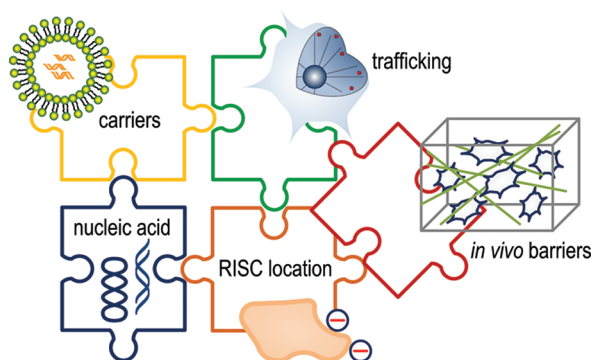# Nucleic Acid Delivery: The Missing Pieces of the Puzzle?

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CONSPECTUS

The delivery of genes or RNA interference (RNAi) agents can increase or decrease the expression of virtually any protein in a cell, and this process opens the path for cures to most diseases that afflict humans. However, the high molecular weight, anionic nature, and instability of nucleic acids in the presence of enzymes pose major obstacles to their delivery and frustrates their use as human therapies.

This Account describes current ideas about the mechanisms in nonviral nucleic acid delivery and how lipidic and polymeric carriers can overcome some of the critical barriers to delivery. Over the last 20 years, researchers have developed a multitude of polymeric and lipidic vectors, but only a small fraction of these have progressed into clinical trials. None of these vectors has received FDA approval, which indicates that the current vectors do not yet have suitable properties for effective *in vivo* nucleic acid delivery.

Nucleic acid delivery is a multistep process and inefficiencies at any stage result in a dramatic decrease in gene delivery or gene silencing. However, the majority of studies investigating synthetic vectors focus solely on optimization of endosomal escape. A small number of studies address how to improve uptake via targeted delivery, and an even smaller fraction examine the intracellular fate of the delivery systems and nucleic acid cargo. The internalization of genes into the cell nucleus remains an inefficient and mysterious process. In the case of DNA delivery, strategies are needed to increase and accelerate the migration of DNA through the cytoplasm and transport it through the nuclear membrane.

siRNA delivery involves fewer barriers. siRNA is more readily released from the carrier and more resistant to enzymatic degradation, and its target is in the cytoplasm; hence, siRNA delivery systems are becoming a clinical reality. With regard to siRNA therapy, the exact cytoplasmic location of RNA-induced silencing complex (RISC) formation and activity is unknown, which makes specific targeting of the RISC for more efficient delivery difficult. Furthermore, we would like to identify the factors that favor the binding of siRNA to Ago-2. If we could understand how the half-life of siRNA and Ago-2/siRNA complex in the cytoplasm can be modulated without interfering with RISC functions that are essential for normal cell activity, we could increase siRNA delivery efficiency.

In this Account, we review the current synthetic vectors and propose alternative strategies in a few cases. We also suggest how certain cellular mechanisms might be exploited to improve gene transfection and silencing. Finally, we discuss whether some carriers that deliver the siRNA to cells could also repackage the siRNA into exosomes. The exosomes would then transport the siRNA into a subsequent population of cells that manifest the siRNA effect. This piggy-back mechanism may be responsible for reported deep tissue siRNA effects using certain carriers.

Introduction

The application of gene and RNAi therapy in the clinic requires safe and efficient vectors. To date, the two main approaches for nucleic acid therapy are based on viral and nonviral vectors. There are a number of safety concerns associated with viral vectors: risks of induced immune responses, unwanted mutagenesis, and cancer. Thus, tremendous effort has gone into developing nonviral vectors based on lipidic or polymeric carriers. In 1965, Vaheri and Pagano introduced diethylaminoethyl modified dextran, the first polymer for gene delivery.[1] In 1987, Felgner introduced DOTMA, the first cationic lipid for DNA transfection.[2] Since then, a multitude of different lipids and polymers have been developed. These, along with technologies for encapsulating nucleic acids in nanosized vesicles,[3] have been extensively reviewed and thus will not be discussed in this Account. By encapsulating, complexing, or binding the nucleic acid into particles, these vectors are able to protect the nucleic acid from degradation and deliver it into certain cellular compartments.

While the optimization of biomaterials for gene delivery was ongoing, the discovery of small interfering RNA (siRNA) by Fire and Mello in 1998 introduced new prospects for the treatment of incurable diseases.[4] Sequence-specific siRNA molecules were shown to target complementary mRNA and induce silencing of the encoded protein. A decade later, tremendous progress has been made in the field of gene silencing, with several RNAi therapeutics in clinical trials. Among the RNAi therapeutics currently being assessed in the clinic are lipid-based or polymeric-based formulations for the systemic treatment of TTR-mediated amyloidosis[5] and cancer.[6−8] Despite these successes, improvements still need to be made to nonviral gene and siRNA vectors.

This Account will focus on the mechanistic aspects involved in lipid-based and polymeric-based gene and siRNA delivery. We discuss the shortcomings of current practices and propose alternative mechanistic approaches that may offer potential means of improving nonviral delivery with synthetic vectors.

