Advanced Review

Challenges in carrier-mediated intracellular delivery: moving beyond endosomal barriers

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The deployment of molecular to microscale carriers for intracellular delivery has tremendous potential for biology and medicine, especially for *in vivo* therapies. The field remains limited, however, by a poor understanding of how carriers gain access to the cell interior. In this review, we provide an overview of the different types of carriers, their speculated modes of entry, putative pathways of vesicular transport, and sites of endosomal escape. We compare this alongside pertinent examples from the cell biology of how viruses, bacteria, and their effectors enter cells and escape endosomal confinement. We anticipate insights into the mechanisms of cellular entry and endosomal escape will benefit future research efforts on effective carrier-mediated intracellular delivery.
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How to cite this article:
WIREs Nanomed Nanobiotechnol 2016, 8:465–478. doi: 10.1002/wnan.1377

INTRODUCTION

Drug and gene carriers have been exploited for intracellular delivery for almost half a century. Such systems have also been referred to as vectors, vehicles, and more recently nanocarriers, nanomedicines, and nanoparticles, depending on their characteristics and applications. Classic examples include agents for gene delivery and nucleic acid transfection, which, apart from enabling decades of basic biomedical research, form the backbone of most gene therapy strategies in the form of viral[1–3] or synthetic nonviral vectors.[4–8] More recently, advances in genome engineering urge the implementation of carriers that can deliver potent molecular gene editing tools to the appropriate intracellular compartments.[9,10] Delivery of therapeutic protein biologics and biopharmaceutical drugs is another frontier, where advances in formulation and intracellular delivery are anticipated to expand the scope of diseases and applications that can be addressed.[11–13] Furthermore, nanocarriers that selectively target specific cell types hold tremendous promise for cancer diagnosis and therapy, and treatment of infectious disease and rare-genetic disorders.[14–18]

Despite the enormous potential of carrier-mediated intracellular delivery, there is currently a paucity of insight into how they interact with cells and deliver their cargo. Until now empirical experimental approaches, trial and error, and large scale screening of physicochemical properties have driven the field. Further new developments may be unlocked, however, by understanding the fundamental mechanisms that govern how the various carriers gain access to the cytosol and other intracellular compartments.[19] In this review, we explore the different types of carriers and examine the current knowledge on their mechanisms of entry. We compare the state of the field with what is known from the cell biology of pathogens, viruses and bacteria, which employ a diverse set of strategies to gain access to the intracellular space. The scope of our exploration is

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Conflict of interest: The authors have declared no conflicts of interest for this article.

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focused on the journey from cell surface to cell interior, as the issues pertaining to the bio-distribution (organ/tissue), circulation time and immune response have been covered elsewhere.

TYPES OF CARRIERS

Carriers used for intracellular delivery comprise a diverse range of synthetic nanoparticles, molecular complexes, shuttles, containers, and bio-inspired systems featuring components of viral and bacterial origin. These vehicles include cell ghosts, viral vectors, reconstituted virosomes, bacterial effectors, polymer-based assemblies (micelles or polyplexes), lipid-based complexes, inorganic nanoparticles, and protein/peptide-based targeting agents (Figure 1). Carrier systems must accomplish specific goals to perform their function. First, they should efficiently package the cargo without adverse damage and protect it from environmental degradation. Second, they must shuttle the cargo into the cell interior and deliver it within reach of the target destination. Third, they are required to release the cargo at the appropriate place and time with suitable kinetics. To achieve this, a variety of architectures have been attempted including protective containers (e.g., liposomes, micelles, polymersomes), blended aggregates (e.g., most cationic-nucleic acid nanoparticles), porous structures (e.g., dendrimers, hydrogels, mesoporous silica), core nuclei for functionalization [e.g., gold and silica nanoparticles, carbon nanotubes (CNTs)], or more compact direct conjugates [e.g., cell penetrating peptides (CPPs), antibodies, and targeting ligands such as N-acetylgalactosamine (GalNAc)]. Hybrid architectures can be produced to confer multifunctional properties, e.g., liposomes functionalized with targeting ligands to engage specific receptors.[20,21] Stimuli-sensitive nanocarriers that respond to intrinsic or extrinsic cues have also been designed. These systems facilitate membrane breakdown or cargo release upon exposure to the stimuli.[21–23]

Owing to the imminent promise of gene therapy, great efforts have gone into optimizing delivery systems for nucleic acids—DNA, which requires nuclear localization for expression, and siRNA, mRNA, and miRNA, which must bind complementary cytoplasmic enzyme machinery to take effect.[4] There are now efforts to tailor nanoparticulate systems toward delivery of a wider range of payloads including proteins, functional molecular probes, hydrophobic drugs, and other bioactive agents.[12] Although several platforms have been devised to deliver diverse sets of cargo, most of the carriers are efficient only for a particular subtype. For example, viral vectors are limited to delivering nucleic acids of a certain size, cationic delivery agents readily complex with negatively charged nucleic acids, and gold nanoparticles usually bind their payload via thiol bonds. Alternatively, the aqueous interior or a cell ghost, liposome, or exosome provides the flexibility to host almost any water-soluble cargo of appropriate size. Naturally-inspired drug delivery systems like viruses and bacteria are being re-examined and re-engineered with synthetic nanocarriers for improved drug delivery.[24] The diverse range of carriers that

