol Doxorubicin | 30 | 18[b,d] | 84 | 0 |
| DSPC/Chol Doxorubicin | 30 | 13[b,d] | 33 | 0 |
| Free Vincristine | 2 | 10[b,d] | 2 | 0 |
| DSPC/Chol Vincristine | 3 | 13.5[b,d] | 38 | 0 |
| Liposomal ara C | 200 | 13.8[b] | 40 | 0 |

[a]Determined in DBA2 and BDF1 mice
[b]Determined in DBA2 mice
[c]Determined in BDF1 mice
[d]Indicates median survival times form one experiment using an n of at least 5 animals
[e]Percentage ILS (Increase in Life Span) Values were determined from mean survival times of treated and untreated control groups. If the animal survived more than 60 days the ILS% was not determined
[f]ND can not be determined based on a 100% survival rate for 60 days

We believe that the unresolved conceptual problems outlined above can best be explained by a fourth, and perhaps most important, dilemma. Liposomal carrier systems that have been optimized for therapeutic use are often designed on the premise that maintenance of high concentrations of drug over extended time periods will facilitate localization of the drug in a diseased site. Such optimization strategies typically result in a formulation that retains drug well following intravenous administration. An obvious benefit to using liposomes that retain drug well is minimizing drug exposure in healthy tissues. The problem with such an optimization approach is that drug sequestered inside the liposomes is not capable of efficiently delivering the drug into tumor cells. This is exemplified best by in vitro studies, which demonstrate that for a well designed liposomal anticancer drug (one that provides optimal circulation lifetime and optimal solid tumor delivery) 10- to 100-fold more drug is required to obtained cell toxicity that is equivalent to free drug. These data are simple to explain: drug must be released from the liposome in order for its bioactivity to be expressed.

## HYPOTHESIS AND ASSUMPTIONS

We argue here that the greatest obstacle to the development of therapeutically effective liposomal anticancer drugs concerns controlling drug release. This argument must also consider when and where drug release should occur. As modeled in Figure 4, for an intravenously administered liposomal anticancer drug to be optimal it must maintain different attributes depending on where the liposome is localized. While in the blood compartment the liposome must retain drug. This will serve two purposes: 1) to minimized systemic exposure of free drug and 2) to maximize delivery of the liposomal drug to sites outside the blood compartment. The latter is a slow process and if the drug release rates are too fast then liposomes that have left the blood compartment may contain little drug. After the majority of liposomes have been eliminated from the blood compartment, the regionally localized liposomes must undergo a transformation process. This process should result in drug release from the liposome and/or target cell specific drug delivery.

It is important to note that the model described in Figure 4 is based on defined assumptions and these drive the development of this carrier technology by our research groups. The primary objective of this overview is to review data that supports our working assumptions. This data is, in turn, discussed in the context of emerging liposome technology. Although the focus is on research developing carriers for small molecules (conventional drugs) there are important comparisons that can be made to delivery systems being developed for proteins and plasmid expression vectors. These comparisons are made where appropriate.

The primary aim of our research programs has been to rationally design therapeutic liposomes with significantly improved versatility (among disease states) and selectivity (between healthy and disease tissue). In this context, our overall hypothesis is that multifunctional liposomes can be prepared and developed as a novel class of therapeutic agents designed to treat a wide spectrum of

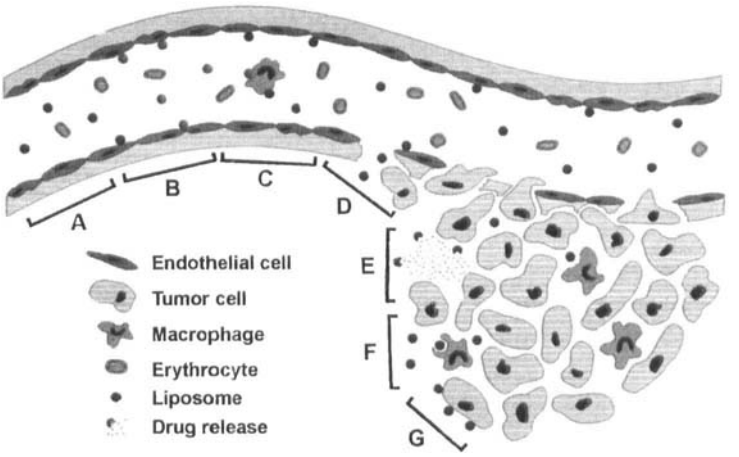


***Figure 4.*** Steps required for targeting to cells outside the vascular compartment following i.v. administration of liposomes. A liposome with surface associated targeting molecules within the blood compartment (A) must escape. This may be a consequence of interactions with vascular endothelium (B) or white cells egressing into a disease site (C). The preferred mechanism of liposome extravasation involves passage through gaps between endothelial cells (D). Following extravasation into a tumor a number of events will determine the efficiency of liposome drug targeting. Drug can be released from liposomes in the interstitial space (E) or the drug loaded liposomes can be internalized by tumor associated macrophages (TAMs) (F). Direct interaction with a target cell population (G) in the tumor will be dependent on access to the cell as well as retention of surface associated targeting molecule as well as encapsulated drug.

diseases. Unlike present technology, which relies on liposomes serving as passive drug carriers, the developing technology will rely on lipid components and membrane specific structural transformations to play an active role in an associated drug's biological activity. Central to testing of the hypothesis is the ability to generate liposomes that exhibit specificity. In addition, these lipid-based carriers must exhibit the potential to change their physical/chemical characteristics at defined time points following in vivo administration. These transformations will allow site-specific expression of properties required for therapeutic activity after the carriers have localized at the disease site.

## THE ACTIVE AGENTS

Before considering the design attributes of liposomal carriers, it is useful to comment on some of the common rules that govern cancer chemotherapy, to reflect briefly on the rationale(s) for developing liposomal anticancer drugs and to identify why lipid-based formulations may be essential for development of next generation pharmaceuticals such a DNA, peptides and proteins.

### Conventional Drugs

We would argue that there are two general reasons for developing a liposomal anticancer drug. First, the drug may be hydrophobic and difficult or impossible

to dissolve in aqueous solutions and a hydrophobic environment is required in order for the drug to stay in solution/suspension. Second, the liposome can serve as a carrier that will improve drug specificity by increasing delivery to the site of disease and/or decrease delivery to a site where toxicity is manifested. The former is an important, perhaps underdeveloped, role for lipid-based carriers. However, the methods and characterization studies required for development of lipid-based formulations optimal for drug solubilization are distinct from those used in the development of liposome drug carrier technology. Differences in the two approaches can be defined primarily through in vivo studies that determine plasma elimination behavior of both drug and liposomal lipid. If the drug dissociates from the liposome immediately following administration then the lipid-based carrier is acting as an excipient for drug solubilization. When drug elimination parameters are dictated by the elimination behavior of the liposomes, then the systems are acting as true delivery vehicles.

This review focuses on use of liposomes developed as drug carriers. The primary consequence of anticancer drug encapsulation is liposome-mediated changes in drug elimination and biodistribution. It is important to recognize that therapeutic responses obtained following administration of anticancer drugs, in free form or associated with a drug carrier, are dependent on tumor physiology and tumor cell heterogeneity. Ideally, an effective drug must access the target cell populations at levels sufficient to cause cytotoxic effects and should be effective in all microenvironments present within tumors. In humans, strategies designed to maximize the antitumor activity of chemotherapeutic agents must, therefore, contend with a heterogeneous population of proliferating cells. Tumor cells are proliferating at different rates, are governed by differences in cell cycle control and are capable of adapting rapidly to the chemotherapeutic stresses exerted on them. In practical terms this means that chemotherapy typically involves the use of multiple drugs that exert antitumor activity via different mechanisms (25). Vincristine is a cell cycle specific agent that acts by destabilizing microtubules and is almost always used in combination with two or three other anticancer drugs. The therapeutic action of vincristine is complemented by drugs such as doxorubicin (an anthracycline that acts as a topoisomerase II inhibitor) as well as cyclophosphamide (a nitrogen mustard pro-drug and strong alkylating agent). The mechanisms of therapeutic action of these drugs are complementary and the toxicity of each drug is sufficiently different such that they can be used in combination without aggravation of any one specific target organ toxicity.

In addition to the necessity of using multiple agents to achieve optimal therapy, another general principle of cancer chemotherapy concerns maximizing dose intensity (26). Tumor cells must be exposed to the highest levels of drug for the longest time periods if maximum therapeutic effects are to be achieved (27). The advantage of anticancer drug carrier technology is based on carrier characteristics that give rise to increased drug exposure in sites of tumor growth. An example of how liposome drug carrier technology can improve the pharmacodynamic behavior of an anticancer agent is evident when evaluating studies with doxorubicin. Efforts to maximize the dose intensity of this chemotherapeutic agent (in free

form) have been limited due to non-specific toxic side effects. For example, doxorubicin is a potent myelosuppressive agent (28). Therapeutic doses must, therefore, be limited to schedules and amounts that do not compromise regeneration of blood cells or cells of the immune system. In addition, doxorubicin exhibits a dose limiting cardiotoxicity (29) restricting the total dose to approximately 450 $mg/m^2$. Myelosuppression can be counteracted using the hemopoietic growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF) (30). Administering the drug in a liposomally encapsulated form, on the other hand, can reduce cardiotoxicity (22–24). It has also been shown that the therapeutic activity of the liposomal drug is greater than or equal to free doxorubicin in a variety of preclinical and clinical studies (15, 30–33).

