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Expert Review

Lipid-based Nanoparticles for Nucleic Acid Delivery

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Abstract. Lipid-based colloidal particles have been extensively studied as systemic gene delivery carriers. The topic that we would like to emphasize is the formulation/assembly of lipid-based nanoparticles (NP) with diameter under 100 nm for delivering nucleic acid *in vivo*. NP are different from cationic lipid–nucleic acid complexes (lipoplexes) and are vesicles composed of lipids and encapsulated nucleic acids with a diameter less than 100 nm. The diameter of the NP is an important attribute to enable NP to overcome the various *in vivo* barriers for systemic gene delivery such as: the blood components, reticuloendothelial system (RES) uptake, tumor access, extracellular matrix components, and intracellular barriers. The major formulation factors that impact the diameter and encapsulation efficiency of DNA-containing NP include the lipid composition, nucleic acid to lipid ratio and formulation method. The particle assembly step is a critical one to make NP suitable for *in vivo* gene delivery. NP are often prepared using a dialysis method either from an aqueous-detergent or aqueous-organic solvent mixture. The resulting particles have diameters about 100 nm and nucleic acid encapsulation ratios are >80%. Additional components can then be added to the particle after it is formed. This ordered assembly strategy enables one to optimize the particle physico-chemical attributes to devise a biocompatible particle with increased gene transfer efficacy *in vivo*. The components included in the sequentially assembled NP include: poly(ethylene glycol) (PEG)-shielding to improve the particle pharmacokinetic behavior, a targeting ligand to facilitate the particle–cell recognition and in some case a bioresponsive lipid or pH-triggered polymer to enhance nucleic acid release and intracellular trafficking. A number of groups have observed that a PEG-shielded NP is a robust and modestly effective system for systemic gene or small interfering RNA (siRNA) delivery.

KEY WORDS: DNA; gene therapy; liposome; oligonucleotide; small interfering RNA.

WHAT DID WE LEARN FROM THE CATIONIC LIPID-BASED GENE DELIVERY SYSTEMS?

Using nucleic acids as drugs has been claimed to be an important future direction of molecular medicine. Non-viral gene therapy or other nucleic acid therapies have been proposed to treat the more serious diseases which require systemic administration for the gene to enter the target cells affected by genetic diseases, viral infections or cancer. To date, the production of effective gene delivery vectors is the bottleneck limiting the success of gene-based drugs in clinical trials. Naked nucleic acids can be delivered locally into specific organs such as muscle or liver by physical methods, such as electroporation (1) or hydrodynamic injection (2). However, these methods are not applicable for systemic gene delivery or are unrealistic for a commercial gene therapy. At the minimum, a synthetic gene vector must be capable of accessing the distal sites of disease following systemic (intravenous) administration (3). Cationic liposomes have been among the more efficient synthetic gene delivery reagents *in vitro* since the landmark publications in the late 1980s (4). Cationic liposomes can condense DNA into a cationic particle when the two components are mixed together. This cationic lipid/DNA complex (lipoplex) can protect DNA from enzymatic degradation and deliver DNA into cells by interacting with the negatively charged cell membrane. Lipoplexes are not an ordered DNA phase surrounded by a lipid bilayer, rather they are a partially condensed DNA complex with an ordered substructure and an irregular morphology (5–7). Since the initial studies, hundreds of cationic lipids have been synthesized as candidates for nonviral gene delivery (3) and a few were used for clinical trials (8,9).

To date, all cationic molecule based systems have failed or been unimpressive in clinical trials due to low gene transfer efficacy and toxicity associated with inflammation and complement activation. Although the origin of toxicity is not fully understood, a combination of unmethylated nucleic acids and the cationic molecules and perhaps the larger diameter of the lipoplexes, play a central role in the induction of the toxicity (10–12).

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The initial distribution of systemically administrated lipoplex is to the lung where pulmonary endothelial cells are transfected. This is proposed to result from the first pass association of the lipoplex with the capillary endothelium of the lung upon the tail-vein injection of the lipoplex (13). Within 10 min after the administration, there is less than 2% of lipoplexes left in the blood circulation system. The critical time period for the DNA being taken up into the pulmonary cells is within 60 min after systemic lipoplex administration (14). In spite of the first pass uptake of lipoplexes in the lung, the liver remains the major elimination organ for the lipoplexes because the lipoplex redistribute from lung to the liver 60 min after injection (15). Although most of the administrated lipoplexes are found in the liver, most of the particles are taken up by the Kupffer cells rather than the hepatocytes. Thus gene transfer by lipoplexes in the hepatocytes is very low.

The cationic surface also mediates strong interactions with plasma proteins upon injection into the blood as well as with the glycocalyx of many tissues (16). These interactions contribute to the rapid elimination of lipoplex (half-life < 5 min) from the circulation by RES (14). The consequences are two fold: inflammatory adverse effects and a decreased access to the target tissue. One lesson from a multitude of *in vivo* distribution studies was the need to overcome rapid elimination from the blood hence the pharmacokinetic behavior of the lipoplexes had to be improved (17).

To overcome the cationic lipid-induced toxicity and extend the *in vivo* circulation time, many groups are encapsulating DNA or other nucleic acid drugs into a PEG-shielded cationic liposomal bilayer using scalable formulation methods (18–23). These PEG-shielded cationic particles differ from traditional lipoplexes by having a bilayer shell around the nucleic acid, smaller particle diameters (< 100 nm) and better stability *in vivo*. These properties enable an extended circulation time *in vivo* of the particles (half-life: 1–10 h) and increase the fraction of injected particles that pass through the target site.