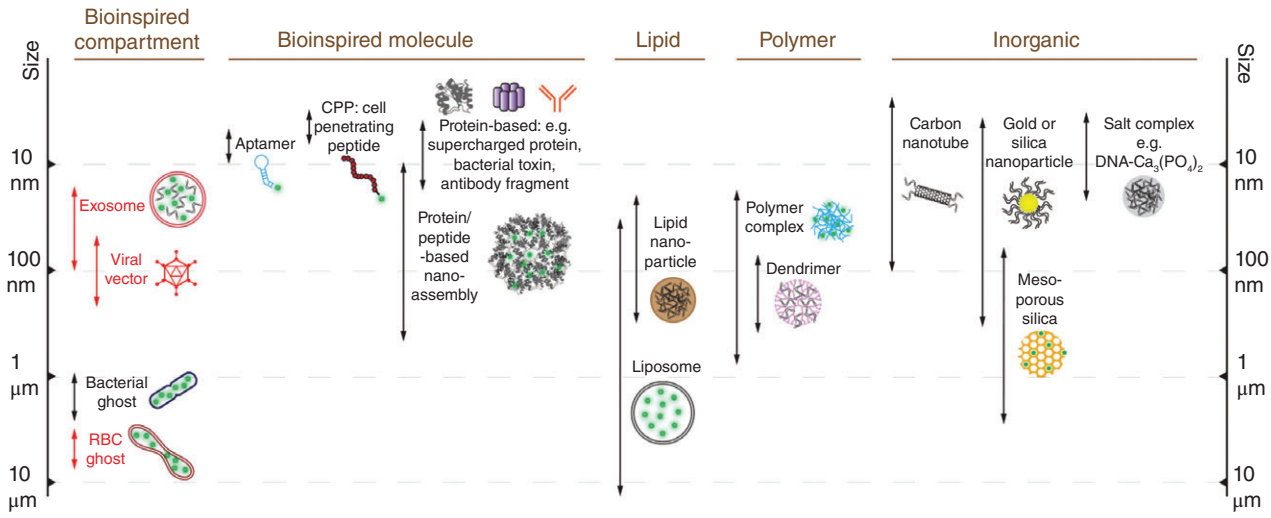

FIGURE 1 | Example of the wide range of carrier architectures employed for intracellular delivery and their approximate size ranges. Carriers with fusogenic potential are shown with red text. The others (black text) generally enter cells via endocytosis. Example cargoes are shown as miscellaneous (green spheres) or nucleic acids (wavy, black lines).

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have been developed are a testament to the alluring potential of carrier-mediated intracellular delivery systems.

CELL SURFACE INTERACTIONS

As a carrier approaches the target cell, the initial interactions at the cell surface are critical, as they govern the subsequent pathway of entry (Figure 2). For hydrophobic, positively charged complexes, such the cationic lipids and polymer vectors used for transfection, the intrinsic negative potential of the plasma membrane and most proteoglycans is thought to facilitate nonspecific binding.[25,26] Beyond that, binding may occur through affinity to different phases of the plasma membrane, such as cholesterol and sphingolipid-rich rafts,[27] or by attachment to extracellular protein domains and carbohydrate moieties.[6,28] For example, glycosaminoglycans at the cell membrane have been shown to be necessary for uptake of several carriers.[6] Engagement of specific receptors may route the vehicle through a defined endocytotic pathway, such as transferrin-conjugates, which traffic through clathrin-mediated endocytosis.[29] Alternatively, fluid phase endocytosis can stochastically capture small particles from the extracellular fluid and into endosomal compartments.[9] It has been shown that varying the density of targeting ligand, such as transferrin, can tune the avidity of the receptor, which can in turn modulate delivery efficiency.[30] As will be highlighted in the next section, specific protein–protein interactions are usually the starting point for fusion of the carrier with the target cell.

FUSION

Cell membrane fusion occurs when two membrane-bound compartments merge (Figure 3). The process has been studied intensively in cell biology where vesicle fusion, cell–cell fusion and cell–virus fusion events are of fundamental importance.[31,32] Juxtaposed membranes are pulled into close contact by specific protein–protein interactions and interfacial water is excluded to promote lipid mixing and fusion. Enveloped viruses use transmembrane viral proteins to mediate fusion with host cell membranes and this mechanism has been harnessed for engineered intracellular delivery. One of the first examples was the use of sendai virus to fuse hypotonically loaded red blood ghost carriers with the plasma membrane of target cells.[33,34] A variation on this technique from Helenius and co-workers involves expression of influenza hemagglutinin (HA) at the target cell membrane, which then binds sialic acid residues on the red blood cell surface to induce fusion.[35] Virosomes, which consist of viral membrane components reconstituted into liposomes or vesicles, have also shown fusion capabilities.[36] Functional virosomes have been constructed with fusion components from sendai,[37] influenza,[38] vesicular stomatitis[39,40] viruses. With the exception of sendai virus, the fusion usually takes place in endosomes where the low pH acts as a trigger for conformational activation of the viral fusion proteins.[41] Recently, it has been shown that exosomes and extracellular vesicles can fuse with target cells.[42] Interestingly, mouse dendritic cells are purported to exchange microRNAs via exosomes that dock, bind, and fuse with acceptor cells.[41] Whether this fusion