## Plasmid Expression Vectors and Antisense Oligonucleotides

Treatment strategies based on the use of gene therapy are considerably more complicated then those involving small drugs, such as doxorubicin. It is important, however, to recognize that there will be common elements used in designing lipid based carriers for these very distinct drug classes. The level of stringency required for carriers to be used for gene therapy applications increases significantly primarily because targeted intracellular delivery is believed to be required for activity. Further, since gene therapy approaches may involve turning off a gene that promotes proliferation, turning on a gene that stimulates programmed cell death or introducing a new gene that will engender a therapeutic response, the end points used to define the activity of these carriers may often be different.

As indicated above, due to tumor heterogeneity, it is often difficult to determine which cells or tissues should become the target for a gene therapy approach. For this reason the first approved clinical trials in gene transfer were aimed at transferring (into target cells within sites of cancer progression) expression cassettes which carry genes that should 1) enhance immune responses to tumors, 2) alter the proliferation rate of cancer cells or 3) sensitize malignant populations to cytotoxic agents or radiation. Most of the therapeutic trials for cancer involving enhanced immune responses consist of introducing one of several cytokine genes into either tumor cells, bone marrow cells or tumor-infiltrating lymphocytes. Alternatively, the antigenicity of the tumor cells has been increased by introduction of a gene encoding for a histocompatability protein. A number of investigators have used this "tumor vaccine" approach, research supported by pre-clinical data suggesting that distant, genetically unmodified tumors can regress following injection of identical tumor cells that have been transfected with an appropriate histocompatibility gene. The clinical strategy (34,35) therefore, consists of regional transfer of a histocompatibility gene, through direct injection of plasmid DNA-liposome complex in a cancerous lesion, with hopes that an immune response will effect therapy at distal sites.

Another approach in cancer gene therapy is to specifically inhibit or block tumor cell proliferation. Much of the research consists of in vitro studies aimed

at inhibiting the expression of oncogenes by the use of antisense oligonucleotides (36–38). Genes that have been targeted include c-myc, c-myb, and bcl-2. The inhibition of expression of these genes is aimed at blocking the translation of mRNA into protein, although inhibition of message production has been reported to be the most likely effect of antisense oligonucleotide delivery. Gene therapy strategies based on enhancing expression of a tumor suppressor gene, such as p53, are comparable to those antisense strategies targeting proteins that augment cell proliferation. The p53 gene product functions as a transcriptional activator of other genes which inhibits the progression of the cell cycle from G1 to S phase in normal cells. p53 protein levels are known to be elevated in response to DNA damage (39), leading to G1 arrest, terminal differentiation or apoptosis (40). Although the function of p53 has been restored efficiently in tumor cells in vitro, it has been less successful in vivo. This is largely due to problems of in vivo targeting of p53 expression vectors to tumor cells. Unlike carriers of conventional small molecules, delivery systems used for plasmid expression vectors, such as ones containing the p53 gene, must facilitate specific and efficient deliver to many if not all the diseased cells. An example of a gene therapy strategy that does not required gene delivery to all cells is the approach relying on use of the thymidine kinase gene (41). Introduction of this gene directly into tumor cells and subsequent expression renders the cell susceptible to killing by the antiviral agent ganciclovir. In the presence of thymidine kinase, ganciclovir is converted to a anti-metabolite that is effective in killing cells expressing the thymidine kinase gene as well as cells that have undergone transfection.

## ACCESSING A TARGET CELL POPULATION

In vivo studies are usually initiated only after one has developed a formulation that exhibits the necessary chemical and physical stability properties to be considered pharmaceutically viable. Subsequent in vivo analysis must then consider the fact that the liposomal drugs will interact with a number of distinct physiological "compartments" and associated barriers between compartments. For the purpose of discussions here, we will focus on systemic administration and, in particular, on the fate of lipid-based delivery systems injected intravenously (iv).

After injection, liposomes are exposed to a variety of circulating protein and cellular components that reside within the central blood compartment, many of which can destabilize the liposomes through interactions with the lipid bilayer or initiate biological processes that lead to increased liposome leakage and/or clearance via the reticuloendothelial systems. To gain access to a disease in an extravascular compartment liposomes must cross the vascular endothelium, the blood vessel lining which is composed primarily of endothelial cells and, in most cases, an underlying basement membrane and associated smooth muscle cells. This vascular barrier represents the greatest obstacle for liposomal drug delivery to extravascular disease sites, however, at the same time it offers properties that can be utilized to differentiate between normal and diseased tissue.

Should liposomes traverse this barrier, a second compartment is encountered consisting of the interstitial space and associated fluids and cells. This compartment can vary significantly not only between normal and disease tissues but also among normal tissues in different organs of the body. Within this compartment, the barriers to liposome movement and distribution are varied and include factors such as interstitial volume, interstitial pressure, and the presence (or absence) of a lymphatic system.

The final physiological compartment(s) are the cells into which liposomes and/or their associated agents are taken up. This includes intracellular organelles that may be involved in processing of the administered agent or that contain the molecular target through which the drug exerts its therapeutic activity. The critical barrier that must be crossed in order to access this final compartment is the cell membrane. Similar to the vascular endothelium, crossing this barrier is a significant obstacle to the development of therapeutically optimized liposomal anticancer drugs.

In the following sections we will follow the fate of liposomes as they enter these physiological compartments and pass through the various barriers. We will focus on specific interactions between liposomes and the biological milieu in the various compartments that directly impact on the delivery of encapsulated agents to their therapeutic target. Further, we will highlight where strategies have been employed to augment conventional liposomes (defined as un-derivatized membrane bilayers composed of naturally occurring lipids) with components that alter these interactions.

## Barriers to Extravasation of Lipid-Based Drug Carriers

While in the circulation, liposomes are continually exposed to cells lining the vasculature. The inner lining, or intima, of blood vessels is composed primarily of endothelial cells that form a contiguous layer on the interior surface of all blood vessels. Underlying this layer is the basement membrane and in larger (non-capillary) vessels the vasculature is supported by smooth muscle cells (42). The endothelial cells in most normal vasculature exhibit intact intercellular junctions and only small molecules are able to readily permeate across capillaries of this type. However, this structure is significantly altered in certain normal tissues, most notably the liver and spleen, as well as in disease sites such as infection and tumor growth. The latter are characterized by the presence of capillaries that are fenestrated or exhibit larger intercellular openings and can be devoid of the basement membrane layer. The gaps in these endothelial layers can range in size from 30 nm for fenestrated capillaries to greater than 500 nm in liver, tumor and inflammation site vascular beds (43,44). In the liver, these openings provide access to sinusoids where the phagocytic Kupffer cells lie. In disease sites, the fenestrated/discontinuous capillary beds and post-capillary venules allow direct exposure of the underlying epithelial cells to the circulation. It is the unique nature

of vascular structures that exist in liver/spleen and disease tissues which permits the movement of liposomes from the blood compartment into extravascular sites.

## Liposome Elimination from the Blood Compartment

The RES has long been recognized as the major site of liposome accumulation after systemic administration. The primary organs associated with the RES are the liver, spleen and lung. The liver exhibits the largest capacity for liposome uptake while the spleen can accumulate liposomes such that the tissue concentration (liposomal lipid/gm tissue) is 10-fold higher than that which can be achieved in other organs. Assuming that liposomes are designed to minimize protein binding and cell interactions, the extent of liposome accumulation in the lung is typically below 1% of the injected dose. Early studies demonstrated that large, as well as charged liposomes (particularly those containing negatively charged lipids like PS, PA or cardiolipin), were removed very rapidly by the liver and spleen with clearance half-lives of less than 1 hour (45). The rate of clearance from the circulation could be reduced to some extent by increasing the administered lipid dose. However, only when small (approx. 100 nm), neutral liposomes containing $>$ 30% cholesterol were utilized at doses of at least 10 mg/kg or more could circulation lifetimes in the range of several hours be achieved (46,47). The removal of liposomes from the blood is attributed to phagocytic cells that reside in the RES and appears to be mediated through direct interactions between the phagocytic cell and the liposomes.

