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Drug Delivery Trends in Clinical Trails and Translational Medicine: challenges and opportunities in the delivery of nucleic acid-based therapeutics

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The development of nucleic acid-based therapeutics has garnered tremendous interest in the past two decades as a new category of biologics. The ability to deliver nucleic acids (e.g., plasmid DNA, antisense oligonucleotides, siRNA) offers the potential to develop potent vaccines and novel therapeutics to cure many diseases that are difficult to treat effectively with traditional therapies, e.g., hereditary diseases, cancer^{1–4}. However, the site of action of nucleic acids requires that these molecules be delivered to the interior of the target cell, which greatly complicates delivery strategies and compromises efficiency. The poor transfection efficiencies of synthetic delivery systems (nonviral vectors, i.e., complexes of polynucleotides with cationic lipids and/or polymers) has forced the majority of clinical trials to employ viral vectors despite the significant safety concerns associated with their immunogenicity and insertional mutagenesis^{5,6}. Although nonviral vectors proficiently transfect cells in culture, it must be appreciated that most cell culture experiments employ rapidly-dividing cells cultured in monolayers that do not accurately mimic the *in vivo* situation. Under these conditions, precipitation can enhance the association of the delivery system with the cell surface which can artifactually elevate transfection rates with delivery systems that are not stable in physiological media. Conversely, delivery systems that are stable in physiological media often do not transfect efficiently in cell culture, leading to the mistaken conclusion that such systems are not worthy of further consideration. Another confounding factor with cell culture experiments is that the nuclear membrane breaks down during cell division, allowing efficient translocation of DNA into the nucleus of rapidly dividing cells that greatly facilitates transfection. These differences between cell culture and *in vivo* gene delivery misled several companies who employed combinatorial chemistry to create tens of thousands of novel delivery agents in the 1990s, only to screen for efficacy in cell culture. Unfortunately, these massive efforts yielded minimal insight into the structure-function relationship of delivery agents that is relevant to the *in vivo* situation. Similar approaches have recently been employed to develop delivery agents for siRNA, and some of the identified “lipidoids” have shown promise in nonhuman primates^{7,8}. Currently, delivery efficiencies of synthetic vectors in the clinic are too low to obtain therapeutic levels of gene expression. As of December 2009, there have been 1579 gene therapy clinical trials worldwide, of which about 25% have utilized nonviral vectors⁹. It should be noted that viral vectors still dominate clinical gene therapy, with the first approved gene therapy product, Gendicine, being the Recombinant Human Ad-p53¹⁰. Besides the approved product in China, some of the viral vector based therapeutics have progressed to phase III clinical trials^{11–13}, such as Rexin-G, a tumor-targeted retrovirus bearing a cytotoxic cyclin G1 construct and ONYX-015, an oncolytic adenovirus. While viruses offer greater efficiency of gene delivery, it is generally agreed that synthetic vectors would be preferable due to safety

concerns, and viral vectors may be more suited for *ex vivo* applications¹⁴. It is clear that the strategies employed by these two classes of vectors appear to be converging¹⁵, but the challenges associated with intracellular delivery are significant. In this review, some key issues in the field of nucleic acid delivery will be addressed for consideration in the development of improved synthetic vectors. Considering that cationic liposomes are the best studied of the nonviral delivery systems, much of the discussion will review findings with these systems, but these issues are also relevant to polymeric systems.

DNA vs. siRNA

Cationic liposomes and polymers are widely used as nonviral vectors both *in vitro* and *in vivo*. Both plasmid DNA and siRNA bind to cationic liposomes and polymers via electrostatic interaction between the anionic phosphodiester backbones and the positively charged group in cationic delivery agents. Indeed, it was recognized by Papahadjopoulos and colleagues^{16–18} that encapsulation efficiency of nucleic acids within traditional anionic liposomes was low, and thus cationic lipids were synthesized in an attempt to improve loading efficiency and promote cellular uptake. The application of this idea by Felgner¹⁹ in 1987 stimulated immense interest in developing nonviral vectors for therapeutic use. At the time of this landmark paper, the mechanism of RNA interference had yet to be discovered, but cationic agents that bind DNA would be expected to interact with RNA via similar electrostatic interactions with the phosphate backbone^{20,21}. However, it is important to recognize the significant structural differences between plasmid DNA and siRNA. The most obvious difference is that plasmid DNA is typically 5000 base pairs or larger whereas siRNA is typically 20–25 base pairs; a 200-fold difference in molecular weight. Work from our laboratory has shown that the affinity of cations for short deoxy-oligonucleotides can vary substantially by sequence, and is significantly different from that to plasmid DNA²². Furthermore, the interaction of plasmids with some multivalent cationic agents causes a remarkable reduction in molecular volume classically referred to as “condensation”^{23–25}. This event is characterized by a molecular collapse that protects the DNA from nucleases and chemical degradation, but true condensation is limited to polynucleotides greater than approximately 400 bases²⁶. DNA condensation has been exploited by Copernicus Therapeutics to collapse single molecules of plasmid DNA into very small structures that facilitate translocation across cellular barriers^{27,28}. Unfortunately, the term “condensation” is frequently misused in the delivery literature to describe the electrostatic association of nucleic acids with a delivery vehicle regardless of polynucleotide size, valency of the cation, or ability of the delivery system to affect molecular collapse. Although the complexation of poly- and oligo-nucleotides with cationic delivery vehicles typically results in nuclease resistance, condensation is rarely achieved.

