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ENDOCYTOSIS PATHWAYS FOR NUCLEIC ACID THERAPEUTICS

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Abstract

The development of nanoscale delivery vehicles for siRNAs is a current topic of considerable importance. However, little is understood about the exact trafficking mechanisms for siRNA-vehicle complexes across the plasma membrane and into the cytoplasm. While some information can be gleaned from studies on delivery of plasmid DNA, the different delivery requirements for these two vehicles makes drawing specific conclusions a challenge. However, using chemical inhibitors of different endocytosis pathways, studies on which endocytotic pathways are advantageous and deleterious for the delivery of nucleic acid drugs are emerging. Using this information as a guide, it is expected that the future development of effective siRNA delivery vehicles and therapeutics will be greatly improved.

Keywords

RNA interference; siRNA; pDNA; nanoparticles; therapeutics; endocytosis

1. Introduction

RNA interference (RNAi) is a naturally occurring pathway within human cells that can be induced both endogenously and exogenously.¹ For therapeutic applications, the exogenous process is most important for development of better therapeutics. In this pathway, double stranded, short, interfering ribonucleic acids (siRNAs), are internalized by cells, typically through the aid of some type of delivery vehicle and trafficked to the cytoplasm. Upon release from the delivery agent, the siRNA duplex is bound by a complex of proteins (Dicer, TAR RNA Binding Protein (TRBP) and Argonaute 2 (Ago2)), that form the RNA induced silencing complex (RISC) and its predecessor the RISC loading complex (RLC).^{2,3} The RLC/RISC selects one strand of the siRNA, the guide strand, to bind the target mRNA by complementarity, leading to sequence specific degradation of the mRNA and a subsequent reduction in protein expression.^{4–7} This allows sequence-specific targeting of mRNAs for the reduction of selected proteins.

Upon its characterization in 1998,⁸ researchers were excited about the potential therapeutic applications of RNAi for regulating specific proteins associated with diseases. The versatility of RNAi makes it possible to suppress a wide variety of targets with high

specificity, making it an ideal complement to small molecule drugs. Currently, there are several on-going clinical trials utilizing siRNA therapeutics for eye diseases, cancer, kidney disorders and antiviral defenses,^{9,10} but none of these candidates have moved from the clinical trials to FDA approval. Additionally, the majority of these trials deal with topical delivery, localized injections, or systemic delivery to liver or kidneys (natural filtering organs that are easily targeted). In short, RNAi therapeutics are not moving through the developmental pipeline as quickly as initially hoped. As a result, major pharmaceutical companies have announced cutbacks in funding for RNAi technologies.^{11,12}

These difficulties can be attributed in part to complications in optimizing siRNA delivery, maximizing RNAi function, while minimizing their cytotoxicity and immune response.¹³ Delivery to the intended target in concentrations high enough for measurable activity is a major shortcoming in siRNA therapeutic design. One facet of this challenge involves determining the optimal cellular internalization and trafficking pathways for delivery of vehicles upon reaching their target cell. While extracellular transport of genetic therapeutics is arguably the foremost challenge to overcome, intracellular targeting issues also represent an area of concern which should be considered concurrently. As such, the content of this review focuses specifically on intracellular transport and delivery mechanisms.

Requirements for extracellular transport of siRNA and plasmid DNA (pDNA) delivery are relatively similar; both needing protection from degradation and the ability to target and enter the cells of interest. Conversely, there are several notable differences between the intracellular trafficking necessary for these nucleic acids, the most evident being siRNA activity occurs in the cytoplasm while pDNA expression requires nuclear delivery. Additionally, a large number of siRNAs are required within a single cell for protein reduction while delivery of a single plasmid molecule is sufficient for successful protein expression. Given these differences, it is likely that the internalization processes that result in strong target silencing by siRNAs are unique from those that lead to strong pDNA expression. However, to date, few studies have been performed that investigate routes of internalization for siRNAs. In this review, we describe various cellular entry pathways, methods for isolating and observing these pathways, current research on the trafficking of nonviral delivery vehicles in general and the extension of these guidelines toward the development of future studies specific to developing highly-active siRNA therapeutics.

