

# Systemic Therapeutic Gene Delivery for Cancer: Crafting Paris' Arrow

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**Abstract:** Tremendous strides have been made in proteogenomics and RNA interference technologies. Hence “personalized” cancer gene therapy has become a foreseeable rather than a predictable reality. Currently, the lack of an optimized, systemic gene delivery vehicle remains a key limiting factor for developing effective treatment applications. Since their introduction by Felgner in 1987, cationic lipids have been an attractive consideration for gene delivery, in view of their biocompatibility, biodegradability, low toxicity, and low immunogenicity. Successful *in vivo* transgene expression by cationic lipid- or cationic polymer-based delivery depends critically on a long circulating half life (>48 h), a definable systemic biodistribution with target-specific cancer localization, and efficient cell entry and internalization. Ideally, the agent should have a hydrophobic, stabilized core that ensures integrity of the therapeutic entity *in vivo*, a biocompatible, neutrally charged shell ( $\zeta$  potential of  $\sim \pm 10$  mv) for enhanced, “stealth” circulation, and a suitable size ( $\sim 50$ -200 nm in diameter) for access into the tumor neovasculature and reduced reticuloendothelial system (RES) uptake. “Smart” receptor-targeting moieties can redirect intracellular trafficking. Additional engineered features have also been incorporated to minimize lysosomal degradation (membrane fusogenic lipids or proton sponge), promote endosomal escape into cytoplasm (cell penetrating peptides, triblock copolymer construction), and enhance nuclear entry and activate the endogenous transcriptional machinery (inclusion of a nuclear localization signal). Improvements in each of these respective areas of study have converged to yield promising *in vivo* results.

**Keywords:** Liposome, small RNA, micelles, nanoparticles, enhanced penetration and retention (EPR), experimental cancer gene therapy, block polymers.

## INTRODUCTION

Most traditional chemotherapeutics have a compromised efficacy due to the narrow window between therapeutically effective and toxicity-producing doses. While small molecule-based targeted therapies (such as imatinib in gastrointestinal stromal tumors, Tarceva in non-small cell lung cancer, cetuximab in colorectal cancers) have produced short term clinical benefits and quality of life improvements, these biotherapeutic approaches have not substantially improved the cancer cure rate [1].

Somatic gene based therapy offers the promise of revolutionizing modern medicine and, in particular oncology, by targeting and modifying the underlying relevant function-dependent pathway[s] of cancer cells, rather than ubiquitously targeting proliferative cells. For gene-based therapeutic approaches directed at the cancer cell, effectiveness is dependent on fulfilling three minimal criteria; 1) identification of a single or linked genetic or epigenetic defect that provides pivotal survival advantage[s] to the overall cancer population (with the corollary that system fragility accompanies target dependency), i.e., essentially the cancer's Achilles' Heel; 2) introduction of a potent and durable gene based agent whose expression can reverse the oncogenic phenotype; and 3) a platform that is effective and specific for global delivery of therapeutic doses of the agent to primary and metastatic tumor foci.

Recent proteomic and genomic advances have led to a remarkably improved understanding of most cancer cell bi-

ologies, and the identification of numerous candidate genes within functionally dominant pathways that impact cancer cell proliferation, apoptosis, and/or immune surveillance. There has been considerable excitement over recent findings of potent and durable oncogene knockdown by small interfering RNAs leading to tumor cures in animal models [2]. However, the lack of a safe and effective systemic delivery platform is widely recognized to be a major bottleneck for translating interference technology-based cancer gene therapy approaches to the clinic [3, 4].

Naked oligonucleotides have half-lives of seconds to minutes when delivered into the bloodstream, even when they are chemically fortified. They are rapidly cleared from the kidneys, because of their small size [5]. Double-stranded siRNA oligonucleotides are resistant to some but not all serum RNases [6]. Cellular uptake of naked siRNA is also limited. siRNA can be administered hydrodynamically by rapidly injecting a large volume of duplex siRNA into the tail vein of mice. However, this strategy is not technically feasible in humans and causes transient heart failure in animals [4, 6, 7].

Ideally, the systemic delivery vehicle should be stable and resist renal clearance and nonspecific uptake while in circulation, but be quickly de-stabilized to release the RNA or DNA transgene once taken up by the target cell [8]. To successfully penetrate the capillary fenestra within the cancer microenvironment, the agent optimally should have a diameter of less than 100 nm (50-100 nm range) although the fenestra have admitted particles as large as 400-500 nm. From a technological standpoint, the vector has to be able to withstand a stringent, large scale production process and be as-

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sembled from simple components using robust methods in order to be cost effective. Currently, none of the available delivery systems can fulfill all of these criteria.

*In vivo* gene delivery can generally be divided into viral and non-viral vectors. Until recently, cancer gene delivery trials have mostly utilized replication-deficient adenoviruses, based on the availability of established and consistent manufacturing processes that generate high yields of viral titers. Adenoviruses also have relatively high transduction efficiency, a capacity to infect non-replicating and replicating target cells, and an episomal mode of transgene expression that obviates the risk of insertional mutagenesis. The application of the more recently developed conditionally replicating adenoviruses (CRADs) has been shown in animal models to be safe and, furthermore, exhibits an additive antitumor effect from both viral oncolysis and transgene-mediated antitumor activities [1]. However, the innate immunogenicity of the viruses contributes to the hepatotoxicity of viral-based constructs and limits their repeat administration. The scaled-up manufacturing process also is technologically challenging and costly, which precludes the widespread acceptance of viral gene delivery [9].

Gene delivery using non-viral assemblies, especially the self-assembled complexes between negatively charged nucleic acids and cationic carriers (lipids or polymers), has gained increased interests as vehicles for cancer gene therapy. The use of cationic lipids was first introduced by Felgner in 1987 [10]. Cationic polymers were introduced in the same year by Wu and Wu and further expanded to second generation, polyethylenimine (PEI) derivatives by Behr and coworkers in 1995 [11]. The attractive features of cationic carriers are their biocompatibility, biodegradability, low toxicity and low immunogenicity [12]. They can incorporate various molecules at high concentrations, which in turn are protected from damage by serum enzymes when given intravenously. Conversely, host cells are protected from the toxicity of the entrapped molecules, particularly from the proinflammatory activity of unmethylated DNAs. However, first generation non-viral gene delivery systems have not lived up to expectations due to drawbacks that include a lack of specificity, cell toxicity, lower than expected site-dependent biodegradability, instability, and low transfection efficiency. In addition, they are rapidly cleared from the blood by the first-pass organs, namely the lungs and liver [13, 14].

Only a limited number of reports have demonstrated the successful, non-viral transfection for metastatic, wide-spread tumors [15-18]. Nonetheless, lipid-based delivery remains an attractive possibility in light of the versatility of the system for technical modifications that can potentially improve targeted localization and transfection efficiency [13-16]. Advances in this field have also benefited from modifications that were initially intended for clinical delivery of conventional chemotherapy [17]. Findings of dose limiting inflammatory toxicity encountered in lipoplex gene delivery have now been attributed, at least in part, to the consequence of unmethylated CpG sequences in the plasmid. Unmethylated CpG sequences putatively interact with toll like receptor 9 (TLR9), resulting in the increased secretion of proinflammatory cytokines IL-1 $\beta$ , TNF $\alpha$ , and IL-12 [18]. Depletion of unmethylated CpG sequences in the plasmid greatly reduced

toxicity, improved therapeutic index and prolonged the apparent expression half-life of the delivered plasmid payload [19]. Replacement of residual prokaryotic elements from the delivered plasmid may further reduce toxicity and extend transgene expression [20].

It is now well established that pharmacokinetics and bio-distribution of the delivered nucleic acid payload are largely a function of the physiochemical properties of the delivery vehicle, namely size, charge, and surface chemistry. Several promising strategies have been developed so as to permit and effective systemic delivery of siRNA oligonucleotides, or plasmid vectors that encode for shRNA with oncogene knockdown activity. They include covalent attachment of neutral peptides such as cholesterol for improved biodistribution, and/or incorporation of antibody-protamine fusion proteins or other receptor/ligand binding motifs for enhanced pharmacokinetics and cellular uptake. Nanoparticle-based delivery vehicles have been developed to accommodate a large payload capacity, and with tunable surface modifications for cell-specific targeting ligands [21-25]. This review summarizes recent advances in formulation technology for encapsulating nucleic acids into cationic carriers and their relevance towards the development of a systemic, clinical delivery vehicle.

