CHAPTER 14

DNA–LIPID AMPHIPHILES FOR DRUG AND GENE THERAPY

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14.1 INTRODUCTION

Gene therapy is defined as the introduction of exogenous genetic materials including both DNA and RNA into a target tissue with the aim of providing therapeutic benefit. Gene therapy holds great promise in treating diseases ranging from inherited disorders, to cancer, to acquired conditions. The genetic material being introduced has encoded genetic sequence functions to replace defective genes. Gene expression occurs by synthesizing functional gene products using the newly introduced genetic material. The introduced genetic material can also function to substitute missing genes, silence unwanted gene expression, or introduce new cellular biofunctions.[1,2] Gene therapy is often hampered by the need to overcome both the extracellular and the intracellular barriers. Before the gene can reach the target cells, it is subject to removal from opsonins, phagocytes, and degradative enzymes. Once it has penetrated through extracellular matrices and reaches the target cells, it is often limited by poor penetration into the cell membrane, lack of recognition characteristics necessary for intracellular transport, degradation within lysosomal compartments, and inability to release from transport vesicles. An efficient delivery carrier is therefore required to protect the genetic materials and facilitate cellular uptake.[2] Gene delivery carriers can be classified as viral and nonviral. In this chapter, the use of viral and nonviral gene delivery carriers will be discussed, with a focus on nonviral cationic liposome gene carriers. This chapter will also discuss on cationic liposomes' therapeutic applications, their structural–functional relationship, preparation methods, characterization methods, toxicity effects, challenges in delivery, their cellular pathways, and performance evaluation.

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14.2 VIRAL AND NONVIRAL GENE CARRIERS

Viruses are known for their ability to penetrate cells to deliver their genome. Viruses have therefore been considered as a gene delivery carrier to deliver foreign genes into cells. Adenoviral vectors are the most commonly employed viral carrier, as they can infect a wide range of mammalian cells with high transfection efficiency. The measure of a gene carrier's performance in terms of transfection is referred to as transfection efficiency. Viral carriers can carry DNA with size up to 7.5 kb and transduce these transgenes into nonproliferating cells.[3] These carriers can be manipulated using recombinant DNA techniques that are well-established protocols. However, the drawback of using viral carriers is that they have low packaging capacity, they are difficult and expensive to manufacture, they raise safety issues such as provoking carcinogenesis, and they can induce an immune response that abolishes transgenic expression.[4,5]

Numerous studies have attempted to develop nonviral, synthetic carriers to mimic the efficient gene delivery capacity of viral carriers. Cationic polymers, cationic lipids, cationic peptides, and recombinant protein are the major types of nonviral carriers. Although nonviral carriers yield lower gene expression compared with viral carriers, the use of nonviral carriers are attractive due to their low toxicity and low immunogenicity, lack of pathogenicity, and ease of pharmacological production.[6] Cationic polymers can be classified into two groups: natural polymers and synthetic polymers. Cationic polymers are composed of protonatable amine residues that enable them to spontaneously associate with negatively charged nucleic acid via electrostatic interactions.[7] Among various gene delivery approaches, cationic liposomal gene delivery was found to be rather effective and safe[8]; the following chapter will therefore discuss the use of cationic lipids for gene therapy.

14.2.1 Cationic Liposomes for DNA Delivery

Negatively charged polyelectrolytes form supramolecular (ion pair) associations with low-molecular-weight cationic surfactants, or lipids, that have single or double chain aliphatic tails. The association between a single anionic site on the polyelectrolyte and a single cationic group of the amphiphile occurs when the latter is molecularly dispersed or when it forms intramolecular suprastructures. Chapter 8 describes the association of DNA types of varying molecular length and rigidity with surfactants, either molecularly dispersed or forming micellar-like structures. Perico and Ciferri[9] described DNA as a rigid, rodlike molecule for molecular lengths corresponding to about 150 base pairs, and as a semirigid molecule for larger contour lengths. It was suggested that the rigidity of short DNA rods interferes with the formation of spherical micelles. However, the rodlike DNA molecules, decorated by the complementary charged surfactants, undergo self-assembly by interdigitation of the hydrophobic tails into a cylindrical symmetry stabilized by hydrophobic inter-

actions. The resulting structure is a hexagonal distribution of DNA rods imbibed in a lattice of less ordered chains (Chapter 8).

In addition to micelles, single- and double-chained amphiphiles are also known to form liposomes, characterized by closed, interdigitated double layers with a distribution of cationic head groups in the external as well as in the internal rim. At variance with spherical micelles, the association of a negatively charged polyelectrolyte can therefore occur also in the internal, water-filled cavity of the vesicle. Cationic liposomes have been extensively used as DNA delivery carriers due to their advantages, including simplicity in preparation, safety in human use, and versatility in compositions.[5] The complexes formed by DNA and cationic liposomes are known as lipoplexes. The introduction of nucleic acids (DNA or RNA) into cells by a nonviral method is referred to as a transfection. If said introduction is performed using a liposome carrier, it is referred as a lipofection, first described by Felgner et al.[10]

Theoretically, there is no limitation on the length of DNA that can be complexed with cationic liposomes.[11] The final size of the lipoplex is determined by the size of the supercoiled DNA plasmid. It is reported that lipoplexes have the potential to transfer DNA with size up to 1 million base pairs.[12] The plasmid DNA can be surrounded by three to five amphiphilic bilayers to form lipoplexes.[13] It is reported that the membrane charge density, σ_M, a measure of average charge per unit area of membrane, can be a key parameter that governs the transfection efficiency of lipoplexes. One of the major problems with cationic liposomal carriers appears to be the tight association of DNA and its inability to dissociate intracellularly.[12] Manipulation the σ_M of lipoplexes can therefore be an important approach to tackle this problem.

14.2.2 Cationic Liposomes for Drug and Small Interfering RNA (siRNA) Delivery

Using the same electrostatic interaction principle, cationic liposomes are also employed as carrier for other negatively charged molecules such as messenger RNA (mRNA), siRNA, synthetic oligonucleotides (ODNs), negatively charged proteins, and peptides.[14] siRNA is a short double-stranded RNA (typically 21-base pair RNA) that can mediate sequence-specific inhibition of gene expression known as gene silencing or RNA interference (RNAi). These siRNA can assemble into RNA-induced silencing complexes (RISCs), which result in cleaving of complementary mRNA. RNAi technology is an emerging field, as RNAi is an important tool for gene function analysis, and has also been evaluated as a therapeutic agent to cure diseases such as cancer, infection, respiratory disease, neuronal disease, and autoimmune disease.[15] Similar to naked DNA, naked siRNA is highly negatively charged and cannot pass through the cytoplasmic membrane on its own; siRNA have a half-time of less than an hour in human plasma.[15] Mammalian cells appear to lack the effective dsRNA uptake mechanism. In addition, the uptake of siRNA by fluid phase endocytosis does not necessary result in the endosomal release of siRNA into

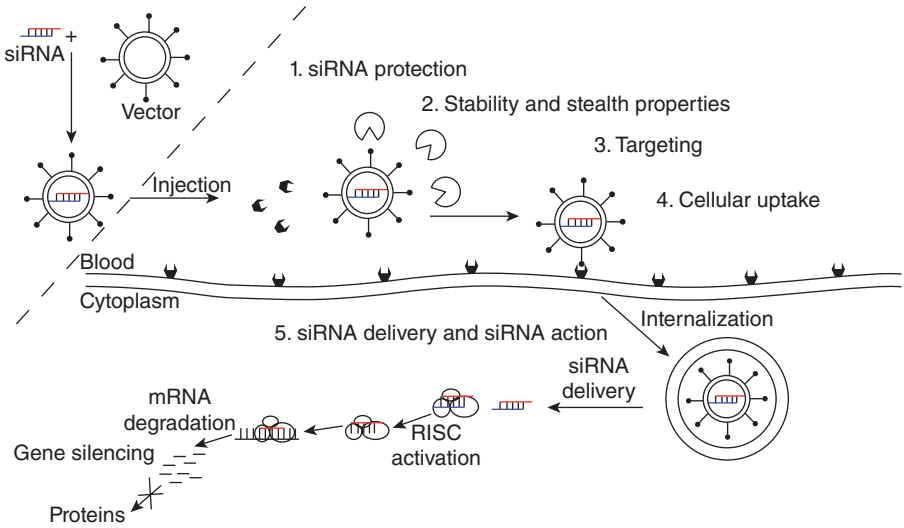

FIGURE 14.1. Illustration of siRNA delivery using a nonviral carrier, which protects siRNA from degradation, facilitates cell uptake and internalization, and releases siRNA into the cytosol. Once the siRNA is free in the cytoplasm, it can assemble into RNA-induced silencing complexes, leading to cleavage of complementary mRNA and knockdown protein expression. Reproduced with permission from David et al.[22] (Copyright 2010 Elsevier)

