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Design of Polymeric Gene Carriers for Effective Intracellular Delivery

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Abstract

Polymeric carriers have emerged as major non-viral alternatives for gene delivery due to their lower immunogenicity and pathogenicity. However, during intracellular delivery of these carriers, multiple barriers have to be overcome, or the efficiency of gene delivery will be impeded. Thorough understanding of these cellular impediments is pivotal to optimizing the efficiency of polymer-based gene delivery. This article delineates the major barriers encountered during intracellular delivery of polyplexes, and discusses possible molecular designs to overcome these barriers. Based on a review of the latest strategies to enhance the intracellular delivery process, we provide insights into further development of polymeric carriers with enhanced efficiency in transfection.

Keywords

Nonviral gene delivery; polymeric vector; nucleocytoplasmic transport; cellular uptake; transfection efficiency

Current Development of Polymeric Carriers

Gene delivery technologies may bring hopes to combat diseases that have been pharmaceutically intractable. Viral vectors entered clinical trials in as early as the 1990's [1]; however, the safety concerns involved have limited their broad use. Over the years diverse non-viral alternatives have been developed from polymers such as poly(lactide-co-glycolide) (PLGA) [2], poly(ethylenimine) (PEI) [3], and bioreducible fluorinated peptide dendrimers [4]. These carriers alleviate the safety risks caused by the use of viral systems, yet their low **transfection** efficiency has impeded their clinical applications. This low efficiency arises in part from the presence of impediments, at the cellular and metabolic levels, which limit the amount of therapeutic nucleic acid materials that can be brought into target sites for action. To enhance the transfection efficiency, one possibility is to perform gene delivery at the time of cell division, in which the **nuclear envelope** disassembles and hence nuclear access can be achieved more readily [5]; however, this approach may impose tremendous limitations to executing gene delivery in terms of timing. More active approaches to enhance nuclear translocation of the transgene are required.

At the moment, a serious discussion on the nature of the barriers to intracellular gene delivery, and the latest advances in technologies to actively tackle each of them, is lacking. Regarding this gap, this review summarizes the current understanding of the mechanisms of intracellular delivery of **polyplexes**, and exploits strategies to overcome major barriers encountered during the delivery process. We hope that with an overview of major impediments that have been limiting the efficiency of polymeric gene delivery, insights into further development of more effective

polymeric carriers can be attained to enhance the future potential of these carriers for execution of **gene therapy**.

Barriers to Intracellular Delivery

Gene delivery mediated by polymeric vectors is a multi-stage process. It involves multiple obstacles to overcome before the nucleic acid material can reach the target site (Fig. 1). The target site here refers to the cytosol for RNA molecules, and the nucleus for plasmids. Contrary to delivery of chemical drugs, in which the therapeutic effect may still be achieved even if the vector fails to undergo cellular uptake but simply releases the drug outside, gene therapy can be executed only if the nucleic acid material can be brought into the target cell [6-8]. Therefore, the first obstacle a polymeric vector will encounter at the cellular level is cellular internalization. This has been demonstrated by Bishop and coworkers [9], who used poly(β-amino ester) for gene delivery to human primary glioblastoma cells and showed that only 0.1 % of the added polyplexes was internalized into cells [9]. This demonstrates that cellular internalization is the rate limiting step during the process of non-viral gene delivery. The mechanism of cellular internalization of the polymeric vector has been modeled by Kopatz, Remy and Behr [10]. According to the model, gradual electrostatic zippering of the plasma membrane onto the vector is sustained by the lateral diffusion of **syndecan** molecules, which cluster into cholesterol-rich rafts after initial cellular binding of the vector [10]. Upon clustering of syndecan molecules, the **protein kinase C (PKC)** activity is triggered. **Actin** binding to the cytoplasmic tail of the syndecan molecule is also induced, followed by engulfment of the polyplexes [10]. This model offers a theoretical perspective on cellular internalization of polymeric gene carriers.

Endocytosis often plays an important role in cellular internalization. It is difficult to assign a specific endocytic pathway to nanocarriers because the endocytic pathway varies with the cell type, the molecular composition of the cell surface, and the physical properties of the nanocarrier [11-14]. For instance, lipoplexes tend to enter the cell through the **clathrin**-dependent pathway [11]; however, when they are coated with serum albumin, they are internalized via **caveolae**-mediated endocytosis [12]. For polyplexes, the mechanism of cellular uptake is determined largely by cell types and the properties of the polymer [11]. In HepG2 cells, cellular uptake of PEI-based polyplexes is achieved via the clathrin-dependent pathway [13]; whereas in HeLa cells, those polyplexes have great preference for the caveolar pathway over the clathrin-dependent pathway [13]. The size of the polyplex also affects the endocytic mechanism. Polyplexes with a size of 200 nm tend to follow the clathrin-dependent pathway; whereas those with a diameter larger than 500 nm tend to be internalized via the clathrin-independent mechanism [14].

