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# Essential cues of engineered polymeric materials regulating gene transfer pathways

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ABSTRACT

Regulating cellular uptake pathways using engineered materials is becoming a vital strategy for efficient gene transfer because the success of gene delivery most often relies on the uptake mechanism and the intracellular fate of the delivery vectors. The uptake of gene carriers can be greatly affected by the various physical, geometrical, chemical, and biological characteristics of the delivery vectors. In the design of gene delivery materials, it is important to understand not only how gene carriers are taken up and transported into cells, but also how the uptake mechanism can be regulated. In this review, we discuss polymeric materials that regulate cellular

*Abbreviations:* 66–34 br-OT, 2:1 M mixture MUS: 3,7 dimethyl octane 1-thiol; 66–34 OT, 2:1 M mixture of MUS and 1-octanethiol; AEM·HCl, 2-aminoethylmethacrylate hydrochloride; AnnA1, annexin A1; APP, aminopeptidase P; ARF6, adenosine diphosphate ribosylation factor 6; BBB, blood–brain barrier; BPS, biodegradable pH-sensitive surfactants; Cav-1, caveolin-1; Cav-2, caveolin-2; CDC42, cell division cycle 42; Chol, cholesterol; CIE, clathrin-independent endocytosis; CLDPD, cationic liposome-coated DNA/protamine/DNA; CMCS, carboxymethyl chitosan; CME, clathrin-mediated endocytosis; Col, collagen; CPPs, cell penetrating peptides; CvME, caveolae-mediated endocytosis; DC, 3β-N-(N,N-dimethylaminoethane)-carbamoyl; DC6-14, O,O'-ditetradecanoyl-N-(alpha-trimethylammonioacetyl)diethanolamine chloride; DNA, deoxyribonucleic acid; DOPE, phosphoethanolamine; DOTAP, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate; DPD, dissipative particle dynamics; DS, degree of substitution; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; Fn, fibrinogen; GPI-APs, glycosylphosphatidylinositol-anchored proteins; GTPase, guanosine triphosphatase; His, histidylated; His-pLK, histidylated polylysine; HUVEC, human umbilical vein endothelial cells; LMWC, low molecular weight chitosan; mAnnaA1, monoclonal antibody to AnnaA1; mAPP, monoclonal antibody to APP; MSCs, mesenchymal stem cells; MUS, 11-mercapto-1-undecanesulphonate; MW, molecular weight; MWCNT, multi-walled carbon nanotubes; NPCS, hydrophobically-modified chitosan nanoparticles; NPs, nanoparticles; OPN, osteopontin; PAMAM, poly(amidoamine); pAntp, Drosophila Antennapedia; PBAE, poly(β-amino ester); pDNA, plasmid DNA; PEG, polyethylene glycol; PEI, polyethylenimine; PHPMA, N-(2-hydroxy propyl) methacrylamide copolymer; PI3K, phosphatidylinositol 3 kinase; PLGA, poly(lactic-co-glycolic acid); PLL, poly(L-lysine); PLT, poly(lactitol-co-PEI); PRINT, particle replication in non-wetting templates; PSOAT, polysorbitol transporter; RVG, rabies virus glycoprotein; siOPN, OPN siRNA; siRNA, small interfering ribonucleic acid; TAT, trans-activator of transcription; TcdB, *Clostridium difficile* toxin B; XGC, poly(xylitol-co-PEI); CRISPR-Cas9, clustered regularly interspaced short palindromic repeats-associated protein 9; SORT, selective organ targeting.

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uptake pathways for highly effective delivery of gene therapeutics, elucidate various routes of cellular uptake that alter the intracellular fate of polymeric gene carriers and finding efficient strategies for overcoming extracellular and intracellular obstacles. We also discuss the structures of polymeric materials in order to understand how they regulate cellular uptake. Lastly, we discuss various strategic approaches, including essential cues on how to regulate the cellular uptake pathways of polymeric carriers and how to control their endocytic trafficking to improve the efficacy of gene delivery.

## 1. Introduction

Gene therapy technology has been showing significant potential in treating a myriad of diseases including inherited and previously-thought untreatable disorders. This technology aims to transfer genetic material into the cells of organ-sites, and express target proteins to generate desired therapeutic effects [1]. Despite diverse bioengineering advances in the design of viral and non-viral vectors, their efficacy in improving or silencing expression of the target genes has been hampered by several delivery barriers [2]. Therefore, the design of an effective and safe gene delivery system has been an extremely important task to avoid unnecessary scrutiny on the field of gene therapy [3,4]. Viral vectors are the most effective gene delivery vehicles which mimic the virus-mediated cellular entry. In the past, viral vectors have caused off-target reactions that hampered successful clinical application [5,6]. Although a few incidents of off-target immune reactions have occurred during the past few decades of virus-mediated gene therapies, including fatal cases and controversial clinical trials [7-11], recent preclinical and clinical advances [12-20] have demonstrated promising bed-side applications, namely the development of vaccines against the 2019 coronavirus disease [16,21]. Viral vectors such as retroviruses, adeno-associated viruses, lentiviruses, and herpes simplex virus (HSV) have been widely considered for gene delivery, and there are already some examples of successful clinical trials [22]. For instance, HSV HF10 showed a good safety and antitumor activity against melanoma when combined with ipilimumab (anti-CTLA-4), showing 68% clinical therapeutic efficacy (NCT02272855) [23]. However, viral vectors should still be used with caution due to side effects including off-target immunogenicity, inflammatory response, toxicity, and the risk of mutagenesis [24].

It is necessary to emphasize that a new gene delivery strategy should meet the safety requirements set by the Food and Drug Administration (FDA); however, poor efficacy limits their effectiveness, preventing their transition to clinical trials and subsequent release to the public. One example of these is the lentiviral mediated gene therapy called “ProSavin,” developed to treat Parkinson’s disease. Although ProSavin caused no adverse side effect, it failed to show any therapeutic effect, thus bringing its progress to a halt [25]. Despite the failure, there have been successful examples of gene delivery, AAV serotype 8 vectors were used to treat Hemophilia-B [11,26] which resulted in the sustained expression of therapeutic factor IX in patients. Other notable treatments include adeno-associated virus serotype 2 vectors to treat Type 2 Leber congenital amaurosis [27-30], an inherited retinal dystrophy caused by RPE65 mutations [31], and blindness associated with Choroideremia caused by mutations in the CHM gene [32]. AAV-mediated gene therapies have also proven to be effective in treating Duchenne muscular dystrophy caused by mutations in the dystrophin gene as well as limb-girdle muscular dystrophies, mutations in the α-sarcoglycan gene [33].

As of yet, there are 4 gene therapies that are commercially available worldwide. In 2003, China was the first country to approve the commercial production of a gene therapy, called Gendicine (recombinant human p53 adenovirus), designed by Shenzhen SiBiono GenTech to treat head and neck squamous cell carcinoma. The treatment uses AAV serotype 5 engineered to express the p53 tumor suppressor gene [34,35]. The second gene therapy developed by UniQure was approved and certified by the European Medicines Agency and the European Commission in 2012. The drug has been named Glybera, intends to treat lipoprotein lipase deficiency using AAV serotype 1 to deliver the lipoprotein lipase gene to muscle cells [36]. In 2016, the third gene therapy, named Strimvelis®, developed by GlaxoSmithKline, was approved in Europe to treat adenosine deaminase deficient-severe combined immunodeficiency using ex vivo hematopoietic stem cell therapy [37]. In 2017, Kymriah® (tisagenlecleucel) developed by Novartis, which was previously known as CTL019, to treat acute lymphoblastic leukemia [38] was approved. Kymriah is a CD19-targeted chimeric antigen receptor-T cell therapy which recognizes and destroys B cells with acute lymphoblastic leukemia. The vector used to transfect the patients’ T cells ex vivo was an HIV-1-derived lentiviral vector [39]. While the technologies in clinical trials have shown some promise by using viral vectors for gene delivery, they are prone to causing undesirable off target effects including insertional mutagenesis and unintended immune responses. To circumvent inherent safety risks of viral vectors, non-viral materials have been extensively studied for gene therapy.

Several non-viral gene delivery vectors have been investigated extensively to replace viral ones [24]. Non-viral vectors have substantial advantages over viral ones. For example, they are easily modifiable, and chemical engineering allows flexibility of the transgene insert, resulting in less immunogenicity [40]. Moreover, they are inexpensive, stable, and easy to scale up and control targeting specificity [41]. However, they offer inadequate transfection capacity compared to viral vectors. Potential toxicity is another concern for non-viral vectors that has often been reported [41]. It is worth noting that increasing transfection efficacy while reducing cytotoxicity is the hardest challenge in developing non-viral gene delivery systems [42].

Each and every non-viral delivery vector has unique advantages and disadvantages, and thus there is no universal delivery system that is suitable for all applications. In other words, a wide range of non-viral vectors such as lipid-based systems, carbon-based vectors, silica-based vectors, gold nanoparticles, peptides and synthetically modified cationic polymers could be applied as carriers in each application to deliver genetic material into target cells. Having said this, it is difficult to directly and objectively compare the different

types of non-viral vectors, because their total behavior *in vivo* varies greatly depending on synthesis procedure, surface functionalization and cellular targets. Lipid-based systems generally have ideal biocompatible characteristics and can provide good transfection efficacy, but they are often immunogenic [42,43]. Conversely, polymer- and dendrimer-based systems are less immunogenic but are more cytotoxic. Carbon-based vectors such as graphene and carbon nanotubes have also been shown to provide a biocompatible delivery system with a high drug loading capacity and easily tunable properties via surface functionalization and modification. Silica-based vectors also offer the same properties since their surface can be easily modified with functional ligands [44]. Silica is resistant to hydrolysis and enzymatic degradation, offering rigorous protection to its drug payload [46]. Furthermore, due to nanoscale properties, mesoporous silica has a large surface area and large pore volume, resulting in an exceptional drug loading capacity, but, like all other non-viral systems, its low transfection efficiency is the main drawback [47]. Gold is yet another inorganic-based delivery system that offers extensive surface area while being inert and generally non-toxic [48-50]. Polymeric materials, on the other hand, are a prime choice for non-viral gene delivery systems due to their well-defined chemical structures and tunable physicochemical properties. In particular, cationic polymers offer several advantages: adequate condensation with nucleotides, stability of the polymer-gene complexes (polyplexes), no limitation on vector size, and reduction of reactogenicity and toxicity when biocompatible and degradable properties introduced. Needless to say, improving their transfection ability while simultaneously reducing cytotoxicity is the key challenge in designing a competent cationic-polymer-based gene carrier system [41].

Designing gene carriers that can regulate cellular uptake has become a leading strategy in improving gene delivery efficacy because uptake pathways determine the intracellular fate of the genes by directing them toward either the digestive or non-digestive route. Designing gene carriers for cellular uptake requires careful consideration of the physical, geometrical, chemical, and biological properties of the polymeric carrier, and structural modification is often mandatory for adding new functional properties. In this review, we describe various engineering approaches to improve polymeric gene delivery materials, conquer cellular barriers, and enhance therapeutic efficacy. As the core part review, we emphasize the structural relationship between polymeric materials and cellular uptake regulation in relation to physical, geometrical, chemical, and biological cues that regulate gene delivery dynamics, including cellular entry and intracellular trafficking.

## 2. Design of polymeric materials and challenges in regulating gene delivery pathways

Deoxyribonucleic acid (DNA) is a genetic information, which contains instructions for development, growth and reproduction of the organism. The messenger that transmits instructions from DNA to control the synthesis of proteins is ribonucleic acid (RNA) while messenger RNA (mRNA) is an extensive group of RNA molecules that transfers genetic information from DNA to the ribosome, where they specify the amino acid sequence of the protein products of gene expression. On the other side, RNA interference (RNAi) is a process in which RNA molecules inhibit the expression or translation of a gene by neutralizing targeted mRNA molecules. A common class of RNAi molecule is the small interfering RNA (siRNAs), which are artificially synthesized using 19–23 nucleotide long double-stranded RNA molecules. The siRNA is commonly used in molecular biology for silencing genes of interest. In gene delivery

**Table 1**
Cellular barriers to polymeric gene delivery and approaches to conquer them.

| Cellular barrier | Effects of barrier | Approaches to conquer the problem | References |
|---|---|---|---|
| **Extracellular barriers** | | | |
| Enzymes (i.e., nucleases) | -Degradation of nucleic acids (DNA or siRNA) | -Development of cationic polymers to condense nucleic acids and protect them from enzymes | [45] |
| Blood components (i.e., serum proteins and red blood cell) | -Interactions with gene carriers and shortened circulation time | -Inclusion of properties (i.e., lowering surface charge) in polymeric carriers through PEGylation or the addition of hydroxyl groups | [87] |
| Poor targetability and binding of cells | –Non-specific cellular targeting and inefficient binding cause failure of gene transfer into cells | -Inclusion of cell targeting ligands (i.e., folate, galactose, mannose, transferrin, IL-2, cholera toxin) to polymeric carriers | [88] |
| Inefficient cellular uptake | -Poor transfection efficiency | -Electroporation and gene gun method<br>-Design of cationic polymeric carriers<br>-Introduction of functional molecules to polymeric carriers to enhance the cellular uptake of particles | [89] |
| **Intracellular barriers** | | | |
| Non-specific intracellular fate and lack of endosomal escape | –Non-specificity can direct particles to the intracellular digestive route, leading to lysosomal fusion and eventual degradation of genes | -Osmotic active polymeric carriers can induce a non-digestive intracellular fate-amine containing polymers (i.e., PEI) with buffering capacity for endosomal escape<br>-Use of endosomolytic peptides | [90] |
| Vector unpacking and inadequate release of gene | -Improper functionality by genetic materials | -Development of biodegradable polymers | [91] |
| Improper nuclear uptake | -Offensive gene expression | -Nuclear localization peptides and nucleotide sequences | [92] |

technology, these above genetic materials (DNA, siRNA or mRNA) are either applied as naked form or using electroporation/ultrasound techniques or encapsulated inside or conjugated to the nanoparticles, which protects the genetic materials until the drug reaches its target site. In this regard, an effective delivery of these genes to the right site at the right time is one of the critical issues to achieve a significant therapeutic index [51]. It is worth noting that depending on the type of the therapeutic modality (e.g., DNA or RNA), the outcome can be completely different with the choice of polymeric carriers. Many polymeric delivery systems have been focused on DNA delivery and have had some promising results in the laboratory [52-56]. However, polymeric carriers are not necessarily effective for siRNA or mRNA delivery due to the inherent structural differences, variations in size, molecular weight, charge density and stability [57-59]. The variation in different polymeric structures also greatly contribute to the condensation, release and delivery of the nucleic acid molecules (more details are included in section 4 of this article). Therefore, the nature of different therapeutic modalities (DNA or RNA) and the structural variations in the choice of polymer are essential considerations when designing and engineering a polymeric gene carrier to overcome specific delivery challenges.

For successful gene therapy, polymeric gene carriers need to overcome several extra- and intracellular barriers before they reach the site of action (Table 1). Although non-viral delivery systems have the potential to address the disadvantages of viral vectors, they cannot conquer certain biological obstacles, which limits their clinical applicability. Extracellular barriers to gene delivery, such as serum stability, inadequate cellular binding, rapid clearance, and poor uptake, take different biological, chemical, and physical forms between the administration of gene carriers and the time they reach the target cells. Once polymeric gene carriers enter the body, they must protect the gene from extracellular enzymatic degradation prior to entering cells. Also, the genes should be protected from any undesired interaction with the biological environment that could damage gene function — for this reason, condensing the gene with a cationic materials (e.g., polymer) is a useful technique for extracellular delivery [45]. Polyplexes are formed by incorporation of plasmid DNA with a cationic polymer. During the process, plasmid DNA goes through a condensation that causes a reduction in the hydrodynamic size from 200 to 300 nm to 100 nm with a doughnut, spherical, or rod shape. Such complexation protects the carrier from the elimination through the reticuloendothelial system in the liver, spleen, and lung [46-48].

Particle size, shape, and surface chemistry are important parameters for efficient extracellular protection [49]. The delivery system must retain stable physicochemical properties in circulation because neutral polyplexes in physiological salt concentrations rapidly form aggregates resulting in unsuccessful gene delivery [47,48,50]. In contrast, if the complexes have a strong positive charge even though they are easily maintained in solution, aggregation will occur in a time- and size-dependent manner and will be expedited by the adsorption of negatively charged molecules, such as complements, which cause aggregation and clearance [51]. Therefore, the surface charge of the polyplexes is an important parameter in preventing unwanted aggregation [52], where hydrophilic and non-ionic polymers are commonly used, such as poly(ethylene glycol) (PEG), a polymer of ethylene oxide. PEG's physical properties depend on the length of its chain although its chemical properties have slight variations. PEG is available in a wide range of molecular weights (400 – 10,000 g/mol) and geometries such as comb, star, and branched PEG. In terms of toxicity, doses of PEG used in clinical applications in PEGylated biologicals are low and far from inducing toxicity [53,54]. PEGylation increases the circulation of polyplexes by masking the surface charge and preventing protein opsonization [55]. Including hydroxyl groups in polymeric gene carriers has also been reported to shield the excessive cationic charge and thus overcome non-specific interactions with other proteins and anionic compounds in circulation [56,57].

Another major barrier to polymeric gene carriers is their inability to increase the circulation half-life and achieve access to extravascular targets by preventing off-target recognition. To conquer this problem, the delivery system must often cross endothelial and epithelial barriers whose permeability differs considerably depending on the type of organ. For instance, adequate particle size is different in some tumor cells; a particle of$<$200 nm can gain access to tumor cells because tumor microvasculature is leaky and disorganized, an effect called enhanced permeability and retention (EPR) [58,59]. Once particles overcome the extracellular barriers, they need to be internalized into the target cells. In general, cells take up foreign materials via endocytosis, an active transport system by which cells transport molecules within themselves via an energy-using process [60]. Most synthetic gene delivery systems possess a net positive charge that targets and binds the anionic cell surface to initiate endocytosis.

In addition to extracellular barriers, there are intracellular barriers in the uptake process including non-specific cellular uptake, poor endosomal escape, and inadequate vector unpacking [61]. The fate of particles in the cell is closely linked to their cellular entry [62], and thereafter endocytic pathways play a major role. Once they are taken up by the cells and after the formation of endosomal vesicles, the particles follow lysosomal tracking for degradation. For successful delivery, the gene carrier must evade this intracellular fate because various degradable enzymes and the low pH of lysosomes will attack the carried genetic materials, leading to severe disruption of gene function. Non-viral carriers cannot readily cross the plasma membrane; instead, they are internalized by endocytosis. In that process, they are surrounded by the plasma membrane, which then buds off inside the cells to form trafficking vesicles. Depending on the uptake mechanism, the vesicles can fuse with lysosomes or avoid them, which affects transfection efficiency. Polymeric gene carriers need to acquire an endosomal escape capacity before they fuse with lysosomes.

There are several hypotheses on how non-viral polymeric gene delivery systems are able to escape the endosome. The first is the "proton sponge effect". Cationic polymeric materials such as polyethylenimine (PEI), which have amine groups, offer a buffering capacity by absorbing protons pumped into organelles and thereby reducing the acidification of the endosome. This event increases the chloride ion influx and is followed by osmotic swelling and the eventual rupture of the endosomal membrane [61]. Another explanation of endosomal escape of cationic polymers in gene delivery is the "umbrella hypothesis" which refers to the volumetric expansion of polymers upon protonation (polymer swelling). Cationic polymers condense negatively charged nucleic acids into compact nanoparticles. Upon acidification of the endosomes, amine groups of the polymer are protonated, leading to the elongation of the polymer chain due to electrostatic repulsion. The terminal branches of the polymer then unfold from a collapsed state into an extended conformation. The endosomal membrane is then destabilized by the elongated cationic polymer chains leading to the

formation of hydrophilic pores in the lipid bilayer. It has been shown that the lack of endosomal escape mechanism is the major reason for poor gene expression and RNAi silencing in polymeric gene transporter system [63]. Therefore, maximal therapeutic benefit from non-viral or polymeric material–based gene therapy will be achieved by adequately designing the carriers to compensate for their lack of endosomal escape. Chemical diversity and the ability to tailor cationic polymers make it easier to give them desirable physiological and physicochemical properties. A wide variety of cationic polymeric materials have been studied as non-viral gene carriers, including PEI [64], poly(L-lysine) (PLL) [65], poly(amino ester) [66], polyanhydride [67], poly(amidoamine) (PAMAM), [68] and chitosan [69]. They all efficiently condense DNA or siRNA and protect them from degradable enzymes [70].

PEI is a linear or branched polymer formed by an amine group and a two-carbon aliphatic $C_2H_4$ spacer. It is polycationic due to primary, secondary, and tertiary amino groups and a weakly basic aliphatic polymer [64]. Available in a large range of molecular masses and forms, linear PEIs consist of only secondary amines, while branched PEIs contain primary, secondary, and tertiary amino groups [71]. At physiological pH, PEIs obtain high cationic charge density and form non-covalent complexes with DNA, siRNA, and antisense oligodeoxynucleotide, and they can get into the cell via endocytosis [72]. In terms of transfection efficacy into cells, the branched form of PEI has shown better results, becoming the preferred structure for gene delivery [73,74]. Due to its low toxicity at controlled doses and its ability to be an effective transfection vector, PEI is the base in the synthesis of new polymeric complexes for a variety of gene delivery purposes [75-77]. PLL specifies several-lysine homopolymer, where the primary Ɛ-amine groups in PLL provide a high positive charge density to the polymer that can be protonated with the negative charged proteins, cell, or phosphate groups of DNA to form soluble complexes. Positive charged PLL binds to the negatively charged ion in the cell membrane. The binding of PLL to DNA is either cooperative or non-cooperative [78]. Condensation between the DNA with the PLA depends upon the PLL chain length, where an increase in the length of the PLL chain increases the condensation [79]. Poly(amino ester) is a functional biocompatible polymer synthesized by conjugation of functional amines to a diacrylate ester [80]. The main physicochemical advantage of poly(amino ester) families is that they degrade into nontoxic bioproducts, and their degradation is related with high transfection efficacy [81]. PAMAM has intrinsic endosomal escape features [82]. PAMAM surfaces are covered with primary amines that can be

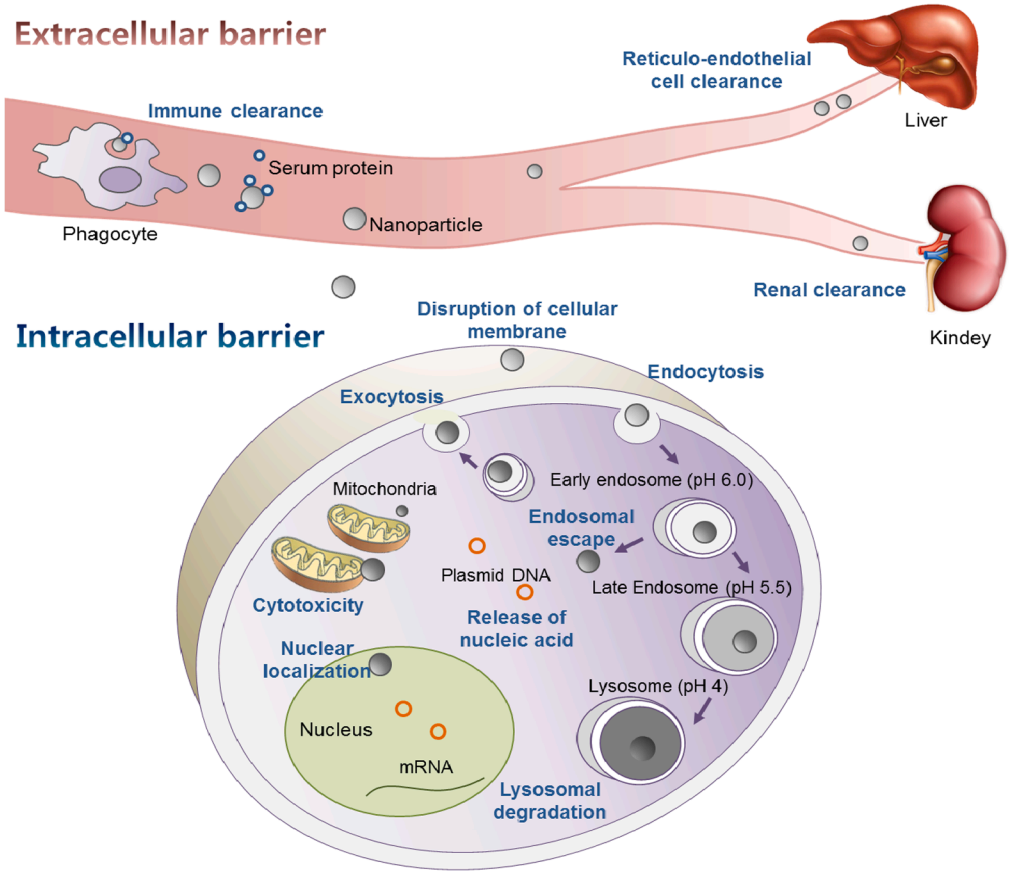



**Fig. 1.** A schematic illustration shows the barriers to extracellular and intracellular uptake of polymeric gene delivery systems. The extracellular barriers include interactions between gene carriers and blood components such as degradable enzymes, serum proteins, and red blood cells; immune clearance; reticuloendothelial cell clearance; and renal clearance. The intracellular barriers include disruption of the cellular membrane; cytotoxicity; poor intracellular fate of the internalized particles depending on the endocytosis pathway; poor endosomal escape ability; vector unpacking ability, which leads to poor release of the target gene; and nuclear uptake barriers that prevent the particles from entering the nucleus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

easily protonated, which allows the interaction with DNA molecules to form nano-sized complexes, while the tertiary amines inside the complexes provide a remarkable endosome buffering capacity that contributes to endosome escape and high transfection [83]. Chitosan is a natural polymer synthesized by deacetylation of chitin [84]. Many previous studies have described the synthesis of chitosan-based vectors for the purpose of gene delivery [84,85].

