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Cellular Uptake Mechanism of Non-Viral Gene Delivery and Means for Improving Transfection Efficiency

Shengnan Xiang and Xiaoling Zhang

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1. Introduction

Non-viral delivery systems usually include mechanical, electrical, and chemical methods. Cationic liposomes and cationic polymers are two typical classes of non-viral vectors. Compared with viral vectors, non-viral ones are considered promising vehicles for gene therapy because of their low toxicity, biocompatibility, and controllability [1, 2], although their low efficacy limits their application as a mature gene delivery system. Improving the efficacy of non-viral vectors necessitates thorough understanding of their *in vivo* key steps. Non-viral vectors can complex with gene materials and help them access the target compartments within cells. Many barriers prevent gene materials from reaching their intended target and performing their functions [3], safe and effective delivery remains an important challenge for the clinical development of non-viral vectors [4].

The delivery of pDNA or siRNA *in vivo* for therapeutic aims has been widely studied in recent years. However, non-viral delivery systems, which exhibit relatively low levels of efficiency, are not clinically applicable. Improving their efficiency is the main task of pDNA- or siRNA-based gene therapy. There are many barriers that hinder pDNA and siRNA from reaching their intended target in the plasma and performing their functions: First, gene materials can be loaded into vectors. After *in vivo* administration, the vectors must be delivered to the blood vessels and should be stable in the blood; otherwise, they will be cleared by albumin because of their high surface charge and may also be uptaken by macrophages. The vectors must then pass through the epithelial tissue of the blood vessels and enter the target tissue. As it is very difficult for nanoparticles larger than 5 nm in diameter to pass through the epithelial tissue of blood vessels [5], it is crucial to study the cellular transport mechanism of epithelial cells through the caveolin-mediated endocytosis (CvME) pathway which is active in epithelial cells [6]. The distance between the extracellular matrix and target cells

is great, and many vectors will be uptaken and cleared by macrophages after they do manage to pass through the epithelial tissue of blood vessels.

Next, the vectors must attach to the cell membrane, which entails other issues altogether. First, the non-viral vectors should be able to identify specific cell types to ensure safety. They then enter cells mainly via endocytosis. Different endocytosis pathways yield different intracellular fates for vectors, which could potentially explain why the same vector differs in its transfection efficiency in various cell modes. After their entry into the cells, vectors must escape from the endosome or avoid the endo-lysosomal (endosomal and lysosomal) pathway through certain endocytosis pathways. After escaping the endosome and then entering the cytoplasm, vectors must release pDNA or siRNA and finally perform their function in the cytoplasm [5]. In addition, pDNA has to be transported into the nucleus. The key steps in non-viral delivery are shown in Figure 1.
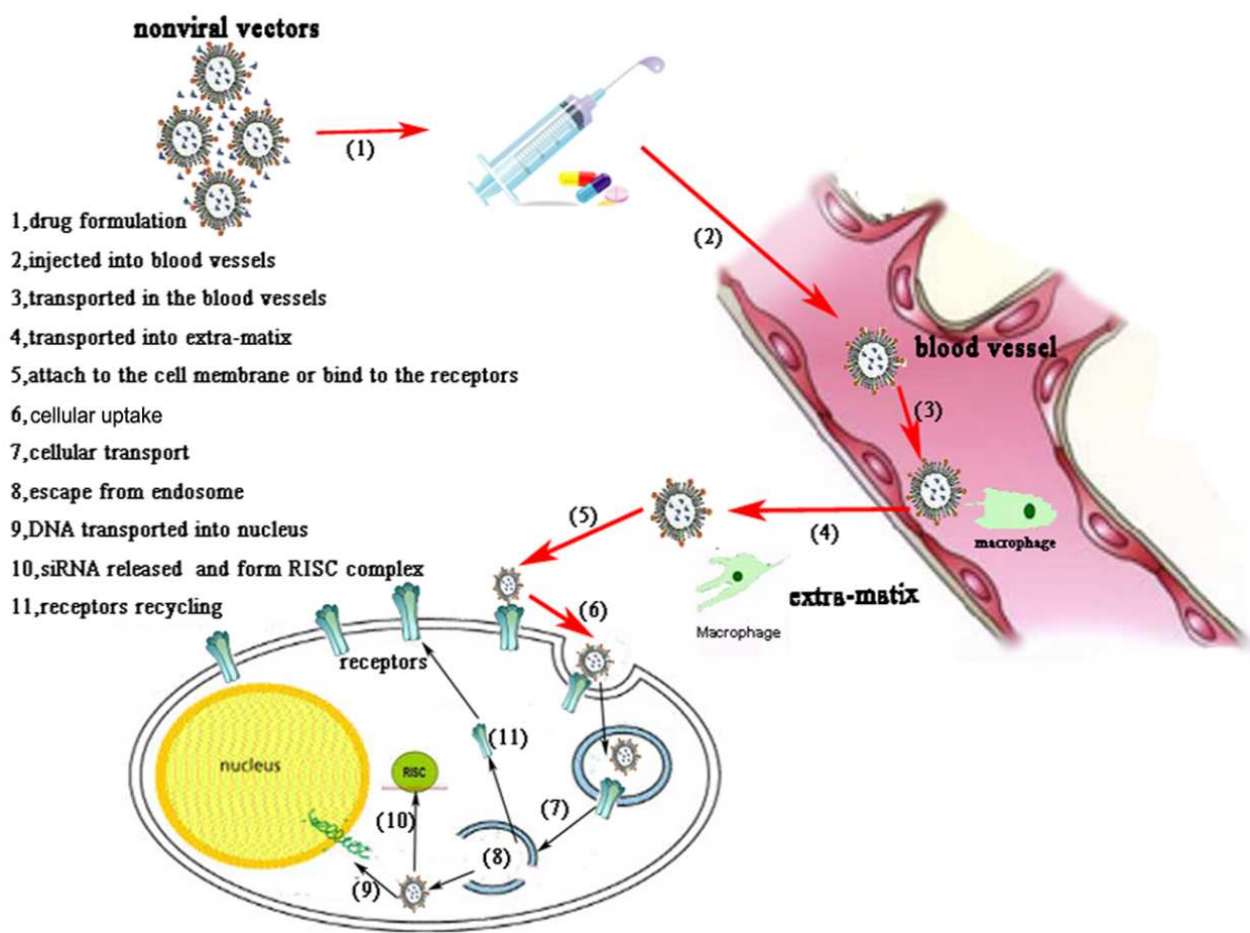