The identification of certain naturally occurring lipids (e.g. ganglioside GM1 and PI) that increase the circulation lifetime of liposomes in which they are incorporated gave rise to what is often referred to as the "second generation" of liposome technology. Analogous to the polymer surfaces that were developed to reduced protein binding to biocompatible materials, it is believed that these carbohydrate containing lipids act by limiting the interaction of liposome surfaces with proteins and this, in turn, inhibited the rate of uptake by phagocytic cells (48,49). A variety of synthetic lipids have been developed to prevent protein binding. The most notable are based on hydrophilic polymers, such as PEG, which are attached to phospholipids such as PE. Perhaps the most widely utilized steric stabilizing lipid is one composed of 2,000 mean molecular weight linear PEG attached to DSPE and it is incorporated at levels of 2 to 10 mol% in the bilayer of conventional liposomes. Inclusion of PEG-PE into conventional empty neutral (PC/cholesterol) liposomes can result in 3- to 20-fold increases in plasma liposome content 24 h after iv injection (50,51). This is accompanied by significant decreases in liposome uptake by the liver and spleen at early times post-injection. It is important to note that the difference in cumulative uptake of liposomes by the RES organs between conventional and sterically stabilized liposomes become less significant over time, indicating that the effect of PEG-PE is to reduce the rate of liposome removal by cells of the RES. It has not been determined whether

eventual removal of these liposomes by the RES is due to time dependent increases in protein association or the loss of PEG from the surface of the liposomes (52).

Although liposome elimination rates differ greatly between conventional and sterically stabilized liposomes in the absence of encapsulated agents, this difference can be significantly reduced for liposomes containing entrapped drugs, particularly drugs that impair the ability of cells to accumulate or process liposomes (53). This is perhaps best exemplified in the case of the anticancer drug doxorubicin. These effects are illustrated in Figure 5, which shows the liposomal lipid levels present in the plasma (24h after administration) as a function of the total lipid dose. These results illustrate two important attributes of drug-loaded lipo-

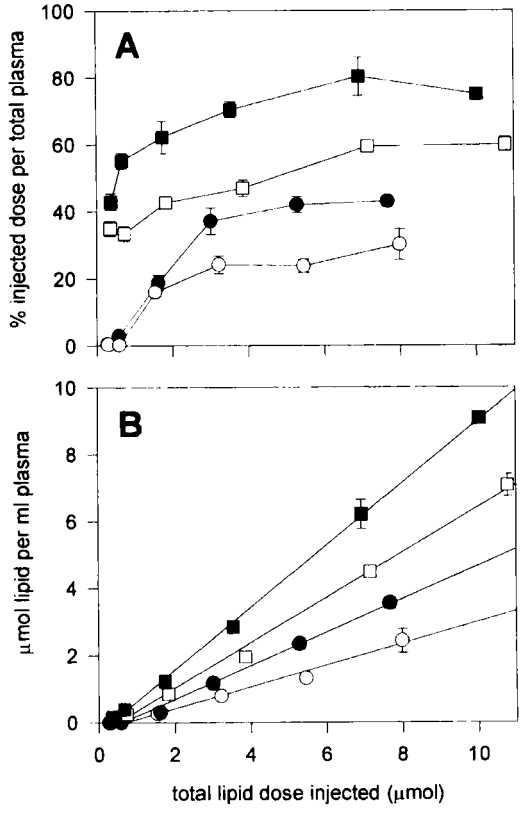


*Figure 5.* Dose titration of the liposomal carrier. Various doses of "empty" or drug loaded (0.2 drug:lipid ratio) liposomes were administered i.v. in a volume of 200 µl. Female BDF1 mice were used and the levels of lipid in the plasma were determined at 24h as described in the Methods. The results shown represent the mean of at least four animals ±S.E.M. per group. If the error bars are not visible they are contained within the space of the symbol. **A**. Plasma recovery at 24 h expressed as percent injected dose per total plasma; DSPC/Chol (open circle); DSPC/Chol + doxorubicin (filled circle); DSPC/Chol/PEG-PE (open square); DSPC/Chol/PEG-PE + doxorubicin (filled square). **B**. Same results as in 'A', expressed as lipid concentration (µmole lipid/ml plasma).

somes and PEG-PE containing liposomes. First, the addition of PEG-modified lipids greatly improved the circulating level of liposomal lipid achieved at 24h for both the empty and doxorubicin loaded liposomes (Figure 5A). As the lipid dose increased the differences between DSPC/Chol/PEG-PE and DSPC/Chol liposomes were still substantial, but these differences (significant at $p < 0.005$ for the 2 μmol lipid per mouse dose) were reduced from 10-fold (observed below the 1 μmol lipid per mouse dose) to less than 3-fold (observed above the 2 μmol lipid per mouse dose) (51). Plotting these data as a function of μmol lipid per ml plasma (shown in Figure 5B) demonstrate that a linear relationship exists between lipid dose administered and the levels of lipid in the circulation at 24 h, regardless of the liposomal formulation used (51). The second important attribute defined by the data presented in Figure 5 is that entrapped doxorubicin significantly increases the plasma blood levels obtained 24 h after i.v. administration of DSPC/Chol/PEG-PE liposomes or DSPC/Chol liposomes. This typically resulted in a 1.5- to 1.7-fold increase in circulating levels of liposomal lipid measured at 24h when comparing doxorubicin loaded liposomes to liposomes without encapsulated drug. However, when the DSPC/Chol liposomes are loaded with doxorubicin, the 24h plasma liposome concentrations are significantly increased and are only 2.8-fold less than those observed for 5 mol% PEG-DSPC containing DSPC/Chol liposomal doxorubicin systems (51).

Significant increases in circulating levels of empty liposomes can also be achieved by pre-dosing animals with a low dose (10 mg lipid/kg) of liposomal doxorubicin (53). This effect, referred to as RES "blockade", has raised concerns over potential harmful side effects resulting from altered RES phagocytic capacity. In vitro studies have demonstrated that liposomal doxorubicin uptake by cultured macrophages can result in cell death and exposure of macrophages in culture to concentrations of doxorubicin that are not cytotoxic significantly impairs the ability of these cells to accumulate particles (M. Bally, unpublished observation) Although a substantial amount of doxorubicin can accumulate in liver tissue (54), indications of significant liver toxicity arising from this uptake have only been observed pre-clinically with high drug doses (80 mg doxorubicin/kg) and in clinical situations where pre-existing liver impairment was a factor (55).

Investigators have been able to demonstrate macrophage and Kupffer cell depletion following administration of high doses of large and/or negatively charged liposomes containing doxorubicin or other agents such as clodronate (56,57). RES blockade induced by low doses (<10 mg/kg lipid and 2 mg/kg drug) of small, uncharged liposomal doxorubicin formulations, however, does not result in complete elimination of Kupffer cells (58). This was determined by histological evaluations of thin sections of liver stained with hematoxylin and eosin as well as on the basis of carbon particle uptake in livers of mice that have been previously treated with liposomal doxorubicin. This information suggests that our understanding of the mechanisms whereby liposomes (particularly small liposomes) are recognized, cleared from the blood and processed may be somewhat simplistic.

In light of the observations cited above, steric stabilizing lipids are likely to provide the greatest RES avoidance benefits at low liposome doses and for liposome formulations containing drugs that do not lead to reduced liposome clearance. Regarding the latter, it has been shown that encapsulation of vincristine, doxorubicin or cisplatin results in a reduction in liposome elimination. In contrast, liposomal mitoxantrone formulations exhibit circulation characteristics identical to liposomes without entrapped drug. It should also be stressed that the theoretical "benefits" arising from decreased liposome elimination by the RES is typically assumed to be related to the increased circulating concentrations of liposomes obtained. However, we suggest that it is not the plasma concentration of liposomes that dictates therapy, but rather the amount of liposomal drug that penetrates the vascular barrier and gains access to diseased tissue.

## Liposome Extravasation

If liposomes are designed in an appropriate manner, whether with respect to size, lipid composition, and/or use of PEG-modified lipids, liposomes can remain in the blood compartment for a period of several days. The fact that under such circumstances the vast majority of liposomes administered can be accounted for in the blood, liver and spleen demonstrates that liposomes are relatively inefficient at crossing the endothelial cell barrier present in most tissues. The property of long circulating liposomes that is exploited for therapeutic purposes relies on changes in the endothelial cell barrier, prevalent in many disease states, that allow liposomes to traverse out of the blood compartment and into the tissue.