Polymer chemistry allows the inclusion of functional domains that can upregulate and improve cellular uptake, offering an improved carrier system for nucleotide transportation. It has been suggested that DNA delivery or RNAi silencing via non-viral polymeric carriers could be improved by targeting a particular cellular uptake process that enhances the delivery efficiency of the target gene [61,86]. Therefore, priority goes to the factors that affect the cellular uptake mechanism of gene carriers and the regulation of their cellular internalization. A schematic illustration (Fig. 1) shows several extracellular and intracellular uptake barriers to polymeric gene delivery systems. Table 1 summarizes various cell barriers to polymeric gene delivery and suggests approaches to addressing the problems.

## 3. Barriers in cellular uptake pathways and the significance of polymeric material engineering

Not only cellular and molecular biologists but also material scientists and biotechnologists have been fascinated by the cellular uptake mechanisms for nanomaterials, especially as they apply to drug delivery systems. The cellular uptake process is difficult to examine for several reasons: (i) a limited availability of markers and specific inhibitors to determine the exact uptake pathways; (ii) limitations in appropriate and specific techniques; (iii) standardization and control of cell homeostasis perturbation and toxicity; and (iv) cross-talk between different cellular uptake pathways and their related molecules [93]. Since polymeric gene delivery materials have a vast potential in gene therapy as a form of nanoparticles, it is important to understand how those particles are taken up and transported into cells and to learn how they can deliver target genes successfully [60]. Cellular uptake is an essential process that determines the intracellular biological fate of particles that internalize and become available for gene expression or silencing. It follows either the digestive or non-digestive route, depending on the cellular entry mechanism [94,95], as shown in Fig. 2. Cellular uptake of particles generally occurs via endocytosis, which is normally classified into two main categories: clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE) [95]. CIE can be further classified into various pathways, including caveolae-mediated endocytosis (CvME), phagocytosis, macropinocytosis, RhoA-mediated endocytosis, CDC42-mediated endocytosis, the ARF6-mediated pathway, and flotillin-mediated endocytosis [93]. The CME, CvME, phagocytosis, and micropinocytosis pathways are

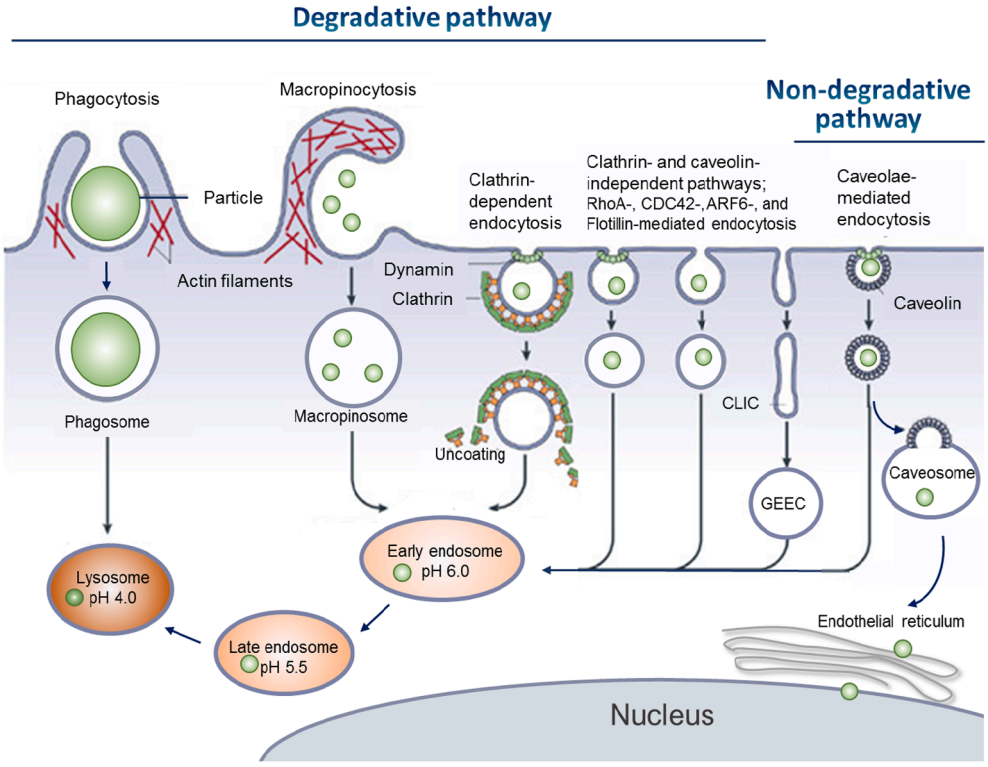



**Fig. 2.** Intracellular fate after cellular uptake of the target particles. The cellular uptake mechanism controls the intracellular fate of the particles to either the digestive or non-digestive route. The digestive route begins with early and late endosome formation followed by lysosomal fusion, whereas the non-digestive route avoids those events. Macropinocytosis, phagocytosis, CME, and CIE generally follow an acidic and digestive route that leads to acidification of the endosomal compartment and fusion with the lysosome. In contrast, CvME governs the cellular internalization of particles toward a non-acidic and non-digestive route, avoiding lysosomal fusion and degradation.

commonly used by mammalian cells for the uptake of macromolecules, particles, and solutes that are impermeable to the plasma membrane [94]. All those endocytosis pathways follow the same basic steps: (i) specific cell surface binding; (ii) budding of plasma membrane and pinching off, (iii) tethering of the vesicle, and (iv) trafficking of the vesicle to a specific subcellular organelle. Table 2 shows the advantages and limitations of these endocytosis mechanisms for gene carriers with a different particle size range.

Studying the uptake of gene delivery vehicles is made possible by the use of chemical inhibitors of endocytic pathways. However, the use of chemical inhibitors is still questionable. Vercauteren *et al.* demonstrated that the cytotoxicity and efficacy of four commonly used endocytosis inhibitors (chlorpromazine, genistein, methyl-β-cyclodextrin, and potassium depletion) to study CME and CIE were cell type dependent (D407, Vero, COS-7, HuH-7, and ARPE-19) [124]. For example, COS-7 and Vero were found to be sensitive to the cytotoxic effect of chlorpromazine, while HuH-7, ARPE-19, and D407 were insensitive to $< 10$ ug/ml to this agent. Methyl-β-cyclodextrin exhibited high cytotoxicity when treated to Vero and HuH-7 types at concentrations that are routinely used for endocytosis studies (<10 mM). They also discovered that these four agents generally lacked the specificity for defined pathways (CME and CIE) and their inhibitory effects are different between cell lines [124]. Some chemical inhibitors that have been used to elucidate the CDC42- and RhoA-mediated pathways have also showed poor specificity and cytotoxicity. Secramine, an inhibitor targeting CDC42 affected the activation of Rho and RAC [125]. *Clostridioides difficile* toxin B (TcdB)10463 inhibits the Rho family (Rho A/B/C), RAC1, and CDC42, whereas TcdB1470 inactivates RAC1 and CDC42 but not Rho (A/B/C) [126]. The use of chemical inhibitors to study the endocytosis pathway should be approached carefully to prevent non-specificity and toxicity. It is therefore important to optimize dose and time period depending on the cell line, and fully characterize their effects on particular cell types [124].

### 3.1. Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) is one of the most studied endocytosis mechanisms. It was discovered in 1964 by the observation of numerous coated pits in thin-section electron microscopy [127]. Eukaryotic cells use the CME pathway mostly to utilize metabolites, hormones, and other proteins [128]. CME sorts transmembrane receptors upon ligand binding, which sequentially triggers the formation of coated pits in the plasma membrane on the cytosolic side. Several cytosolic proteins assemble in those coated pits, and clathrin is the main protein unit. In the CME pathway, the coated pits originate as a polygonal clathrin lattice with the help of adapter proteins. Then, the clathrin-coated pits are pinched off from the plasma membrane, forming intracellular clathrin-coated vesicles ranging from 100 to 150 nm in diameter [96]. The clathrin coats then depolymerize to form an early endosome, followed by fusion with the late endosome and a lysosome. After internalization, the particles experience an acidic condition (pH 5–6), and they travel toward the late endosome that eventually merges with a lysosome, where the particles containing genetic material could be degraded. Therefore, polymeric gene carriers that gain entry to the cell via the CME pathway must be able to prevent lysosomal degradation through an endosomal escape mechanism. Otherwise, the particles should take a different endocytic pathway to prevent lysosomal fusion. It is worth noting that the CME pathway can be inhibited by chlorpromazine and potassium depletion agents that dissociate clathrin from the cell surface membrane [97,98].

**Table 2**
Advantages and limitations of major endocytosis mechanisms for non-viral gene carriers.

| Endocytosis mechanism | Particle size | Advantages | Limitations | References |
|---|---|---|---|---|
| Clathrin endocytosis pathway | < 300 nm | -Most of the small particles followed this route of cellular entry | -Cannot prevent lysosomal fusion and degradation | [96-98] |
| Caveolae-mediated endocytosis | < 80 nm | -Avoids acidic and digestive route by avoiding lysosomal fusion | - Controlling CvME is not straightforward: caveolae are mainly influenced by mechanical stimuli | [93,99-102] |
| Phagocytosis | Particle-type dependent | -Effective for larger particles<br>-Allows targeting of immune cells | -Lysosomal degradation<br>- Phagocytic cells only | [93,103-105] |
| Macropinocytosis | < 2000 nm | -Does not require any receptor or coated compound for uptake<br>-Suitable for large amounts of external fluid | -Lysosomal degradation | [93,106-110] |
| RhoA-mediated endocytosis | < 200 nm | -Controls the actin cytoskeleton dynamics and filament rearrangement | * | [93,111-116] |
| CDC42-mediated pathway | < 50 nm | -Regulates actin cytoskeleton and cell stress signaling<br>-Controls cell differentiation and maturation | * | [93,117,118] |
| ARF6-mediated endocytosis | < 100 nm | -Identified as a clathrin- and caveolae-independent endocytosis process | * | [119,120] |
| Flotillin-mediated endocytosis | < 100 nm | -Resembles caveolae-like structure<br>-Described as a clathrin- and caveolae-independent process that regulates domain organization of other pathways | * | [121-123] |

* Mechanism and function still unknown, and thus more studies are needed to be carried out.

### 3.2. Clathrin-independent endocytosis

#### 3.2.1. Caveolae-mediated endocytosis

Caveolae-mediated endocytosis (CvME) is the most studied CIE mechanism. It is involved in numerous biological functions, including vesicular transport, lipid regulation, and cellular signaling. CvME consists of small hydrophobic domains rich in glycosphingolipids and cholesterols [99]. Caveolae are primarily composed of caveolin-1 (Cav-1) and caveolin-2 (Cav-2). Cav-1 is responsible mainly for the structural formation of the caveolae, but the exact function of Cav-2 remains unclear [129]. Caveolae have also been reported to function as membrane tension regulators [130]. Several non-enveloped viruses, such as Simian virus 40 [131], and certain molecules, including cholera toxin [132] and folic acid [133], enter cells through CvME. How CvME works is of major interest in polymeric gene delivery research because it has several advantages for gene therapy [100]. CvME, in contrast to CME, leads the endocytosed materials through a non-digestive, non-acidic route [134]: particles taken up via CvME are carried into early endosomes or caveosomes in a neutral pH environment and do not fuse with lysosomes [101]. Thus, CvME could serve as a unique pathway in polymeric gene delivery. Besides, extravasation through caveolae is even called caveolae pumps because of the quick and effective movement of molecules within seconds from blood across endothelium, even against a concentration gradient [102]. Genistein inhibits CvME by disrupting the action network and preventing the dynamin recruitment essential for this cellular uptake pathway [135]. Methyl-β-cyclodextrin, which depletes cholesterol in the plasma membrane, has also been shown to inhibit CvME, suggesting that cholesterol could be a fundamental component of caveolae [136].

#### 3.2.2. Phagocytosis

Phagocytosis (meaning 'to devour' in ancient Greek) is a cellular process by which solid particles are engulfed by the cell membrane to form an internal phagosome. It is primarily a mechanism to remove cell debris and pathogenic microbes from phagocytic cells, including macrophages, dendritic cells, and neutrophils. The event begins as a cup-shaped membrane distortion, continues by sorting the particles inside the cells, and ends with the formation of a phagosome followed by phagolysosome [103]. Phagocytosis is not generally expected to play a key role in gene delivery to non-phagocytic cells because phagosomes are supposed to occur only in phagocytes for the uptake of large particles. However, binding to anionic syndecans (heparin sulfate proteoglycans), a common strategy of internalization used by many pathogens, has been proposed to trigger actin-mediated phagocytic uptake of cationic gene carriers [104]. The inhibition of protein kinase C, an important transducing signal controlling heparin sulfate proteoglycan–dependent phagocytosis, decreased the uptake of the gene carrier, demonstrating the phagocytosis-mediated engulfment of the gene carrier. On the other hand, an RNAi technique used a different chemical inhibitor to distinguish phagocytosis from macropinocytosis of the polyplexes [105]. Knock-down of ARF6, a key protein in macropinocytosis, had no inhibitory effect on polyplex uptake, but protein activated kinase 1, involved in actin rearrangement in the phagocytosis pathway, did affect the uptake efficiency, suggesting the involvement of phagocytosis in the uptake of the polyplexes. A further mechanistic study was performed using treatment with sodium chlorate (ATP-dependent sulfurylase) and heparinase III to observe the effects of their interaction with heparin sulfate proteoglycans on the uptake of cationic polyplexes [105]. These studies underline the importance of electrostatic interaction between cationic polyplexes and negatively charged heparin sulfate proteoglycans for phagocytic uptake of cationic gene carriers.

#### 3.2.3. Macropinocytosis

Macropinocytosis is a cellular uptake process that internalizes an extracellular medium non-selectively via cell membrane protrusions that fuse with and collapse onto the cell membrane [109]. It engulfs external fluid by creating waving sheet-like extensions of the plasma membrane to form a large organelle (>200 nm) called a macropinosome [106]. Macropinocytosis is an actin-regulated endocytosis pathway, unlike other endocytosis mechanisms, that is neither coated nor connected with any cargo or receptor during internalization [106]. It can be initiated by specific signals such as growth factors, chemokines, cationic peptides, and amyloid assemblies [110]. It has been suggested that the activity of the Rho family, guanosine triphosphatase (GTPase), and phosphatidylinositol 3 kinase (PI3K), influences macropinocytosis by regulating actin rearrangements [107,108]. Wortmannin, a chemical that inhibits PI3K activity, is known to inhibit macropinocytosis [137].

#### 3.2.4. RhoA-mediated endocytosis

RhoA belongs to a Ras homolog gene family whose prime role is to control actin cytoskeleton dynamics and sequentially regulate gene expression, actin filament rearrangement, proliferation, and the shape of cells [111,112]. RhoA-mediated endocytosis was reported to control ricin uptake on the apical side of polarized kidney cells and the entry of baculovirus in non-phagocytic cells [113,114]. RhoA was inhibited by bacterial toxins (C3 transferase and *C. difficile* toxin B (TcdB)) and dominant negative genes in a study of its role on PEI-mediated gene transfer [115]. C3 selectively inhibits RhoA, B, and C by modifying the asparagine at the effector region of GTPase, and TcdB inactivates the Rho, RAC, and CDC42 pathways by glycosylation of threonine residue [116]. Moreover, Rho GTPase inhibition led to a significant decrease in transgene expression [115]. Although the exact mechanism is unclear, whether RhoA is essential for sorting the receptor only at the early stage or during the entire endocytic process, RhoA is clearly a key molecule regulating actin cytoskeleton dynamics [138,139].

#### 3.2.5. CDC42-mediated pathway

Cell division cycle 42 (CDC42) is also a member of the Ras superfamily. CDC42 is involved in a variety of cellular processes that control the differentiation, maturation, and apoptosis of cells by regulating the actin cytoskeleton and cell stress signaling. CDC42 was first recognized during the observation of small GTPase in the cellular uptake of glycosylphosphatidylinositol-anchored proteins (GPI-

APs) [117]. GPI-APs anchor to the extracellular membrane through lipid chains and form nano-clusters on the living cell surface. These clusters generate a sorting signal for selective endocytosis through interaction with the CDC42 protein [118]. Endocytosis via CDC42 is modulated by changes in plasma membrane tension depending on vinculin as a mechanotransducer, and the mechanisms are upregulated under conditions where cells need to rapidly internalize excess plasma membrane such as under hypotonic condition [110]. CDC42-mediated endocytosis can be inhibited by treatment of ML 141 by inducing the dissociation of guanine nucleotides from the active site of CDC 42 [110].

#### *3.2.6. ARF6-mediated endocytosis*

Adenosine diphosphate ribosylation factor 6 (ARF6) belongs to an ARF family of 6 small GTPase expressed in all eukaryotic cells and related to the Ras gene [119]. Although the exact mechanism of ARF6 in the endocytosis pathway is not yet fully understood, it serves as an important experimental paradigm for studying the CME and CvME pathways [120]. ARF6-mediated endocytosis is associated with a tubular morphological structure rather than a vascular shape [140]. A recent report demonstrates that ARF6 binds to GTP and generates a membrane curvature that eventually initiates membrane budding [141]. Following internalization into a vesicle, the cargo is transferred into an endosome that can fuse with endosomes generated via the CME pathway and go on to lysosomal degradation [120]. Cytochalasin D, which disrupts the actin cytoskeleton resulting in the redistribution of ARF6, has been used in an ARF6 cellular trafficking study [142]. At present, RNAi-mediated ARF6 reduction has been used as an alternative to cytochalasin D to overcome the problem of non-specificity [105].

#### *3.2.7. Flotillin-mediated endocytosis*

Flotillin, originally discovered in neurons during axon degeneration, is a marker of lipid membrane domains and forms a hairpin structure when bound to the plasma membrane [122]. The conformation is similar to that of Cav-1, which was confirmed by its localization in caveolae-like structures, suggesting the possibility of an analogous role in lipid sorting [143]. However, several reports suggest that flotillin takes a dynamin-dependent CIE pathway [143,144], although its function as a different endocytosis pathway has yet to be explained. Another study showed that flotillin regulates the domain organization of other pathways independent of clathrin and caveolae [123]. Inhibition of flotillin-mediated endocytosis is commonly determined using the flotillin-1 RNAi technique; however, the cellular trafficking of the flotillin pathway and its downstream signaling have not yet been demonstrated clearly [144,145].

Different endocytic pathways thus have different modes of action for extracellular and intracellular uptake and different delivery processes for gene expression. The mechanisms of cellular uptake along the different major endocytosis pathways are shown in Fig. 2. Because cellular uptake is an indispensable step in gene delivery, being able to regulate the uptake mechanism will provide great benefits in overcoming cellular barriers and enhancing gene transfer efficacy.

### *3.3. Non-endocytic pathways*

Almost all nanoparticles internalized via endocytosis become entrapped in an endosome and then either fuse with lysosome for degradation or somehow escape to the cytosol [146]. Thus, among the endocytosis pathways, non-digestive pathways such as CvME have been targeted for the safe delivery of therapeutic genes [134]. Another pathway could be a non-endocytic route that bypasses the endocytic vesicle and directly locates the gene in the cytosol without lysosomal degradation [147]. Non-endocytic pathways can be classified into invasive and non-invasive systems. The efficacy of invasive systems, including microinjection, microfluidic delivery, and electroporation, is better than that of most of carrier-mediated non-invasive systems. However, invasive systems are difficult to apply in clinical settings because they are associated with pain, risk of infection, and challenging techniques [148,149]. Researchers wishing to improve non-invasive systems have targeted factors that determine the non-endocytic delivery of nanoparticles, such as size, surface charge, amphipathic property, and conjugated ligands. Dokka and Rojanasakul provided an excellent review of nucleic acid delivery methods that bypass the endocytic pathway [149]. Successful non-endocytic delivery of cationic peptides with protein transduction domains, including hyperphenylalaninemia 1 (Hph-1) and *trans*-activator of transcription (TAT), directly penetrates the cell membrane without extra help from, for example, an ATP-dependent pathway. The advantages of a non-endocytic pathway using Hph-1-conjugated QD-PEI have been demonstrated: not only does it improve the uptake rate and prevent endosome formation; it also accelerates the intracellular unpacking of nucleic acids [150]. TAT-conjugated nanoparticles also help transverse strong cellular barriers, such as the blood–brain barrier (BBB), because the internalized nanoparticles bypass lysosomal degradation and efflux via P-glycoprotein [151]. Gold-nanoparticles covered by shells of amphipathic cell penetrating peptides (CPPs) slipped by the membrane more successfully than nanoparticles covered with a non-ordered amphipathic shell [152]. Cationic PAMAM formed a hole on the cell membrane using its strong positive charge and diffused into the cells non-specifically [153]. However, the significance of cationic polymer-mediated cellular uptake via a non-endocytic pathway remains an unanswered question; various evidence suggests that the endocytosis mechanism is the dominant route for cationic polymers, including PAMAM, PEI, and chitosan [154,155]. The advantages of a non-endocytic pathway have been repeatedly described over the years; however, the practical use of such a system will require further evaluation and optimization.

## 4. Engineered polymeric materials and their structural relationship to cellular uptake pathways

Improving the structural relationship between polymers and cellular uptake regulation requires an understanding of the effects of chemical structure, pH, charge ratio, molecular weight, degradability, composition, hydrophobicity, and molecular configuration on cellular uptake. That information provides a basis for the further development and optimization of novel engineered polymers as

effective gene carriers. In this section, we illustrate the relationship between polymer structure and various endocytosis uptake pathways to promote understanding when designing new polymeric materials for gene delivery [156-158].