The intention of this review is to summarize the formulation technology for encapsulating nucleic acids into a

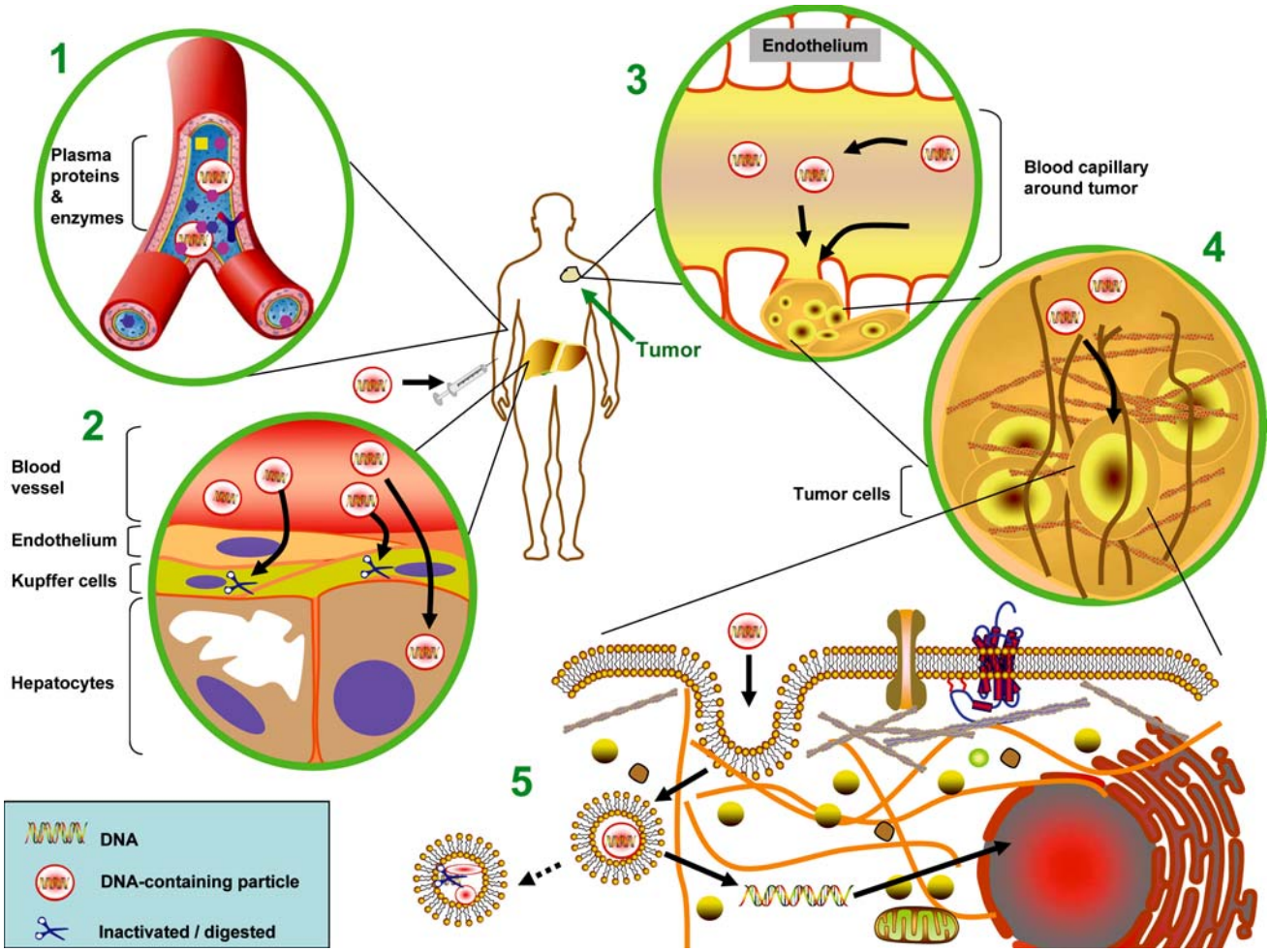

Fig. 1. Illustration of barriers for intravenous gene delivery. DNA-containing NP are injected intravenously into human body. Serum proteins may bind to the particles, crosslink them and increase the particle size. This can result in rapid particle elimination (*Insert 1*). The Kupffer cells (RES) may take up particles, leading to rapid NP elimination from circulation and decrease their access to the hepatocytes (*Insert 2*). Circulating NP may extravasate in tumor tissue through the leaky tumor vessels (the EPR effect) (*Insert 3*). Particles then need to pass through the crowded extracellular matrix to contact the cell surface (*Insert 4*). When the particles are internalized into cells, DNA must escape from the endosome and find its way into the nucleus (*Insert 5*).

nanosized particle for delivery *in vivo*. The nucleic acids include plasmid DNA, siRNA and antisense oligonucleotide (ODN). These nucleic acids have a high linear negative charge density hence the initial formulation parameters can be similar for the various nucleic acids in spite of the large differences in molecular weight among them. However, molecular weight differences among the various nucleic acid drugs may require formulation modifications.

BARRIERS FOR SYSTEMIC GENE DELIVERY

The barriers for gene delivery are illustrated (Fig. 1). Liposomes are the prototype for NP design and are cleared rapidly from the circulation by RES unless the particles have a diameter less than 100 nm and a neutral surface charge or the particle surface is protected by polymers such as PEG (24). If the surface is cationic, such as found in cationic lipoplexes, the surface is reactive toward the high concentration of serum proteins such as serum albumin an anionic protein at physiological pH (Fig. 1 insert 1). This results in clot-like masses in the blood due to the cross-linking and aggregation with proteins. Subsequently these large aggregates are either entrapped in the lung endothelial capillary bed or taken up by RES (Fig. 1 insert 2). For cationic liposomes, serum proteins rapidly interact with the liposome bilayer and alter its stability (25). In addition to the correlation of the total amount of bound protein to the liposome clearance *in vivo*, individual proteins such as opsonins attached to liposomes promote the clearance (25).

Small particles (with diameter less than 500 nm, usually less than 150 nm) have an enhanced permeation and retention (EPR) in inflammation sites and solid tumors because the vasculature and endothelial junctions in those tissues become leaky. This permits small particles to extravasate from the blood stream into the disease site (Fig. 1 insert 3). The EPR effect dramatically decreases if the particle sizes are bigger than 500 nm. To exploit the EPR effect for passive targeting, the particles should have a small diameter, neutral surface charge and remain stable to size growth *in vivo*.

The disease tissue is not solely made of cells. A substantial part of the tissue volume is extracellular space, which is filled by the extracellular matrix (Fig. 1 insert 4) composed of a variety of polysaccharides and proteins forming a network over the surface of cells that produce them (26). The extracellular matrix serves as a scaffold and forms a highly hydrated, gel-like network where the fibroblasts are embedded. This network may permit rapid diffusion of small molecules and proteins between the blood and the tissue cells but not large particles. Here again, the smaller the particle size, the better the particle is able to diffuse through the extracellular matrix to reach the cell surface.

