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Advancing Polymeric Delivery Systems Amidst a Nucleic Acid Therapy Renaissance

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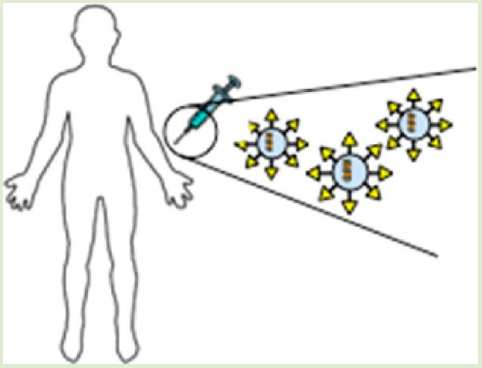
ABSTRACT: Nucleic acid therapeutics are attracting renewed interest due to recent clinical advances and product approvals. Most leading programs use chemical conjugates, or viral vectors in the case of gene therapy, while several use no delivery system at all. Polymer systems, which have been at the periphery of this renaissance, often involve greater molecular complexity than competing approaches, which must be justified by their advantages. Advanced analytical methods, along with biological tools for characterizing biotransformation and intracellular trafficking, are increasingly being applied to nucleic acid delivery systems including those based on polymers. These frontiers of investigation create the opportunity for an era where highly defined polymer compositions are optimized based on mechanistic insights in a way that has not been previously possible, offering the prospect of greater differentiation from alternatives. This will require integrated collaboration between polymer scientists and those from other disciplines.



The development of innovative new classes of therapeutics is a time-consuming and risky endeavor. Proof of concept in humans can take a decade or more, with infrequent opportunities to reflect on which approaches have proven most fruitful. Nucleic acid therapeutics, including antisense, short interfering RNA, and plasmid-based gene therapies, represent a case in point. Although long recognized as an attractive potential class of drugs, nucleic acids have yet to make a meaningful impact on the pharmacopeia. A number of recent developments suggest this situation may be about to change. While the first gene therapy, Gendicine, was approved in China in 2004, the west saw its first gene therapy approval in 2012 with Glybera, a gene therapy for lipoprotein lipase deficiency.[1] This milestone is accompanied by recent clinical advances in gene therapies for β-thalassaemia,[2] leukemia,[3] Canavan disease,[4] and Leber congenital amaurosis.[5] Antisense oligonucleotides are similarly emerging from a long product drought with the 2013 U.S. FDA approval of mipomersen (Kynamro), an antisense against apolipoprotein B for treatment of homozygous familial hypercholesterolemia.[6] Two oligonucleotide therapies for Duchenne's muscular dystrophy, eteplirsen and drisapersen, have shown very promising results in delaying progression of a devastating disease and are advancing rapidly through clinical development.[7] Further, progress in the clinic has brought renewed interest in RNA interference as a potential basis of new therapies.[8]

Novel drug delivery systems face a development latency period similar to that seen with alternative therapeutic modalities. Polymer systems have been the subject of much investigation as a technology solution to one of the most significant hurdles facing nucleic acid therapies: their safe and effective delivery to the site of action. These efforts notwithstanding, the clinical impact of this approach has been modest. Polymer delivery systems are not included among the recognized vectors used in the 1800 gene therapy clinical trials to date,[9] and the aforementioned gene therapy successes utilized viral vectors as opposed to synthetic vehicles. However, polymers have had an impact with synthetic oligonucleotide cargo, particularly siRNA. The RONDEL siRNA delivery system from Calando (a unit of Arrowhead Research) represents a noteworthy achievement, having reached phase I clinical testing (Figure 1A).[10] The Dynamic PolyConjugate technology developed by Mirus, now also part of Arrowhead Research, is slated for clinical entry for a candidate hepatitis B therapy (Figure 1B). While encouraging, are these achievements sufficient to form the basis for a future stream of nucleic acid based therapeutics based on polymeric delivery systems? What lessons for the next generation of polymeric systems can be gleaned from the track record to date?