### 4.1. Chemical structure

Most cationic polymers used as non-viral gene carriers can be specifically tailored for a particular gene delivery application by adjusting their chemical structures, such as a linear or branched type, homopolymer or copolymer, and block, alternative, or graft copolymer. However, only a few studies have considered the relationship between chemical structure and cellular uptake because experimental design is difficult due to the large number of structural parameters. Nonetheless, this issue is of utmost importance in elucidating how a polymer's chemical structure should be designed and engineered to regulate cellular uptake mechanisms and enhance gene delivery efficacy. To evaluate the relationship between a polymer end group and endocytosis, Kim *et al.* synthesized poly (β-amino ester) (PBAE) end-capped with 2-(3-aminoprophylamino)ethanol and 1-(3-aminopropyl)-4-methyl-piperazine (Fig. 3A), and compared its cellular uptake with that of PBAE without the end groups [159]. Cellular uptake of PBAE/DNA polyplexes depended on small changes in the end-group even though the base polymer structure, size, and zeta potential of the polyplexes were essentially the same. Interestingly, PBAE with 1-(3-aminopropyl)-4-methyl-piperazine was more efficient in both the CME and CvME pathways than PBAE with 2-(3-aminopropylamino) ethanol (Fig. 3B). Although the exact mechanism is unclear, it might have resulted from a difference in the nanoparticle polydispersity. In another study, PBAE was synthesized in a reaction of 1,4-butanedial (or 1,6-hexanediol) diacrylate with 1-aminobutanol and varying amine/diacrylate stoichiometric ratios to make polymers with either amine or acrylate end-groups, and transfection efficiency between chain end-groups was examined [72]. None of the acrylate-terminated PBAE could induce appreciable levels of transfection due to an inability to enter the target cells. Only amine-terminated PBAE was suitable for use as a gene delivery carrier, although the exact configuration of the chemical structure required for cellular uptake was not described. Acrylate-terminated PBAE showed significantly less transfection efficiency than amine-terminated PBAE, without any significant difference in their physicochemical properties [160], suggesting that small-molecule end-group modification of the polymer is a critical parameter for cellular uptake regulation.

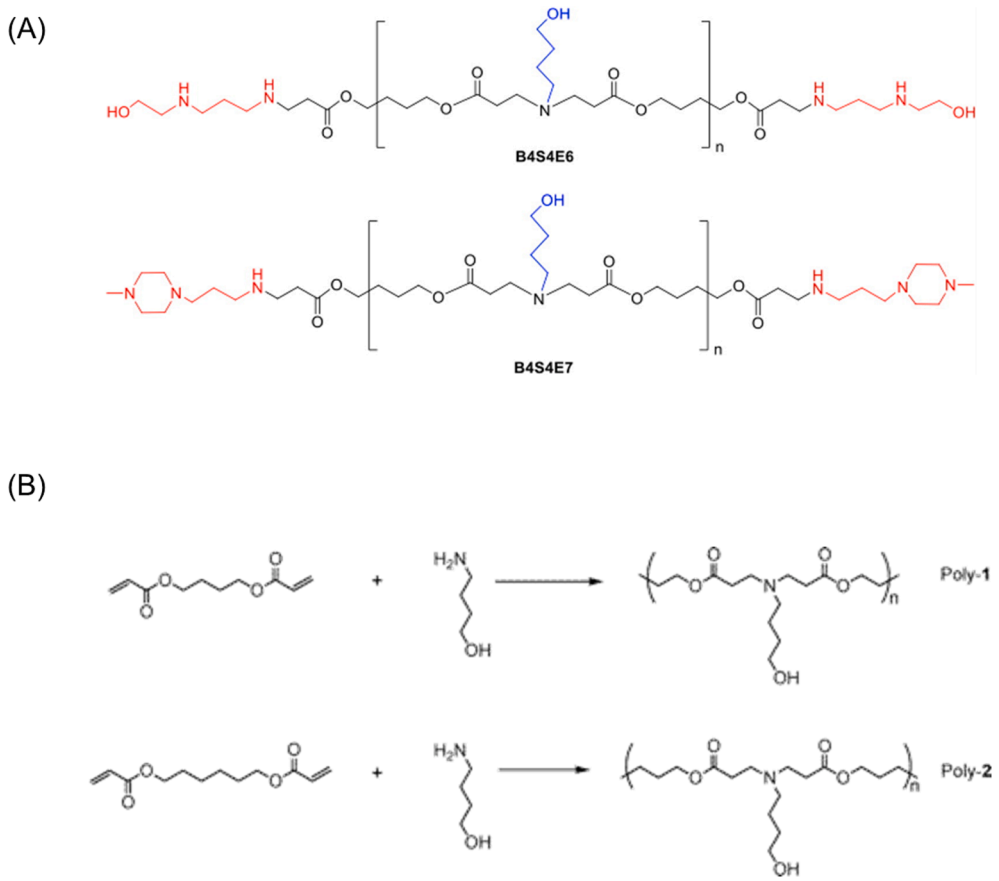



**Fig. 3.** Structures of PBAEs. (A) B4S4E6 and B4S4E7 have the same B4S4 base polymer but different end groups. Copyright permission obtained for the figure, ref. [159] from American Chemical Society. (B) Structure of PBAEs (Poly-1 and Poly-2), adding 1,4-butanediol diacrylate and 1,6-hexanediol diacrylate, respectively, to 1-aminobutanol. Copyright permission obtained from Ref. [72].

### 4.2. Molecular weight

The molecular weight (MW) of a cationic polymer has a major influence on its biological and physicochemical properties [161] because MW can modulate gene expression efficiency. The MW of the polymer induced a chain entanglement effect on the formation of the polymer/gene complexes [162]. Less chain entanglement contributes to complex formation as the MW of the polymer decreases, whereas high MW polymers more easily entangle the gene following the initial electrostatic interaction. In general, polymers with high MW showed high gene expression efficiency because of stable gene complexation, increased polyplex stability, and uptake efficacy [163], although risks of cytotoxicity remain due to strong interactions with cell membranes. It has been reported that high-MW PBAE was more likely to use CvME as the major uptake pathway because of the increased monodispersity of the polyplex size compared to the low-MW PBAE [159].

### 4.3. Degradability

The degradability of cationic polymers is one of the most important factors in ensuring their safety and efficacy before clinical trials because it reduces cytotoxicity by degrading the polymers to small MW molecules so that they can be easily eliminated through excretion pathways *in vivo*. Degradable polycations used as gene carriers can be prepared using several degradable linkages, such as ester, disulfide, imine, carbonate, amide, and ketal. The characteristics of degradable linkages based on various structural features and their effects on gene delivery have been elaborately explained elsewhere [164]. However, it remains unclear how the degradability of the gene carriers affects the cellular uptake mechanism. Laterally stabilized complexes of DNA with linear reducible polycations with degradable linkages has been reported. The polyplexes were coated with a hydrophilic copolymer of *N*-(2-hydroxy propyl) methacrylamide and methacryloylglycylglycine 4-nitrophenyl ester (PHPMA) (Fig. 4). A surface coating of PHPMA induced an intracellular reducing capacity and reversed the lateral stabilization, facilitating release of the delivered gene [165]. This suggests that the intracellular reductive condition of the polymer caused by the disulfide linkage increased cellular uptake activity.

### 4.4. Composition

Among cationic polymers, PEI has been used as the golden standard because of its capacity to allow endosomal escape of the genes delivered [166]. However, PEI has several disadvantages, including non-degradability, high cytotoxicity, and instability of the polyplexes, although its cytotoxicity depends on its MW and structure [167]. To overcome PEI's non-degradability and toxicity, researchers have synthesized degradable PEI in copolymer systems. Degradable poly(xylitol-co-PEI) (PXP) copolymer (Fig. 5) crosslinks low-MW PEI (1.2 kDa) with xylitol diacrylate, examined whether the high transfection efficiency of the polyplexes in target cells could be attributed to a specific endocytosis pathway induced by the high osmotic polyxylitol portion in the copolymer [168]. It is notable that the transfection efficiency of the PXP gene carrier neither change in the presence of different endocytosis inhibitors (chlorpromazine, wortmannin, methyl-beta-cyclodextrin, genistein, filipin III and nystatin) nor differ from that of the unmodified PEI (25 kDa) used as a control, suggesting that the transfection efficiency of the polyplexes is not correlated with particular cellular uptake pathways. PXP has a high osmotic polyxylitol part (3.9 wt%) that shows 1.5-fold more osmolarity than PEI 25 kDa (Fig. 6A-B). Interestingly, the volume ratio of endosome to PXP/DNA polyplexes was 1.8-fold higher than that of PEI/DNA, indicating that endosomes containing PXP/DNA polyplexes swelled more than those containing PEI/DNA polyplexes (Fig. 6C-H). Therefore, the high

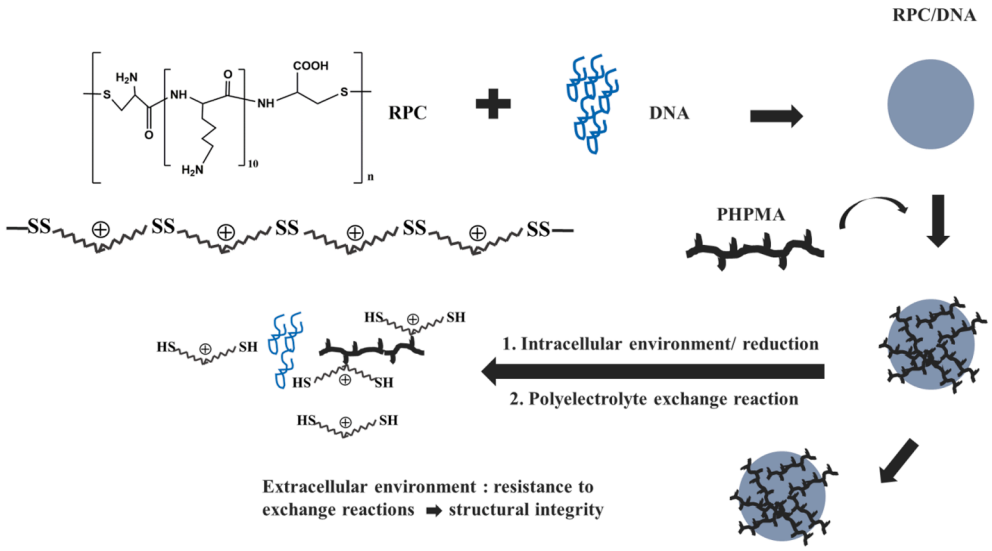



**Fig. 4.** Reversible lateral stabilization of DNA complexes. Copyright permission obtained from Ref. [165].

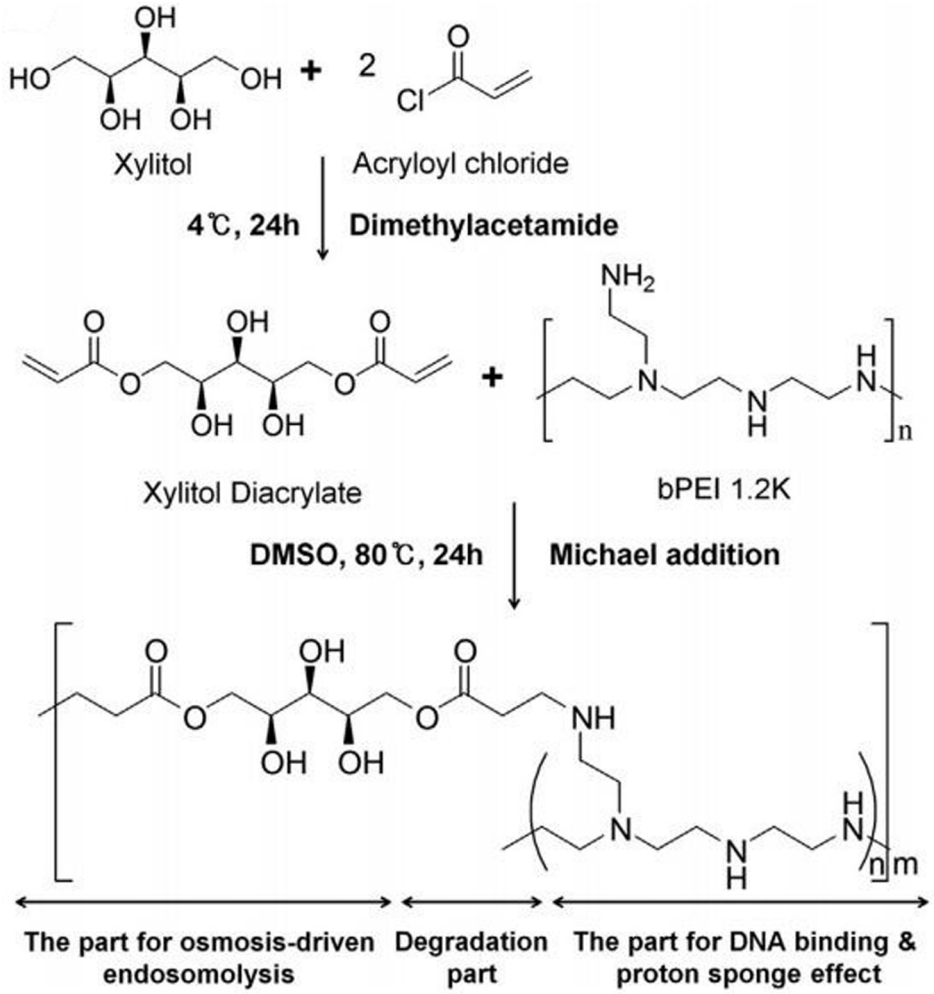



**Fig. 5.** Scheme for the synthesis of poly (xylitol-co-PEI). Copyright permission obtained from Ref. [168].

osmosis of endosomes caused by PXP enhanced endosomal escape of the delivered genes resulting in high transfection efficiency [168].

As shown in Fig. 7, the effect of PEI content in poly(triglycerol-co-PEI)/DNA complexes on various endocytic pathways was evaluated to understand the cellular uptake and intracellular processing of the polyplexes [169]. A higher content of PEI (91 mol-%) in the copolymer was found to induce the use of all possible pathways (CME, CvME, and macropinocytosis) into cells. It is worth noting that CvME-mediated transfection using a high PEI content in the copolymer was lower than that of unmodified PEI (25 kDa), whereas the use of macropinocytosis was higher in this regard. Interestingly, transfection with a lower content of PEI in the copolymer did not affect CME or CvME; rather, it entered cells predominantly through the macropinocytosis pathway. The low transfection efficiency found with a low content of PEI in the co-polymer was related to the insufficient acidic condition of the endosome, which was not enough to allow gene release to the cytosol [169].

Regarding PEI composition, Cortez *et al.* studied a circular form of PEI and compared it with its linear counterpart with the same molecular weight as the circular form. Higher transfection efficiency was demonstrated for each circular PEI sample compared to its linear PEI analogue, in addition to reduced toxicity (60–80% viability in HFE-1 cells) relative to the branched PEI, the "golden standard" control (40–60% viability in HFE-1 cells). These results show how the architecture of PEI critically influences transfection efficiency and cell toxicity [74].

### *4.5. Hydrophobicity*

Hydrophobic segments in polymeric materials are expected to enhance gene transfection efficiency by modulating interactions with cells, by causing adsorption on cell surfaces and subsequent facilitation of cellular uptake [170]. The balance between the hydrophobic and hydrophilic properties of gene carriers is an essential parameter for increasing transfection efficacy because hydrophobic modification of the carriers affects cytotoxicity. Hydrophobically-modified glycol chitosan nanoparticles using β-cholanic acid as a hydrophobic group took three different endocytic pathways: CME, CvME, and micropinocytosis [171]. In a similar study,

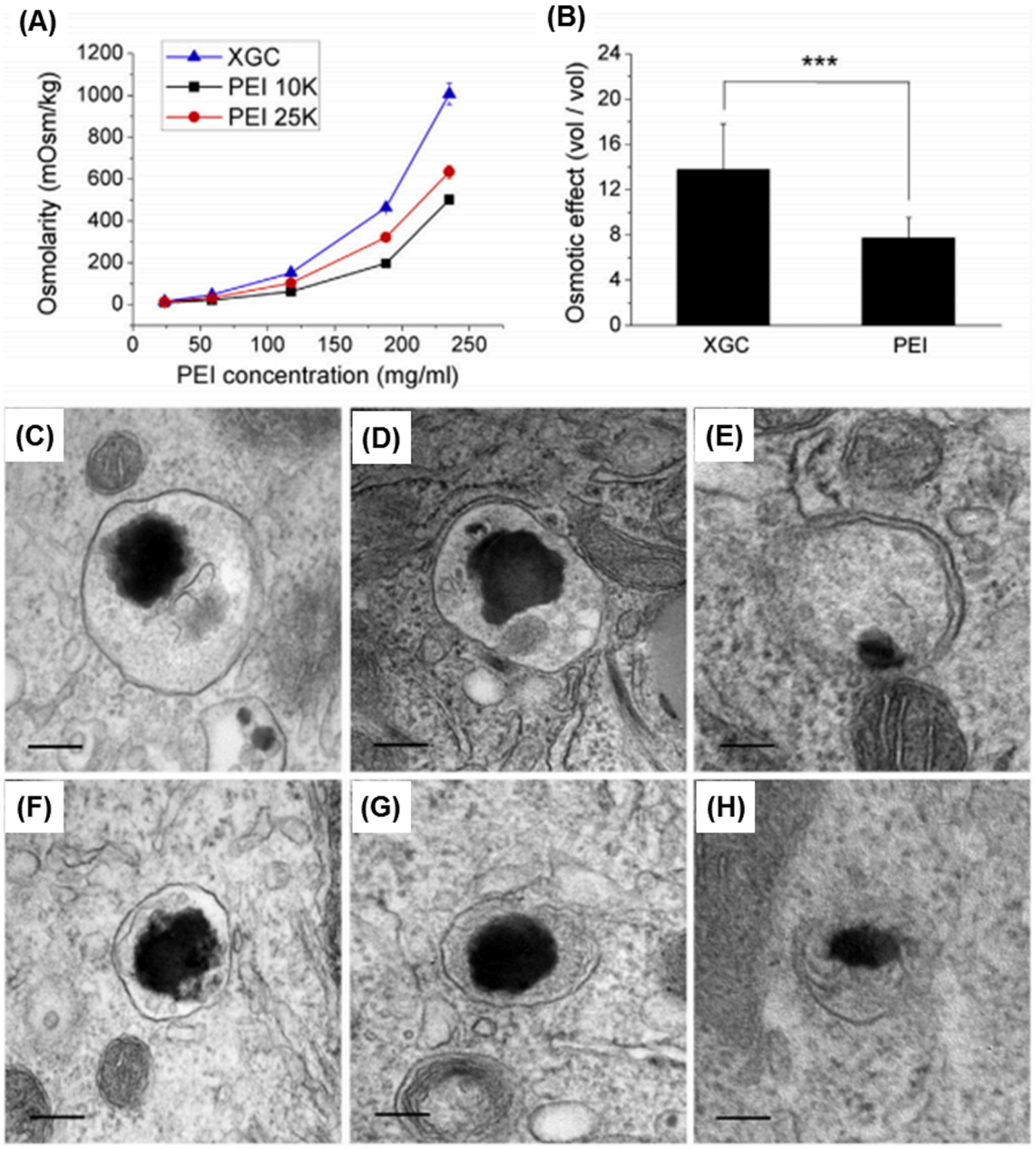



**Fig. 6.** Osmotic properties of XGC. (A) Osmolarity of XGC, PEI 10 kDa, and PEI 25 kDa measured by osmometer (n = 3, error bar: SD). (B) Volume ratio of endosome to polyplexes from the TEM images of XGC/DNA and PEI/DNA polyplexes (n = 35, error bar: SD; ***P < 0.001, one-way ANOVA). (C) and (D): TEM images of swelled endosomes containing XGC/DNA polyplexes. (F) and (G): TEM images of swelled endosomes containing PEI/DNA polyplexes. (E) Endosomal escape of XGC/DNA polyplexes. (H) Endosomal escape of PEI/DNA polyplexes [(C)–(H) scale bar = 100 nm]. Copyright permission obtained from Ref. [168].

hydrophobically-modified chitosan nanoparticles (NPCS) (Fig. 8) that used palmitic acid as a hydrophobic group were examined for their endocytic pathway. Cellular uptake of NPCS increased proportionally to the degree of substitution (DS) in the palmityl group due to enhanced interaction with the cell membrane. Endocytosis of NPCS was clearly related to the lipid raft-mediated route. Significantly more NPCS entered cells via CvME with increased DS in the NPCS [172]. These findings indicate that the DS of hydrophobic materials in a nanoparticle system can greatly affect endocytosis regulation. For the purpose of testing the effects of the hydrophobic part on cellular uptake regulation, amphiphilic poly(oxazoline) copolymers were synthesized [173]. The cellular uptake increased without cytotoxicity in an energy-dependent manner with an increase in the hydrophobic parts of the copolymers (Fig. 9). Recently, Jiang *et al.* prepared modified pullulan (CHP) nanoparticles using cholesterol as a hydrophobic group to test the cellular uptake mechanism in HepG2 cells [174]. The results indicated that about 88% of the nanoparticles entered the cells through an energy-dependent

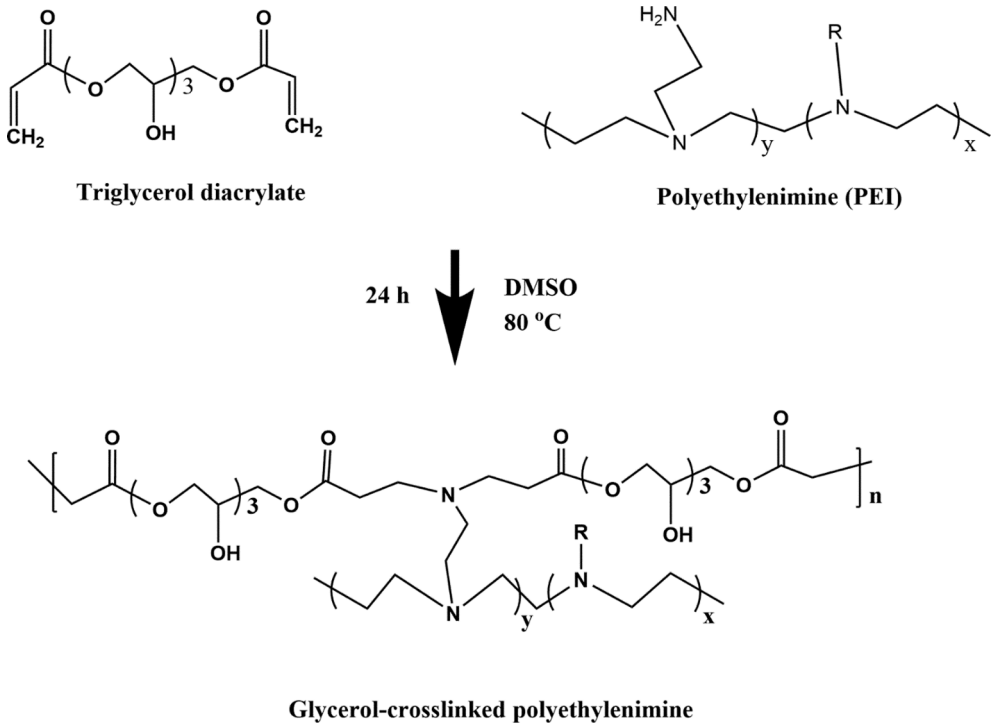



**Fig. 7.** Synthesis scheme of glycerol-crosslinked PEIs. Triglycerol diacrylate was reacted with PEI at the ratio of 1:2 and 2:1 to yield LG-PEI and HG-PEI, respectively. Copyright permission obtained from Ref. [169].