2. Delivery Vehicle Development

The direct administration of siRNAs has shown some success when delivered topically or through localized injection.¹⁴ However, this requires direct and sometimes intrusive access to the tissues of interest. When delivered systemically, naked siRNAs have minimal *in vivo* success due to degradation by serum nucleases and early filtration through the renal system.^{15,16} Additionally, naked siRNAs cannot be targeted directly to the tissues/cells of interest. As a result, it is expected that siRNA therapeutics will need to be encapsulated in some type of carrier for clinical application.

Delivery approaches are commonly divided between viral and nonviral methods. Adenoviruses (ADs), adeno-associated viruses (AAVs), or retroviruses are common examples of viral-based delivery for nucleic acid therapeutics,^{17–19} which are known for their highly efficient delivery. However, viral delivery systems also have the potential for immunogenicity,^{20,21} adaptive immunity,¹⁷ or insertional mutagenesis with active viruses or untargeted cells.¹⁹ Concern over these risk factors has driven the development of biologically safe, synthetic, nonviral vectors.

Nonviral delivery vehicles are also categorized into several different sub-groups according to their chemical or physical properties. These vehicles often form nanoscale complexes

with the siRNA on the order of 50–200nm in size but can also include larger or smaller complexes. Lipids,^{22–25} polymers^{26–29} and solid-core particles^{30–32} have all shown success in cell culture and in some *in vivo* studies.^{33,34} However, vehicles synthesized to contain similar chemical characteristics can have widely differing silencing efficiencies,^{24,35} indicating that modifications on chemical composition alone are insufficient for creating optimized vehicles. Attributes such as size, surface charge and complex structure likely also play a role in determining the utility of a molecule for siRNA delivery.

The chemical and physical variety among the carrier molecules that have been developed to date provides no clear guidance on how best to approach synthesizing improved delivery vehicles for siRNA delivery. It is worthwhile to restate that due to their differences in trafficking, function, and, not least of all, size, data for optimization of vehicles for pDNA delivery is not necessarily helpful in the development of siRNA delivery vehicles. Long-term, systematic analysis of chemical and physical variables (e.g., complex size) individually as well as potential interactions among them will be required to characterize those properties that contribute most strongly to effective delivery vehicles. Polymeric delivery vehicles, with their potential for modification and varied functional groups, are playing and will continue to play a key role in studying and optimizing the desired properties for siRNA delivery vehicles.

3. Cellular Uptake Mechanisms

Phagocytosis, macropinocytosis and endocytosis/pinocytosis are the three major mechanisms of uptake for extracellular molecules. Endocytosis can be further sub-classified into clathrin-dependent, caveolae-dependent, or clathrin- and caveolae-independent methods (Fig. 1).^{36–38} The vesicles that form in each of these processes have different intracellular destinations and fates, in many cases precluding all access to the cytoplasm. As such, depending on the drug being delivered, the preferred uptake pathway will vary.

Phagocytosis is an actin-dependent pathway most commonly used by white blood cells such as macrophages, monocytes and neutrophils for initiating the adaptive immune response.³⁸ Phagocytosis has been utilized for targeting and delivery of magnetic nanoparticles to macrophages.³⁹ Macro-phages can internalize larger particles, on the order of 5–10 μm , with increased internalization of ellipsoid- over spherical-shaped particles.⁴⁰ Despite this ability, it is not a general mechanism by which one can expect to deliver drugs to cells outside of these specific types.