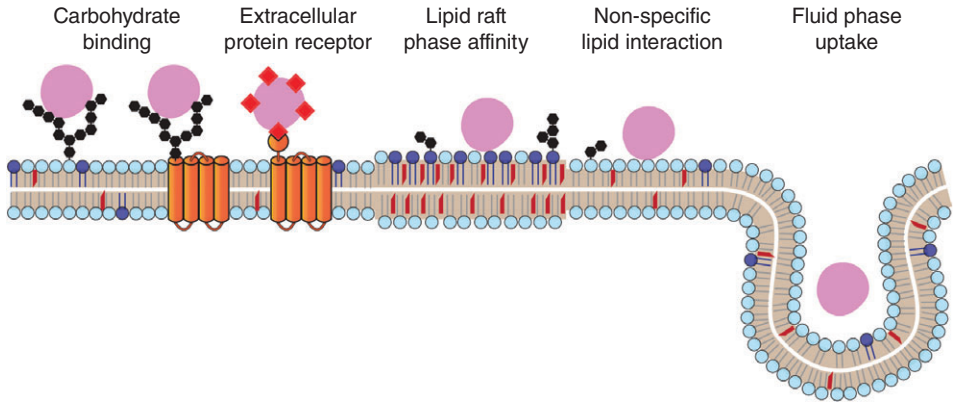

FIGURE 2 | Cell surface interactions direct the pathway of uptake. Carriers interact with the cell surface by specifically or nonspecifically binding exposed carbohydrate moieties from lipids or proteins, extracellular proteins domains/receptors, or different phases of the plasma membrane, such as cholesterol-rich lipid rafts. Alternatively they remain unbound and are taken up by fluid phase endocytosis. Illustrated are generic carriers (purple), ligands (red squares), cholesterol (red/brown wedges), lipid heads (light blue, dark blue circles), bilayer (light brown strip), carbohydrate residues (black hexagons), and membrane proteins (orange).

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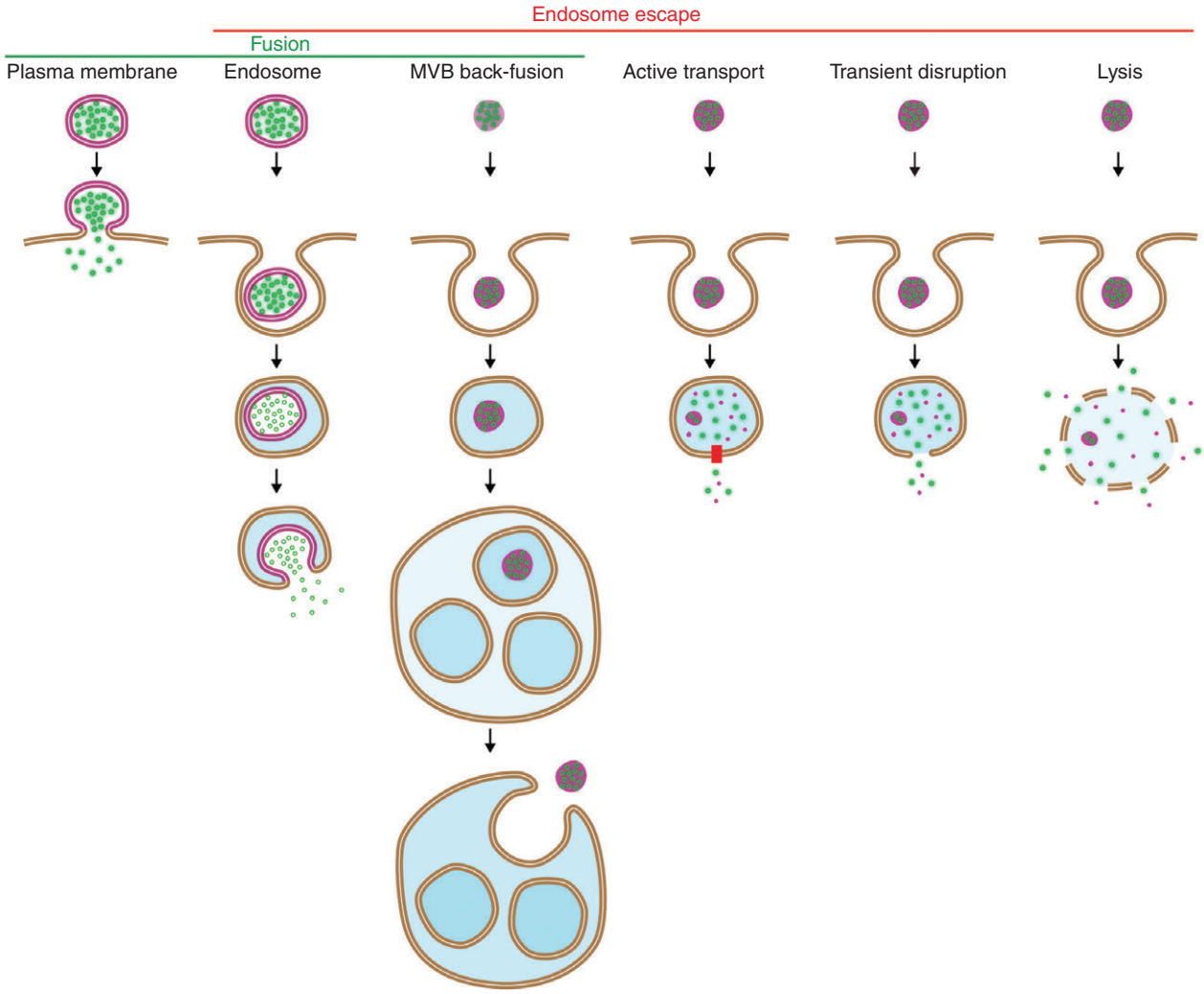