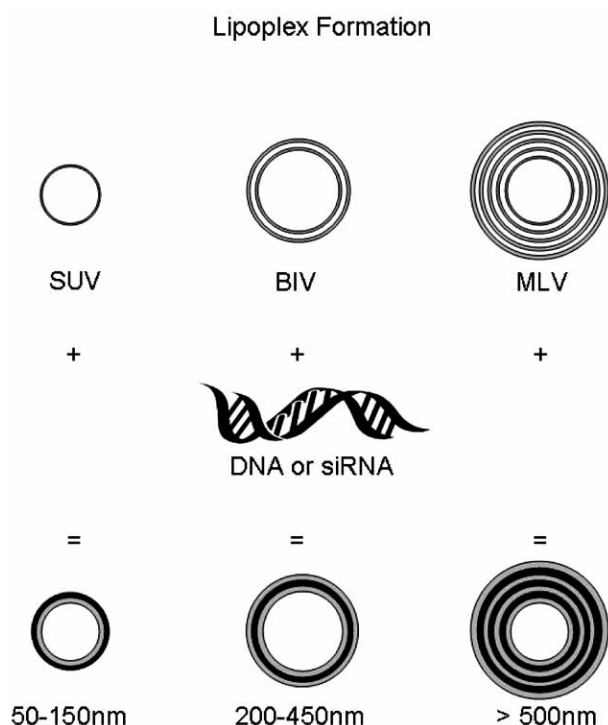
## CATIONIC LIPOPLEXES, NANOPARTICLES, AND POLYIONIC MICELLES

Since the landmark publications in the late 1980s, positively charged (cationic) liposomes and cationic polymers have been used extensively as efficient, synthetic gene delivery reagents *in vitro* [8, 10, 26, 27]. Cationic liposomes can condense DNA when these two components are mixed together. The resulting lipoplex is not an ordered DNA phase surrounded by a lipid bilayer, rather a partially condensed DNA complex with an ordered substructure and an irregular morphology [28, 29]. The hydrophobic nucleic acid backbone of free siRNA limits its ability to penetrate the cell membrane [17] and hence its gene silencing activity due to low bioavailability. In contrast, cationic lipoplexes can bind to negatively charged mammalian cell membranes, and induce intracellular uptake of the incorporated nucleic acid. Hundreds of cationic lipids have been synthesized as candidates for non-viral gene delivery [30]. However, the few that have been used for clinical trials have exhibited low gene transfer efficacy [31, 32].

Among the variety of currently available liposomal formulations, DOTAP (Dioleoyl 3-Trimethylammonium-Propane) has emerged as the most utilized, because of its ease of use and robust transfection efficiency [33]. According to the study by Ramesh and coworkers [34], enhanced systemic transgene expression and significantly elevated cancer growth inhibitory outcomes can be achieved by extrusion processing of the liposome components that reduces aggregate formation, and yielded DNA: liposome complexes of 300-325 nm in size.

Liposomes can form three different structures depending on the manufacturing process. Generally, powdered lipids are dissolved in an organic solution and dried into a thin sheet or "cake". These lipids rehydrate in an aqueous solution, and form large, multi-lamellar vesicles (MLVs). The

MLVs can be reduced in size and structure by sonication or extrusion resulting in bi-lamellar vesicles (BIVs) or small unilamellar vesicles (SUVs). SUVs and BIVs are most commonly used for transfections due to their smaller size. The most common method of preparing DNA-lipoplexes is to directly mix the negatively charged DNA with the cationic liposome (Fig. 1). The interaction spontaneously forms ready to use lipoplexes that are unique to the original type of liposome. However, small oligonucleotides such as siRNA are not enveloped by liposomes, but merely coat the surface of the lipid. Therefore, it is common to use a detergent or organic solution (such as ethanol) to dissociate the liposomes into monomers during the mixing process. Then the unwanted detergent or organic material can be removed by dialysis or evaporation followed by rehydrating the oligonucleotide-lipoplex in the final aqueous buffer.



**Fig. (1). Formation of Lipoplexes.** Powdered lipids form large, multi-lamellar vesicles (MLVs) upon re-hydration. The MLVs can be reduced in size and structure by sonication or extrusion resulting in bi-lamellar vesicles (BIVs) or small uni-lamellar vesicles (SUVs). The most common method of preparing DNA-lipoplexes is to directly mix the negatively charged DNA with the cationic liposome.

An improved understanding of cationic liposomal bio-distribution and cellular uptake has led to the identification of two key limiting factors in systemic lipoplex delivery [35]. At the cellular level, the positive charge of cationic lipoplexes facilitates binding to negatively charged mammalian cell membranes, and induces the uptake of the associated nucleic acid into cells. When introduced *in vivo*, however, lipoplexes bind to negatively-charged serum proteins such as serum albumin (opsonization). The resulting, altered surface charge in turn leads to cross-linking of the nucleic acid-

carrier complexes. Aggregate formation (commonly with diameters of 400-500 nm) [36, 37] destabilizes the integrity of the siRNA-carrier complexes [37] and the opsonized liposomes are sequestered by Kupffer cells in the liver.

Clumped oligonucleotide-carrier complexes also contribute to toxicity. Transient embolism in the lung capillaries has been reported following IV administration, after which the dissociated particles subsequently redistributed to the liver [38]. Thus cationic lipoplexes often display a pharmacokinetic profile of initial, rapid clearance from the blood, followed by accumulation primarily in the lung and the liver.

When the biodistribution of liposomes of different sizes (30-400 nm) were analyzed following IV injection [39], complexes of 100-200 nm in diameter were found to have the best circulatory half life (60% remaining in peripheral blood after 4 hr). These complexes displayed a lower RES uptake in the liver (20% of injected dose) as compared with smaller (<50 nm) or larger (>250 nm) lipoplexes. These findings were extended in a study by Mareira, where tumor uptake for lipoplexes of 100-120 nm was markedly improved as compared with liposomes that were less than 50 nm or larger than 170 nm [39, 40].

Hence the minimal release criteria of a lipoplex should incorporate careful considerations of its chemical composition, size, and zeta potential. The zeta potential is the electrokinetic potential in a charged colloidal system which, in lipoplexes, stems from the electrostatic interaction between the cationic lipid, the anionic DNA, and the surrounding buffer. Thus, the charge ratio of lipid to DNA (+/-) has an effect on the size (due to compaction) and zeta potential of the final lipoplex [16]. Generally, an equal ratio of lipid to DNA results in a final lipoplex that is neutral, while an excess of liposome results in a positive zeta potential [41]. For lipoplexes of approximately 200 nm in size, negatively charged complexes ( $\zeta$  potential of  $\sim -40$  mv) were more rapidly cleared from the blood than neutral complexes ( $\zeta$  potential of  $\sim \pm 10$  mv) [42]. Negatively charged lipoplexes also showed an increased liver RES uptake presumably due to opsonization. Further, a mildly positive lipoplex may have a better electrostatic interaction with the negatively charged cell membrane resulting in higher transfection efficiency. The excess liposome is believed to protect the nucleic acid from degradation.

Nanoparticles (NPs) of a reduced and more uniform size (usually less than 150 nm) have recently been developed as an alternative for reducing lipoplex toxicity [43-46]. NPs can be liposome-based, or comprised of polymers, silica, or hybrid materials [47]. NPs that are made up of liposome-polycation-DNA complexes (LPD) can be prepared by mixing cationic liposomes, a polycationic peptide (protamine), and nucleic acids at fixed ratios [32]. For the delivery of siRNAs, eukaryotic DNA (such as calf DNA) is often added as a carrier in order to reduce particle size. The nucleic acid is complexed by protamine to form a compact core, which is then coated by the two cationic lipid bilayers. The inner bilayer is supported by charge-charge interaction of the cationic lipids and the negatively charged complex core [48]. However, some of these reagents are not applicable to clinical use and therefore are limited in utility.