Another mechanism for internalizing particles is through macropinocytosis, the encapsulation of extracellular fluid in vesicles with diameters in excess of 150–5000 nm.³⁸ Unlike most forms of endocytosis, macropinocytosis results from the cell membrane reaching out and enveloping extracellular contents. The pathway is actin driven, characterized by outward-directed actin polymerization (surface ruffling).⁴¹ Unlike clathrin or caveolar mediated endocytosis, macropinocytosis does not rely on special protein coatings or concentrated receptors on the membrane surface, although it may depend on the presence of cholesterol.⁴² Macropinocytotic vesicles do not undergo transition into acidic lysosomes or merge with other endocytotic pathways;⁴³ rather, some contents leak into the cytosol prior to the vesicle recycling back to the cell surface.³⁶ Macropinosome trafficking has also been shown to vary by cell type.⁴²

Clathrin-mediated endocytosis (CME) is the most common and universal endocytotic pathway among cells.^{38,41,44} CME is characterized by the presence of clathrin protein pits which form a polygonal lattice structure around a portion of the cell membrane, pinching it off to form an internalized vesicle.^{38,44,45} Particles taken up by CME can range in size from 100–150 nm in diameter. The process is energy and dynamin dependent, however there are

conflicting reports on the role of actin in this process.^{36,45,46} It is likely that while actin may not be required for formation of clathrin vesicles, it is necessary for further trafficking of clathrin endosomes within the cell.⁴⁷ Although CME is not the only form of receptor-mediated endocytosis, receptors for transferrin and low density lipoprotein (LDL) also concentrate within these organelles.³⁸ Clathrin-coated endosomes transition into late endosomes and acidified lysosomes, a process which often occurs closer to the nucleus,⁴⁸ a possible benefit for pDNA delivery vehicles.

The second type of protein coated endocytosis is caveolae-mediated endocytosis. These vesicles are 50–60nm in diameter, contain hydrophobic domains high in cholesterol and glycosphingolipids, called lipid-rafts, and typically have a flask-shaped formation⁴⁷ but can also have flat or tubular structures.³⁶ Activation of this pathway is both dynamin and actin dependent.⁴⁷ Caveolae are most abundant in epithelial and adipocyte cells³⁸ but not always present in others, such as HepG2 cells.⁴⁹ Folate⁵⁰ and insulin receptors⁵¹ are among those that localize on the cell membrane within caveolar regions. Trafficking of caveolae typically follows a nonacidic route,^{36,38} with vesicles processed toward the golgi or endoplasmic reticulum organelles.^{43,50,52} However, caveolar vesicles have been shown to occasionally undergo acidification.⁴⁸

The final uptake pathway identified within cells involves the formation of lipid rafts but does not require clathrin or caveolin proteins. These clathrin- and caveolin-independent pathways form cholesterol- rich structures 40–50nm in size and may be both dynamin and actin independent.^{38,47,52} While less well-understood than the classical pathways, it is believed that these independent vesicles have the ability to merge with other pathways during endosome maturation.³⁸

Historically, the proton sponge hypothesis has been used to explain the release of nucleic acid delivery vehicles from endosomal vesicles into the cytoplasm.^{53–55} According to this theory, amine containing vehicles, most specifically polymers containing many amines, are internalized through some type of endocytotic pathway. As the endosome acidifies, the buffering capacity of the amines draws in an excess of protons followed by an excess of chloride ions. Osmotic swelling then causes the endosome to burst, releasing the contents into the cytoplasm of the cell.^{53–55} Other delivery vehicles are reported to utilize fusogenic peptides to create more potent and active endosomal escape.⁵⁶ For this mechanism to hold, these vehicles would need to rely heavily on clathrin-mediated endocytosis. Discerning the exact internal cellular trafficking of delivery vehicles is critical for achieving active silencing and is emerging as an important area of study. Designing vehicles to target specific endocytic pathways as well as understanding how these pathways affect their ultimate destinations are essential to overcoming some of the hurdles in the efficiency of current delivery vehicles.

4. Endocytotic Inhibitors and Other Analytical Methods

Pharmacological inhibitors for isolating specific routes of endocytosis have long been used as a means for studying the spread of viruses among cells and are currently being employed for similar analyses of delivery of nucleic acid therapeutics (Table 1).^{36,41} It is important to note that the mechanistic similarity among many of the endocytotic pathways makes it difficult to ensure the specificity of inhibition for these molecules.^{57,58} As a result, concentrations and treatment times for all inhibitors require optimization based on cell type as well as the delivery vehicle being studied. Additional methods for confirming specific pathway inhibition, typically through microscopy, must therefore be used.