After cellular internalization, polyplexes have to undergo endosomal escape before nuclear translocation [15]. Low efficiency of endosomal escape is considered to be a barrier second to cellular internalization for effective intracellular gene delivery. The third obstacle, particularly to plasmid transfer, is cytosolic transportation, in which **cytoskeleton** components play important roles. Random bindings with actin, keratin and vimentin largely compromise the transfer of the plasmid to the nucleus [16-18]. Finally, nuclear import of the delivered transgene can be hindered by the nuclear pore complex (NPC), which regulates macromolecular exchange between the nucleus and the cytoplasm (Box 1) [19].

Engineering Physical Properties to Enhance Transfection

To enhance the efficiency of intracellular gene delivery, one strategy is to modulate the physical properties of polyplexes. The two major properties that can be easily modulated are polyplex size and surface charge. As polyelectrolyte complexation is a major process of polyplex formation, these parameters can be manipulated simply by increasing the polymer charge density, which can facilitate nucleic acid condensation to generate polyplexes with a smaller size and more positive **zeta potential**. Particle shape is the third parameter that should be taken into account because of its impact on cellular uptake and the length of blood circulation [6]. Its impact on cellular uptake has been hinted at by gold nanorods, whose efficiency of cellular internalization is reduced by an increase in the aspect ratio [20]. Regarding the length of blood circulation, Ghandehari's team reported that, after 6 hours of post-intravenous administration, the plasma concentration of PEGylated rod-like gold nanoparticles in mice was around 11 % of the injected dose [21]; whereas that of the spherical counterparts was below 1% [21]. This result demonstrates the high relevance of particle geometry to the biological performance.

One of the techniques that may help to manipulate the geometry of polymeric particles is **layer-by-layer assembly** [22, 23]. Upon selection of suitable deposition conditions and polymers [24], chemical, mechanical and surface properties of the particles can be tuned. By adopting a sacrificial template with the desired geometry, manipulation of the shape of the particles can be achieved as well. The feasibility of this strategy has been demonstrated by a previous study, which adopted the layer-by-layer assembly approach to generate rigid hydrogen-bonded tannic acid/polyvinyl pyrrolidone (PVP) hemispherical capsules, which were shown to be internalized by macrophages two times more efficiently than the spherical counterparts [24]. Another study employed the layer-by-layer approach to fabricate red blood cell-mimicking multilayer discoidal capsules, which exhibited 60 % lower cellular internalization efficiency compared to spherical capsules [25]. A similar observation on the effect of particle geometry on the cellular internalization efficiency has more recently been reported by Xu and coworkers [26], who generated micrometer-sized particles of various shapes by cross-linking poly(methacrylic acid) (PMAA) with cystamine within the PMAA/PVP multilayers on sacrificial mesoporous templates. They found that spherical particles lead to more rapid internalization at the initial step of cell-particle interactions as compared to cubical particles. Other than the layer-by-layer method, **step and flash imprint lithography** (S-FIL) has been used to generate square, triangular and pentagonal polymeric nanoparticles, which undergo enzyme-triggered degradation for controlled release of antibodies and nucleic acids [27]. Apart from the layer-by-layer technique and S-FIL, there are numerous other reported strategies to manipulate the geometry of polymeric nanoparticles [28-31]. Examples include the mini-emulsion technique, film stretching, particle replication in non-wetting templates, and template-assisted assembly. With these available technologies, optimizing the performance of polymeric carriers by manipulating the geometry of polyplexes in the future is both theoretically and technically feasible.

In addition to modulating the physicochemical properties of polyplexes, engineering the structure of the carrier may facilitate nuclear translocation of the delivered transgene. This has been demonstrated by an earlier study [32], in which a UV-degradable carrier was synthesized by crosslinking low-molecular-weight branched PEI with *o*-nitrobenzyl urethane (NBU) linker molecules. Upon UV irradiation, the carrier degrades, leading to the formation of anionic carbamic acid groups and nitrosobenzyl aldehyde groups [32]. These groups allow the polyplexes to interact with nuclear transcription proteins, thereby increasing the final gene expression level [32]. More

recently, alveolar epithelial type I (ATI) cell-specific nuclear import of plasmid DNA has been achieved by incorporating the plasmid with a DNA sequence that can bind with ATI-enriched **transcription factors** (e.g., HNF3 and TTF-1) [33]. This finding demonstrates that, besides engineering the structure of the carrier *per se*, gene delivery and expression can be restricted to particular cell types by engineering the sequence of the plasmid.