Changes in the Composition of Lipoplexes or Polyplexes upon *in Vivo* Administration

For reproducible *in vitro* and *in vivo* gene and siRNA delivery, the formulation of liposomes, lipoplexes, and polyplexes containing nucleic acids requires precise composition of the transfection reagents. Although several studies have investigated how blood components can destabilize lipidic and polymeric nanoparticles,[9,10] little is known about the final composition of the delivery systems that mediates gene delivery or silencing in the cells. The composition of polyplexes and lipoplexes undergoes constant changes after systemic administration into the bloodstream. Excessive polymer chains or liposome components not strongly attached to the complexes[11,12] will be shed from the particles, and new components, such as lipoproteins, can adhere to the surface of the complexes. This not only can lead to destabilization of the particles but also can alter the biodistribution or promote clearance *in vivo*.[13] Understanding how polyplex and lipoplex composition changes at each stage of delivery *in vivo* (at the administration site, during circulation in the bloodstream, in the extracellular matrix of organs and tissues, and finally upon entry into target cells) could potentially allow for the design of synthetic vector systems with higher stability. Although single particle tracking in whole animals is not yet possible with the current resolution of luminescence and fluorescence imaging, advances in multiphoton excitation microscopy or confocal microscopy may allow lipoplex and polyplex composition to be fully characterized from administration to final destination in the target cells.

Controlling Intracellular Uptake by Less Explored Mechanisms

Nonviral, synthetic vectors are shown to enter cells by endocytosis. This can be divided into clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis, and clathrin- and caveolae-independent endocytosis. In contrast to viruses, synthetic vectors still suffer from low intracellular uptake and low transfection efficiency. One strategy to increase intracellular uptake is to attach targeting ligands to nonviral vectors to induce receptor-mediated endocytosis. These ligands mostly target nutrient uptake receptors such as transferrin, folate, and low-density lipoprotein receptors (LDL).[12,13] Although this leads to improvement in cellular uptake and gene delivery, other more powerful strategies should be taken into consideration. Invading viruses often activate signaling cascades of cells to induce their intracellular uptake.[14,15] Enhanced uptake is triggered by binding to signaling receptors that regulate the endocytic machinery. Influenza viruses, for example, initiate the formation of new clathrin-coated pits (CCP) at their site of binding. There is 20 times as much CCP formation at the site of virus binding than at other sites of the cells.[16,17] Vaccinia viruses trigger their uptake via macropinocytosis by activating kinases and GTPases.[18] The challenge remains to engineer synthetic vectors with ligands that bind to signaling
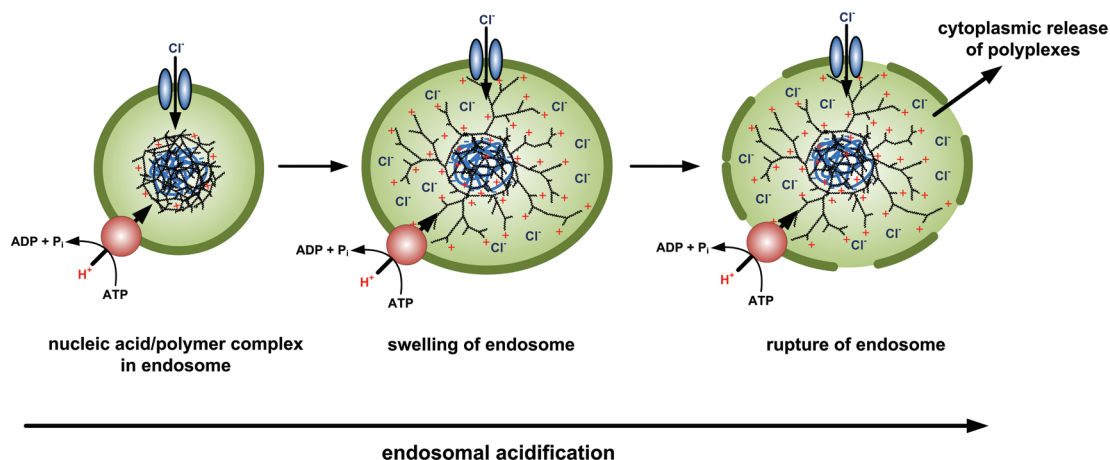
FIGURE 1. Endosomal rupture is mediated by polymers with a buffer capacity in the endosomal pH range which can trigger (a) the proton-sponge effect and (b) polymer swelling according to the umbrella hypothesis.

receptors to enhance endocytosis without inducing an immune response, a severe effect often caused by viruses. The type of ligands can also be used to influence intracellular fate and to sort vectors into two distinct populations of early endosomes: a fast maturing population that is transported rapidly on microtubules toward the perinuclear region and a static population that hardly moves.[19] The population that is targeted will determine how quickly the vectors will be transported to the perinuclear region (important aspect for DNA delivery) or how long they will stay at the cell periphery. This might have consequences on the efficiency of gene silencing or expression. Influenza viruses preferentially choose the fast maturing populations, most likely as a means of transportation to the perinuclear region as fast as possible.[19]