The three major classifications of nucleic acid delivery constructs are viral carriers, nonviral carriers, and unencapsulated nucleic acid conjugates. Clinically approved Gendicine and Glybera are both viral (adenovirus and adeno-associated virus, respectively) formulations. Polymer carriers, together

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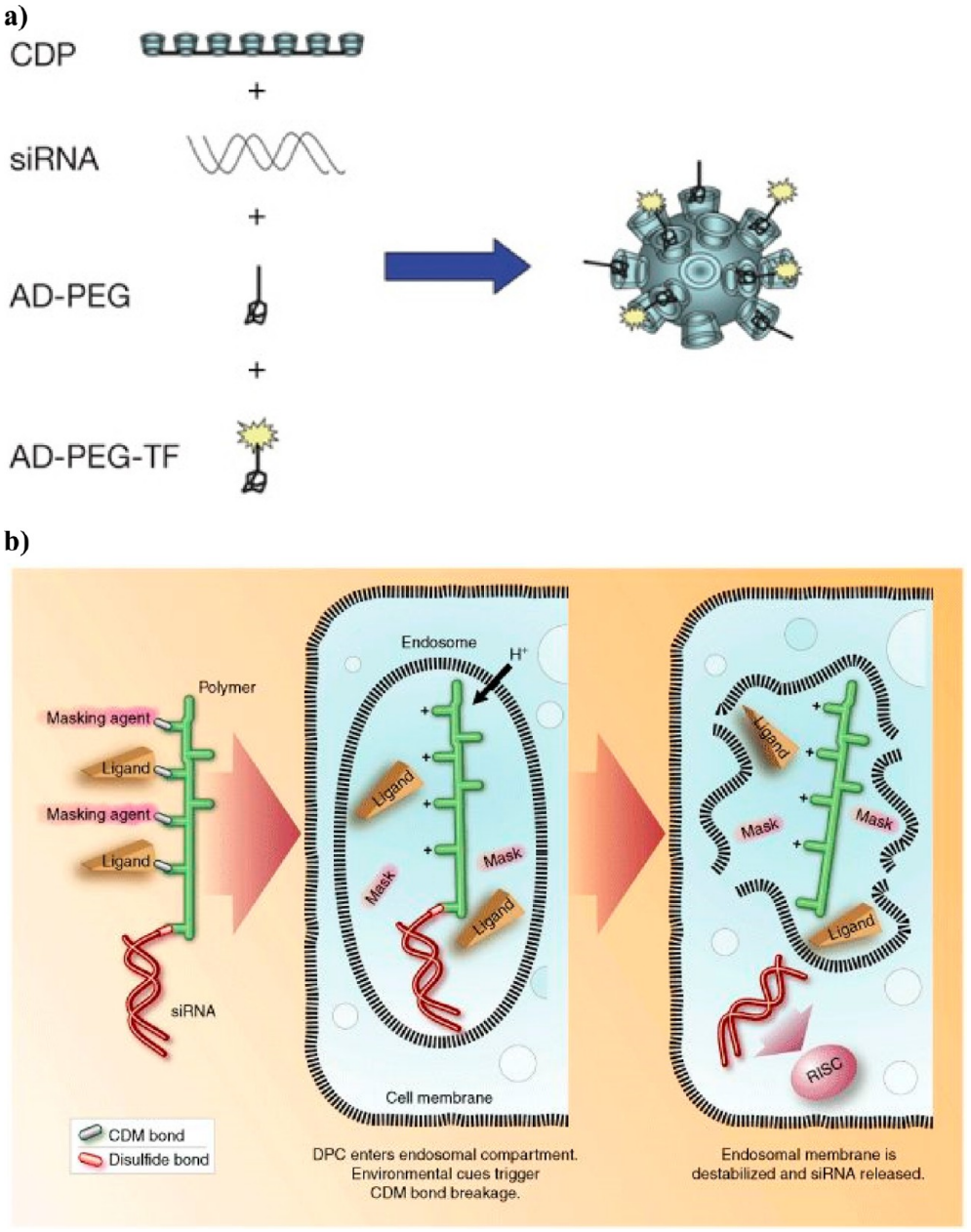