Chemical Conjugation as a Strategy for Carrier Design

Engineering the physical properties as mentioned above is one possible method to increase the efficiency of gene delivery, but chemical conjugation is a more versatile approach. The process of cellular internalization can be facilitated by covalently binding ligands (e.g., transferrin, RGD peptide, lactose, mannose, anti-CD3 antibody, OV-TL16, PSMA-specific monoclonal antibody J591, HER-2 antibody, GRP-78 targeting peptide, and folic acid) to the polyplex surface to stimulate receptor-mediated endocytosis. In addition to covalent binding, surface modification might be achieved using microfabrication technologies. This has been hinted at by a recent study [34], in which a microfluidic chip containing a particle exchanger was adopted for continuous surface functionalization of micro- and nanoparticles in flows. The exchanger has two fluidic channels that overlap over a short section, in which a dielectrophoretic force perpendicular to the direction of the fluid flow is applied to the dielectric particles. Those particles move from a buffer solution to a reagent and then back to the clean buffer for one cycle of surface modification. This modification strategy might be applied to future modification of polyplexes; however, due to the nature of polyelectrolyte complexation, the stability of polyplexes has first to be secured when polyplexes are subjected to such manipulation to modulate surface properties.

Endosomal escape can be facilitated by chemical modification of polymeric carriers, too. By incorporating moieties (e.g., PEI, chitosan, and imidazole groups) that possess unprotonated amines groups into the carrier backbone, endosomal swelling and rupture may be induced by the **proton sponge effect** [35-36]. The success of this strategy has been supported by Zhang and coworkers, who generated nanoassemblies using the solvent evaporation method, in which phenylboronic acid-modified cholesterol (Chol-PBA) was coupled with 1,3-diol-enriched oligoethylenimine (OEI-EHDO) (Fig. 2) [37]. Upon the lysosome-acidity induced assembly destruction via the proton-sponge effect, the gene payload can be effectively released after endocytosis, thereby promoting gene transfection [37]. Apart from the proton sponge effect, pH-sensitive fusogenic peptides (e.g., penetratin, trans-activating transcriptional activator peptide, transportan, ppTG1, ppTG20 and KALA), which undergo conformational changes in a mildly acidic environment, have been exploited to disrupt the endosomal membrane via the membrane fusion activity to improve the transfection efficiency [15, 38, 39]. More recently, melittin, a lytic peptide extracted from honey bee venom, has been exploited for endosomal membrane dissociation. Unfortunately, cells often hardly afford the damage imposed by melittin to cytoplasmic membranes at the physiological pH. To tackle this, dimethylmaleic anhydride has been used to cover the cationic charge so that the lytic activity is hindered at the physiological pH and is elicited only at the pH of 5 [40]. Different mechanisms of endosomal escape induced by the fusogenic or lysogenic peptides, or by the proton sponge effect, are summarized in Fig. 3.

The final stage of plasmid delivery is nuclear import, which can be enhanced by conjugating the carrier with peptides that can import large nanoparticles across the nuclear membrane. This has been shown by Wu and coworkers [41], who attached the cell-penetrating peptide TAT to large-

pore ultrasmall mesoporous organosilica nanoparticles for nuclear translocation. Apart from TAT, **nuclear localization signal (NLS) peptides** have been widely employed for conjugation. This has been reported by a recent study which incorporated a histone-derived NLS into polyplexes to facilitate interactions of the polyplexes with histone effectors [42]. The polyplexes first accumulate in the endoplasmic reticulum (ER) prior to mitosis, followed by re-distribution into the nucleus after cell division (Fig. 4) [43]. The success of using NLS peptides has also been reported by Chen and colleagues, who developed a carrier based on a hydroxyl-terminal poly(amidoamine)(PAMAM) dendrimer derivative (PAMS). The derivative contains a β-thiopropionate bond, which is degradable under acidic conditions. The carrier can interact with DNA, and an NLS peptide, to generate PAMS/DNA/NLS polyplexes [44]. The peptide facilitates the binding of the polyplexes to importins (Fig. 5) [44]. In GTPase-activating protein1 (RanGAP1)-overexpressing cells, both the transfection and nuclear import of the polyplexes are enhanced [44]. This provides evidence to the dependence of the NLS-mediated nuclear import on RanGAP1, and offers insights into the possibility of increasing the transfection efficiency by pre-treatment with RanGAP1 prior to transfection. This possibility has already been corroborated by the observation that mice pre-injected with polyplexes containing a RanGAP1-encoding plasmid show higher expression efficiency of PAMS/DNA/NLS polyplexes [44]. Apart from RanGAP1, pre-treatment to inhibit the histone deacetylase (HDAC6) activity may increase the transfection efficiency by stabilizing acetylate microtubules [16-18], which are cytosolic peptides necessary for proper nuclear translocation.