Major diseases, such as bacterial infection, inflammation and cancer, have the common feature of altered vasculature permeability at the site of disease progression. The mediators that lead to increased permeability of the vascular barrier are quite distinct for different disease states. For example, chemotactic factors and adhesion molecules over-expressed at sites of inflammation attract infiltrating lymphocytes and granulocytes that subsequently release factors which can directly damage endothelial cells and/or cause defects in intercellular junctions (59). In hypoxic environments, such as those that arise during rapid cell proliferation or through vascular injury, cells can release vascular endothelial growth factor (VEGF) (60,61). VEGF is an endothelial cell specific mitogen and its release can lead to the development of neovasculature. VEGF is identical to vascular permeability factor (62), a protein first identified as a factor capable of inducing defects in the permeability barrier of blood vessels. An approach to increase delivery of liposomal anti-cancer agents to a site of tumor growth was developed based on IL-2 induced changes in blood vessel structure and function (Figure 6). Although IL-2 caused a non-specific increase in plasma elimination of i.v. injected liposomes, there were also IL-2 induced increases in drug delivery which resulted in improved therapeutic activity. This approach, based on inducing changes in blood vessels that promote movement of drug carriers to diseased sites, may be beneficial if the increases in vascular permeability can be achieved locally. Re-

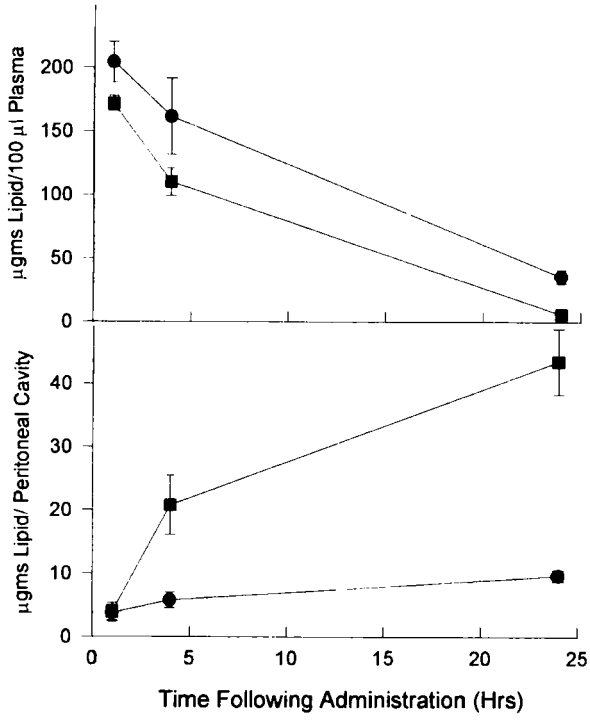


*Figure 6.* Plasma elimination (top) and liposomal lipid accumulation in the peritoneal cavity (bottom) of mice following i.v. administration of DSPC/Chol (55:45 mol ratio) at a dose of 100 mg lipid/kg. Mice were pre-treated with saline (filled circles) or with a single dose of IL-2 ($3 \times 10^6$ units) (filled squares) 24 hrs prior to liposome administration. Lipid levels were determined using the non-exchangable, non-metabolizable lipid marker [$^3$H] Cholesteryl hexadecyl ether. Each data represents the mean (± standard deviation) obtained from at least 4 mice.

gardless of the mediator, the end result for all of these conditions is the presence of blood vessels that are permeable to large molecules. This may be a consequence of fenestrations or "gaps" occurring between adjacent endothelial cells through which macromolecules can pass (63) or, alternatively, may involve increases in endothelial cell mediated transcytosis (64).

Increases in vascular permeability give rise to the selective accumulation of small liposomes in sites of infection, inflammation and tumor growth. However, this is not a selective process and there is also a general increase in extravascular fluids in these regions. The hydrostatic pressure within these sites is elevated relative to the vascular pressure, resulting in a pressure gradient that impedes movement of molecules from the blood into the tissue interstitium (65). We must therefore assume that additional features lead to selective accumulation of macromolecules in the diseased extravascular space. Studies, for example, have demonstrated that the lack of a developed lymphatic system in conjunction with the large openings in the vascular endothelial cell lining may lead to an extravascular

"trapping" phenomenon (66). In the absence of lymphatic drainage, interstitial diffusion of molecules leads to egress from the disease site and this diffusion rate is dependent on molecule size, small molecules exiting more rapidly than large molecules.

Designing liposomes that will exhibit maximal extravasation in disease sites associated with leaky vasculature is of considerable interest and is an area of some controversy. The inclusion of PEG-modified lipids in conventional liposomes can significantly increase the circulating liposome levels over extended times by decreasing the rate of clearance by the RES. It has generally been assumed that increases in the concentration of liposomes in plasma over time will lead to increased accumulation of liposomes in the extravascular disease sites and experimental evidence supporting this has been reported (67,68). Videomicroscopy has also suggested that the permeability coefficient of tumor vasculature is greater for PEG-PE containing liposomes compared to conventional liposomes (69). In contrast, studies conducted in our laboratories as well as others have demonstrated that although plasma levels of PEG containing liposomes are several fold higher than for comparable conventional liposomes, this often does not result in increased extravasation and accumulation in solid tumor tissue (51).

As shown in Table 2, we have examined the tumor uptake properties for conventional and steric stabilized liposomal formulations of doxorubicin, vincristine and mitoxantrone in a variety of tumor models. Three important observations can be made on the basis of the comparative biological properties of conventional

***Table 2.*** Tumor Accumulation Efficiency (Te) for Conventional and Steric Stabilized (PEG-Containing) Liposomal Anticancer Drug Formulations

| TUMOR MODEL | PREPARATION[a] | PLASMA AUC[b] | TUMOR AUC | $T_e$[c] |
|---|---|---|---|---|
| Lewis Lung | DSPC/Chol[d] | 2,118 μgh/ml | 819 μgh/g | 0.39 |
| (murine solid tumor) | DSPC/PEG-PE/Chol[d] | 7,910 μgh/ml | 1,432 μgh/g | 0.18 |
| Fsa-N fibrosarcoma | DSPC/Chol[d] | 10,560 μgh/ml | 2,981 μgh/g | 0.28 |
| (murine solid tumor) | DSPC/PEG-PE/Chol[e] | 18,500 μgh/m | 2,892 μgh/g | 0.16 |
| P388 | DSPC/Chol[e] | 16,530 μgh/ml | 1,720 μgh/peritoneum | 0.10 |
| (murine ascitic tumor) | DSPC/PEG-PE/Chol[e] | 37,600 μgh/ml | 2,037 μgh/peritoneum | 0.05 |
| | SM/Chol[f] | 5,116 μgh/ml | 206 μgh/peritoneum | 0.041 |
| | SM/PEG-PE/Chol[f] | 6,762 μgh/ml | 184 μgh/peritoneum | 0.027 |

[a]Area under the curve (AUC) values were calculated as trapezoidal AUC over the time period 0–24h.
[b]All liposomes were 100 nm in size and contained 45 mol% cholesterol. PEG-DSPE was incorporated at 5 mol% when utilized.
[c]Tumor Accumulation Efficiency was calculated as the 0–24hr liposome AUC in the tumor divided by the 0–24hr liposome AUC in plasma.
[d]Empty liposomes injected at a dose of 100 mg/kg.
[e]Liposomal doxorubicin preparations constituted by pH gradient encapsulation at a drug to lipid weight ratio of 0.2:1.
[f]Liposomal vincristine preparations constituted by pH gradient encapsulation at a drug to lipid ratio of 0.1:1.

and sterically stabilized liposomes. First, sterically stabilized liposomes uniformly display increased circulation longevity compared to conventional liposomes, regardless of the presence of encapsulated drug. Second, the rate and extent of liposome accumulation in tumor tissue are often comparable for both conventional and sterically stabilized liposomes. Third, the tumor targeting efficiency or TE (defined as the mean AUC in the tumor divided by the mean AUC in plasma) is higher for conventional liposomes compared to sterically stabilized systems. It can be suggested from these data that inclusion of lipids such as PEG-DSPE appears to decrease the efficiency of liposome extravasation from the blood into tumor.

It should be noted that our comparisons are typically based on extended AUC measurements of total tumor liposome uptake (following a non-exchangeable, non-metabolizable lipid label and correcting for blood volume contributions) and we place great emphasis on measuring both liposomal lipid and drug over the specified time course. Simultaneous measurements of drug and liposomal lipid can be used to assess drug retention, which is a determining factor in terms of accumulation of entrapped contents in tumors (see following section).

It should not be unexpected that conventional and sterically stabilized liposomes exhibit different efficiencies in extravasation. Videomicroscopy studies with steric stabilized liposomal doxorubicin systems have identified that some endothelial cells can take up liposomes (69). Endothelial cell interactions may contribute to the extravasation process either directly via transcytosis or indirectly by facilitating an increase in the local liposome concentration at the endothelial cell surface, thereby increasing access to openings in the vasculature. Given the effects of PEG on inhibiting liposome-cell interactions, this polymer may reduce endothelial cell interactions and this, in turn, would reduce the rate of extravasation. In contrast, conventional liposome extravasation could be facilitated through increased interactions with the endothelial cell lining of the neovasculature in tumors. This is, of course, highly speculative but is consistent with the surface properties of conventional liposomes compared to steric stabilized liposomes. A logical extension of this argument, however, is that improved extravasation may be possible by designing liposomes which interact more extensively with vascular endothelium in tumors.