Antibody specific labeling of either clathrin or caveolin can provide insight into the involvement of these pathways following inhibitor treatments.^{59,60} Fluorophore-labeled

aids such as dextran for analysis of bulk fluid endocytosis (pathway nonspecific),^{43,60} transferrin or LDL for clathrin-based pathways,⁴⁶ cholera toxin for caveolae⁶¹ or lysosomal compartment sensing dyes,⁶² can be combined with confocal microscopy as a secondary verification of specific pathway function. Endocytotic pathways can also be characterized by transmission electron microscopy (TEM) for higher magnification.^{50,63} For higher selectivity, siRNA delivery itself may be used to down-regulate both types of protein mediated pathways.⁵⁹

5. Internalization Studies

Currently, there are relatively few published studies on mechanisms of uptake for nucleic acid delivery complexes. The results of these studies support the idea that preferred uptake pathways vary depending on complex composition, surface charge and particle size, in addition to cell type (Table 2). In many cases, the pathway most prominent in particle uptake is not necessarily the pathway which permits nucleic acid activity.^{59,60,62,64} It has also been reported that for lipid delivery vehicles, pDNA and siRNA activity may result, independent of any endocytotic events. This suggests that cytoplasmic access of active lipid-containing complexes occurs through direct fusion with or transport across the lipid bilayer membrane, resulting in delivery directly to the cytoplasm of the cell.^{60,62} Studies reporting caveolae-mediated endocytosis as the pathway utilized for active plasmid delivery with polymeric NPs^{59,60,65} question whether endosome acidification is a requirement for particle release. Upon ruling out endosomal buffering as a means of release to the cytosol for cases of caveolar-dependent mechanisms, it remains unclear how delivery vehicles ultimately escape from various endosomal compartments to reach their final destination. Recently, pDNA-PEI complexes including histone tail peptides were reported to utilize caveolar vesicles for retrograde trafficking to the Golgi followed by the endoplasmic reticulum, presumably en route to the nucleus.⁶⁵ However, it remains to be shown if the pathways required for active plasmid delivery mimic the pathways required for active delivery of siRNA when using the same type of delivery vehicle. Advancing beyond cellular delivery, an early study involving translocation of delivery vehicles across epithelial cells⁶¹ supports the idea that vehicle design must account for intracellular and extracellular trafficking differences to ensure maximal activity of the delivered cargo.

Early attempts at systematic studies of internalization based upon single variables such as particle size,⁶⁶ shape,⁶⁷ or surface charge³⁷ are difficult to compare given variations in other characteristics of the vehicles that were not characterized. Further systematic studies of all variables to determine the best structural and chemical complex characteristics for each cellular entry pathway in concert with the determination of the most efficient trafficking pathway for each type of cargo will be necessary in order to piece together the results of current studies and provide insight towards future design of improved vehicles.

6. Future Directions

The development of successful nucleic acid therapeutics is clearly hindered by design challenges related to delivery and transport at both the cellular and *in vivo* scales. Despite the multitude of candidates, there is no single delivery vehicle developed that guarantees reliable, consistent siRNA delivery to all cell types. This is attributable both to a lack of studies that focus on the relationships between chemical and physical characteristics of the vehicles and the function of the cargo and a lack of good model systems for evaluating vectors for their likely success *in vivo*. It is reasonable to believe that the best vehicle choice may continue to vary depending on the disease and cellular target. Nevertheless, improvements in research identifying the exact mechanism(s) required for the transport of delivery vehicles across the cell membrane, leading to cytoplasmic release of accessible

siRNA cargo, will benefit the design of higher-efficiency vehicles. Upon these developments, future investigations for improving delivery will need to expand toward improving mechanisms within *in vivo*-like environments.