Spatiotemporal Confinement of Gene Expression

Chemical conjugation is a prevailing approach to achieve active targeting, but techniques enabling spatiotemporal control over the expression of the delivered nucleic acid material will be the future. Achieving this requires physicochemical changes of the polymeric carrier in response to bio-stimuli that are linked with any step of the intracellular gene delivery process. Such a concept has already been verified to be practicable over the past decade. For instance, by using the engineered red/far-red light-switchable protein, phytochrome B (PhyB), and its red light dependent interaction partner, phytochrome interacting factor 6 (PIF6), Suh's team has engineered an adeno-associated virus (AAV) for light-controlled gene delivery [45]. Upon red light irradiation, the AAV can bind to NLS-tagged PhyB, and its translocation into the host cell nucleus can be facilitated [45]. Intriguingly, by using projected patterns of co-delivered red and far-red light, gene delivery can be spatially controlled [45]. Together with recent advances in photonics [46], spatiotemporal control of polymeric gene delivery may be just around the corner.

With the emergence of photoactivable molecules that can release payloads at specific sites upon UV irradiation, spatiotemporal control of the release of the delivered nucleic acid material becomes more sophisticated [47]. Unfortunately, owing to the toxicity and low tissue penetration power of UV, the application potential of these molecules in gene delivery is limited. This problem may now be ameliorated by the use of **upconversion nanoparticles** (UCNPs), which can convert near-infrared (NIR) or visible light to UV *in situ* and have been used to control gene expression in tumor cells transplanted in adult zebrafish [48]. Both gene over-expression and the onset of RNA interference can be regulated using this approach. This is exemplified by a study in which a carrier of small interfering RNA (siRNA) was prepared by first encapsulating Yb^{3+}/Tm^{3+} co-doped nanocrystals in a silica shell with surface amine groups, followed by surface functionalization with cationic photocaged linkers [49]. NIR light irradiation can cleave the photocaged linker on the

nanoparticle surface [49], thereby initiating siRNA release in a temporal-spatial manner. More recently, a similar method has been adopted by Guo and colleagues for site-specific gene silencing [50]. They caged siRNA molecules using light-sensitive 4,5-dimethoxy-2-nitroacetophenone (DMNPE), and delivered those molecules with $NaYF_4$:Yb,Tm UCNPs. Upon irradiation with NIR light, emission of the upconverted UV light was stimulated to uncage DMNPE. These technologies have paved the way to precisely manipulate gene expression.

Further Optimization for Preclinical Use

So far, we have focused our discussions on strategies to enhance transfection at the cellular level (Table 1). Future research, however, may be directed to enhance the translation of bench results into preclinical and clinical use. To achieve this goal, a few extra properties of the polyplex have to be considered. One example is polymer biodegradability and toxicity, which has attracted extensive research interests over the years for a search of solutions. For instance, an earlier study copolymerized dextran with lactic acid oligomers to enable polymer degradation [51]. Such degradation occurs via either random hydrolysis of the ester bonds in the lactate grafts, or cleavage of the lactate graft from the dextran backbone by the attack of OH^-. This remarkably enhances the biodegradability of the polymer under physiological conditions [51]. Recently, by lowering the charge density of PEI via **graft polymerization** with polysorbate 20, the cytotoxicity of the nanoparticles was shown to be reduced [52]. Many of these efforts devoted to the development of polymers with low toxicity and high biodegradability have been reviewed in detail elsewhere [53, 54].

Another factor to be considered is the blood circulation time. One strategy to lengthen the blood circulation time is to hydroxylate a polymeric carrier. This can offer the carrier stronger tolerance to serum-induced transfection inhibition by inhibiting protein adsorption [55]. Another strategy is **PEGylation**. Owing to the hydrophilic nature and brush-type polymer crowding of polyethylene glycol (PEG) [56], PEGylated carriers are, in general, less prone to **opsonization** and reticuloendothelial system (RES) uptake [56]. This has been observed in the chitosan-based carrier. After being incorporated with PEG (Mw = 5 kDa, grating degree = 9.6 %), the carrier has exhibited less polyplex aggregation even in the presence of bile and serum [57]. *In vivo* studies reported that, on day 1 after bile duct infusion and day 3 after portal vein infusion, PEGylated chitosan produces much higher transgene expression in rat liver compared to unmodified chitosan [57]. This is partly ascribed to the reduction of opsonization, as well as the prolongation of the blood circulation time, of the polyplex after PEGylation. This postulation has been verified by Zhang and colleagues [58], who demonstrated that PEGylation can reduce Kupffer cell-mediated clearance of chitosan-based polyplexes. Following caudal-vein infusion into Sprague-Dawley mice transplanted with hepatic carcinoma cells, the PEGylated polyplexes were found to mediate higher transgene expression in hepatoma tissues than did the non-PEGylated polyplexes [58].