The use of NPs for parenteral drug delivery enhances selective tissue uptake. This enhanced homing to the targeted organ site by NPs with a diameter less than 400-500 nm (optimally less than 150 nm) has been attributed to the enhanced permeation and retention effect (EPR) in inflammatory sites and solid tumors where the vasculature and, in particular, the larger endothelial junction, is "leaky". In addition to wide fenestrations, lack of a smooth muscle layer, and impaired angiotensin II receptors, vascular hyperpermeability is also attributed to overexpression of cytokines, such as vascular permeability factor (VPF), vascular endothelial growth factor (VEGF), as well as other factors such as the basic fibroblast growth factor (bFGF), bradykinin, nitric oxide and peroxy-nitrate in tumor tissues [49]. EPR permits small particles to extravasate from the blood stream into the disease site [8] through the extracellular matrix, a highly hydrated, gel-like network which serves as scaffolding between the blood vessels and the tissue cells. The small size of the NPs facilitates their diffusion through the extracellular matrix to the cell surface [8].

NP condensates have now been produced that comprise single DNA molecules in a particle size of <25 nm [50]. Encouraging data were observed for localized, intravitreal injections by compacted NPs that comprised of a 30-mer lysine peptide conjugated to an N-terminal cysteine [51]. Intravitreal injection resulted in strong reporter gene expression in the retina, various ocular tissues and lens, indicating that this delivery approach is applicable for a variety of retinal degenerative disorders. In the recently completed clinical trial with sirna-027, a NP-delivered siRNA against VEGFR, intravitreal treatment was safe and well tolerated without dose-limiting toxicities. The trial represented the first clinical documentation of siRNA biological activity in humans, with 8 of the 26 patients displaying visual acuity stabilization and clinically significant improvement eight weeks after a single injection [52].

A number of Japanese studies has recently described the use of atelocollagen, a highly purified type I collagen of calf dermis, as platform for DNA-NPs [53, 54]. The positively charged atelocollagen is low in immunogenicity and toxicity, and has been used in the clinic for a wide range of purposes including wound healing and vessel prosthesis [53]. Atelocollagen-based complexes exhibit many desirable characteristics, including a tunable size based on atelocollagen concentration, a prolonged pharmacokinetics of up to 1 week, and resistance to nuclease [55]. Atelocollagen-based NPs has been shown to be highly effective in the delivery of siRNAs to skeletal muscles by the systemic route [54], and demonstrated efficacy in the systemic delivery of an siRNA against the enhancer of zeste homolog (EZH2) oncogene to bone metastatic prostate cancer cells in a murine model [53]. Currently, the precise mechanism by which atelocollagen achieves selective tumor uptake is unestablished.

Returning to the issue of systemic delivery, however, the cationic nature of the NPs clearly impacts biodistribution and the potential toxicity from aggregation with serum proteins and red blood cells [56]. NPs that are <50 nm may be used to minimize capillary clogging, but remain subject to glomerular excretion and RES uptake in the liver, spleen, and lung [57]. Glomerular excretion can be avoided by using NPs that

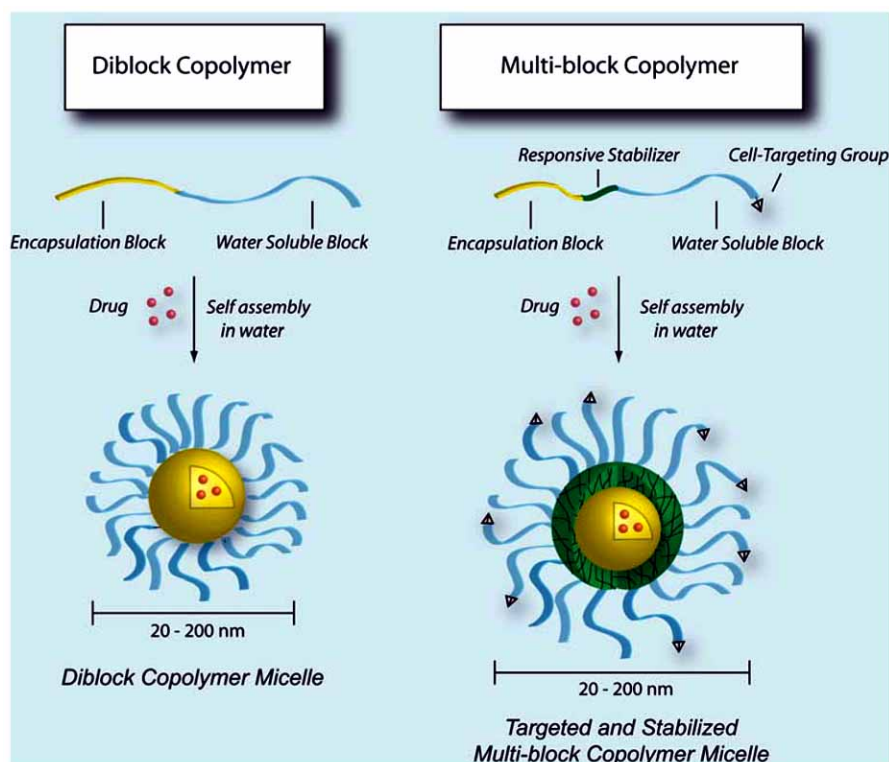
exceed the filtration threshold (>14 nm, although the threshold may be higher for non-globular and deformable complexes) and RES recognition can be minimized by using polyplexes with an improved biocompatibility with particle sizes of between 100-200 nm and endowed with stealth capabilities [58].

The polyionic (PIC) micelle is a recent candidate vehicle for systemic chemotherapy drug delivery (Fig. 2), and has been proposed for oligonucleotide delivery with improved biocompatibility [59]. PIC micelles are comprised of amphiphilic block copolymers that interact electrostatically with DNA. Each copolymer comprises a "block" of repetitive hydrophobic monomeric units (such as *block* poly-L-lysine, or b-PLL) linked to a block of hydrophilic polymers (such as polyethylene glycol, or PEG). The prototypic PIC micelle is assembled spontaneously from mixtures of DNA and PEG-b-PLL through hydrophobic and electrostatic interactions between the charged block copolymers and oppositely charged macromolecule. The resulting assemblage is a unique core-shell structure in which the inner core serves as a hydrophobic nanocontainer surrounded by an outer hydrophilic polymer shell. When loaded with plasmid DNA, the PEG-b-PLL micelle has a small size (~100 nm), a low zeta potential, and excellent colloidal stability [60]. The condensed plasmid DNA remains structurally and functionally intact within the PEG-b-PLL micelle core after incubation in serum-containing media, supporting the applicability of PIC micelles for *in vivo* gene delivery. PIC micelles, with an expected size of 50-100 nm, are likely to circumvent glomerular excretion and RES uptake by virtue of the high density of the PEG shell [56]. An additional issue for the circulating PIC micelles is its critical association concentration (CAC), as defined by the threshold concentration for its assembly. However, the remarkably low CAC for PIC micelles ( $10^{-6}$  to  $10^{-7}$  M) allows them to circulate in the bloodstream after IV injection until accumulation at target tissues. PICs have circulatory half lives of up to 18 hrs [61], and interact minimally with blood cells according to blood: tissue volumetric calculations.

## STEALTH DELIVERY FOR IMPROVED BIODISTRIBUTION

In animal studies, lipoplexes accumulate in the lung following tail vein injection, presumably from association with the pulmonary capillary endothelium [62]. Less than 2% of lipoplexes were detectable in circulation within 10 min. Several possible mechanisms may contribute to this rapid clearance [63]. Large and hydrophobic particles interact with and are taken up quickly by cells of the RES [64]. The cationic surface of lipoplexes also bind nonspecifically to plasma proteins and glycocalyx of many tissues through ionic interactions [65]. Thus serum circulating proteins, in particular the negatively charged albumin, function as opsonins [66] for ternary hydrophobic complexes, leading to excessive aggregation [67]. Filtering by capillary beds was also proposed to contribute to rapid clearance from the blood.