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References

1. Carthew RW, Sontheimer EJ. Cell. 2009; 136:642. [PubMed: 19239886]
2. MacRae IJ, et al. Proc Natl Acad Sci USA. 2007; 105:512. [PubMed: 18178619]
3. Maniatakis E, Mourelatos Z. Genes Dev. 2005; 19:2979. [PubMed: 16357216]
4. Hammond SM, et al. Nature. 2000; 404:293. [PubMed: 10749213]
5. Rivas FV, et al. Nat Struct Mol Biol. 2005; 12:340. [PubMed: 15800637]
6. Elbashir SM, Lendeckel W, Tuschl T. Genes Dev. 2001; 15:188. [PubMed: 11157775]
7. Leuschner PJF, et al. EMBO Rep. 2006; 7:314. [PubMed: 16439995]
8. Fire A, et al. Nature. 1998; 391:806. [PubMed: 9486653]
9. Davidson BL, McCray PB. Nat Rev Genet. 2011; 12:329. [PubMed: 21499294]
10. Malefyt, AP., et al. siRNA therapeutic design: Tools and challenges. In: Mallick, B., editor. Regulatory RNAs. Springer; 2012.
11. Pollack A. Drugmakers' fever for the power of RNA interference has cooled. New York Times. 2011
12. Krieg AM. Mol Ther. 2011; 19:1001. [PubMed: 21629254]
13. Peer D, Lieberman J. Gene Ther. 2011; 18:1127. [PubMed: 21490679]
14. Whitehead KA, Langer R, Anderson DG. Nat Rev Drug Discov. 2009; 8:129. [PubMed: 19180106]
15. Soutschek J, et al. Nature. 2004; 432:173. [PubMed: 15538359]
16. van de Water FM, et al. Drug Metab Dispos. 2006; 34:1393. [PubMed: 16714375]
17. Ghosh SS, Gopinath P, Ramesh A. Appl Biochem Biotechnol. 2006; 133:9. [PubMed: 16622281]
18. Coura RD, Nardi NB. Virol J. 2007; 4:99. [PubMed: 17939872]
19. Cockrell AS, Kafri T. Mol Biotechnol. 2007; 36:184. [PubMed: 17873406]
20. Kaiser J. Science. 2007; 317:580. [PubMed: 17673625]
21. Hartman ZC, Appledorn DM, Amalfitano A. Virus Res. 2008; 132:1. [PubMed: 18036698]
22. Ren T, et al. Gene Ther. 2000; 7:764. [PubMed: 10822303]
23. Akinc A, et al. Nat Biotechnol. 2008; 26:561. [PubMed: 18438401]
24. Whitehead KA, et al. Mol Ther. 2011; 19:1688. [PubMed: 21750531]
25. Alvarez-Erviti L, et al. Nat Biotechnol. 2011; 29:341–U179. [PubMed: 21423189]
26. Burke RS, Pun SH. Bioconjug Chem. 2008; 19:693. [PubMed: 18293906]
27. Lynn DM, Langer R. J Am Chem Soc. 2000; 122:10761.
28. Rozema DB, et al. Proc Natl Acad Sci USA. 2007; 104:12982. [PubMed: 17652171]
29. Siegwart DJ, et al. Proc Natl Acad Sci USA. 2011; 108:12996. [PubMed: 21784981]
30. Hom C, et al. Small. 2010; 6:1185. [PubMed: 20461725]
31. Rosi NL, et al. Science. 2006; 312:1027. [PubMed: 16709779]
32. Veisheh O, et al. Biomaterials. 2011; 32:5717. [PubMed: 21570721]
33. Shim MS, Kwon YJ. Febs J. 2010; 277:4814. [PubMed: 21078116]
34. Davis ME. Mol Pharm. 2009; 6:659. [PubMed: 19267452]
35. Green JJ, Langer R, Anderson DG. Acc Chem Res. 2008; 41:749. [PubMed: 18507402]
36. Khalil IA, et al. Pharmacol Rev. 2006; 58:32. [PubMed: 16507881]