FIGURE 3 | Pathways for carrier-mediated endosomal escape and cytosolic delivery of drugs/nucleic acids. Fusion can occur between a membrane-bound carrier and the plasma membrane, or inside endosomal compartments. Alternatively, back-fusion of a smaller vesicle (ILV) inside a limiting multivesicular body may inadvertently release the cargo. Other purported mechanisms of endosomal escape may involve active transport via membrane proteins (red), passage through transient disruptions or pores, or complete lysis of the containing endosomal compartment.

proceeds via lipids or proteins remains to be seen. Cationic arginine-functionlized gold nanocapsules stabilized with fatty acid nanodroplets are a recently reported example of siRNA delivery directly to the cytosol via proposed fusion mechanism.[43] However, due to relative low amounts of cytosolic delivery through nonviral vectors, it is often difficult to decipher whether cell fusion is the primary mechanism for cytosolic delivery.

ENDOCYTOSIS

In lieu of direct fusion with the plasma membrane, carriers generally enter target cells via endocytosis (Figure 4). Nanocarriers enter cells through a variety of regulated portals, which includes: clathrin-mediated endocytosis, caveolae-mediated, clathrin- and caveolin and micropinocytosis.[29,44,45] Antibody targeted nanocarriers have been utilized to assess these gateways as a mean to cross the endothelial barrier.[46,47] The initial internalization pathways of different carriers have been cataloged in recent reviews.[28,29,48,49] Once inside cells, macromolecules are captured by the pleomorphic early endosomes (EEs). EEs serve as a primary sorting station from where macromolecules are assigned to the late endosomes (LEs) or toward the recycling compartment. Macropinosomes can bypass EEs and shuttle cargo directly into the LEs.[48] Direct mass transfer from the LE to the lysosome contributes to the majority of nanocarriers being trapped inside the multilamellar

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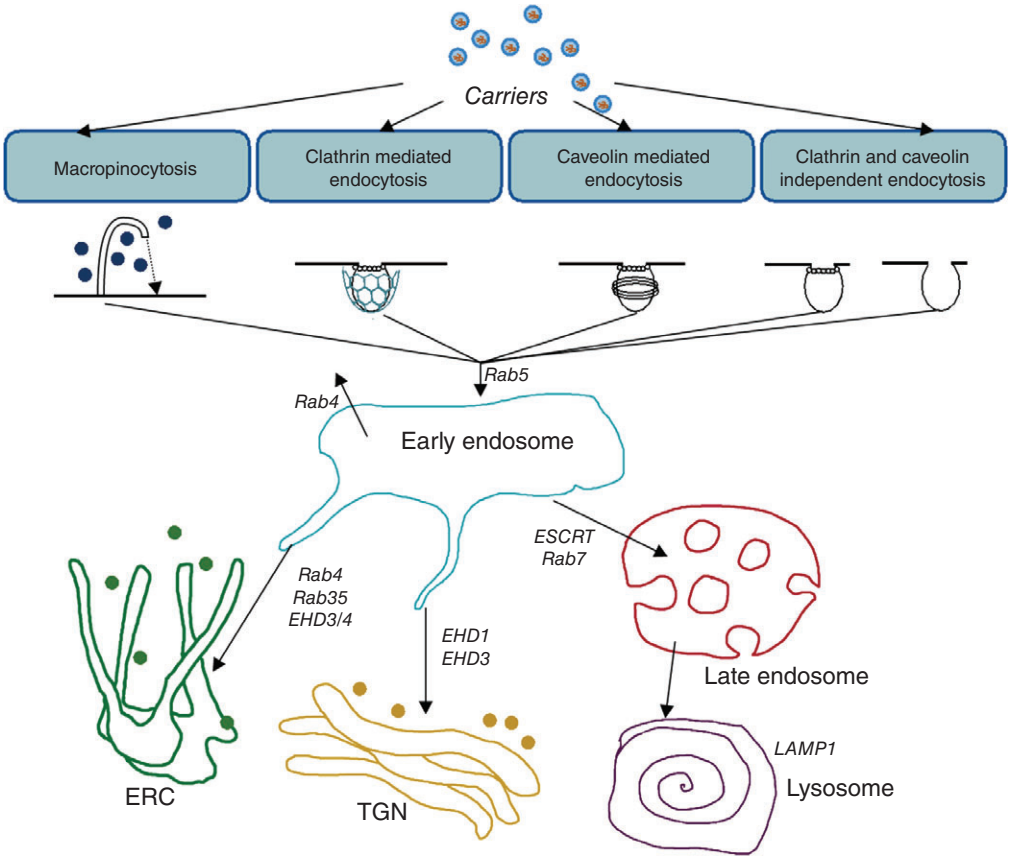

FIGURE 4 | Intracellular transport of macromolecules in the endo/lysosomal system. Nanoparticles can utilize multiple pathways to enter cells, once inside the cargo faces a changing environment of the maturing endosome, i.e., decrease in pH and shape change, the unilamellar EE, becomes multivesicular, followed by a multilamellar lysosome. Several effector proteins bind to the cytosolic end of the endosomal lumen and transport the vesicles to different subcellular organelles. Genetic manipulation of these components has been used to unravel endosomal transport of different bacteria and viruses. Investigation of nanocarriers–endosome interactions using these methods can reveal new methods to enhance endosomal escape.

lysosomes, preventing any endosomal escape (Figure 4).[50] Upon engulfment into the membrane trafficking system, the carrier and cargo may either escape via a fusion event or other escape mechanisms (Figure 3). Even for bio-inspired membrane-bound carrier types that exhibit the potential for direct fusion, such as viruses and exosomes, the majority still enter through endocytosis.[42,51,52] For nonmembrane-bound carriers and synthetic nanocarriers the uptake mechanisms are almost always through endocytosis.