Finally nucleic acids are unable to permeate the mammalian cell membrane because they are highly anionic, hydrophilic macromolecules. Thus, the delivery systems must have the ability to increase the intracellular delivery of nucleic acids. The endocytosis route is generally considered to be the predominant pathway for the uptake of particles into cells (Fig. 1 insert 5). The topics of endocytosis, subsequent endosome escape, and intracellular trafficking into the nucleus have been extensively reviewed by others (8,27,28) and will not be discussed here.

FORMULATION OF NANOSIZED PARTICLE FOR GENE DELIVERY

The requirements for a successful gene vector are copied from virology. A DNA-containing particle should have a diameter less than 100 nm to be suitable for systemic gene therapy. The particle should be stable and resist nonspecific uptake in the circulation, but be quickly de-stabilized to release DNA once it is taken up into the target cells. For commercial purposes the particle must be produced at a large scale, from simple components using robust methods, to be cost effective.

The above contradictory requirements, make vector design and production a challenge. A particle composed of multi-components, each with a documented mechanism, may theoretically fulfill the various functions required for effective delivery; but the properties of such a multi-component particle may depend on how it is assembled. The components must be organized in an appropriate manner for the functions to be manifest at the right time and correct place. The formulation variables to prepare a particle to fulfill these functions include: the nature and number of components, if a cationic condensing agent is used, the cation to nucleic acid phosphate charge ratio (+/-), the concentration of components, the order and rate of mixing of the components, the concentration of detergents or percentage of organic solvent used in the assembly process, ionic strength, temperature of preparation and temperature of assembly.

In viral vectors, DNA is condensed by various mechanisms to save space and to regulate gene function. Without macromolecular condensation, DNA is an extending coil *in vitro*. The free length of 5,000 base-pair linear DNA is about 1.7 μm, with a diameter of 2.2 nm. Most nucleic acid drugs are sensitive to enzyme digestion and condensation protects the nucleic acids from enzyme attack. In an NP, cationic components or multivalent cations are employed to condense DNA so it can be packaged.

COMPONENTS

Usually, the nanosized lipid particle is constructed from a combination of the following components: lipids, PEG-lipids, lipopolymers, pH or reduction-sensitive components and targeting ligands.

Cationic Lipids

A multivalent cation is the essential component to condense negatively charged nucleic acid by charge–charge interaction into a small particle. For most of the NP cationic lipids are the key element used to package the DNA. An added advantage of the cationic lipids is that they can bind to negatively charged mammalian cell membranes and induce the uptake of the associated nucleic acid into cells. This advantage is the reason that cationic lipoplexes are the most

widely used transfection reagent for cell culture. This *in vitro* advantage becomes a detriment for *in vivo* studies because of the previously mentioned adverse effects of cationic lipids: immune reactivity, aggregation with blood components and enhanced uptake by RES. To overcome these effects, one approach is to use titratable cationic lipids that are charged at acidic pH but uncharged at pH 7.4 (29); an alternative approach is to covalently modify a cationic group to convert it to an anionic or neutral group after the particle is formed; and a third approach is to design a disulfide-linked cationic lipid which can be used to encapsulate DNA into small particles and, upon a disulfide exchange reaction, the particle surface charge can be exchanged to charge neutral or negative (30).

Neutral Lipids/Helper Lipids

Neutral lipids such as the fusogenic lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and cholesterol are usually used as helper lipids which may increase transfection activity of the DNA-containing liposomal particles. For example, DOPE has a phosphoethanolamine head group whose size is smaller than its hydrophobic diacyl chain; and DOPE can induce membrane fusion by facilitating the formation of fusion intermediate structures (31). DOPE is widely used in the cationic lipid based gene delivery system to enhance the DNA escape from endosome membrane through the membrane fusion and disruption. In a recent publication, diacyl phosphocholine lipid, a neutral lipid but less fusogenic, has been used as the building block to improve the stability of the DNA-containing NP (32).

Anionic Lipids

Due to the toxicity and rapid clearance of cationic lipoplex *in vivo*, anionic lipidic particles are an alternative to reduce the nonspecific interaction with negatively charged serum proteins such as albumin and the extracellular matrix. In this approach, DNA is usually pre-condensed by cationic polymers such as polyethyleneimine (PEI), protamine sulfate or polylysine (PLL) to form a nanometric cationic core; then the anionic liposomes or lipids are added into the system to form a surface coat and form an anionic or charge–neutral lipid particle (33,34).

PEG-lipids

PEG is a biocompatible and inert polymer which minimizes the interaction between the particle and the cell surface (21). In a gene delivery vector, PEG-lipid substantially decreases the *in vitro* uptake of DNA particles into the cells by reducing the interaction with cell membranes and reduces gene transfer. Nevertheless, PEG-lipid may substantially improve gene transfer and the safety of the delivery system by extending the circulation time, reducing the toxicity and stabilizing the particle *in vivo* (35).

Targeting Ligands

A major problem associated with the PEG stabilized NP is the low uptake of the particles into the target cell. Although NP with a diameter less than 100 nm may passively accumulate into a tumor, they may be unable to enter the tumor cell. Thus an ligand to an internalizing receptor would be expected to improve the binding and uptake of the DNA-containing particles into the cells. Although tremendous efforts are spent on designing suitable ligand to date, ligands have modestly increased *in vivo* gene transfer in the target tissue compared to the unliganded NP.

One well characterized receptor–ligand interaction occurs between peptides containing the RGD motif (arginine–glycine–aspartic acid) and several members of the integrin receptors on cell surface, such as the αvβ3-integrin (36,37). RGD peptide motif promotes the transfection of lipid–protamine–DNA (LPD) complex into cells expressing appropriate integrin receptors such as the MDA–MB-231 cell line (38).

Folic acid is another widely investigated ligand that has improved NP gene delivery *in vitro*. It targets the DNA-containing particles to cancer cells that over-express the folate receptor (39). Folic acid-targeted liposomes deliver ODN or DNA into tumor cells *in vivo*. Importantly, both the targeted and non-targeted liposomes have the same level of uptake into tumor tissue (40) but the targeted liposomes have higher DNA transfection activity than the non-targeted particles. It is thought that the long circulation of NP (diameter < 100 nm) dominates the biodistribution to the target site *in vivo*, and the active targeting increases the particle internalization efficiency and subsequently improves particle uptake into the target cell at the site so the gene is more efficiently delivered (41).