Endosomal Escape

Polymeric Vectors: The Proton Sponge Effect and the Umbrella Hypothesis. Once taken up into the cells, polyplexes have to escape from the endosomal pathway to release the siRNA or DNA into the cytoplasm. The endosomal pathway starts with the early endosomes, which become progressively acidic as they mature into late endosomes. The proton pump vacuolar ATPase generates acidification by accumulating protons in the vesicle until the pH drops to pH 5−6. Usually this would end with the fusion of the late endosomes with the lysosomes, where the pH reaches 4−5 and the content would be degraded by enzymes. The ability of many cationic polymers to mediate efficient nucleic acid delivery is mainly attributed to their strong buffering capacity in the pH range from 5 to 7. It is hypothesized that these strongly buffering polyamines

prevent acidification of the endosomes by acting as "proton sponges" (Figure 1). This leads to an increase in proton influx followed by an enhanced accumulation of Cl^- and osmotic swelling.[20−22]

An extension of the proton-sponge effect was introduced with the umbrella hypothesis, which describes the ability of polymers to expand volumetrically when protonated at lower pH (pH 5−6). According to the "umbrella effect" the polymer unfolds from a collapsed state into an extended conformation after protonation of the amine groups (Figure 2). When the polymer forms complexes with DNA, compaction of both into small particles based on electrostatic interactions occur. After being taken up into cells and transported along the endosomal pathway, excess protons in the endosome lead to protonation of the tertiary amines in the interior of the polyplexes. Due to the electrostatic repulsion of the neighboring charged amine groups, the terminal branches of the polymer spread out and adopt a fully extended conformation if not restrained by steric hindrances. Tang et al. have shown that this increase in volume and space caused by polymer swelling contributes to endosomal escape of the polyplexes. This was shown using a set of intact and fractured polyamidoamine (PAMAM) dendrimers that differ in their degree of flexibility and their ability to volumetrically expand with decreasing pH. It was found that fractured dendrimers with optimal flexibility of their branches mediate superior transfection efficiency compared to intact dendrimers that are sterically constrained. Taken together, the proton-sponge hypothesis and the umbrella effect suggest that the requirements for endosomal escape of cationic polymers are titratable amine groups at pH 5−7 and a highly flexible
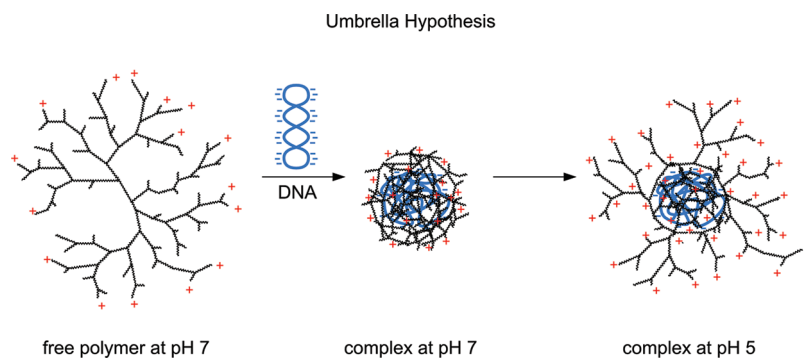
Umbrella Hypothesis

free polymer at pH 7   complex at pH 7   complex at pH 5

FIGURE 2. Schematic representation of the umbrella hypothesis induced by polymer swelling. Cationic polymers form a complex with negatively charged nucleic acid. At lower pH in the endosomes, the complex partially unfolds. Due to the protonation of the terminal amine groups and electrostatic repulsion, the terminal branches of the polymer spread out and adopt a fully extended conformation.
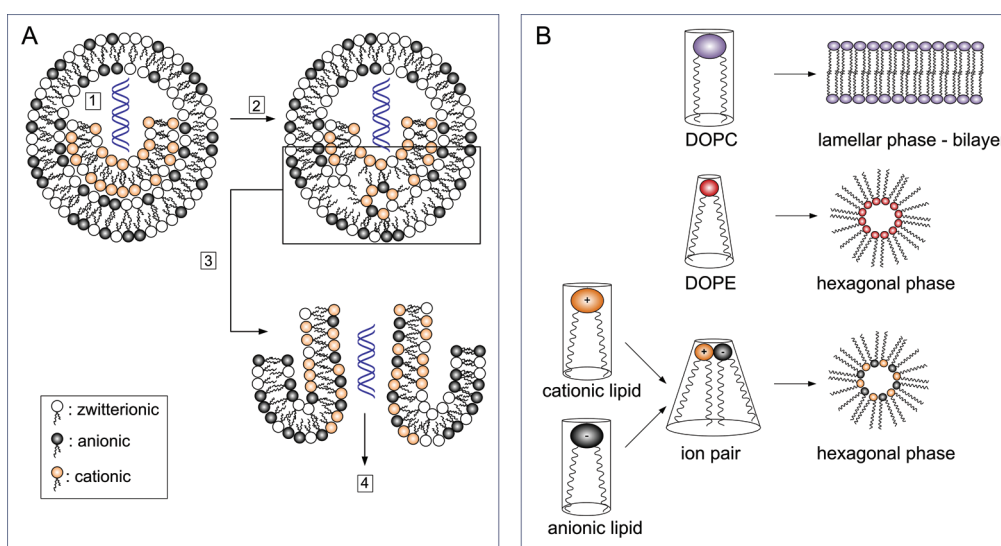