Figure 1. Schematic representation of RONDEL (a) and dynamic polyConjugate (b) targeted siRNA delivery systems. (a) Polyethylene glycol (PEG) molecules are terminated with adamantane (AD) that form inclusion complexes with surface cyclodextrins to decorate the surface of the nanoparticle with PEG for steric stabilization and PEG-transferrin (TF) for targeting. Adapted from Davis, M. E.; Zuckerman, J. E.; Choi, C. H. J.; Seligson, D.; Tolcher, A.; Alabi, C. A.; Yen, Y.; Heidel, J. D.; Ribas, A. *Nature* **2010**, *464*, 1067 and used with permission. (b) Schematic showing the siRNA Dynamic PolyConjugate, with a polymer backbone and pendant targeting ligands and siRNA. CDM (carboxylated dimethyl maleic acid) masking groups shield the positively charged polymer backbone. Following cellular uptake, the conjugate disassembles in the low pH environment of the endosome, with release of the siRNA into the cytoplasm. Adapted from Wolff, J. A.; Rozema, D. B. *Mol. Ther.* **2008**, *16*, 8 and used with permission.

with lipid carriers, comprise the majority of investigated nonviral constructs. The third category includes modified nucleic acids and molecular conjugates. For example, Alnylam recently announced favorable results from ALN-TTRsc, an N-acetyl galactosamine conjugate of an siRNA targeting transthyretin (TTR), for the treatment of TTR-mediated amyloidosis.[12] The Mirus Dynamic PolyConjugate, originally designed with a disulfide bond linking the polymer and siRNA drug cargo (Figure 1B) has been deconstructed as a cargo-free polymeric delivery excipient, which is coadministered with an siRNA conjugated to cholesterol.[13]

Development of polymeric nucleic acid carriers continues to be an active area of investigation because these materials offer potential advantages in safety, manufacturing, and pharmacokinetics over the other systems, such as viruses and lipids. Immunogenicity and insertional mutagenesis remain unresolved issues for most adenoviral and retroviral vectors, respectively.[14] In contrast, pre-existing antibodies and integration are typically not concerns for most polymer gene carriers. Scale-up and commercial manufacturing are known challenges for liposomal formulations such as the marketed cytotoxic Doxil. Lipid-based nucleic acid delivery systems, which can

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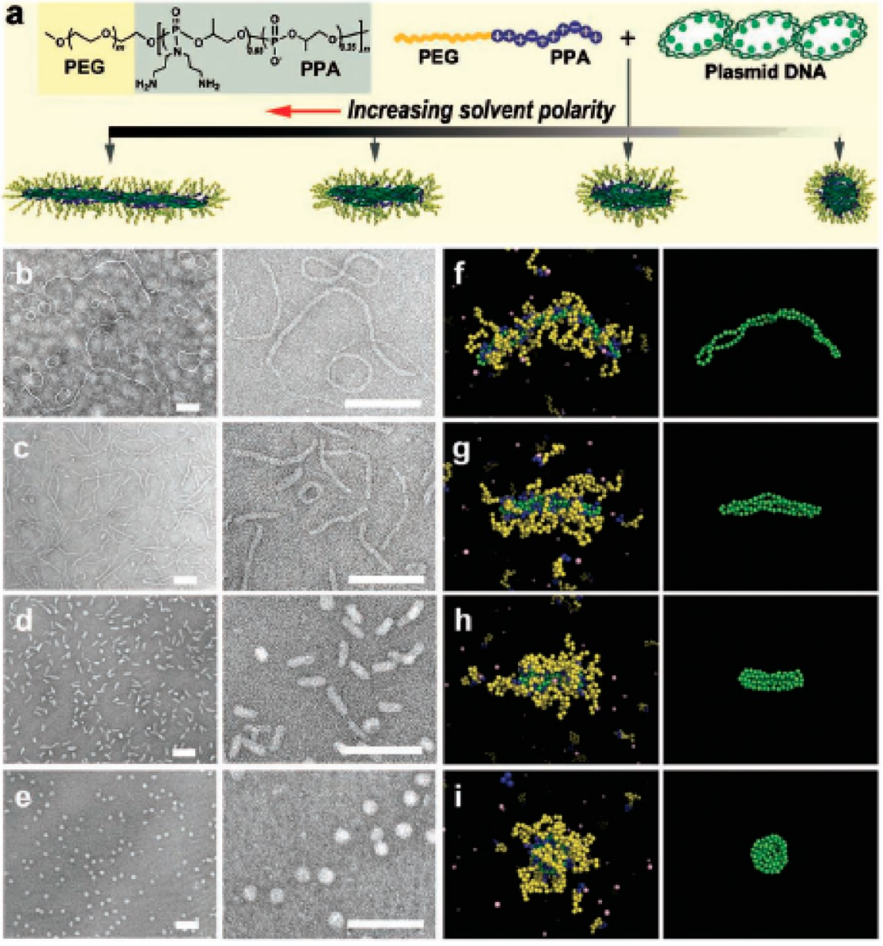