## Other Methodology Considerations

For many applications, liposomal delivery systems are employed to improve the therapeutic index of encapsulated agents by selectively accumulating in extravascular disease sites. Further, there is increasing evidence indicating that drug released from liposomes in the circulation does not contribute significantly to therapeutic activity of liposomal anticancer agents. There is no question that liposomes can provide sustained exposure of therapeutic agents in the blood compartment through controlled release kinetics of encapsulated drugs, however it is difficult to justify development of liposomal drugs using a rationale that involves sustained systemic exposure. This is largely due to significant advances made in the area

of drug infusion technology. Compact and cost effective infusion pumps are now widely used and these can provide well-controlled systemic drug exposure over several days. We maintain that the most significant advantage for the use of liposome drug carriers arises as a consequence of disease specific changes in vascular permeability that favor accumulation of the intact liposome and associated drug into the site of disease progression. We differentiate this property from the benefits of drug infusion technology, which are primarily concerned with maintenance of circulating blood levels of free drug. As indicated in the previous section, in terms of methods used to characterize liposome formulations it is critical to assess the biological fate of the lipid/liposome carrier as well as the drug. We have reviewed this methodological consideration elsewhere (70).

## DISSOCIATION OF ACTIVE AGENT FROM THE CARRIER: THE CRITICAL PARAMETER

Once liposomes have moved through the vascular endothelial barrier, their fate in the interstitial spaces is tissue specific. Generally, negligible levels of liposomes extravasate into tissues such as muscle and kidney (71). Presumably the liposomes that have distributed into these sites migrate slowly through the intercellular matrix until they are removed via the lymphatics. Interestingly, liposomes administered i.v. do appear to accumulate to high levels in lymph nodes (on a per weight basis), where combined filtration and presence of phagocytic cells act to concentrate liposomes (72). In liver and spleen, fixed macrophages actively take up liposomes and these cells process the carrier via the intracellular phagolysosomal system. However, for the purpose of this discussion we will focus on the behavior of liposomes that have extravasated into disease sites, and in particular, into solid tumors.

The distribution of liposomes that have extravasated into the tumor interstitium is heterogeneous. This is not unexpected given the irregular and often redundant organization of tumor vasculature. Tumor vascular structure often engenders highly variable blood flow properties and evaluations of histological sections from tumors reflect this heterogeneity. This would be more apparent for liposomes compared to unencapsulated small molecules due to the decreased diffusion through the interstitial space for large macromolecules. This slow diffusion after extravasation has been documented by fluorescence video microscopy where fluorescently labeled liposomes could be seen to accumulate in the perivascular spaces primarily associated with the roots of capillary sprouts (73). Diffusion away from these sites was observed to be very slow and significant perivascular clustering was observed for several days. This is consistent with the data from several tumor models that demonstrate that tumor accumulation levels of liposomes reached a maximum approximately 24h after injection and these levels are maintained for extended time periods. Importantly, evaluations of drug accumulation properties can reveal remarkably different behavior, where drug release from the liposomes in the extravascular site results in greater drug penetration into the tissue and more rapid loss of the drug from the site when compared with the loss of liposomal lipid.

The preferential extravasation and accumulation of liposome encapsulated anticancer drugs in solid tumors results in tumor drug levels that can be an order of magnitude higher than that achieved with free (non-liposomal) drug (51,74–76). In addition, the prolonged residence of liposomes in tumors also significantly increases the duration of tumor drug exposure and AUC relative to free agents (51,77). In some tumor models, such properties have been shown to correlate with increased antitumor activity for liposomal formulations of drugs such as doxorubicin and daunorubicin. It is not clear from these studies, however, what the relative increase in therapeutic potency is in the context of tumor drug delivery improvements. Specifically, studies have typically compared the efficacy and tumor drug accumulation following administration of equal doses of free and liposomal drug. A comparison of efficacy under conditions where tumor drug accumulation is comparable for free and liposomal drug has not been completed, but would likely demonstrate that the liposomal drug is less potent. Other studies have demonstrated comparable antitumor efficacy for free and liposomal doxorubicin under conditions where tumor drug levels were as much as 5-fold higher for liposomal systems (51). Such observations have raised obvious questions about the bioavailability of anticancer drugs carried inside liposomes that have extravasated into solid tumors as well as the mechanisms that lead to drug release in the interstitial compartment.

The consensus emerging from studies in several laboratories on the mechanism of action of liposomal anticancer drug formulations is that liposomes exert their effect on therapeutic activity by providing an in situ drug infusion reservoir within the tumor. Once released, the anticancer drug can diffuse through the tumor and has direct access to tumor cells where it can act in a manner that presumably is similar to drug in the absence of a liposomal carrier. In vitro studies have demonstrated that macrophages can engulf doxorubicin-loaded liposomes, process them and re-release doxorubicin extracellularly in free form (78). In view of the high macrophage content residing in some tumors (79), such phenomena led to the proposal that liposomal anticancer drug release may involve macrophage processing after extravasation. However, recent studies have shown that in solid tumors there are limited interactions between tumor-associated macrophages and extravasated liposomes (80). Although macrophage enriched tumors do accumulate higher levels of liposomal doxorubicin, this effect appears more related to increased vascular permeability rather than direct uptake and processing of the liposomes by the macrophages. This was further supported by the fact that both conventional and sterically stabilized liposomes displayed comparable distribution properties (as determined by fluorescence microscopy of tumor thin sections) after extravasation into the tumor.

## Drug Release—*In vitro* versus *In vivo*

The ability of adsorbed blood proteins to increase liposome permeability properties has been demonstrated by several laboratories (81–84). Such interactions can be simply modeled by determining the drug release kinetics for lipo-

somes suspended in serum compared to protein-free buffer. An example of this is shown in Figure 7. The leakage of vincristine from DSPC/Chol liposomes is approximately 5-fold faster in the presence of serum. Interestingly, comparison of these results with the release kinetics of vincristine from DSPC/Chol liposomes after i.v. administration (as determined by monitoring changes in the circulating drug-to-lipid ratio) reveals that drug leakage is further increased in vivo (Figure 7). These differences are not simply due to the presence of a "tissue sink" into which the released vincristine is absorbed since increased dilutions or extended

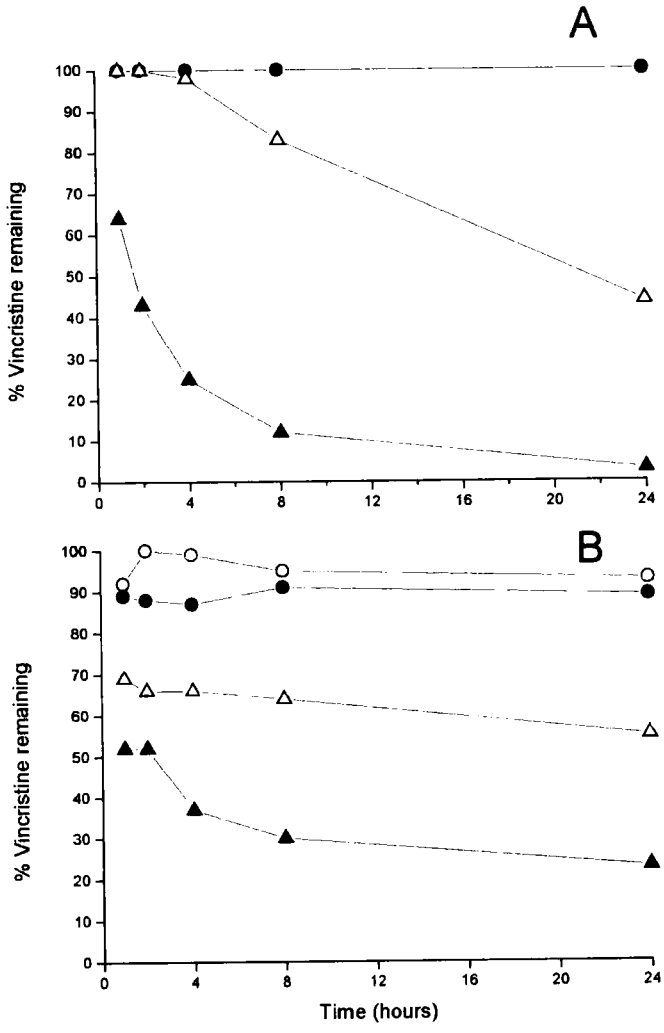


*Figure 7.* Vincristine release from 100 nm DSPC/Chol vesicles incubated in buffer (A) and mouse serum (B) at 37°C for internal pH of 2.0 (open circles), 3.0 (filled circles), 4.0 (open triangle), and 5.0 (filled triangle). Internal buffering capacity was 300 mM citrate for all systems. Initial drug/lipid ratios were 0.1/1 (wt/wt).