To gain insight into endosomal escape processes and methods of re-engineering it is therefore imperative to understand the biogenesis, structure and microenvironment within the distinct compartments that play a role in trafficking of carriers. In the following sections we provide an overview of the key endocytic agents and regulators that determine the biogenesis and compartmentalization of endosomes and discuss the putative sites for nanoparticle–endosome interactions (Figure 4).

Early Endosomes

After the sequestration of nanoparticles from the cell surface, EEs are usually the first set of distinct compartments that accept macromolecules.[53] EEs are pleomorphic structures that serve as a focal point through which cargo is routed to either the recycling compartment, LE/lysosomes or delivered to the trans-golgi network (Figure 4).[54] The EEs develop unique micro-domains within their structure that are maintained by select endocytic proteins and lipids that sort macromolecules to different destinations. Ras-associate protein (Rab)5 is the most well studied molecule required for biogenesis of EEs; its binding to different effector proteins on a vesicle leads to maturation into an EE. Overexpression of Rab5's active

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forms potentiates fusion between distinct EEs, resulting in an enlarged vesicle. Similarly, inhibition of all three different isoforms of Rab5 (a, b and c), causes a complete inhibition of endo/lysosomal system *in vitro* and *in vivo*.[55] Several kinesins bind to Rab5 which, in turn, interact with microtubules to route macromolecules toward the LEs or to the cell periphery.[53] EE formation is very dynamic, thus observing interactions of nanoparticles with this compartment has proven to be difficult. However, use of mutant Rab5 that causes formation of enlarged EEs with extended lifespan allowed visualization of nanoparticle–EE interaction and facilitated visualization of endosomal entrapment of select materials within this compartment.[56] Small molecules that inhibit EE progression toward lysosomes have been proposed to improve nucleic acid delivery.[57,58] It is yet to be seen whether the lipid composition or unilamellar structure of the EE makes it an ideal location for nanoparticles to escape. Therefore, the EE remains an exciting target for increasing cytosolic delivery of nanoparticles before they are destined for downstream degradation.

Recycling Endosomes

Recycling from the cell occurs in two phases.[59] First, an early/fast-recycling event occurs as soon as a molecule (e.g., transferrin receptor) reaches the EE. Rab4 and Rab35 have been identified as the key regulators that orchestrate fast recycling, silencing of these components have been shown to increase accumulation of transferrin. Second, a late/slow recycling event that transfers the cargo from EE's to a tubular compartment known as the endocytic recycling compartment (ERC) (Figure 4). Rab11 governs the biogenesis and transport from the juxta-nuclear ERC. A molecule that enters through a clathrin independent pathway is generally recycled through an Arf6 (ADP-ribosylation factor 6) mediated endocytic compartment. Rab11 and Arf6 recruit EHD (C-terminal Eps15 Homology Domain ATPase) proteins responsible for fission and tubular morphology of the recycling compartments.[60] Poly(lactic-co-glycolic acid) based nanoparticle have been shown to recycle through this pathway;[61] however, the interactions of carriers with the ERC are not clearly understood.

Late Endosomes

LEs are multivesicular structures formed from the maturation of EEs (Figure 4).[62] The intraluminal vesicles (ILV) that form within the lumen of an endosome during its maturation into a LE gives them this unique morphology. It is the endosomal sorting complexes required for transport (ESCRT) complex, which constitutes four oligomeric proteins (ESCRT 0, I, II, III), that organizes ILV formation. The ESCRT complex choreographs the inward invagination of the outer limiting endosomal membrane, followed by its pinching into the lumen to form ILVs.[63] The formation of ILVs leads to attenuation of cell signaling of promiscuous proteins that remain active at the luminal membrane of an endosome even after internalization. Epidermal growth factor receptor (EGFR) is sequestered inside ILVs to prevent interaction with cytosolic signal transduction molecules and destined for lysosomal degradation.[64] Genetic mutation of the ESCRT complex, such as the silencing of Hepatocyte growth factor tyrosine kinase, caused inhibition of ILV formation and a unilamellar LE is observable via electron microscopy.[65]

There are several other components of the LE that have been implicated as essential to the ability to escape from this compartment. The LE houses the 13 transmembrane protein Niemann Pick Type C-1 (NPC1) on its endosomal surface. The sterol sensing domain of NPC1 interacts with cholesterol and other lipids to facilitate their recycling from the cell. NPC1 deficient cells have been observed with increases in number and size of LE's, which improves cytosolic delivery of siRNA.[66] Rab27a or Rab27b are responsible for the transport of LE's toward the cellular periphery in macrophages; they are involved in cell–cell communication by secreting ILVs, which then become exosomes, to the cell surface.[67] Inhibition of Rab27 isoforms inhibit exosome secretion and was recently shown to improve lipid-based delivery of siRNA.[66] Exosomes are now being investigated for cell specific delivery of nucleic acids and proteins, especially to treat neurological disorders.[68,69] However, the potential endosomal escape capabilities of exosomes remain a point of active research. Finally, several studies suggest LE to be the site for release of oligonucleotides from endosomes,[66,70,71] yet it is possible that escape from LEs may be cell type dependent and/or based on the type of the carriers or cargo.