FIGURE 3. (A) Proposed mechanisms of cationic lipid/nucleic acid complexes after endocytosis along the endosomal pathway: (1) cationic lipids interact with anionic lipids in endosomes by forming ion-pairs; (2) the lipid bilayer is destabilized; (3) the hexagonal phase is formed; (4) the nucleic acid is released into the cytoplasm. (B) The formation of the hexagonal phase is triggered by lipid molecules with a cone shape shown here for DOPE and a charged lipid ion-pair that transits from a cylindrical shape into a molecular cone shape. Helper lipids (i.e., DOPC) with a cylindrical shape mediate bilayer stabilization.[8,24,26]

structure that can increase in volume after protonation in the endosome.[23]

Lipidic Vectors: Membrane Destabilization, Ion Pair Formation and Nucleic Acid Release. The escape of cationic lipidic vectors from endosomes is mainly mediated by their interactions with anionic phospholipids from the endosomes and their ability to transit from the lamellar to the hexagonal phase. This model introduced by Xu and Szoka identified factors important for cytoplasmic delivery (Figure 3).[24] First, the nucleic acid/lipid complexes are transferred into endosomal vesicles where the close proximity of the lipoplex and endosomal membrane promotes an electrostatic interaction between the cationic lipids in the lipoplex and anionic lipids in the endosomal membrane. The lipid bilayers are destabilized due to the formation of

cationic−anionic ion pairs, and the nucleic acid is released from the lipoplex. The ion pairs adopt a molecular cone shape, which promotes the transition from a lamellar phase to an inverted hexagonal phase with delivery of the nucleic acid into the cytoplasm.[25,26] The ability of cationic liposomal systems to mediate transition into the hexagonal phase is generally triggered by lipids whose molecular shapes exhibit high curvature. This can be controlled and enhanced by the following factors: (i) the geometry of the headgroup and the lipid tail of the cationic lipid; (ii) the addition of helper lipids, such as DOPE and cholesterol, to further enhance the adoption of a nonbilayer structure; and (iii) the bilayer-to-H_{II} transition temperature of the liposomal formulation.[8,25,27]

The same endosomal escape mechanisms proposed for cationic liposomes can be applied to ionizable lipids with
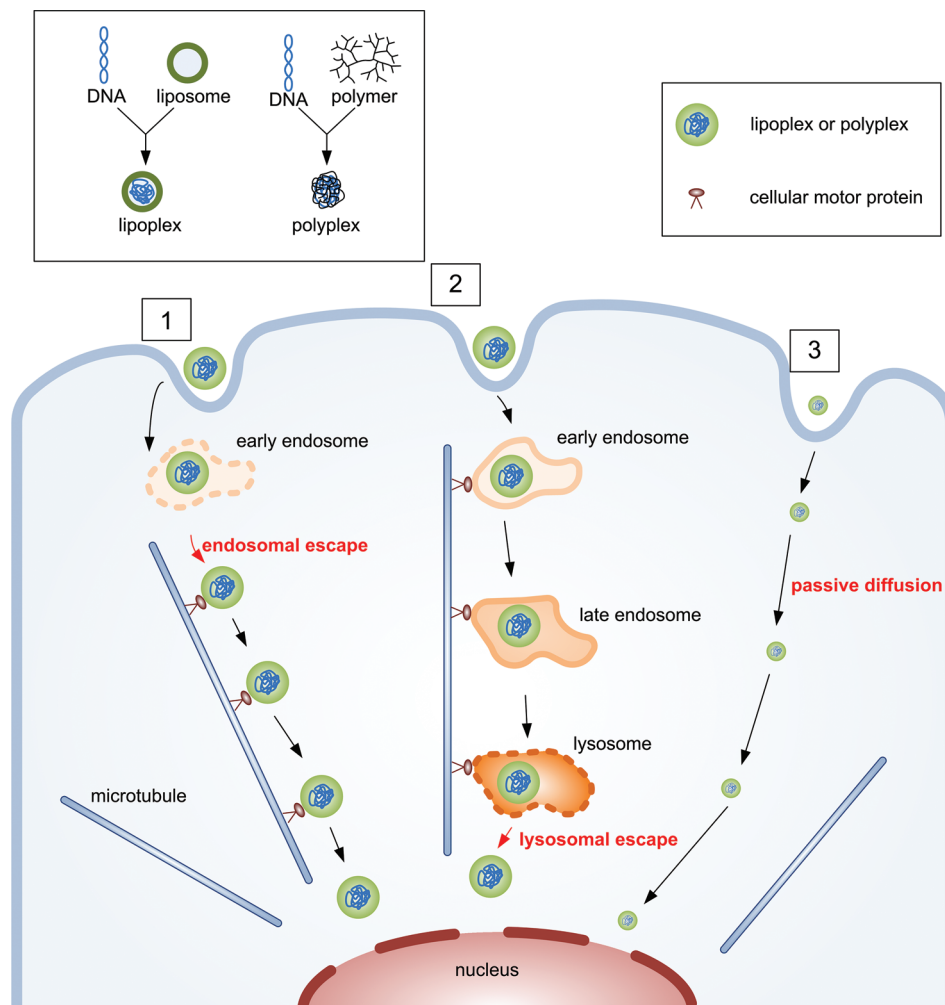
FIGURE 4. Cytoplasmic transport of synthetic vectors. (1) After endocytosis, synthetic vectors are released from the early endosomes at the cell periphery into the cytoplasm. For efficient transport of synthetic vectors through the cytoplasm, a mechanism for facilitating MT transport would be required. (2) Lysosomes are transported along the MT pathway to the perinuclear region. Synthetic vectors designed to exploit the lysosomal pathway have to be able to protect the DNA against the degrading enzymes in the lysosomes. (3) To migrate efficiently through the cytoplasm by passive diffusion, synthetic vectors need to be smaller than 30 nm.