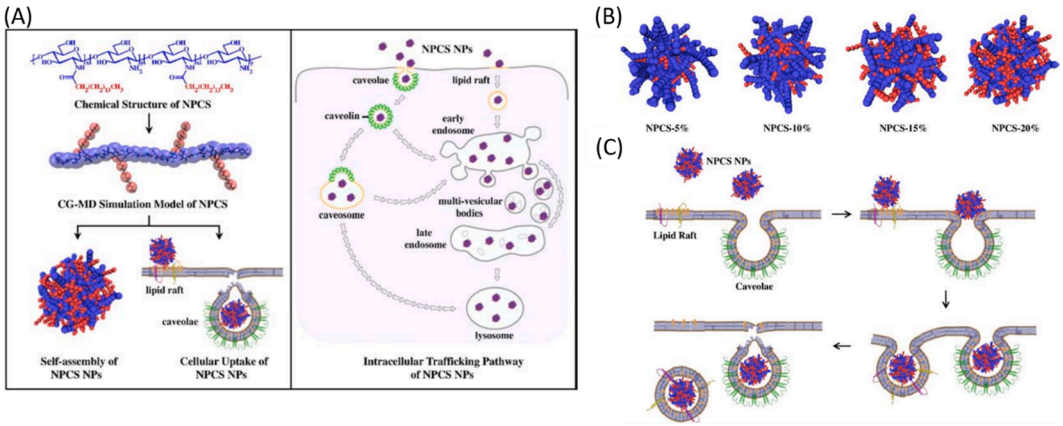



**Fig. 8.** Cellular uptake and intracellular trafficking of nanoparticles made of hydrophobically-modified chitosan. (A) Schematic illustrations showing the self-assembly of N-palmitoyl chitosan (NPCS) polymers into nanoparticles (NPs) via coarse-grained molecular dynamic simulations, along with the potential cellular uptake mechanism and the intracellular trafficking pathway of NPCS NPs. (B) Results of coarse-grained molecular dynamic simulations of the self-assembly of NCPS with different degrees of substitution into NPs at pH 7.4; (C) schematic illustrations of the interaction between NPCS NPs and the cell membrane (lipid rafts and caveolae). Copyright permission obtained from Ref. [172].

endocytosis process, and about 12% entered through physical adhesion or diffusion. The internalization of CHP nanoparticles involved both CME and macropinocytosis.

### *4.6. Molecular configuration*

A 3D polymeric configuration for efficient gene delivery has been suggested because branched polymers with 3D architecture have several advantages over linear structures: enhanced interaction between polymers and genes, improved formation of polyplexes, and protection of the complexed genes from degradation by enzymes coincident with increased cellular uptake of polyplexes due to the multiple functional terminal groups [175]. A series of degradable branched poly(dimethylaminoethyl methacrylate) copolymers,

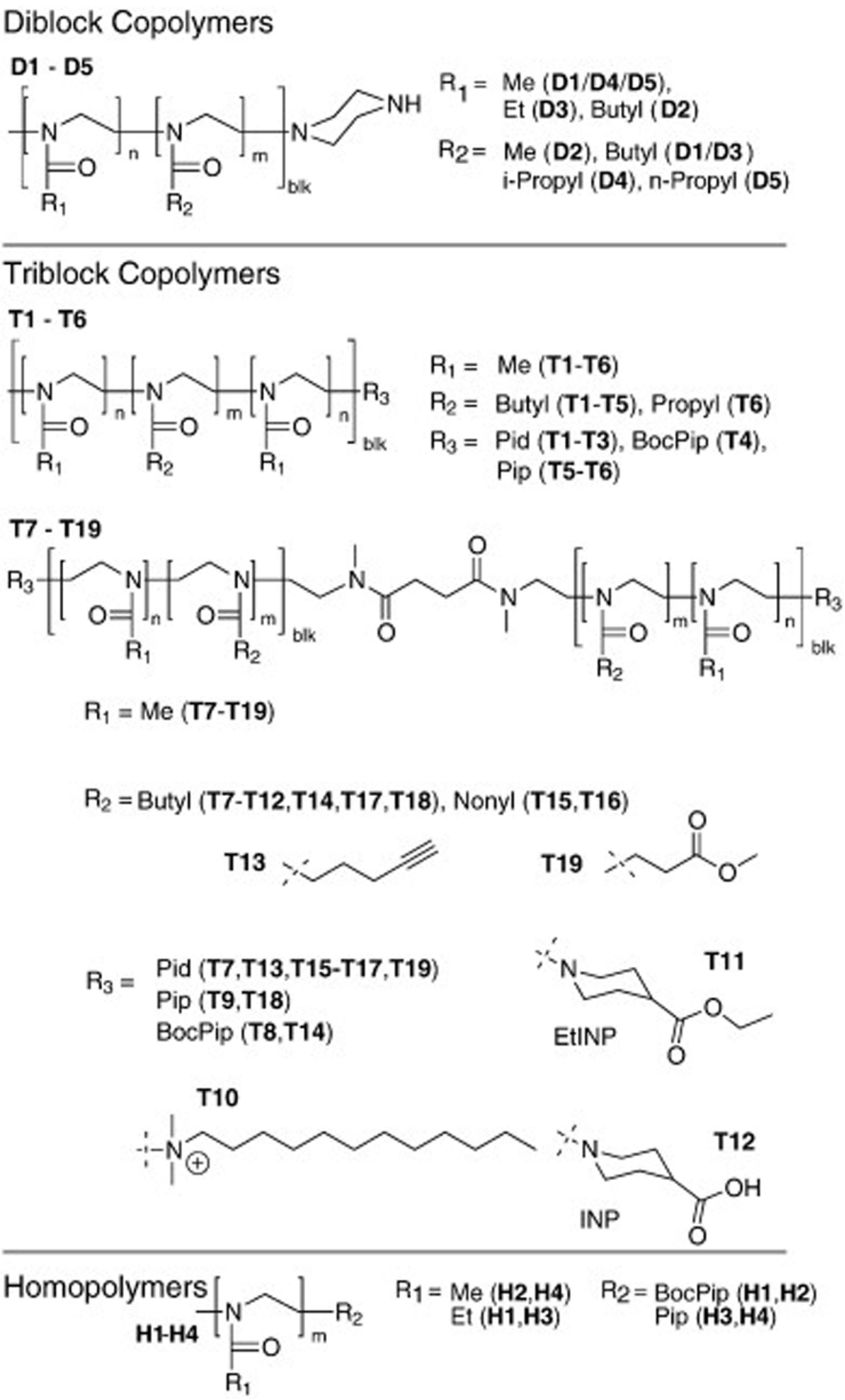



**Fig. 9.** Different architectures of diblock, triblock copolymers and homopolymers were investigated. Triblock copolymers were prepared from bisfunctional initiators as well as monofunctional initiators. Moreover, polymers with different termini were prepared and studied (T7-T12). Water soluble homopolymers were synthesized from 2-methyl-2-oxazoline and 2-ethyl-2-oxazoline. Copyright permission obtained from Ref. [173].

formed by controlled radical cross-linking copolymerization, were compared for transfection efficiency in different cell types [176]. The high degree of branching provided better functionality and cell viability. Higher gene transfection efficiency and biocompatibility were observed in keratinocytes treated with highly branched PBAEs than in those treated with the linear counterpart due to their high

number of end groups and relatively high molecular flexibility (Fig. 10) [175,177,178]. Furthermore, researchers synthesized a novel family of highly branched PBAEs via a new type of Michael-addition reaction using 4-amino-1-butanol as an A2 type monomer, trimethylolpropanetriacrylate as a B3 type monomer, and bisphenol A ethoxylate diacrylate as a C2 type monomer. The transfection efficiency between branched PBAEs and linear ones was compared in different cell lines (HeLa, rADSC, and SHSY-5Y) *in vitro* as well as *in vivo* in C7 null mice for the curing of skin diseases [179]. The branched polymers, which have molecular weight around 8 kDa to 10 kDa, improved 3- to 8-fold in higher transfection efficiency compared with LPAE [linear poly (β-amino ester)s] with minimal cytotoxicity (over 75% cell viability even at the highest w/w ratio of 30, while LPAE showed only 30%) and actively delivered COL7A1 gene to restore the expression of functional proteins in mouse skin [179]. This report is particularly important because it reveals the effects of different molecular configurations in polymers in increasing the cellular delivery of the target gene.

Various up-to-date technologies and the availability of reagents to test the endocytosis mechanism make it possible to define gene carriers and a particular uptake route. A proper understanding of the relationship between polymeric structure and the cellular uptake pathway would help in the design of effective gene delivery systems by regulating the cell entry mechanism, which would allow more personalized nanotherapeutics for effective gene therapy.

## 5. Physiochemical and biological cues: Control of cellular uptake and endocytic regulation

The regulation of cellular uptake for an intended application is an ideal strategy to enhance gene expression or silencing efficacy of polymeric gene carriers [180]. In this section, we discuss various physical, chemical, and biological cues that can be used to engineer polymeric modifications and regulate cellular uptake pathways (Fig. 11).

### *5.1. Physical cues*

#### *5.1.1. Mechanical cues*

*5.1.1.1. Osmotic pressure.* Osmotic activity is one of the key factors to improving the cellular uptake process of polymeric gene carriers [181,182]. Polymeric carriers with hyperosmotic activity showed enhanced cellular uptake and transfection activity, resulting in increased target gene expression [181]. Another study demonstrated increased transient gene expression in non-synchronized cells after the post-transfection osmotic shock [182]. Both studies offer a sense of the prominent effect of osmotic activity in improving the cellular uptake process. Ideally, it would be possible to regulate and direct the endocytosis pathway to CvME because it follows a non-acidic and non-digestive route without lysosomal fusion and degradation. It is interesting to note that cell exposure to a hypertonic solution selectively stimulated CvME by down-regulating CME and macropinocytosis uptake [183]. This is further supported by the fact that cells under osmotic stress phosphorylate Cav-1 protein by the activity of the Src-kinase enzyme, which is important for budding and pinching off from the plasma membrane [184]. Hypertonic solutes, such as sorbitol and mannitol, are non-ionic osmolytes that do not penetrate the plasma membrane [184]. Treatment with hypertonic solutes causing osmotic imbalance induced hypertonic stress followed by cell shrinkage because the non-penetrating osmolytes tended to draw water from the intracellular space for homeostatic balance. Because signal transduction and CvME are tightly linked in caveolae, osmosensing transduction will affect this endocytosis pathway [185]. Therefore, the use of hypertonic activity could be a unique strategy in gene delivery to precisely regulate endocytosis toward CvME.

Sorbitol (D-glucitol) is a major osmolyte widely produced in plants, especially in the Rosaceae family, including cherries, apples, and pears. It is also commercially produced through the reduction of 4-lactone, D-glucose, or D-glucono-1, and is extensively applied in the food industry because of its complete solubility in water without any discernible toxic side-effects [186]. It was reported that a sorbitol-based molecular transporter containing guanidine moieties enhanced cellular uptake and intracellular localization properties [186]. Furthermore, a guanidine-coated lipidated sorbitol–based molecular transporter increased transfection efficiency using plasmid DNA (pDNA) and enhanced silencing by siRNA [187]. Although exactly how these transporters enhanced cellular uptake has not been explained thoroughly, the sorbitol backbone played an important role in the cellular uptake process. Considering this, our group recently reported the inclusion of osmotic sorbitol properties in a cationic gene carrier that showed superior application for non-viral

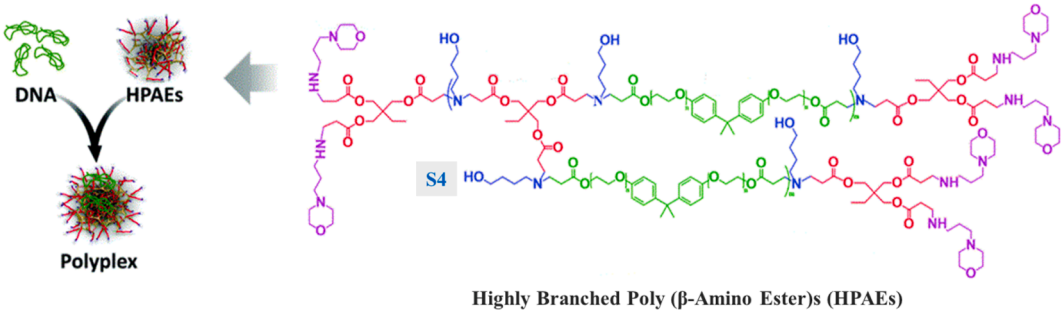



**Fig. 10.** Synthesis of HPAEs for gene delivery via an A2 + B3 + C2 type Michael addition reaction. Copyright permission obtained from Ref. [177].

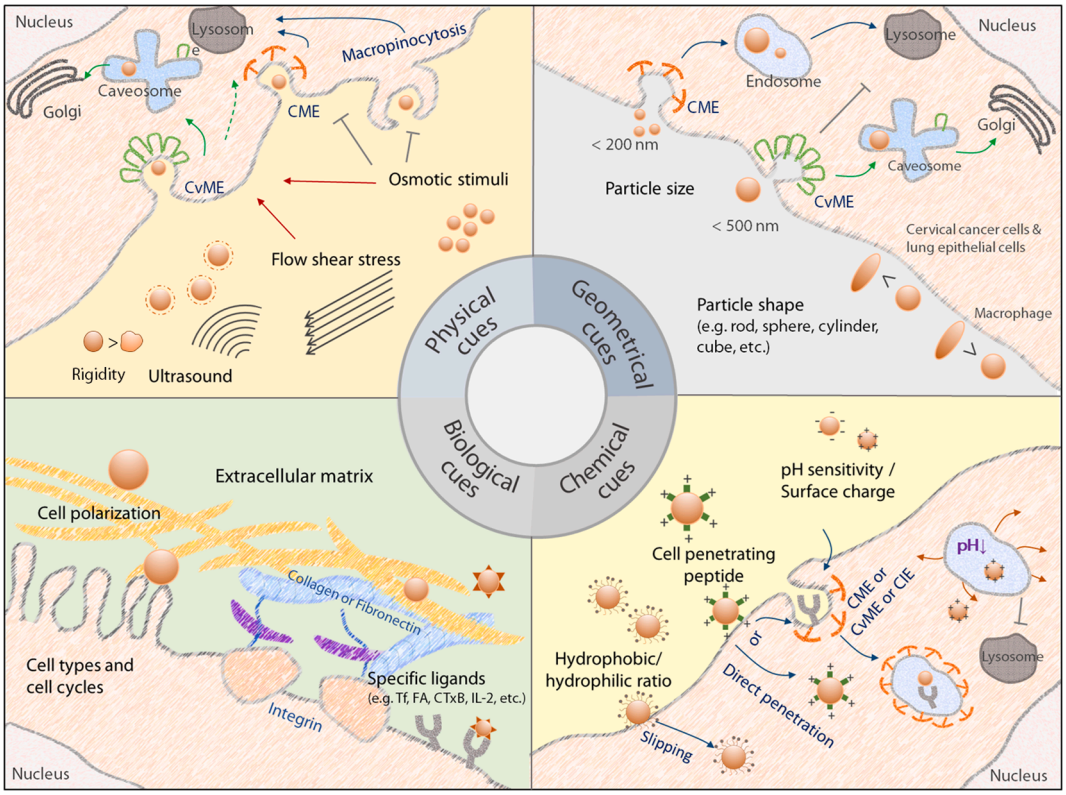



**Fig. 11.** Schematic illustration of essential cues affecting the regulation of cellular uptake and endocytic trafficking of engineered polymeric gene carriers.

polymeric vectors to deliver DNA [62,188,189], siRNA [190,191], microRNA [192], and protein antigens [193].

The state-of-the-art cationic vector, low-MW PEI, was cross-linked with sorbitol as a reactive diacrylate or dimethacrylate by forming degradable ester linkages between sorbitol's acrylate groups and PEI's amino groups, which produced a polysorbitol carrier with polyvalent capacity, as shown in Fig. 12 [164]. This polyvalent property of polysorbitol more effectively accelerated cellular uptake than monovalent sorbitol, thus improving transfection efficiency [62,188,189] as well as silencing osteopontin [190] and p62 (Fig. 13) [180] or the c-myc gene using an RNAi system [192,194]. Because sorbitol is a prime osmolyte, the osmotic polysorbitol carrier selectively directed the cellular uptake mechanism of the particles to CvME. The transfection ability of the polysorbitol gene carrier was affected only by the caveolae uptake inhibitors (genistein and methyl-β-cyclodextrin), avoiding attraction to other endocytosis inhibitors such as chlorpromazine and wortmannin. The osmotic activity of the polysorbitol transporter caused COX-2 expression by the activation of osmosensing molecules and regulated COX-2 activity with osmolyte-dependent adaptation of cells [195,196]. It is important to mention that cells which express high COX-2 exhibit better cellular uptake than cells with low or no COX-

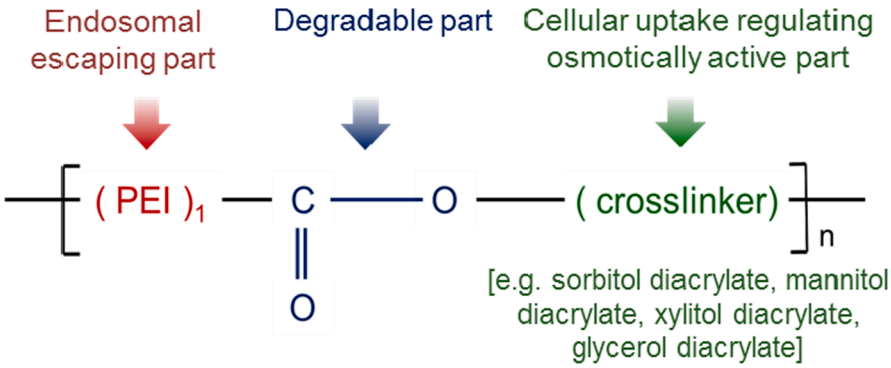



**Fig. 12.** A schematic illustration of the design of an osmotically active gene transporter based on low-MW PEI with an osmolyte backbone as a form of diacrylate cross-linking chain. Different parts of the transporters show different functional properties. Copyright permission obtained from Ref. [164].

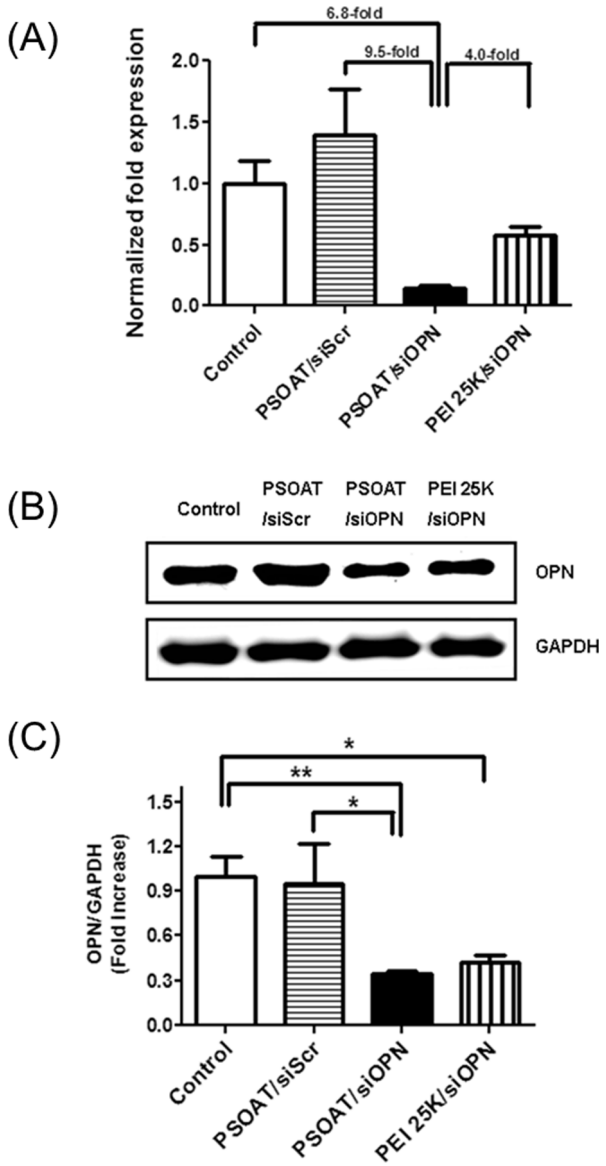



**Fig. 13.** Silencing of osteopontin (OPN) expression through the polysorbitol transporter (PSOAT)-mediated delivery of OPN siRNA (siOPN) compared with PEI 25 kDa-mediated delivery in A549 cells. Cells (80% confluence) were transfected with PSOAT/siOPN and PEI 25 kDa/siOPN complexes and examined for OPN expression silencing efficacy by (A) RT-qPCR analysis, and (B) Western blot assay. Data are expressed as the mean ± SEM from three independent experiments, and representative bands are shown. The significance was analyzed using paired t-tests; *P < 0.05 and **P < 0.01, respectively. Copyright permission obtained from Ref. [190].

2, demonstrating the importance of COX-2 [197]. Because the CvME pathway and its signal transduction are closely linked with caveolae, COX-2 expression along with selective CvME showed the unique properties of the osmotic polysorbitol-based transporter in accelerating the transport of DNA and siRNA. Moreover, the osmotic polysorbitol backbone contains many hydroxyl groups, which help reduce cytotoxicity and improve stable transfection [198].

The presence of hydroxyl groups in polymers such as PEG [55] and poly(3-amino-2-hydroxypropyl methacrylate) [57] reduced the transfection capability of gene carriers *in vitro*. However, the polysorbitol-based transporter accelerated the cellular transfer of the nucleotide cargo system to enhance gene expression or silencing because it could regulate the cellular uptake pathway toward the non-digestive track (such as CvME) and induce regulatory molecules such as COX-2 in response to hyperosmotic activity. This was the best combinatorial feature of the polysorbitol-based transporter, offering a novel gene carrier system for future applications, as shown in the schematic representation in Fig. 14.

Our group also designed a polymannitol gene transporter, another osmotic gene carrier system that showed selective stimulation of CvME, leading to efficient DNA delivery into cells [90]. The polymannitol-based transporter/DNA complexes avoided lysosomal fusion

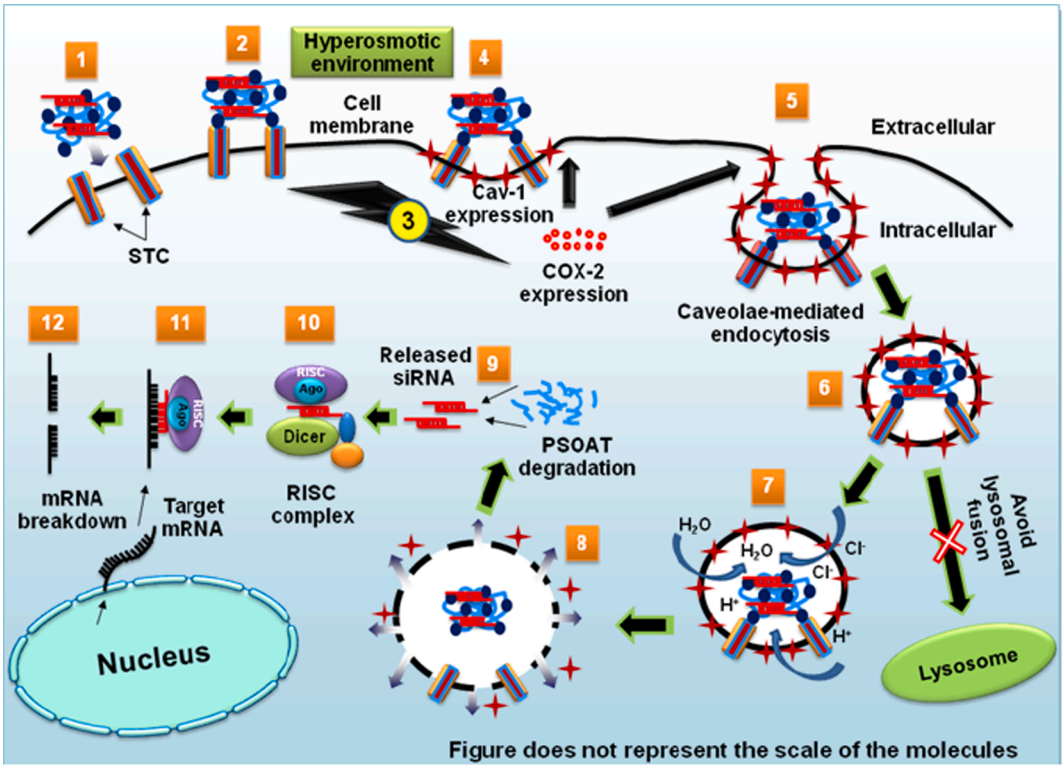



**Fig. 14.** A schematic representation of the function of polysorbitol transporter (PSOAT). Graphical presentation shows selective CvME of PSOAT/siRNA complexes following hyperosmotic pressure that induces Cav-1 and COX-2 expression, which accelerates the efficacy of the transporter for RNAi silencing. **(1–2)** PSOAT/siRNA complexes recognize and bind to the sorbitol transporting channel on the extracellular membrane and create a hyperosmotic environment. **(3–5)** The osmotic pressure-sensitive PSOAT/siRNA complexes induce Cav-1 expression and selectively stimulate CvME. This event subsequently generates COX-2 expression under osmotic stress. At this stage, Cav-1 expression augments and a mature caveolae structure is formed where COX-2 might work on disrupting the joint surface of particle deposition and accelerate CvME. **(6)** Selective CvME allows the caveolae endosomes (caveosomes) containing PSOAT/siRNA complexes to avoid lysosomal fusion. **(7–8)** The endosomes containing PSOAT/siRNA complexes swell and eventually burst due to the proton sponge effect of LPEI, which allows the complexes to escape into the cytosol. **(9)** Due to the nature of degradable linkages, PSOAT degradation occurs and siRNA is released. **(10–12)** The released siRNA recognizes and breaks down the target mRNA at the post-transcriptional level through an RNAi mechanism. Figure does not represent the scale of the molecules. Copyright permission obtained from Ref. [190].

and degradation by selectively regulating the cellular uptake process via CvME, as shown in Fig. 15. The mechanism by which the polymannitol gene transporter shifted the cellular uptake pathway toward CvME involved the transduction of mechanical stress induced by an osmotic polymannitol backbone. The mechanosensing signal transduction activated the protein kinase cascade of phosphorylation in Cav-1, which stimulated the trafficking of the caveolae vesicles (Fig. 15). Furthermore, the structural features of the polymannitol gene transporter also helped it penetrate the BBB [199]. Delivery of therapeutic genes to the brain is one of the biggest challenges for successful brain gene therapy because the BBB permits selective entry of only a few substances. As a molecular Trojan horse to ferry therapeutic genes across the BBB to the brain, we functionalized the polymannitol gene transporter using a BBB-permeable rabies virus glycoprotein (RVG) via a PEG link, generating R-PEG-PMT. The RVG ligand led to BBB penetration through receptor-mediated transcytosis via nicotinic acetylcholine receptors expressed on the BBB. In a mechanistic study, stimulation of receptor-mediated caveolar transcytosis by the osmotic polymannitol backbone improved the BBB penetration of the R-PEG-PMT/siRNA complexes, implying that the introduction of a physical stimulus cue is a promising approach to overcoming the BBB. Furthermore, an osmotically active multifunctional gene transporter, poly(lactitol-co-PEI) (PLT) was reported [200] (Fig. 16). By using lactitol composed of D-galactose and D-sorbitol as a crosslinker, the generated PLT has (i) liver cell targeting specificity from the galactose part, (ii) selective stimulation of CvME from the sorbitol part, and (iii) induction of rapid endosomal escape from the PEI part. The asialoglycoprotein receptors recognized by D-galactose are localized in detergent-resistant lipid-rafts in liver cells; thus, PLT/DNA complexes are internalized through CvME. We found that the sorbitol part of PLT prompted CvME *in vitro*, showing a trend resembling those of previously reported osmotically active gene transporters. Systemic gene delivery using PLT showed 9-fold higher transfection efficiency than available using PEI. Further investigation of the osmotic activity–induced cellular uptake regulation could be highly beneficial to the design and development of osmoregulated polymeric gene carriers because targeting a particular step during the process of cellular uptake is a sound strategy for improving gene delivery or silencing efficacy of gene carriers.

