Endosomal escape for cell-targeted proteins. Going out after going in

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Abstract:

Protein-based nanocarriers are versatile and biocompatible drug delivery systems. They are of particular interest in nanomedicine as they can recruit multiple functions in a single modular polypeptide. Many cell-targeting peptides or protein domains can promote cell uptake when included in these nanoparticles through receptor-mediated endocytosis. In that way, targeting drugs to specific cell receptors allows a selective intracellular delivery process, avoiding potential side effects of the payload. However, once internalized, the endo-lysosomal route taken by the engulfed material usually results in full degradation, preventing their adequate subcellular localization, bioavailability and subsequent therapeutic effect. Thus, entrapment into endo-lysosomes is a main bottleneck in the efficacy of protein-drug nanomedicines. Promoting endosomal escape and preventing lysosomal degradation would make this therapeutic approach clinically plausible. In this review, we discuss the mechanisms intended to evade lysosomal degradation of proteins, with the most relevant examples and associated strategies, and the methods available to measure that effect. In addition, based on the increasing catalogue of peptide domains tailored to face this challenge as components of protein nanocarriers, we emphasize how their particular mechanisms of action can potentially alter the functionality of accompanying protein materials, especially in terms of targeting and specificity in the delivery process.

Keywords:

Protein nanocarriers, Protein engineering, Targeting, Endosomal Escape.

1. Introduction:

Drug targeting is an urgent and unmet need in molecular medicine and especially in oncology, where most of the administered drugs display potent cytotoxic effects (Bazak et al., 2015). In this sense, cell-specific drug delivery is usually explored through the development of nanoscale drug carriers (Raj et al., 2021), that are functionalized with ligands for specific cell surface molecules overexpressed in target cells (Jiang et al., 2019). For that, a nanoscale size reached through the self-assembly of building blocks into homo-oligomers is particularly convenient to favor the intended biodistribution, to exploit the enhanced retention and permeability effect (EPR), and to allow multiple display of the cell ligand for cooperative receptor binding, resulting into highly selective cell penetrability through endosomal routes (Duncan and Gaspar, 2011).

In this context, cell-targeted delivery systems, in form of functional nanoparticles, are being developed as promising nanomedical tools. Among the diversity of materials explored to construct suitable nanoparticles, proteins represent an emerging and very useful category of biomaterials. This is because of their full biocompatibility, biodegradability and easy production in cell factories(Corchero et al., 2014), together with their capability to tailor many of the functions valuable in nanomedicine such as self-assembly or specific molecular recognition and binding. In addition, the extraordinary plasticity of protein materials allows the recruitment of different functional domains such as targeting ligands, architectonic tags or nuclear localization signals, in a single multifunctional polypeptide by simple genetic fusion (Ferrer-Miralles et al., 2015; Vazquez et al., 2009). In this regard, protein drugs can be also incorporated to such constructs resulting in unique species of protein-only, self-organized nanosized therapeutics (Casanova et al., 2019; Serna et al., 2018).

Cell-targeted protein delivery usually relies on receptor-mediated endocytic processes, in which early endosome vesicles rapidly mature into late endosomes and finally fuse with lysosomes. This fusion leads to the complete degradation of the engulfed protein material by lysosomal enzymes and acidic conditions (Bareford and Swaan, 2007; Huotari and Helenius, 2011). Since most of delivered drugs, including proteins, do not perform their activities in lysosomes but in the cell cytoplasm or in other subcellular compartments, internalized nanomaterials usually need to be efficiently released from endosomes to reach their intracellular therapeutic target (Lee et al., 2019). Thus, the entrapment of protein-based therapeutics into endo-lysosomes is a main reason for their limited efficacy (Erickson et al., 2006; LeCher et al., 2017; Pirie et al., 2011). Some strategies as the incorporation of non-natural amino acids are intended to delay protein degradation within the lysosomes (Kato et al., 2016; Yamashita et al., 2014) . However, these strategies could complement but not replace the incorporation of efficient endosomal escape (EE) agents, including peptides or protein domains, which are mandatory to protect their final functionality. To such end, peptides with endosome-disrupting capacities have been recruited into protein constructs through genetic fusion to act in cis, fitting the modular setting (Ahmad et al., 2019; Varkouhi et al., 2011). However, most of the described EE peptides act through fusogenic or pore-forming activities, that being receptor-independent usually promote unspecific cell interactions and penetration, which can potentially impair the highly desired specific cell binding capacities of targeted constructs (Sánchez-García et al., 2017; Serna et al., 2019; Varkouhi et al., 2011). Therefore, although current nanomedical approaches have