In addition to molecular size, it is important to realize that DNA assumes a B conformation in physiological solutions in contrast to RNA which assumes a less-hydrated, more compact A conformation characterized by a relatively narrow major groove and shallow minor groove. It follows that these structural differences that alter the spacing of phosphates in the backbone might also affect the interaction of cations with siRNA. This idea is consistent with experiments showing a dramatically reduced binding stoichiometry of cations for ribo- versus deoxyribo- oligonucleotides (20 base pairs), which may be relevant to incorporation into cationic delivery systems²⁹. Furthermore, a reduced binding of cationic agents to RNA could potentially explain why much higher levels of cationic agents (i.e., +/- ratio) are generally needed to deliver RNA as compared to DNA³⁰. Reduced interactions of RNA with cationic agents could also play a critical role in the release of siRNA from the delivery vehicle into the cytoplasm, which would influence the ultimate biological effect.

Aside from the physicochemical differences between DNA and RNA, it should be noted that plasmid DNA needs to be transported into the nucleus for gene expression, which requires crossing two biological barriers, i.e. the plasma/endosomal and nuclear membranes. The endosomal release of DNA and transport into the nucleus pose additional challenges for delivery efficiency. In contrast, siRNA only needs to be transferred across the plasma membrane in order to accomplish silencing in the cytoplasm. This may explain why siRNA-induced silencing does not require endocytosis, but is thought to be mediated by direct fusion with the plasma membrane³¹. Not surprisingly, it has been suggested that an optimized siRNA formulation procedure is quite different from the pDNA formulation, and that parameters such as toxicity and efficacy can be controlled by the development of specific protocols for lipid/siRNA complex formation³². Similar results were reported in a study by Malek *et al.* using PEGylated polyethylenimine (PEI)³³. It was shown that siRNA-mediated gene silencing is much less dependent on the N/P (PEI nitrogen/nucleic acid phosphate) ratio than is DNA transfection. These studies, in addition to the physicochemical differences noted above, strongly suggest that delivery agents and protocols need to be optimized for siRNA delivery. To date, the delivery of siRNA has predominantly utilized cationic agents that were developed for gene delivery, suggesting that delivery reagents should be specifically developed for siRNA delivery. Although novel vehicles tailored to siRNA may lead to more efficient delivery, all systems must overcome many common problems that are discussed below.

Serum stability and PEGylation

While there are some applications for which local administration might suffice (e.g., non-resectable lesions, vaccine inoculation), most studies to date have focused on systemic delivery via intravenous injection. One major problem associated with the use of cationic delivery vehicles is their strong interaction with blood components, which can dramatically lower the transfection efficiency^{34–40}. Serum has been reported to exert its inhibitory effect by binding serum proteins to charged particles, which leads to structural reorganization, aggregation and/or dissociation of the delivery vehicle^{37,41–43}. The presence of nuclease in the serum can also degrade nucleic acids, resulting in a loss of supercoil content and biological activity⁴⁴. Many early studies on gene delivery focused on developing tight associations of the delivery vehicle with DNA such that nuclease resistance was imparted. However, such studies ignored the fact that these delivery systems needed to be capable of disassembly within the cell; an issue that continues to receive insufficient attention. In fact, the difficulty of extracting DNA from pure cationic lipid formulations suggests that the neutral helper lipid contributes significantly in dissociation/release. This physical effect of the helper lipid is generally not appreciated, and the role of helper lipids is typically thought to be limited to promoting hexagonal phase formation to enable endosomal escape⁴⁵. By promoting fusion with the endosomal membrane, the helper lipids also facilitate mixing with anionic lipids that allows DNA to be released from the delivery vehicle within cells⁴⁶.

In the case of oligonucleotides (e.g., siRNA, antisense, aptamers) that are produced by chemical synthesis, modifications (e.g., phosphorothioates, amides, 2'-O-methyl, locked nucleic acids) can be introduced that greatly enhance nuclease resistance⁴⁷. Because plasmid DNA is produced in bacteria, incorporation of modified bases is not possible. Although most studies on stability in blood have focused on serum proteins, it should also be recognized that delivery vehicles can bind to erythrocytes, a major constituent of blood cells, both *in vitro* and *in vivo*⁴⁸. Such interactions can result in association/fusion of the delivery vehicle with erythrocytes, which reduces transfection efficiency and accelerates removal of the delivery vehicle from the circulation via liver and spleen. In this regard, studies have shown that the helper lipid DOPE promotes fusion with erythrocytes to a much greater extent than

cholesterol, and thus cholesterol is more commonly used as a helper lipid for *in vivo* experiments⁴⁸.

The adverse effects by blood components can be circumvented in cell culture by replacing serum-containing media with serum-free media. Unfortunately, this has become a standard practice in nucleic acid delivery studies, and this media alteration is sometimes not even mentioned in the methods section of publications. Partially because of the interactions with serum proteins mentioned above, the use of serum-free media yields misleading results with regard to the transfection efficiencies that can be expected *in vivo*. Some researchers describe transfection rates “in serum”, but these studies typically employ media fortified with only 10% fetal bovine serum as is typically used in cell culture. Studies have clearly documented that stability and delivery efficiency are progressively compromised at increasing levels of serum, although the detrimental effects of serum are less dramatic as the serum content is increased above 50%^{38,49}. Considering that approximately half of the blood volume is serum, 50% (v/v) serum is often referred to as “full-strength serum”. Similarly, delivery vehicles are often diluted with an equal volume of serum to achieve 50% (v/v) serum, and this is often described as “exposed to 100% serum”. Because the non-serum fraction of blood is occupied by cells whose volume is not available for dilution, intravenous administration exposes delivery vehicles to 100% (v/v) serum. In conducting serum stability studies, the volume in which the delivery vehicle is suspended should be considered when reporting the serum concentration to which vectors have been exposed. For practical reasons, it is difficult to perform extensive transfection and biophysical characterization studies in > 50% (v/v) serum, i.e., a 1:1 dilution of the delivery vehicle in serum. However, as stated above, results from such experiments can provide a more realistic estimate of *in vivo* performance than the serum-free conditions that are typically employed.