The liver remains the major elimination organ for the lipoplexes despite initial uptake in the lung, after a redistribution process at approximately 60 min after injection [68]. Most of the particles are taken up by Kupffer cells (part of



**Fig. (2). Polyionic (PIC) micelles for drug or gene delivery.** Spherical, nanosized capsules (20 - 200 nm in diameter) are formed by the assembly of block copolymers in water. Each copolymer comprises a “block” of repetitive hydrophobic monomeric units linked to a block of hydrophilic polymers PIC micelle is assembled spontaneously through hydrophobic and electrostatic interactions between the charged block copolymers and oppositely charged macromolecule. The resulting assembly produces a unique core-shell structure, in which the inner core serves as a nanocontainer of hydrophobicity, surrounded by the outer shell of hydrophilic polymer. Late generation multi-block polymers may incorporate additional features (responsive stabilizers) to temporarily “lock” the micelle, enhancing the stability of the polymer micelle after injection, and “unlock” to release the payload in diseased tissue or upon entry into diseased cells.

RES) rather than the hepatocytes, such that lipoplex-mediated gene transfer to hepatocytes is low. Uptake by Kupffer cells also induces cytokine production [69, 70], that include tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon  $\gamma$  (IFN $\gamma$ ), [71, 72], leading to inflammatory adverse effects, liver injury, and a reduced biodistribution to the target tissue [73].

One of the most direct approaches for minimizing the undesired interaction with serum proteins is to incorporate neutrally charged, “helper” lipids in the lipoplex formulation [74, 75]. As shown by Templeton and others, incorporation of cholesterol (chol) increased lipoplex stability, presumably by stabilizing bilayers and complexes in the plasma against mechanical breakage upon adsorption of plasma components [29]. The incorporation of DOPE was shown to promote the transition of lipoplexes from the lamellar, bilayer phase to the hexagonal phase, providing stealth properties that preclude clustering of the complexes [76]. The most commonly used formulation, DOTAP: chol, has been shown to facilitate transfection efficiency in presence of serum *in vitro* [14, 77] and *in vivo* [13, 29].

Attachment of hydrophilic polymers such as polyethylene glycol (PEG) and poly(N-2-hydroxypropyl-methacrylamide) (pHPMA) at the positively charged particle surface

can also reduce interactions with proteins and cells through a mechanism referred to as steric stabilization [64, 78]. With increased incorporation of PEG, LPDs showed a stripping of the outer lipid bilayer, with PEG inserted into the outer leaflet of the inner lipid bilayer [48]. This modification resulted in complete charge shielding, which abolished liver sinusoidal uptake in an isolated liver perfusion experiment [48]. PEG-encapsulated lipoplexes have better stability *in vivo* [79], resulting in an extended circulation time (half-life: 1-10 h). An increase in the fraction of injected particles passing through the target site was also observed [8]. These features are similar to those of the FDA-approved nanoparticle formulation, Doxil (liposomal doxorubicin), used for cancer therapy. Doxil has a size of around 100 nm, a  $\zeta$  potential within 10 mV, with PEG grafted onto the surface of the NPs. However, incorporation of shielding molecules during the initial lipoplex self-assembly with DNA has not consistently demonstrated an improved distribution kinetics *in vivo* [78, 80]. The neutralized PEGylated lipoplex may have reduced electrostatic interactions with the negatively charged cell membrane, but this modification may also reduce cellular uptake and siRNA expression [81], leading to low silencing activity [47].

Similarly, the incorporation of neutral lipid (cholesterol, or dioleoyl-phosphatidyl-ethanolamine, or DOPE) onto

the polymer backbone of NPs confers hydrophilic and biocompatible properties, resulting in a water-soluble lipopolymer that can impair or even avoid RES recognition [58]. The outcome is such that the DNA can be readily packaged, the polyplex circulatory half life is extended, and DNA release from the endosome is also improved (to be further discussed in a later section) [16, 82]. At least with regard to regional (intratracheal) administration, covalent modification of the lysine peptide has shown enhanced *in vivo* stability of unimolecular DNA-NPs. In the lung, organ distribution was achieved using a condensing peptide consisting of a 30-mer lysine peptide with an N-terminal cysteine coupled to a 10 kDa PEG [83]. The introduction of lateral stabilization with multivalent coating polymers may provide better stability than simple steric stabilization [78]. However, the *in vivo* advantage of these modifications remains unestablished.

In drug delivery systems, PEG-lipoplexes have a longer blood circulation half life (>48 hrs) than PIC micelles (24 hrs), but the smaller sized PIC micelles appear to have a higher tumor infiltrating ability [84]. Cross-linking of the PIC core through formation of disulfide bonds between thiol-modified copolymers can be used to stabilize *in vivo* transfection efficiency without impacting intracellular release [85]. The cross-linked micelles were expected to be stable during blood circulation, but plasmid DNA was released efficiently once taken up as the glutathione concentration in the cytoplasm is 50-1,000 times higher than that of the extracellular media [56]. Thus far, cross-linked PIC has been shown to generate a uniform expression in the liver following intravenous injection [85]. An extended half life (5 days) can also be achieved with a worm-shaped nanoparticle that was generated from diblock copolymers [86]. Prolonged circulation time appeared to be related to the strong drag force experienced by the worm-shaped NPs that limited macrophage uptake.

#### **ENHANCED PASSIVE CANCER VASCULAR-MEDIATED DELIVERY**

As noted above, the EPR effect, coupled with a vehicle characterized by a prolonged plasma residence time and the lack of an effective tumor lymphatic drainage system, is largely responsible for the tumor selective localization of NPs [49]. The EPR effect can be augmented through targeted barrier alteration [87]. By further increasing vascular permeability, decompressing intratumoral vessels [88] and decreasing high interstitial fluid pressure [89], effective barrier alteration can elevate effector penetration and intratumoral convective transport [90]. In particular, the proinflammatory cytokine tumor necrosis factor (TNF) has been instrumental in facilitating chemo-penetration via EPR as exemplified by the results of isolated limb perfusion (ILP) [91, 92]. This cytokine presumably acts through TNF-R1 activation [93], deactivation of  $\alpha_v\beta_3$  integrins, and induction of tissue factor (TF) [94]. The tumor uptake of stealth liposome doxorubicin is also significantly augmented by TNF [95]. TNF-mediated liposome uptake has been shown to produce a homogeneous distribution throughout the targeted tumor and interestingly, to promote intact liposome uptake [96]. Unfortunately, the therapeutic window for systemically administered TNF is effectively zero, with dose limiting toxicities that include life threatening hypotension, fever, hepatotoxicity and severe malaise as observed in the early phase I studies [97, 98].

Recent studies indicate that TNF-decorated pegylated colloidal gold particles can be used to augment the EPR effect. Colloidal gold nanoparticles are compelling delivery vehicle candidates. They are able to bind protein biologics without altering their activity [99], they are pliable to a variety of synthetic methods producing size distributions of 1-150 nm [100], they demonstrate rapid cellular uptake kinetics, apparently by clathrin mediated endocytosis, both with and without decoration [101] and they are non-toxic [101, 102]. Cationic mixed monolayer protected clusters (MMPC) with a 2 nm gold core functionalized with quaternary ammonium chains are able to bind plasmid through electrostatic interactions and subsequently release the bound DNA by GSH produced in the cytoplasm [100, 103]. The reduction of 4% HAuCl<sub>4</sub> with 1% sodium citrate in deionized water followed by filtration produces colloidal gold nanoparticles [99]. TNF and thiol-derivatized PEG can then be directly bound *via* covalent bonds, in the same relative amounts, to the surface of the nanoparticle as separate entities sharing available binding sites. The final vector has a mean diameter of  $32.6 \pm 3$  nm with a PDI of 0.106 and  $\zeta$  potential of -2.94 mV. Compared to native TNF following IV administration in C57/BL6 mice implanted with MC-38 colon carcinoma cells, there was significantly higher intratumoral accumulation of PEG-thiol cAu-TNF (PT-cAu-TNF) as well as duration of TNF concentration over time. Furthermore, the wide *in vivo* therapeutic window of PT-cAu-TNF was demonstrated by effective tumor reduction without concomitant host toxicity. More importantly, with regard to EPR, PT-cAu-TNF exhibited rapid and selective reporter fluorescence uptake through the tumor neovasculature with no measurable change in perfusion, thereby emphasizing the role of increased permeability [104]. Preliminary results from a phase I trial of CYT-6091 (PT-cAu-TNF) in patients with a variety of tumors have already exceeded the MTD of native TNF without dose limiting toxicity [105], suggesting the potential for CYT-6091 induced EPR enhancement of liposome and/or nanoparticle bound therapeutics.