already developed a huge armamentarium of EE peptides, it is critical to evaluate which of them are potentially suitable to be incorporated into targeted delivery platforms, without affecting their cell specificity throughout the delivery process.

Taking all these points into consideration, the rational design of protein nanocarriers devoted to targeted intracellular delivery of protein-based and chemical drugs or imaging agents, that are sensitive to lysosomal degradation, should look forward to the incorporation of efficient EE peptides that do not interfere into receptor-mediated internalization. In addition, the fine evaluation of EE also requires very accurate analytical tools especially refined for such purpose. Altogether, this review aims to discuss the different mechanisms of action employed by currently described EE peptides, focusing on their potential interference over cell-surface receptor targeting. Moreover, we will also discuss the recent advances in the methods used to track these processes, highlighting the EE protein domains that have already been empirically tested for receptor-targeted delivery approaches as well as the ones predicted to be especially suited for that.

2. Mechanisms of endosomal escape

Understanding the particular mechanisms used by different peptides and protein domains to control the EE of proteins is a key element for the proper rational design of protein-based nanomedicines (Mejia et al., 2022; Pei, 2022). For that, it is important to note that in the context of cell-targeted proteins, EE peptides are usually recruited within the same modular polypeptide (in cis) in order to induce the EE of the whole protein after receptor-mediated endocytosis. In this sense, although some particular peptides are supposed to induce the EE of accompanying proteins in trans, they could also potentially induce the release of their neighbor internalized polypeptides when incorporated in cis in a fusion protein. In some cases, the incorporation of a furin cleavable site between the different protein modules is also mandatory to allow the endosomal cleavage of the EE domain from the polypeptide to further induce the EE of the cargo protein in trans. All together, the most studied approaches involve the use of membrane fusion peptides (Ahmad et al., 2019) or pore-forming peptides (LeCher et al., 2017) among other more sophisticated strategies.

2.1 Pore formation.

Highly cationic peptides are widely known to induce pore formation in lipid bilayers. They act through three main mechanisms accounted by the barrel stave, carpet or toroidal models (Figure 1A) (Matsuzaki, 2019; Priyadarshini et al., 2022). The barrel-stave model involves the self-assembly of a pore-forming peptide on the surface of the membrane for the subsequent internalization of the protein or oligomer through the hydrophobic region of the lipid bilayer, creating a channel (Figure 1A, i). The carpet model, however, implies a dual-step mechanism in which the oligomerization of the protein is not necessary to create the pore. Instead, the pore-forming peptide binds to lipids on the membrane through electrostatic interactions, covering the surface. Eventually, upon reaching an adequate concentration, a consequent micellization occurs and leads to the membrane dissolution (Figure 1A, ii). Finally, the toroidal mechanism also involves a dual step process in which the pore-forming peptide is first oriented in parallel to the lipid bilayer to be then reoriented perpendicularly to penetrate the hydrophobic region of the membrane. Thus, along with some lipid molecules, they form

lipid-lined pores and contiguous membrane leaflets that result in an irreversible rupture of the membrane (Figure 1A, iii).

2.1.1 Pore-forming peptides in endosomal escape.

Some authors have claimed the use of pore-forming peptides as EE promoters. However, it is important to note that being highly cationic, most of them can also interact with extracellular membranes. Arginine-rich cell penetrating peptides (CPP) such as TAT, Penetratin or 8R are clear examples of this kind of pore-forming motifs, which show interesting endosomolytic activities and can also induce unspecific cell uptake via micropinocytosis (Lönn and Dowdy, 2015). In this regard, the dimeric fluorescent TAT (dfTAT) has been engineered as a potent and non-toxic endosomolytic agent that efficiently induces the cytosolic leakage of accompanying molecules in late endosomes (Erazo-Oliveras et al., 2016, 2014). However, dfTAT was proven to also penetrate cells majorly through micropinocytosis (Allen et al., 2018), which makes it a poor candidate as a partner domain in targeted delivery settings.