In order to overcome the adverse effects of serum protein binding, there is an intense effort to develop lipid and polymer based systems that efficiently transfer nucleic acids in the presence of serum. Among the pursuits toward this goal, the predominant strategy is to utilize PEGylated components to sterically shield the delivery vehicles from blood components^{50–52}. This strategy has been utilized for decades in the development of liposome-based formulations and has been shown to allow the accumulation of liposomes containing small molecule drugs in tumors⁵³. It is well documented that the steric stabilization provided by PEGylation reduces particle aggregation in serum and extends circulation lifetime. It is typically assumed that the extended circulation lifetime can be attributed to the reduction or prevention of serum protein adsorption. However, there is little evidence that the presence of PEG at the surface of a vehicle actually reduces serum protein binding. Although some studies suggested that PEG on liposome surfaces can reduce specific protein interactions^{54,55}, others have indicated that the clearance of liposomal formulations is not correlated with plasma protein binding to liposomes⁵⁶. In a recent study by Dos Santos *et al.*⁵⁷ it was clearly demonstrated that the adsorption of total and specific serum proteins were not significantly affected by the presence of either PEGylated lipids or cholesterol, in spite of significant differences in their circulation lifetimes. Interestingly, liposomal formulations containing saturated phosphatidylcholine with adsorbed proteins had long circulation lifetimes. These results show that the increase of circulation lifetime cannot be directly associated with either PEGylation or protein binding, despite the observation that PEG-containing formulations typically have longer circulation lifetimes than liposomes lacking PEG. In fact, it has been suggested that the binding of proteins to PEGylated liposomes may benefit the prolonged circulation time of the vectors to some extent. Other studies have shown that PEG on the surface can result in a “dysopsonization” effect by which binding of proteins to a PEG-coated surface could reduce uptake by target cells^{58–60}.

Regardless of the role of protein adsorption, PEGylation has a clear benefit to circulation time after intravenous administration, and thus it is commonly used for delivery studies in animal models. However, PEGylation is known to interfere with trafficking involved in intracellular delivery, and render vectors more susceptible to agitation-induced damage during processing^{52,61–65}. Other studies have reported that PEGylation significantly lowers the cellular interaction and uptake of the lipid nanoparticles, resulting in reduced biological activity^{66,67}. Similarly, Remaut *et al.*⁶⁸ observed that PEGylation lowers the transfection efficiency of DOTAP/DOPE liposomes carrying nuclease stable phosphothioate ONs (PS-ONs) or nuclease sensitive phosphodiester ONs (PO-ONs). Furthermore, the authors concluded that the PEG chains inhibited the endosomal escape of the degraded ONs, which consequently caused a dramatic decrease in transfection efficiency. To overcome these shortcomings of PEGylation, strategies such as using PEG-chains that are removable in the endosomal compartment have been adopted in order to allow endosomal escape. It is known that the extent of PEGylation and the rates at which particles lose PEG shielding have significant effects on formulation pharmacokinetics⁶⁹. One of the approaches is to adopt a pH-sensitive linker between the PEG moiety and lipid. The PEG chains are then removed in the endosomal compartment due to acid-catalyzed hydrolysis^{70,71}. Although this strategy could potentially be used to trigger PEG release and cell uptake in hypoxic tumors, the pH of such environments is not sufficiently low (pH $\approx$ 6.5) to efficiently trigger hydrolysis of the PEG chains. A more promising tactic utilizes the matrix metalloproteinase that is present at high levels in the extracellular environment of the tumor to release PEG from the vector⁷². Another approach is to exchange PEG-lipid by modulating the hydrophobicity of the PEG-lipid conjugate. This can be achieved by varying the length of the alkyl chain of the lipid anchor⁷³ because the lipid portion of the conjugate determines its affinity for the lipid delivery vehicle. This approach has been described as “programmed release”, but there is no distinct trigger to release the PEG-lipid conjugate at the target site.

It is worth pointing out that PEGylation of soluble biomolecules (e.g., peptides, small proteins, ribozymes, siRNA, antisense) differs fundamentally from the complications encountered by particulate formulations described above. While PEGylation is used to extend circulation times in both cases, the conjugation of PEG to soluble molecules is designed to increase their molecular weight above the threshold for glomerular filtration and urinary excretion. Therefore, it is critical that discussions of the merits of PEGylation distinguish between these two distinct mechanisms of extending circulation times.

Encapsulation of nucleic acids for efficient delivery

The landmark paper by Felgner *et al.*¹⁹ in 1987 demonstrated the ability of cationic agents to bind DNA, and electrostatic interactions continue to be exploited to associate nucleic acids with current delivery systems. It should be recognized that cationic agents also facilitate interactions with anionic moieties on the cell surface (e.g., proteins), and the ability of cationic lipids to target chemotherapeutics to tumor vasculature is being developed by MediGene as a commercial product^{74–76}. In addition to creating a positive surface charge that promotes cell and serum protein binding, cationic components are typically toxic even when biodegradable linkages are employed. It follows that the ideal gene delivery vector would not rely on cationic components to foster an interaction with the nucleic acid. Such an approach would not only avoid the problems associated with the toxicity and protein binding, but would also facilitate the release of the nucleic acid from the delivery vehicle within the cell. In order to achieve this goal, encapsulation of the payload (i.e., nucleic acids) inside the delivery vehicle is a promising strategy which would also prevent access of nucleases to the encapsulated cargo (assuming that the delivery vehicle remains intact during delivery to the target site). If one reads the literature, it is easy to conclude that “encapsulation” is routinely achieved with virtually all delivery systems. This becomes