Protein ligands such as antibody (42) and transferrin (16) have also been explored to target DNA-containing lipoplexes for gene delivery with modest levels of successes. Overall, more effective ligands are required for the targeted delivery of DNA-containing NP *in vivo*. Since it appears easier to deliver siRNA into the cytoplasm than to deliver DNA into the nucleus, currently available ligands may prove adequate for delivery of siRNAs.

Lipid–polymer Conjugates

Cationic polymers such as PEI are effective transfection reagents *in vitro*. Modifications of PEI with lipid molecules, such as cholesterol, conjugated onto the polymer backbone to create a water-soluble lipopolymer have been proposed to combine the advantages of both cationic liposomes and polycations; the DNA can be readily packaged and DNA is also able to escape from the endosome into the cytoplasm or perhaps across the plasma membrane into the cytoplasm (43,44).

FORMULATION METHODS

NP are formed in solution; the trick is to keep the components separated, then allow them to self-associate in a controlled manner. This approach relies on kinetic control to permit small particles that consist of cationic lipids, neutral lipids, PEG-lipids and nucleic acid to ultimately form. In the simpler direct mixing approaches, kinetic control is achieved by adjusting the concentration, charge ratio, ionic strength of the solution and rate of mixing of the cationic lipid and

nucleic acid. In the more sophisticated methods, the components are kept from aggregating using either a detergent or an organic solvent–water mixture to disrupt the bilayer structure. This reduces or eliminates the multivalent interaction between the cationic lipid and the nucleic acid. The detergent or the organic solvent is removed by dialysis or filtration and as the concentration of the detergent/solvent decreases the lipids self-assemble with the nucleic acid into an NP (Table I).

Direct Mixing

Direct mixing is used to formulate traditional lipoplexes. The procedure consists of rapidly mixing aqueous suspensions of preformed cationic liposomes with plasmid DNA. Gao and Huang used polycations such as polylysine to precondense the DNA prior to incorporating the DNA into a cationic lipid complex. This resulted in condensed DNA surrounded by a cationic bilayer. An advantage of this protocol is that it minimized the particle diameter (45). When protamines, low molecular weight proteins with a high content of positively charged arginines, are used as the polycations in the formulation, the particle is named a LPD complex. The diameter of LPD particles is about 110 nm (46). This nonviral vector demonstrated high *in vivo* gene transfection activity (47). The idea behind this approach is that some polycations, including protamines, can effectively condense DNA into a highly compact structure, then cationic liposomes may cover the condensed DNA core to form a virus-like vector. Protamines condense sperm DNA to form a compact structure and may play a unique role in the LPD by enabling release of the DNA from the particle inside the cell and thus contribute to the enhanced delivery efficiency reported by the Huang group.

An unusual improvement on the direct mixing method was made by the Pardridge group (48–50). The method employs three steps. The first step is the same as the traditional formulation of lipoplex: the lipid mixture, including neutral lipid (POPC), cationic lipid (didodecyldimethylammonium bromide (DDAB)), PEG-lipid (PEG–DSPE), and trace amount of maleimide–PEG–DSPE, were dried from the organic solvent. The lipids were rehydrated into a buffer and mixed with DNA in the buffer. In the second step, the liposome/DNA dispersion was frozen and thawed ten times and extruded through 400-, 200-, 100-, and 50-nm pore size polycarbonate membranes each for ten times. The final product has a particle diameter <100 nm. The plasmid DNA absorbed to the exterior of the liposomes was digested by DNase. In the third step, a monoclonal antibody (mAb) targeting to transferrin receptor was conjugated onto liposomes through the maleimide–PEG-lipid at 50 copies per liposome. The particle, named a pegylated immunoliposome (PIL), can be further purified by gel filtration. PIL can target plasmid DNA to brain using the mAb ligand, which binds to the transferrin receptor on the blood–brain barrier. Tissue-specific gene expression in brain is observed after the i.v. administration of the PIL that delivered plasmid DNA containing tissue-specific gene promoters. Tissue-specific expression in brain and peripheral organs of different exogenous genes (β-galactosidase and luciferase) is significantly higher than that observed using the non-targeted control liposome at 1–3 days after i.v. injection in adult mice (48). PIL were further used to deliver plasmid DNA encoding anti-sense RNA (49) or siRNA (50) via the intravenous route into a murine brain tumor model.

Detergent Dialysis

The detergent dialysis method (51,52) was initially used for preparing relatively stable cationic lipid–DNA particles by dissolving DNA and cationic lipid mixture in a detergent solution, which is followed by a subsequent dialysis process to remove the detergent. The resulting particles, did not have higher *in vitro* transfection activity than the simply mixed lipoplex but they were stable for a prolonged period and were more active in the presence of serum containing medium (40,41). However this method did not improve the *in vivo* performance. The lessons learned from these experiments are: (a) a detergent solution can be used as an arresting medium for the particle formulation because both DNA and lipids are soluble; and (b) ocytyl glucoside (OG) is a good detergent choice since it has high critical micelle concentration (CMC) and is a non-ionic detergent which would not interfere with the charge–charge interaction between DNA and cationic lipid.

When a PEG-lipid is included in the composition, a particle known as a stabilized plasmid–lipid particle (SPLP) is created using the detergent dialysis method. This method comprises two steps: (a) mixing a plasmid DNA with a cationic lipid, neutral lipid and PEG-lipid in a detergent (OG) solution to provide a complex composed of these components; and (b) removing the detergent from the solution/suspension formed in step a. This results in a mixture of 60 to 100 nm diameter particles with encapsulated DNA, DNA on the surface of the particle, and free DNA. SPLP has substantial advantages for parenteral gene delivery (21,22,53,54). First, the SPLP are water-soluble NP smaller than 100 nm with circulation half-life in a range of 5–15 h in the mouse (35,55). The long circulation time permits a greater fraction of injected SPLP to extravasate through the leaky blood vessels and reach the tumor by the EPR effect. Second, the particles deliver genes *in vivo* at a detectable level into solid tumor. Although the gene expression is low, it is higher than the lipoplex control and had no observed toxicity after single dose administration in mice (54). Recently, Judge *et al.* investigated the antibody response to PEGylated liposomes (56) and sequence-dependent innate immune response to siRNA (10), which raises a concern on the immune response of PEG-lipid-based NP particularly when multiple doses are needed to treat the disease.