Despite the seemingly promising effect of PEGylation, every coin has two sides. PEGylation can extend the time of polyplexes in blood circulation, but may also impair the DNA compaction ability of the carrier. The latter has been suggested by the observation that PEGylated polyplexes may experience premature polyplex unpackaging in blood, resulting in less efficient gene transfer to liver [59]. Davis's group also reported that PEGylation can confer salt stability to polyplexes but may hamper the cellular uptake and intracellular trafficking of them [60]. In this regard, and considering the fact that characteristics (e.g., density, conformation, molecular weight, and

flexibility) of the PEG moiety are the predominant factors determining the effect of PEGylation [56], optimizing the degree of PEG grafting as well as the coating size is indubitably imperative in constructing PEGylated carriers.

Concluding Remarks and Future Perspectives

Viral vectors remain the most effective candidates for gene delivery. Yet, safety concerns (particularly with regard to the acute immune response and insertional mutagenesis [61, 62], along with the limitation on the size of the payload) promote a search for non-viral substitutes. Polymers are widely studied substitutes because of their ease of modification and flexibility in structural design. In this article, we have presented an overview of advances in polymeric gene delivery, whose practical potential is anticipated to be further escalated by incorporating multiple functionalities for more extended applications in the future. Such feasibility has been exemplified by a recent study [63] that obtained cationic carbon quantum dots (QDs) from alginate. The QDs, on one hand, display strong fluorescence for functioning as an imaging probe [63]. On the other hand, they can deliver genes by internalizing into cells via both caveolae- and clathrin-mediated endocytosis [63]. In the coming decade, we foresee a few technical problems that still have to be solved for streamlining the clinical translation of the developed carriers (see Outstanding Questions), but carrier development will continue as technologies (e.g., S-FIL and layer-by-layer deposition) for materials fabrication advance. These technologies can help to extend the flexibility and versatility in design of polymers for gene delivery. Along with the increasing understanding of the mechanisms of cellular internalization and intracellular trafficking, the emergence of more effective polymeric carriers with promising application potential is anticipated.

Disclaimer Statement

The authors declare that they have no conflicts of interest.

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Figure Legends

Fig. 1 Intracellular gene delivery mediated by a polymeric gene carrier. Major processes include (a) electrostatic interactions, (b) cellular internalization, (c) endosomal escape, (d) nuclear translocation, and (e) polyplex dissociation.

Fig. 2 "Superfast" transnuclear gene translocation and gene transfection mediated by the OEI-EHDO/Chol-PBA nanoassembly. (a) Fabrication and structures of OEI-EHDO and Chol-PBA. (b) A schematic diagram depicting the process of transnuclear gene translocation and gene transfection mediated by the nanoassembly. The process is proposed to be achieved via lysosomal-acidity-responsive disassembly. (Reproduced from ref. 37 with permission from Elsevier)

Fig. 3 Different mechanisms of endosomal escape. (a) During maturation of early endosomes to late endosomes, acidification occurs. An electrical gradient is obtained by V-type ATPases, which drive the ATP-driven transport of H^+ ions across the endosomal membrane. This electrical gradient is subsequently balanced in part by the influx of counter-ions, presumably Cl^- ions. (b) Upon acidification, cationic carriers induce phospholipids on the outer endosomal leaflet to flip to the luminal side of the endosome, causing membrane destabilization. If fusogenic or lysogenic peptides are present, these peptides may also undergo conformational changes in acidic environments to destabilize the endosomal membrane. (c) Pore formation is resulted upon membrane destabilization. (d) When polymeric carriers possessing the pH-buffering capacity are present in the endosomal lumen, an osmotic gradient and internal pressure will be generated due to the proton-sponge effect, causing rupture of the endosome. (e) In the case of cationic liposomes, fusion with the endosomal membrane may occur, resulting in escape of the cargo into the cytosol. (Reproduced from ref. 15 with permission from Elsevier)

Fig. 4 A proposed mechanism undertaken by H3-targeted polyplexes for cellular uptake and intracellular trafficking. Key regulators include Rab5, Rab9, H3K4MT, and Rab6. Abbreviations: ER, endoplasmic reticulum; H3K4MT, H3 lysine 4 methyltransferase. (Reproduced from ref. 43 with permission from Elsevier)

Fig. 5 The process of nuclear transport undertaken by PAMS/DNA/NLS polyplexes. (a) A schematic diagram showing the formation of PAMS/DNA/NLS polyplexes. NLS binds to DNA to form the DNA/NLS complex (DNA/NLS), which can interact with PAMS to generate PAMS/DNA/NLS polyplexes. (b) PAMS/DNA/NLS degrades in the endosome. After endosomal escape, DNA/NLS binds to importins (Ims) to form a complex, whose nuclear import necessitates the involvement of RanGAP1. (Reproduced from ref. 44 with permission from Elsevier)

Trends

Incorporation of multiple functionalities, ranging from stimuli-responsiveness to imaging properties, into the development of polymeric carriers will be the future trend in gene delivery research. Such a research trend will enable spatiotemporal control of transgene expression during gene delivery, and will open up a vista of new possibilities in theranostics and treatment development.