the cytoplasm. Due to these reasons, the addition of naked siRNA to mammalian cells does not result in effective gene silencing.[16] Therefore, various cationic liposomes have been employed as a carrier for siRNA delivery.[17–20] Issues involving the use of liposomes for siRNA and plasmid DNA delivery are similar, with the exception that the targeted delivery site for siRNA is the cytoplasm, where the targeted site for plasmid DNA is the nucleus.[21] Figure 14.1 summarizes how a nonviral carrier protects siRNA in the extracellular matrix, follows by delivery, and facilitates siRNA internalization into target cells, after which the siRNA is released into the cytosol and activates the gene silencing pathway.[22]

Apart from siRNA, biologically active ODNs are short nucleic acids that are commonly used as gene function examining tools, and have also found therapeutic applications as polynucleotide drugs. Problems relating to administration, delivery, and cellular uptake of ODNs are similar to those of siRNA. The use of polycation conjugates with amphiphilic copolymers for ODN delivery has been attempted to increase dispersion stability of polymer/DNA and reduce serum inhibition. Representative examples include conjugates of hydrophilic nonionic polymers poly(ethylene oxide) (PEO), amphiphilic block copolymers of ethylene oxide (EO) and propylene oxide (PO), copolymer of lipophilic poly(propylene oxide) (PPO), and hydrophilic PEO. These polycation conjugates consist of lipophilic segments and hydrophobic segments that

can form micelle-like structures with ODNs in a similar fashion as lipoplex. Cationic copolymers of hydrophilic polyethylenimine (PEI) and amphiphilic Pluronic (BASF, Mount Olive, NJ) have been reported to form stable suspensions of small micelle-like particles with ODNs, and have shown increased cellular uptake and increased sequence-specific activity of antisense ODNs.[23]

Liposomes have been extensively studied as drug delivery systems, and macromolecules including protein and anticancer agents (e.g., doxorubicin, donaumycin) can be encapsulated in liposomes by hydrophobic interaction. Wang et al.[24] demonstrated that gene expression can be enhanced by the synergistic effect of drug and gene delivery both *in vitro* and *in vivo*, where attempts have been made to codeliver drug and DNA using a biodegradable amphiphilic copolymer containing cholesterol side chains to form liposome-like polymeric nanoparticles.

14.3 CHEMICAL STRUCTURE OF CATIONIC AMPHIPHILES

A prototypical cationic lipid amphiphile molecule contains three basic domains: a positively charged polar head and a hydrophobic part connect through a linker. Figure 14.2 shows a typical structure of an amphiphile DOTAP representation.[25] The hydrophobic moiety of DOTAP consists of two unsaturated diacyl side chains, the linker consists of an ester group, and the positively charged group consists of a propylammonium group.[25]

14.3.1 Hydrophobic Chain

The hydrophobic part of cationic lipid amphiphiles generally consists of either aliphatic chains or cholesterol-based derivatives.[26] Traditionally, it is believed that a cationic lipid with a single aliphatic chain has lower transfection

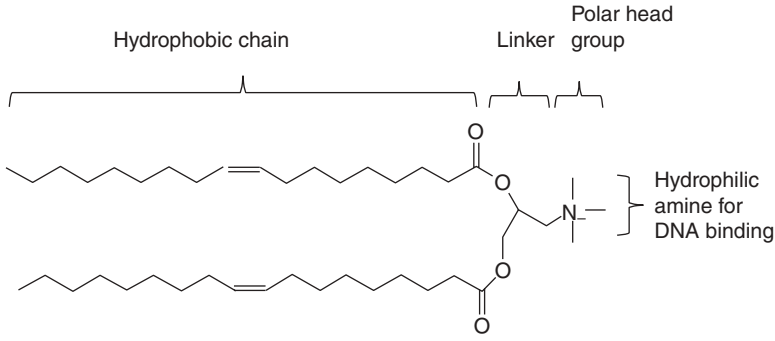

FIGURE 14.2. Schematic representation of a cationic amphiphile DOTAP. A typical cationic amphiphile, DOTAP consists of a hydrophobic part and a polar head group anchored by a linker. Adapted from El-Aneed.[25]

efficiency compared with a cationic lipid with double chains. In additional, single-chain cationic lipids such as CTAB are less efficient and induce higher cytotoxicity compared with their double-chain analogs, such as DOTMA[15]. However, some studies demonstrated that single-chain LHON is more efficient and less cytotoxic compared with double-chain DOTAP. There are two major types of double chains: unsaturated and saturated chains. Representative examples of unsaturated double chains are DOTMA, DOSPA, DOTAP, and DOSPER. For saturated double chains, representative examples are DOGS, DDAB, TM-TPS, and DMRIE. Both single and double aliphatic chains can form stable liposome by itself, but the incorporation of helper lipids in the formulation is not uncommon.[27] It was shown by Felgner et al.[28] that by decreasing the carbon number of the acyl chain from 18 to 14, the phase transition temperature (T_m) of lipids can be lowered. A cationic lipid that exhibits T_m below 37°C can result in higher transfection.[28–30] The presence of the double bond has a similar effect as acyl chain shortening and gives rise to a less rigid bilayer.[31] Obika and coworkers[32,33] have attempted the synthesis and utilization of cationic triglycerides for lipofection, and it was found that the transfection activity of symmetrical cationic triglycerides is comparable with asymmetrical cationic triglycerides.

The cholesterol-derived cationic lipids generally cannot form stable bilayers on their own. To overcome this, these lipids can be formulated with helper lipids such as neutral lipids.[27] Neutral lipids such as DOPE, cholesterol, and DOPC are the three most commonly used helper lipids. It is believed that the incorporation of helper lipids facilitates lipid membrane destabilization and allows higher transfection activity. For instance, DOPE is known to destabilize the endosome membrane and facilitate endosomal escape of lipoplexes.[8] It is reported that antisense ODNs carried by the DC-CHOL/DOPE formulation were preferentially transfected into target cells' nuclei.[8] Despite the need for helper lipids, the use of cholesterol-based hydrophobic moiety is still attractive due to its role in membrane fusion during lipofection. The effect of lipid hydrophobic moiety on cytotoxicity has not been fully understood, though it is postulated that their cytotoxicity effect is dependent on the physiochemical features of the positively charged head group and linker.[26]

14.3.2 Positively Charged Group

The positively charged group of cationic lipid amphiphiles is responsible for the ionic interaction with negatively charged molecules such as peptide and phosphate groups in nucleic acid (including DNA, mRNA, siRNA, and ODNs).[14] Typically, amine group substitution on the lipid chain is carried out via methylation.[31] These positively charged groups generally consist of primary, secondary, and tertiary amines, or quaternary ammonium salts with varying degrees of substitution. Polyvalent cationic lipid amphiphiles have also been prepared using polyamine to improve DNA binding and transfection efficiency; some well-known examples are DOGS and DOSPA[8]. Guanidine- and

imidazole-containing groups have also been used as the positively charged group on cationic lipid amphiphiles. The cytotoxicity of cationic lipid amphiphiles is mainly caused by the positively charged head group; it is also known that tertiary amine is less toxic compared with quaternary amine. The cytotoxicity can be reduced significantly by substituting the linear amine head group by a heterocyclic ring such as pyridinium and guanidine, which spreads the positive charge of the cationic group.[26]

14.3.3 Linker

The linker of cationic lipid amphiphiles can be composed of amide, ester, carbamate, ether, or a phosphate bond. Besides functioning as an anchor for both the hydrophobic group and the positively charged group, the linker group also has some influence on stability, biodegradability, and transfection activity. It is known that the substitution of amide or urea linkages for the carbamate group can yield lower transfection activity due to the reduced chemical stability.[31] Cationic lipid amphiphiles with an ether linker tend to give better transfection activity compared with those with an ester or amide bond.[26] The use of ester linkage enables fast hydrolysis after transfection, which reduces cytotoxicity of cationic lipid amphiphiles.[32] Generally, cationic lipid amphiphiles that are composed of ether linkages are more toxic compared with those that are composed of biodegradable linkages, such as ester, amide, and carbamoyl bonds. The use of a linker segment with a length of 3–6 atoms can optimize transfection activity[34] as well as lower cytotoxicity, given that the linkage is degradable.[26] Figure 14.3 shows some other commonly used cationic amphiphiles for nucleic acid delivery.