Some other pore-forming peptides have exhibited intrinsic cytotoxic effects, which makes them also unsuited for general drug delivery purposes. This set includes the antimicrobial peptide (AMP) GWH1 and other bacterial peptides such as the pneumococcal pneumolysin (Browne et al., 1999), streptococcal streptolysin O (SLO) (Browne et al., 1999) or the *Listeria* listeriolysin O (LLO) (Plaza-GA et al., 2019). Similar problems were reported for the bee venom-derived peptide melittin, whose cell membrane-disrupting activities produced high cell toxicity at neutral pH (Kandil et al., 2019; Paray et al., 2021). However, the engineering of novel peptide analogs with reduced cell cytotoxicity and enhanced endosome-selective pore-forming activity has recently generated growing interest (Paray et al., 2021). In this regard, the substitution of basic residues for glutamic acids in melittin and two other membrane-disrupting AMPs (LL-37 and bombolitin V) produced pH-sensitive analogs with selective membrane disrupting activity at low pH (pH 5.0), with no evidence of cell membrane permeability or toxicity at neutral pHs (Ahmad et al., 2015). The same occurs with the novel synthetic leucine zipper endosomolytic peptide (LZEP), whose pore-forming ability is obtained only under acidic conditions(Ahmad et al., 2021). In a similar way, Aurein 1.2 is an AMP of animal origin that selectively disrupts endosomal membranes via the carpet mechanism (Fernandez et al., 2012). Thus, it enhances the EE of cargo proteins *in vitro* and *in vivo* with no toxicity over mammalian cells (Li et al., 2015). Recently, Aurein 1.2 has been successfully conjugated to an EGFR-targeted fluorescent protein, promoting improved intracellular protein accumulation without affecting its specificity or functionality (Lieser et al., 2022).

Related to that, in an attempt to selectively target other pore-forming peptides to cancer cells, the AMP Ceratotoxin A (CtxA) was also recently conjugated to folic acid and tested over folate-receptor overexpressing KB cancer cells (Mayer et al., 2019). In this sense, CtxA is a 36-aa cationic peptide that efficiently forms pores into cell membranes following the barrel stave model. However, and opposed to expected, folic-acid targeted CtxA (CtxA-FA) showed no difference over KB cancer cells compared to wild type CtxA (Mayer et al., 2019). Similar results were also reported for a Her2-targeted lysteriolysin O (LLO) (Fotoohi-Ardakani et al., 2019). In contrast, recent work targeting LLO to LHRH-receptor showed some extend of target-cell selectivity (Kheirandish et al., 2019).

Nevertheless, all these AMPs exerted their pore-forming activity over external cell membranes, showing no specificity for endosomes.

In summary, despite being quite useful to promote the cytosolic delivery of some accompanying proteins, cationic pore-forming peptides still display important cell penetrating properties that are generally accompanied with unspecific targeting and cell membrane-lysis associated toxicities. Therefore, unless adapted to exhibit endosome-selective activities, they may not be the most appropriate choice when dealing with targeted delivery approaches. Related to that, the described attempts to selectively target pore-forming peptides to cancer cells were in general not very encouraging either (Fotoohi-Ardakani et al., 2019; Kheirandish et al., 2019; Mayer et al., 2019).