Figure 1. Biological key steps of non-viral vectors

As discussed above, the cellular process (including uptake, transport, endosomal escape, and nuclear localization) is one of the most important steps for non-viral gene delivery. In 2001, Hideyoshi Harashima et al. stated that novel strategies of medical treatments, such as

gene therapy, highlight the importance of studying the intracellular fate of macromolecules, such as DNA and siRNA. In particular, in the case of gene therapy, intracellular events would be expected to be the major factors controlling the fate of the introduced gene and the efficiency of its expression. These authors attempted to establish an intracellular pharmaco-kinetic model of genes to study the intracellular events involved in gene therapy [7]. Understanding the intracellular fate of a gene or vector is important for us to overcome the cellular barriers of DNA or siRNA delivery and rationally design efficient systems thereof.

Of all intracellular events, the cellular uptake mechanism of non-viral vectors is the most essential to their efficiency and intracellular fate. Different cellular uptake pathways have different intracellular fates. As the gene materials will be degraded in the endo-lysosomes (endosomes and lysosomes). One good example is that some endocytic pathways involve endo-lysosomes, but others that can bypass the endo-lysosomes have higher levels of delivery efficiency. Polyethylenimine (PEI) is one of the most promising non-viral vectors [8]. Some researchers have shown that cellular uptake of PEI polyplexes affects other cellular processes and, consequently, transfection efficiency [9, 10]. These differences may depend on such factors as the size, surface properties, and shape of the particles [11], as well as different cell lines [9].

Research has shown that polyplexes and lipoplexes have different uptake mechanisms in A549 pneumocytes and HeLa cells. Lipoplex uptake proceeds only by clathrin-mediated endocytosis (CME), whereas polyplexes are taken up by two mechanisms — one involving caveolae and another using clathrin-coated pits [10]. As the caveolae-mediated uptake mechanism has slower kinetics, the transfection process of polyplexes is slower than that of lipoplexes in A549 pneumocytes and HeLa cells. However, as the polyplexes uptaken via the caveolae escape the lysosomal compartment, polyplexes have a high level of transfection efficiency [10]. Taken together, these findings highlight the importance of studying the cellular uptake of non-viral vectors, their intracellular fate, and their effects on transfection efficiency. Understanding cellular uptake mechanisms is crucial to engineering successful reagents or vectors for non-viral gene transfection [12].

2. Cellular uptake pathways of non-viral gene delivery

The uptake pathways are divided into two groups: endocytic pathways and non-endocytic pathways. Inside endocytic group, there are two types of pathways: phagocytosis and non-phagocytosis pathways [11].

2.1. Phagocytosis

Phagocytosis is a special type of endocytic pathway which primarily exists in professional phagocytes such as macrophages, monocytes neutrophils, and dendritic cells (DCs) [13]. In comparison, other nonphagocytic pathways such as clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME), and macropinocytosis occur in almost all kinds of cell types [14]. Phagocytic pathway is mediated by cup-like membrane extensions that are

usually larger than 1 μm to internalize large particles such as bacteria or dead cells. Understanding of the mechanism of phagocytosis is very helpful to the non-viral gene therapy of macrophage-dominated immune diseases such as rheumatoid arthritis. In addition, a phagocytosis-like mechanism was proposed for the uptake of large lipoplexes and polyplexes that are larger than can be taken up by the classic CME pathway [15, 16].

Phagocytosis depending on opsonins can be called as opsonic phagocytosis. There is also another phagocytosis which is opsonins independent. This will be discussed later. First, for opsonic phagocytosis, the complexes will be recognized by opsonins in the bloodstream. Then, the opsonized complexes adhere to professional phagocytes and are ultimately ingested by them [11]. Opsonization is the key step of the phagocytosis pathway. It involves complexes tagged by some major opsonins including immunoglobulins G and M (IgG and IgM), as well as complement components C3, C4, and C5 in the bloodstream [11, 17]. These opsonized complexes become visible to macrophages and bind to their surface through the interaction between receptors (such as fragment crystallizable receptors (FcR) and complement receptors (CR)) and the constant fragment of particle-adsorbed immunoglobulins.