37. Dausend J, et al. *Macromol Biosci.* 2008; 8:1135. [PubMed: 18698581]
38. Conner SD, Schmid SL. *Nature.* 2003; 422:37. [PubMed: 12621426]
39. Kamat M, et al. *Bioconjug Chem.* 2010; 21:2128. [PubMed: 20977242]
40. Champion JA, Mitragotri S. *Proc Natl Acad Sci USA.* 2006; 103:4930. [PubMed: 16549762]
41. Medina-Kauwe LK, Xie J, Hamm-Alvarez S. *Gene Ther.* 2005; 12:1734. [PubMed: 16079885]
42. Kerr MC, Teasdale RD. *Traffic.* 2009; 10:364. [PubMed: 19192253]
43. Hewlett L, Prescott A, Watts C. *J Cell Biol.* 1994; 124:689. [PubMed: 8120092]
44. Wang LH, Rothberg KG, Anderson RG. *J Cell Biol.* 1993; 123:1107. [PubMed: 8245121]
45. Kaksonen M, Toret CP, Drubin DG. *Nat Rev Mol Cell Biol.* 2006; 7:404. [PubMed: 16723976]
46. Macia E, et al. *Dev Cell.* 2006; 10:839. [PubMed: 16740485]
47. Kirkham M, Parton RG. *Biochim Biophys Acta.* 2005; 1745:273. [PubMed: 16046009]
48. Huotari J, Helenius A. *EMBOj.* 2011; 30:4481.
49. Fujimoto T, et al. *J Cell Sci.* 2000; 113:3509. [PubMed: 10984441]
50. Pelkmans L, Helenius A. *Traffic.* 2002; 3:311. [PubMed: 11967125]
51. Gustavsson J, et al. *FASEB J.* 1999; 13:1961. [PubMed: 10544179]
52. Damm EM, et al. *J Cell Biol.* 2005; 168:477. [PubMed: 15668298]
53. Akinc A, et al. *J Gene Med.* 2005; 7:657. [PubMed: 15543529]
54. Cho YW, Kim JD, Park K. *J Pharmaceut Pharmacol.* 2003; 55:721.
55. Sonawane ND, Szoka FC, Verkman AS. *J Biol Chem.* 2003; 278:44826. [PubMed: 12944394]
56. Kwon EJ, Bergen JM, Pun SH. *Bioconjug Chem.* 2008; 19:920. [PubMed: 18376855]
57. Lamaze C, Schmid SL. *Curr Opin Cell Biol.* 1995; 7:573. [PubMed: 7495578]
58. Ivanov AI. *Meth Mol Biol.* 2008; 440:15.
59. Gabrielson NP, Pack DW. *J Control Release.* 2009; 136:54. [PubMed: 19217921]
60. McLendon PM, Fichter KM, Reineke TM. *Mol Pharm.* 2010; 7:738. [PubMed: 20349982]
61. Yacobi N, et al. *Am J Respir Cell Mol Biol.* 2010; 42:604. [PubMed: 19574531]
62. Lu JJ, Langer R, Chen JZ. *Mol Pharm.* 2009; 6:763. [PubMed: 19292453]
63. Parton RG, Joggerst B, Simons K. *J Cell Biol.* 1994; 127:1199. [PubMed: 7962085]
64. van der Aa M, et al. *Pharmaceut Res.* 2007; 24:1590.
65. Reilly MJ, Larsen JD, Sullivan MO. *Mol Pharm.* 2012; 9:1280. [PubMed: 22420286]
66. Rejman J, et al. *Biochemical J.* 2004; 377:159.
67. Gratton SE, et al. *Proc Natl Acad Sci USA.* 2008; 105:11613. [PubMed: 18697944]
68. Alam M, et al. *Nucleic Acids Res.* 2008; 36:2764. [PubMed: 18367474]
69. Alam M, et al. *Oligonucleotides.* 2010; 20:103. [PubMed: 20038250]
70. Goncalves C, et al. *Mol Ther.* 2004; 10:373. [PubMed: 15294184]
71. Zuhorn IS, Kalicharan R, Hoekstra D. *J Biol Chem.* 2002; 277:18021. [PubMed: 11875062]
72. Rejman J, Bragonzi A, Conese M. *Mol Ther.* 2005; 12:468. [PubMed: 15963763]
73. Huth S, et al. *J Gene Med.* 2004; 6:923. [PubMed: 15293351]
74. von Gersdorff K, et al. *Mol Ther.* 2006; 14:745. [PubMed: 16979385]
75. Phonphok Y, Rosenthal K. *FEBS Lett.* 1991; 281:188. [PubMed: 1901801]
76. Hansen S, Sandvig K, vanDeurs B. *J Cell Biol.* 1993; 121:61. [PubMed: 8458873]
77. Yoshimori T, et al. *J Biol Chem.* 1991; 266:17707. [PubMed: 1832676]
78. Reilly MJ, Larsen JD, Sullivan MO. *Mol Pharm.* 2012; 9:1031. [PubMed: 22280459]
79. Liu Y, Reineke TM. *Bioconjug Chem.* 2006; 17:101. [PubMed: 16417257]