14.3.4 Helper Lipids

Neutral lipids such as DOPE, DOPC, and cholesterol are often used in synergy with cationic lipid amphiphiles as helper lipids. Figure 14.4 shows some commonly used neutral lipids. Cationic liposome formulated from equimolar amounts of neutral lipid and cationic lipid has been reported to give high gene transfection. The incorporation of neutral lipids can also decrease toxicity of the liposome formulation, which contributes to higher transfection levels.[26] The choice of helper lipids has a strong influence on the activity of liposomes. For example, cholesterol as a helper lipid can enable the formation of more stable liposomes with cationic lipids but results in less efficient transfection compared with a DOPE-containing formulation.[35] The improved gene transfection may partly result from reducing the DNase I susceptibility of liposomes in the presence of helper lipids. Neutral lipids such as DOPE can facilitate membrane fusion and aid the destabilization of endosomes, while DOPC does not destabilize lipid bilayers.[26] Some studies have reported that DOPE can also facilitate the disassembling of lipopolexes to enable DNA escape from endocytotic vesicles, which contributes to higher transfection levels.[35]

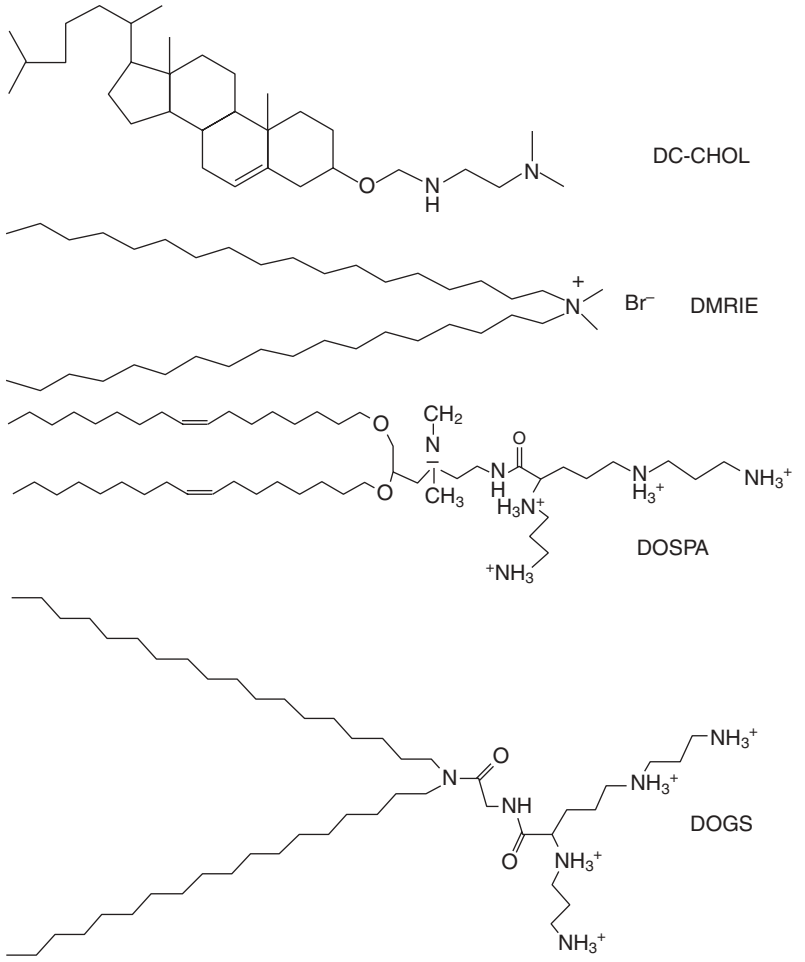

FIGURE 14.3. Structures of some other commonly used cationic amphiphiles for nucleic acid delivery: DC-CHOL, DMRIE, DOSPA, and DOGS.[12,27,34]

14.4 COMPLEXATION

In gene therapy, plasmid DNA is the most commonly employed nucleic acid due to its advantages compared with retroviral vectors. Plasmid DNA is an autonomous, extrachromosomal, circular DNA that exhibits two topological forms: supercoiled and nicked relaxed (open circular). Plasmid preparation is simple, quick, safe, and inexpensive; plasmid DNA can be modified to contain a promoter and the gene coding for the protein of interest. Plasmids can be expressed in the transfected cell nucleus in a transient or stable (in rare events) manner.[36]

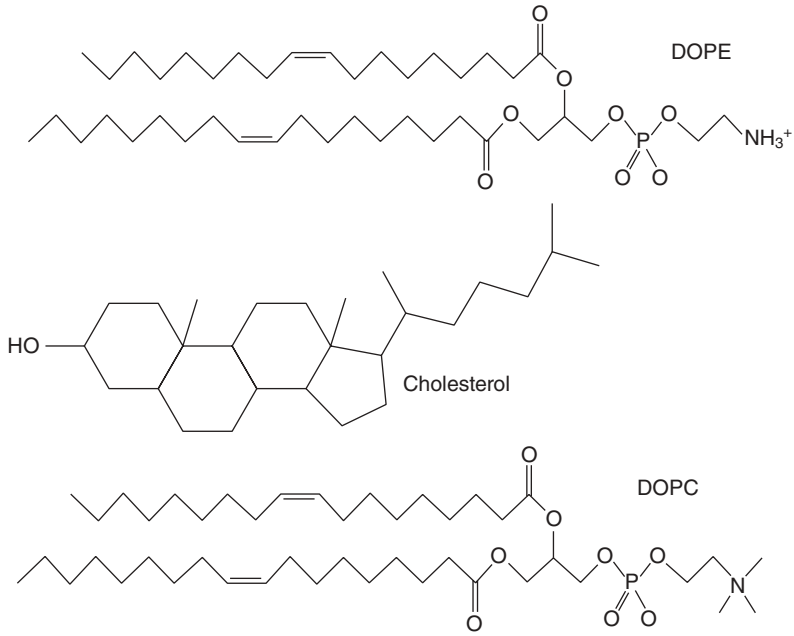

FIGURE 14.4. Structures of some commonly used neutral lipids: DOPE, cholesterol, and DOPC.[12,96]

When the solutions of cationic lipid amphiphiles and DNA are combined, they can spontaneously self-assemble to form complexes via electrostatic interaction. These complexes can be bilayered lipoposomal structures, depending on the lipid compositions; some lipoplexes form multilayered structures consisting of plasmids sandwiched between cationic lipid.[7] The driving force of this process comes from the release of counterions that were tightly bound to the cationic lipid and negatively charged DNA[37]. The complex formation involves a three-step mechanism. The DNA plasmid first interacts with the lipid amphiphile monolayer, then unwraps itself into lamellae with lipid amphiphile molecules. At the end, the wrapped DNA assemble with lipid amphiphiles to form a complex.[11] The resulting complex known as lipoplex can be anionic, neutral, or cationic depending on the charge ratio (ρ_{chg}) of cationic lipid to DNA. For lipofection, a positively charged ($\rho_{chg} > 1$) is generally preferred, as the lipoplex can be attached on the negatively charged cell surface proteoglycans by electrostatic attractions.[37]

The mixing method[18,38–41] and lipid film hydration method[42–45] are the most commonly used methods for the preparation of lipoplex for nucleic acid delivery. Figure 14.5 illustrates the two lipoplex preparation methods using siRNA as the representative nucleic acid.[46]

The mixing method involves simply mixing solutions of plasmid DNA and cationic liposomes under serum-free conditions. The lipid film hydration