Other receptors that mediate phagocytosis pathway have also been reported. Mannose receptor (MR) has been used in gene vaccine by targeting human DCs and macrophages through the phagocytic pathway [18]. Scavenger receptor (SR)-mediated delivery of anti-sense miniexon phosphorothioate oligonucleotide to leishmania-infected macrophages is proved to be selective and efficient in eliminating the parasite [19]. SR-A, macrophage receptor, and CD36 are the three SR subtypes. CD36 can mediate non-opsonic phagocytosis of pathogenic microbes [20]. Unlike opsonic phagocytosis, non-opsonic phagocytosis is directly mediated by the receptors on the cell surface without the help of opsonins. This kind of mechanism can also be used for gene delivery.

Then the activated Rho-family GTPases trigger actin assembly and cell surface extension formation. This surface extension finally zippers up around the complexes and engulfs them [11]. The phagosomes carrying the complexes fuse with lysosomes to form mature phagolysosomes [11]. In phagolysosomes, the complexes undergo a process of acidification and enzymatic reaction. As the intracellular fate of phagocytosis is the transportation of complexes into the lysosome, the gene materials will be degraded by the nucleases inside it [21]. Endosomes and lysosomes (endo-lysosomes) are very important biological barrier for gene delivery. The vectors should have capability to escape form them, if gene materials loaded vectors entry into the cell via phagocytosis. The mechanisms of endo-lysosome escape will be discussed later.

2.2. Non- phagocytosis pathways

Non- phagocytosis pathways mainly include clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME), and macropinocytosis. CME is the best-characterized type of endocytosis, which is receptor-dependent, clathrin-mediated, and GTPase dynamin-required [22, 23]. The uptake of low-density lipoprotein and transferrin is typically via this endocytic pathway, and they are often used as the CME probes in many studies [24, 25]. Transferrin has also been used as a ligand of non-viral vectors to improve the endocytosis of

complexes [26, 27]. In this pathway, a series of downstream events are activated after the recognition of ligands by receptors on the cell surface. Clathrins assemble in the polyhedral lattice right on the cytosolic surface of the cell membrane, which helps to deform the membrane into a coated pit with a size about 100–150 nm [27]. This process is mediated by GTPase dynamin. As the clathrin lattice formation continues, the pit becomes deeply invaginated until the vesicle fission occurs. In the next step of the CME pathway, the endocytosed vesicles internalized from the plasma membrane are integrated into late endosomes and finally transported to lysosomes.

CvME begins in a special flask-shaped structure on the cell membrane called caveola, which is a kind of cholesterol- and sphingolipidrich smooth invagination [28]. CvME usually happens in the vessel wall lining monolayer of endothelial cells [7]. Caveolae have a diameter range of 50–100 nm [11] and are typically between 50 and 80 nmwith a neck of 10–50 nm [6]. CvME is also a type of cholesterol, dynamin-dependent, and receptor-mediated pathway [29]. The fission of the caveolae from the membrane is mediated by the GTPase dynamin, which locates in the neck of caveolae and then generates the cytosolic caveolar vesicle [11]. Some receptors located in caveolae, such as insulin receptors [30] and epidermal growth factor receptor (a type of receptor in ovarian cancer) [31], can mediate CvME [32]. The vesicle budding from the caveolae, a type of caveolin-1-containing endosome is called caveosome [29]. The intracellular fate of the caveosome differs from that of CME. Compared with CME, CvME is generally considered as a alternative pathway which can deliver the vectors into Golgi and/or endoplasmic reticulum, thus avoiding the normal lysosomal degradation

Macropinocytosis is a type of distinct pathway that nonspecifically takes up a large amount of fluid-phase contents through the mode called fluid-phase endocytosis (FPE) [33]. Macropinocytosis is a signal dependent process that normally occurs when macrophages or cancer cells are in response to colony-stimulating factor-1 (CSF-1), epidermal growth factor (EGF) and platelet-derived growth factor or tumor-promoting factor, such as phorbol myristate acetate respectively [34-36]. However, this process occurs constitutively in antigen-presenting cells [37]. Macropinocytosis occurs via the formation of actin-driven membrane protrusions, which is similar to phagocytosis. However, in this case, the protrusions do not zipper up the ligand-coated particle; instead, they collapse onto and fuse with the plasma membrane [11]. The macropinosomes have no apparent coat structures and are heterogenous in size, but are generally considered larger than 0.2 μm in diameter [38, 39]. During this process, the small GTPase, Ras-related in brain (Rab) proteins are essential for the vesicle fission from the cell membrane [40]. The relationship between the macropinocytosis and lysosome is still unknown. This will be discussed later.