somewhat of a semantic argument regarding the definition of “encapsulated”, and this term is now commonly used to refer to any cargo (e.g., DNA or RNA) that cannot be degraded by nucleases or separated from the delivery vehicle by washing. In this sense, nucleic acids that are associated with the delivery vehicle by whatever means (e.g., surface adsorption, electrostatic and/or hydrophobic interactions) are described as “encapsulated”, and there is no distinction made among “encapsulated”, “associated”, and “complexed”. The Merriam-Webster dictionary defines “encapsulate” as “to enclose in or as if in a capsule”, which clearly implies the lack of an interaction with the encapsulating material. This definition is consistent with the traditional use of the term in liposomal studies wherein encapsulation refers to molecules that are enclosed within lipid bilayers and reside inside the aqueous interior of liposomes. In this classic definition, the DNA or RNA would be essentially “free-floating” in the aqueous space enclosed by the liposome, not exposed to the exterior surface, and would freely diffuse away from the delivery vehicle upon disruption/fusion; highly desirable attributes for intracellular delivery. Therefore, it is impossible to achieve nucleic acid encapsulation (by the traditional definition) in delivery vehicles that employ cationic components.

In the case of polymers such as poly(lactic-co-glycolic acid (PLGA) that are used for gene delivery, cationic components are often avoided, but DNA or RNA is embedded in the polymer matrix rather than encapsulated. While this approach has many of the same advantages as encapsulation (e.g., nuclease protection, no interaction with the encapsulating material, release of naked nucleic acid), problems associated with degradation within the micro/nano spheres are significant, especially considering that nucleic acids can reside within these systems for weeks prior to release. Although this latter property could be a significant advantage of these systems, prolonged release will require that nucleic acids be stable within the polymer matrix for extended periods at body temperature. Furthermore, efficient entrapment within the polymer matrix during micro/nano sphere preparation typically requires an association with the hydrophobic polymer which ultimately contributes to the extended release⁷⁷. For this reason, it is advantageous to formulate nucleic acids with cationic components that have nonpolar moieties which can associate with the PLA or PLGA matrix. In addition, the cationic components help to compact the DNA so that it can be embedded within nanospheres that are small enough to allow efficient cellular uptake. Some studies have been able to entrap plasmids within very small nanospheres without the use of cationic components⁷⁸. Presumably, the ability of polymers at high concentration to condense DNA contributes to their incorporation within such small delivery systems^{79,80}, and the presence of a plasmid (especially at pharmaceutically-acceptable loading capacities) likely has a significant effect on the polymer matrix and consequent release kinetics.

Considering the large size and polyanionic nature of DNA and RNA molecules, the conventional method of loading small molecule drugs into liposomes cannot be applied because nucleic acids are unable to cross lipid bilayers by diffusion⁸¹. Loading of plasmid DNA has been made possible by a detergent-dialysis approach for the formation of liposomal DNA carriers known as stabilized plasmid-lipid particles (SPLP)^{43,64}. SPLP are formed from mixtures of plasmid and lipids (cationic and zwitterionic) by a detergent-dialysis procedure involving octyl-glucopyranoside (OGP). Details of experimental procedures for small and large scale production of SPLP are outlined elsewhere⁸². The particles are small (about 70 nm), relatively monodisperse, and have been shown to accumulate in distal tumor sites with subsequent gene expression in mouse tumor models following intravenous injection⁸³. Meanwhile Maurer *et al.*⁸⁴ developed a new formulation that utilizes an ionizable lipid [1, 2-dioleoyl-3-dimethylammonium propane (DODAP)] and an ethanol-containing buffer for loading large quantities of antisense oligonucleotide in lipid vesicles. The resulting particle is known as a “stabilized antisense-lipid particle” or SALP. The SALP is a multilamellar vesicle with a small diameter (70–120 nm), which exhibits

extended circulation half-life, ranging from 5–6 h for particles formed with PEG-CerC₁₄ to 10–12 h for particles formed with PEG-CerC₂₀^{84,85}. Essentially this same loading technology has been broadened to include other nucleic acids (SNALP – stable nucleic acid lipid particles) and has been used to successfully deliver siRNA in non-human primates^{86,87}. This approach was originally developed by Protiva Biotherapeutics (now Tekmira) and is currently being employed by Alnylam Pharmaceuticals for some of their clinical trials on siRNA delivery. More recently, a modified SPLP method has been developed to load pre-condensed plasmid DNA within a lipid bilayer using a controlled mixing process⁸⁸. This method allows for the incorporation of either poly-L-lysine or poly(ethyleneimine) (PEI) to condense the DNA, which results in “pre-condensed stable plasmid lipid particles” (pSPLPs) with small size (104 ± 3 nm) and low surface charge. Furthermore, both SPLP and PEI-pSPLP formulations exhibited significant luciferase expression in tumors and low levels of gene expression in liver and lung⁸⁸.

Encapsulation of nucleic acids offers many advantages for delivery, and a process which does not rely on cationic components would be a significant breakthrough for the field. Unfortunately, the current techniques described above still employ some cationic lipids in the formulation, and thus raise concerns about opsonization, toxicity, and release of the nucleic acid inside the cell. However, attempts to encapsulate DNA without utilizing cationic lipids have proven to be inefficient⁸⁹. One study by Bailey and Sullivan⁹⁰ employed calcium to encapsulate DNA in zwitterionic lipids at reasonably high efficiencies, albeit at low DNA-to-lipid ratios. Other approaches have utilized the nucleic acid as a nucleation site for liposome formation, but have had to rely on electrostatic interactions and the use of cationic lipids⁹¹. An interesting alternative has been investigated by Fernandez *et al.*⁹² in which association of vehicle components with the nucleic acid is accomplished by intercalation instead of electrostatics. While this clever strategy does not require cationic components, the ability of the intercalated components to dissociate from the nucleic acid may be problematic. In contrast, true encapsulation in which the nucleic acid is not bound to the vehicle would offer many advantages, and new methods of encapsulation should be explored. To this end, it should be theoretically possible to form a “shell” of neutral material around nucleic acids, however the nucleic acids would need to be sufficiently small (e.g., condensed DNA, antisense, siRNA) for the size of the resulting particle to be compatible with intracellular delivery. Hopefully, novel methods of physical encapsulation can provide opportunities for nucleic acids to be enveloped within a neutral material without the use of cationic components in the near future.