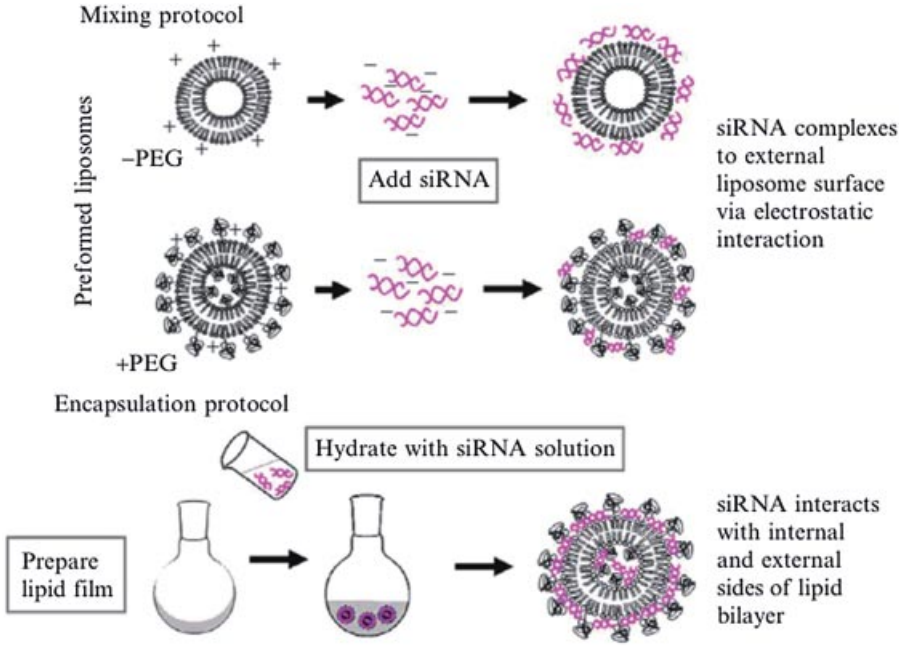

FIGURE 14.5. Preparation of lipoplex for nucleic acid (e.g., siRNA) delivery using (a) the mixing method and (b) the lipid film hydration method. Reproduced with permission from Podesta and Kostarelos.[46] (Copyright 2009 Elsevier)

method involves dissolving the lipid in a volatile solvent such as chloroform, and evaporating the solvent to obtain a thin film, which will then be hydrated to obtain crude liposomes. The resulting liposomes are heterogeneous in size and can be mechanically fragmentated or extruded to obtain liposomes of a more uniform size. Next, a plasmid DNA solution will be added dropwise to the aqueous liposome to form a complex.[47] Both of the methods are amenable to cationic lipid and/or neutral helper lipid substitutions. Some studies have simplified the lipid film hydration procedure; for example, Podesta and Kostarelos[46] reported a preparation method where the lipid film is hydrated directly with an siRNA solution, followed by extrusion. Regardless of the method being employed, it should be noted that the complexation between liposomes and nucleic acids may result in localized precipitation. To overcome this, the nucleic acid should be added to the liposomes with rapid mixing, not vice versa.

The following protocols for preparation of lipoplexes are adapted from Whitemore et al.[47] and Girao da Cruz et al.[38] The lipoplexes should be prepared using sterile solutions under aseptic conditions; these complexes should be used while fresh to avoid aggregation. All solutions and glassware used should be DNase-free or RNase-free, whichever is applicable. DNase-free water can be prepared by autoclaving deionized water. RNase-free dH_2O can

be prepared by treating the water with 0.1% (v/v) diethylpyrocarbonate (DEPC) overnight, followed by autoclaving the solution at 121°C for a minimum of 20 minutes.[46] The protocol is amenable for liposome/siRNA complexation.

Reagents and Solutions

1. *5× Dextrose Stock Solution:* Dissolve 2.6 g dextrose in 10.0 mL sterile water; sterile filtrate the solution through a 0.2-μm membrane.
2. *Cholesterol Stock Solution 20.0 mg mL^{-1} (51.7 mM):* Dissolve 100.0 mg cholesterol into 5.0 mL chloroform solvent.
3. *DOTAP Stock Solution (20 mg mL^{-1}):* Dissolve 20 mg DOTAP in 1 mL chloroform solvent.
4. *Dextrose 5.2% Buffer Solution:* Dissolve 2.6 g dextrose in 50 mL sterile water; sterile filtrate the solution through a 0.2-μm membrane.

Preparation of DOTAP/DNA Complexes Using the Mixing Method

1. Prepare DOTAP liposome stock solution by adding 1.0 mL DOTAP stock solution to 0.55 mL cholesterol stock solution; follow by swirl mixing.
2. Prepare liposome/polycation solution by adding 30.0 μL of 5× dextrose, 43.0 μL DOTAP liposomes, 3.0 μL protamine sulphate, and 74.0 μL sterile water into a 50-mL sterile conical tube; mix the solution by swirling.
3. Prepare DNA solution by adding 50.0 μL plasmid DNA, 30.0 μL of 5× dextrose, and 70.0 μL sterile water to a sterile microcentrifuge tube; follow by tapping the tube to mix.
4. Add DNA solution dropwise to a gently swirling liposome/polycation solution.
5. Incubate the complexes at room temperature for 10–15 minutes. Use the complexes immediately.

Preparation of Cationic Lipid/DNA Complexes Using the Lipid Film Hydration Method

1. Rinse a 30.0-mL Corex glass centrifuge tube three times with chloroform.
2. Mix 1.0 mL DOTAP stock solution to 0.55 mL cholesterol stock solution into the Corex tube; follow by swirl mixing.
3. Dry the chloroform from DOTAP/cholesterol in a chloroform mixture under a vacuum using a rotator evaporator. Alternatively, blow-dry the chloroform using N_2 gas to form a thin lipid film on the glass while rotating the tube by hand.
4. Dry the lipid film completely in vacuum desiccators for another 2–3 hours.

5. Hydrate the lipid film to suspension by adding 2.0 mL of 5.2% dextrose buffer solution to the film and vortex several times.
6. Incubate the suspension for 2–3 hours at room temperature.
7. To disperse lipid aggregates, the resulting lipid suspension can be bath sonicated, vortexed, or extruded through a 100-nm polycarbonate filter hereafter, until lipids are entirely resuspended.
8. The resulting liposomes can be diluted with distilled water, and the phospholipid concentration can be determined by a phosphate assay.
9. Add predetermined volume of DNA solution to the liposomes to obtain desired lipid/DNA charge ratio.
10. Incubate the complexes for 15 minutes at room temperature. Use lipolexes immediately after being prepared.

14.5 CHARACTERIZATION OF CATIONIC LIPID CARRIER

14.5.1 Agarose Gel Electrophoresis

The ability of a cationic lipid amphiphile to bind DNA is usually evaluated using agarose gel electrophoresis, followed by staining with nucleic acid stain. Agarose gel electrophoresis is a method used to separate DNA or RNA molecules by size. The agarose gel electrophoresis protocol can be divided into three steps: (1) prepare agarose gel with concentration appropriate for the size of DNA to be separated; (2) load the lipoplexes and controls (DNA molecular ladder, naked DNA) into the sample wells and run the gel at a voltage and time period to obtain optimal separation; and (3) stain the gel by a nucleic acid stain and follow by visualization using UV light illumination. Standard protocols for running agarose gel electrophoresis can be found in Voytas[48] and Armstrong and Schulz.[49] The commonly used nucleic acid stains are ethidium bromide, or fluorescent cyanine DNA dyes such as SYBR Green I and SYBR Gold. The use of SYBR Green I dye is gaining popularity due to several reasons: Its concentration-dependent fluorescence intensity is identical for the nicked-relaxed form and supercoiled form DNA, it has higher sensitivity for both double-strand and single-strand DNA compared with ethidium bromide,[50] and because ethidium bromide is more mutagenic.[51,52]

Using the agarose gel electrophoresis technique, the mobility of the DNA in the presence of various formulations is monitored and compared with the control (naked DNA).[53] This assay usually cannot provide quantitative information, but qualitative conclusions can usually be drawn from the results. Figure 14.6 illustrates the separation of DNA on an agarose gel. The separation is achieved by using an electric field to move negatively charged nucleic acid through the agarose matrix. Lane 1 represents a DNA ladder that is a molecular weight size marker that provides a scale for size estimation. The molecular weight of DNA is inversely proportional to its migration rate, as smaller molecules can move faster than longer molecules. Lane 2 represents naked plasmid

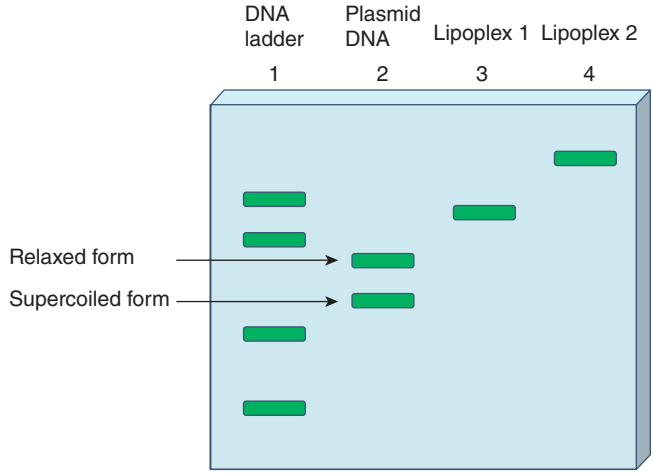

FIGURE 14.6. Illustration of DNA separation on agarose gel.