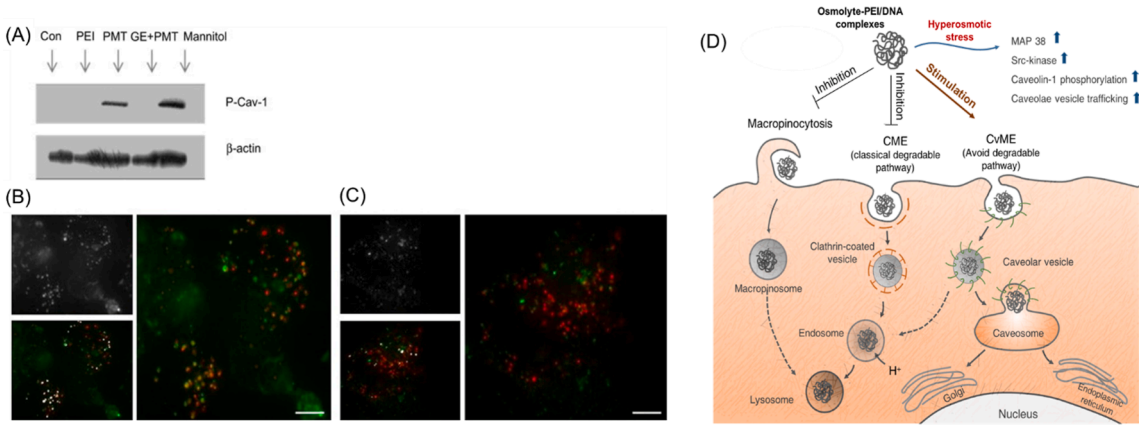



**Fig. 15.** Phosphorylation of Cav-1 on tyrosine 14 induced by polymannitol-based gene transporter (PMT)/DNA complexes and a co-localization study of PMT/DNA complexes with lysosomes. (A) A549 cells were (lane 1) untreated or treated with (lane 2) PEI/DNA or (lane 3) PMT/DNA complexes (N/P 20) for 30 min, or (lane 5) mannitol (600 mM) for 10 min. To inhibit Src tyrosine kinase, genistein was applied before transfection with PMT/DNA complexes (lane 4). Western blot analysis of cell lysates was conducted with phospho-Cav-1 antibody and β-actin antibody as a control. B–C: A549 cells were pre-incubated with fluorescent dextran for lysosome staining (red) and transfected with (B) FITC-PEI/DNA (green) or (C) FITC-PMT/DNA complexes (green) for 3 h. Then, co-localization of lysosomes and polyplexes was observed with fluorescent microscopy. Upper left images represent polyplexes detected with FITC, and lower left images represent co-localization spots analyzed by the ImageJ program. The overlay of polyplexes and lysosomes appeared in yellow, as shown in the right image. Scale bar represents 5 μm. Copyright permission obtained from Ref. [90]. (D) A schematic illustration of the concept of targeting CME by a polymannitol-based gene transporter (PMT) to control the intracellular fates of non-viral vectors. The PMT/DNA complexes maintain the nature of an osmolyte activate Src-kinase and selectively stimulate CvME by inhibiting clathrin-mediated endocytosis, which efficiently avoids lysosomal degradation of the complexes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

*5.1.1.2. Rigidity.* The effects of nanoparticle hardness on the choice of internalization pathway for gene delivery has been quite unexplored; however, given that rigidity can dramatically alter cellular uptake efficiency, it is worth investigating. Several research groups have used dissipative particle dynamics (DPD) simulations to investigate the effects of nanoparticle hardness on cellular internalization by reproducing the dynamic behavior of the lipid bilayer. Soft nanoparticles are less prone to wrapping than rigid particles because soft particles initially spread along the membrane without significant membrane deformation, which results in an abrupt rise in elastic energy at the later stage of wrapping for complete endocytosis [201]. Recently Guo *et al.* demonstrated the role of nanoparticle elasticity to improve tumor uptake [202]. They synthesized alginate nanolipogels composed of identical lipid bilayers with tunable elasticity which exhibited Young's moduli ranging from 45 ± 9 to 19,000 ± 5 kPa. In neoplastic and non-neoplastic *in vitro* cell models, the soft nanolipogels showed greater uptake with Young's modulus $< 1.6$ MPa compared to their elastic counterparts with Young modulus $> 13.8$ MPa. In an *in vivo* orthotopic breast cancer model, the soft nanolipogels exhibited significantly enhanced distribution in tumors, whereas the elastic nanolipogels accumulated much in the liver, suggesting that particle rigidity and elasticity play a significant role in regulating their delivery [202]. In another study, a library of silica nanocapsules (SNCs) was synthesized with a wide range of elasticity (Young's modulus ranging from 560 kPa to 1.18 GPa) to explain the effect of elasticity or rigidity of the particles in interaction with phagocytic and cancer cells [203]. The nanocapsules were modified with methoxy-PEG and folate-PEG to investigate non-specific and specific particle-cell interactions, respectively using the softest (560 kPa) and rigid (1.18 GPa) SNCs. Folate-PEG modified SNCs had no significant cellular uptake by macrophages whereas SK0V3 cancer cells had greater uptake, because of high level of folate receptors. The rigid SNCs showed higher (3-time) uptake by macrophages than the soft SNCs. The soft and the rigid SNCs (PEG-modified) exhibited equal non-specific uptake by SKOV3 cells. In contrast, the rigid SNCs (folate-PEG–modified) showed 4-fold higher uptake by SKOV3 cells than the soft one. It was also found that the folate-PEG–modified SNCs entered into SKOV3 cells mainly via a CME pathway, whereas the rigid SNCs stayed spherical in the course of either binding to the cell membrane or internalization. On the other hand, the soft SNCs were deformed during both binding and uptake processes. The uptake of SNCs in the macrophages mainly depended on phagocytosis/micropinocytosis. The softest SNCs did not flatten on the surface of macrophages, demonstrating no meaningful biological force on the cells. However, the softest SNCs did bend during the cellular uptake process and the distended pseudopodium configurations suggested the use of the phagocytosis/micropinocytosis pathway. It is likely that the distortion of soft SNCs slows their cellular uptake rate, leading to poorer macrophage internalization [203].

Previously, three models have also been suggested for the analysis of cellular internalization patterns: polymeric nanoparticles, liposomes, and solid nanoparticles with increasing hardness. The polymeric nanoparticles were constructed by a backbone chain linked with several branched chains, whereas the liposome model was developed via a self-assembly technique after attaching the beginning and the end of the polymeric nanoparticles. The solid nanoparticle model was constructed by eliminating all bonds in the liposome, making its structure a rigid solid. Solid nanoparticles can achieve complete endocytosis; however, the endocytosis processes for polymeric nanoparticles and liposomes are inhibited or frustrated by rapid depletion of the free ligands of soft nanoparticles, and the shape deformation caused by the ligand–receptor attraction slows down the wrapping process (Fig. 17) [204]. However, it is

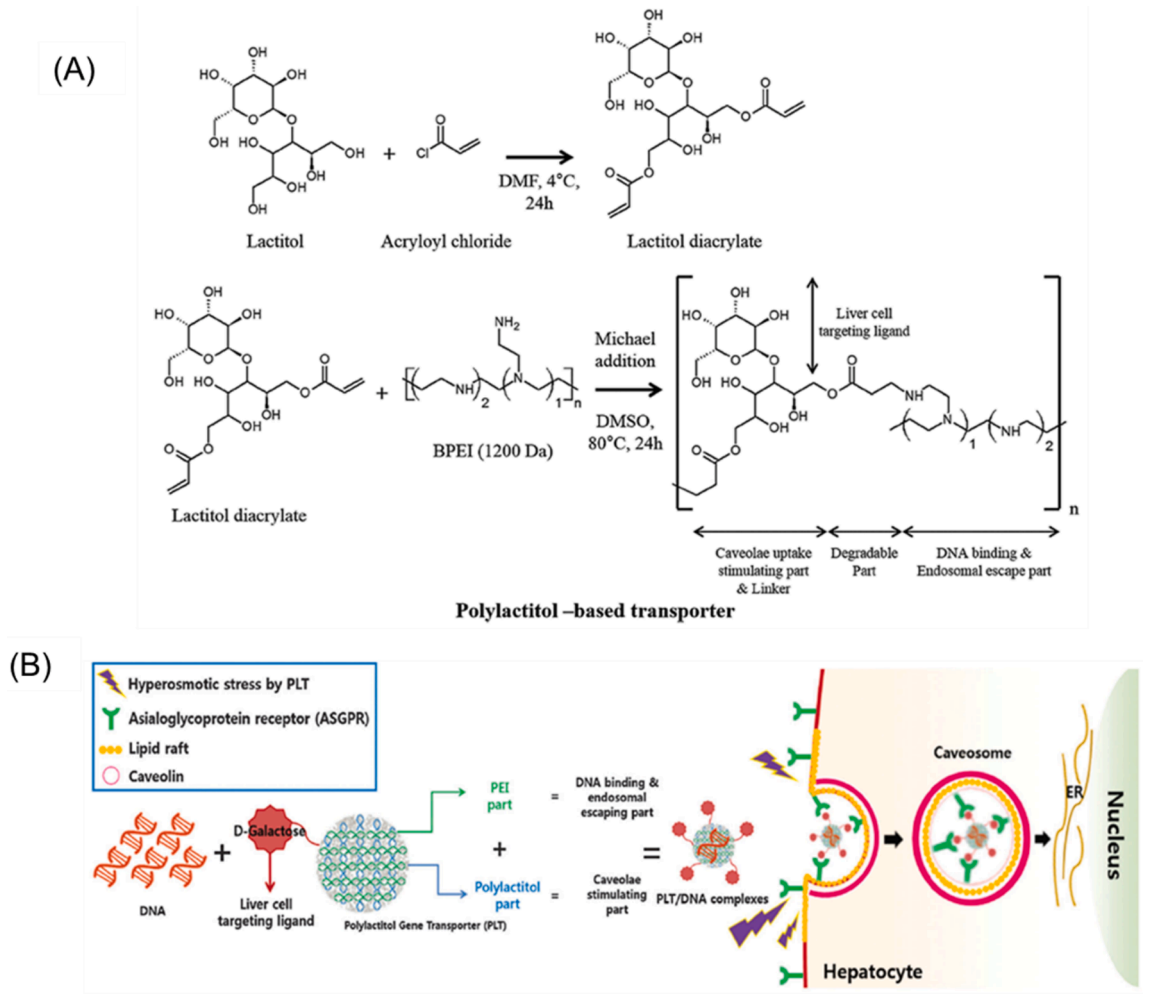



**Fig. 16.** (A) Scheme of PLT synthesis. (B) Schematic illustration of osmotically active PLT-mediated hepatocyte gene delivery. PLT provides synergistic effects that increase cellular uptake and result in liver cell targeting and rapid release of the gene from the endosome because the hyperosmotic property of the polysorbitol part selectively stimulates CvME, the polygalactose part provides liver cell targeting, and the PEI part assists in the rapid endosomal escape of the gene due to its proton sponge effect. Copyright permission obtained from Ref. [200].

important to note that the effect of rigidity on the endocytosis pattern likely depends on the cell type. Rigid nanoparticles were taken up by phagocytic cells in larger amounts and at higher rates than soft nanoparticles, whereas no significant difference was observed in non-phagocytic human umbilical vein endothelial cells (HUVEC). Upon the injection of nanoparticles in mice bearing cancer, soft nanoparticles exhibited a longer circulation half-life and higher accumulation in the tumor region, which has profound implications for the benefit of deformability in polymeric particles [205]. On the other hand, Sun *et al.* showed that rigid nanoparticles provided higher drug delivery efficiency in HUVEC [206]. Those conflicting results might be due to different chemical and physical characteristics of the materials used to formulate the nanoparticles. Although many important questions remain to be answered, regulating cellular pathways by tuning the rigidity of nanoparticles could be an interesting approach to achieve better gene delivery efficacy.

*5.1.1.3. Flow shear stress.* The consideration of shear stress is important in intravenous gene delivery because the cellular uptake of nanoparticles by the vascular endothelium depends on shear stress adaptation and flow exposure conditions [207]. The effect of flow on transfection efficiency can be explained by the balance between hydrodynamic shearing forces and the strength of adhesive bonding [208,209]. High fluid shear stress disrupts the nanoparticle–cell bond via hydrodynamic forces; however, a certain range of flow influences the membrane fluidity, which results in more accessibility of cationic nanoparticles relative to anionic syndecan molecules. The different attachment of PEI/DNA polyplexes on lung microvascular endothelial cells under shear stress has been addressed in a certain range of flow rates (0.06–2 dyn/cm$^2$) and the static condition [209]. A physiologically relevant flow rate range (0.3–1 dyn/cm$^2$) increased the binding rate of polyplexes compared to the static condition; however, under higher shear stress (2 dyn/cm$^2$), the polyplexes hardly adhered to endothelial cells at all. The shear stress on endothelial cells increases the formation of actin-based cytoskeletal structures, including stress fibers and membrane ruffles, which are associated with nanoparticle endocytosis [210].

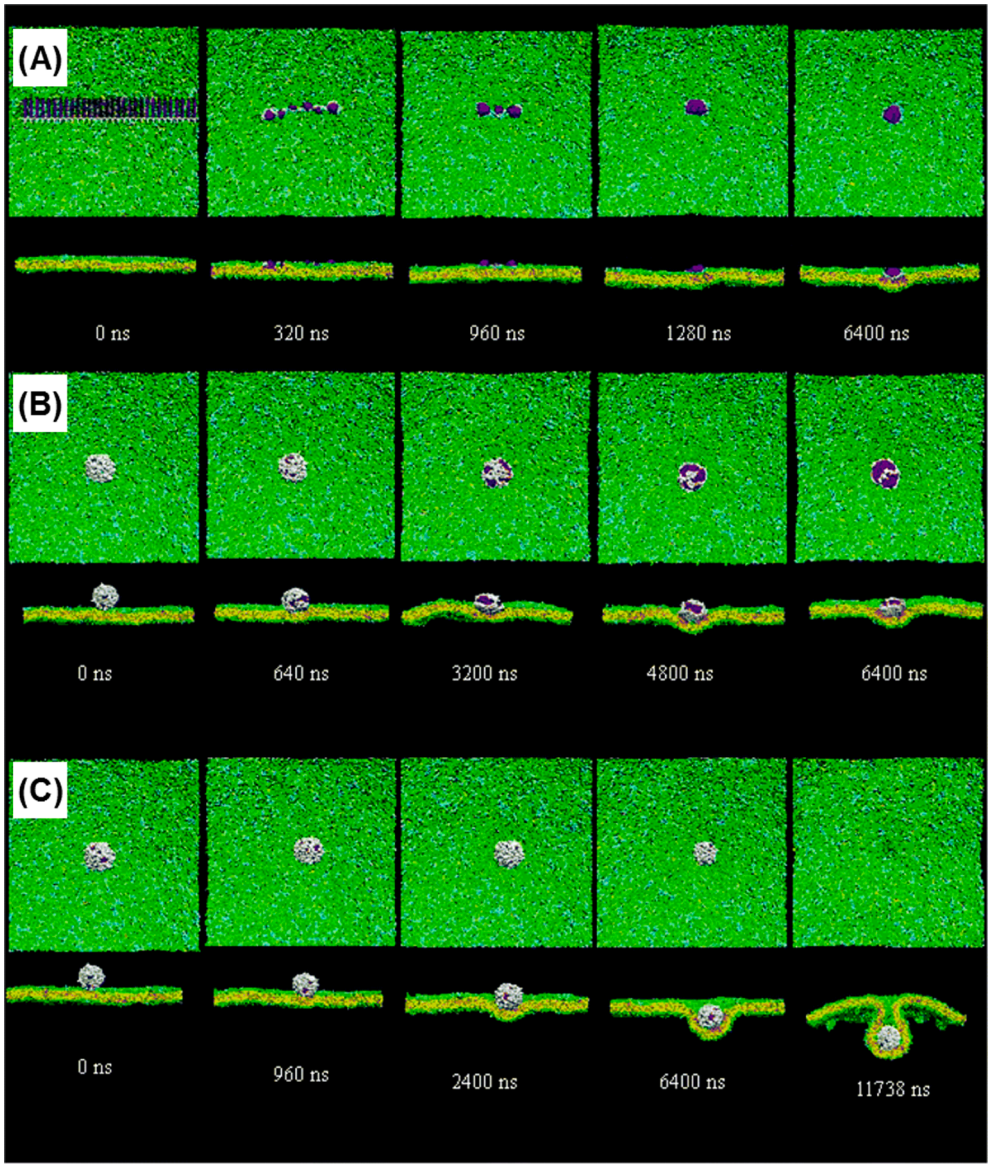



**Fig. 17.** Several typical snapshots of the endocytosis processes of polymeric NPs (A), liposomes (B), and solid NPs (C). Water molecules are not shown in the snapshots for clarity. The volume of the liposomes and solid NPs is 162 $nm^3$, whereas that for the polymeric NPs is 41 $nm^3$. Copyright permission obtained from Ref. [204].

Considering the different cellular uptake mechanisms seen under static conditions and dynamic flow [211], the application of a microfluidic cell culture system (e.g., organ-on-a-chip) mimicking a physiological environment is needed to promote understanding of the cellular uptake of nanoparticles *in vivo* and to achieve more reliable therapeutic benefits [212]. Moreover, to develop an endothelium targeted gene delivery system, the phenotype of the endothelium under shear stress needs to be considered. Shear flow is important for leukocyte adhesion in blood vessels via selectin (E, L, and P types) and other cell-adhesion molecules expressed on endothelium, such as PECAM, VCAM, and ICAM [213]. Modifying the particle surface with a selectin ligand, platelet glycoprotein Ibα [214], or P-selectin ligands, including Sialyl $Lewis^x$ [215] and LFA-1 [216], enhanced the cellular uptake of particles under physiological flow conditions. Using the same mechanism of action, antibodies against P-selectin [78], E-selectin [78], ICAM-1 [207,217], and PECAM-1 [218,219] are also effective in improving the cellular uptake of particles. The effect of flow was inhibited by disrupting the cholesterol-rich plasma domain, showing that caveolae play an important role in translating mechanical signals into biological responses [219]. To enable the fine-tuning of intracellular delivery during exposure to the circulation, detailed research is needed into the molecular mechanisms of flow effects on endocytosis patterns.

*5.1.1.4. Ultrasound.* The application of ultrasound to enhance the cellular uptake of genes has been showing some progress for more

than two decades; however, the biological and biophysical mechanisms of its cellular uptake is not thoroughly understood. Also, the effects of intensity variation (low or high, short or prolonged pulses) and their relationships with the molecular size of the drug, cellular uptake, and intracellular regulation must be further defined. A short or intense ultrasound pulse can induce pore generation on cells and facilitate direct drug uptake, whereas low intensity ultrasound induces endocytosis-mediated uptake, suggesting that ultrasound intensity is vital for the change of cell surface conformation and subsequent uptake mechanisms. Small molecular drugs can readily cross the small pores; however, larger drugs, such as gene complexes or nanoparticles, would need high ultrasound intensities for direct cellular entry [220]. Generally, ultrasound is applied to cells in the presence of microbubbles. Although, the application of ultrasound alone is uncommon, some reports have demonstrated that its application alone improves the cellular delivery of DNA [221-223], proteins [224], and chemical drugs [225-229].

Ultrasound alone can exert biophysical effects, such as cavitation and acoustic streaming, which subsequently provide pore formation as well as endocytosis of the particles. High-intensity, low-frequency ultrasound alone stimulated pore formation on cells with no involvement of endocytosis [230,231]. Other studies showed endocytosis-mediated cellular uptake (especially CME and CvME) when low intensities were applied with high frequencies [232-234]. Selective CME activity in endothelial cells was induced by ultrasound exposure alone with the frequency and intensity range currently used in clinical diagnostic methods but at a high mechanical index (1.2 MHz) [233], suggesting that proper optimization of ultrasound strategy can effectively regulate the cellular uptake pathway toward a desired mechanism.

The application of ultrasound in the presence of microbubbles to enhance cellular uptake is well studied. Enhanced cellular uptake of fluorescent-labeled dextrans with different MW (4.4 to 500 kDa) by regulating the uptake mechanism has been shown for two major endocytosis pathways (CME and CvME) and macropinocytosis by pore formation [235]. It is worth noting that CME and macropinocytosis were involved in the cellular uptake of ultrasound microbubbles for all sizes of dextran, whereas CvME mainly occurred at 155 and 500 kDa, but not at 4.4 and 70 kDa. The dextran vesicles (500 kDa) showed co-localization of Cav-1 and clathrin in primary bovine aortic endothelial cells. Moreover, the ultrasound and microbubble-targeted dextran induced an influx of $Ca^{2+}$ [235] and the release of preloaded dextran post-exposure, with subsequent stimulation of pore formation. Precious study also demonstrated that ultrasound-targeted microbubble destruction stimulated the CME mechanism for adeno-associated virus delivery by stimulating the formation of clathrin-coated pits, suggesting that endocytosis pathways can be regulated by ultrasound treatment [236]. The effect of low intensity ultrasound on biocompatibility and the cellular uptake of chitosan tripolyphosphate nanoparticles [237], suggests that ultrasound treatment facilitated cellular membrane leakiness and improved the cellular uptake of chitosan nanoparticles. It has been also suggested that ultrasound microbubble treatment promoted both CME and fluid phase endocytosis of receptor-bound macromolecules such as transferrin receptors [238]. Ultrasound microbubble treatment stimulated clathrin-coated pits to induce CME and enhanced the rate of fluid-phase endocytosis, but with a delayed onset relative to CvME stimulation, indicating that these two endocytosis processes are distinctly regulated by ultrasound treatment.