In an attempt to improve the gene delivery efficiency, our group has reported the synthesis of an acid-labile PEG–diorthoester-lipid conjugate (POD) (57), and the use of this lipid to create a pH-sensitive SPLP composed of POD/DOPE/DOTAP. This composition was selected because it rapidly disintegrates when the pH was reduced from 7.0 to 5.0 (20), the pH found in the endosome. The acid-sensitive POD SPLP could be prepared with a good DNA encapsulation yield using the 200 mM OG to dissolve the lipids including POD composed of PEG 2,000 and a lipid diacyl chain. The POD SPLP is stable at basic conditions (pH 8.5)

Table I. Examples of Nanosized Systems for Gene Delivery

Particle name[a]	Typical components[b]	Formulation/construction
SPLP (21,22,35,53,54,81,82)	PEG-lipid/cationic lipid/neutral lipid: PEG–ceramide/DODAC/DOPE = 10/6/84	Detergent dialysis (200 mM OG)
LDP (18)	PEG-lipid/cationic lipid/neutral lipid: PEG–DSPE/DODAC/DOPE = 10/50/40	Detergent dialysis (35 mM OG)
SNALP (10,23,56,65–67)	PEG-lipid/cationic lipid /neutral lipid/neutral lipid: PEG–S–DSG/DODMA/cholesterol/DSPC = 10/15/55/20	Ethanol dialysis
Genosphere™ (32,83)	PEG-lipid/Cationic lipid/neutral lipid/neutral lipid: PEG–DSPE/DDAB/POPC/cholesterol = 0.21/6/15/10/ or PEG–DSG/DOTAP/POPC/cholesterol = 0.62/6/15/10	Ethanol dialysis
NLP (19,30,68)	PEG-lipid/cationic lipid/neutral lipid: POD/DOTAP/DOPE = 10/50/40	Detergent dialysis (28 mM OG)
	Ligand–PEG-lipid/PEG-lipid/cationic lipid/neutral lipid: TAT–PEG–DSPE/POD or PEG–DSPE/TCL or DOTAP/DOPE = 0.3/10/50/40 or 0.3/10/30/60	Ethanol dialysis
$(C_{12}CCP)_n$/DNA complex (71–75)	a cationic detergent containing a bicysteine group: $C_{12}CCP$	Direct mixing and spontaneous cross-linking
PIL (48–50)	PEG-lipid/cationic lipid/neutral lipid: PEG–DSPE/DDAB/POPC = 4/2/94	Directly mix lipids with DNA and extrude the mixture through membranes after multiple cycles of freeze-thawing
	In addition, mAb targeting to transferrin receptor was conjugated onto the liposome surface at 50 copies mAb per liposome	Targeting ligand (mAb) was conjugated onto the liposome surface

[a] The abbreviation for the particle name includes: stabilized plasmid–lipid particle (*SPLP*), lipid–DNA particle (*LDP*), stable nucleic-acid–lipid particle (*SNALP*), nanolipoparticle (*NLP*), and pegylated immunoliposome (*PIL*).
[b] The indicated ratios are molar ratio between molecules.

but rapidly collapses at low pH and exhibits better *in vitro* transfection activity than does a pH-insensitive SPLP (20). The particle diameter was influenced by the molecular weight of the PEG on the lipid (21,22); and, when either PEG 750 or PEG 5,000 was used, small particles were not produced (19).

To overcome this problem, we developed a low concentration OG dialysis method (18) to prepare the DNA-containing lipid particles and designated them nanolipoparticles (NLP) (19). In this method the concentration of OG (28 mM) used to solubilize the lipids and DNA is substantially lower than that used in the earlier reports (21,22) and only slightly higher than the CMC of OG, 23 mM (58). In the modified dialysis condition, the detergent is reduced below a critical value where the components are able to form small particles upon detergent removal before they can form large aggregates (Fig. 2 and Table II). The formation of the NLP is under kinetic control and it appears that there is a particle intermediate that is formed in the presence of detergent (19). The low detergent concentration permits the entrapment of DNA into a small diameter NLP from the preformed particle as the detergent is removed (Fig. 2). Since the detergent concentration only has to decrease from 28 mM to 23 mM, the NLP do not have a chance to form large aggregates before the PEG coating is able to shield the particle surface and inhibit particle–particle interaction. The low detergent method uses 75% less total lipids for DNA encapsulation yet results in an increased DNA encapsulation as quantified using a DNase protection assay from 40–50% in the high detergent dialysis method to 80–100% in the NLP (Table II).

One tactic to improve the biocompatibility of the cationic NLP is to remove the cationic charge on the lipid after the particle is formed. We used a disulfide-containing cationic cholesterol derivative to generate an NLP with detergent dialysis (30). Afterwards, the positive surface charge of the NLP can be selectively converted to a neutral or negative charge by the treatment with an appropriate reducing reagent. It should be noted that this process may not be applicable to the traditional lipoplex formulated with the disulfide-containing cationic lipid because the lipoplex has an unordered and discontinuous lipid/DNA structure and is more permeable to the hydrophilic reducing reagents. Access of the reducing reagent to all of the lipids can rapidly disintegrate the particles. Subsequently, a targeting ligand which usually is linked to a PEG-lipid can be incorporated onto the surface to enhance the particle–cell recognition, and substantially increase the transfection activity of NLP. This strategy, called sequential assembly, permits one to tailor the