dialysis times in the presence of serum do not increase in vitro drug release rates (L. Mayer, unpublished observations). This effect is also not unique to vincristine. Studies with mitoxantrone suggest that the phase transition temperature (Tc) of the phospholipid species does not markedly affect mitoxantrone loading or release characteristics (85). In vitro drug release studies (Figure 8) with DMPC (1,2-Dimyristoyl-sn-glycero-3-phosphocholine)/Chol and DSPC/Chol demonstrate no difference in drug release from either liposomal formulation (85). The in vitro release assay used is based on dialysis against a large volume (1L) of buffer with (results not shown) and without 10% fetal bovine serum. Less than 2% drug release was observed from the liposomal formulations over a 72-hour incubation period at 37°C. Figure 9 show that the plasma elimination of liposomal lipid following i.v. administration of mitoxantrone loaded DMPC/Chol and DSPC/Chol liposomes is similar (Figure 9A). An estimation of the amount of mitoxantrone retained in the liposomes remaining in the circulation can be made by determining the ratio of mitoxantrone-to-lipid at the indicated time points; an estimation that assumes the level of free drug in the plasma of animals given liposomal mitoxantrone is negligible. The results shown in Fig. 9B demonstrate greater release of mitoxantrone from DMPC/Chol liposomes than DSPC/Chol liposomes. For DMPC/Chol liposomes, 73% of the mitoxantrone originally associated with the carrier has been released within 48 hours. In contrast, less than 5% of the drug was released from DSPC/Chol liposomes. These results are consistent with those

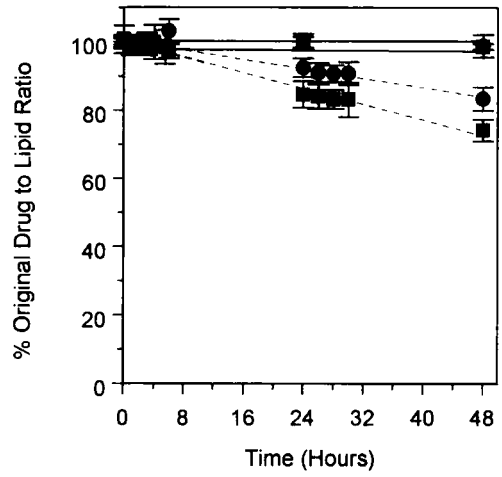


***Figure 8.*** Release of Mitoxantrone from DSPC/Chol (filled circle) and DMPC/Chol (filled square) liposomes in HEPES Buffered Saline at 37°C. Solid lines indicate the absence of Nigericin. Dashed lines indicate the addition of Nigericin at time zero. Samples (100 μl) were taken from the dialysis bags and applied to Sephadex G50 mini spin columns in duplicate and spun at 500 × g for 2 minutes. Duplicate samples were taken from the resulting mixture and [$^{3}$H] and [$^{14}$C] were measured to assess the non-exchangable, non-metabolizable lipid marker [$^{3}$H] Cholesteryl hexadecyl ether and [$^{14}$C] labeled mitoxantrone. Data represents the average values ± SD of at least four measurements for studies in the presence of Nigericin.

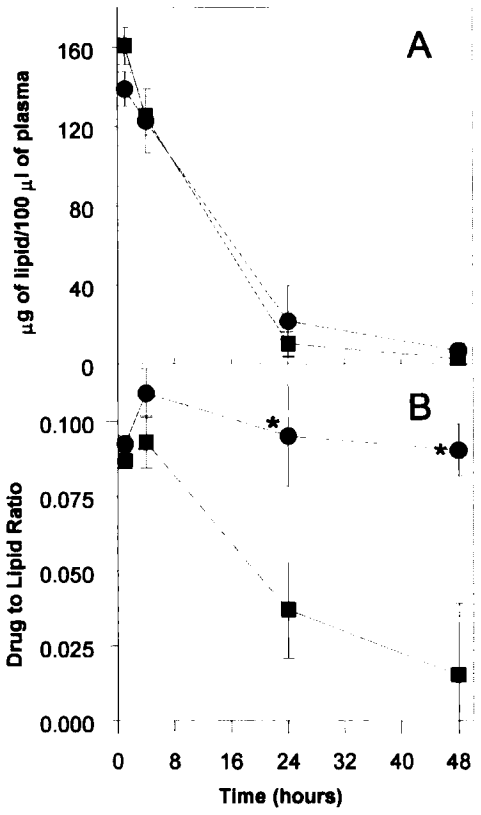


***Figure 9.*** *In vivo* release of mitoxantrone from DSPC/Chol (filled circle) and DMPC/Chol (filled square) liposomes. Liposomes were loaded with mitoxantrone at a drug to lipid weight ratio of 0.1 (wt:wt). Female CD1 mice were injected at a 10 mg/kg drug dose i.v. via a lateral tail vein. Panel A shows elimination of lipid from the plasma compartment over 48 hours. Panel B shows the change in the drug to lipid ratio over the 48 hour time period. Data represents the mean and standard deviation obtained from at least 4 animals.

obtained using entrapped doxorubicin (75) and clearly demonstrate that control of in vivo mitoxantrone release rates can be achieved through simple changes in liposomal lipid composition. Importantly, we believe that in vivo drug retention properties as well as comparisons of drug release kinetics for different liposomes cannot always be predicted simply on the basis of in vitro data.

In addition to increasing the permeability of liposome bilayers in the blood, protein adsorption can also lead to increased susceptibility to transmembrane stresses caused by ion gradients or high levels of encapsulated drugs. The high concentrations of buffer components and/or drug entrapped in liposomes often result in significant osmotic gradients across the liposome membrane when exposed to physiological fluids. While most liposomes can withstand a significant transmembrane osmotic gradient in the absence of extraneous proteins, exposure of liposomes exhibiting large osmotic gradients to plasma or purified lipoprotein

fractions results in a burst of leakage from the liposomes while osmotic balance is re-established (83). This effect is more pronounced with less ordered membranes where, for example, DSPC/Chol liposomes can withstand osmotic gradients of far greater magnitude than EPC/Chol liposomes in the presence of proteins (83). This may, in part, explain the differences observed between DSPC/Chol and EPC/Chol liposomal doxorubicin formulations in vivo where the circulating drug-to-lipid ratio (used to assess drug leakage) observed for EPC/Chol liposomes drops approximately 50% within 1h of injection and subsequently decreases to a release rate comparable to that observed for DSPC/Chol.

### Drug Release—Importance of Drug Type

As suggested by the data shown in Figures 7 through 9, drug release rates must be empirically determined for each drug of interest. It is not suitable to determine release rates using a trapped "marker" (e.g. radiolabeled inulin) to predict the release characteristics for an encapsulated therapeutic agent. The therapeutic benefit of controlling drug release rates is also dependent on the nature of the entrapped drug. For example, reducing the drug release rate is advantageous for encapsulated formulations of vincristine but are of questionable benefit for doxorubicin. In contrast, encapsulated mitoxantrone therapeutic activity is dependent on use of formulations that release drug steadily following i.v. administration. When doxorubicin is encapsulated in the liposomal formulation that is optimal for mitoxantrone (DMPC/Chol) it results in a formulation that is significantly more toxic then the free drug (75). These drug affects are discussed in more detail below.

Liposome encapsulation can significantly reduce the toxicity of doxorubicin by decreasing drug accumulation in drug sensitive normal tissue, presumably by decreasing peak levels of free doxorubicin that are experienced after administration in the conventional (unencapsulated) form (29). The degree of toxicity buffering is directly related to the ability of the liposomes to retain their entrapped doxorubicin where increased phospholipid acyl chain saturation results in decreased toxicity (75,86,87). The antitumor activity of liposomal doxorubicin, however, is much less sensitive to drug leakage or circulation longevity. Liposomal formulations with widely varying doxorubicin retention properties have been shown in some preclinical models to exhibit comparable antitumor activities when compared on an equal dose basis (75,86). In this case, increased efficacy for the less permeable liposomes is achieved by the ability to administer elevated drug doses due to their reduced toxicity. Further, while the inclusion of PEG-PE increases the circulation longevity of liposomal doxorubicin (51,80), the magnitude of increased liposome levels in the blood (compared to conventional liposomes) is far less than that observed for empty (drug-free) liposomes (51,80). This is related to the RES blockade effect described previously for doxorubicin loaded conventional liposomes

In contrast to the observations made with doxorubicin, altering the physical properties of liposomal vincristine formulations results in dramatic changes in

antitumor activity while only minimally affecting drug toxicity characteristics. Increasing the retention of vincristine inside 100nm liposomes by changing the phosphorylcholine-containing lipid component from EPC to DSPC to sphingomyelin (while maintaining cholesterol content at 45 mol%) leads to dramatic increases in antitumor activity, particularly when compared to the efficacy obtained with free vincristine (74,77). This is consistent with the steep dependence of vincristine antitumor potency on the duration of drug exposure (88) as well as the fact that retention of vincristine in most tissues, including tumors, is rather poor (89). In this case it appears that the ability to prolong the exposure of vincristine in vivo is more important than peak drug concentrations. Furthermore, although inclusion of PEG-PE in the liposomes increases the circulating liposomal lipid levels at extended time periods, this steric stabilizing lipid does not improve the vincristine pharmacokinetic or therapeutic properties over conventional DSPC/Chol or sphingomyelin/Chol systems (74). This is due to the fact that PEG-PE increases the permeability of the lipid bilayer to vincristine, thus offsetting the potential benefits provided by increased longevity of the liposomal carrier. The reasons for this increased drug leakage are not well understood. It may be related to the fact that PEG-modified phosphatidylethanolamine is negatively charged and this may alter drug-partitioning properties at the inner monolayer membrane surface. In addition, it is not yet clear whether this phenomenon is specific for vincristine encapsulated via pH gradient techniques employing citrate buffers, compared to ammonium sulfate entrapment systems (90).