Reporter gene transfection by the MMPC with gold core is optimal at a MMPC:plasmid molar ratio of 2200:1, in which the cationic charge is still retained. Somewhat counterintuitive is the finding that gold nanoparticle-oligonucleotide complexes, composed of anti-sense oligonucleotides (ASODN) conjugated to a 13 nm gold nanoparticle surface which produces a densely packed poly-anionic monolayer of DNA, was not only inherently non-toxic but shows greater transfection efficacy in EGFP- C166 cells than Lipofectamine 2000 equally loaded [106]. It is likely that the surface density of ASODN and serum protein adsorption are largely responsible for this provocative effect [107]. In addition to ASODN, RNAi mediated silencing in HuH-7 cells has also been demonstrated with an effector in which thiolated siRNA was conjugated to a polyethylene glycol-copolymer (PEG-PAMA) gold nanoparticle complex [108].

#### **“SMART” CANCER-SPECIFIC TARGETED DELIVERY**

Positively charged NPs and PIC micelles accumulate by virtue of EPR in the tumor microenvironment [109, 110], and achieve cell surface binding largely by nonspecific ionic interactions with the negatively charged cell membrane. This

process is believed to engage the negatively charged cell surface components such as heparin sulfate proteoglycans, integrins [111], and syndecans, the transmembrane family of proteins that cluster and form focal points in lipid rafts of the plasma membrane [112].

Incorporation of targeting moieties such as antibodies and their single chain derivatives, scFv), carbohydrates, or peptides have been shown to facilitate transgene expression in the target cell [15, 16, 47, 113, 114]. This approach is particularly relevant to the delivery of DNA or siRNA oligonucleotides, which have low membrane permeability [114] and appears to be uniformly advantageous for lipoplex-, polyplex-, or LPD NP-based delivery [47, 115, 116]. Notably, Bartlett showed that siRNAs that were delivered systemically by tumor-targeted NPs were significantly more effective in inhibiting the growth of subcutaneous tumors, as compared to undecorated NPs [3]. This and other studies showed that targeted delivery did not significantly impact pharmacokinetics or biodistribution, which remain largely an outcome of the EPR effect [115]. Improved gene delivery apparently occurred from enhanced cellular uptake [115-117]. A brief discussion of promising targeting moieties that are relevant to human cancers is provided below.

### Targeting the Transferrin Receptor

The ubiquitous cell surface transferrin receptor interacts with transferrin (Tf), an iron-transporting protein [118]. Iron is essential in cell metabolism and its pro-oxidant capacity, and tumor cells generally have high levels of iron uptake compared to normal tissue. Iron uptake correlates with the binding of the serum iron carrier protein, transferrin, to transferrin receptor 1 (TfR1) [119]. As expected, TfR1 is expressed at high levels in most cancer cells [16]. Increased expression correlated directly with disease aggressiveness or proliferative activity [120]. TfRs recycle after internalization in rapidly dividing cells such as cancer cells [120], which contributes further to the uptake of TfR-targeted moieties. A second TfR-like molecule, known as transferrin receptor 2, also binds iron but with lower affinity. Its expression appears to be regulated by the cell cycle. In B lymphoma cells, TfR1 was a critical downstream target of c-myc [121] and found to be pivotal in promoting c-myc-dependent tumorigenesis.

TfR targeting of human cancers has been studied extensively with the use of specific antibodies [122, 123], or by incorporation of Tf onto PEGylated or unmodified liposomes [82, 124]. Moruyama initially showed that transferrin-conjugated, long circulating liposomes have a significantly prolonged residence in tumor tissues, in spite of similar profiles of blood clearance and tumor localization as nontargeted liposomes [125]. Studies with a Tf-targeting, <sup>64</sup>Cu-labeled, cyclodextrin-containing polycation (CDP) showed that the systemically delivered anti-luciferase siRNA molecule achieved enhanced cellular internalization in Neuro2A-Luc tumor cells [21]. This feature, as opposed to increased localization, was the primary advantage of the targeting moiety. Similarly, administration of the Tf-targeted CDP for delivery of EWS-FLI1 siRNA produced an enhanced, though transient reduction of tumor growth in a mouse Ewing's sarcoma model [126].

Pirollo and coworkers [127] recently utilized a nanosized immunoliposome complex that incorporated a TfR-1-targeting, single chain antibody fragment of the variable region (scFv) for systemic, targeted delivery of an anti-HER-2 siRNA (the scL delivery systems) [30]. Decoration of the DOTAP:DOPE lipoplex with TfR-reactive scFv was found to achieve a smaller aggregate size than Tf or the parental mAb, thereby facilitating small capillary penetration and improving ease of production. Following systemic (IV) injection, the 200 nm TfRscFv immunolipoplex delivered the fluorescently labeled siRNA specifically and efficiently to xenografts of orthotopic human prostate and pancreatic tumors, and human breast tumors metastatic to the lung. Transgene expression was also detected in micrometastases in the vicinity of larger tumor nodules, indicating that even tiny nodules composed of only a few tumor cells can also be reached and transfected. These findings confirm the applicability of TfRscFv as a tumor-selective targeting moiety and the subsequent efficient delivery of the siRNA by the scFv-nanocomplex.

The enhanced transfection efficiency of TfR targeted lipoplexes through improved internalization has now been validated in a variety of dividing and non-dividing cells [13, 16, 82]. Tf can also trigger cytoplasmic delivery of the carried nucleic acids through destabilization of the endosomal membrane under acidic conditions, thus further improving the transfection process [77]. Subsequent to favorable findings in mice [3, 22] toxicity studies were conducted in non-human primates by intravenous administration of Tf-targeted, CDP-based NPs to deliver siRNA doses up to 27 mg/kg [22]. The Tf-targeted NPs were well-tolerated, with no significant complement activation or immune mobilization at siRNA doses of up to 9 mg/kg [126].

### Chlorotoxin Decoration

Chlorotoxin (CTX) is a 36 amino acid peptide derived from scorpion venom [128]. CTX has been shown to specifically bind to normal and malignant tissues of neuroectodermal origin, including glioma, medulloblastoma, prostate cancer, sarcoma, and intestinal (GIST) cancer [128]. TM601, the synthetic version of CTX, demonstrated high selectivity and binding affinity for gliomas and other neuroectodermal tumors, and has been examined extensively for its tumor targeting properties [129, 130].

Cell binding and internalization of CTX is believed to culminate in the loss of functional chloride ion channel [131, 132], leading to cell death. However, the cell surface receptor for CTX is undefined. It is thought to be a protein complex comprising matrix metalloproteinase-2 (MMP-2), membrane type-I MMP, and transmembrane inhibitor of metalloproteinase-2 (TIMP2). CIC-3 chloride ion channels and other lipid raft-anchoring proteins were also implicated [131, 132]. MMP-2 (gelatinase) is a proteolytic enzyme that degrades extracellular matrix (ECM) during embryologic development and a critical component for cancer invasion of normal tissue [133], although its specific role in targeting and trafficking is unelucidated.

CTX-conjugated nanoprobe exhibit an enhanced capacity for targeting gliomas *in vitro* and *in vivo*, as compared with non-neoplastic cells or normal brain [129, 132, 134].

The profound difference between targeted and non-targeted nanoprobe in tumor labeling efficiency was attributed to the ligand–receptor-mediated nanoprobe internalization. Treated animals exhibited no neurologic or behavioral deficits, and postmortem studies revealed no evidence of neuropathology, further supporting the selective targeting by CTX.