In addition, future efforts will continue to be devoted to searching for novel ligands to enhance the cell targeting capacity of polymeric carriers. Collaborations between chemists and biologists are expected to be more frequent to fulfill the multidisciplinary nature of this search.

Glossary

Actin: A cytoskeletal protein that plays an important role in diverse functions in cells, ranging from regulating cellular motility to determining cell morphology

Aprataxin: A nuclear protein with a role in DNA repair

Caveolae: Flask-shaped invaginations of the plasma membrane

Clathrin: A cytoplasmic protein which plays an important role in endocytosis and intracellular trafficking

Cytoskeleton: A highly dynamic supramolecular network that consists of protein components such as actin filaments, intermediate filaments, and microtubules. This network involves in regulating cell morphogenesis and motility

Endocytosis: A process that transports molecules from the extracellular milieu into cells via vesicle formation at the plasma membrane

Gene therapy: A procedure to manipulate the genetic component of living cells for treating or improving the health condition of a patient

Graft polymerization: A polymerization process in which macromolecular chains are covalently immobilized on a polymer substrate

Layer-by-layer assembly: A technique that involves a sequential assembly of polymers onto sacrificial substrates before substrate dissolution is performed

Nuclear envelope: A complex membrane structure that forms the boundary between the nuclear and cytoplasmic compartments in eukaryotic cells

Nuclear localization signal (NLS) peptides: Peptides that can localize polymeric carriers to the cellular nucleus and allow the carriers to be actively transported across the nuclear pore complex

Nuclear pores: Pores in the nuclear envelope, regulating the transport of molecules in and out of the nucleus

Opsonization: A process in which opsonins interact with the exogenous entities to make the entities more susceptible to phagocytosis

PEGylation: A technique to lengthen the blood circulation time by covalently attaching PEG to a given molecule

Polyplexes: Polyelectrolyte complexes formed between a polycation and the delivered nucleic acid material

Protein kinase C (PKC): A protein that plays an essential role in signal transduction and hence is regarded as an important regulator of cell functions

Proton sponge effect: A widely-accepted hypothesized mechanism of osmotic endosomal disruption mediated by an agent which can swell upon protonation

Step and flash imprint lithography (S-FIL): A nanomolding process that enables replication of the topography of a quartz template, with a photocurable macromer solution being used as a molded material

Syndecan: An integral membrane proteoglycan that can transmit changes in the extracellular environment into changes in cellular behavior

Transcription factor: A protein that involves in the transcription of genetic information from DNA to messenger RNA

Transfection: A procedure to introduce exogenous genetic materials into a cell for subsequent expression of the encoded protein

Upconversion nanoparticles (UCNPs): Nanoparticles in which trivalent lanthanide ions are dispersed in a selected dielectric host lattice. These nanoparticles can emit outcome photons with energy higher than the incident photons

Zeta potential: The electrical potential at the boundary of the hydrodynamic shear plane of a charged particle

Additional Materials

Box 1. The structure and working mechanism of the nuclear pore complex

A nuclear pore complex (NPC) is a soluble protein channel embedded in a nuclear envelope [64]. It has a mass of around 15 times that of a ribosome, and is a supramolecular assembly consisting of multiple copies of approximately 30 different nucleoporins (Nups). Each NPC has three major parts: the central structure, cytoplasmic components (i.e., cytoplasmic ring and filaments), and nucleoplasmic components (i.e., nuclear ring and nuclear baskets) [65, 66]. The innermost part of the central structure contains phenylalanine-glycine (FG)-repeat Nups, which expose directly to cargoes during nucleocytoplasmic transport (NCT). FG-repeat Nups are also part of the peripheral structure that extends from the channel towards the cytoplasmic and nuclear space. Adjacent to the FG-repeat layer is the scaffold layer that stabilizes the structure. The outermost part comprises transmembrane Nups that anchor the pore to the nuclear envelope. A schematic illustration of the structure of an NPC is shown in Fig. 1.

To explain the selectivity mechanism of the NPC, few models have been proposed. According to the virtual gate model, noncohesive and entropic bristles are constructed from FG-repeat filaments. These bristles prevent non-karyophilic molecules from passing through the NPC [67]. On the other hand, the selective phase model holds that FG-repeat Nups interact with each other via hydrophobic interactions to form a selective meshwork that sieves molecules by size exclusion [68]. Owing to the presence of these barriers, NCT of macromolecules usually necessitates the involvement of import and export receptors (collectively termed karyopherins) as well as translocation signals [i.e., nuclear localization signal (NLS) and nuclear export signal] [69]; however, as suggested by the reduction of dimensionality model [70] and the spaghetti oil model [71], small molecules and ions having a size below the 40-60 kDa size exclusion threshold could be transported across the **nuclear pore** via passive diffusion.