A final example, derived from recent reports describing liposomal formulations of mitoxantrone, illustrates how a balance between efficient liposome delivery to the disease site and controlled drug release can work synergistically to achieve optimum therapeutic results (85,91). Mitoxantrone is less cardiotoxic than doxorubicin and is not capable of generating free radical mediated toxicity on non-dividing cell populations (92,93). The liposome mediated increases in mitoxantrone MTD observed for formulations (phosphorylcholine and cholesterol based systems) described by Chang et al. (91) and Lim et al. (85) are comparable to those reported for liposomal mitoxantrone formulations prepared using an anionic lipid-drug complex (94). In contrast to the results of Schwendener et al., liposomal mitoxantrone formulations prepared using DSPC or DMPC and cholesterol (45-mol%) exhibit significantly better drug retention characteristics. This is reflected in higher blood levels and improved circulation lifetimes for mitoxantrone encapsulated in the PC/Chol based liposomal carriers. These differences may be due to protein binding and rapid clearance of anionic liposome formulations. Alternatively, differences in drug release characteristics may, as suggested above for vincristine, be a consequence of the use of anionic lipids, which have been shown to enhance release of the anthracycline doxorubicin even in the absence of serum (95).

Studies evaluating the therapeutic activity of DSPC/Chol and DMPC/Chol liposomal mitoxantrone focused on treatment of an iv L1210 and/or P388 tumor model, where cells seeded primarily in the liver and spleen following iv adminis-

tration (85,91). These studies illustrated how controlled drug release effected significant improvements in therapeutic activity of the anticancer drug mitoxantrone. It is well established that the liver is a primary site of liposome accumulation, and that the rate of accumulation for DSPC/Chol liposomes in liver is comparable to DMPC/Chol liposomes. Based on this information, a relatively simple question was asked: Is a liposome (DSPC/Chol) which retains drug following iv administration therapeutically more active than a liposome (DMPC/Chol) that releases drug when tested against a tumor that progresses in the liver? Despite being less effective in terms of delivering drug to the site of tumor progression, the DMPC/Chol liposomes, which release drug steadily following administration, were strikingly more efficacious then the DSPC/Chol formulations. A natural extension of the previous question was: What effect would incorporation of PEG-modified lipids have on the therapeutic activity of either of these formulations when used to treat disease in the liver? For both formulations, addition of PEG-PE resulted in significant reductions in antitumor activity (Lim et al. unpublished observation). It can be concluded from such data that it is not necessarily sufficient to develop drug carriers that accumulate at the disease site to high levels; one must also engineer appropriate drug release rates. Controlled drug release must, however, be balanced with liposome mediated drug delivery to the site of tumor growth.

## Lipid-Based DNA Formulations—Importance of DNA Release

Although it is difficult to correlate encapsulated drug release with the attributes of lipid-based DNA formulation, we believe that is important to draw a connection between the two technologies. Our working hypothesis for development of effective delivery systems for plasmid expression vectors is that release of DNA following internalization is a key factor controlling transfection activity of these formulations. The structures that have been proposed for lipid-based DNA formulations are as varied as the procedures used to prepare them (19,96,97). For simplicity it is perhaps easiest to imagine that these formulations are complex mixed lipid micelles where associated DNA is protected by bound lipids. In this context micelles are defined, as suggested by Tanford (98), as "any water-soluble aggregate spontaneously and reversibly formed from amphiphiles." It is not clear whether bound lipids are organized in a bilayer structure or whether a hydrophobic-complex of bound lipids and DNA is surrounded by a lipid monolayer. It is important to elucidate the role of lipid constituients in the self assembling process that leads to formation of these complex micelles, particularly since this information will be required to develop rationalely designed formulations for therapeutic applications. It is equally important that we gain a better insight into the disassembly process that results in release of DNA from the macromolecular structure, a requirement following cell uptake.

We believe that there are four critical events that are required for plasmid delivery to occur. First, the active agent (the plasmid expression vector) must

be protected from degradation by nucleases in the tissue culture media, serum and sites of injection or interstitial spaces. Second, the lipid-DNA complex must come in contact with the target cell. For in vivo applications, access to the target cell will be dependent, in part, on the route of administration. Third, the carrier system must interact with the target cell membrane and subsequently undergo internalization or cytoplasmic delivery. After a lipid-based DNA formulation interacts with a cell membrane, DNA enters the cell either directly through the plasma membrane or indirectly following endocytosis. Both entry routes require membrane destabilization and, regardless of whether the destabilized membrane is the plasma membrane or the endosomal membrane, the entry process must also involve dissociation of the plasmid expression vector from the lipid-based carrier. Once again we must contend with conflicting roles for the lipid-based carrier following internalization. The lipid-based DNA formulation must protect the DNA against enzymatic degradation, but dissociation is required for gene expression. The problem of nuclear delivery is further confounded by the presence of nucleases in the cytoplasm and strategies will have to be developed to facilitate protection of the DNA as it transferred into the nucleus.

Xu and Szoka authored a pivotal manuscript addressing potential mechanism(s) of cationic liposome/DNA formulation mediated membrane destabilization in 1996 (99). These investigators developed a model that accounts for membrane destabilization reactions, required for DNA release into the cytoplasm, as well as reactions that lead to dissociation of DNA from the cationic lipids used to prepare lipid-based formulations. We argue that the latter is likely one of the most important attributes, citing evidence that suggests that the transfection enhancing role of certain lipids is a consequence of specific interactions with cationic lipids used in lipid DNA preparation, rather than roles involving membrane fusion. The lipid composition will play an important role in effecting the ability of the DNA to be released from bound lipid following entry into the cell. This may be affected by the strength of the ionic/hydrophobic interactions of the lipid with the DNA, influencing DNA stability as well as DNA release and delivery to the nucleus. Regardless, if endosomal escape is not achieved then the lipid-based carrier, with its associated DNA, will eventually be degraded as it is transported from early to late endosomes, along the lysosomal degradation pathway (100). It is currently thought that only a small proportion of DNA delivered via cationic liposomes escapes the lysosomal degradative pathway. It has yet to be established how many plasmids are required to reach the nucleus in order to achieve efficient transgene expression and expression will be under the control of factors that are independent of nuclear delivery.

## THE FUTURE

It is our belief that the same advances in liposome technology that have given rise to the first generation of clinically proven drug formulations may unfor-

tunately limit further increases in therapeutic activity. Specifically, the drug retention properties required to minimize systemic exposure of drugs encapsulated inside long circulating liposomes significantly limit bioavailability of the agent once it has reached the disease site. This conclusion arises from results in several model systems that show that significant increases in disease site drug delivery often translate into only incremental increases in drug potency. It has been demonstrated in pharmacodynamic studies with liposomal anticancer agents that the circulating drug pool itself has little direct impact on therapeutic activity. Instead, it appears that once extravasated, the lipid carrier provides a localized source of drug infusion within the disease site. While the liposomal drug formulations used to date have engendered significant improvements in therapeutic activity, many results suggest that drug within the tumor is not freely bioavailable. In vitro studies measuring the doxorubicin concentrations necessary for 50% inhibition of growth (IC50) of tumor cells in culture indicate a range in doxorubicin IC50's of 100 nM in MCF-7 breast tumor cell line (101) to 190 nM and 24 μM in parental and DOX-resistant P388 cells, respectively (102). We have demonstrated that drug concentrations of 250 nmoles per gram tumor can be achieved using doxorubicin loaded drug liposomes and it can be suggested that drug concentrations within the tumor are in excess of that required to achieve maximum cytotoxic effects, even for drug resistant tumors. However, calculated rates of drug release from liposomes in tumor (0.60 to 0.65 nmol drug/μmol lipid/h for doxorubicin encapsulated in DSPC/Chol liposomes) may not be sufficient for inhibition or elimination of the tumor cells (51).