DNA control; two DNA bands can usually be found, one corresponds to the relaxed form and the other corresponds to the supercoiled form. The supercoiled form can migrate further through the gel due to its complex structure compared with the relaxed form. Lane 3 represents a formulation of lipoplex; the change in mobility relative to the control is an indication of complexation. Lane 4 represents a different formulation of lipoplex; the DNA band is retarded at the origin, indicating that the corresponding cationic lipid amphiphiles can form stable complexes with DNA.

Lipoplex stability generally increases with the number of charges in the positively charged amine group. Cationic lipid amphiphiles composed of more hydrophobic bile acid skeletons can retard DNA mobility more efficiently due to their ability to form more stable complexes compared with their hydrophilic counterparts.[53] Even-Chen and Barenholz[50] reported that cationic liposomes preferentially bind to plasmid DNA in nicked-relaxed form over the supercoiled form, as the binding of supercoiled DNA is weaker and easier to dissociate from the lipoplex. The negative charges of the nicked-relaxed DNA are more readily available and can induce changes in positive charge distribution during complexation.

14.5.2 Size and Surface Charge of Lipoplex

The size of a lipoplex is an important parameter that affects transfection efficiency, as it determines the cell internalization pathway via endocytosis, which will be discussed further in a later section. The type of liposomes employed, the charge ratio, incubation time in polyanion-containing media, and the presence of serum can all influence the size of lipoplex, and thus the transfection efficiency. The lipoplex size and heterogeneity usually increase with increasing

lipid-to-DNA ratio. Lipofection of lipoplexes with size less than 250 nm is known to be inhibited by the presence of serum. Lipoplexes of size larger than 700 nm are known to induce efficient transfection in the presence or absence of serum. For this reason, many transfection protocols in the literature employ a serum-free reagent such as Opti-MEM (GIBCO, Invitrogen, Carlsbad, CA) for gene transfection.[18,54–57] The size inhibition effect of serum is suggested to contribute to the serum resistance to ethanol-dried lipid-DNA (EDL). Large cationic lipoplexes may be able to delay the dissociation of DNA from lipids, and can therefore overcome EDL. In addition, it is postulated that larger liposomes can increase phagocytic activity due to their maximum contact with cells. Upon cellular internalization, the formation of large intracellular vesicles can be more easily disrupted to enable endosomal escape of DNA into cytoplasm. The *in vitro* criteria for effective gene transfection may not be applicable to *in vivo* transfection due to bioavailability problems; lipoplexes of size 200–400 nm are optimal for *in vitro* transfection, while smaller lipoplexes of size 40–90 nm are better for *in vivo* transfection.[58]

Another important parameter that determines the transfection efficiency is the surface charge of the lipoplex.[59] The surface charge of lipoplex increases as the ratio of cationic lipid to DNA increases, and higher charge ratios are generally more toxic to a variety of cells. Some nonspecific efficacious issues are due to the toxicity of lipoplex and should be taken into consideration during lipoplex design.[60] Lipoplexes that carry different surface charges display different structures: positively charged lipoplex exhibits aggregated multilamellar structure where DNA is encapsulated within the lipid coat, while negatively charged lipoplex exhibits free plasmids or protruding DNA-strings structure where DNA is bound to the exterior of the complex.[58]

The surface charge often determines the biodistribution of lipoplex *in vivo*. Chonn et al.[61] reported that surface charge is the key determinant of complement-activating liposomes. Complement activation by negatively charged liposomes containing phosphatidylglycerol, phosphatidic acid, cardiolipin, phosphatidylinositol or phosphatidylserine occurred via the Ca^{2+}-dependent classical pathway. Neutral liposomes such as PC:cholesterol (CHOL) and PC:CHOL:DPPE are less likely to trigger complement activation. Complement activation triggered by positively charged liposomes containing stearylamine occurred via an alternative pathway.

Experimentally, the particle diameter (*z*-average diameter) of lipoplexes and the surface charge (ζ potential) of lipoplexes can be measured using photon correlation spectroscopy (PCS)/dynamic light scattering and laser Doppler electrophoresis (LDE), respectively. PCS is a technique that measures the time-dependent fluctuation in light scattering intensity caused by particles in suspension that undergo Brownian motion. LDE is a technique that measures the frequency shift caused by electrophoretic mobility of particles in suspension under the influence of an electric field.[62] A polydispersity index can be calculated at the same time; this parameter gives an indication of the homogeneity of the liposome population.[46]

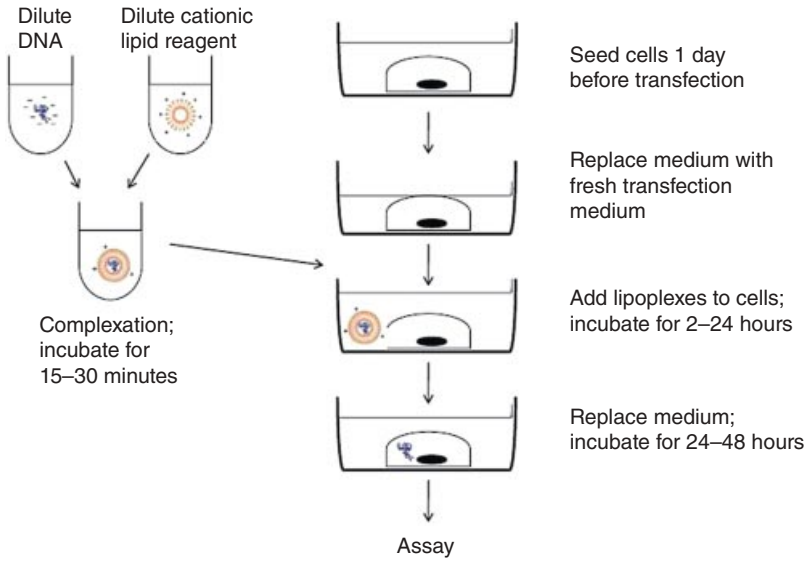

FIGURE 14.7. Diagram demonstrating lipoplex *in vitro* transfection protocol adapted from Hawley-Nelson et al.[66]

14.5.3 Transfection of Mammalian Cells *In Vitro*

Many studies performed *in vitro* transfection using cultured mammalian cells in order to study the performance and optimize the lipoplex formulation prior to *in vivo* transfection using animals.[63–65] This section describes the basic protocol of how to transfect adherent mammalian cells (primary cells or cell lines) with lipoplexes (Fig. 14.7).

The following protocol for the preparation of lipoplexes is an adaptation from Hawley-Nelson et al.[66] It should be noted that the preparation and transfection experiments should be carried out using sterile solutions under aseptic conditions with the aid of a biosafety cabinet. All mammalian cell cultures should be incubated at 37°C in a humidified 5% carbon dioxide incubator. Table 14.1 shows the recommended working volumes for lipoplex transfection in culture well plate of different sizes. The protocol is amenable for liposome/siRNA transfection.