2.3. Non-endocytic pathways

There are three technologies that are designed to mediate the non-endocytic pathways and successful transfect the gene. One is microinjection, by which each cell is injected with the gene materials using glass capillary pipettes. The second one is permeabilization by using pore-forming reagents such as streptolysin O or anionic peptides such as HA2 subunit of the influenza virus hemagglutinin. The third one is electroporation,

which uses an electric field to open pores in the cell. All of them are highly invasive and not ideal for in vivo gene delivery.

However, There are evidences which can prove the existing of other non-endocytic pathways. One pathway is related to the formation of holes in the cell membrane, called "penetration". A class of cationic peptides with the protein transduction domains (PTDs), such as TAT, has the ability to be taken up without endocytic events [41]. These peptides can directly penetrate cell membranes in a receptor-, and energy-independent way. In 2004, Hong et al. studied the hole formation on the cell membrane induced by poly(amidoamine) (PAMAM). The results indicated that the hole formation can be induced by positively charged PAMAM, and labeled PAMAM can diffuse into the cells through small holes in the membrane. This mechanism is considered a nonspecific pathway, which is not receptor-mediated and lacks selective cellular uptake [42]. In 2010, Lee et al. used a PTD called Hph-1 to conjugate vector PEI to deliver siRNA. The result showed that the complexes entered the cells through the non-endocytic pathway, which has a quicker dynamic behavior compared with the endocytosis pathways and is energy-independent because it has high transfection efficiency even in low temperature [43]. Another non-endocytic pathway is called "fusion", which is special for lipoplexes, as it can cause a direct release of DNA to the cytoplasm before entering the endocytic pathways. However, more and more evidences suggest that fusion with the cell membrane contributes minimally to the overall uptake of lipoplexes, while the CME plays an important role in the uptake of lipoplexes [44]. there have been few studies on non-endocytic pathways, and more efforts are needed to have a comprehensive understanding of these pathways for the improvement of non-viral gene delivery.

Pathways	GTPases	Relationship with lysosome	Receptors
Phagocytosis	Rho	Dependent	Dependent
CME	Dynamin	Dependent	Dependent
CvME	Dynamin	Independent	Dependent
Macropinocytosis	Rab	In dispute	Non-specific
Non-endocytic	Independent	Independent	Non-specific

Table 1. Cellular uptake mechanisms.

3. Factors that influence the uptake pathways of non-viral gene delivery

There are many factors that are involved in the selection of uptake pathways of non-viral gene complexes. These factors include particle size, particle surface charge, particle shape, cell type, and even culture condition. Because the complexes of non-viral gene vector/DNA or siRNA are usually a group of heterogeneous particles with diverse sizes, surface charges, and shapes, several uptake pathways may be involved in the internalization of one kind of

complexes into a single cell type. For example, transfection by branched PEI25kDa/DNA polyplexes was mediated by both CME and CvME pathways in HUH-7 and Hela cells [9]. Later, Hansjörg Hufnagel reported that the macropinocytosis is also very important for the uptake of branched PEI25kDa/DNA polyplexes into Hela and CHO-1 cells due to the existence of large particles of polyplexes (>500 nm) [12]. Therefore, the heterogeneity of complexes has to be taken into consideration when the results are analyzed. Particle size is a very important factor for the pathway selection of complexes. As mentioned above, the labeled cationic PAMAM can induce hole formation in the cell membrane. The holes induced by PAMAM are 15–40 nm in diameter [42]. The particle including the gene complex, which is smaller than these holes, can diffuse through the holes and be taken up by nonspecific non-endocytic rather than specific receptor mediated endocytic pathways. PEI/DNA complexes with sizes smaller than 500 nm are mainly taken up by CME and CvME according to a previous study [10]. While PEI/DNA complexes with sizes >500 nm are mainly internalized by macropinocytosis pathway [10].

The charge density of a complex is also an important factor for its uptake. The cell membrane consists of a bilayer of lipid and anionic membrane proteins. These anionic proteins are very helpful to the uptake of cationic complexes. However, once the net positive charge falls to neutral, the uptake efficiency will be inhibited a lot. This is because the neutral charge density will weaken the interaction between complexes and membrane proteins, and it will also increase the aggregation of complexes, which will make them large and hard to be internalized. This change can be caused by the anionic proteins in the in vivo circulation of blood, and the serum used in the in vitro transfection medium. The modification of polyethylene glycol (PEG) can solve this problem with its high hydrophilicity, electrical neutrality and steric-repulsive propensity [45].

As to the relationship between the shape of particles and the pathway selection, few studies have been made about this issue. A group once reported that the uptake of protein-coated spherical gold nanoparticles is more efficient than rod-shaped ones in Hela cells, SNB19 cells, and STO cells [46, 47]. However, as to the relationship of nonviral gene complexes and their uptake efficiency, it is not easy to draw such a conclusion, because the non-viral gene complexes are usually a group of nanoparticles with heterogeneous shapes, and their shapes are dependent on the experimental conditions. Taking chitosan as an example, the fraction of complexes that have nonaggregated, globular structures increases with increasing chain length of the chitosan oligomer, increasing charge ratio and reduction of pH (from 6.5 to 3.5) [48]. Because of this, this complicated issue leaves much room for researchers to discuss.