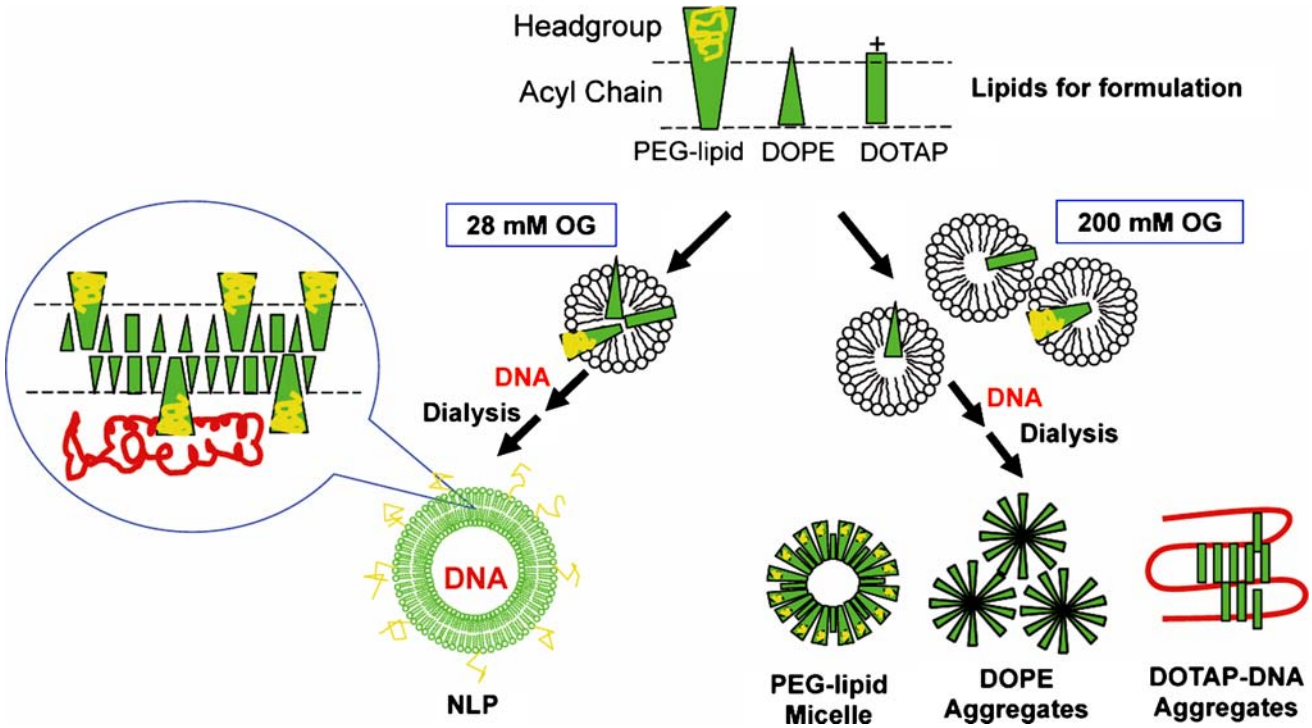

Fig. 2. Proposed model for the formation of NLP from detergent suspension (19). *Left side*: Lipids (PEG-lipid (POD), DOPE and DOTAP) were dispersed into a buffer of 28 mM OG as mixed micelles. When DNA was added into the system, the cationic micelles interacted with the DNA template and spontaneously formed a DNA–lipid intermediate (19). Upon detergent removal, the bilayer became continuous and stable NLP (particle diameter~100 nm) were formed. The 28 mM OG concentration is slightly higher than the Critical Micelle Concentration of OG (23 mM). The inserted figure on the left is used to show the lipid arrangement on the membrane of NLP. *Right side*: When the OG was raised to >36 mM, such as 200 mM, the DNA particles precipitated (particle diameter > 1 μm) as the detergent was removed. Thus, there is a critical window of OG concentration which permits the formulation of small diameter NLP at the certain lipid compositions and lipid concentrations. The NLP formation is under kinetic control and the critical low detergent concentration permits entrapment of DNA into small diameter NLP. With the dialysis conditions employed, the detergent is reduced below the critical value for component rearrangement before the particles can rearrange into PEG-micelles and larger aggregates (DOPE hexagonal phase or DNA/DOTAP aggregates). More details were described in Li *et al.* (19).

Table II. Formulation of DNA-encapsulated PEG–liposomal NP at Different Detergent Concentration: A Case Study

Properties	200 mM OG dialysis (Choi *et al.* (20))	28 mM OG dialysis (Li *et al.* (19))
Lipid mole composition [a]	PEG-lipid/DOTAP/DOPE = 20/12/68	PEG-lipid/DOTAP/DOPE = 10/50/40
Charge mole ratio [b]	2/1	2/1
Size (particle diameter)	~60 nm	80–150 nm
DNA encapsulation ratio	30–50%	90–100%
Purification recovery (anion-exchange column)	~20%	~50%
Surface potential	Neutral	Neutral
Transfection activity	Medium	High
pH-sensitive collapse (84)	~100 min (pH 5)	~10 min (pH 5)
Applicable	PEG2000 lipid	PEG2000 lipid PEG750 lipid PEG5000 lipid

[a] Lipid mole composition is the lipid mole ratio at which ratio the lipids are mixed before dialysis. Lipid ratio was optimized for each dialysis method of different OG concentration based on the resulting particle size and DNA encapsulation ratio. The lipid mixture in 200 mM OG can form precipitates after dialysis if its composition is identical to that in 28 mM OG.
[b] Charge mole ratio is the mole ratio between cationic lipid/DNA phosphate at which ratio the lipid and DNA are mixed together for dialysis.

surface charge, recognition elements and other surface characteristics of the nanoparticle with retention of DNA inside the particle and holds forth the possibility of increased gene transfer activity. The initial disulfide exchange provides a pathway to minimize the nonspecific charge–charge interaction of the gene carrier with negatively charged plasma protein or with anionic cell surfaces (30).

Ethanol Dialysis

Although the detergent dialysis method shows considerable potential for parenteral gene delivery, it has limitations. The method is very sensitive to: the ionic strength of the formulation buffer, the temperature of dialysis and the detergent concentration that, if not optimized, will result in the formation of particles with much larger particle size or decreased DNA encapsulation. In addition, the high cost of OG and other detergents limits the scale-up potential of this method. Ethanol has been used to form small unilamellar liposome (diameter about 60 nm) by injecting the ethanolic lipid mixture into water (59). Cationic liposomes formed via ethanol injection exhibit a similar transfection activity as liposomes obtained by standard evaporation/sonication method when mixed with DNA to form lipoplexes (60). Ethanol is also a solvent that can condense DNA into compact structures such as toroids, rods or fibers in the presence of a critical concentration of multivalent cations (61,62).

Bailey and Sullivan described a method for trapping plasmid DNA inside unilamellar liposome as a particle they termed neutral lipid complexes (NLCs) with average diameter less than 200 nm and DNA encapsulation yield up to 80% (63). The entrapment is induced by the addition of calcium and ethanol into the DNA and a neutral liposome (such as DOPC or DOPC/DOPE liposome) mixture in buffer, which is followed by dialysis to remove the ethanol and calcium. No cationic lipid is used in this method (63). The *in vivo* behavior of NLCs is similar to the liposomes of the same composition and size without DNA. About one-third of the injected dose remained in circulation at 1 h following intravenous injection; and the serum level of NLCs is much greater than found using the traditional cationic lipoplex (63).