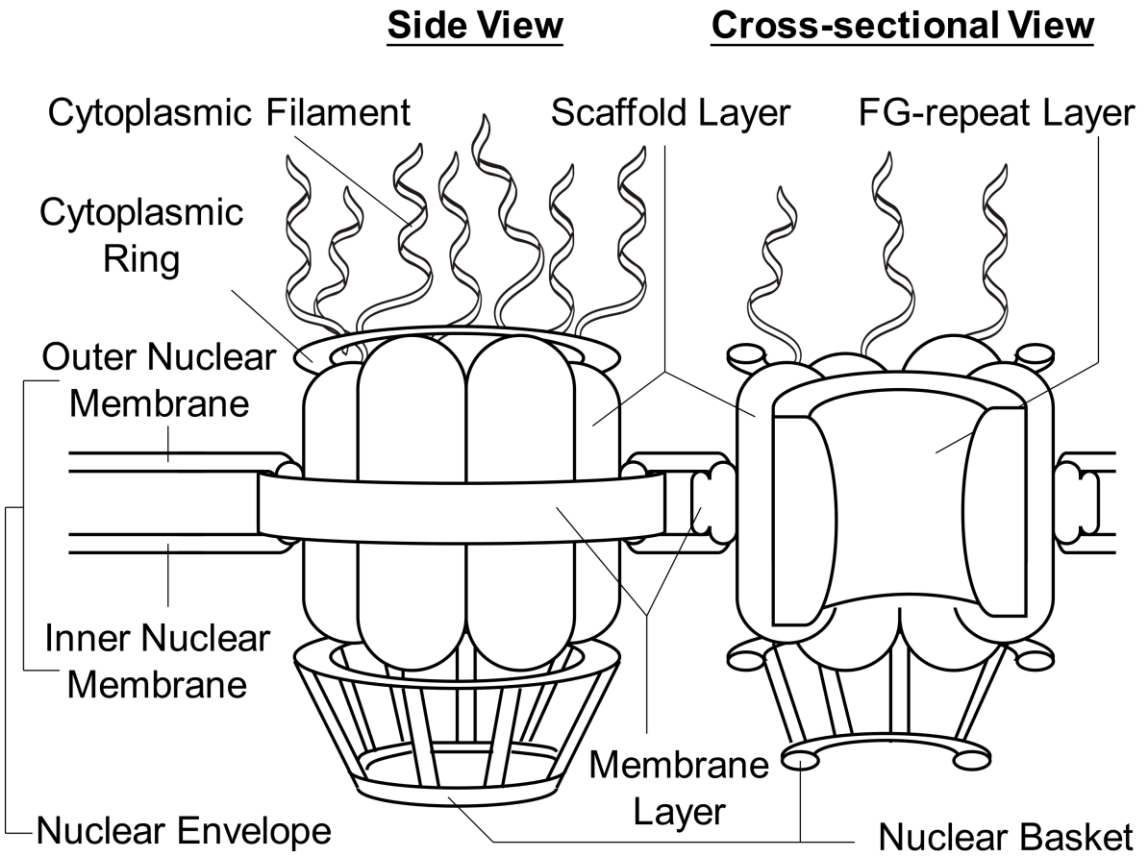

Fig. 1 A schematic illustration of the nuclear pore complex structure.

NCT can tightly control the directionality of the transport. This is often achieved by governing the concentration gradient of the GTP- and GDP-bound forms of Ran (a Ras-related small guanosine triphosphatase) between the nucleus and cytoplasm. In the cytoplasm, Ran GTPase activating protein (RanGAP) hydrolyzes GTP, leading to conformational changes of Ran to yield RanGDP. On the other hand, Ran guanine nucleotide exchange factor (RanGEF) replaces GDP with GTP to regenerate RanGTP in the nucleus. The action of RanGEF and RanGAP leads to the dissociation of import and export complexes, causing the cargo to be released in the nuclear or cytoplasmic space. The dynamics of NCT has already been reviewed elsewhere [72, 73]. Readers are referred to those reviews for details.

Because the NPC serves as a conduit for macromolecular exchange across the nuclear envelope and involves in fundamental cellular events such as chromatin organization [74], a dysfunction in the NPC may lead to pathological manifestations. For instance, a malfunction of the scaffold Nup, ALADIN(I482S), can compromise the karyopherin-α/β-mediated import of **aprataxin** and DNA ligase I, subjecting the affected cells to DNA damage [75]. Moreover, changes in the expression of the oxalate binding protein Nup62 (a FG-repeat Nup at the central gated channel [76]) have been reported to be associated with nephrotic syndrome [77]. All these corroborate the importance of proper functional regulation of the NPC to normal physiological functioning.