Cell type is another important factor that influences the pathway selection of non-viral gene complexes. Different types of cells can take up a kind of complex in different pathways. Most of the studies focused on COS-7 cells, which were used as a well-established model cell for gene delivery researches [28]. Some researchers also used other cell lines such as A549, Hela, and HUH-7 cells. Caveolae, which are a very important structure for CvME pathway, are present in many cell types, but they are particularly abundant in the vessel wall lining monolayer of endothelial cells. As a result, endothelial cells have been especially used in studies on CvME pathway. A study tested the endocytosis pathways involved in the

transfection of PEI/DNA complexes with different cell lines. The result showed that in COS-7 cells, the clathrin-dependent pathway was the main contributor to the transfection process for both linear and branch PEIs [9]. Another study suggests that macropinosomes have a higher propensity to deliver PEI/DNA cargo than do endosomes in CHO and Hela cells [12]. Therefore, different cell lines involve different endocytic pathways, and cell type is the important factor that must be considered in such studies.

4. Tools for the study of uptake pathways

The study on the mechanisms of uptake pathways is important to the rational design of non-viral gene vectors because this step can determine the intracellular fate of complexes. However, because there are many factors that influence the pathway selection, how to conduct these studies is also a very complicated problem that needs to be discussed in detail.

Inhibitors are the effective tools to block specific pathway in order to determine whether it plays an important role in the uptake of complexes. However, none of the commonly used inhibitors of different uptake pathways is absolutely specific. All of them either affect the actin cytoskeleton with their side effects, or interfere with alternative uptake pathways simultaneously. In addition, they usually show cell type variations. The scope of the usage of commonly used inhibitors will be introduced according to the classification of uptake pathways in the following paragraphs of this section. The most direct way to distinguish endocytic pathways and non-endocytic pathways is to use the inhibitor or method of energy depletion, because most endocytic pathways are energy dependent. The commonly used inhibitors and methods are: low temperature (4 °C) and sodium azide (an ATPase inhibitor). Low temperature and ATP inhibitor should be used together in some conditions because some of the non-endocytic pathways are also sensitive to low temperature [42, 49].

To distinguish the phagocytic and macropinocytic pathways with CME and CvME pathways, the commonly used inhibitors and methods for phagocytic and macropinocytic pathways are: inhibitors of sodium-proton exchange "amiloride and its derivatives", F-actindepolymerizing drugs "cytochalasin D and latrunculins", inhibitors of phosphoinositide metabolism "wortmannin and LY290042", and protein kinase C activator "phorbol esters". Except phorbol esters, the specificity of all the inhibitors is still in doubt as depolymerizing F-actin and inhibition of phosphoinositide metabolism may also disrupt the other two endocytic pathways. For example, cytochalasin D is also used as the inhibitor for CvME [50]. Within these inhibitors, amiloride and its derivatives may be considered as the first choice for their fewest side effects. Rottlerin, a novel macropinocytosis inhibitor which is rapid acting, irreversible, and selective, was discovered in 2005. In 2009, Hufnagel et al. found that rottlerin can specifically inhibit the transfection efficiency of PEI (25 kDa)/DNA complexes on Hela and CHO-K1 cells up to 50%, which verified the important role of FPE in the non-viral gene delivery by PEI (25 kDa) [12].

The commonly used inhibitors and methods for clathrin-mediated endocytosis are: Hypertonic sucrose (0.4–0.5 M), potassium depletion, cytosolic acidification, chlorpromazine (50–

100 μM), monodansylcadaverine (MDC), phenylarsine oxide. However, all of them have been shown to be able to inhibit macropinocytosis, thus cannot be used to distinguish the clathrin-mediated endocytic pathway and the macropinocytic pathway. Besides this, all these inhibitors can influence the cortical actin cytoskeleton more or less, which can cause non-specific cytotoxicity. However, potassium depletion, chlorpromazine, and MDC are the relatively better choices than the other ones for the initial discrimination of clathrin-mediated endocytic pathway [51].

As to caveolae-mediated endocytic pathway, the commonly used inhibitors and methods are: statins, methyl-β-cyclodextrin (MβCD), filipin, nystatin, genestein, and cholesterol oxidase. Among them, the incubation with filipin, nystatin, and cholesterol oxidase produce the fewest side effects. The chronic inhibition of cholesterol synthesis by statins or acute cholesterol depletion by MβCD nonspecifically disrupts intracellular vesicle trafficking and the actin cytoskeleton. Also, the specificity of genestein is still in doubt for its nonspecific disruption of the actin network. That being so, appropriate controls should be included when filipin, nystatin, and cholesterol oxidase are used [51].