2.2. Fusion with lipid membranes.

Some peptides are able to induce the release of the endosomal content by driving the fusion of lipid membranes they are embedded in with the endosomes. This class of peptides are usually rich in bulky hydrophobic residues and show large proportions of glycine and alanine residues (Pei and Buyanova, 2019). Thus, a change in the pH during the endosome maturation process induces a conformational shift in the peptide, which exposes the hydrophobic residues that interact then with the endosomal membranes. This particular mechanism of action is extensively present in viral proteins (Pal, 2021; Somiya and Kuroda, 2020). As a representative example (Figure 1B), the well-known fusogenic protein from the influenza virus hemagglutinin-2 (HA2) undergoes a conformational change at acidic pH due to the protonation of glutamate and aspartate residues. Consequently, the hydrophobic residues of the peptide become exposed and trigger the fusion of the free HA2 end to the endosomal membrane. Thus, both ends of the same HA2 polypeptide become embedded in two parallel (viral and endosomal) membranes. Finally, the protein folds into a hairpin-like conformation that approaches both protein ends leading to a close contact of the two membranes which eventually fuse (Worch, 2014).

2.2.1 Fusogenic peptides in endosomal escape

Most of the EE peptides described in the literature act as fusogenic peptides. This category includes peptides with viral (HA2, L2, gp41, gpH, WNV-ENV, INF-7 or DEN), animal (B18), synthetic (KALA, GALA or GALA3) or even human (S19) origin (Varkouhi et al., 2011). As stated, the HA2 peptide and its derivatives (as INF-7) are the most representative members of this group(Lin et al., 2014; Sung et al., 2013). However, although having shown potential in targeted delivery approaches (Liu et al., 2017; Sala et al., 2019) there are still important concerns about whether the peptides remain attached to endosomal membranes after promoting their lysis (Lee et al., 2011) and the influence of these agents over the selectivity of proteins intended to be delivered in a targeted way (Lee et al., 2011; Lieser et al., 2022; Sánchez-García et al., 2017).

Similar worries arise with the use of GALA, a synthetic amphipathic peptide consisting in EALA (glutamic acid, alanine, leucine, alanine) repetitions, whose activity is based on glutamic acid protonation at acidic pH (Nakase et al., 2011). Thus, the incorporation of specific acidic amino acids (Glu) in the sequence has served to generate a pH-sensitive peptide, whose charge completely changes from physiological to acidic pH, resulting in a pH-selective membrane interaction activity (Ahmad et al., 2019). In this sense, despite

maintaining some specificity in a targeted approach(Lieser et al., 2022), GALA has failed to induce the cytosolic release of some proteins due to its high hydrophobicity and tendency to aggregate on the plasma membrane (Li et al., 2020). A rationally-modified version (GALA3) with reduced repeated units of EALA has successfully solved this problem and has efficiently induced the EE of a non-targeted fusion protein (Li et al., 2020). However, further exploration is required to determine the suitability of this new version for targeted delivery settings.

Related to that, the PreS2-domain of the hepatitis-B virus surface antigen is one of the first reported fusogenic peptides that despite being described as a cell permeable motif (Oess and Hildt, 2000) has been used to promote the cytosolic delivery of an EGFR-targeted protein (Saporin), reducing its side effects and enhancing its antitumor activity in vivo (Fuchs et al., 2007). The 19-aa peptide DEN (from the Dengue virus envelope protein) is another recently reported fusogenic peptide (Huang et al., 2010) that, by showing selective affinity for specific lipids present in the late endosomes (Zaitseva et al., 2010), has also been successfully used in targeted delivery approaches (Kiesgen et al., 2014). In this sense, the addition of DEN efficiently promoted the *in vitro* cytotoxic activity of an EGFR-targeted Ranpirnase (RNase) without affecting its cells selectivity (Kiesgen et al., 2014). Therefore, enlightened by the promising applicability of these peptides in targeted delivery, their potential immune reactivity, derived from their viral origin, needs to be still addressed.

A similar concern was raised regarding the use of the 18-aa fusogenic peptide (B18) from the sea urchin fertilization protein (animal origin) that was successfully used for the intracellular delivery a of TAT-guided GFP protein (Niikura et al., 2015) and in an EGFR-targeted approach (Niikura et al., 2016). To solve this problem, a potentially less immunogenic 19-aa fusogenic peptide (S19) derived from the human protein syncytin 1 has been recently described by the same group and successfully used to enhance the intracellular delivery of different proteins including GFP, SNAP-tag or β-galactosidase into mammalian cells in absence of cytoxicity (Sudo et al., 2017). However, although the reported assays suggested an endosome-specific affinity of S19, cell uptake was still induced by TAT-mediated micropinocytosis in the tested proteins. Therefore, its real influence over a targeted setting is yet to be determined.