#### *5.1.1.5. Geometrical cues*

*5.1.1.5.1. Particle size.* The cellular membrane and endocytic machinery have well defined flexibilities and geometries that can regulate or restrict cellular uptake, depending on the size of the particles [251]. A particular size of nanoparticle can trigger gene carriers to use the endocytic route that gives the highest gene transfection and expression efficacy [251]. Knowing the suitable particle sizes could be very useful in effectively regulating uptake pathways for gene carriers and achieving high transfection efficacy. Rejman *et al.* investigated the effect of polyplex particle size (10–1000 nm) on the pathway of entry into non-phagocytic B16 cells. Using a latex base fluorescent vector, it was observed that particles up to 200 nm entered the cells via CME route and particles with sizes above 200 nm through CvME pathway [252]. Other polymeric carriers, such as polystyrene particles, exhibited a linear relationship between particle size and cellular uptake [253]. It was also observed that larger silica nanoparticles (160 nm) showed higher antitumoral miRNA delivery efficiency than small particles (60 nm) because of larger contact area to the cell membrane and simultaneously a larger number of targeting ligands allowing for a maximum degree of endocytosis [254]. In contrast, poly(lactic-co-glycolic acid) nanoparticles, which are smaller, showed 25 times higher transfection efficiency than larger particles in COS-7 cells [255]. Lai *et al.* demonstrated that carboxyl-modified polystyrene nanoparticles (24.4 nm) could be directed toward a unique non-caveolae and non-clathrin pathway while particles with a size of 43 nm were internalized via CME in HeLa and primary HUVEC cells. This suggested that small particles ($< 25$ nm) enable effective intracellular trafficking allowing for the avoidance of enzymatic degradation of the endolysosomal pathway [256]. Such a discovery seems to overcome the problem of polyplex degradation while crossing the nuclear membrane. Although this discovery may improve polymeric nanoparticle-mediated drug delivery into the nucleus, making such a small particles$<$25 nm will be challenging with nucleic acids such as DNA or mRNA because of their large size and length. To estimate the efficacy of oral delivery of nanoparticles in relation to particle size, oleoyl alginate ester nanoparticles with a narrow size distribution for their cellular uptake was determined. Endocytosis of 50–120 nm particles mainly occurred via CME, whereas larger nanoparticles, around 420 nm, occurred via CvME [257]. Moreover, particles 50–100 nm in diameter were easily taken up by the cells within 30 min and found in the lysosomal compartment at a later phase. On the other hand, the larger particles took a span of hours and were not co-localized with the late endosome, suggesting that they trafficked slowly via a non-digestive CvME pathway, unlike the smaller particles, which were involved with the CME mechanism. Therefore, size is an important factor in directing the entry pathway and intracellular fate of particles. A matter of particular importance is that the type of the endocytic pathway may depend on the cell type and property of materials as well as the size of the particles. It would be interesting to apply this knowledge in the design of non-viral polymeric gene carriers to maximize the gene delivery efficiency in specific target cells.

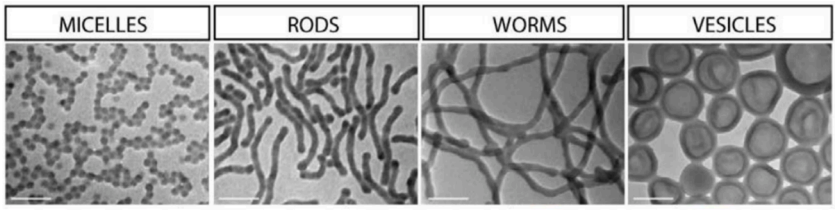
*5.1.1.5.2. Particle shape.* In general, nanoparticles are designed to have a spherical shape for effective gene transfer into cells.

However, many studies have reported that the particle shape is an important factor in controlling cellular uptake and intracellular trafficking. Using correlation microscopy, Hinde *et al.* examined the cellular uptake and route of intracellular transport of poly(oligoethylene glycol methacrylate)-block-poly(styrene-co-vinylbenzaldehyde) block copolymer nanoparticles with different shapes including micelles (20 × 20 nm), vesicles (l00-300 × 5–10 nm), rods (400–700 × 5–10 nm), and worms (100 × 100 nm) (Fig. 18) [239]. The study showed that polymeric nanoparticles with different shapes, but identical surface chemistries, moved across the cellular barriers at different rates and accessed nuclear envelope with different efficiency. The study concluded that rods and worm shaped nanoparticles with the highest aspect ratios (14–28 and 57–71) delivered agent more actively into the nucleus than spherical (aspect ratio of 1) nanoparticles and they are more toxic when loaded with equal amounts of doxorubicin [239]. In another study, Gratton *et al.* reported on the internalization mechanism of non-spherical particles. For this purpose, the group made three distinct series of cationic PEG-based particles: micrometer-sized cube (2, 3 and 5 μm), μm-sized cylindrical (0.5 μm with aspect ratio of 2 and 1 μm with aspect ratio of 1), and finally cylindrical shaped nanoparticles (diameters of 200, 100 and 150 nm with aspect ratio of 1, 3, and 3, respectively) using particle replication in non-wetting templates (PRINT) method [240] (Fig. 19). The PRINT is a top-down particle fabrication technique used in the microelectronics industry, originally to make transistors, and offers precise control over a wide range of particle sizes (20 nm–100 μm), shapes (spheres, cylinders, discs, toroids), composition (organic/inorganic, solid/porous), cargos (therapeutics, biologics, proteins, oligonucleotides, siRNA), and surface properties (targeting peptides, antibodies, aptamers, cationic/anionic charges, stealth PEG chains). The findings in the study suggest that rod-like particles had accelerated internalization to non-phagocytic cells, similar to the infection strategy used by many rod-shaped bacteria [240,241]. This is consistent with the observation that amphiphilic block copolymer/DNA complexes with a rod-shape are internalized more efficiently than spherical particles. Shape-dependent uptake might differ for nanoparticles with varying geometries because the size of the shielded part of the hydrophobic blocks affects interactions with cell membranes [242].

Uptake studies with porous silica particles in difference shapes found that the rod-like nanoparticles were more efficient at internalization in Caco-2 cells but not in HeLa cells, suggesting that the shape of the carrier influences the uptake in a cell-dependent manner [243]. The reason for this is because the dynamics in the plasma membrane and development of endocytosis pathways vary depending on the cell types of origins [244]. Chithrani *et al.*, also determined that traditional spherical-shaped gold particles entered cells more efficiently than rod-shaped nanoparticles in HeLa cells [245]. It has been postulated that the slower uptake process of the rod-shaped particles might be related to the wrapping time. It took longer to wrap the entire rod than it did to wrap traditional spherical nanoparticles, coincident with a higher fraction of exocytosed nanoparticles [246]. A dissipative particle dynamic (DPD) simulation of the uptake of PEGylated nanoparticles previously showed that spherical nanoparticles need to overcome a minimal membrane bending energy barrier (8 πκ) compared to rod- (12 πκ) or disk-like particles (17.2 πκ) [241]. In another simulation study, it was also shown that spherical nanoparticles can be internalized with the least Monte Carlo steps-receptor diffusion, particle translation or rotation, bond formation or breakage, and membrane surface evolution, indicating the higher efficiency [247]. On the other hand, it was demonstrated that prolate ellipsoidal particles can be internalized with fewer bonds of ligand-receptor bond compared to spherical shaped particles, which are excellent candidates for transcellular drug delivery [247]. Because the internalization of nanoparticles facilitates interactions between the cell membrane and the particle surface, shape together with the endocytic dynamics of target cells must be considered for the best drug delivery strategy.

The shape of nanoparticles also influences the efficiency of phagocytosis [248]. Champion *et al.* prepared worm-like particles with

(A)




(B)

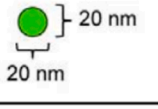
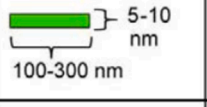
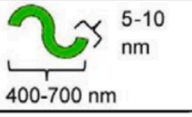
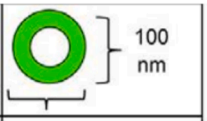
| PARTICLE DIMENSIONS (nm) |   |   |   |   |
|---|---|---|---|---|
| POLYMERISATION TIME (h) | 8 | 12 | 18 | 48 |
| ASPECT RATIO | 1 | 14-28 | 57-71 | 1 |
| IC $_{50}$ (μM) | 2.193 | 0.302 | 0.796 | 1.928 |

**Fig. 18.** Characteristics of nanoparticles of four different shapes to target doxorubicin to the nucleus. (A) Transmission electron micrographs of the four types of polymeric nanoparticles with different shapes. Scale bar = 100 nm. (B) Physical properties of the four differently shaped polymeric nanoparticles: nanoparticle dimensions (end diameter and length), polymerization time (the reaction of the block polymer mixture to create nanoparticles of different shape), IC50 values of nanoparticle cytotoxicity when loaded with doxorubicin (concentration of doxorubicin that was required to induce cytotoxicity in 50% of a population of MCF7 cells). The chemical properties of the four different nanoparticle shapes are identical. Copyright permission obtained from Ref. [239].

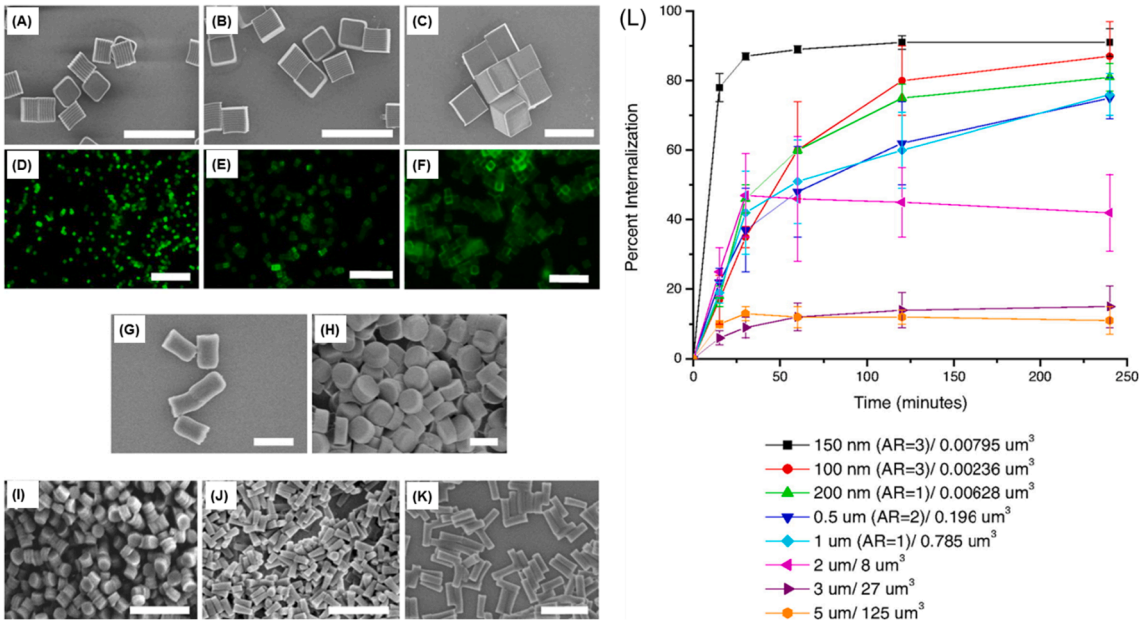



**Fig. 19.** Micrographs of PRINT particles varying in both size and shape. The particle composition for these experiments was 67 wt% trimethylolpropane ethoxylate triacrylate (MW = 428 g/mol), 20 wt% poly(ethylene glycol) monomethylether monomethacrylate (MW = 1,000 g/mol), 10 wt% 2-aminoethylmethacrylate hydrochloride (AEM·HCl), 2 wt% fluorescein-O-acrylate, and 1 wt% 2,2-diethoxyacetophenone. (A–C) Scanning electron micrograph of the cubic series of particles [diameters equal to 2 μm (A), 3 μm (B), and 5 μm (C)]. (D–F) Fluorescence micrographs of the cubic series of particles [diameters equal to 2 μm (D), 3 μm (E), and 5 μm (F)]. (G and H) Scanning electron micrographs of the cylindrical series of microparticles having the same height (1 μm), but varying diameters [diameter = 0.5 μm (G) and 1 μm (H)]. (I–K) Scanning electron micrographs of the series of cylindrical nanoparticles [diameter = 200 nm, height = 200 nm (I); diameter = 100 nm, height = 300 nm (J); diameter = 150 nm, height = 450 nm (K)]. (Scale bars: A–F, 20 μm; G–K, 1 μm). (L) Internalization profile of PRINT particles with HeLa cells over a 4-h incubation period at 37 °C. Legend depicts the particle diameter per particle volume. Copyright permission obtained from National Academy of Science [Ref. 241].

high aspect ratios (> 20) and tested their effects on the phagocytosis process. The decreased high curvature regions (Ω) of the worm-shaped particles induced a negligible uptake by macrophages, suggesting that shape dependency varies considerably depending on the role of actin in phagocytosis and endocytosis. Various shapes, such as disks and UFO-like nanoparticles, have also been suggested to boost the immune system [249]. Rod-shaped nanoparticles conjugated with trastuzumab, a HER2 (human epidermal growth factor receptor 2)-targeting antibody, enhanced a specific uptake pathway in cells compared to spherical particles [250]. The orientation of the particles attached to the cells appears to be an important factor because it affects the internalization time and the complexity of the actin structure that forms the curvature for uptake. Elucidating the mechanism by which the shape and dimensions of nanoparticles affecting cellular uptake could lead to new ways of controlling geometrical gene delivery vehicles.

## 5.2. Chemical cues

### 5.2.1. Surface charge and pH-sensitivity

Cationic nanoparticles have long been used to deliver drugs and genes because they are good at inducing cell membrane permeability. They elicit better (i) adherence to the negatively charged outer surfaces of cells, (ii) formation of nanoscale holes in the lipid bilayer, and (iii) internalization by endocytic machinery depending on the surface charge density. More importantly, the proton sponge effect, which promotes endosome osmotic swelling, is caused by cationic polymers; thus, much interest has focused on controlling the surface charge to adjust cellular trafficking. A positive charge on the surface of nanoparticles apparently increases their internalization into cells because it induces them to move into clathrin-coated pits on the plasma membrane [251]. That finding is consistent to the reports described PEI-coated superparamagnetic iron oxide nanoparticles [252], positively charged mesoporous silica nanoparticles [253], and chitosan-coated nanoparticles had enhanced cellular uptake efficiency via CME [254]. Incorporating a negative charge on the nanoparticles greatly affected their intracellular route and targeted them to the degradable pathway [255]. The surface charge of chitosan plays an important role in both cellular internalization and intracellular trafficking. Positively charged chitosan nanoparticles tend to localize at the nucleus, possibly because of electrostatic interaction with the negatively charged nucleus [256]. The surface charge of gene carriers can significantly affect intracellular uptake, localization, and biological function; thus, surface charge is a critical factor for the design of nanoparticles. However, several researchers have also demonstrated that a strong positive surface charge can lead to apoptotic cell death via membrane potential perturbation [257] and endolysosomal disruption [258]. Balancing unintended cytotoxicity with intended cellular uptake by surface charge modulation, therefore, is the key to

synthesizing a safe polymeric gene delivery material.

Several researchers have demonstrated enhanced cellular uptake using a pH-sensitive surface-charge-switched gene carrier [259-261]. The pH-sensitive gene delivery vehicles exploit pH conditions not only in the circulation (to provide stability and prevent interaction with serum components, thereby offering prolonged circulation of gene carriers and improved cellular uptake), but also in the cellular microenvironment for uptake regulation [261]. Carboxymethyl chitosan (CMCS) and cationic liposome-coated DNA/protamine/DNA (CLDPD) complexes have been reported as pH-sensitive multifunctional gene carriers [261]. The outermost CMCS layer was pH-sensitive, containing a cationic charge at pH $< 6.5$ and an anionic charge at pH $> 7.0$. The anionic charge protected the CLDPD complexes from interactions with serum components in the blood, providing prolonged circulation ability to the gene carrier. When the CLDPD complexes reached the microenvironment of tumor cells (pH $< 6.5$), the CMCS took on a cationic charge, repelled the CLDPD, and fell off. The cationic CLDPD complexes then interacted and combined with the negatively-charged tumor cells and entered the cells via endocytosis, demonstrating a profound example of cellular uptake regulation using the pH-sensitivity of non-viral gene carriers. Another study reported the cellular uptake of a nucleic acid using biodegradable pH-sensitive surfactants (BPS) in liposomes [260]. As a member of the BPS family, dodecyl 2-(1́-imidazolyl)-propionate showed that an acidic pH could trigger BPS activation, which induced fusion with the cellular membrane [260].

HUVEC cells follow three major endocytosis mechanisms, CME, CvME, and macropinocytosis, to internalize pH-sensitive liposomes, and COS-7 cells use CME and CvME but not micropinocytosis [260], suggesting that the endocytosis pathway depends on cell type. Thus, it is necessary to study and optimize the cell uptake behavior of polymeric-material-based gene carriers in appropriate cells [260]. A pH-sensitive stealth coating using LMW chitosan for tumor-specific drug delivery enhanced cellular internalization at tumor-specific sites in an acidic environment; in other words, pH-sensitive stealth coating materials delivered a drug to specific tumor sites

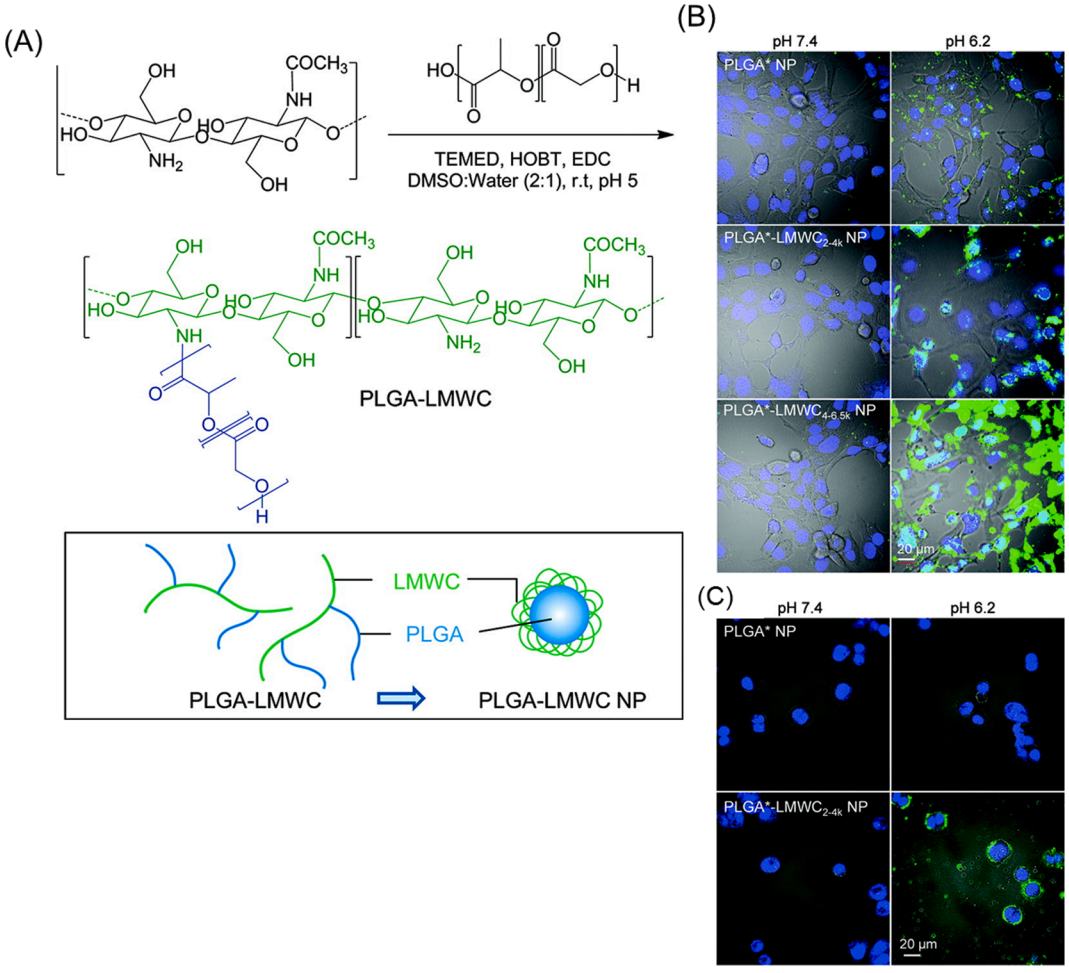



**Fig. 20.** The pH dependence of cellular association of poly (D, L-lactide-co-glycolide) (PLGA) NPs and PLGA-LMWC NPs. (A) Synthesis of PLGA-LMWC and Schematic Diagram of PLGA-LMWC NP. Confocal images of (B) SKOV-3 ovarian cancer cells or (C) NCI/ADR-RES multidrug resistant ovarian cancer cells were taken after 3 h of incubation with the NPs (overlaid images of NP (green), nuclei (blue), and transmission images). Copyright permission obtained from Ref. [259]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[259] (Fig. 20). Many researchers have demonstrated that pH could influence the delivery of DNA and siRNA after cellular internalization [49,261]. Poly(β-amino ester)/DNA complexes based on self-assembled carboxymethyl poly(L-histidine) have been reported for DNA delivery [49]. The carboxymethyl poly(L-histidine), with a large number of pH-sensitive imidazole groups, protected the DNA from lysosomal degradation through efficient intracellular delivery by enhancing endosomal escape through the proton sponge effect [49]. In another study, pH-sensitive polyion complex micelles prepared using methacrylic acid copolymers and PAMAM provided stable and efficient delivery of siRNA [261]. Endosomal escape of the polyion complex micelles was enhanced because the acidic pH in the endosomal network protonated the amine groups of the poly(amidoamine) and induced the proton sponge effect, causing efficient intracellular delivery of siRNA and target gene silencing [261]. Polyspermine imidazole-4, 5 imine (PSI) synthesized in a reaction between bisformaldehyde imidazole and spermine has a pH-responsive linkage, the bisimine bond conjugated with the imidazole ring [262]. That linkage has a pKa value in the range of 5–6, providing stability to the PSI and also promoting dissociation in the endosomal acidic compartment, thereby propagating the proton sponge effect and leading to the endosomal escape of siRNA and subsequent downregulation of gene expression [262]. In a previous report, polyaspartamide derivatives with a repetitive array of amino groups in the side chain were introduced for the delivery of siRNA with enhanced stability [263]. The pH-responsive disruption of the cytoplasmic membrane facilitated efficient endosomal escape with less cytotoxicity than comparable methods and high efficacy of RNAi silencing [263]. Controlling the pH-responsiveness of gene carrier is not always feasible for every cell type. T cell, for example, has slow acidification of endosome, which reduces the gene transfer efficiency of polymers designed for pH-triggered endosomal escape [264]. All these evidences suggest that pH-responsiveness is an important factor that could greatly affect the regulation of cellular uptake and intracellular delivery, leading to efficient gene expression or silencing effects. It is also important to note that pH-sensitivity in cellular uptake regulation is not specific to a particular uptake route but rather depends on the polymeric material design.