Figure 2. (a) Assembly of PEG_{10K}-*b*-PPA_{4K} block copolymer in solvents with different polarities with results in different nanoparticle shapes. (b–e) Morphologies of polyplexes prepared in deionized water at different *v*/*v* ratios: (b) 3:7, (c) 5:5, (d) 7:3, (e) DMF–water mixtures at N/P ratio of 8. Scale bars = 200 nm. (f–i) Representative images of polyplexes obtained in molecular dynamics simulations corresponding from the conditions in panels (b)–(e). DNA is represented in green and the PEG and PPA blocks are in shown in yellow and blue, respectively (monovalent counterions shown in pink). The left most panel represents the DNA conformation in the polyplex. Figure and caption reprinted with permission from Jiang, X.; Qu, W.; Pan, D.; Ren, Y.; Williford, J.-M.; Cui, H.; Luijten, E.; Mao, H.-Q. *Advanced Materials* **2013**, *25*, 227.

exhibit heterogeneity in size, composition, and potency[15] are susceptible to similar challenges. Polymeric systems offer an attractive potential advantage in having fewer components and with the composition determined by synthetic rather than process steps. Finally, unencapsulated oligonucleotides, due to their low molecular weight, typically have poor circulation half-lives, requiring high and frequent dosing. Polymer carriers offer the possibility of prolonged blood circulation while also facilitating intracellular delivery of nucleic acid cargo.

Given these advantages, why do polymer carriers lag alternatives in clinical impact?

Efficacy (which is closely linked to potency) and manufacturability are two key hurdles for translation of any drug product and merit specific focus for future investigation. The relatively low delivery efficiency in vivo achieved by current carriers is a known drawback. To address this issue, structure–function relationships must be better understood and optimized, requiring inputs from key analytical and biological steps. The recent development with the Dynamic PolyConjugate system, where potency of a cholesterol conjugated drug cargo was observed in nonhuman primate, in absence of direct covalent attachment to the polymeric carrier,[13] illustrates the need in this arena. Material characterization, reproducible formulation, and thorough vehicle characterization are necessary to precisely define the drug product, to ensure that preclinical studies are indeed comparable, and to guide scale-up and manufacturing. Three specific frontiers requiring broader exploration to position polymer systems for greater future impact in the nucleic acid space are highlighted here: analytical characterization, particularly to better define dispersity; "biotransformation" (in this context referring to the chemical and physical changes to the delivery formulation that occurs after in vivo administration); and intracellular trafficking to the site of action. Recent work in each of these areas illustrates their importance to the continued maturation of polymeric delivery systems.