The inhibitors for the study of intracellular fates of complexes are also very important. Monensin, bafilomycin A can inhibit the acidification of endosomes, thus preventing their maturation and fusion into lysosomes [52, 53]. Chloroquine is another inhibitor that accumulates in endosomes/lysosomes and causes the swelling and disruption of endocytic vesicles by osmotic effects [21]. Last but not least, the cell-dependence of inhibitors should be noted when experiments are carried out. For example, chlorpromazine treatment inhibited the uptake of transferrin, a marker for CME by ~50% in D407 and HUH-7 cells. However, it showed no or little significant inhibitory capacity in ARPE-19 and Vero cells or even an enhanced effect in COS-7 cells [54, 55]. Therefore, a range of concentration with lowest cytotoxicity and sufficient inhibitory efficiency should be determined first when the inhibitor is used on the cell for the first time. Then, the lack of absolute specificity can be compensated by the combined application of biological methods such as siRNA silencing, transient or stable expression of dominant-negative proteins, and reconstruction of proteins by knockout mutants, all of which aremore specific than classical chemical inhibitors. For example, mutant dynamin has been successfully used to prove the necessity of dynamin in the endocytic pathways of transferrin receptors and EGF receptors [55]. A constitutive knockdown technique through RNAi has been used to prove the role of an essential accessory protein "epsin" in the CME pathway [56]. Another efficient way of making up the pitfalls of nonspecific inhibitors is the combined usage of fluorescently labeled gene complexes and fluorescent probes that are specifically internalized through certain uptake pathways.

Except for inhibitors, molecular probes and markers are also important tools for the study of uptake pathways for non-viral gene complexes. They can be used together with the classical chemical inhibitors or biological inhibitors to make the results more convincing. There are several classical molecular probes that are known to be specifically internalized through each uptake pathway. Transferrin is often used as a probe of CME pathway in many studies [12, 57, 58]. Transferrin receptor (TFR) mediates transferrin uptake by CME, so that it can be used as a CME marker and detected by anti-TFR [59].

Cholera toxin beta subunit (CTBs) is commonly used as a probe for CvME [12, 57]. However, Lisa et al. argued that CTBs binds receptors that are contained in lipid-rich areas and are internalized via a mechanism similar to CvME, because CTBs uptake is unaffected by a clathrin inhibitor and 33% uptake remains after treatment with a specific caveola inhibitor. Therefore, CTBs may enter into the cells via another unknown clathrin-independent mechanism [60]. In addition, caveolin-1 is also an important marker for CvME, as it is specifically involved in the formation of caveosome [29].

Dextran is the popular probe for macropinocytosis in some studies because it can accumulate in the endo-lysosome compartment [57]. As to phagocytosis, large (2 μm) microspheres are usually used as the probes. To solve the issue about the intracellular fate of complexes, a group of the specific markers or biological dyes are necessary to colocalize the non-viral gene complexes and intracellular organelles. TFR is used as a classical early endosome marker because it is transported into an early endosome when transferrin is internalized. EEA-1 is a hydrophilic peripheral membrane protein present in cytosol and membrane fractions. It colocalizes with TFR, and immunoelectron microscopy shows that it is associated with tubulovesicular early endosomes [61]. The lysosome-associated type 1 membrane glycoproteins LAMP-1 and LAMP-2 are localized primarily on the periphery of the lysosome, and can be used as markers for lysosome [62, 63]. The different roles of EEA-1 and LAMP in the endolysosome pathway allow us to know the stage in which the uptake carries on. Other endosome or lysosome markers are the Rab family proteins. They are small GTPases that control multiple membrane trafficking events in the cell, and there are at least 60 Rab genes in the human genome [64]. Inside the Rab family, Rab5 and Rab7 are the most studied Rab variants, in which Rab5 is found to be the marker for early endosomes as it in part controls the invagination at the plasma membrane, endosomal fusion, motility, and signaling [63], and Rab7 is found to be the marker for late endosomes and lysosomes as it controls the aggregation, fusion, and maintenance of perinuclear lysosome compartment [65].

Pathways	inhibitors	markers
Phagocytosis	Amiloride, cytochalasin D, latrunculins, wortmannin, LY290042, sodium azide	Large microspheres (2 μm)
CME	Chlorpromazine, monodansylcadaverine, phenylarsine oxide, sodium azide	Transferrin, lactosylceramide, TFR
CvME	Filipin, nystatin, cholesterol oxidase, statins, genestein, MβCD, sodium azide	CTBs, caveolin-1
Macropinocytosis	Rottlerin, amiloride, cytochalasin D, latrunculins, wortmannin, LY290042, sodium azide	Dextran

Table 2. Inhibitors and markers

The organelle specific dyes are other ideal tools for the detection of colocalization, and they are relatively convenient. LysoTracker (red) and Lyso Sensor (green) are the widely used

dyes for lysosomes. Cell light (red or green) are the widely used dyes for early endosomes. Combined with the confocal imaging technology, the colocalization of labeled non-viral gene complexes and intracellular compartments can be viewed intuitively. However, the classical confocal imaging technology can only provide the monolayer images, the information from which is not convincing enough. A novel three dimensionally integrated confocal technology is so strong that it can provide the intact information of a whole cell by scanning layer by layer.

5. Application of cellular uptake mechanism.

Based on the current understanding of cellular uptake mechanisms, one can rationally design vectors and improve their efficiency. Each pathway has advantages that need to be optimized and disadvantages that should be avoided (Table 3).

Pathways	Advantages	Disadvantages
Phagocytosis	Specific cell-type targeting Specific receptors	Lysosome involved In vivo clearance
CME	Specific receptors	Lysosome involved
CvME	Bypass the lysosome Specific receptors	Membrane structure dependent Slower cellular uptaking
Macropinocytosis	Larger particles uptaking.	Non-specific
Non-endocytic	Bypass the lysosome	Non-specific

Table 3. Characteristics of pathways.