The inability to differentially control drug release rates in the plasma compartment and disease site is perhaps the most significant limitation of presently available liposomes. Ideally, one would be able to completely eliminate drug leakage in the circulation and then increase the release rate at the disease site to a level that would provide the optimal concentration vs. time profile for the specific drug being utilized. Early attempts to selectively increase drug leakage at tumor sites centered on the fact that liposomes can be constructed to become leaky in the acidic interstitial pH of some solid tumors (103), which can drop to values of 6.5. More direct evidence of the importance of site-specific drug release has been obtained using localized hyperthermia (104–108). Liposomal doxorubicin preparations, for example, can be prepared such that there is an increase in drug release at 42°C, compared to 37°C. These liposomes are administered iv to tumor bearing mice and the tumor site is then heated using a topical microwave heating device placed on the subcutaneous tumor. Application of a transient heating pulse after the liposomal doxorubicin had accumulated into the solid tumor resulted in a significant increase of therapeutic activity compared to free drug with hyperthermia and liposomal doxorubicin in the absence of heating. Although hyperthermia may not be applicable to many multifocal or deep-seated tumors, this technique provides encouraging indications that liposomes exhibiting controlled or triggered release of their contents will significantly augment the pharmacological improvements provided by liposomes.

We have suggested that significant advances in the use of liposomes for therapeutic purposes will require the development of liposomes that contain features specific for stability to blood components, controlled circulation lifetimes, disease site localization, controlled drug release following extravasation and/or target cell specific delivery. Such liposomes will have to exhibit many different functional components in order that each of the desired attributes can be expressed optimally. Our approach to this multifunctional carrier is based on the premise that liposomes can be designed to transform their physical characteristics so that properties required during the delivery phase of treatment can be differentiated from those required for therapy. The technical capabilities for constructing such liposomes have been established based on recent results that show that PEG-modified lipids can undergo spontaneous transfer between membranes (52, 109,110). The PEG-lipids can, if used as a bilayer stabilizing component in a lipid mixture consisting of non-bilayer lipids, therefore, act as regulators of liposome stability. Loss of the PEG-modified lipid, which occurs at a rate that is dependent on the acyl chain length of the PEG-lipid anchor, leads to formation of a highly fusogenic, unstable, liposome. These liposomes have been referred to as programmable fusogenic vesicles or PFVs (109).

Based on experience with conventional liposomal drug carriers, and in accordance to the model shown in Figure 4, certain attributes are also known to decrease the likelihood of lipid-based DNA transfer systems to interact with cells in an extravascular site. Larger, aggregate structures (>200 nm) are eliminated rapidly following intravenous administration in comparison to small (<200 nm) structures (111). Those structures that exhibit a positive or negative surface charge are eliminated more rapidly in comparison to neutral systems (112). For lipid-based DNA formulations exhibiting a cationic surface charge, anionic serum protein binding will certainly result in alterations of the surface characteristics. Protein binding is often associated with increases in plasma elimination rates, increased nonspecific cell binding, increased phagocytic cell uptake, perturbations of the membrane structure and complement activation, all of which compromise the utility of systemically administered lipid-based drug carriers.

Lipid-based DNA formulations which aggregate under physiological conditions and exhibit a net charge (whether positive or negative) will have reduced access to cells outside the blood compartment simply as a consequence of mechanisms that enhance elimination. Elimination is often restricted to select organs in the body, such as those with i) microcapillary beds that can filter out macromolecular structures (e.g. lung), ii) fenestrated or discontinuous blood vessels that encourage removal of large systems (e.g. spleen and liver) and/or iii) cells capable of recognizing, binding and phagocytosis of foreign particulates (e.g. Liver Kuppfer cells, Tumor-associated macrophages). It is not surprising, therefore, that significant levels of transgene expression are often observed in these organs following i.v. administration (113,114). It is important to note that it is unclear which cells are transfected in these organs and that phagocytic cell uptake will likely result in DNA degradation.

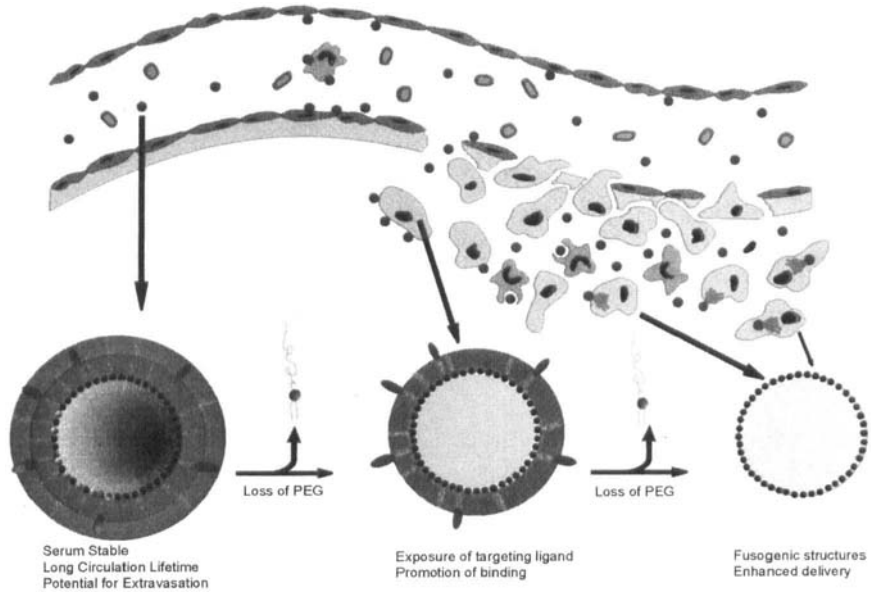


***Figure 10.*** The transformable liposome. Advances in the use of lipid-based DNA delivery technology will require the development of structures that contain features specific for stability to blood components, controlled circulation lifetimes, disease site localization and target cell specific delivery. Such liposomes must exhibit many different functional components such that each of the desired attributes can be expressed optimally. Our approach to this multifunctional carrier is based on the premise that liposomes can be designed to transform their physical characteristics so that properties required during the delivery phase of treatment can be differentiated from those required for a therapeutic effect. PEG-lipids, which can undergo spontaneous transfer between membranes, can act as regulators of the lipid-based DNA carrier attributes.

In order to redirect lipid-based DNA formulations to cells in other sites it will be necessary to develop methods that result in small (<200 nm), neutral or charge-shielded structures. We believe that this can be achieved through use of surface grafted polyethylene glycol (PEG). As suggested for conventional liposomal drug formulations, significant advances in the use of lipid-based DNA transfer technology for plasmid delivery and gene therapy will require the development of macromolecular structures that contain features specific for stability in blood, controlled circulation lifetimes, disease site localization, and target cell specific binding and delivery. These formulations will contain many different functional components in order that each of the desired attributes can be expressed optimally. Our approach to this multifunctional carrier is based on the premise that carriers can be designed to transform their physical characteristics so that properties required during the delivery phase can be differentiated from those required for cell binding and internalization. In particular, it is known that PEG-modified lipids can be used to protect and stabilize lipid structures (115,116), including liposomes prepared with non-bilayer forming lipids (109,117) as well as emulsions of triacylglycerol (118). Protection is facilitated by shielding of the membrane surface.

This, in turn, reduces the rate of protein adsorption, inhibits aggregation reactions mediated between surfaces with multiple reactive groups (119), prevents (delays) interaction with cells (120) and effects significant increases in carrier circulation longevity. As indicated above, it is known that PEG-lipids can also undergo spontaneous transfer between model lipid membranes (110,117). For this reason the PEG lipids can be used as regulators of the surface properties and cell-binding attributes of lipid-based DNA formulations (Figure 10). Studies with PEG-containing lipid based DNA formulations have recently been described by other researchers (121).

## CONCLUDING COMMENTS

As our understanding of the processes that dictate the fate of liposomes after i.v. injection has increased, we have been better able to design formulations that will optimize the selectivity of action for associated therapeutic agents. Inclusion of additional components into conventional liposomes can now be done on the basis of extensive data describing the in vivo behavior of various liposome types. Although some questions still remain in areas such as the uptake and processing of liposomes in extravascular sites, we can now more reliably predict how such specific manipulations of liposomes should affect therapeutic activity. This increased understanding has also helped to identify new directions that may improve the therapeutic activity of liposomal drug formulations. Greater control of drug leakage rates within disease sites and the use of targeted and/or fusogenic liposomes for intracellular delivery offer opportunities to dramatically increase the efficiency and specificity of lipid-based delivery systems. The challenge for the future will be to develop systems that are actually therapeutically superior and not just technologically sophisticated.

## ABBREVIATIONS

RES, reticuloendothelial system; MLV, multilamellar vesicles; PC, phosphatidylcholine; chol, cholesterol; DSPC, distearoylphosphatidylcholine; Chol, cholesterol; PE, phosphatidyl-ethanolamine, HBSS, HEPES buffered saline solution; VEGF, vascular endothelial growth factor; PEG, polyethylene glycol; PEG-PE, distearoylphosphatidylethanolamine derivatized with 1900 molecular weight polyethylene glycol; s.c., subcutaneous; RES, reticuloendothelial system; LUV, large unilamellar vesicles; PFVs, programmable fusogenic vesicles; QELS, quasielastic light scattering; IgG, immunoglobulin G.; FDA, Food and Drug Administration

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