An unexpected positive finding was the extended half life of the internalized probe. Gliomas were distinguishable from normal tissues for 14 days after bioconjugate injection, and well after peak serum levels were reached. In a recent phase I/II clinical trial of human brain cancer therapy, <sup>131</sup>I-TM-601 targeted gliomas displayed approximately 1.3-fold higher radiolabel retention as compared with adjacent brain tissue [134, 135], and confirmed the safety profile observed in pre-clinical studies [135]. Currently, there is no available information regarding biodistribution of CTX-targeted lipoplexes following systemic administration. However, the cited findings demonstrate their promise for enhanced cationic carrier delivery.

### **Nucleolin-Targeting**

Nucleolin is an RNA- and protein-binding protein ubiquitously expressed in exponentially growing eukaryotic cells [136]. It is found at multiple intracellular locations, including the nucleolus, where it controls many aspects of DNA and RNA metabolism; and in the cytoplasm, where it shuttles proteins into the nucleus and provides a posttranscriptional regulation of strategic mRNAs [137, 138]. In addition, nucleolin is expressed on the cell surface, where it serves as a low affinity receptor for HIV-1 and various growth factors that interact with the RGG domain of nucleolin, such as midkine, pleiotrophin (PTN) and lactoferrin [137-140]. Binding of these ligands results in clustering of cell-surface nucleolin in lipid raft membrane microdomains before endocytosis of the ligand-nucleolin complex by an active process [139, 141].

Nucleolin binding has been implicated in events that are pivotal for cell proliferation, differentiation, adhesion, mitogenesis and angiogenesis. Cell surface expression of nucleolin was first reported in hepatocarcinoma cells. Elevated levels of cell surface nucleolin has now been documented in multiple tumor cell types and in angiogenic endothelial cells within the tumor vasculature [138, 142, 143], where it serves as a pivotal receptor for tumorigenesis and angiogenesis pathways [144, 145].

Nucleolin-binding by growth factors such as midkine and PTN led to cell transformation, as well as mitogenic and angiogenic events for endothelial cells [146]. Binding by laminin-1 induces cellular differentiation, whereas ligation and co-internalization by urokinase impacts pericellular proteolysis, cell-surface adhesion, and mitogenesis [147]. Hepatocyte growth factor, which regulates angiogenesis, invasion and growth of carcinoma cells, also utilizes surface nucleolin as an alternative receptor [148]. The tumor homing peptide F3 that binds both endothelial and tumor cells is internalized via surface nucleolin, while the anti-angiogenic endostatin binds nucleolin on endothelial cells before translocation to the nucleus [143, 149]. Exposure to vascular endothelial growth factor (VEGF) upregulates cell surface nucleolin expression in endothelial cells, whereas blockade of nucleolin

function or expression inhibits their migration and prevents capillary-tubule formation [150]. The capacity of surface nucleolin to bind pathogens and a diverse range of ligands (including low density lipoproteins) suggest that it may serve as scavenger receptor [137, 138]. In addition to its function as cell surface receptor, cytoplasmic nucleolin also binds to the 3'-untranslated region of matrix metalloproteinase-9 (MMP-9) and bcl-2 oncogene mRNAs, a process that contributes to their stability and translational efficiency [151, 152].

Encouraging anti-tumor and anti-angiogenic activities were recently demonstrated with the use of a specific pseudopeptide (HB-19) that binds the C-terminal tail of nucleolin [136]. In athymic nude mice, HB-19 treatment markedly suppressed the progression of established human breast tumor cell xenografts, and in some cases eliminated measurable tumors while displaying no toxicity to normal tissues. This potent antitumoral effect is attributed to the direct inhibitory action of HB-19 on cell surface nucleolin of tumor and endothelial cells without affecting nuclear nucleolin function, illustrating the dual inhibitory effects on tumor development and neovascularization. However, the mechanism by which HB-19 downregulated surface nucleolin remains to be elucidated.

Systematically administered HB-19 is rapidly cleared from blood. However, a significant proportion of the active form persisted in tissues that expressed elevated levels of surface nucleolin even after 24 hours [153]. There is a threshold for the tissue uptake of HB-19, thus precluding any eventual toxic effects at increased doses [153]. The acceptable safety profile of the nucleolin-targeting HB-19 ligand and its tumor selective activity are attractive features for its consideration as a tumor targeting moiety.

Following systemic injection, non-targeted, PEGylated PEI NPs localized in cells that expressed cell surface nucleolin [154]. Surface nucleolin-depletion reduced the expression of reporter genes delivered by these NPs. These findings indicate that nucleolin targeting can enhance DNA NP uptake and transgene expression. Further studies are clearly needed to better understand the pathophysiologic outcome of the delivered siRNA or plasmid against the backdrop of membrane nucleolin ligation for individual, targeted cancer cell types.

### **Other Antibody-Targeted Cell Surface Ligands**

Full length and single chain antibodies can be added to DNA lipoplexes via covalent linkage or steric interactions. Various tumor-associated antigens have been validated as targets for antibody-based cancer therapy [155]. The epithelial cell adhesion molecule (EpCAM) is a transmembrane protein involved in calcium independent cell adhesion, and has been shown to be overexpressed in several solid tumors. EpCAM has been identified as a target for immunoliposome (IL) delivery [156]. A single chain antibody against EpCAM was covalently linked to a cationic lipid and used to deliver a modified siRNA against bcl-2 and bcl-xl. The immunolipoplex demonstrated a 10 – 20 fold increased binding *in vitro*, as compared with non-decorated lipoplexes. The immunolipoplex entered the cell via receptor-mediated endocyto-

sis, and increased apoptosis by 2-5 fold when used in conjunction with doxorubicin.

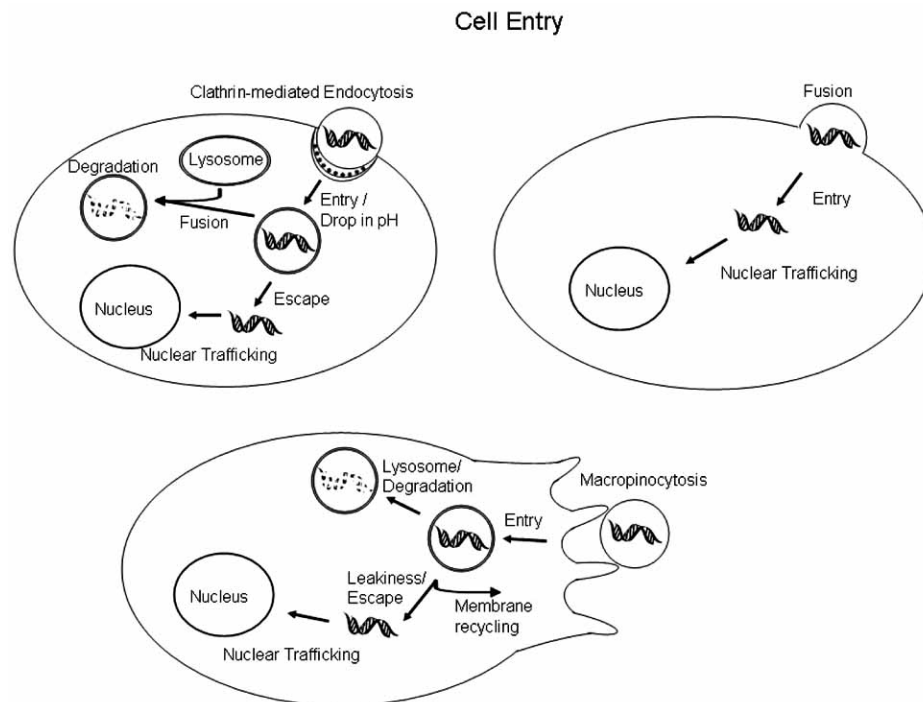
Epidermal growth factor receptor (EGFR) is another popular target for immunolipoplex delivery, based on the high expression levels of EGFR in solid cancers [157]. Monoclonal antibody fragments against EGFR (matuzumab and cetuximab) were linked to liposomes and used to deliver chemotherapeutic drugs [158]. The EGFR-IL bound specifically to colorectal cancer cells more efficiently, and was significantly more tumor-toxic than nontargeted lipoplexes. By altering the PEG chain that linked the Fab' to the liposome, Mamot showed that longer PEG chains promoted cellular uptake [159]. Lipoplexes carrying cetuximab (delivered IV) had a circulation half life of 21 hours, and efficiently accumulated in tumor bearing mice [159]. Similar to other targeted delivery moieties, there was no difference in tumor accumulation between the non-targeted vs. EGFR-ILs. However, internalization of EGFR-IL (92%) was significantly increased as compared with the non-targeted lipoplexes (<5%), indicating more effective cellular uptake *via* cell surface EGFR ligation. This difference has translated into an improved delivery of chemotherapeutic drugs and more effective tumor reduction. An independent study by Zhou

found that nature of the antibody-targeted EGFR epitope and the decorating ligand density both contributed to the efficiency of the immunolipoplex [160]. By using a yeast display library, EGFR scFvs were selected, based on their binding affinities. The use of currently approved antibodies such as cetuximab (anti-EGFR), trastuzumab (anti-HER2) or bevacizumab (anti-VEGF) to generate highly decorated immunoliposomes are sufficient for tumor targeting and cellular uptake.