Maurer *et al.* investigated the effect of ethanol and the presence of PEG-lipid on the interaction of ODN with cationic PEG-liposome (64). In their study, the PEG-liposomes composed of PEG-lipid/cationic lipid/neutral lipid were first dissolved in 25–40% (v/v) of ethanol before or after the 100-nm membrane extrusion. Then the ODN solution was slowly added to the ethanolic liposome suspension while it was mixed on a vortex mixer. The ethanol was removed by a dialysis protocol. This procedure produces ODN-entrapped particles with the diameter from 90 to 130 nm and the ODN encapsulation yield 5–90% depending on the initial ODN/lipid ratio and ethanol concentration. One conclusion from this work is that the incorporation of PEG-lipid as high as 10 mol% does not interfere with ODN binding to the cationic lipid domain in presence of ethanol and, in turn, the PEG-lipid provides a stabilized environment to minimize the particle size after removing ethanol.

Recently Jeffs *et al.* (23) reported a scalable process for the DNA encapsulation based on modifications to the ethanol dialysis method used for the ODN encapsulation (64). The lipid composition is 10/15/75 (PEG-lipid/cationic lipid/neutral lipids; molar ratio). In this procedure, the lipid solution in 90% ethanol in one reservoir and plasmid DNA in buffer in a separate reservoir are combined using a mixing tee for scale-up purpose. The lipid intermediate suspension in 45% ethanol has approximately 60% DNA encapsulation. The liposomal suspension is further diluted with buffer using a mixing tee to 22.5% ethanol. This step was used to create particles that significantly increased the DNA encapsulation from 60 to 80–90% (23). The particles are further purified to remove free DNA and diafiltered to remove ethanol. The particle size is in a controllable range from 100 to 150 nm. This method is used to prepare a 225 mg DNA batch of particles from start (dissolution lipids) to finish (sterile filtration) in 1 day; the authors claimed that the process is faster for the preparation of particles than the detergent dialysis method (23). The particles were renamed as stable nucleic-acid–lipid particles (SNALP) (65).

The ethanol removal method has been used by the scientists at Protiva Biotherapeutics to formulate SNALP containing an siRNA anti-hepatitis B virus (HBV) sequence (65) or a serum cholesterol-lowering siRNA sequence (66).

Recently this group reported the systemic efficacy of siRNA in primates (67). The apolipoprotein B (ApoB)-specific siRNAs, when delivered systemically in the SNALP formulation, knocked down expression of ApoB in monkeys. A single intravenous injection of siRNA resulted in dose-dependent reduction of ApoB messenger RNA expression in the liver 48 h after administration. Significant lowering in serum cholesterol and low-density lipoprotein levels were observed as early as 24 h after treatment and persisted for 11 days. These results strongly support that NP can effectively deliver therapeutic amounts of siRNA (67). This group of scientists also investigated the immunogenicity and safety of SNALP using either DNA (56) or siRNA (10,66) as the payload. They observed that the *in vivo* efficacy was reduced and adverse events of SNALP were increased following repeat administration. This was attributed to the induction of an antibody against PEG that results from the strong adjuvant effect of the plasmid DNA–cationic lipid components in the SNALP (56). The good news is that the immune response and toxicity of siRNA are abrogated by chemical modification of the nucleosides to avoid the use of immunostimulatory motifs (10,66) or predosing patients with dexamethasone.

Similar formulations to SNALP have been described by Hayes *et al.* (32) and Li *et al.* (68). These two reports describe the solublization of the lipid and DNA in separate ethanolic solutions prior to mixing. The two ethanolic solutions are mixed and, upon removal of ethanol by dialysis, homogenous and stable nucleic acid–lipid NP are formed. The DNA is well protected inside the particles. These particles, named as Genosphere™ by Hayes *et al.* (32), can be stably stored under a variety of conditions including a lyophilized state where no significant increase in particle size or DNA degradation by DNase was observed following reconstitution in water. Low-pH sensitive PEG-lipid and disulfide-containing cationic lipid can be incorporated in the formulation which produces a DNA-encapsulated bioresponsive NLP with smaller size (68) than the NLP formulated using the detergent dialysis method (30).

Other Organic Solvents

Organic solvents other than ethanol have also been employed for the encapsulation of DNA. Murphy *et al.* combined both the surfactant (cholate) and organic solvent (dimethylformamide) in the formulation of a DNA-containing liposome (69,70). In this procedure, plasmid DNA and a cationic peptide–lipid conjugate in dimethylformamide and anionic cholate micelle are mixed at an appropriate ratio to form a stable and condensed DNA–micelle complex with diameter between 30 and 60 nm. Then neutral liposomes in dimethylformamide were added to the mixture, followed by dialysis to remove the detergent and organic solvent. This resulted in the formation of 80 nm liposome particles with DNA condensed inside (69).

Template Directed Assembly

Cationic detergent usually are not considered as a useful DNA condensation vector because either (a) it dissociates *in vivo* if the CMC of the detergent is too high or (b) it forms large aggregates with DNA if the CMC is too low. However, several groups have introduced a template directed assembly that assembles particles directly on the DNA template using cationic detergents that can dimerize as lipids on the DNA via disulfide formation (71–75). To adjust the stability of the detergent micelle, a biscysteine detergent was synthesized and used to form a soluble micelle complex with DNA, and then the monomer detergents covering DNA will react with a neighbor to form multi-cationic multi-acyl lipids linked by disulfide bond (71). This detergent-to-lipid transformation can increase the stability of the DNA complex but avoid large aggregates in certain conditions. This method leads to a formation of 30 nm diameter neutral particles when formulated at equal molar ratio between the DNA phosphate group and cationic group on the detergent. Theoretically each single particle only encapsulated monomolecular DNA

