CHAPTER 9

Liposomal Formulations for Nucleic Acid Delivery

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CONTENTS

9.1 LIPOSOMES FOR THE DELIVERY OF NUCLEIC ACID DRUGS

Liposomes are artificial vesicles made up of one or more bilayers of amphipathic lipid encapsulating an equal number of internal aqueous compartments. They are distinguished on the basis of their size and the number and arrangement of their constituent lipid bilayers (Figure 9.1).

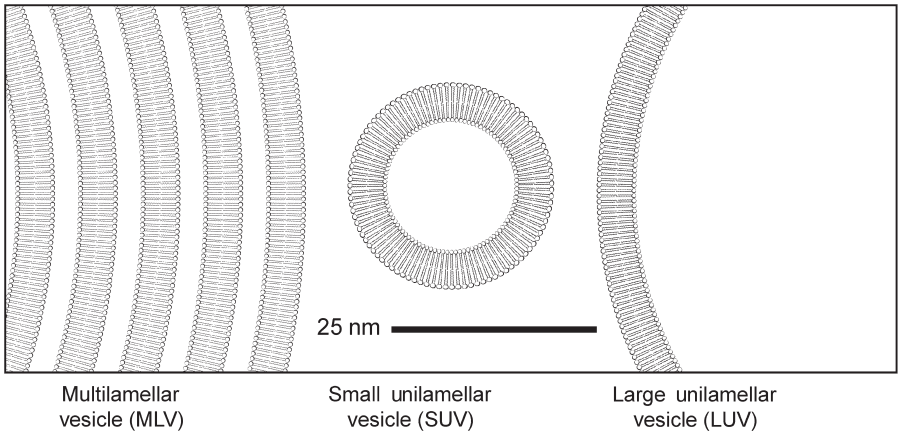

Figure 9.1 Liposomes. Mulilamellar vesicles (MLVs) are large (hundreds of nm in diameter) complex structures containing a series of concentric bilayers separated by narrow aqueous compartments. Large unilamellar vesicles (LUVs) are between 50 and 500 nm in diameter, while the smallest liposomes namely small unilamellar vesicles (SUVs) are <50 nm. LUVs are the preferred systems for delivery of NA drugs. Lipids are drawn roughly to scale.

Multilamellar vesicles (MLVs) are formed by the aqueous hydration of dried lipid films. Typically hundreds of nanometers in diameter, they are large, complex structures containing a series of concentric bilayers separated by narrow aqueous compartments. Simple unilamellar vesicles between 50 and 500 nm in diameter are referred to as large unilamellar vesicles (LUVs) while the smallest liposomes, vesicles smaller than 50 nm in diameter, are small unilamellar vesicles (SUVs).

Liposomes have received attention not only for their utility as model membrane systems, but also for use in drug delivery. Typically, liposomes are used as drug carriers, with the solubilized drug encapsulated in the internal aqueous space formed by the liposomal lamellae. Liposomal drug formulations can be used to overcome a drug's nonideal properties, such as limited solubility, serum stability, circulation half-life, biodistribution, and target tissue selectivity. Experience with conventional small molecule drugs has shown that the drugs which benefit the most from liposomal delivery, are those that are chemically labile, subject to enzymatic degradation and have an intracellular site of action [1]. For this reason, there is considerable interest in exploiting liposomes as carriers of nucleic acids (NAs), either as plasmid vectors for gene therapy applications or to deliver smaller NA species such as antisense oligonucleotides, ribozymes and, more recently, siRNA for the purposes of downregulating target genes. Because of their ability to achieve favorable drug/lipid ratios and their more predictable drug release kinetics LUV are the preferred liposome delivery system for NA drugs.