DLin−DMA headgroups. The headgroup of DLin−DMA lipids contains tertiary amines (pka 6−7). These are protonable at lower pH and become cationic in the endosomes. Due to their neutral charge at physiological pH, they appear to be less immunoreactive than cationic lipids with a fixed charge such as DOTAP.[8]

DNA Delivery: Cytoplasmic Transport to the Nucleus and the Impact of Spatiotemporal Factors

Endosomal escape is often believed to be the most critical step in gene delivery, and vectors are designed to mediate endosomal release at the earliest time possible to avoid enzymatic degradation in the late endosomes or lysosomes. A closer look at the endosomal pathway and the vectors' journey from the cell periphery toward the nucleus,

however, indicates that particles or DNA released at the perinuclear region have the best chance of entering the nucleus.[28,29] The transport of endocytic vesicles is organized by a network of microtubules (MTs). MTs radiate from a MT organizing center (MTOC) near the nucleus toward the periphery of the cells. Transport along the MTs is regulated by motor proteins such as dynein and kinesin.[30] Particles that escape from the endosomes prematurely close to the cell membrane have to travel the longest distance before reaching the nucleus. Passive diffusion of particles or DNA through the highly viscous cytoplasm is slow and constrained by the high concentrations of proteins in the cell. Prolonged exposure of particles or DNA to the cytoplasmic environment can lead to destabilization of the complexes and degradation of the DNA by DNase.[31] However, as long as particles stay inside endosomes, they can exploit the

endosomal pathway to receive active directional transport along the MTs toward the nucleus (Figure 4).

The optimal time for endosomal escape would be when the complexes reach the perinuclear region, right before the lysosomes start to accumulate into clusters.[32] When the lysosomes concentrate and aggregate, the speed of diffusion decreases and the escape of complexes from clustered lysosomes becomes more difficult.[28] In contrast to viruses, current synthetic vectors are not capable of fully utilizing the cell machinery to their advantage: (1) the timing and location of endosomal escape of synthetic vectors is not optimized; (2) once released from the endosomes, synthetic vectors are not capable of utilizing motor proteins for active transport on MTs; (3) because the complexes formed by synthetic vectors are large (80–500 nm), passive diffusion through the cytoplasm is slow.

One way to define the optimal time-point for endosomal escape is to incorporate a pH-sensor into the synthetic vectors, a mechanism used by adenovirus serotype 7 (Ad7). It was suggested that the fiber protein of Ad7 serves as a pH sensor and triggers lysosomal escape at $\sim$ pH 5. This ensures that Ad7 accumulates in the perinuclear region, enabling efficient nuclear entry.[33] Vectors designed to target the lysosomal pathway for gene delivery need to protect the DNA against degrading enzymes in the harsh environment of the lysosomes.

If the synthetic vectors are designed to escape the endosomes at an early time-point at the cell periphery, a pathway exploited by adenovirus serotype 5, incorporating a mechanism facilitating MT transport would improve migration through the cytoplasm. Ligands with high affinity to dynein as a motor protein (adenovirus hexon monomers are one example[34]) may be capable of mediating active transport along the MT through the cytoplasm. Directional transport could be further accelerated by stimulating the signaling cascades of cells such as protein kinase A (PKA) and P38/ MAPK pathways.[35] Adenoviruses utilize this mechanism to boost minus-end motility of the MT network for transport toward the nuclear region.

According to Stokes−Einstein, diffusion is a function of diameter; hence smaller particles move faster than larger ones. Thus, another way to optimize gene delivery to the nucleus would be to decrease the size of the particles to increase the velocity of passive diffusion through the cytoplasm. It has been shown that viruses and synthetic particles, which are small in diameter (<30 nm), are able to move through the cytoplasm independently from the MT network.[36,37] Since DNA plasmids are large in size, packaging and complexing them into small particles can be difficult and would require more sophisticated encapsulation methods. The passage through the nuclear pore complex (NPC) is another critical barrier in gene delivery. Strategies to overcome the NPC have been thoroughly described by Lam et al.[38]

Once inside the nucleus, the copy number of DNA and its accessibility for the transcription machinery determines the level of transgene expression. Studies have shown that the reported minimum number of plasmids delivered to the nucleus required for measurable transgene expression depends on the type of vectors and varies between 75 and 4000 plasmid copies.[39,40] Comparisons between different delivery vehicles showed that higher copy numbers of DNA molecules in the nucleus do not necessarily correlate with higher transfection efficiency. At similar plasmid/nucleus copies, lipofectamine mediated 10-fold higher transfection efficiency than PEI. This suggests that the DNA delivered by PEI is biologically less active than the DNA delivered by lipofectamine. It also emphasizes that a deeper understanding of the nuclear events in gene delivery is required for future progress.