1. Trypsinize and count adherent cells 1 day prior to transfection. Seed cells into a sterile well plate with complete cell culture medium (with serum) to obtain 50–80% confluent on the transfection day.
2. On the day of transfection, dilute DNA into dilution medium in a polystyrene or polypropylene tube. Dilute cationic lipid reagent into dilution medium. Mix diluted DNA and diluted cationic lipid reagent, and allow mixture to incubate for 15–30 minutes at room temperature.

TABLE 14.1. Suggested Working Volumes for Lipoplex Transfection for 48–72 Hours Study (Adapted from Hawley-Nelson et al.[66])

Well Plate	Seeding Density (cells per well)	DNA (μg)	Dilution Medium (μL)	Cationic Lipid Reagent (μL)	Transfection Medium (μL)
96 wells	$0.5–1 \times 10^4$	0.05–0.4	10–25	0.075–1.5	0.08–0.1
48 wells	$1.5–2 \times 10^4$	0.1–0.8	12–37	0.25–5	0.1–0.2
24 wells	$3–4 \times 10^4$	0.2–1.6	25–50	0.5–10	0.2–0.5
12 wells	$6–8 \times 10^4$	0.4–3.2	50–100	1–20	0.4–1
6 wells	$15–16 \times 10^4$	1–8	100–250	2.5–50	0.8–2.5

3. While complexation is taking place, carefully replace culture medium with fresh transfection medium without scratching adherent cells. Avoid the use of serum and antibiotics during transfection.
4. Add lipoplex into each well. Incubate for 2–4 hours at 37°C in 5% CO_2 incubator.
5. Remove medium, add fresh cell culture medium (with serum), and allow cells to cultivate for another 24–48 hours before assay.

The cell morphology during transfection interval should be monitored, especially when cells are maintained in a serum-free medium during lipoplex treatment. Some cells may lose viability under these conditions; in such cases, the lipoplex/serum-free medium transfection time should be reduced. The transfected cells should generally be harvested 24–72 hours post transfection for gene expression or protein expression analysis. To optimize transfection efficiency, one can vary the charge ratio of cationic lipid to nucleic acid; the amount of DNA or RNA added to each well can also be varied.

14.6 EXTRACELLULAR BARRIERS

The use of cationic liposomes to deliver nucleic acid can increase drug availability due to the prevention of nucleic acid degradation. However, these nucleic acid carriers are limited by extracellular barriers such as the opsonization process, in which opsonin proteins present in the blood serum often bind quickly to conventional nanoparticles; these opsonin-binded nanoparticles can be recognized by macrophages.[67,68] For this reason, the administration route should be carefully selected; for instance, these carriers have limited applicability for intravenous administration routes,[69] as they are subjected to clearance by the reticuloendothelial system (RES), a complex system that consists of macrophages, dendritic reticular cells, lymphocytes, and plasma cells that contribute to pathogenic microorganism resistance.[70] About 90% of any immunological recognized particles will be taken up by the liver macrophage within 5 minutes, 1–5% of these particulates will be taken up by spleen macrophage,

and the rest may be taken up by macrophage population in lung and bone marrow. Larger particles are taken up by the RES faster than small particles. Particles with higher charge are taken up by the RES faster than lower or noncharged particles. Particles that display higher surface hydrophobicity tend to enhance phagocytosis due to their hydrophobic interactions with phagocytes. In addition, the surface hydrophobicity also affects the amount and composition of apolipoproteins adsorbed to the particles and leads to binding with apolipoprotein receptors.[71]

The most common approach to minimizing RES uptake is to modify the lipid by polyethylene glycol (PEG), also known as pegylation, to reduce the opsonization of liposomes. Pegylation has also been employed in various nanoparticle designs to improve circulation half-time and enhance drug delivery.[72] Nonpegylated liposomes often encounter phagocyte system uptake and undergo rapid clearance by the RES compared with pegylated liposomes. Uptake by the RES results in irreversible sequestering of liposomes and can induce toxicity or acute impairment of the phagocyte system in some cases.[73]

The effectiveness of a drug or gene carrier can be assessed by measuring the *in vivo* biodistribution of the liposomes. Usually, it can be carried out by intravenously injecting animals using a lipid and/or DNA marker. The animals are sacrificed and blood samples are withdrawn from the heart at different time intervals post injection. Organs including the liver, spleen, lung, kidney, and heart will be excised and the amount of marker can be quantified according to a calibration curve of the administered particles, after subtracting the background measurement of untreated animals.[74]

14.7 INTRACELLULAR BARRIER: CELL ADHESION, INTERNALIZATION, AND INTRACELLULAR TRAFFICKING

14.7.1 Cell Adhesion

Whether a lipoplex can be transfected into cells is determined by several critical factors in addition to the nucleic acid's own innate biological activity. Once overcoming extracellular barriers, the lipoplex is required to overcome a few intracellular barriers before the encapsulated gene can be expressed. The first intracellular barrier is the cell membrane (Fig. 14.8; see also Chapter 13). The first step of successful lipofection involves the binding of lipoplex to negatively charged membrane-associated proteins such as sialyated glycoproteins or proteoglycans, mainly via electrostatic interactions. It is therefore possible to enhance cell adhesion by manipulating the charge ratio of cationic lipid to nucleic acid. Following cell adhesion, the internalization of lipoplex occurs through the fusion of liposome and plasma membrane.[31] Although the mechanisms involved are still under debate, it is generally accepted that endocytosis and endocytosis-like mechanisms are the main internalization pathways of lipoplex; these mechanisms will be discussed in the following sections.

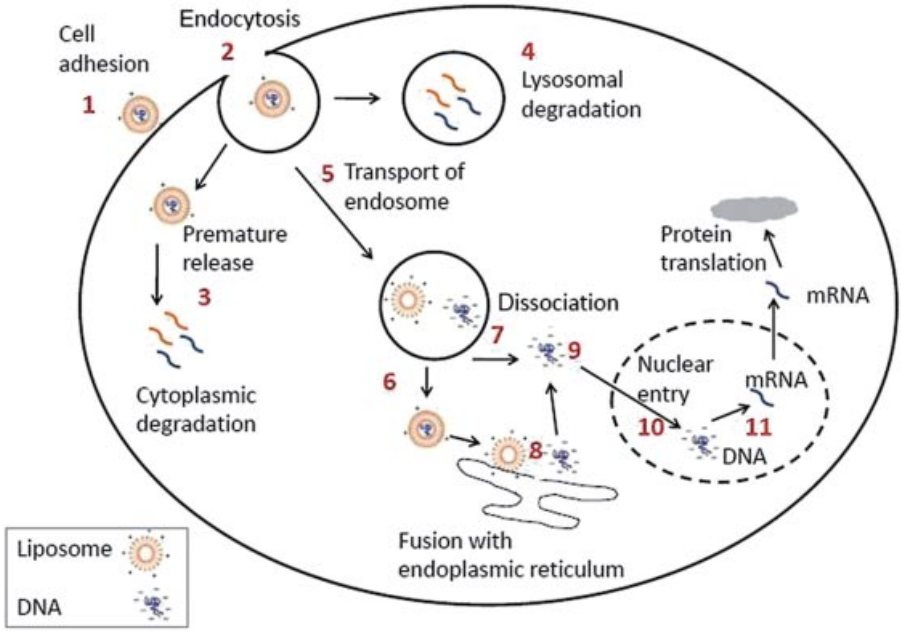

FIGURE 14.8. Summary of intracellular barriers involved in lipofection: (1) the lipoplex needs to bind to the membrane surface for cell adhesion; (2) the lipoplex may internalize inside the cell by endocytosis; (3) if the lipoplex can be degraded by cytoplasmic nucleases before reaching the nucleus if it is released too early from endosomes; (4) the lipoplex may be degraded in lysosomes if it is unable to escape from endosomes; (5) cytoplasmic transport of endosomes can bring the lipoplex near the perinuclear region; (6) the lipoplex can escape from endosomes if osmotic rupture of endosomes occur; (7) the DNA may dissociate from liposomes during endosomal escape; (8) the DNA may dissociate from liposomes in the cytoplasm; (9) the plasmid may diffuse through the cytoplasm; (10) the DNA may translocate to the nucleus if nuclear entry is successful; and (11) successful gene expression will lead to DNA transcription to mRNA followed by protein translation. Diagram adapted from Barron and Szoka[31] and Elouahabi and Ruysschaert.[77]

14.7.2 Cell Internalization

Once the lipoplex has successfully adhered to the cell membrane, the lipoplex has to overcome the next intracellular barrier, where it will have to be internalized into the cell cytosol in order for it to reach the cell nucleus. Phagocytosis and pinocytosis are two of the major endocytosis mechanisms that have been described as possible pathways of internalization of lipoplex. Phagocytosis is a specific cell surface receptor and signal cascade-mediated process that is highly regulated. Phagocytosis occurs only in specialized mammalian cells including macrophages, monocytes, and neutrophils. The major function of phagocytosis is to remove large pathogens or debris. The phagocytosis is acti-

vated by antibodies bound to surface antigens on pathogen cells such as bacteria; next, a signal cascade triggers actin assembly and the formation of cell surface extensions that enclose and engulf antibody bond pathogens.[75]

Pinocytosis, also known as fluid-phase uptake, can occur in many cell types, and there are five morphologically distinct pinocytosis mechanisms: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, lipid raft-mediated endocytosis, and clathrin- and caveolae-independent endocytosis. The cargo molecule and its receptor determine which pinocytic pathway they will use to internalize into the cell.