An advantage of liposomal drug delivery is that the pharmacokinetics, biodistribution, and intracellular delivery of the liposome payload are largely determined by the physicochemical properties of the carrier. For example, the biodistribution of a NA entrapped within a small, long circulating liposome is independent of the type of NA, which can be a relatively stable double-stranded plasmid DNA molecule or single-stranded antisense DNA, or one of the more labile ribonucleotide molecules such as ribozymes or a duplex siRNA. This is only true if the liposome is truly acting as a carrier, rather than a mere excipient. Liposomes function as excipients when used to formulate hydrophobic drugs that would otherwise be difficult to administer in aqueous dosage form. Hydrophobic drugs rapidly exchange into lipoproteins or other lipid-rich environments soon after injection, resulting in comparably uncontrolled pharmacology. In the context of NA drug delivery, liposomes are considered excipients if used to enable vialing and aqueous dosing of hydrophobic lipid–NA conjugates [2–5]. (These applications are not considered in this chapter, nor are those that use preformed, cationic lipid-containing vesicles to form "lipoplex" or "oligoplex" systems.)

An objective inherent in all pharmaceutical development is to minimize the risks associated with treatment while maximizing the benefit to patient health. The most important risk to patients is the toxicity associated with the administration of poorly tolerated compounds, often exacerbated by attempts to increase efficacy by escalating the administered dose. A well-designed liposomal delivery system will be capable of reducing the toxicity and increasing the potency of NA-based drugs by optimizing NA delivery to target tissues. Liposomal NA delivery will be determined by the physical and biochemical properties of the liposome including stability, size, charge, hydrophobicity, interaction with serum proteins, and interaction with nontarget cell surfaces. Ideally, liposomal carriers for NA delivery will have the following properties: (i) they will be safe and well tolerated; (ii) they will have appropriate pharmacokinetic attributes to ensure delivery to intended disease sites; (iii) they will mediate effective intracellular delivery of intact NA; (iv) they will be nonimmunogenic, enabling the use of multidosing treatment regimes; and (v) they will be stable upon manufacture so that large batches can be prepared with uniform, reproducible specifications. In this chapter we discuss the physical makeup, manufacturing methods, and pharmacological considerations specific to liposomal systems for the delivery of NA-based drugs, with emphasis on those that enable systemic delivery of synthetic polynucleotides such as antisense ODN, ribozymes, and siRNA.

9.2 LIPOSOME CONSTITUENTS

NA encapsulation was first described in the late 1970s, prior to the development of cationic lipid-containing lipoplex, using naturally occurring, neutral lipids to encapsulate high-molecular-weight DNA [6–8]. The first reports of low-molecular-weight oligo- or polynucleotide encapsulation similarly used passive techniques to entrap NA in neutral liposomes [9–11]. The advent of cationic lipid-mediated lipofection [12] saw a shift in emphasis away from encapsulated systems in favor of "lipoplex" or "oligoplex" systems. More recently, improvements in formulation technology have allowed for a return to encapsulated systems that contain cationic lipids as a means of facilitating both encapsulation and intracellular delivery. More advanced systems typically contain multiple lipid components, each of which play a role in determining the physical and pharmacological properties of the system as a whole.

9.2.1 Cationic Lipids

Cationic lipids play two roles in liposomal NA formulations. In the first case, they encourage interaction between the lipid bilayer and the negatively charged NA, allowing for the enrichment of NA concentrations over and above that which would be achieved using passive loading in charge neutral liposomes. Cationic lipids allow for encapsulation efficiencies greater than 40% when using coextrusion methods, and greater than 95% when using more sophisticated techniques [13–15]. Cationic lipids also function by providing the liposome with a net positive charge, which in turn enables binding of the NA complex to anionic cell surface molecules. The most abundant anionic cell surface molecules, sulfated proteoglycans and sialic acids, interact with and are responsible for the uptake of cationic liposomes [16–18]. The role of cationic lipids in liposomal uptake presents a dilemma: highly charged systems are rapidly cleared from the blood, thereby limiting accumulation in target tissues. Particles with a neutral charge however, display good biodistribution profiles, but are poorly internalized by cells. This supports the concept of a modular delivery solution, that is, an engineered nanoparticle with individual components fulfilling different functions in the delivery process, and in particular, a system which responds to the microenvironment in a manner that facilitates transfection. Titratable, ionizable lipids are components that allow for the adjustment of the charge on the system by simply changing the pH after encapsulation [19]. At reduced pH when the system is strongly charged, NAs are efficiently encapsulated. When liposomes containing titratable, ionizable lipids are at a pH closer to the pK_a of the cationic lipid, such as