In the pharmaceutical industry, an appropriate battery of analytical measures to stringently characterize a potential product for reproducible structure, formulation, and biological activity ensures quality, efficacy, and safety in patients. In addition, manufacturing processes require maintenance of characteristics within an acceptable range. Complexity is compounded when a system has multiple components, such as polyplexes. This means that careful analytical characterization and strict controls must be in place to ensure homogeneity; for multicomponent assemblies, this includes a

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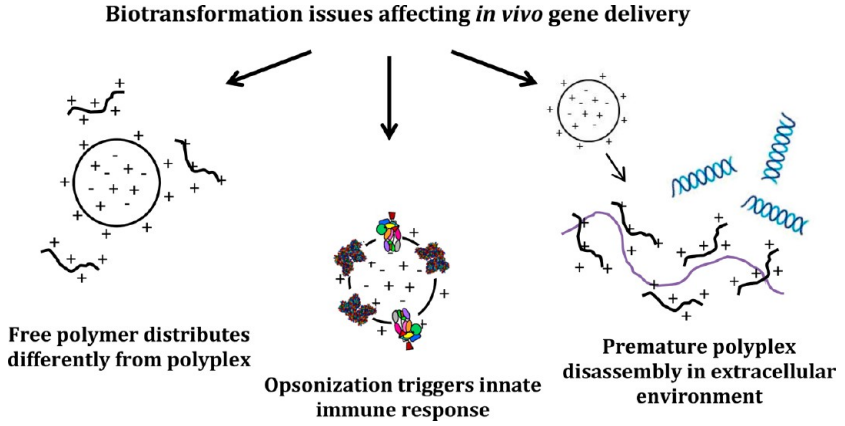

Figure 3. Three reported changes in polyplexes after in vivo administration.

thorough structural understanding of each individual material and the assembled nanosystem.

While conventional small molecule and many biologic drugs are discrete molecules, polyplex systems are associated with complexity and variability. Dispersity in these systems arises during several stages of development. First, the polymer carrier can have a dispersity associated with molecular weight during synthesis. Fortunately, dispersity can be decreased using the many convenient controlled polymerization routes now available. For example, reversible fragmentation chain transfer polymerization (RAFT) and atom transfer radical polymerization (ATRP) techniques allow control in molecular weight, dispersity, and polymerization of functional monomers containing reactive groups.[16] Dispersity can arise during postpolymerization conjugation chemistry as well. For instance, if a targeting group or other chemical label is being added to a material, the number of molecules added per polymer is often difficult to determine and poorly characterized, hindering development. Careful materials design should be used; conjugating functional molecules directly to polymerization initiators or directly as a monomer facilitates careful control in labeling number.

Second, the assembled complexes of polymers with nucleic acid cargos can have dispersity associated with the formed nanocomplexes in terms of particle size, aggregation number (number of nucleic acids and number of polymers), and concentration of free (uncomplexed) polymer in solution. Classic characterization methods such as isothermal titration calorimetry, and laser light scattering should be used to carefully characterize polycation and polyanion binding affinity and polyplex composition, respectively.[17−19] Free polymer in solution is also important to characterize to understand its role in increasing both toxicity and delivery.[20]

Third, variability in the phase behavior and solution conformation of both the polynucleotide and the polymer can cause variability in nanocomplex formation and size, in particular, when solvent composition, ionic strength, and pH variables are considered. For example, a recent study revealed that slight alterations in pDNA morphology in solution (caused only by differing solvent polarity, with everything else consistent) can lead to large differences in polyplex shape ranging from long cylinders to spheres (Figure 2).[21] Understanding the fundamental phase behavior of polyplexes in the solution state is also essential for clinical advancement, yet significantly lacking in this area.

Polymer phase behavior is traditionally characterized in both bulk and solution states under many conditions (temperature, concentration, solvent, ionic strength, pH, etc.),[22] facilitating product development and improvement for a large number of applications. Many characterization techniques collectively aid fundamental studies of polymer phase behavior including dynamic and static light scattering, cryo transmission electron microscopy, small-angle X-ray scattering (SAXS), and small angle neutron scattering (SANS). However, in only a few examples have these techniques been fully applied to understanding complex formation and phase behavior of polyplexes. For example, Prevost et al. recently used a combination of dynamic light scattering, SAXS, and SANS to characterize polyplex structure in high resolution showing that the packing of linear DNA changes from a loose to structured fractal network to finally laterally packed rods with increasing polycation concentration during polyplex formation.[23] A high level of fundamental understanding of material behavior and assembly can be gained from these techniques.