Lysosomes

Lysosomes serve as a ubiquitous catabolic site for the molecules that have entered the cells. These compartments receive material for degradation from the endosomal system, autophagy-based processes, and phagosomes from immune cells.[72,73] Antigen presentation and microbial killing is triggered through lysosome-dependent degradation, which can be severely attenuated through defective endo/lysosomal

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transport. Consignment to lysosomes has significant impact on the ability of nanoparticle carriers to deliver their cargo. Recent studies have suggested that enhanced stabilization of nucleic acids can prevent degradation of therapeutic molecules in lysosomes and other compartment, hence improving their efficacy.[74] Unique membrane proteins like LAMPs and Arl (Arf like protein) have been identified that maintain the integrity, transport and biogenesis of the lysosomal compartment.[75] Whether perturbation of these regulators influences nanoparticle-mediated drug delivery, or whether altered lysosomal structure affects progression of carriers to the cytosol, is under investigation in several labs, including ours.

Autophagy

Autophagy is a catabolic process through which proteins that are prone to cytosolic aggregation and unneeded cellular organelles are degraded.[76] Different autophagy-based genes elicit membrane-derived sequestration of defective proteins and defective organelles in autophagosomes. Subsequently, the autophagosomes fuse with LEs to form amphisomes which are directed toward the lysosomal pathway for the destruction of these cytosolic components (Figure 5). Polymeric nanoparticles have shown the ability to induce autophagy in macrophages, which may be associated with toxicity related to these systems.[77] Lipoplexes used to deliver DNA are captured by autophagosomes and these carrier systems have an ability to promote autophagy in cells. Interestingly, the absence of autophagy-based genes, led to a fivefold increase in gene delivery and expression, suggesting that autophagy pathways can be an additional barrier for nanocarriers for gene delivery.[78] However, other studies have suggested that, as the endosomal escape occurs at an earlier time frame of trafficking, targeting autophagosome-sequestered nanocarriers may not alter efficacy of nucleic acid delivery.[79]

In light of the contradictory reports it is likely that endosomal escape processes are highly dependent on carrier material and cargo type. To pinpoint a single compartment that is responsible for endosomal escape for different groups of nanocarriers remains an unmet challenge.

MECHANISMS OF ENDOSOMAL ESCAPE

To achieve substantial endosomal escape the carrier must first induce significant endosomal uptake. Key physicochemical properties identified in determining uptake propensity are size, shape, material composition, charge, and surface chemistry.[29] For example, multiple studies have determined the optimal particle size for promoting efficient endocytosis to be 50–100 nm.[6,29] However, for many of these rules, variations between cell types make it difficult to apply broad models.[29] Indeed, despite a multitude of

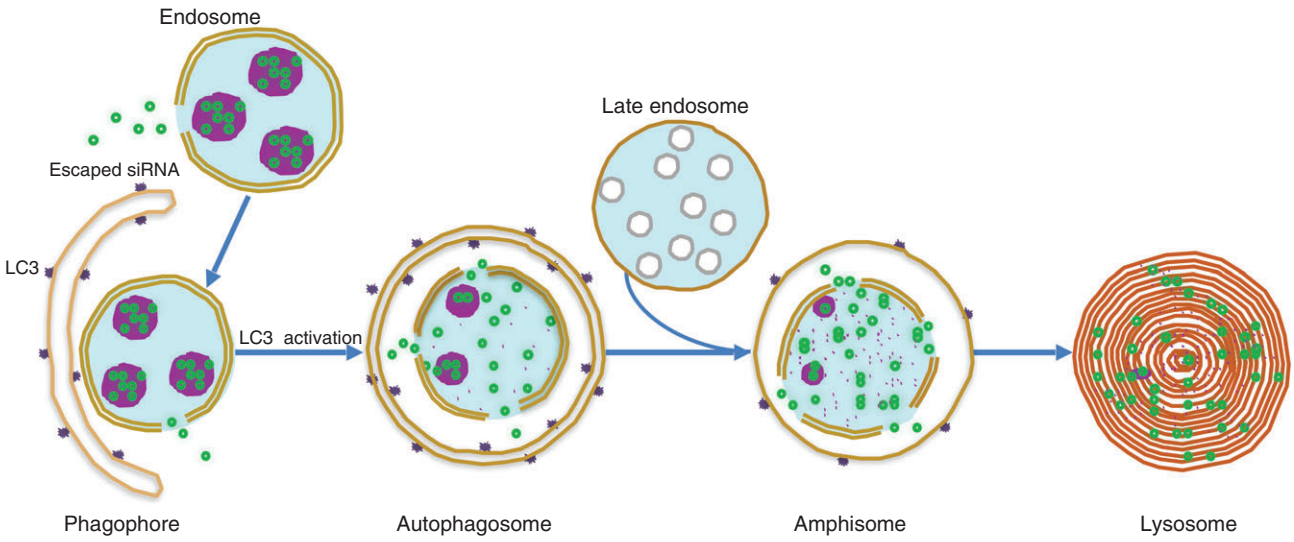

FIGURE 5 | Autophagy-based capture of transiently disrupted endosomes. Activation of autophagy leads to the formation of a, phagophore, that recruits microtubule-associated protein 1A/1B-light chain 3 (LC3), onto its surface, leading to sequestration of cytosolic components or organelles within the cell into a double membrane-auto-phagosome, which fuses, with the endo/lysosomal system for degradation of its constituents. Few nanoparticles can penetrate endosomal membranes and cause transient vesicular disruption, initiating autophagy. These vesicular compartments containing large amounts of drug-carriers are quarantined in autophagosomes and directed to lysosomal degradation.