Anti-HER-2 and anti-transferrin receptor antibodies have been coupled to lipoplexes to improve delivery and uptake in tumor cells, *in vivo* and *ex vivo* [161, 162]. In both scenarios, the immunolipoplexes were stable for a longer period of time when compared to naked lipoplexes, and demonstrated greater target specific delivery with enhanced cellular expression.

### ENDOSOMAL ESCAPE

In addition to a high level of tumor localization, bioavailability to the tumor also depends critically on the site of release of the gene therapy payload [44, 163]. The internalization mechanism of lipoplexes currently is not well under-



**Fig. (3). Putative Routes of lipoplexes Entry.** Most of lipoplex uptake likely occurs by endocytosis through one or more of three morphologically distinct pathways, namely clathrin-mediated endocytosis (CME), clathrin independent caveolae macropinocytosis, or fusion. The first step of internalization through CME is the strong binding of a ligand to a specific cell surface receptor, as exemplified by the binding of TfR by iron-laden Tf. The ligand-receptor complexes clusters in plasma membrane coated pits which invaginate and pinch off to form intracellular clathrin-coated vesicles (CCVs). CCVs carry the concentrated receptor-ligand complexes into the cells and are characterized by the presence of a polygonal clathrin coat. The clathrin coat then depolymerizes, resulting in early endosomes, which fuse with other endosomes to form late endosomes that further fuse with lysosomes. This process is accompanied by a rapid drop in pH from neutral to pH 5 in the lysosomes. Macropinocytosis is the process of large endocytic vesicles (macropinosome) formation, and usually is accompanied by cell surface ruffling upon stimulation by growth factors or other signals. Macropinosomes vary in size and shape, and are generated by actin-driven plasma membrane invagination. Macropinosomes have no coat and do not concentrate receptors, and best exemplified by the uptake of soluble antigen by dendritic cells. Intracellular access by caveolar macropinocytosis may escape lysosomal degradation.

stood. Early reports suggest that fusion with the plasma membrane is responsible for delivering DNA directly to the cytosol [164]. However, most of the uptake appears to occur through endocytosis, according to recent blocking studies with endocytosis inhibitors, lysomotropic reagents and electronic microscopic imaging [12]. Endocytosis occurs by at least three morphologically distinct pathways, namely, clathrin-mediated endocytosis, clathrin independent endocytosis or macropinocytosis that is either dependent or independent of caveolin, and membrane fusion (Fig. 3). Clathrin-mediated endocytosis (CME) is the major and best-characterized endocytic pathway [165], where clathrin, the main assembly unit of cytosol coat proteins, forms a polygonal lattice (clathrin coat) on the surface of the membrane upon ligand binding. CME can be targeted by the use of certain ligands, such as transferrin, which triggers receptor-mediated endocytosis, resulting in an increased internalization of the formulation as well as offering the possibility to target specific cells that substantially overexpress the receptors [166]. The ligand-receptor complex clusters in plasma membrane coated pits, which then invaginates and pinches off to form intracellular clathrin-coated vesicles (CCVs) [167]. The clathrin coat then depolymerizes, giving rise to early endosomes which fuse to form late endosomes. These endosomes either fuse with lysosomes for degradation, or recycle their contents back to the cell surface. The progression from early endosomes to fusion with lysosomes is accompanied by a rapid drop in pH, from neutral to pH 5.9-6.0 in the lumen of early endosomes, and further reduction to pH 5 in lysosomes [168]. To achieve cellular expression, the transgene must avoid degradation in lysosomes, be released from intracellular vesicles into the cytosol, and/or access the nucleus [12]. Therefore escape from endosomes is essential for efficient transfection.

By comparison, uptake of PEI-based polyplexes may involve both caveolar entry as well as CME. Macropinocytosis is the process of actin-dependent, large endocytic vesicles (macropinosome or caveosomes) formation which usually accompanies cell surface ruffling upon cell stimulation by growth factors or other signals. It has now been shown that pinocytic pathways are involved in receptor-ligand interactions [169]. Macro-pinosomes are less likely to be funneled into an endosomal or lysosomal construct, an explanation for the apparent, improved gene delivery efficiency of polyplexes *in vitro* [170] through escape from lysosomal degradation. According to the studies of Rejman and others [76], the alternatives of caveolar- or clathrin-mediated macropinocytosis are particle size dependent, with polyplexes that are >200 nm gravitating towards the preferred, macropinocytic pathway.

The enhanced transfection and payload expression efficiency of the DNA compacted PEGylated poly-L lysine (PLL-g-PEG) NPs may partly be attributed to macropinocytic trafficking [171]. The rhodamine-labeled reporter plasmid deposited in the perinuclear area following delivery by the neutral-charged, rod-like and toroid-like NPs. There was no colocalization with lysobisphosphatidic acid (LBPA) that identifies late endosomes /lysosomes, while pretreatment with amiloride, a specific inhibitor of the  $\text{Na}^+/\text{H}^+$  exchange required for macropinocytosis, markedly inhibited NP entry, indicating that significant NP entry occurred by lipid-raft

mediated macropinocytosis. Subsequent endosomal escape from lysosomal degradation likely contributed to improved perinuclear localization [171], as supported by a recent study with the PLL-g-PEG DNA NPs in COS-7 cells [172].

To withstand lysosomal degradation, the backbone of the siRNA payload can be modified to be resistant to RNase, thereby reducing loss of activity following CME [173]. Some neutral lipids such as DOPE (dioleophosphatidylethanolamine) have membrane fusogenic properties. Their incorporation endows micelle-forming lipids (such as oleic acid or CHEMS) the capacity to fuse with other lipids when exposed to low pH as found in endosomes, aiding in cytosol release and expression of the associated DNA transgene (126, 173). Given the *in vivo* toxicity of DOPE, it is worthwhile noting that the intracellular fate of Tf-liposomes was also improved by adding GALA, a pH-sensitive fusogenic peptide, which enhances the endosomal escape in response to the low pH in endosomes [173, 174].

Based on the concept of pH dependent endosomal escape, some investigators [126, 175] incorporated linear and branched histidine-lysine (HK) polymers of varying lengths onto the surface of cationic lipoplexes. The histidine component is expected to function as a "proton sponge" by protonating under acidic pH in the endosome, leading to increased endosomal pH and contributing to its rupture. Such HK copolymers significantly increased the transfection efficiency of cationic liposomes [175]. A small linear pH-sensitive peptide, HoKC [176] incorporated into the HK copolymer further increased the effective cytoplasmic concentration of the siRNA payload. An efficient knockdown of the targeted gene was observed, as well as augmented tumor growth inhibition in combination with standard chemotherapy [30].

Polycationic polymers, such as the high transfectable PEI polyplexes with a neutral  $\text{pK}_a$  ( $-\log_{10}$  of the acid equilibrium constant  $K_a$ ) can also achieve a proton sponge effect for enhanced endosomal escape [177]. These polyplexes usually contain multiple neutral amine groups with a  $\text{pK}_a$  of approximately 6.5. When internalized into the endosome (pH 5-6), protonation of the amine groups under acidic pH in effect reduces free proton concentration, resulting in an increased endosomal pH. A continuous influx of protons and chloride counterions (in order to maintain the low pH of the endosome) leads to a rapid rise in osmotic pressure, eventually rupturing the endosome and releasing the NPs [47, 56]. This proton sponge effect has been incorporated into siRNA delivery by PIC micelles [60]. Itaka and coworkers utilized a PEG-b-polycation with a diamine structure and having 2 distinct  $\text{pK}_a$  (PEG-b-DPT). The primary amino group provides the basis for PIC formation, while maintaining a buffering capacity of the secondary amino group for the proton sponge effect. PEG-b-DPT delivered siRNA against the endogenous gene lamin A/C demonstrated a significant gene silencing activity even after a 30 minute preincubation in 50% serum. Whether this mechanism applies *in vivo*, however, is presently unclear in view of the expected dilutional effect during *in vivo* administration.