Targeted delivery of nucleic acids

It has been known for decades that liposomes with extended circulation times accumulate in tumors⁵³. In any discussion of “targeting”, it is important to distinguish between accumulation/localization at the target tissue from uptake/internalization by the target cells. Most studies simply quantify plasmid/siRNA levels in tissues without determining the extent of uptake by target cells. As mentioned above, PEGylation has been the predominant strategy employed to prolong circulation times, but the influence of PEGylation on cellular uptake and intracellular trafficking of particles compromises the ultimate delivery efficiency. Recent studies by Li *et al.*⁶⁷ on siRNA delivery concluded that PEGylation reduces delivery efficiency in cell culture by approximately 10-fold, and *in vivo* studies have also observed decreased tumor accumulation with PEGylated gene delivery systems⁹³. Additional studies have demonstrated that enhancement of “passive accumulation” via the enhanced permeation and retention effect (EPR) requires targeting ligands to be attached to the distal end of the PEGylated components such that the ligand is projected beyond the PEG “shield” to allow binding to receptors on the target cell surface^{94–96}. In some cases, the conjugation of the ligand to the delivery vehicle is performed after the loading of nucleic

acid, and unreacted ligand is subsequently removed. Such manipulations are suitable for academic studies, but greatly complicate commercial development.

In studies of ligand-assisted uptake, the effect of protein binding on the surface conjugated ligand is typically ignored. However, fouling of the ligand could result in inefficient/inadequate presentation to the receptor on the target cell. Since most studies employing targeting ligands also utilize PEGylation, it is assumed that protein binding is reduced to the extent that ligand fouling is not a concern. However, as discussed above, PEGylation does little to reduce serum protein binding, and thus even ligand conjugated to the distal end of PEGylated components may be fouled by adsorbed protein. The fact that many of these studies report increased cell internalization after intravenous administration suggests that ligands are still able to bind to cell receptors regardless of fouling, but the diminished capacity for binding and uptake is difficult to assess. One interesting alternative approach to utilizing PEG is to employ lipid domains that appear to resist serum protein binding, and thus may be an ideal location for presenting ligands on the particle surface⁹⁷. Preliminary studies with this approach indicate that location of the ligand within lipid domains greatly enhances transfection rates⁹⁸.

Site specific delivery of lipoplexes requires the identification of cell surface receptors and the design of appropriate ligands for receptor-mediated endocytosis, however this strategy can traffic the delivery vehicle into the lysosomal pathway which necessitates efficient endosomal escape. The most popular approach is to use liposomes or polymers with specific ligands such as antibodies, transferrin, folic acid, RGD peptide, anisamide, *etc.*^{67,99–104}. Studies have shown that the use of antibodies as targeting ligands are especially effective in this regard because they are large molecules that bind to specific cellular sites^{105,106}. This approach clearly demonstrates the proof-of-concept that targeting ligands can be utilized to enhance the uptake of particular delivery systems by specific cells. However, the use of proteins as targeting ligands compromises one of the primary advantages of employing synthetic delivery systems, i.e., the lack of a specific immune response. Although humanized antibodies could potentially be produced to minimize ligand immunogenicity in clinical trials, it should be recognized that the general strategy of attaching delivery systems to PEGylated components that also need to be conjugated to protein ligands would be virtually impossible to realize on a commercial scale. To simply produce such an intricate delivery system, the structural integrity of the protein ligand would need to be preserved during manufacturing, conjugation, processing, and storage. In addition, the cost of the ligand, and the complications associated with conjugation, purification, etc. virtually prohibit such a strategy from commercial development. Recent studies have utilized human transferrin (approx 80 kD with significant secondary structure) as a targeting ligand for a cyclodextrin-based siRNA delivery system¹⁰³. Although the ability of this system to efficiently deliver siRNA is currently being tested in clinical trials by Calando Pharmaceuticals¹⁰⁷, it is unlikely that a protein-based ligand like transferrin would ultimately be used in a commercial drug delivery product. Furthermore, many academic studies employing protein ligands use exceedingly high levels of ligand to achieve targeted delivery. In this regard, it is common to express the level of ligand in terms of a molar ratio (e.g., lipid/transferrin = 40/1) that disguises the fact that the ligand comprises 50–90% of the delivery system on a weight basis. Although the literature typically depicts these delivery systems as elegant spherical capsules with ligand molecules protruding from the surface, a more accurate illustration would be that of many large protein molecules being randomly adhered to nucleic acid, with the cationic “vehicle” being used as an electrostatic “glue”. Thus, the particles in such preparations would be more accurately depicted as polydisperse molecular complexes of ligand and nucleic acid instead of the tightly-organized, highly-oriented nanoparticles they are typically portrayed to be.