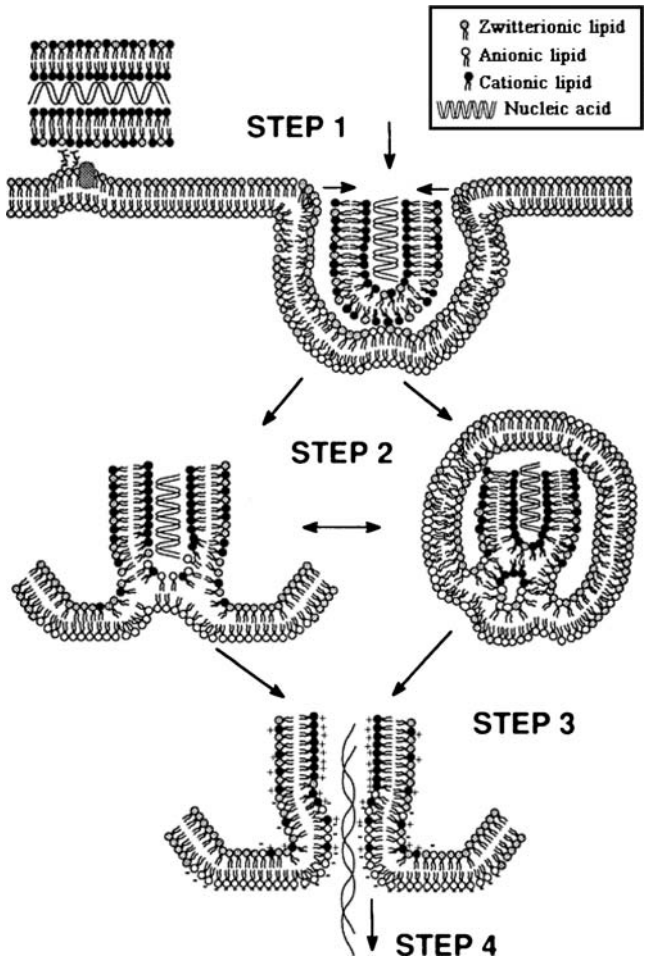

Fig. 3. Mechanism of uptake and release of nucleic acid (such as plasmid DNA and siRNA) from the nucleic acid/lipid particles. After electrostatic interaction with the cell membrane, nucleic acid/lipid particles are endocytosed (*Step 1*). In the early endosome, membrane destabilization results in anionic phospholipid flip-flop (*Step 2*). The anionic lipids diffuse into the particle lipids and form a charge neutral ion pair with cationic lipids (*Step 3*). The nucleic acid dissociates from the complex (particle) and is released into the cytoplasm (*Step 4*). Reproduced with permission by American Chemical Society, from (78).

(71). These methods have not been shown to have *in vivo* activity following a low volume intravenous injection.

Molded Fabrication

Lithography is normally used in the design of inorganic nanomaterials which are more resistant to extreme processing techniques (high temperature or ion etching etc.) derived from the microelectronics fabrication. Rolland *et al.* developed a particle fabrication technique, called as "Particle Replication in Nonwetting Templates (PRINT)," to fabricate various polymer particles for protein or ODN encapsulation (76). This technique uses the fluorinated polymers to make a fabrication mold that is nonwetting to organic liquid. The non-wetting property enables the isolation of the monomer/polymer liquid precursor inside the features of the mold. This imparts a precise shape and composition control to the particles but without harsh processing conditions (76). The diameter/length of the particles range from about 100 nm to 5 μm. This process has been used to encapsulate ODN with polymers, and presumably DNA could also be encapsulated in these particles. A major advantage of this molded fabrication is that it appears to be general and versatile regardless of the chemical properties of the particle materials. It traps the shape and dimension of the particles in a mold so that they cannot aggregate, at least during the formation stage. A challenge for this procedure is to maintain the DNA suspended within the particle-forming materials when the mold is filled. We expect "forced assembly" methods could be used to fabricate DNA or ODN encapsulated lipidic NP for gene delivery in the near future.

PERSPECTIVES

This review of recent progresses on the methods to formulate lipidic NP to encapsulate nucleic acid therapeutics has revealed that steady progress is being made in the development of methods to prepare small diameter lipid particles that stably encapsulate nucleic acids. These formulation methods have been applied with substantial success for the *in vivo* delivery of siRNA. Clearly the development of improved non-cationic NP for siRNA delivery should be the focus of research due to the promising therapeutic activity of siRNA in various animal disease models.

The delivery of siRNA may progress faster than DNA delivery. The question that will be asked is why it is easier to deliver small ODN such as siRNA than it is to deliver plasmid DNA. In our previous studies (77–79), we proposed that after the nucleic acid/cationic lipid complex (or particle) is internalized by endocytosis it destabilizes the endosomal membrane (Fig. 3). This destabilization induces flip-flop of anionic lipids from the cytoplasmic facing monolayer, which laterally diffuse into the complex (or particle) and form a charge neutral ion-pair with the cationic lipids. This process results in the displacement of the nucleic acid from the cationic lipid and subsequent release of the nucleic acid into cytoplasm of the cells (77–79). Following this theory, the answer becomes simple—that you only have to break, for example, 40 ionic interactions to release a 20 mer siRNA from the association with cationic lipids in the lipid-based delivery system. Whereas to release one 4,000 base pair plasmid DNA, you have to release the major fraction of 8,000 ionic pairs. For instance, if you broke 4,000 ion pairs, the plasmid would still be retained on the cationic lipid of the delivery system; but if you broke 4,000 ionic pairs in an siRNA-encapsulated cationic lipid particle, you would release 100 siRNA molecules (Fig. 3). Thus one might observe an siRNA effect from an siRNA-encapsulated lipid delivery system but not induced transfection from a plasmid DNA delivery system under similar release conditions. Furthermore, when siRNA is released into the cytoplasm, siRNA has lower molecular weight than plasmid DNA, which permits it a faster diffusion in the crowded cytosol (80). The target of siRNA is located in the cytosol, rather than the cell nucleus, so a nuclear barrier does not exist for siRNA delivery. In addition, the techniques for chemically manipulating the synthetic siRNA molecules are more developed than plasmid DNA; thus siRNA backbone can be modified to be more stable and resistant to RNase, which reduces the loss of activity in the delivery pathway.

NP are not yet an efficient delivery vector for DNA. A significant unanswered question is how can the multiple components be engineered to work synergically in the particle. These components may work quite well in their individual model systems, but often do not work well together. There is ample work for multiple laboratories both in the preparation of superior components and in the development of better ways of assembling them.

There is also a need for industrial groups to develop methods to stabilize the formulations and robust analytical methods to characterize formulations during formation and storage. Without the pharmaceutical industry to participate in nucleic acid drug development, the dream for gene related therapy will not come true.

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