Because of the presence of the NPC, small particles with a diameter of less than 40 kDa can pass through the pores by passive diffusion; whereas larger particles need to possess an NLS in order to enter the nucleus. Some FRET analysis has suggested that sometimes plasmids have not been dissociated from polyplexes even after nuclear import [78, 79]. This leads to confusions because nuclear pores are expected to be possibly amplified only to 39 nm, which is remarkably smaller than the typical diameter of polyplexes (70-300 nm). On the other hand, in the case of liposomes, lipoplexes have not been found to be present inside the nucleus, suggesting the complete dissociation of lipoplexes prior to nuclear import. Recent studies have proposed that such complete dissociation is the reason explaining the higher transfection efficiency of liposomal formulations [80, 81]. Nevertheless, till now the mechanism of nuclear import of polyplexes has been ill-defined. Follow-up research unraveling the quantity and location of the plasmid and the polymeric carrier on both sides of the nuclear pore, as well as the interactions of polyplexes with nuclear membrane complexes, may offer insights into future molecular design of the polymeric carrier to enhance the efficiency of transfection.

Table 1 Major strategies to enhance intracellular gene delivery mediated by polymeric carriers

Strategy	Approach	Working Principle	Strength	Limitation	Upcoming Trend	Ref.
Optimization of the timing of transfection	Transfection at the time of cell division	Facilitate nuclear import of the delivered gene by matching with the time of nuclear dissociation during cell division	• Applicable to different transfection agents • No manipulation of the polyplex is required • Easy to operate	• May not be applicable to non-dividing cells • Proper timing of transfection is passively determined by the stage of the cell cycle • May not be effectively applicable to the *in vivo* context	• Applicability of this strategy will continue to be confined to *in vitro* applications • Potential use of this strategy in biological interventions will be limited	5
Cell pre-treatment	Pretreatment with RanGAP1, HDAC6 inhibitors, or other agents that can intervene with the physiology of the cell	Manipulate the physiological process relevant to intracellular gene delivery prior to the delivery process	• No manipulation of the polyplex is required • Effective in *ex vivo* and *in vitro* applications • Applicable to different transfection agents	• Changes in the physiology of the cell will be resulted • Sophisticated prior knowledge of the physiological mechanism of intracellular gene delivery is required	• Practical use of this method in preclinical studies may be enhanced by the continuous advances in the development of carriers for co-administration of multiple drugs • More molecular targets will be identified as the understanding of cell physiology increases	16-18, 44
Manipulation of the physical properties of polyplexes	Size manipulation	Optimize the polyplex size for effective cellular uptake	• The most straightforward approach to optimize the transfection performance • Strategies to engineer different physical properties of polyplexes are available	• Proper understanding of the relationships between the polymer structure and polyplex properties is required • Active targeting can hardly been achieved by sole manipulation of physical properties of the carrier	• Efforts to optimize polyplex properties will continue to be a major strategy in carrier development • Flexibility in geometry manipulation is projected to be enhanced by the rapid advances in materials engineering	6
	Zeta potential manipulation	Optimize the zeta potential of the polyplex for effective electrostatic interactions with the anionic plasma membrane				
	Geometry manipulation	Engineer the geometry of the polyplex to facilitate cellular internalization				
Chemical conjugation	Targeting ligand	Ligand conjugation to enhance cellular uptake via receptor-mediated endocytosis	• Diverse well-developed bioconjugation techniques are available	• Immunogenicity may be resulted upon conjugation with peptides and proteinaceous ligands	• Chemical conjugation will continue to be a major and convenient	15, 38, 39, 42, 43

	Fusogenic peptide	Facilitate endosomal escape via the membrane fusion capacity of the peptide	• A track record of the applications of chemical conjugation in carrier development is available • Convenient to carrier development because one ligand can be used in multiple carrier designs	• Incorporation with ligands and peptides may change the physical properties of the polyplex and may require additional consideration during carrier design • The repertoire of ligands and peptides currently available for selection is limited	approach in carrier design • More ligands and peptides will be discovered as studies on cell surface markers accumulate • More carriers entering preclinical studies are anticipated to be surface-modified for receptor-mediated endocytosis	
	Cell penetrating peptide and NLS peptide	Facilitate nuclear import of the polyplex to enhance transgene expression				
Polymer engineering	Structural engineering of the polymeric gene carrier	Manipulate the polymer structure and properties for better interactions with major biomolecules (e.g., nuclear transcription factors) in cells for more effective transfection	• Well-supported by the availability of sophisticated knowledge of synthetic chemistry • Strategies to engineer the structure and properties of polymers are available • High flexibility in molecular design	• Proper understanding of the structure-activity relationship is required for molecular design • Sufficient prior knowledge of organic synthesis is needed	• Versatility in polymer engineering will be further enhanced by the development of materials fabrication technologies • New polymers with diverse structures, ranging from straight chains to dendrimers, will continue to be developed and reported in the literature	32, 36