The choice of spacer/linker is another consideration when developing a targeting ligand conjugate. PEGs with different molecular weight have been commonly used as a linker between the targeting ligand and its anchor¹⁰⁸. For example, a certain distance between the folate moiety and the lipid particles is needed for folate receptor targeting. This is believed to be due to the need for folate to enter the binding pocket of the receptor on the cell surface. Ward *et al.*¹⁰⁹ reported that a folate-linked PEG₈₀₀ polymer-modified pLL/DNA complex did not lead to a significant increase in transfection *in vitro*. Additional studies by Leanon *et al.* optimized the targeting activity of liposomes by modifying the length of the PEG-linker, and found that PEGs as small as a molecular weight of 1000 could function as effective linkers¹¹⁰. The length of PEG₁₀₀₀ is estimated to be approximately 2.5 nm¹¹¹, therefore a spacer length of > 2.5 nm might be essential for folate receptor targeting. Schaffer *et al.*¹¹² also found that lengthening the crosslinker arm used to attach EGF (a 74 kD protein) to the conjugate significantly enhances the specific binding of conjugates to the EGF receptor, which translates into more efficient gene delivery *in vitro*. More precisely, Handl *et al.* showed that an optimal linker length of 2.5 ± 1.0 nm is needed for ligand binding to its corresponding G-protein coupled receptors¹¹³. In short, studies consistently show the need for a spacer/linker of approximately 2.5 nm for proper presentation and flexibility of the ligand to facilitate binding to the target cell, regardless of whether the ligand is a large protein or a small molecule.

With the goal of developing a commercial product, it is important to keep in mind that the components of the delivery system will need to be manufactured and assembled on a commercial scale that is very different than laboratory- or research-scale production. For this reason, many of the approaches utilized in basic research are not conducive to further development. For example, the cost of manufacturing just a relatively simple liposome product is considerable, and companies who have developed such products have needed to negotiate much lower prices from lipid manufacturers in order to achieve commercial viability. If we consider the added cost of the nucleic acid, the targeting ligand, conjugation/purification, and other molecules to enhance delivery (e.g., nuclear localization sequences, endosomolytic peptides), many of the strategies that appear promising in the literature must be viewed as academic exercises rather than viable technologies that will lead to commercial development. In particular, the cost associated with producing components (e.g., plasmid, targeting ligand) in bacteria, purifying each component, and assembling a vector with uniform physical characteristics is excessive. However, some current enzyme replacement and antibody-based therapies can cost hundreds of thousands of dollars per year, and therefore it is possible that expensive gene therapies may be commercially-viable alternatives for some conditions. Regardless, components that can be chemically synthesized (e.g., siRNA, small molecule ligands) offer the best hope for regulatory approval (potentially avoiding regulation as a “biologic”) and commercial development if loading into the delivery system can be sufficiently controlled to achieve tight product specifications.

It is worth mentioning that delivery systems that are touted as “self-assembling” are often very difficult to control on a commercial scale. In practice, many of these self-assembling systems require dilute conditions to achieve reasonably uniform particle characteristics. This significantly complicates bulk manufacturing, and requires that even lab-scale preparations be concentrated prior to administration. To illustrate this point, it should be appreciated that self-assembling preparations of nonviral gene delivery vehicles typically require relatively dilute conditions (e.g., ≈ 0.1 mg DNA/mL) to achieve sufficiently small and uniform particle size. Therefore, many early studies in gene delivery utilized large volume injections into animal models (e.g., 1 mL bolus injection into a mouse having a blood volume of approximately 2 mL) that ultimately helped fuel speculation that nonviral gene therapy was ready for the clinic. Years later, the ability of hydrodynamic pressure to facilitate transfection is well-documented, and potentially has limited clinical application^{114,115}.

However, this artifact was standard practice for years in the field of gene therapy, and results from studies utilizing this approach were commonly presented at conferences without mention of the extreme conditions needed to achieve such high levels of gene expression in mouse models. Many research studies still utilize this approach for target validation, promoter assessment or effects of sequence modifications, but realistic evaluation of *in vivo* gene delivery efficiency requires preparations to be concentrated in order to reduce injection volumes. In this respect it is important to realize that most particle preparations are amenable to concentrating *after* sufficient time is allowed for self-assembly, and can even be incorporated into processes that are compatible with clinical applications, e.g., rehydrating lyophilized samples with reduced volumes of diluent^{116,117}.

Biodistribution and Immunogenicity

It is well known that cationic lipid- and polymer-based nucleic acid formulations accumulate predominantly in lung and liver after intravenous injection. It has been shown that cationic lipoplexes and polyplexes interact with serum proteins in the circulation, resulting in uptake by the cells of the mononuclear phagocyte system (MPS), classically known as the reticuloendothelial system^{118–121}. Although some studies have exploited this non-specific accumulation for lung and liver delivery, it is clear that accumulation of the delivery vehicle in lung or liver does not necessarily indicate that uptake of the nucleic acid into cells of these organs (e.g., hepatocytes) has occurred. In fact, much of the delivery to these sites has proven to be uptake by macrophages and endothelial cells lining the blood vessel instead of the therapeutic target. Regardless, accumulation into lung and liver also presents complications for delivery into other tissues such as tumors. Considering the predominant accumulation of nanoparticles in the liver, it is surprising that studies continue to tout rapid liver accumulation as effective ligand-induced, liver targeting for hepatic diseases. One creative approach to avoiding MPS uptake has exploited drag forces to hinder engulfment by macrophages and to extend the circulation time of polymeric micelles¹²².