Biotransformation, the understanding of how complex delivery systems behave and change in the biological environment, represents a second frontier. Recent developments illustrate the criticality of both a more detailed understanding of biotransformation and the incorporation of this information in delivery system design. Three major changes have been reported to significantly affect delivery efficiency in vivo: first, the separation of free polymer from cargo-associated polymers; second, interaction with plasma proteins; and finally, destabilization of polyelectrolyte complexes by competitive interaction with other biological macromolecules (Figure 3).

Most in vitro transfection studies using polyplexes formulate these particles at a high excess of cationic polymer relative to anionic nucleic acid. The Wagner and Mely groups showed using two independent methods that polyethylenimine (PEI)/DNA complexes are composed of polymer and nucleic acid mixtures at N/P (nitrogen to phosphate) ratios just above neutrality (N/P < 3) with excess polymer free in solution.[20,24] The excess free polycation that is present in solution is critical for achieving significant levels of gene transfection, reducing interaction of polyplexes with cell surface glycosaminoglycans (GAGs) and also improving endo/lysosomal escape.[25−27] The latter may be especially critical for polymers such as PEI that are hypothesized to escape from endosomes via the concentration-dependent "proton-sponge" effect.[28] The dependence of efficient delivery on either the presence of excess polycation or a critical internalized polycation concentration is

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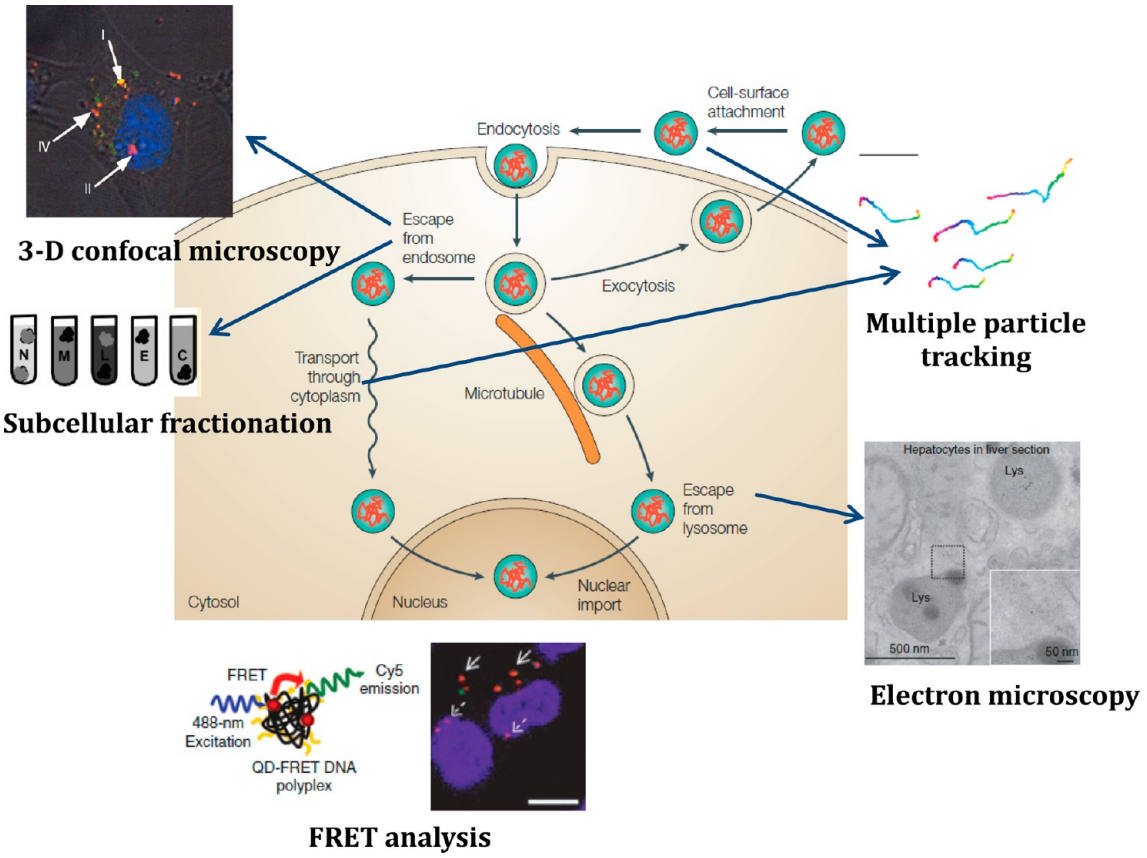