With advances in proteomics, single particle tracking, and electron microscopy, it should be possible to fully identify every single component and protein participating in molecular motor binding and the MT transport in the near future. This will aid in a mechanism-based design of synthetic gene delivery systems.

Suitability of 2D *in Vitro* Cell Cultures for Predictability of *in Vivo* Results

Vectors mediating high transfection efficiency *in vitro* often fail to achieve similar results *in vivo*. One possible reason for this is that lipidic and polymeric vectors are optimized *in vitro* using two-dimensional (2D) cultures that lack extracellular *in vivo* barriers and do not realistically reflect *in vivo* conditions. While cells *in vitro* grow in monolayers, cells *in vivo* grow in 3D tissue layers held together by the extracellular matrix. Vectors delivered *in vivo* by systemic administration not only have to withstand the bloodstream but also have to overcome the cellular matrix to reach all cell layers of the tissue. While large particles seem to have an advantage in *in vitro* transfection due to a sedimentation effect on cells, efficient delivery of particles deep into organs requires particles <100 nm. Small particles (40 nm) diffuse faster and more effectively in the extracellular matrix and inner layers of tissues, whereas larger particles (>100 nm) are restricted by steric hindrance.[41]

Another aspect that influences nucleic acid delivery is the difference in cell geometry and morphology between *in vitro* and *in vivo* environments. Cells grown on 2D cultures are
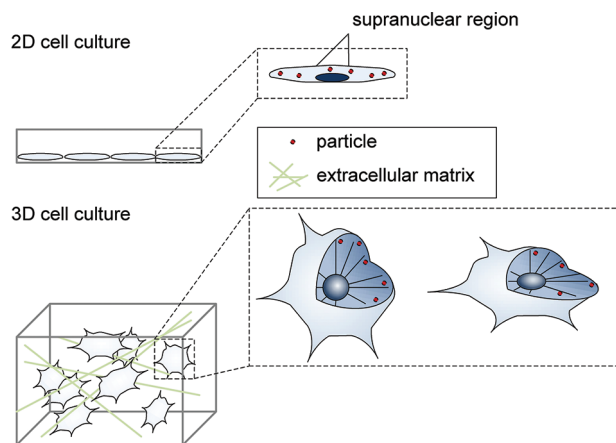
FIGURE 5. Comparison of cells grown under 2D conditions *in vitro* and cells under *in vivo* conditions: Cells cultured under 2D conditions grow in a monolayer and exhibit a flattened morphology. Particles taken up in the supranuclear region have the shortest distance to the nucleus, whereas particles taken up in the cytoplasmic area have to travel a longer distance. In contrast, cells grown under 3D cell culture conditions exhibit a more circular geometry, where no points at the cell membrane are particularly close to the cell nucleus.[28]

monolayers and usually adapt a flattened morphology (Figure 5). This results in cells with reduced thicknesses but larger widths and lengths. Particles that are taken up directly above the nucleus (supranuclear region) have the shortest transport distance to the nucleus ($4-8$ μm) and hence a greater chance of delivery success. Dinh et al. have shown that size and morphology of cells have a large influence on the spatiotemporal distribution of carriers and on transfection efficiency (transfection of flattened cells is up to an order of magnitude greater than spherical cells for the same input of DNA).[28] The spatiotemporal distribution of carriers, however, determines the optimal time for endosomal escape and the optimal intracellular pathway.[28] Because most cells *in vivo* are more spherical and not flattened, no particular region of the cellular membrane is especially close to the nucleus. Consequently, optimization of carriers under *in vitro* conditions may not be applicable to *in vivo* conditions. Three-dimensional *in vitro* models that culture cells in an extracellular matrix and that take the spatial organization of cells into account may present a more viable cell culture method for the optimization of synthetic vectors.[42]

siRNA Delivery: Targeting to Specific Locations of RISC Activity and Assembly

The extensive investigations and optimization of DNA delivery systems has translated into rapid progress being made for siRNA delivery. However, similar to DNA delivery,

a major limitation of siRNA-based therapeutics is the inability to deliver a significant fraction of the dose to the target site after intravenous administration. The most easily accessed organ is the liver, and even in this tissue recent studies have shown that less than 0.1% of the total siRNA dose reaches the target site, the remaining 99.9% of the siRNA is being degraded or lost on its way to the cytosol of the target cells.[43,44] Hence, strategies that do more than optimize endosomal escape are needed to further increase gene silencing efficiency.