Fig. 1.

Entry pathways for mammalian cells. Routes of cellular internalization vary by cell type but can include phagocytosis (macrophage specific), macropinocytosis (actin dependent), clathrin-mediated (dynamin and clathrin dependent), caveolae-mediated (actin-, dynamin-, and caveolae-dependent) or clathrin- and caveolae-independent routes (some dynamin and actin dependency). In addition to variations in size, these pathways vary by receptor localization as well as internal processing pathways.

Table 1

Mechanism of action for reported endocytotic inhibitors.

Pathway(s) Inhibited	Inhibitor	Mechanism of action	References
Actin dependent pathways (caveolin, macropinocytosis, phagocytosis)	Cytochalasin D	depolymerizes actin, disrupts actin filaments, inhibits membrane ruffling	36, 37, 57, 58, 60–63, 68–71
	Latrunculin A	sequesters actin monomers	52, 61, 69
Caveolin, lipid raft	Genistein	tyrosine kinase inhibitor, prevents phosphorylation of caveolin	36, 37, 59, 64, 66, 70, 72
	Nystatin	binds and sequesters cholesterol	36, 52, 58, 62, 73
	(m β CD) Methyl- β -Cyclodextran, (β CD) β -Cyclodextran	sequesters cholesterol on the cell surface	36, 37, 58, 59, 61, 64, 66, 68–71
	Filipin complex III	binds and sequesters cholesterol	36, 37, 57, 58, 60, 62, 66, 70–72, 74
	Progesterone	inhibits cholesterol synthesis	52, 73
Clathrin	Amantadine	blocks budding of clathrin-coated vesicles	59, 75
	Chlorpromazine	inhibits formation of coated pits (clathrin accumulate in late endosomes)	36, 37, 41, 44, 57–62, 64, 66, 70, 72, 74
	Potassium depletion	removes clathrin lattices from the membrane	36, 41, 57, 58, 72, 76
Dynamin dependent pathways (clathrin, caveolin, macropinocytosis)	Dynasore	inhibits dynamin GTPase activity	37, 46, 60, 61
Endocytosis	Lowered temperatures (4°C)	slows energy dependent processes	36, 37, 62
Lysosomes	Bafilomycin A1	inhibits vacuolar type H ⁺ -ATPase that drives proton pumps	41, 59, 77
	Chloroquine	prevent endosome acidification, enhances membrane permeability	36, 59, 73
Macropinocytosis	Amiloride, dimethylamiloride(DMA), or 5-(N-ethyl-N-isopropyl amiloride (EIPA)	blocks the Na ⁺ /H ⁺ exchanger, inhibits ruffling	36, 37, 52, 57, 58, 60, 62, 66, 69, 70
	Wortmannin	inhibitor of phosphatidyl inositol-3 phosphate kinase (PI3K)	36, 58, 64, 70
Microtubules	Nocodazole	dissociates microtubules	37, 41, 52, 64, 70, 73

Table 2
Summary of results from reported mechanism of entry studies for polymer, lipid, and peptide-based delivery vehicles.