#### 5.2.2. *Hydrophobicity/hydrophilicity ratio*

The surface hydrophobicity/hydrophilicity ratio is one determinant factor controlling cellular internalization and biodistribution. Surface hydrophobicity results in the formation of a protein corona, which drastically decreases cellular uptake and enhances phagocytosis by immune cells *in vivo*. Many approaches have been proposed to prevent a protein corona by controlling surface hydrophobicity/hydrophilicity, including PEGylation [265-267] or incorporation of zwitterions such as amino acids [268] and polybetaines [269] onto the polymeric nanoparticle surface. Ironically, however, a more hydrated layer on the nanoparticle surface leads to lower affinity with the lipid bilayer of cells, also preventing cellular uptake. Less hydrophobic packing surrounding a quaternary ammonium chain of gold nanoparticles resulted in lower transfection efficiency [270]. Therefore, a well-balanced hydrophobic/hydrophilic ratio is needed to minimize protein corona formation and enhance cellular uptake [271]. Because the cell membrane is composed of an amphipathic lipid bilayer, designing an amphipathic gene carrier might be feasible [272].

Some biomolecules, such as amphipathic CPPs, can penetrate the membrane; however, it is important to note that no synthetic nanoparticles can pass through membranes without disrupting the membrane integrity. A mechanism for internalizing amphipathic nanoparticles has been suggested that is not mediated by endocytosis but occurs by slipping through the membrane without disruption [273]. Interaction between the self-assembled striped nanoparticles as micelles with the phospholipid head of the membrane produces cylindrical micelles that then assemble into a stable bilayer and release their particles into the cytoplasm [272]. The consideration of surface amphipathicity is an effective strategy for designing nanoparticles to deliver therapeutic nucleotides without disrupting the membrane while also reducing cytotoxicity.

#### 5.2.3. *Cell penetrating peptides*

The discovery of CPPs is a remarkable breakthrough in the transportation of large cargo molecules such as oligonucleotides into cells. CPPs were introduced in the late 1980s during the discovery of the human immunodeficiency virus type 1-encoded TAT peptide [274] and the penetrating peptide from Drosophila Antennapedia (pAntp) [275]. Recently, composites of different lengths and properties of cationic hydrophilic peptides with hydrophobic tails have opened the door for the synthesis of new materials for the delivery of plasmids [276], siRNAs [277] or the clustered regularly interspaced short palindromic repeats (CRISPR)/associated protein 9 (CRISPR/Cas9) [278,279]. Based on the peptide origin, CPPs can be classified into three groups: i) protein derived peptides such as TAT and penetratin, commonly called protein transduction domains; ii) chimeric peptides that contain motifs from other peptides; and iii) synthetic peptides such as the polyarginine family. Another classification of CPPs is based on the peptide sequence and properties for binding to membrane lipids. That classification also contains three groups: primary amphipathic, secondary amphipathic, and non-amphipathic CPPs [280].

Cellular uptake mechanisms are not the same among different groups of CPPs, and most of them can take multiple pathways [280-282]. CPPs have two main cellular uptake mechanisms: direct penetration and endocytosis. Direct penetration occurs by the interaction of cationic CPPs with anionic membrane components, such as heparin sulfate and the phospholipid bilayers, followed by stable or transient membrane destabilization associated with peptide folding on the lipid membrane [283]. Cellular internalization of CPPs via direct penetration depends primarily on the peptide sequences, peptide concentration, and the lipid composition of the membrane [280]. The other major mechanism for cellular uptake of CPPs is endocytosis, with the process directed by CME/CvME or an independent pathway [284]. CPPs can also be taken up by phagocytosis for large particles and by pinocytosis (mainly macropinocytosis) for solutes [284,285]. According to a recent report, CPPs, including TAT and HA2, influence the intracellular delivery of nanoparticles; however, they do not lead to cytosolic localization because they lack an endosomal escape mechanism, which implies that cellular internalization of CPPs depends more on endocytosis than direct penetration [286].

Several factors affect the regulation of the cellular uptake of CPPs, depending on their physicochemical properties [280]. The net charge differences of CPPs can regulate their uptake, with positive charges (especially arginine residues) being more favorable than

lysine for effective delivery [287]. The conformation (including alpha helix and beta sheets) and size of CPPs also regulate their cellular uptake. Therefore, the different amphipathic CPPs show different cellular uptake pathways. In this regard, the primary and secondary amphipathic CPPs show direct penetration of the cell membrane at low micromolar concentrations, whereas non-amphipathic CPPs mainly use the endocytosis pathway [288]. The concentration of CPPs also seems to affect their cellular uptake: they enter into cells by direct penetration at higher concentrations and by endocytosis at low concentrations. Other factors affecting the cellular uptake of CPPs include cell type, temperature, and type of cargo [280]. It is important to know the exact uptake mechanism (e.g., by direct penetration or endocytosis) and other related factors, such as the influence of cargo molecules, that affect the bioavailability of CPPs. Moreover, a major problem with CPPs and related peptides is their susceptibility to degradable proteases. They could follow a non-specific cellular uptake mechanism, depending on their type and properties. Therefore, it is necessary to improve the stability of these molecules and to define precisely their cellular uptake pathway for each specific application which can benefit to trigger their clinical implementation.

### 5.3. Biological cues

#### 5.3.1. Extracellular matrix proteins

The cellular microenvironment can be engineered to regulate and enhance gene delivery to a variety of cell types [289]. Mooney and colleagues reported how the stiffness of an extracellular matrix (ECM) protein bound with cells affected DNA transfer and internalization process (Fig. 21) [289]. Fibronectin (Fn) and collagen (Col) have been studied as ECM proteins to regulate non-viral gene transfer. Fn has previously been applied to mesenchymal stem cells (MSCs) to increase the gene transferability of non-viral gene carriers [290,291]. Gene-transfer to Fn-bound cells was more efficient than to cells plated on bovine serum albumin, Col I, or laminin [292]. On the other hand, Col IV showed significantly enhanced gene transfer compared to that of Fn, Col I, laminin and PLL [293]. Although the ECM microenvironments showed increased gene transfer capacity, the exact mechanism by which they regulate non-viral gene transfer has not yet been fully addressed; a few investigations have indicated that the cellular uptake pathway is affected by the ECM environment [292-294]. Fn was found to increase gene transfer to fibroblasts in correlation with endocytosis

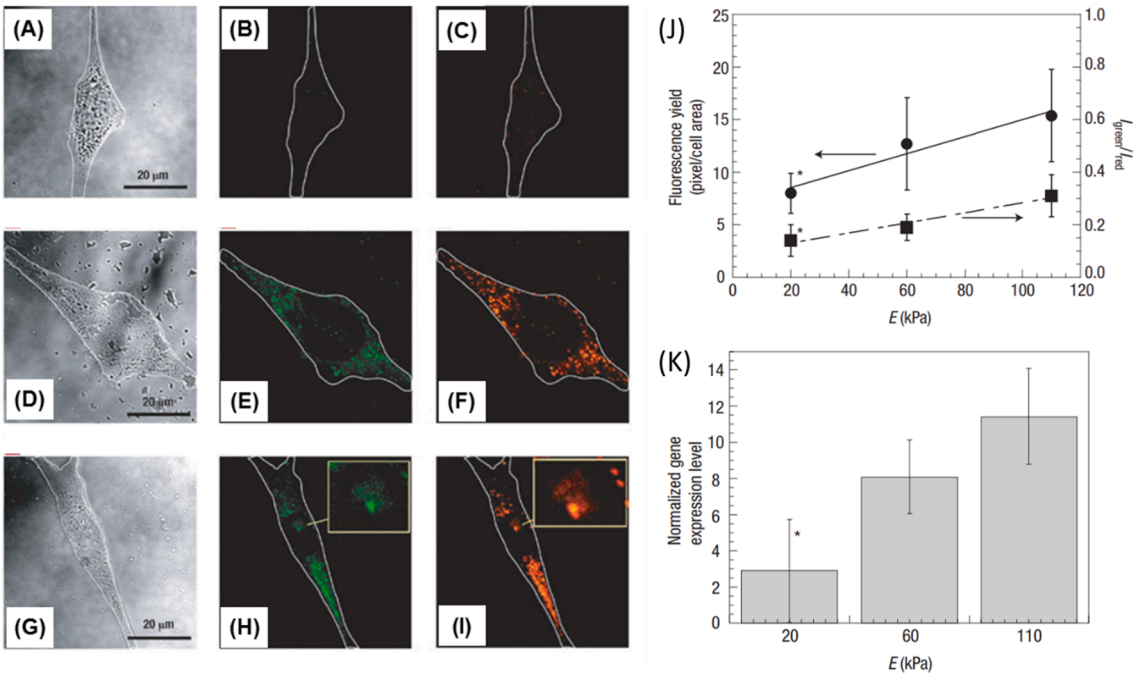



**Fig. 21.** The levels of gene transfer and expression were dependent on the stiffness of cell-adhesive hydrogels. (A-I), Increasing the elastic moduli (E) of hydrogels to which cells adhered, from 20 (A-C) to 60 (D-F) and 110 kPa (G-I)), enhanced the cellular uptake of pDNA-PEI condensates. Photos, A, D and G are bright-field images of the cells, and photos, B, E and H are fluorescent images of the same field collected through the green channel; photos, C, F and I were prepared by overlaying fluorescent images collected through the green channel with those collected through the red channel. The white line in each image represents the outline of the cells' adherent to the gels. Inserts in photos H and I are magnified images of pDNA condensates transported into the nucleus. (J) Quantification of fluorescence yield and the dissociation between pDNA and PEI (measured with $I_{green}/I_{red}$) confirmed the qualitative effects noted in images. (K) The level of expression of -galactosidase increased in proportion to (E) of the gel. Images of cells were captured following exposure to pDNA condensates for 24 h, and gene expression levels were quantified by normalizing the luminescent reading to the number of cells. In (J) and (K), the values for cells on the gels of 20 kPa (denoted by the asterisk) were statistically different ($p < 0.05$), as compared with values for cells on the gels of 60 and 110 kPa. Error bars represent standard deviation. Copyright permission obtained from Ref. [289]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[292], whereas collagen IV-mediated enhancement of gene transfer in PC12 cells was regulated by the relative projected area of the plated cells [293]. Those studies postulate the possible modulation of cellular processes by the ECM environment, which could enhance non-viral gene transfer capability. Dhaliwal *et al*. demonstrated differential cellular uptake regulation of PEI/DNA complexes in bone-marrow-derived MSCs bound on Fn or Col-coated surfaces [294]. They found that Fn promoted CME of the complexes, whereas Col I led to cellular internalization through CvME, suggesting that the cellular microenvironment can be engineered with ECM proteins to regulate the gene transfer mechanism of the polymeric gene carrier. Recent studies also indicate the interplay of the biophysical properties of ECM and the cell internalization process of nanoparticles [295-297]. The stiffness of ECM influences the assembly of the cell cytoskeleton by mechanotransduction, and therefore, affects the cellular uptake of nanoparticles [298]. Panzetta *et al*, confirmed that nanoparticles were internalized in larger amounts when they are decorated with RGD integrin ligands, which activated an internalization pathway, and when cells are cultured on stiffer substrates promoting the formation of a more structured cytoskeleton [296]. These evidences suggest the ECM stiffness as additional parameter to be considered.

### 5.3.2. Nature of cells

Cellular uptake mechanisms also depend on cell type. The uptake of human transferrin (a specific marker for CME) by D407 (retinal pigment epithelial cell) and HuH-7 (human hepatocellular carcinoma cell) cells was inhibited about 50% in the presence of chlorpromazine, which showed little or no inhibitory effect in ARPE-19 (retinal pigment epithelial cell), Vero (African green monkey kidney epithelial cell), and COS-7 (African green monkey kidney fibroblast cell) cells. Similarly, the presence of potassium depletion buffer had no significant effect on the uptake of human transferrin in COS-7, ARPE-19, or Vero cells; however, in D407 and HuH-7 cells, cellular uptake decreased by $>$ 80%. Cell-type-dependent endocytosis mechanisms were also pronounced when using CIE inhibitors such as genistein and methyl-β-cyclodextrin in the above cell lines [124]. HepG2 cells (human hepatocellular liver carcinoma cell) provided lower transfection efficiency after transfection with PEI/DNA polyplexes, most of the internalized DNA degraded at the intracellular compartments. On the other hand, 293 cells (transformed human embryonic kidney cell) exhibited high transfection activity and no intracellular degradation [299]. The differences occurred because the HepG2 cells could not internalize the polyplexes via CvME because they lack endogenous caveolin [300]; thus, they facilitated CME, which guided the polyplexes to the lysosomal compartment for degradation.

Transfection efficiency and cellular uptake can also be affected by the cell cycle phases. The G1 is a cell cycle phase in which cells mostly remain in a resting state; thus, it is less effective for cell transfection. However, PEI-based polyplexes can transfect cells in the G1 phase, although their transfection ability varied according to the type of PEI. The transfection efficiency of branched PEI-based polyplexes was several log-units (2 or $>$ 3 log-units) higher in the S/G2 phase than in the G1 phase in HeLa cells, although the transfection was not significantly affected when using linear PEI-based polyplexes in both the G1 and S/G2 phases [301]. Moreover, Mannisto *et al*. reported that cellular uptake of branched PEI and PLL-based polyplexes by synchronized D407 cells was higher at the S phase (80 to 90%) than at the G1 phase (5 to 30%), and the uptake of branched PEI was higher than that of PLL [302]. After the delivery of pDNA using a branched PEI system, the cytoplasmic pDNA content was similar in each cell cycle phase. Interestingly, however, the number of pDNA per nucleus varied significantly: ~8 at G1, ~32 at S, and ~170 at G2/M. This result suggests that nuclear envelope disruption during cell division significantly contributed to nuclear localization of the pDNA, allowing the cytosol-distributed pDNA to be engulfed by the nucleus at the time of its re-formation [302]. Because polyplexes can be taken up by various endocytosis mechanisms, it will be interesting to determine cell-cycle phase-dependent uptake pathways because it has been shown that the expression of Cav-1 was significantly higher at the G0 phase than after the S phase [303].

### 5.3.3. Cellular polarization

The cellular uptake of polyplexes and their transfection ability differ greatly between polarized and non-polarized cells due to their unique features. Polyplexes in the circulation can be taken up by the apical side membrane of polarized cells, whereas non-polarized cells do not contain this membrane compartmentalization, suggesting that the cellular interactions, endocytosis process, and intracellular trafficking of polyplexes depend on cell polarization [304]. Previously, a comparative study of polarized and non-polarized cells investigated the uptake process and transfection efficiency of PEI polyplexes and histidylated (His) PLL polyplexes [305]. PEI polyplexes were more transfection efficient than His polyplexes in both polarized and non-polarized cells. The uptake mechanism that the PEI polyplexes mainly used was the CME endocytosis pathway, regardless of the cell polarization state. The uptake of His polyplexes mainly occurred via CME, with the additional involvement of a cholesterol-dependent route in non-polarized cells, whereas in polarized cells their endocytosis mechanism was predominantly affected by CIE pathways [305]. This finding suggests that cellular polarization affects the regulation of cellular uptake, and those pathways also depend on the characteristics of polymeric materials that compose the polyplexes.

### 5.3.4. Specific ligands

Understanding of the regulation of cellular processes has progressed considerably with the advancement of techniques and mechanisms that work at the molecular level. Substantial details about the key features of cellular uptake regulation are now understood in this regard, such as the involvement and importance of small-molecule ligands in this regulatory function [306]. The regulatory functional mode of cellular uptake pathways has also been explored and delineated to promote the success of gene delivery, with particular gains in understanding cell targeting, cellular uptake, and the process of intracellular gene delivery [307]. The cell-specific targeting ligands useful to tag with gene carriers include antibiotics, sugar molecules, vitamins, hormones, and other small molecules, all of which have been popularly used to confer cellular specificity [308-311].

Specific interactions between cellular receptors and their cognitive ligands generally improve the cellular uptake efficiency of gene

**Table 3**
The advantages and limitations of physical, geometrical, chemical, and biological cues that affect the cellular uptake regulation of polymeric gene carriers.

| Factors affecting regulation of cellular uptake | Polymeric materials | Advantages | Limitations | References |
|---|---|---|---|---|
| Physical cues | | | | |
| Osmotic pressure | -Polyester-amine<br>-Polysorbitol<br>-Polymanitol<br>-Polylactitol-Poly (sorbitol-co-PEI)-Poly (mannitol-co-PEI)-Complex of copolymers of poly (ethylene glycol), poly((2-dimethyl amino)ethyl methacrylate) and poly (2-hydroxyethyl methacrylate) | -Enhances cellular uptake process<br>-Specific to CvME uptake mechanism by downregulating CME and other CIE<br>-Avoids lysosomal fusion by regulating the cellular uptake mechanism toward CvME | - Including and optimizing osmotic segments in copolymer is highly challenging | [62,90,181-200] |
| Rigidity | -PLGA<br>-PVA | -Enhances the cellular uptake of nanoparticles | -Requires extensive studies to determine uptake specificity | [201,202,204-206] |
| Flow shear stress | -PEI,<br>-Histidylated polylysine<br>-Polystyrene<br>-PLGA<br>-PEG | -Increases the formation of actin-based cytoskeletal structures, which is associated with nanoparticle endocytosis<br>-Related to caveolae signaling | -Requires detailed research into molecular mechanism of flow effect on endocytosis pattern to fine-tune intracellular delivery | [78,207-210,214-219] |
| Ultrasound | -Chitosan-tripolyphosphate | -Distinctly regulates CME and fluid-phase endocytosis | -Increased microbubble concentration poses a risk of embolism | [220-238] |
| Geometrical cues | | | | |
| Particle size | -Polystyrene<br>-Alginate | -Size-dependent internalization of particles determines the use of the CME or CvME pathway | -Requires extensive studies to determine uptake specificity | [247,250,337-339] |
| Particle shape | -poly((2-dimethylamino)ethyl methacrylate) (PDMAEMA)<br>-PEI<br>-Polystyrene | -Accelerated internalization of rod-shaped particles, depending on the cell type | -Controlling particle shape is difficult | [240,242,243,245-250] |
| Chemical cues | | | | |
| Surface charge and pH-sensitivity | -PEI<br>-Chitosan<br>-PEG-PLA<br>-Polyaspartamide<br>-PAMAM | -Improved internalization via ionic interaction of positively charged particles with plasma membrane and nucleus<br>-Exploits pH conditions for cellular uptake regulation | -Strong positive surface charge leads to apoptosis via membrane potential perturbation | [49,251-264] |

(*continued on next page*)

**Table 3** (*continued*)

| Factors affecting regulation of cellular uptake | Polymeric materials | Advantages | Limitations | References |
|---|---|---|---|---|
| | -PVPBr<br>-DADE-dextran<br>-DADMAC<br>-PLL<br>-CMCS-poly (L-histidine) coated poly (beta-amino ester)<br>-Methacrylic acid copolymers<br>-Polyspermine<br>-Imidazole-4,5-imine | | | |
| Surface hydrophobicity/ hydrophilicity ratio | - PEGylated polylactide<br>-β-cyclodextrin-crosslinked PEI | -Orderly amphipathic nanoparticles can slip through the plasma membrane without disruption | -The mechanism by which amphipathic particles are internalized remains to be elucidated | [152,265-270,272,273] |
| Cell-penetrating peptides | -Glycosaminoglycans | -Highly efficient internalization into cells | -Susceptible to protease degradation<br>–Non-specific uptake mechanism | [280,285-288] |
| Biological cues | | | | |
| Extracellular matrix proteins | -PEI | -Can regulate cellular uptake route (i.e., fibronectin promotes CME, whereas collagen controls CvME) | –Non-specific uptake mechanism | [292-297] |
| Nature of cells | -PEI<br>-PLL | -Can regulate uptake mechanism | -Difficult to control specific cell-cycle phases in a particular cell type | [124,299-303] |
| Cellular polarization | -PEI<br>-polylysine | -Can regulate cellular uptake pathways by modulating cell polarization state | - Non-specific uptake mechanism | [304,305] |
| Specific ligands | -Galactosylated -polyethylenimine-graft-poly (vinyl pyrrolidone)<br>-Mannosylated chitosan-graft-polyethyleniminepolylysine-Folate conjugated poly (ester amine)<br>(FP-PEA)-Folate-Poly(ethylene glycol)-Poly (L-lysine) | -Have specific cell and tissue targetability-Most ligands (i.e., transferrin) follow CME, but others (i.e., folic acid and cholera toxin) use CvME route | - Selective biomarkers for targeting diseased organs and diseases which guides preferred endocytosis pathway must be identified. | [102,133,200,306-327] |

carriers; commonly, the mechanism is called receptor-mediated endocytosis. Therefore, targeted gene delivery using specific ligands can significantly increase therapeutic efficacy at a much lower dose [312]. At present, many ligand/receptor-mediated non-viral gene delivery systems demonstrate gene transfer with high specificity better and more persuasively than viral vectors. Galactose moieties, as specific ligands targeting the asialoglycoprotein receptors of hepatocytes, provide specific gene transfection to liver-parenchymal cells [200,313,314]. Mannose confers mannose-receptor-mediated gene transfection in macrophages that express those receptors on the surface [315,316]. Several researchers used transferrin, an iron-binding glycoprotein, as a tumor-targeting ligand because rapidly dividing cells over-express transferrin receptors due to their cellular need for iron [317], although that could be problematic for newly divided normal cells. Folic acid can be used to target folate receptors over-expressed on many other tumor cell surfaces [309]. In all ligand-receptor-interaction-mediated targeting, the surface receptors of the cells allow them to respond to their ligands and exhibit responses for targeted gene delivery. Once ligand-receptor binding occurs, a conformational change within the receptors leads them to form vesicles and bud off from the inside of the cell surface to the cytosol. The resulting vesicles carry the content to the early endosome where the ligands and their receptors can be dissociated. The vesicles can be recycled to the cell surface or sent out to lysosomes, leading to the acidification of the endosomal network and eventual degradation of endosomal content [312].

Although, as previously mentioned, many ligands enter cells through CME and CIE, they can also use ligand-receptor-mediated endocytosis. Folic acid and transferrin differ in their cellular uptake mechanisms, resulting in distinct intracellular fates. Transferrin is taken up by the CME pathway, involving endosomal acidification followed by trafficking to lysosomes [318]. On the contrary, folic acid internalizes via CvME and avoids trafficking to lysosomes [133,319]. Parton *et al.* reported that the cholera toxin binding subunit takes the CvME pathway and is transported toward Golgi complexes via the early endosome [320]. Furthermore, the internalization of IL-2 into lymphocytes was partially inhibited using a CME pathway inhibitor, suggesting CIE for efficient targeting of IL-2 receptors and their internalization [321]. Thus, it is clear that the uptake can be modified and regulated based on the purpose and uses of these ligands. Although it is a promising approach for targeted gene delivery, clathrin-dependent internalization significantly limits transfection activity because of poor intracellular trafficking. Therefore, enhancement of endosomal escape is highly desirable for ligand-based gene carriers in the clathrin pathway. The exploration of a new ligand as a gene carrier to trigger CIE, especially CvME, could be a valuable approach because not only is CvME generally unaffected by lysosomal degradation, but it helps accelerate the extravasation.

Vascular endothelial cells pose a significant problem in nanoparticle drug/gene delivery systems, acting as a barrier, preventing access to target tissue. Most systems rely on passive transport to travel across the endothelium, requiring a large concentration gradient for movement to occur [322]. Consequently, for an effective dosage of the drug to reach target cells, higher concentrations of NPs must be administered, increasing the likelihood of adverse side effects [323]. This is a major challenge to overcome; otherwise, the majority of administered drugs remain in circulation to be eliminated by the liver or kidneys. This limits the extravasation of NPs and reduces the overall therapeutic efficacy of the drug [324]. To achieve more effective targeted delivery, one approach is to target not the tumors themselves, but instead the caveolae, which can function as trafficking vesicles across the endothelium barrier. It has been shown that it is possible to target vascular endothelium caveolae to deliver therapeutics to targeted tissue. Caveolae present themselves as prime candidates for targeted drug and gene delivery to facilitate their movement across the endothelial barrier and enhance access to solid tumor tissue. A common approach to targeted delivery is the use of immunotoxins that bind antigens expressed exclusively on the surface of cancer cells. This method has been proven *in vitro* to be effective in eliminating cancer while not harming healthy cells. However, this approach does not seamlessly translate into the clinic to treat solid tumors due to the resilient endothelial barrier [325]. The endothelial barrier paired with increased interstitial pressure presents difficulties for tissue penetration of the delivered drug and consequently prevents access to diseased cells across the vascular endothelium. The endothelial barrier is a formidable hurdle to overcome and is responsible for poor accumulation of intravascularly administered tumor-specific antibodies within solid tumors [326].