An alternative solution to circumvent issues of *in vivo* stability and toxicity as related to free polycations is to utilize an A-B-C type tri-block copolymer for PIC construction. The proton sponge effect can be maintained in a polyplex

micelle comprising PEG, poly[3-orphanopropyl) aspartamide] (PMPA) as a low pKa polycation, and the hydrophobic PLL [178]. The resulting polyplex micelle, made up of an inner core of the plasmid DNA-PLL wrapped with an intermediate layer of the low pKa PMPA segment and an outer layer of the biocompatible PEG segment, displayed a >10-fold higher transfection efficiency to human hepatoma HuH-7 cells than the PEG-PLL copolymer micelle *in vitro* [178]. The *in vivo* applicability of this triblock copolymer is currently being examined.

Recently, there has been considerable interest in short peptides known variously as cell penetrating peptides (CPPs), membrane translocation sequences (MTS), "Trojan peptides", or protein transduction domains (PTDs). PTDs are arginine-rich, positively charged peptides of 7-30 amino acids that are capable of transmembrane delivery of large biologically active molecules [179]. The PTD peptides share the presence of several arginine residues in their sequences, which are shown to be responsible for the activities [180]. The octaarginine (R8) peptide is regarded to be prototypic of the PTD peptides, as the optimum number of arginine was around 8.

Decoration of liposomes with the R8 peptide enhanced cellular uptake as well as improved the intracellular trafficking towards a more efficient endosomal escape [179]. Modification with low or high density of the R8 peptide significantly enhanced the cellular association of liposomes [179]. At a high R8 density, complex uptake induced internalization away from CME, while initiating ruffle formation and stimulating macropinocytosis [179] as well as cytoskeleton rearrangement [181]. Conversely, blocking macropinocytosis-mediated internalization reduced the transgene expression by R8 modified NPs by >90%, indicating that redirection to macropinocytosis was the main contributor for increased gene expression [182]. The shunted, R8-modified liposomal trafficking is similar to the internalizing process of adenovirus, which triggers macropinosome formation and leakage into the cytoplasm in order to initiate viral escape and the subsequent infectious process [183].

## NUCLEAR ENTRY

siRNA oligonucleotides need only to be delivered to the cytosol, its putative site of activity, whereas transcription of the shRNA-encoding plasmid DNA requires access to the cell nucleus. After having overcome the lysosomal degradative process, free DNA molecules of >2,000 bp are mostly detained in the cytoplasm [184]. For cells that are active in mitosis, integrity of the nuclear membrane is lost transiently to allow transgene access. This is illustrated in the study by Ogris [185], where cell cycle was shown to impact gene expression of TfR or EGFR receptor targeted PEI liposomes. Transfection efficiency was highest during the late S/G2 phase. Therefore, transgene expression would be expected to be more pronounced in rapidly dividing cells, such as tumors, over slowly dividing somatic cells, where nuclear trafficking in nondividing cells is limited to passive transport of molecules with  $\leq 70$  kDa molecular mass (approximately 10-25 nm in diameter) through the nuclear pores [12, 186]. This constitutes a significant physical barrier for transgenes. Although compacted DNA NPs with neutral charge and small

size ( $\leq 25$  nm in diameters) are likely to gain nuclear access [50, 154], these smaller sized NPs are, counter-intuitively, more susceptible to renal glomerular filtration.

In transfection studies with post-mitotic lung epithelium, retina, and brain cells *in vivo*, cell surface nucleolin binding apparently facilitated nuclear transport and uptake of DNA-carrying NPs in a nondegradative pathway [43]. Alternatively, incorporation of a nuclear localization signal (NLS) may be utilized to achieve active transport through the nuclear pore complex [187, 188] in a cycle independent manner. Enhanced transgene delivery to the nucleus was attained through incorporation of NLSs which are basic amino acids (~100 peptides/1,000 bps of DNA) that can be recognized by cytosolic factors to mediate active transport [187]. For polyplex delivery, the coupling of NLS to the DNA payload facilitated nuclear access, but not when NLS was integrated into the polycation shell. Studies with tiopronin-protected gold nanoparticles functionalized with the Tat protein-derived sequence GRKKRRQRRR were effectively targeted to the nucleus of HTERT-BJ1 fibroblasts as demonstrated by transmission electron microscopy [188]. Although Tkachenko *et al.* [189] were unable to detect nuclear entry of Tat complexed gold nanoparticles in HeLa, 3T3/NIH murine fibroblastoma cell or HepG2 hepatocarcinoma cells, they were able to do so with the adenovirus NLS and the integrin binding domain.

## CONCLUDING REMARKS

Extensive studies over the last decade have identified the requisite physiochemical features of cationic delivery vehicles for optimal gene delivery. These include an extended circulatory half life with minimal cytotoxicity, a biodistribution profile that favors the selective targeting of primary and metastatic tumor lesions, and biochemical properties to achieve defined intracellular uptake, cytosol trafficking and nuclear entry. While multiple investigators have offered promising solutions for each scientific problem, most findings have not progressed beyond the proof of principle stages *in vitro*, or, if *in vivo*, used approaches with limited clinical applications (intratumoral, intramuscular, intraperitoneal). To achieve the capabilities of penetrating small capillaries with enough structural integrity to withstand degeneration, rapid RES uptake and renal clearance, the optimized delivery vehicle needs to be a stealthed (e.g., PEGylated) NP of approximately 100 nm with a  $\zeta$  potential of within 10 mV [12, 47, 127]. Nonetheless, typical accumulation at the target tissue represents only 2-10% of input dose [47]. Hence further maneuvers are needed to maximize the bioavailability to the targeted tissue. Recent *in vivo* findings have shown promise in the use of cancer-targeted molecules of well defined intracellular trafficking properties (e.g., nucleolin), coupled with multiple, triggered release mechanisms that further enhance endosomal release, lysosomal escape, and nuclear entry. These approaches have not been extended to clinical formulations. Future work will involve the integration of multiple, well characterized components onto a single, robust, and proven formulation, and the validation of its physiochemical features according to *in vivo* performance.

Beyond the intrinsic variables of formulation physiochemistry, the unique properties of the target cell and the

molecular impact by the delivery vehicle have to be clearly addressed. For considerations of specificity and toxicity, the biodistribution profile of targeted lipoplexes or polyplexes should be determined by animal models with orthotopic tumor xenografts following systemic administration, in order to demonstrate an improved homing with limited lung, liver and spleen accumulation. The impact of EPR on delivery vehicle trafficking is another process that can be exploited, where the relevant transgene can be delivered concurrently to the tumor and its vasculature by vehicles decorated with distinct targeting moieties. Head to head comparisons of the same prototypic tumor model with a well defined phenotype will be needed to assess the relative efficacy of different formulations. Studies that integrate all of these methodologies have been limited. The evaluation of tumor specific parameters, such as accessibility, tumor target load, and growth rate can then be followed by assessment of treatment-specific parameters (knockdown threshold, dosing requirements for cytostatic *vs.* cytotoxic targets, long-term *vs.* short-term therapeutic outcomes) in optimizing the design of cationic lipoplex based cancer treatment strategy [3]. RNAi technology has the potential for establishing a new therapeutic paradigm in medicine and, particularly, in oncology where tumor processing robustness implies susceptibility to selective dominant pathway fragility, i.e., an Achilles Heel for tumor growth and progression. As safety and effectiveness of clinical applications of RNAi-based therapeutics are being validated, successful target modification ultimately depends on selective, specific and functionally intact systemic effector delivery-crafting Paris' arrow.

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