Basis	Vehicle	Nucleic acid	Formulation	Complex characteristics		Cell Type(s)	General uptake mechanism(s)	Prominent mechanism for active delivery	Ref.
				Charge	Size (diameter)				
Polymer	Polystyrene NPs	None	75 µg/ml	+59 mV and -60 mV	Positive-113 nm Negative-121 nm	HeLa	Both utilize energy, dynamin and actin dependent pathways. Positive particles utilize macropinocytosis more significantly	n/a	37
	PEG cubes and cylinders	None	1 mg/ml	+35 mV and -34 mV	100–5000 nm	HeLa	Low internalization for µ-sized, and negative particles. Cylindrical particles internalized faster. Multiple pathways utilized	n/a	67
Polymer/Lipid	Latex beads	None	Concentration not specified	n/a	50, 100, 200, 500, 1000 nm	B16-F10	<200 nm–clathrin, 200–500 nm – caveolar, >500 nm – none	n/a	66
	LPEI, folate and transferrin targeting	pDNA	polymer:DNA 0.5 (wt), 1 µg plasmid	n/a	n/a	HeLa	Folate–primarily caveolar, Transferrin–primarily clathrin	Caveolar	59
	LPEI, pDMAEMA	pDNA	PEI - N:P 6, pDMAEMA N:P 5, 1 µg plasmid	Cationic	n/a	COS-7	Caveolar and Clathrin pathways	Caveolar	64
	LPEI, BPEI	pDNA	LPEI - N:P 6, BPEI- N:P 6 and 10, 1.25 µg/ml plasmid	Cationic	100–1000 nm, depending on buffer	HUH-7, COS-7, HeLa	n/a	COS-7–clathrin (LPEI & BPEI), HUH-7– clathrin (LPEI) clathrin & caveolar (BPEI), HeLa – clathrin & caveolar (LPEI & BPEI)	74
Lipid	histidylated-PLL	pDNA	polymer:DNA 3 (wt)	+18 mV	110 nm	HepG2	Clathrin and Macropinocytosis	Clathrin	70
	histone H3 peptide- PEI	pDNA	N:P 10, 20 µg/mL plasmid	+20 mV	110 nm	CHO-K1	Caveolar and Clathrin pathways	Caveolar	65, 78
Peptide	PEI coated iron oxide	pDNA	polymer:DNA 2 (wt), 0.5 µg plasmid	n/a	>200 nm	HeLa, BEAS-2B, HepG2	Multiple pathways	Cell-line dependent	73
	Poly(glycoamidoamine), JetPEI	pDNA	PGAA - N:P 20, JetPEI - N:P 5, 20 µg/ml plasmid	n/a	PGAA -75–150 nm in water	HeLa	Mostly caveolar and clathrin, macropinocytosis minorly	PGAA–Caveolar, JetPEI–independent of specific mechanism	60, 79
Lipid	DOTAP, LPEI	pDNA	lipid:DNA 6 (wt), polymer:DNA 30mM/µg plasmid	n/a	n/a	A549, HeLa	DOTAP–clathrin, LPEI–caveolae and clathrin	DOTAP–clathrin, LPEI–caveolae	72
	SAINT-2/DOPE (synthetic amphiphile)	pDNA	lipid:DNA 2.5 (wt)	n/a	~200 nm	COS-7	Clathrin	Clathrin	71
Peptide	DharmaFECT1	siRNA	4uL lipid, 100nM siRNA	n/a	n/a	BSC-40	Multiple endosomal pathways	Independent of endocytosis, membrane fusion event	62
Peptide	RGD ligand	antisense	100 nM	Antionic	MW ~8–9 kDa	A375SM-Luc705-B	Caveolar (actin dependent, dynamin dependent)	Not clathrin or macropinocytosis (caveolar not measured)	68, 69