Macropinocytosis is a membrane ruffling process that is generally considered to be a nonreceptor-mediated process, where cells internalize a large volume of extracellular fluids. In this process, an actin-driven formation of membrane protrusions is triggered by a signal cascade stimulated by growth factors or other signals. Unlike phagocytosis, these protrusions collapse onto ligand-coated particles and fuse with the plasma membrane to generate endocytic vesicles known as macropinosomes. Macropinosomes do not fuse into lysosomes, so particles that are internalized via this pathway can therefore avoid lysosomal degradation, and macropinosome escape occurs due to their leaky nature.[75,76]

Clathrin-mediated endocytosis occurs in all mammalian cells and functions to continuously uptake essential nutrients, antigens, growth factors, and pathogens. Clathrin-mediated endocytosis was previously known as receptor-mediated endocytosis, and plays a crucial role in intercellular communication during tissue development. Clathrin-coated vesicles with size ranging from 100 to 150 nm in diameter can carry concentrated receptor–ligand complexes into cells. Clathrin-mediated endocytosis is also involved in regulating the internalization of membrane pumps and membrane transport of ions and small molecules, which play an important role in cell and serum homeostasis. Molecules internalized through clathrin-mediated endocytosis experience a pH change from neutral to 5.9 or 6.0 in the lumen of early endosomes, followed by transition to pH 5 from late endosomes to lysosomes.[76]

Caveolae are flask-shaped invaginations of the plasma membrane that are present on many cells and constitute 10–20% of the cell surface. After activation, caveolae are slowly internalized to form small vesicles 50–60 nm in diameter. Caveolin, a dimeric protein that binds cholesterol in caveolae, can self-associate on the surface of membrane invagination to form a striated caveolin coat. It is postulated that caveolae may be involved in intracellular cholesterol trafficking and intracellular cholesterol homeostasis. Caveolae-mediated endocytosis is nonacidic and nondigestive, and is receptor-mediated. It could be advantageous if it is utilized as a drug delivery route; however, this internalization route has a low capacity uptake, which can only internalize the fluid phase with small volume.[75]

Lipid rafts are small, cholesterol-free and sphingolipid-rich planar microdomains of cell surfaces that lack caveolin and caveolae. These 40–60-nm-diameter lipid rafts are found in lymphocytes, many human cancer cells, and rodent

TABLE 14.2. Multiple Endocytic Pathways with Regard to Size of the Endocytic Vesicle (Adapted from Prokop and Davidson[76])

Triggering Mechanism	Vehicle Size	Entry Ports
Receptor mediated, signal cascade mediated	1–10 μm	Phagocytosis
Growth factors, other signals	1–5 μm	Macropinocytosis
Receptor mediated	120–150 nm	Clathrin-mediated endocytosis
Receptor mediated	<60 nm	Caveolae-mediated endocytosis
Hydrophobic interaction, scavenger receptor mediated	40–60 nm	Lipid rafts
Unclear	20–300 nm	Clathrin- and caveolin-independent endocytosis

macrophages. Anionic, neutral, and hydrophobic particles can be internalized into cells via lipid raft-mediated endocytosis. Lipid raft-mediated endocytosis includes hydrophobic uptake via lipid membrane fusion, and uptake of lipophilic and anionic groups via scavenger receptor-mediated membrane fusion. Unlike caveolae-mediated endocytosis, lipid raft-mediated endocytosis lacks an energy requirement and the uptake process bypasses the lysosomal compartment, so it is therefore a potential route for gene delivery.[76]

Apart from caveolae, there are other cholesterol-rich microdomains 40–50 nm in diameter, referred to as rafts, that diffuse freely on the cell surface. Clathrin- and caveolae-independent endocytosis mechanisms are poorly understood. It is only known that these raft structures have unique lipid compositions that are specific for membrane protein and glycolipid sorting.[75] Table 14.2 summarizes the different mechanisms for entry of nanovehicular drugs/carriers.

14.7.3 Intracellular Trafficking

Once lipoplexes internalize into cells, they need to overcome another intracellular barrier, where they need to escape from endocytic vesicles and diffuse through the cytoplasm before they can import into the nucleus. If the endosome release of lipoplex occurs too early, the nucleic acid will be degraded by cytoplasmic nucleases before reaching the nucleus. Cytoplasmic transport of endosomes can determine the success of lipoplex; endosomal release near the perinuclear region provides a better chance for the nucleic acid to enter the nucleus.[77]

Endosomal escape of lipoplex can occur via three major mechanisms: (1) endosomal membrane destabilization; (2) cationic lipid and anionic phospholipid exchange that occurs mostly on the cytoplasm-facing monolayer of cell membrane; and (3) endosomolysis induced by osmotic swelling.[78]

Dissociation of nucleic acid may occur during endosomal escape or after fusion of released lipoplexes with the endoplasmic reticulum (ER), where nucleic acid will separate from the carrier. Lipoplexes that fail to escape from

endosomes will end up being degraded by the lysosome. The DNA may translocate into the nucleus during mitosis depending on the cell division activity and the plasmid lifetime. The nuclear translocation efficiency of plasmid DNA, determined as the nuclear fraction divided by the fraction of plasmid DNA that escaped from the endosome,[79] is estimated to be 1/1000. If the DNA is encoded with specific sequences that mediate its interaction with the transcription factor, the DNA–protein complex can be translocated into the nucleus via a nuclear localization signal-mediated transport mechanism. It is also postulated that the lipoplex-enclosed endosomes may fuse with the nuclear membrane and facilitate the direct entry of DNA into the nucleus.[77] The cell binding, internalization processes, and intracellular barrier presence are summarized in Figure 14.8.[31,77]

14.8 CYTOTOXICITY OF LIPOSOMES

Cationic lipids can cause *in vivo* and *in vitro* toxic effects; for example, they may induce cell shrinking, reduced number of mitoses, and vacuolization. Their toxicity effect is often dose-dependent.[26] In order for gene therapy to be successful, a cationic lipid amphiphile with low cytotoxicity is required. The cytotoxicity of cationic lipid or liposomes can be evaluated by measuring the cell viability. Traditionally, cell viability is measured using thiazolyl blue formazan (MTT) assay.[80,81] More recently, other viability assays such as tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] reduction assay (MTS) and Alamar Blue™ (Trek Diagnostic Systems, Cleveland, OH) have gained popularity due to their simplicity of use.[38,82–87] Unlike MTT, Alamar Blue assay is nontoxic and is less likely to interfere with normal metabolic activity of cells, and can therefore give more reliable measurements.[88] Alamar Blue assay can be reduced by $FMNH_2$, $FADH_2$, NADH, DADPH, and cytochromes, whereas MTT assay cannot be reduced by cytochromes. The reduction in Alamar Blue causes a color change that is detectable by both UV absorbance spectrometer and fluorometer. The fluorescence is measured at 530–560 nm excitation wavelength and 590 nm emission wavelength,[89,90] whereas the absorbance is determined at 570 and 600 nm. The following section describes the general procedure for determining the viability of transfected mammalian cells using the Alamar Blue assay, adapted from Chan et al.[82]

1. Seed cells into a sterile 96-well plate with complete cell culture medium (with serum) to obtain 50–80% confluent on the transfection day; perform lipofection using the transfection protocol described above.
2. Incubate cells at 37°C in 5% CO_2 incubator for 3 days.
3. Remove spent medium. Add 250 μL of 10% Alamar Blue (in complete medium) to each well.
4. Return well plate to incubator for 3–4 hours; measure the absorbance using a microplate reader.