One strategy is to better identify the cytoplasmic location of the RNA-induced silencing complex (RISC), which contains different argonaute (Ago) family proteins responsible for mRNA knockdown. After escaping from the endosomes, the siRNA needs to be released from the polymeric or lipidic particles into the cytosol to be able to bind to the RISC. Upon loading into RISC, the passenger strand of the siRNA is degraded. The guide strand base-pairs with the target, complementary mRNA sequence and mediates its cleavage and degradation.[4] Of the different Ago family proteins, only Ago2 is capable of catalyzing mRNA degradation.[45] Although great progress has been made in characterizing and identifying the main components of RISC, little is known about where in the cytoplasm RISC assembly and activity takes place. With current synthetic vectors, siRNA is released into some random locations of the cytoplasm. The cytoplasm constitutes a large part of the entire cell with different organelles and compartments including the golgi apparatus, endoplasmic reticulum, mitochondria, endocytic vesicles, and perinuclear region. Thus, knowledge about the exact intracellular location of RISC and Ago2 would provide powerful insights into how to effectively target the siRNA to the site of action. Several groups have shown that RISC and Ago2 are not randomly distributed in the cytoplasm but are concentrated in specific centers of the cytoplasm.[46−48] Sen et al. demonstrated that Ago2, a main component of RISC, is localized to cytoplasmic P-bodies, regions where mRNA degradation occurs. They suggest two models for the RISC location. In the first, Ago2/RISC stays permanently in P-bodies (Figure 6). In the second model, the Ago2/RISC binds to the siRNA and mRNA in the cytoplasm and serves as a shuttle between the cytoplasm and P-bodies. Where the Ago2/RISC binding of siRNA occurs and whether the process is signaling-based or stochastic-based are unknown factors. More recent studies suggest that the endosomal trafficking pathway is involved in silencing by small RNAs.[47] They found Ago2 and RISC colocalized with GW-bodies associated with late endosomes called multivesicular bodies
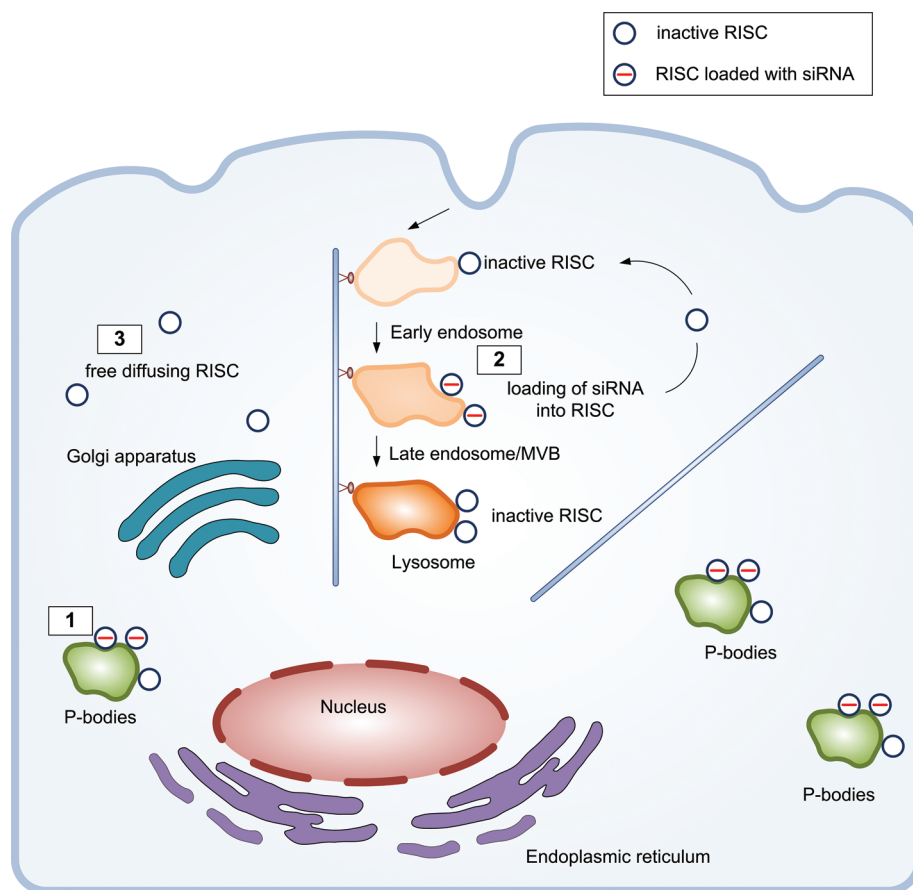
FIGURE 6. Models of RISC location and activity in the cytoplasm: (1) RISC is concentrated in cytoplasmic bodies, such as P-bodies; (2) RISC is associated with late endosomes (MVBs); (3) RISCs freely diffuse in the cytoplasm.

(MVB).[46,47,49] Lee et al. suggest that MVBs control gene silencing by promoting the turnover of RISC and its loading competence for miRNA or siRNA.[47] Thus, in analogy to DNA targeting into the nucleus, siRNA targeting into the RISC rather than depending upon diffusion in the cytoplasm might enhance activity.