All in all, the use of fusogenic peptides that undergo conformational changes at acidic pH and that therefore become active at late endosomes, is an attractive strategy because of their small size, presumable endosome-specificity and lack of cell toxicity. Nevertheless, it is still unclear how peptides that promote fusion between lipidic membranes (which makes them extremely appealing for membrane-based delivery approaches such as liposomes) can assist the release of proteins without a lipidic envelope from the endosomal lumen to the cytosol. Therefore, although some promising peptides as B18, DEN or S19 have already been reported, further exploration is needed to better understand their particular mechanism of action and determine if their general use in targeted delivery settings is a near reality or not.

2.3 Proton-sponge mechanism

Basic amino acids are hydrophilic, polar and cationic in nature. In this sense, histidine is the only basic amino acid that is found deprotonated at physiological pH and becomes protonated at slightly lower pH (pKa = 6) (He et al., 2020). During the acidification

process of endosomes, the imidazole ring of histidine gets protonated, accepting hydrogen molecules from the medium and neutralizing the endosomal pH. This fact results in an increased active transport of hydrogen protons inside the endosomes along with an influx of chloride ions to reach the desired pH. In consequence, a passive transport of water molecules into the endosomes promotes their osmotic swelling and subsequent lysis. This event is known as the proton sponge effect (Figure 1C). Based on such principle, the presence of histidine residues in a given protein sequences has already been reported to assist its endo-lysosomal escape (Liu et al., 2022; Váňová et al., 2022).

2.3.1 Histidine-rich peptides in endosomal escape

Genetic fusion of histidine tags, mainly hexahistidine, is a very common procedure in biotechnology, not only to allow immobilized metal affinity chromatography (IMAC) of recombinant proteins but also, as an architectonic tag for protein assembly as nanoscale entities (López-Laguna et al., 2022). In this context, the role of polyhistidines as EE peptides has gained increasing interest and has been extensively studied, especially in targeted drug delivery, as such peptides do not interfere with the targeting properties of the protein (Dhankher et al., 2021; Yu et al., 2021). Interestingly, and as an additional feature, the incorporation of hexahistidine tags in a carrier protein allows its direct release into the cytosol through the proton sponge effect, avoiding any need to interact with lipid bilayers. Being then hexahistidine peptides appealing in drug delivery, their potential immunogenicity has been recently addressed by the generation of humanized histidine-rich variants that retain endosomolytic properties while still allowing an efficient purification via IMAC (López-Laguna et al., 2020).

Other histidine-rich peptides have also been used to induce the cytosolic release of proteins intended for targeted delivery. As an example, the peptide ppTG21 successfully promotes the endosomal escape of CRISPR-Cas ribonucleoproteins in trans in a cell-targeted system (Rouet and Christ, 2019). Histidine residues have also been incorporated into previously described pore-forming peptides to improve their endosomolytic properties. In this regard, EB1 is a synthetic analog of penetratin in which certain residues have been substituted for histidines to yield an amphipathic alpha helix upon protonation at acidic pH (Lundberg et al., 2007).

Therefore, the use of histidine-rich peptides appears as a very promising strategy to induce the cytosolic release of targeted fusion proteins. In this regard, the major challenge related to the use of this type of EE peptides is determining the minimal amount of histidine residues that need to be incorporated to promote an effective proton sponge effect within the endosome.

2.4 Protein translocation to the cytosol

This particular EE mechanism has been mainly described in the diphtheria toxin (DT) from *Corynebacterium diphtheriae* (Ladokhin et al., 2021). DT is a potent toxin composed of three different domains including a receptor (R domain), a translocation (T domain) and a catalytic domain (C domain). Thus, the R domain servers as a ligand for the receptor-mediated endocytosis of the toxin. Then, a furin cleavable site located between the T and C domains, drives the separation of both domains at early endosomes, which remain attached through an intermolecular disulfide bond. Later, at

acidified endosomes, the T domain undergoes a conformational change that results in its endosomal membrane insertion, forming a small pore through which the C domain is specifically transported to the cytosol. There, the intermolecular disulfide bond is immediately cleaved by cytosolic thioredoxin reductase and the C domain is finally refolded back into its active conformation (assisted by cytosolic Hsp90 chaperone) to catalyze the ADP-ribosylation of the elongation factor 2 and promote cell death (Figure 1D) (Schuster et al., 2017).