physiological pH, they become more charge neutral and are able to avoid opsonization by blood components [19]. More recently, the use of novel, pH titratable cationic lipids with distinct physicochemical properties that regulate particle formation, cellular uptake, fusogenicity, and endosomal release of NA drugs have been described [20]. The chemical and biological properties of pH-titratable cationic lipids are influenced by their degree of lipid saturation. In particular, the phase transition properties, as measured using ^{31}P-NMR, are affected. Above the phase transition temperature, T_c, lipids adopt the more highly fusogenic reverse hexagonal H_{II} phase [20–22]. By noting the temperature at which this phase transition occurs, the relative ease with which lipids form the H_{II} phase and become "fusogenic" can be determined. On this basis it has been shown that the fusogenicity of liposomal systems increases as the titratable cationic lipid becomes less saturated. The lipid pK_a also correlates with the degree of saturation. pK measurements confirm that saturated lipids carry more residual charge at physiological pH. For this reason, liposomes containing the more highly saturated cationic lipids are taken up more readily by cells in vitro [20]. However, liposomes containing the more fusogenic unsaturated cationic lipids DLinDMA and DLenDMA are more effective at mediating RNA interference in both in vitro cell culture systems and in vivo. The apparently conflicting results between cellular uptake and silencing potency are a reminder that cellular uptake per se is insufficient for effective delivery of NA. Cellular uptake, fusogenicity, and endosomal release are distinct processes, each of which need to be enabled by the delivery vehicle and each of which are profoundly affected by the physicochemical properties of the cationic lipids used.

9.2.2 The Role of Helper Lipids in Promoting Intracellular Delivery

Although we have just shown that cationic lipids may have inherent fusogenic properties of their own, cationic lipids were originally believed to require fusogenic "helper" lipids for efficient NA delivery [23–26]. Fusogenic liposomes facilitate the intracellular delivery of complexed plasmid DNA by fusing with the membranes of the target cell. Fusion may occur at a number of different stages in delivery, either at the plasma membrane, endosome or nuclear envelope. Fusion of first-generation, nonencapsulated lipoplex systems with the plasma membrane is expected to be a particularly inefficient method of introducing NA into the cytosol. Since lipoplex-NA is predominantly attached to the surface of the liposome, lipoplex fusion events resolve with NA, formerly attached to the liposome surface, deposited on the outside surface of the plasma membrane. Encapsulated systems are significantly different from lipoplex in this respect. Upon fusion with either the plasma or endosomal membrane(s), encapsulated carriers deliver their contents directly into the cytosol.

Lipids that preferentially form nonbilayer phases, in particular the reverse hexagonal H_{II} phase, such as the unsaturated phosphatidylethanolamine DOPE, promote destabilization of the lipid bilayer and fusion. Similar to fusogenic cationic lipids, decreasing the degree of lipid saturation increases the lipid's affinity for the fusogenic H_{II} phase [27–32]. However, some cationic lipids can function in the absence of these so-called helper lipids, either alone [24,25] or in the presence of the nonfusogenic lipid cholesterol [33]. This would suggest that either these lipids have properties which promote delivery through a mechanism which does not require membrane fusion, or that their own fusogenic properties are adequate to support delivery. As described above, cationic lipids are readily designed for optimal fusogenicity by controlling lipid saturation. This provides for multiple opportunities for modulating the fusogenicity of a liposomal lipid bilayer [20].

Attempts to address the role of fusogenic lipids in vivo have yielded confounding results. In this regard it is important to distinguish the effect of fusogenic lipids on NA delivery to target tissue from their effect on intracellular delivery. Fusogenic formulations are more likely to interact with the vascular endothelium, blood cells, lipoproteins, and other nontarget systems while in the blood compartment. For this reason there may be an advantage to transiently shield the fusogenic potential of systemic carriers using shielding agents such as polyethylene glycol (PEG).