Another issue that needs to be addressed is that non-proportional amounts of siRNA are required for increased levels of knockdown: in a liver targeting mouse model, 370 copies of siRNA mediated 50% knockdown, whereas 2200 copies of siRNA were required for 80% gene silencing.[43] Several factors will need to be investigated to understand this: siRNA delivery needs to be optimized with regard to the kinetics of siRNA loading into Ago2; the half-life of Ago2/siRNA complexes must be identified; the factors that determine the binding affinity of siRNA to Ago2 should be explored.

Exosomal Pathway as Potential Means for Deep Tissue Delivery of Nucleic Acids

One of the limitations of current nucleic acid delivery systems is their inability to deeply penetrate tissues and organs such as solid tumors and brain. Typically, only the outer layer of cells can be reached and transfected thus resulting in poor therapeutic efficacy. The Epstein–Barr Virus (EBV), a human tumor pathogen, seems to overcome this by hijacking the exosomes for intercellular communication in the tumor microenvironment. Exosomes are small membrane vesicles (40–200 nm) of endocytic origin. After being released into the extracellular environment, they can fuse with neighboring cells because of the presence of cell recognition molecules on their surface.[50] Hence, it is hypothesized that by going through several cycles of cell internalization and release, exosomes are able to cross several layers of tissues.[51] Exosomes serve as shuttles of mRNA, small RNAs (miRNA), and signaling factors between cells. They can be secreted by a number of cells, including tumor cells, dendritic cells, B cells, T cells, epithelial cells, and neurons.[50,52] Furthermore, it has been suggested that exosomes may be used by viruses (such as EBV and HIV) and other pathogens (such as prions) to promote infectivity.[53] Recently it was observed that EBV repackages its viral miRNA and viral-encoded proteins into exosomes to infect neighboring cells in

the tumor microenvironment and to manipulate tumor growth.[54]

This process might already be responsible for the observed silencing effect of certain siRNA carriers. If not, synthetic vectors might be engineered to exploit the pathway to create carriers capable of delivering nucleic acids from the periphery to the center of tumor tissues or other organs. One potential way to utilize the exosomal pathway would be to repackage liposomal nucleic acid into exosomes. This could be achieved by targeting membrane proteins specific to exosomes such as tetraspanins and annexins and initiating a fusion event between the liposomes and exosomes.[55] A deeper understanding of the biogenesis of exosomes and the factors that control their formation may open possibilities for engineering vectors capable of piggybacking on the exosomal pathway.

Summary and Perspectives

While tremendous progress on nonviral vectors for DNA and siRNA delivery has been made over the past three decades, clinical advances have been slow to arrive. A major limitation of current systems *in vivo* is their inability to effectively deliver a high dose to the target site. Thus, we believe the following developments, which can be divided into cellular transport and *in vivo* cellular accessibility, will be necessary to advance gene and RNAi therapy in humans:

- New strategies to enhance uptake of particles into cells should be developed. The signaling receptors and cascades that regulate the endocytotic machinery of cells might be exploitable for this end.
- A more detailed understanding of RISC functioning must be developed to improve siRNA delivery. This will entail identifying the exact cytoplasmic location of RISC formation and RISC activity as well as the factors regulating RISC loading, RISC turnover, and the affinity of siRNA to Ago2.
- The passive diffusion of particles or nucleic acids through the cytoplasm needs to be minimized. Novel mechanisms that utilize the active transport of particles or nucleic acids through the cytoplasm along the microtubules are required.
- More representative *in vitro* cell culture models that precisely predict *in vivo* nucleic acid transfer should be developed.
- For the precise engineering of nucleic acid delivery systems, new *in vivo* imaging technologies are required so that changes in the composition of particles can be characterized in circulation after intravenous administration.

- Novel strategies for the deep tissue delivery of nucleic acids must be identified. Repackaging nucleic acids into exosomes of circulating cells in the body may provide a way of accomplishing this.
- A challenge remains for researchers to develop systems with few components capable of performing multiple functions (such as nucleic acid encapsulation, targeting, and transfer) in parallel. This is especially important for product manufacturing given the analytical and stability challenges that exist for even the current nontargeted nanomedicines, such as the FDA-approved Doxil.[56]

The optimization of synthetic delivery systems has been largely based on empirical approaches. A better understanding of the intracellular mechanisms and molecular bases of nucleic acid transfer, however, will enable a more rational and mechanism-based design of vectors. This knowledge will provide the missing pieces of the puzzle for effective nucleic acid delivery in animals and humans.

BIOGRAPHICAL INFORMATION

Juliane Nguyen received her pharmacy degree and her Ph.D. in Pharmaceutical Sciences from the Philipps-Universität Marburg (Germany). She is currently a postdoctoral fellow in the research group of Professor Dr. Szoka, University of California, San Francisco. Her research interests include nanoparticle-based nucleic acid delivery systems using biodegradable polymeric and lipidic carriers.

Francis C. Szoka received his M.S. (Microbiology) from the University of Maryland and his Ph.D. (Biochemistry) from the State University of New York (SUNY). His major research interests include applying chemical, biochemical, and biophysical approaches to the study of membrane fusion/destabilization and developing drug/gene delivery systems based upon defined physicochemical mechanisms of membrane destabilization and intracellular trafficking.

FOOTNOTES

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The authors declare no competing financial interest.

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