The cell viability can be calculated as a percentage of Alamar Blue reduction using nontransfected cells as the control group, according to the equation, [62]:

$$\% \text{ Cell viability} = \left(\frac{A_{570} - A_{600}}{A_{570,\text{control}} - A_{600,\text{control}}} \right) \times 100,$$

14.9 DETERMINATION OF TRANSFECTION EFFICIENCY

Various experiments can be performed to assess the transfection efficiency of cationic liposome carriers. The transfection efficiency is a measure of target gene or protein expression compared with the gene or protein expression result from that of a control. Usually, naked DNA/siRNA transfected cells are employed as negative control, and cells that are transfected by DNA/siRNA complex with a commercial carrier are employed as positive control. The commercial carrier Lipofectamine™2000 (mixture of DOSPA : DOPE) (Invitrogen, Carlsbad, CA) is the most commonly used positive control for both DNA and siRNA delivery.[91] A cationic liposome carrier is considered to be efficient if its resulting gene expression or protein expression is higher than that given by the controls.

14.9.1 RNA Expression

Post transfection, the targeted gene products may require isolation of RNA or protein prior to analysis. RNA expression level can be quantified by assaying mRNA using reverse transcription polymerase chain reaction (RT-PCR). For quantification by RT-PCR, the total RNA is first extracted and isolated from cell lysates. Next, a reverse transcriptase will be employed to synthesize complementary DNA (cDNA) from the isolated total RNA at a temperature range of 42–60°C. RNA extraction and isolation and cDNA conversion can be performed with the aid of commercial kits such as the RNeasy Mini Kit (Qiagen, Valencia, CA) and SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). The cDNA will be amplified by PCR and quantified using a thermocycler. The target gene expression level will be normalized to the amount of total RNA by comparing with an endogenous reference gene expression level.[62]

14.9.2 Protein Expression Quantified by Western Blot

Protein expression can be quantified using enzymatic assays or immunoassays such as Western blotting. For Western blot, protein extracts are obtained from transfected cells, and a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) will then be performed. After electrophoresis, the separated protein bands will be transferred to a polyvinylidene fluoride (PVDF) membrane, and the protein of interest will be detected using the immunodetection method. The target protein's band intensity is normalized against refer-

ence protein expression to correct for variation in total protein content, where β-actin is the most commonly used reference protein. The transfection efficiency is usually expressed as percentage of protein intensity with respect to nontransfected controls.[62]

14.9.3 Flow Cytometry and Confocal Microscopy Studies

For analysis of lipoplex internalization or intracellular distribution, it is often useful to perform the transfection using fluorescently labeled DNA or siRNA, or fluorescently labeled liposomes. Post transfection, fluorescent cells are first washed with phosphate buffered saline (PBS), trypsinized, and fixed by a reagent such as paraformaldehyde. The intracellular trafficking of the lipoplexes can then be visualized by laser confocal scanning microscopy (LCSM).[92] Figure 14.9 shows an example of intracellular trafficking of lipoplexes being visualized using LCSM; the plasma membrane of target cell was stained with PKH67 (green fluorescent dye), and the lipoplexes were labeled with red fluorescent dye, so the cellular uptake and nuclear entry of lipoplex can be observed.[93]

For DNA delivery, it is useful to employ a plasmid that encodes reporter genes, such as green fluorescence protein (GFP) or luciferase, which allows the transfection efficiency to be measured using simple assays. For siRNA delivery, it may be useful to employ cell lines that stably express reporter gene GFP and luciferase, so that the transfection efficiency and the degree of gene knockdown can be measured easily.[62] Post transfection, the GFP expressing

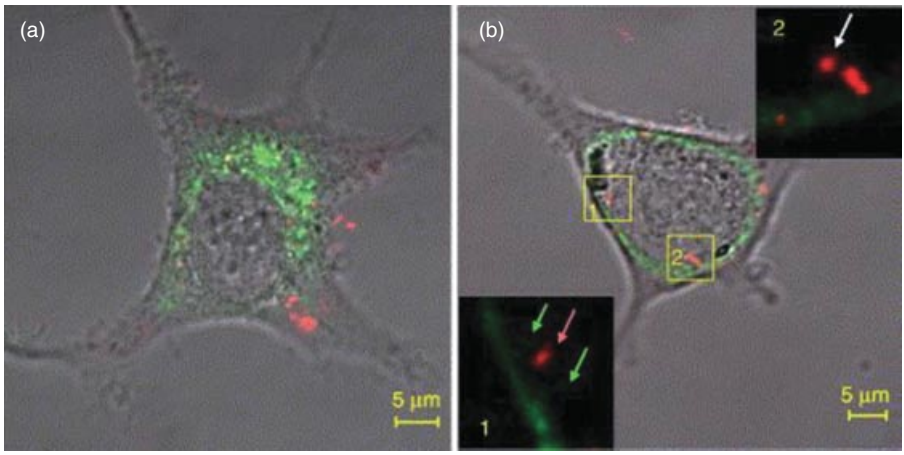

FIGURE 14.9. Imaging of (a) cellular uptake and (b) intracellular distribution of lipoplexes by confocal laser scanning microscopy. Plasma membranes were stained with PKH67 (green fluorescent); the labeled lipoplexes are detected as red clusters The white and red arrowheads (in inset 1 and 2) indicate intracellular uptake of lipoplex; the green arrowhead represents plasma membrane signals. Reproduced with permission from Iwasa et al.[93] (Copyright 2006 Elsevier)

cells will be washed with PBS, trypsinized, resuspended in PBS, and can be quantified by flow cytometry.[92] Flow cytometry is an important technique that simultaneously measures and analyzes multiple cell characteristics including size, quantity, granularity, and relative fluorescence intensity as cells flow through the laser beam detector. The principle of this method is to determine the different characteristics of cells by measuring how the cell scatters incident laser light and emits fluorescence.[62]

14.9.4 Luciferase Gene Expression

Genetic reporters are commonly used to study transfection efficiency, wherein a reporter gene-encoded plasmid is transfected into mammalian cells using the delivery vehicle, and the reporter protein expression is evaluated. Firefly luciferase is widely used as a reporter as its activity is available immediately upon gene translation, where protein posttranslational processing is not required. Luciferin gene expression can be measured by a luminescence assay. The procedure involves harvest and lyses transfected cells, followed by mixing cell lysate with a luciferin-based reagent. The firefly luciferase expressed by cells will catalyze luciferin oxidation through an electron transition using ATP. With Mg^{2+} as a cosubstrate, light is produced from the formation of product oxyluciferin.[94,95] The luminescence light emission can be quantified using a luminometer, and the intensity is normalized by the amount of total protein content to correct for variation in cell number. The total protein content can be measured using bicinchoninic acid protein assay (BCA).[92]

ABBREVIATIONS

CHOL	cholesterol
CTAB	acetyl trimethylammonium bromide
DC-CHOL	3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride
DDAB	dimethyldioctadecylammonium bromide
DMRIE	2,3-di(myristyloxy)propyl(2-hydroxyethyl)dimethylammonium bromide
DOGS	dioctadecylamidoglycylspermine
DOPC	dioleoylphosphatidylcholine
DOPE	1,2-dioleoyl-*sn*-glycerophosphatidylethanolamine
DOSPA	1,3-dioleoyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate
DOSPER	1,3-di-oleoyloxy-2-(6-carboxy-spermyl)-propylamid
DOTAP	1,2-dioleoyl-3-trimethylammonium propane
DOTMA	*N*-[1-(2,3-dioleyloxy) propyl]-*N*,*N*,*N*-trimethylammoniumchloride

DPPE	dipalmitoylphosphatidylethanolamine
LHON	6-lauroxyhexyl ornithinate
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]
MTT	1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan, or thiazolyl blue formazan
PC	phosphatidylcholine
TM-TPS	*N*,*N*1,*N*2,*N*3 tetramethyltetrapalmitylspermine

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