Caveolae targeting for therapeutic applications of drug and gene delivery presents a promising prospect. Delivery vectors can be designed to target and bind molecules and receptors that exist exclusively in tumor endothelial caveolae. Several studies demonstrate the speed and efficacy with which caveolae transport specific antibodies across the epithelial barrier and into underlying tissue. The methods used involve fluorescence imaging, mainly through observation of the uptake of fluorophore-antibody conjugates. Oh *et al.* determined that aminopeptidase P (APP) exists in high concentrations within caveolae, more so than other proteins [102]. Thus, monoclonal antibodies to APP (mAPP) were generated to enable caveolae-specific targeting. The results showed that intravenous injection of mAPP in mice accumulated within murine lung tissue, reaching a maximum intensity in a matter of 30 s. The mAPP first rapidly bound to endothelial cells and subsequently crossed the endothelial barrier to the underlying tissue. Moreover, fluorescence imaging showed that mAPP did not enter any surrounding tissue. Within 1 min of injection, it could be seen that mAPP spread all throughout lung tissue. Further, *in vivo*, real-time imaging agreed with these results, demonstrating persistent mAPP presence over 2 days. For comparison, several other antibodies, including control monoclonal antibody (mIgG), mCD34 and caveolin antibody, were tested, and none other than mAPP demonstrated rapid uptake against a concentration gradient. Even at 10 times lower dosage, mAPP displayed significantly greater fluorescence intensity than the other antibodies, which remained in circulation within blood vessels for extended periods of time. This indicated that the mechanism by which mAPP entered target tissue was not via leaky vasculature or general cell surface binding but rather specific active transcytosis. Further investigation indicated that caveolin-1 expression was necessary for active caveolae transport across the epithelium. Lentiviral silencing of caveolin in mice showed significantly less mAPP uptake, indicating 98% less mAPP transport than non-caveolin knockdown mice [102].

Another protein that Oh *et al.* discovered as a viable caveolae target was annexin A1. Antibodies specifically generated for annexin A1 (AnnA1) were used for caveolae transendothelial transport [324]. AnnA1 is found specifically in human and rodent tumor caveolae, hence it's useful as a delivery target. Similar to mAPP, intravital imaging *in vivo* demonstrated rapid, effective transendothelial

transport of fluorophore-conjugated AnnA1 from blood to tissue of mammary, prostate, and lung tumors. This discovery makes it possible to target tumor caveolae for transendothelial transport and localized accumulation in solid tumors. This manner of targeting can facilitate selective endocytosis for tissue-selective gene expression or for destroying the endothelial cells themselves. Such damage has the potential to starve tumors by instigating local intravascular clotting. Moreover, therapeutics delivered via this system have enhanced tissue penetration due to its improved transport across the endothelium barrier, improving the overall efficacy of the therapy. The advantage of endothelial-specific targeting is that endothelial cells are genetically stable, whereas cancer cells frequently mutate, having changes in protein receptor and antigen expression, allowing tumors to potentially evade targeted therapy. Taking this into consideration, variance does exist among caveolar targets within tumor endothelial cells; therefore, several different molecules should be used for targeting to improve the proportion of cancer cells affected, reducing the likelihood that heterogenic tumors evade targeted therapeutics. The challenges encountered with the implementation of caveolae targeting are the need for unique biomarkers that allow for exclusive targeting of diseased cells. In the aforementioned works, the rapid transcellular transport of mAPP and AnnA1 across the epithelial barrier into underlying lung tissue was likely as effective as it was because the lungs receive essentially all cardiac output. In addition to this, the endothelial cell to surface area ratio is considerable in the lungs, contributing to the effectiveness described [327]. For this reason, the uptake speed and efficacy of mAPP and AnnA1 seen in the lungs may not be possible in other organs.

To fully take advantage of CvME for drug and gene delivery, caveolae biomarkers for selected organs and diseases must first be identified. One optimistic prospect for caveolae targeting is the design of delivery vectors that can target and bind tumor endothelium caveolae in addition to tumor cell surface antigens. This approach would allow delivery vectors to selectively bind to tumor caveolae, move across the endothelium barrier, and proceed to selectively accumulate within tumor tissue. This would result in targeted tissue dosages that far exceed the local concentrations possible with current delivery systems that rely on passive extravasation. Therapy involving caveolae pumping is an exciting approach that requires a lower administered dose, limiting toxic effects while also promising in rapid and effective transendothelial transport, improving tissue specific concentrations of the delivered therapeutic. The advantages and limitations of various physical, geometrical, chemical, and biological cues that potentially affect the cellular uptake regulation of

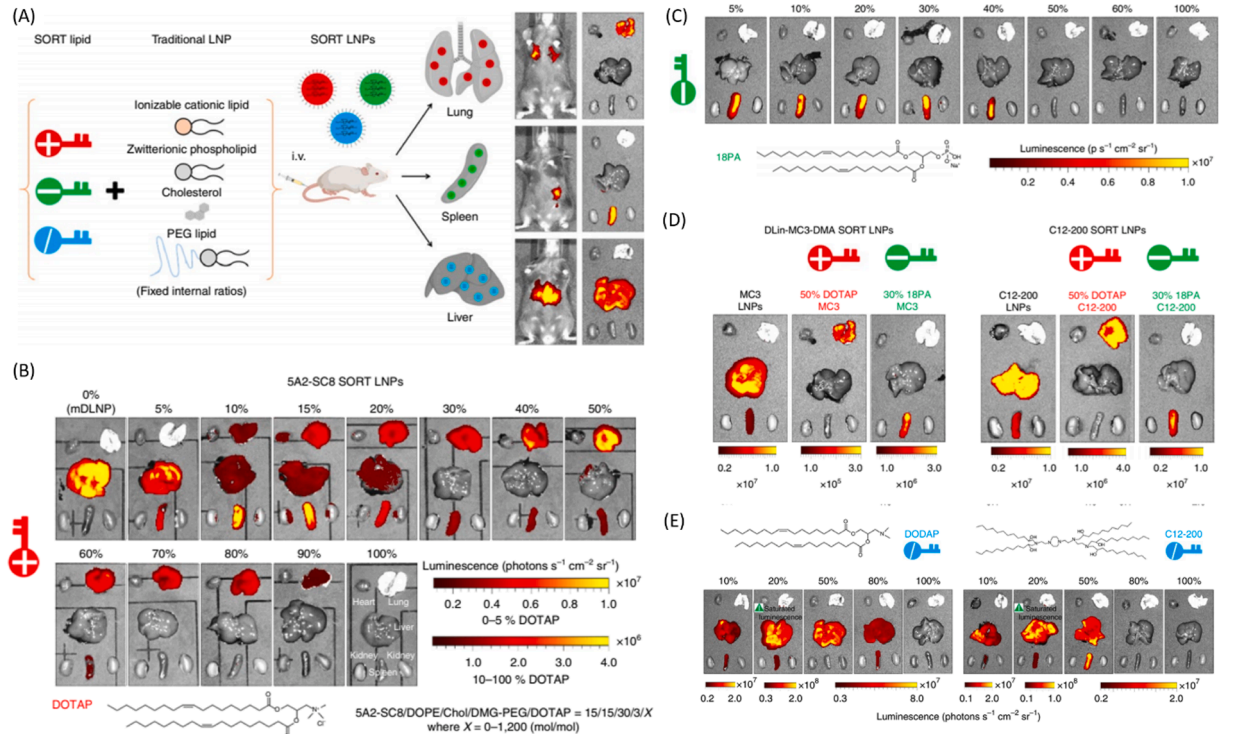



**Fig. 22.** SORT allows LNPs to be systematically and predictably engineered to accurately deliver and regulate the uptake of mRNA into specific organs. (A) Addition of a supplemental component (termed a SORT molecule) to traditional LNPs systematically alters the *in vivo* delivery profile and mediates tissue-specific delivery as a function of the percentage and biophysical property of the SORT molecule. This methodology successfully redirected multiple classes of nanoparticles. (B) 5A2-SC8 SORT LNPs were formulated as indicated to produce a series of LNPs with 0% to 100% SORT lipid (fraction of total lipids). Here, inclusion of a permanently cationic lipid (DOTAP) systematically shifted luciferase protein expression, from liver to spleen to lung, as a function of DOTAP percentage. (C) Inclusion of an anionic SORT molecule enabled selective mRNA delivery to the spleen. Luciferase expression was only observed in the spleen when 18PA lipid was introduced into mDLNPs up to 40%. (D) Ex vivo images of luminescence in major organs of DLin-MC3-DMA SORT LNPs and C12-200 SORT LNPs (0.1 Luciferase mRNA mg $kg^{-1}$, i.v., 6 h). (E) Ionizable cationic SORT lipids with tertiary amino groups (DODAP, C12-200) enhanced liver delivery without luciferase expression in the lungs (0.1 mg $kg^{-1}$, 6 h). Copyright permission obtained from Ref. [391].

polymeric gene carriers are summarized in Table 3.

#### 5.3.5. Cytoplasmic trafficking and nuclear entry

Last but not least, to significantly increase the efficiency of non-viral vectors, it is important to understand how pDNA induces transgene expression and is transported from outside the cell to the nucleus [328]. pDNA, naked or encapsulated into a non-viral vector, must go through a series of barriers such as plasma membrane, cytoplasm, and nuclear envelope [329]. Despite the fact that naked DNA can reach the nucleus from the cytosol through the nuclear membrane [330], it is still challenging for naked pDNA molecules to cross the nuclear membrane without being degraded by the cytoplasmatic nucleases, resulting in reduced transfection efficiency [331,332]. Pollard *et al.* demonstrated that $< 1\%$ of pDNA reached the nucleus when injected directly into the cytoplasm [333]. Therefore, cytoplasmic trafficking across the nuclear envelope is critical for the success of non-viral vectors in gene delivery. Three different routes for pDNA entries into the nucleus have been proposed: a) transfection during cell division such as during the mitosis process when the nuclear envelope breakdown takes place; b) nucleus entry via nuclear pores; and c) diffusion across the nuclear membrane by means of karyophilic proteins used as transfer carriers [328,334-336].

### 5.4. Other miscellaneous gene delivery materials that control cellular uptake

The cellular uptake mechanism of lipoplexes was investigated extensively to improve the transfection capacity by controlling their intracellular fate. In early studies, release of genes from the lipoplexes was expected to occur through direct fusion with plasma membranes [340]. On the other hand, the involvement of energy-dependent endocytosis during uptake has also been observed. Thus, both membrane fusion and endocytosis are involved in the entry of lipoplexes into cells. The effects of a fusion inhibitor (Z-Phe-Phe-Gly) mixed with endocytosis inhibitors (antimycin A, NaF, and sodium azide) on the transfection efficiency and cellular trafficking of O,O'-ditetradecanoyl-N-(alpha-trimethylammonioacetyl)diethanolamine chloride (DC6-14)/DNA lipoplexes were examined. The study demonstrated that fusion process was necessary for the internalization of large lipoplexes before endocytosis but not at the time of intracellular trafficking [341]. Furthermore, the fusion process involved in lipoplex entry was partially dependent on the cholesterol-content of the lipoplexes [342].

The dominant factors regulating the endocytosis of lipoplexes are the chemical composition, particle size, and cell types. Some studies suggest that CME is the preferential entry for most efficient lipoplex formulations [343-345]. The endocytosis pathway between PEI/DNA polyplexes and N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP)/DNA lipoplexes has been examined. The cellular uptake and transfection capacity of DOTAP/DNA lipoplexes, in contrast to PEI polyplexes, were inhibited by treatment with chlorpromazine and potassium depletion agent, indicating involvement of the CME pathway [343,344].

Macropinocytosis is a major pathway responsible for the internalization of DOTAP-dioleoylphosphocholine and 3β-N-(N,N-dimethylaminoethane)-carbamoyl (DC)-cholesterol (Chol)-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) lipoplexes [346]. Recently, the transfection efficiency of Lipofectamine 2000 was found to be significantly inhibited by cytochalasin D in immature dendritic cells, suggesting preferential use of macropinocytosis for cellular entry [347]. Interestingly, the uptake of lipoplexes was inhibited by methyl-β-cyclodextrin, which correlates their internalization, at least partially, with the lipid-raft compartment and CME [347]. Moreover, lipoplex-mediated transfection increased with higher molar fractions of cholesterol in lipid formulation, mediated by cholesterol–cholesterol interactions on the plasma membrane.

Particle size is a critical factor in the efficient transfection of lipoplexes. Sakurai *et al.* evaluated the effective size of N-(1–2,3-dioleyloxypropyl)-N,N,N-trimethylammonium/dioleoylphosphatidylethanolamine (DOTMA/DOPE) lipoplexes for transfection. They found that the largest lipoplexes (1000 nm) transfected most efficiently in caveolae-enriched HUVEC cells [348], suggesting that non-degradative CvME might be involved in the transfection of large lipoplexes [349]. The intracellular trafficking of lipoplexes is also of great interest because lysosomal trafficking and the degradation pathway are the key rate-limiting steps in gene delivery efficiency [350]. Some researchers reported the mechanism for the endosomal escape of lipoplexes to be destabilization of the endosomal membrane by the formation of a hexagonal conformation, but they did not determine the fraction of escaped lipoplexes [351]. It has been argued that about 70% of dissociated siRNA from late endosomes or multivesicular late endosomes is exocytosed through endocytic recycling [352]. On the other hand, Gilleron *et al.* found that only a minor fraction (1–2%) of endocytosed lipoplexes escaped from endosomes [353]. Those two recent reports suggest the importance of endosomal escape to gene delivery by lipoplexes and highlight the opportunity to enhance transfection efficiency by optimizing the chemical composition of lipoplexes, focusing on endosomal destabilization.

The mechanism of cellular uptake for inorganic platforms, such as metallic nanoparticles, carbon nanotubes, and mesoporous silica nanoparticles, as potent gene carriers has also been investigated. Among the determinant factors in the uptake mechanism of inorganic carriers, physiochemical characteristics such as size, shape, and surface charge are of major importance. It has been confirmed that the quantitative uptake of gold nanoparticles depends considerably on size and shape [245,246]. Once inside the cells, endosomal escape or direct penetration of the endosomal vesicle is required to deliver genes to target sites [354]. The chemical modification of gold nanoparticles, such as by conjugation with membrane penetration peptides (CPPs) or the use of liposomes, was useful for successful intracellular delivery by providing efficient escape from the endosomal membrane [355].

The cellular uptake mechanism of carbon-based nanostructures has also been documented [356,357]. The advantage of carbon nanotubes, compared to most of the polymeric delivery systems, is their capacity for energy-independent cellular entry that does not require an endosomal escape process once they are inside the cells [357,358]. Both phagocytosis and energy-independent cellular uptake pathways appear to be associated with the uptake mechanism of functionalized multi-walled carbon nanotubes. The results showed that 30–50% of them entered the cells by an energy-independent pathway, such as direct-membrane translocation, membrane

wrapping of an individual tube, and in bundles within vesicular compartments. On the other hand, 50–70% of them entered through an endocytic pathway, mostly phagocytosis [359]. More investigations of the intracellular trafficking of directly penetrated nanotubes will greatly help the advanced engineering of functionalized nanotubes.

Mesoporous silica nanoparticles of inherent biocompatibility, low toxicity, and easy surface modification have also been investigated as potential gene carriers that can regulate the uptake mechanism [360], which generally occurs by energy-dependent endocytosis. It was demonstrated that CME was a major route for their cellular entry without surface modification [361]. Therefore, control of the uptake mechanism via the surface properties of mesoporous silica nanoparticles was investigated for efficient intracellular trafficking [210-212]. Materials with a more negative charge were endocytosed via CME and had a better buffering capacity than those with a less negative charge [362]. Similarly, a strong relationship was observed between morphology and the endocytosis kinetics of mesoporous silica nanoparticles. The rate of endocytosis for spherical nanoparticles was faster than that for rod-shaped nanoparticles, which was attributed to different aggregation abilities and the surface area of particles to bind to the cellular membrane [363].

## 6. Current status of polymeric delivery systems for genome editing

In recent years, gene editing technologies have enabled researchers to make permanent DNA modifications to any gene in a wide range of organisms and cells. Through genome editing technology, it is possible to add, remove, or alter genetic material at particular locations in the genome. There are three different editing nucleases currently available: Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (CRISPR-Cas9). Genome editing kicked off with the discovery of Kim et al., in 1996, that the zinc finger protein domain coupled with the FokI domain, acted as a site-specific nuclease that cut DNA [364]. Even though the ZFNs have disadvantages such as high cost and difficulty with handling, it has been used in combination with polymeric material for the purposes of gene editing.

PLGA is used to be a polymeric vector for drug delivery [365]. One of the challenges with using polymeric materials for gene editing is the encapsulation process of negatively charged payloads. This problem is also seen in PLGA-mediated nucleic acid delivery systems. This problem was overcome by using chitosan to modify PLGA, which improved the encapsulation of DNA and siRNA, demonstrating efficient delivery to various target tissues in animal models [366]. Mahiny *et al.* utilized chitosan-coated PLGA to deliver ZFN mRNA targeting the transgenic surfactant protein B (SP-B) cassette to the lungs of a mouse model with SP-B deficiency [367]. This formulation allowed successful site-specific genome editing *in vivo*, showing prevention of the decline in lung function and large-scale edema [367].

TALENs, analogous to ZFNs, have two domains: a DNA binding domain and a C-terminal FokI endonuclease cleavage domain. TALENs, are isolated from the pathogenic bacteria Xanthomonas [368]. TALENs have remarkable advantages compared to ZFNs, such as lower cytotoxicity [369] and higher targeting range [370]. However, there are no single site-specific TALENs with long arrays due to the lack of the linkage between repeats. These TALENs repeats are nearly identical in sequence. When TALENs are fused to the FokI endonuclease, TALENs bind and cleave the target DNA in pairs. Direct delivery of TALENs is not possible since the proteins cannot penetrate the cellular membrane when administered alone [371]. The role of non-viral delivery carriers for TALEN encoding plasmids is highly important given the large genomic size of the cargo. TALEN plasmids complexed with cationic polymers provided a positive therapeutic result *in vivo* in a study led by Hu *et al.* [372]. The researchers used a commercial mix of cationic polymers known as TurboFect® to encapsulate TALEN plasmids to target human papillomavirus (HPV). The system was injected into the cervix of transgenic mice displaying HPV infection and cervical cancer. The therapy successfully reduced viral loads and tumor size. Off-target mutations were not detected, and there were no signs of inflammatory responses [372].

The latest genome editing system is the CRISPR-Cas system. Its RNA-based bacterial defense mechanisms are designed to recognize and eliminate foreign DNA from invading bacteriophages and plasmids. They consist of a Cas endonuclease that is directed to cleave a target sequence by guide RNA (gRNA). To date, the most widely used system is the wide-type CRISPR system in Streptococcus pyogenes (SpCas9). In 2020, Nobel Prize in Chemistry awarded to scientists who discovered CRISPR-associated protein 9 (Cas9) and pioneered the revolutionary gene-editing technology. CRISPR–Cas9 systems edit the genome using the Cas9 protein for all target sequences, whereby Cas9 is accurately guided by a single guide RNA (gRNA) via base pairing to the target sequence [373]. This system facilitated genome engineering, for the genetic modification of bacteria, plants and animals, and opened the prospect of direct gene correction therapy.

Efficient and safe *in vivo* genome editing relies on the spatially and temporally controlled delivery of the gene editing machinery into the nuclei of the target cells. Viral-based delivery systems have been suggested to deliver the CRISPR-Cas9 machinery [374-376], however, there are several drawbacks. Persistent expression of CRISPR-Cas9 in target cells may induce anti-Cas9 immune responses [377], and furthermore, viral vectors (AAV) themselves were shown to have severe toxicity to non-human primates [378] implying that the host response to the viral vector system is difficult to predict. Therefore, polymeric CRISPR/Cas delivery systems have received increasing attention because they can be easily tailored for delivering different forms of the CRISPR-Cas9 system. Cas9 nuclease can be delivered as DNA, mRNA or protein form [379]. Using polymeric gene carriers, Cas9 protein or Cas9-gRNA ribonucleoprotein, Cas9 mRNA with gRNA and plasmid DNA encoding Cas9 can be delivered into the cells [379]. They have shown promising gene editing efficiency *in vitro* [380-385], however, *in vivo* delivery of CRISPR/Cas systems is still nontrivial because of the physiological barriers [386,387]. A recent study used hierarchical nanocomposites with NPs featuring arginine headgroups for delivery of E-tagged Cas9 protein and sgRNA. The results of the study showed $>$ 8% gene editing efficiency in macrophages of the liver and spleen [388]. In another study, Cas9 protein and gRNA embedded in PEI hydrogel were encapsulated by lipid membranes, and further decorated with CPPs to enhance the tumor delivery efficiency *in vivo*. The NPs reached 30% of tumor cells of xenograft model at 3 days after tail-vein injections, in addition, when CRISPR/Cas9 targeting polo-like kinase1 (PLK1) was delivered, PLK1 expressions were inhibited $>$ 60% and survival of tumor-bearing mouse was improved [389]. Lao *et al.*, suggested self-assembled micelles, composed of quaternary

ammonium-terminated poly(propylene oxide) (PPO-$NMe_3$) and amphiphilic Pluronic F27 for delivery of plasmid encoding Cas9 and gRNA against human papillomavirus18-E7 to inhibit the downstream cancerous activity [390]. In a mouse model using xenograft HeLa tumors, the optimized micelles resulted in reduced tumor growth by 60% compared to the negative control group [390]. Despite some initial positive data using polymeric carriers, the target tissues have been limited to liver and tumors so far, where gene delivery efficiency is relatively high because of the highly permeable vessels. Interestingly, in a recent report, Siegwart and colleague developed selective organ targeting (SORT) lipid NPs for tissue-specific mRNA delivery and CRISPR-Cas gene editing by only tuning the percentage and charge of the lipid composition in the NPs with defined biophysical properties [391] (Fig. 22). To improve the tissue specificity and gene editing efficiency for clinical application of CRISPR/Cas9, the polymeric delivery vehicles should be designed to overcome substantial extracellular and intracellular barriers. In particular, the regulation of intracellular trafficking of the nanoparticles utilizing geometrical, chemical, and biological cues will enhance the CRISPR/Cas9 activity in target organs.

## 7. Conclusion

Improving transfection or target gene silencing capabilities with minimal side-effects are the most essential tasks for successful application of polymeric carriers to use as non-viral gene delivery system. The efficiency of gene transfection largely depends on the mode of cellular uptake of the gene carriers. Endocytosis is a general process of cellular uptake, and different endocytosis pathways vary in their mechanisms. Gene delivery vectors, including polymeric carriers, use various endocytic routes into the endosome, with some pathways leading to fusion with lysosomes followed by enzymatic degradation, which could significantly reduce gene expression or silencing effects. Therefore, development of an improved gene transporter is a priority demand to efficiently regulate cellular uptake and the intracellular fate of delivered genetic materials. We have provided a comprehensive summary of physicochemical and biological cues that can control cellular internalization routes and the intracellular fate of polymeric gene carriers and improve therapeutic efficacy. Developing more potent polymeric carriers will require an in-depth understanding of all these essential cues because the internalization of particles follows a combination of the dynamics described in various parts of this article. Understanding these essential cues and concerns to improve cellular uptake and the intracellular functionality of genes will allow polymeric gene carriers not only to create new problem-solving solutions but also to open up exciting possibilities for future biomedical applications, such as targeting sub-cellular organelles (endoplasmic reticulum, mitochondria, and nucleus). Although this field still encounters challenges to developing more effective polymeric materials and associated gene carriers, several promising reports shed light on advances in gene delivery technology. In conclusion, we anticipate that advanced strategies for the critical investigation and analysis of various cues that regulate polymeric gene delivery pathways and intracellular targeting will produce potent nanomedicines for useful clinical applications in the near future.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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