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screens seeking to tune the physicochemical properties of nonviral nanocarriers for optimal cellular entry, most material remains trapped in endosomes post-uptake.[28,29,48,49] For polymer- and lipid-based vectors commonly used for nucleic acid transfection, quantitative studies have consistently indicated maximum delivery efficiencies of 1–2% due to entrapment within endosomes[57,80] or recycling back to the extracellular space.[66] Not only is this wasteful, but it leaves ~98% of vector material to exert toxic effects.[49] Thus, a long-standing challenge for many carrier-mediated technologies has remained efficient delivery beyond endosomal compartments.

Exogenous material taken up by endocytosis is destined either for degradation in autolysosomes or recycling back to the extracellular space. Endosomal progression is characterized by a drop in pH, from 6.5 in EEs to 4–5 in lysosomes.[81] The shape of the vesicles themselves also changes, from unilamellar structures to multivesicular bodies (MVBs) and finally to multilamellar lysosomes (Figure 4). The changing endosomal environment can serve as a trigger for escape of both pathogens and synthetic carriers. Indeed, the effectiveness of the much-used polymer PEI as a delivery vector was originally ascribed to the 'proton sponge' model, where it was thought that an abundance of proton buffering amine groups retard pH decrease and facilitate osmotic bursting of endosomes.[82] However, recent mechanistic studies have struggled to observe the expected disruption events and revealed inconsistencies with this concept.[83,84] In the case of lipid carriers, disassembly of the internalized material is proposed to free up the exogenous lipids to perturb native phospholipid structure of the endosomal membrane.[85] Dynamic poly-conjugates (DPCs) have been proposed to deliver siRNA by triggering escape through endosomal lysis.[86] Again, such models remain difficult to verify and appear to require revision. The mechanisms of escape from endosomes remain elusive partly due to lack of measurable escape from vesicular confinement.

Far more is known about how viral and bacterial components mediate egress from vesicular compartments. Most fundamental discoveries have been elucidated from studies of pathogens.[52,87] In general, endosome escape strategies can be categorized into endosome disruption by (1) transient breakdown or (2) complete lysis; (3) active transport of small molecules; and fusion either through (4) back-fusion of MVBs with the outer limiting membrane or (5) merging of the carrier with the endosomal membrane (Figure 3). Vaccinia and Influenza virus respond to the declining pH of maturing endosomes either by triggering lysis of EEs or fusion with the outer limiting membrane.[52] Ebola virus has been shown to utilize the cholesterol transporter Niemann Pick Type C-1 to escape LEs/lysosomes, and deficiency of NPC1 leads to attenuation of Ebola infection. More recently, endosomal calcium channels known as two-pore channels (TPCs) have been implicated in infection caused by Ebola in target cells.[88] Nakamura et al. demonstrated the existence of peptide transporters that mediate active transport of bacterial muramyl dipeptide from EEs.[89] Vesicular stomatitis virus is sequestered in the MVBs of the LE and triggers back-fusion to promote escape.[87] A haploid screen revealed that the sialyltransferase ST3GAL4 was required for the interaction of the Lassa virus glycoprotein with LAMP1 to achieve endosomal escape. Interestingly, Lamp1-deficient mice show resistance to Lassa virus, further suggesting that molecular transporters have an important role in endosomal escape. Studies of endogenous cell death have also yielded insights. The immune pore-forming toxin perforin is thought to enable the delivery of granzymes by stimulating endocytosis of plasma membrane followed by partial or full rupture of enlarged endosomes, as observed by dispersion of endosomal cargo in real time.[90] Another example is the involution of mammary epithelial cells, where milk fat globules containing triglycerides are trafficked into swollen vacuolar lysosomes and broken down by acid lipases into products including the membrane-perturbing oleic acid. These breakdown products then permeabilize the lyosozomal membrane to trigger release of cell death-inducing proteases.[91] Such studies of endosomal escape in other biological contexts thus reveal possible models that carriers may inadvertently utilize to gain access to the cytosol.

Recent mechanistic studies on the cell biology of carrier entry are now complementing the aforementioned scenarios. In the case of siRNA delivery, for example, small lipophile-siRNAs conjugates can be shuttled across membranes by the transporter Sid-1.[92] Gilleron et al. demonstrated siRNA-lipid nanoparticles perturb membrane trafficking progression by inducing the formation of early/late hybrid compartments, from which delayed trafficking may boost the probability of escape *in vitro and in vivo*.[57] Sahay et al., have shown that endocytic recycling back to the cell exterior limits the escape of siRNA-lipid carriers and that precise perturbations of membrane trafficking genes can significantly modulate delivery efficiency.[66] Gilleron et al., and Yang et al., leveraged high throughput screening to identify specific small molecule enhancers that improve escape of nucleic acid cargo from EEs and LEs, respectively.[58,71] In addition, small molecules were