5.1. Endo-lysosomal escape

Endo-lysosomal escape is one of the most crucial issues in non-viral vector design. Non-viral delivery systems, such as polyplexes and lipoplexes, will be trapped and degraded in the lysosomes if their cellular uptake pathways involve endo-lysosomes. As discussed above, some of the uptake pathways involve endo-lysosomes, such as CME and phagocytosis. CvME is known to bypass the endo-lysosomes. Similarly, macropinocytosis is known to not have any associations with endo-lysosomes [66, 67], but some studies have suggested that it involves lysosomes [67, 68]. These contradictory data may be dependent on cell type. Stimulating special pathway to bypass endo-lysosomes is a novel direction to improve efficiency. This will be discussed later.

A non-viral delivery system uptaken by endo-lysosomes dependent pathways must be capable of escaping endo-lysosomes. From early endosome to late endosome transport, a maturation process involving compartment acidification by proton pumps located on the endosomal membrane exists. Some non-viral vectors exhibit the ability to escape the endo-

lysosome, called *proton sponge*, such as PEI [10, 69, 70]. PEI contains a nitrogen atom that can be protonated, and this serves to consume endosomal protons because endosomes acidify their microenvironment. As a result, an increase in endosomal chloride anion, which diffuses into the endosomes with the protons, leads to an increase in osmotic pressure, thus inducing osmotic swelling [69]. Therefore, the endosome might break down and release PEI. This mode of action has been widely incorporated in recent non-viral vector designs. However, a pDAMA-based vector with endosomal buffering capacity has been reported to show no endosomal escape activity in cell-based assay, indicating that the proton sponge hypothesis may not be applicable in some cases. These findings warrant further elucidation and investigation of the mechanism of non-viral gene delivery [71].

For lipoplexes, the cationic liposome can interact with the anionic cytoplasmic facing monolayer lipid of endosome and release the DNA from the endosome through the flip-flop mechanism [72]. 1,2-Dioleoylsn-glycero-3-phosphatidylethanolamine (DOPE), the pH-sensitive fusogenic lipid additive, is very helpful to the displacement of the anionic lipds from the cytoplasm-facingmonolayer of the endosomal membrane to the opposite direction via a flip-flop mechanism. However, the serum components are known to inactivate and destabilize the lipoplex structures that contain DOPE [73].

Viruses have the ability to destabilize the endosomal membrane, which explains why many proteins from different viruses are being used [69]. Some viruses are well known to use fusogenic peptides to cross the endosomal membrane and reach the cytosol [21]. The process by which viruses destabilize endosomal membranes in an acidification-dependent manner has been mimicked with synthetic peptides containing the amino-terminal 20-amino-acid sequence of the influenza virus HA [70]. Generally, short sequences of only 20 amino acids are needed for membrane destabilization, and they usually contain a high content of basic residues [74].

Cell-penetrating peptides (CPPs) are used to enhance endosomal escape. The HIV-1 Tat protein is the first CPP to be discovered. It transactivates the transcription of the HIV-1 genome, has been observed to cross the plasma membrane by itself, leading to the identification of a peptide fragment (49–59 amino acids) that confers cell permeability to the protein (Tat peptide), and is one of the better characterized CPPs [75]. Most of the CPPs contain a high density of basic amino acids (arginines and/orlysines), which are proposed to interact with the anionic surface of the plasma membrane and enhance internalization of the peptides [75]. These peptides adopt an a -helical structure at endosomal pH leading to hydrophobic and hydrophilic faces that can interact with the endosomal membrane to cause disruption and pore formation [74].

5.2. Optimization of CvME

CvME is considered an alternative pathway that can bypass the endo-lysosomes. As gene materials will not be degraded in the lysosomal compartments, we can take advantage of CvME to improve the efficiency of transfection. For example, Nathan P. et al. targeted complexes (PEI–DNA) in CvME and CME with folic acid and transferrin, respectively; however, only vectors via CvME successfully delivered genes, as CvME is avoidant of lysosomes.

These data demonstrate that the uptake mechanism and subsequent endocytic processing are important design parameters for gene delivery materials [76]. However, the key is controlling the uptake mechanism.

Particle size is a very important factor for uptake mechanisms. In a previous study, three particles (20, 40, and 100 nm) were investigated for their uptake efficiency via CvME in endothelial cells. The results showed that the uptake efficiency levels of the 20- and 40-nm nanoparticles were 5–10 times greater than that of the 100-nm particles [6], indicating that small particles can be uptaken by CvME more efficiently compared with large ones. However, another study found that the uptake of microspheres with a diameter <200 nm in non-phagocytic B16 cells involved CME. With increasing size, a shift to a mechanism that relied on a caveolae-mediated pathway became apparent, which became the predominant pathway of entry for particles measuring 500 nm in size [77]. This can be attributed to the fact that the mechanism of CvME is cell type dependent in some cases. According to the target cell type, the mechanism must be fully studied before designing a vector.