Figure 4. Methods for intracellular trafficking analysis. Figure adapted, compiled, and reprinted with permission from (a) Pack, D. W.; Hoffman, A. S.; Pun, S.; Stayton, P. S. *Nat. Rev. Drug Discov.* **2005**, *4* (7), 581−593; (b) de Bruin, K.; Ruthardt, N.; von Gersdorff, K.; Bausinger, R.; Wagner, E.; Ogris, M.; Brauchle, C. *Mol. Ther.* **2007**, *15* (7), 1297−1305; (c) Akita, H.; Ito, R.; Khalil, I.; Futaki, S.; Harashima, H. *Mol. Ther.* **2004**, *9*, 443−451; (d) Shi, J.; Chou, B.; Choi, J. L.; Ta, A. L.; Pun, S. H. *Mol. Pharmaceutics* **2013**, *10* (6), 2145−2156; (e) Chen, H. H.; Ho, Y. P.; Jiang, X.; Mao, H. Q.; Wang, T. H.; Leong, K. W. *Mol. Ther.* **2008**, *16* (2), 324−332; (f) Gilleron, J.; Querbes, W.; Zeigerer, A.; Borodovsky, A.; Marsico, G.; Schubert, U.; Manygoats, K.; Seifert, S.; Andree, C.; Stoter, M.; Epstein-Barash, H.; Zhang, L.; Koteliansky, V.; Fitzgerald, K.; Fava, E.; Bickle, M.; Kalaidzidis, Y.; Akinc, A.; Maier, M.; Zerial, M. *Nature Biotech.* **2013**, DOI: 10.1038/nbt.2612.

detrimental for in vivo applications where free polycation may be rapidly separated from polyplex. Therefore, formulations that do not require free polycation are likely to be more effective for in vivo use. Multilayer complexes have been shown to effectively deliver plasmid in vitro in the absence of free polycation by using layer-by-layer complexation to increase the polymer to nucleic acid ratio in purified polyplexes.[29] Alternatively, membrane-active polymers that directly disrupt vesicular structures have been shown to be effective in vivo nucleic acid transfer agents.[11,30]

The biodistribution, toxicity, and efficacy of injected delivery vehicles are significantly impacted by plasma protein-binding profiles. Protein binding can trigger opsonization of particles with consequent ingestion by phagocytic cells of the innate immune system, activate the complement cascade, cause toxicity due to particle/protein aggregation, or prevent access to desired cells or molecular targets. Adsorption of complement proteins to synthetic vectors and subsequent activation of the complement system can trigger adverse reactions such as those reported for other nanoparticle carriers.[31] A seminal study by Merkel and co-workers recently showed that PEI conjugates show dose-dependent cardiopulmonary reaction when tested in pigs despite differential complement activation when tested in in vitro assays.[32] Results such as this highlight the importance of full efficacy and toxicity evaluation of potential carriers in relevant in vivo models.

Despite the obvious importance of protein adsorption on the in vivo activity of synthetic polymer vectors, there are few published studies on this topic: most nanoparticle−protein studies have used instead model nanoparticles. A study worth mentioning conducted by Niidome and co-workers identify key cytosolic proteins that bind to PEI/DNA polyplexes by incubating polyplexes with isolated cytosol from mouse liver, precipitating the complexes, and analyzing bound proteins through 2-D gel electrophoresis and peptide mass fingerprinting analysis.[33] The reader is referred to an excellent recent review that discusses the effect of protein binding on the pharmacokinetics and toxicity of synthetic drug delivery systems.[34]