As mentioned above, PEGylation of lipid- and polymer- based formulations has been widely used to improve the circulation lifetime and reduce the lung accumulation after intravenous injection. The mechanism for this appears to be two-fold: PEGylation prevents the formation of aggregates that can be trapped in pulmonary capillary beds, and also reduces the surface charge that has been linked to lung accumulation^{123,124}. In this respect, it is important to mention that commonly-used PEGylated lipids (e.g., DSPE-PEG) are conjugated through the cationic ethanolamine headgroup such that the zwitterionic phospholipid is converted to an anionic lipid. Therefore, the incorporation of these anionic components into a cationic lipid formulation will reduce the positive zeta potential via charge neutralization; an effect that is typically attributed only to steric stabilization by PEG. Accumulation in the liver is thought to be more closely related to particle size because the pore size of liver fenestrae are approximately 100 nm¹²⁵. Therefore nanoparticles with diameters < 100 nm rapidly accumulate in the liver. In fact, it is often jokingly stated that the definition of a nanoparticle is “anything that accumulates in the liver”. In contrast, these small particles generally do not accumulate in the spleen unless aggregation occurs such that the particle size increases to > 300 nm¹²⁵. In considering both surface charge and particle size, it is important to recognize that particles can be carefully prepared with the desired properties, but interaction with blood components dramatically changes these properties upon IV administration^{36–38,42}. Therefore, the particle properties that are relevant for intravenous delivery are size and surface charge *after* blood exposure, but these properties are rarely measured and/or considered¹²⁶.

Although accumulation in the lung, liver and spleen remains a significant problem for nucleic acid delivery systems in terms of both toxicity (e.g., hepatotoxicity) and poor

distribution to the target site (e.g., tumor), studies have relied on the extended circulation times provided by PEGylation to enhance tumor accumulation. However, as discussed above, PEGylation is also known to alter cellular uptake and trafficking. Attempts to overcome this drawback of PEGylation have conjugated a targeting ligand on the distal end of PEGylated lipids to further improve the uptake into target tissues. While this strategy has improved internalization at the cellular level, it has not increased the amount of material that accumulates in the tumor tissue^{67,124,127}. Instead, distribution to the tissues appears to be largely governed by the EPR effect, which is predominantly affected by particle size. From this perspective, delivery vehicles will extravasate through openings of sufficient size, as is evident from the reduced distribution to lung and liver in tumor-bearing as compared to tumor-free animals^{67,93}. Further distribution within tissues (i.e., after the initial accumulation due to EPR) appears to be very dependent on particle size, and particles of 100 nm or smaller are better able to distribute throughout tumor¹²⁸. Again, because surface properties will largely determine the interaction of the drug delivery vehicle with proteins and other blood components upon injection, properties such as surface charge and hydrophobicity can have a dramatic effect on the size of the delivery vehicle that ultimately circulates and accumulates in tissues. Moreover, even the size of the particle can affect the corona of proteins that adsorb to the particle surface¹²⁹. Accordingly, more attention should be paid to particle size and surface properties *after* exposure to physiological fluids if significant improvements in toxicity, accumulation/localization and internalization are to be achieved. Similarly, studies that monitor accumulation and or activity (e.g., gene expression, silencing) only within the tumor ignore the significant toxicity associated with liver accumulation which will likely limit therapeutic potential. Researchers need to be upfront about these issues, and avoid burying unflattering data (e.g., elevated liver enzymes) in supplementary material that is difficult to access (i.e., supplementary information available only online can have file sizes greater than 50 megabytes that discourage downloading).

While efforts are clearly needed to improve delivery efficiency, vector safety must be assured if nucleic acid-based therapeutics are to become commercial products. It is known that although viral vectors possess higher transfection efficiency, safety concerns associated with their use in clinical trials makes the nonviral vector an attractive alternative because synthetic vectors elicit low immunogenicity compared to viral vectors¹³⁰. However, it is known that cationic lipoplexes can induce innate immune response and tissue damage after systemic injection^{131–136}. Intravenously-injected cationic lipoplexes induce an acute inflammatory response characterized by cytokine (TNF- α , IFN- γ) release in the serum that is attributed to the unmethylated CpG motifs of the plasmid DNA used in lipoplexes and polyplexes, which is recognized by Toll-like receptors of immune cells^{135,137,138}. Removal of CpG motifs in plasmid DNA greatly reduces cytokine production^{136,139}, which should be taken into account when developing a gene delivery system. In this sense, siRNA delivery offers a significant advantage over plasmid-based approaches because the nucleic acid is chemically synthesized and can be modified to reduce immunogenicity and increase nuclease resistance. In particular, studies have shown that incorporation of 2'-O-methyl modifications can virtually eliminate immunogenicity in animal models^{140,141}. However, the use of CpG-containing DNA can act as a potent stimulator of the immune system that may be useful for developing improved vaccine technology. In fact, the ability of this innate immune response to shrink tumors was conclusively demonstrated a decade ago by Dow and colleagues¹³⁷, yet studies continue to report tumor shrinkage in response to gene delivery without considering the effects of the immune response elicited by the plasmid backbone. It is also worth noting that sequential administration of the DNA and lipid components (as opposed to a lipoplex) greatly reduces the cytokine response, suggesting that presentation of CpG motifs on a particle (and the nuclease protection associated with complexation/adsorption) is capable of enhancing the immune response¹²³.

Furthermore, it is generally expected that PEGylation of nonviral vectors would attenuate the level of immunogenicity due to its surface shielding. Controversially, evidence from recent studies showed that intravenous administration of PEGylated liposomes causes the second dose of PEGylated liposomes (injected a few days later) to lose their long-circulating profile and accumulate extensively in liver^{142–147}. This phenomenon is referred to as the “accelerated blood clearance (ABC) phenomenon”¹⁴² and has been observed in mice, rats and a rhesus monkey. Because repeated injections of PEGylated formulation are likely in the case of nucleic acid-based therapies, these findings raise concerns about the development of PEGylated formulations and their clinical application. The mechanism behind this ABC phenomenon has been proposed to involve anti-PEG IgM that is produced in the spleen in response to the first dose of PEGylated liposomes, and subsequently binds to the PEG on a second dose of these liposomes injected several days later¹⁴⁸. Once the PEGylated liposomes reach the spleen, they bind and crosslink to surface immunoglobulins on PEG (or PEGylated liposome)-reactive B cells in that organ, and consequently trigger the production of an anti-PEG IgM. Further investigation indicated that the ABC phenomenon is dependent on the physicochemical properties of injected liposomes as well as the time interval between injection and lipid dose¹⁴⁸. However, it is important to recognize that PEGylated liposome products that are currently on the market (e.g., DOXIL) have not reported any problems or reduced efficacy associated with repeated administration every few weeks.