9.2.3 PEG–Lipids

An ideal delivery system would be one that is transiently shielded upon administration, facilitating delivery to the target site, yet becomes increasingly charged and fusogenic as it reaches the target cell. PEG lipids partially address this challenge. PEG–lipid conjugates are readily incorporated in liposomal NA formulations. They provide a benefit during the formulation process, stabilizing the nascent particle and contribute to formulation stability by preventing aggregation in the vial [13]. PEG conjugates sterically stabilize liposomes by forming a protective hydrophilic layer that shields the hydrophobic lipid layer. By shielding the liposome's surface charge they prevent the association of serum proteins and resulting uptake by the reticuloendothelial system when liposomes are administered in vivo [34,35]. In this way, cationic liposome NA formulations are stabilized in a manner analogous to PEGylated liposomal drug formulations that exhibit extended circulation lifetimes [36–41]. Although this approach has been investigated with a view towards improving the stability and pharmacokinetics of lipoplex containing either plasmid DNA [42] or antisense oligonucleotides [43], PEG–lipid-containing lipoplex systems suffer from the heterogeneity and suboptimal pharmacology common to most nonencapsulated NA–cationic lipid complexes.

Although PEG–lipid-containing systems are promising with respect to their ability to deliver NA to disease sites, improvements are required to increase their potency. Early PEGylated liposomes for the delivery of small molecule chemotherapeutic drugs utilized stably integrated PEG lipids such as PEG-DSPE [39]. These systems are designed to function as carriers that facilitate the accumulation of active drug compound at disseminated disease sites. The drug is released at the cell surface at a "leakage rate" determined by the liposomal bilayer composition. NA-based drugs differ in this respect in that they require effective *intracellular* delivery, hence the use of the cationic and fusogenic lipids described earlier. PEGylated systems typically exhibit relatively low-transfection efficiencies. This is mainly due to the ability of the PEG coating to inhibit cell association and uptake [23,44,45]. Ideally, PEG–lipid conjugates would have the ability to dissociate from the carrier and transform it from a stable, stealthy particle to a transfection-competent entity at the target site. Various strategies have been applied to this problem. A number of investigators have explored the use of chemically labile PEG–lipid conjugates [46–52], in particular those that are "pH sensitive." Typically, these systems invoke a chemically labile linkage between the lipid and PEG moieties that reacts via acid-catalyzed hydrolysis to destabilize the liposomes by removal of the sterically stabilizing PEG layer. Although this approach results in improved performance both in vitro and in vivo, it may be regarded as suboptimal for two reasons. First, pH-sensitive PEG lipids are designed to be rapidly hydrolyzed in the reduced pH environment encountered within the endosome, but since PEG lipids are known to inhibit cellular uptake, a prerequisite to endosomal localization and hydrolysis, their use actually limits the amount of material delivered to the endosome [53]. Second, the incorporation of pH-sensitive or otherwise chemically labile lipids results in a truncation of formulation shelf life relative to systems that use more stable PEG–lipids. An alternative to the use of acid-labile PEG–lipids involves the use of chemically stable, yet diffusible PEG lipids.

The concept of diffusible PEG lipids arose from the observation that the length of the PEG lipid anchor has an influence on PEG lipid retention and the stability and circulation lifetime of empty lipid vesicles [54]. It has been found that by modulating the alkyl chain length of the PEG lipid anchor [55–59], the pharmacology of encapsulated NA can be controlled or "programmed" in a predictable manner. Upon formulation, the liposome contains a full complement of PEG in steady-state equilibrium with the contents of the vial. In the blood compartment, this equilibrium shifts and the PEG–lipid conjugate is free to dissociate from the particle over time, revealing a positively charged and increasingly fusogenic lipid bilayer that transforms the particle into a transfection-competent entity. Diffusible PEG lipids differing in the length of the their lipid anchors have been incorporated into liposomal systems containing plasmid DNA (SPLP) [13,55], antisense oligonucleotides (PFV, SALP) [19,56,60], and siRNA (SNALP) [14,15,61]. This approach may help to resolve the two conflicting demands imposed upon NA carriers. First, the carrier must be stable and circulate long

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