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identified that drive the efficient intracellular delivery of native proteins from the extracellular solution. These molecules trigger macropinocytotic uptake and intracellular release of extracellularly applied macromolecules.[93] Moreover, materials that undergo breakdown into constituent components in endosomes may make them more amenable to active transport or leakage through transient disruptions and leaks.[57,66,92] In congruence with this notion, the recent success of small conjugates may be in part due to their compact, well-defined, and stable structures and minimal vector material.[4] Furthermore, for most nanocarriers, insights into how their surfaces bind absorbents from extracellular fluids is guiding our understanding of their interactions with cells.[94] For example, in the cardiovascular system *in vivo*, endogenous small micro RNAs have been found to complex with high-density lipoproteins to facilitate intracellular uptake via specific cellular transport processes.[95] In addition, different classes of bacterial pore-forming proteins have been employed to trigger endosomal escape, although the potent cytotoxicity of these molecules and potential immune response remain a concern with this technology. Recently, however, Yang et al., designed and engineered a bispecific, neutralizing antibody against Perfringolysin O a bacterial pore-forming protein that targeted the endosomal compartment and mediated endosomal release of gelonin construct with high specificity and minimal toxicity *in vitro*.[96]

For carriers that traffic through endocytosis, one of the prime challenges has been the difficulty in observing membrane perturbation events and cargo release in real time. New tools are currently being implemented that can capture intracellular events with sufficiently high spatiotemporal resolution, such as multiresolution 3D tracking.[97] Wittrup et al. recently developed a sensitive time-lapse imaging method with large dynamic range to detect cytosolic siRNA release from cells treated with lipid carriers.[79] They found that siRNA release occurs in a 5–15 min post-uptake 'window of opportunity' from maturing endosomes, and not from LEs or lysosomes. The fast diffusion (~10 s) of fluorescently tagged siRNA throughput the cell cytosol suggested it was released in free (uncomplexed) form. Release was also detected as a discreet event, with the majority of cargo remaining in an intact endosome, indicating recovery of the endosome. This would fit with a 'transient disruption' model illustrated in Figure 3. Interestingly, Wittrup et al., found that galectins recognized the compromised endosome and targeted it for autophagy, although inhibiting autophagy did not enhance siRNA release. In future, analyzing galectin recruitment to endosomes is an example of an assay that could be used to track transient disruption events. Establishment of assays that yield mechanistically rigorous data are expected to serve as platforms for screening the effectiveness of carrier systems arising from new advances in materials science, nanotechnology and chemistry.[57] Such strategies may pave the way toward exploiting endogenous pathways to overcome the decades old challenges of endosome escape and poor delivery to recalcitrant cells and tissues.

CONCLUSIONS

Future studies on the cell biology of carrier uptake are anticipated to bring insights that improve delivery of macromolecules to the intracellular space. Such advances will be critical to emerging therapies and biomedical applications that rely on intracellular delivery of carriers. The field should benefit greatly from deeper studies on how viruses and bacterial toxins, and immune components appropriate host cell membrane trafficking and cellular distribution systems to gain access to intracellular targets.[19] Efforts must be taken to decode the rules governing targeting of synthetic carriers to membrane subdomains, endocytic pathways and compartments, major organelles of the endoplasmic reticulum, golgi, and mitochondria, the cytosol, and nucleus.[27] With the advent of genome editing, haploid screens and RNAi, it is now relatively simple to block specific endocytic effectors that can result in modified vesicular size, structure and shape; which in turn can reveal productive sites for endosomal escape. Although poorly understood, manipulation of endosomal pH has been widely utilized to boost cytoplasmic release until now. On the other hand, the different array of lipid components in endocytic compartments may provide clues that lead to development of new methods to potentiate cytosolic delivery. Improved cooperation between the disciplines of cell biology and drug delivery research should foster agreed standards and protocols to define and determine the mechanisms of carrier-mediated uptake and release.[28,98]

Another way to circumvent the problems of endosomal entrapment is direct fusion. However, the realization of carriers that efficiently fuse with the plasma or endosomal membrane has been elusive. First, although some nanoparticles have been reported to fuse with, or perturb, the membrane for direct entry,[43,99,100] it is unclear to what extent nonmembrane-bound carriers are indeed capable of fusion. There are previous cases where carriers

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purported to proceed through fusion or plasma membrane disruption, such as cell-penetrating peptides, were later shown to enter via endocytosis.[101,102] Secondly, even the rules for fusion of membrane-bound carriers, such as with extracellular vesicles and exosomes, are still being decoded.[41,51] As our knowledge expands, it will be interesting to see whether bio-inspired vesicles and exosomes that fuse directly with the plasma membrane represent a viable approach.

As the development of gene therapy and nanomedicines move forward, carriers that can safely and efficiently deliver to the intracellular space are crucial. Current trends are leaning toward more compact, minimalist structures for carriers, such as those becoming popular for delivery of siRNA therapeutics in clinical trials.[4,79,103] These trials are expected to provide key lessons on intracellular delivery in a clinical scenario. We suggest a major thrust in the field should be to toward identifying the molecular mechanisms of intracellular delivery and re-engineering the most promising carriers based on fundamental cell biology, so that effective intracellular delivery can move beyond endosomal barriers to reach drug targets.

ACKNOWLEDGMENTS

The authors wish to thank College of Pharmacy at Oregon State University for their start up grants (G.S.) and funding for collaborative efforts with the Oregon Translational and Development Institute (OTRADI) (G.S.). M.P.S. is supported through advanced postdoc mobility Swiss NSF fellowship P300P3_151179. As we try to fathom the richness of cell biology to guide development of novel technologies that target deep within a cell, the authors G.S., J.D., and M.S. would like to thank our mentors: Prof(s). Robert Langer (MIT), Daniel Anderson (MIT), Klavs Jensen (MIT) and Alexander Kabanov (UNC), for their continued support and mentorship, which positions us toward moving in this exciting new direction.

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