Perspectives/Prospectives

Nucleic acid based therapeutics as a new category of biologics is still in its early stages. Lessons have been learned in the frantic course of gene therapy research during the 1990s when the potential of gene therapy was hyped as the ultimate panacea, which actually made the barriers to success even greater¹⁴⁹. From the FDA database (www.clinicaltrials.gov), there are 80 clinical trials (completed and active) of antisense technology, and 70 clinical trials of plasmid DNA based therapeutics; most of the latter are vaccine therapy. In fact, two of these plasmid therapeutics are in late-stage phase III clinical trials (Collatogene by AnGes Inc. and Allovectin-7 by Vical Inc.). Since the demonstration of RNA interference in mammalian cells in 2001, siRNA has become the predominant focus for development, and has rapidly progressed to clinical trials. Starting from 2005, there are already 14 clinical trials of siRNA based therapeutics. It should be recognized that therapeutics based on synthetic oligonucleotides (i.e., antisense and siRNA) are significantly more amenable to development than is plasmid DNA, and the ability to silence specific genes has applications to a wide variety of diseases. However, it was just over a decade ago when gene therapy was in a similar spotlight, and the inability to demonstrate clear efficacy in clinical trials suggests that the hype outpaced the science. We must learn from this experience, and honestly present both promising and problematic issues associated with nucleic acid delivery in the scientific literature¹⁵⁰. To this end, it is counterproductive to present data in an unconventional way to make the results appear more promising. For example, presenting nucleic acid accumulation on a per tumor basis can be misleading if tumor sizes are abnormally large. Conversely, accumulation in very small tumors is misleading if presented as “percent injected dose *per gram*” because the percent in the tumor is multiplied by a large number (the inverse of a relatively small tumor weight) in order to be presented on a ‘per gram’ basis. Direct comparison of such values to other tissues (e.g., liver, lung) obscures the fact that a large percentage of the injected dose (> 90%) accumulates in organs with weights many-fold greater than that of the tumor. Instead, researchers in the field should present their data in a consistent, straightforward manner that does not disguise the problems with current technologies. Clearly, many of the issues revolve around delivery¹⁵¹, and novel technologies are needed to avoid MPS uptake, accumulate therapeutic levels of nucleic acid at the target site, facilitate distribution within the target tissue, and stimulate internalization by target cells.

It is also worth noting again that PEGylated liposomes have been linked to accelerated blood clearance and an immune response after repeated injection¹⁴⁸. Considering these issues, as well as the decreased cellular interactions and altered trafficking noted above, it would be beneficial to develop alternative strategies to PEGylation for increasing circulation lifetimes. Unfortunately, PEGylation has become the standard method for achieving prolonged circulation, and surface modification by alternative hydrophilic polymers (e.g., poloxamers) are also likely to result in a decreased interaction with the target cell surface and altered trafficking. As discussed above, PEGylation does not appear to reduce protein binding, and thus the mechanism by which PEGylation increases circulation time is unclear. It has been suggested that PEGylation serves to shield the surface charge, and thereby reduce rates of liposome clearance¹⁵². Alternatively, PEGylation could prevent particle-particle interactions that result in rapid clearance in liver and lung, thereby leading to prolonged circulation times⁵⁷. In this context, it is interesting that high levels of cholesterol in lipid-based formulations reduce aggregation in serum and result in longer circulation lifetimes^{38,41}. Furthermore, work from our lab has shown that high cholesterol formulations accumulate in tumors to levels greater than PEGylated formulations⁹³. This approach has the potential advantage of avoiding the problems associated with PEGylation, and other alternative strategies for increasing circulation times should be investigated.

In addition to the issues associated with serum stability, targeting, and biodistribution, the significant hurdles to developing a regulated, marketable, pharmaceutical product should not be ignored. It is important to recognize that the late-stage plasmid therapeutics mentioned above are relatively simple products in which development is not complicated by elaborate multi-component delivery systems. While the added cost associated with producing a delivery system may be a significant barrier to commercial development, it must be recognized that dosing and stability also represent formidable obstacles. For example, animal dosing of nucleic acids is typically in the 0.2–1 mg/kg range, translating to a single dose in the tens of milligrams for an adult human. Also, these doses are often administered at least weekly in animal studies, raising concerns about the expense and toxicity surrounding long term clinical deployment. Furthermore, the stability issues associated with particulate delivery systems are substantial, and more work on the stability of nucleic acid formulations on pharmaceutically-relevant timescales is clearly warranted, especially since the stability of RNA and many nucleic acid modifications are virtually unknown^{67,153,154}. In particular, the prospect of developing a two-vial formulation in which vectors are assembled by mixing in the pharmacy is wholly unrealistic considering the significant effects that mixing parameters (e.g., speed, order of mixing, dilution, ionic strength) are known to have on vector properties. To this end, we reiterate that many of the current academic studies on nucleic acid delivery must be viewed as “proof of concept” (even if they advance to clinical trials), with the realization that such elaborate molecular assemblies are incompatible with scale-up and commercial development. Progression to marketed pharmaceutical products will require understanding the mechanisms exploited by the intricate delivery systems currently extolled in the literature, and identifying more robust vehicles with fewer components that can duplicate their delivery performance. In this regard, conjugate/polymeric delivery systems offer a significant advantage by reducing the number of components while endowing the delivery vehicle with multiple functionalities^{104,151,155–157}.

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