CvME is a kind of receptor-mediated endocytosis pathway. As a result, some specific ligands can mediate CvME via ligand–receptor binding. The insulin receptor [30], epidermal growth factor (EGF) receptor [31], transforming growth factor beta (TGFbeta) receptor [78] have been found to mediate this pathway. Another study used the cyclic Asn–Gly–Arg peptide to enhance gene transfection efficiency in CD13-positive vascular endothelial cells via CvME [79]. However, cyclic RGD ligands have been reported to facilitate CvME of thiolated c(RGDfK)-polyethylene glycol (PEG)-b-PLL micelles without high endosomal-disrupting properties and thus improve transfection efficiency [80]. The cyclic RGD peptide ligands c(RGDfK) can selectively recognize $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin receptors on the cell surface. The receptors can mediate CvME and bypass endo-lysosomes. The $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin receptors overexpressed on endothelial cells of tumor capillaries and neointimal tissues. As a result, the vectors with cyclic RGD peptide ligands can be used for cancer gene therapy.

Cellular stress can also be used to control the cellular uptake mechanism. Heat shock and hyperosmotic shock can stimulate caveolin internalization [81]. Recent research has shown that hypertonic exposure of alveolar cells caused down-regulation of CME and fluid-phase endocytosis while stimulating CvME. An osmotic polymannitol-based gene transporter that can increase caveolae-mediated endocytosis was designed taking advantage of this mechanism [82]. The possible mechanisms have been discussed. Non-penetrating osmolytes tend to draw water from the intracellular space through an osmotic gradient, cause cell hypertonic stress accompanied by cell shrinkage. Responding the cellular hypertonic stress, phosphorylation of caveolin-1 is mediated by Src-kinase. Src-kinase-mediated phosphorylation of caveolin-1 is required for caveolae budding. Finally the CvME is stimulated.

5.3. Inhibition of phagocytosis

After *in vivo* administration, the non-viral delivery system can be uptaken by macrophages and then cleared. This macrophage clearance effect mainly via phagocytosis is one of the main barriers for non-viral gene delivery. Numerous methods are used to avoid phagocytosis of macrophages in vector design. Antibodies are being widely used for tar-

geting non-viral gene delivery. However, the constant fragments can be recognized by phagocytosis and then uptaken by macrophages. Therefore, antibodies that lack constant fragments are sometimes used to help non-viral vectors avoid recognition and clearance by macrophages *in vivo* [83].

Other vectors can also be recognized by macrophages. As discussed above, some cationic polyplexes or lipoplexes will be tagged by some opsonins and then recognized *in vivo*. PEGylation is widely used to avoid the *in vivo* clearance effect by phagocytosis. The highly hydrophilic nature of PEG produces a hydration shell around its conjugated partner, hence reducing intermolecular interactions and, consequently, toxicity [84]. As an effect of reducing intermolecular interactions, PEGylation can effectively avoid phagocytosis; moreover, *in vivo* studies have reported on long circulating half-life of PEGylated vectors [85].

However, some studies have shown that PEGylation can reduce the efficiency of vectors [84] possibly because PEGylation may inhibit cellular uptake and endosomal escape of the vectors. One study compared non-PEGylated and PEGylated liposomes, with the data showing that PEGylated liposomes have poor endosomal escape capability as non-PEGylated liposomes can escape from endosome efficiently [86]. The inhibitory effects of PEGylation depend on some factors. A study about PEGylated cationic liposomes demonstrated that acid-labile PEGylation liposomes have higher transfection efficiency than acid-stable PEGylation ones, which can be ascribed to the more efficient endosomal escape activity of acid-labile PEGylation liposomes [87]. The possible mechanism involved here is that the PEG of acid-labile PEGylation liposomes can be cleaved under low pH (endosomal compartments), allowing the vector to fully interact with the endosomal membrane. So other biodegradable shielding methods should be better than classical PEGylation. According to this hypothesis, recently, an alternative to PEGylation was designed. This work reports, for the first time, the use of hydroxyethyl starch (HES) for the controlled shielding/deshielding of polyplexes. Non-viral delivery systems can be protected by HES shielding, and the HES can then be degraded *in vivo*, indicating that HES shielding has less influence on the efficiency of vectors compared with PEGylation [88].

6. Conclusion

In summary, cellular uptake is the most important intracellular process. Understanding cellular uptake mechanisms is essential to determining the limits of gene delivery. Different pathways have different intracellular fates. Some vectors can enter cells via endo-lysosomal pathways. Thus, some methods have to be used to protect genes against degradation in lysosomes. Optimizing CvME can successfully deliver genes by avoiding endo-lysosomes. Each pathway has its own disadvantages, and learning how to inhibit certain pathways is significant in some cases. In conclusion, taking advantage of cellular uptake mechanisms and knowing how to control them hold considerable potential for improving the efficiency of gene delivery.

Acknowledgements

Author details

Shengnan Xiang and Xiaoling Zhang

*Address all correspondence to: xlzhang@sibs.ac.cn

The Key Laboratory of Stem Cell Biology, Institute of Health Sciences, Shanghai Jiao Tong University School of Medicine (SJTUSM) & Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences (CAS), Shanghai, China

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