For in vivo use, polymer carriers both protect the nucleic acid from nuclease degradation in the extracellular environment and release active cargo in the appropriate intracellular space. Premature release of nucleic acid results in rapid clearance and poor delivery efficiency. PEGylation of polyplexes reduces the extent of protein adsorption but has also been shown to reduce serum stability of complexes in vivo.[35] In addition, extracellular matrix (ECM) can potently compete for polycation binding. Both liver ECM and the glomerular basement membrane in kidney have been shown to destabilize polyplexes by binding to polycation and releasing free nucleic acid before cellular entry.[36−38] Some approaches to increase extracellular stability of polymeric carriers are inclusion of hydrophobic domains in

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polycations,[39,40] incorporation of nucleic acid-intercalating molecules into polymers,[41] and direct conjugation of nucleic acid cargo to carrier.[11,30,42]

Upon reaching the target cell, polymeric delivery vectors must facilitate cellular uptake and nucleic acid delivery to the cytoplasm (for oligonucleotides) or nucleus (for plasmids). While cell interaction is necessary for internalization, selective membrane interactions may be important in decoupling the often noted correlation between delivery efficiency and cytotoxicity. For example, while permeabilization of endosomal and nuclear membranes by polycations such as PEI might contribute toward effective nuclear delivery,[35,43] permeabilization of the plasma and mitochondrial membranes is likely to cause cellular toxicity[44,45] and lead to apoptosis.[43] Polymers with masked or triggered membrane-active domains can mitigate toxicity without compromising delivery efficiency by revealing membrane-permeabilizing activity only within certain intracellular locations.[11,46]

The method of nanoparticle internalization is now appreciated to affect intracellular distribution, trafficking, and ultimately delivery efficiency (Figure 4). For example, several reports now indicate that caveolae-mediated uptake of synthetic carriers may be more productive than clathrin-mediated uptake, which shuttles cargo rapidly toward degradatory compartments.[47] The differential trafficking pathways result in differences in expression (plasmid) or downregulation (oligos) kinetics as well as in cytotoxicity.[48] The various internalization pathways and their effect on gene delivery vectors was recently covered in a review by El-Sayed and Harashima.[49] This review also includes a wise warning against relying on inhibitor studies alone to determine internalization pathways due to pharmacological side effects from these drugs.

While efficient cytosolic or nuclear delivery is desired, it is difficult to achieve. Understanding the correlation between polymer structure and intracellular distribution is key to designing more effective carriers. Single or multiple particle tracking reveals the intracellular pathway and velocities of carriers. One interesting discovery from particle tracking analysis revealed that EGF targeting of synthetic vectors increased the rate of initial vector internalization.[50] Intracellular distribution has been quantified by both quantitative 3-D confocal laser scanning microscopy[51] and by subcellular fractionation combined with either radiolabeling or quantitative PCR.[52,53] Electron microscopy has been used to image detailed interactions of synthetic vehicles with cellular compartments and even to quantify low levels of endosomal escape not detectable by fluorescence microscopy.[54,55] Finally, fluorescent resonance energy transfer (FRET) between dual-labeled polymer/nucleic acid has been used to monitor polyplex unpackaging in live cells.[56] Currently, intracellular trafficking studies have been primarily investigated in 2-D cultured, transformed cells. There remains a need to expand these studies into more relevant in vitro 3-D models and into in vivo situations.

Conclusion: Recent development milestones suggest the field of nucleic acid therapeutics may be about to experience a renaissance. This could lead to greater future opportunity for polymeric delivery systems, which are an inherently attractive platform of materials. However, the enhanced function provided by these systems also come with a certain degree of complexity with respect to both physicochemical characteristics and biological interactions. Systematic optimization requires input from each of these, and the examples detailed above illustrate how sophisticated analytical and biological tools can provide that input. Nevertheless, advanced analytics are often difficult to access, and the typical polymer lab is not equipped for detailed biological characterization. A higher level of integrated cross-disciplinary collaboration will be required to better position polymer-based nucleic acid delivery systems for their ultimate aim, the enablement of important new medicines for the benefit of patients who desperately need them.

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Notes

The authors declare no competing financial interest.

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