A similar strategy was reported for the anthrax toxin from *Bacillus anthracis*. However, here, the protective antigen (PA) is both the receptor-binding and the pore-forming subunit (Friebe et al., 2016). Thus, once internalized by receptor-mediated endocytosis, the PA undergoes a conformational change at early endosomes that leads to pore formation and subsequent translocation of the enzymatic subunits across the membrane (Friebe et al., 2016). In this regard, although PA has already served for the translocation of some proteins (Bachran et al., 2013; Liao et al., 2014), its use in a targeted setting is not indicated because the PA itself shares both, intrinsic cell-targeting and EE properties.

2.4.1 Diphtheria-toxin assisted translocation in endosomal escape

Fused to the N-terminal catalytic domain of the toxin, several proteins of multiple sizes and structures including Sumo, α-amylase or m-Cherry, have been efficiently delivered to the cytoplasm of cells, hijacking the translocation process of the toxin (Auger et al., 2015). However, this strategy requires the use of both T (21.6 kDa) and C (20.4 kDa) domains of the toxin in order to transport other accompanying proteins to the cytosol.

The T domain alone has also been reported to be sufficient to promote EE of targeted proteins (Gilyazova et al., 2006). Nonetheless, it is still unclear how the T domain might assist the translocation of other proteins in absence of their disulfide linking. In this regard, a small region of the catalytic domain that includes its target cysteine to allow the disulfide-bond formation has been also incorporated to some protein designs (Aullo et al., 1993). Going still further, direct disulfide-conjugation to the diphtheria T-domain has also allowed the successful cytosolic delivery of the catalytic domain from the toxin ricin (Sundan et al., 1982). However, a partial interference of the T Domain over the receptor-specificity of a tumor targeted oligomeric protein nanoparticle has been also recently reported (Voltà-Durán et al., 2022).

Therefore, the diphtheria toxin T domain appears as an attractive tool in targeted proteins delivery since it shows high protein specificity, exclusively transporting the tagged protein without disrupting endosomes. However, its potential interference over the cell-targeting of some protein constructs needs to be closely watched. Also, the minimal domain segment required for such process needs to be still fully determined and its potential immunogenicity, derived from its large size and bacterial origin, needs to be also addressed.

2.5 Lysosomal bypassing

Some pathogens and many plant and bacterial toxins such as *Pseudomonas aeruginosa* exotoxin A, ricin, cholera toxin, Shiga toxin, pertussis toxin or cytolethal distending toxins, hijack the Endoplasmic Reticulum-Associated Degradation (ERAD) mechanism to reach the cell cytosol and avoid lysosomal degradation (Morito and Nagata, 2015;

Nowakowska-Gołacka et al., 2019). This protein clearance mechanism allows misfolded, misassembled, or metabolically regulated proteins to be specifically dislocated from the endothelial reticulum into the cytosol to be degraded. Thus, once internalized by receptor-mediated endocytosis, toxins are retrogradely transported to the endoplasmic reticulum (ER), where the enzymatically active subunit is disassembled from the holotoxin and delivered to the cytosol (Figure 1E) (Nowakowska-Gołacka et al., 2019). It is important to note that the process of retrograde transport, extraction from the ER and refolding in the cytosol is particular for each toxin and it involves complex procedures in which different receptors, chaperones and other proteins take part (Mantis, 2012; Sowa-Rogozińska et al., 2019; Tsai et al., 2001).

2.5.1 Lysosomal bypassing in endosomal escape

Taking advantage of the ERAD pathway, some protein fragments from the above-mentioned toxins have been recently used to drive the lysosomal bypassing of cargo proteins. For example, the translocation domain of *P. aeruginosa* exotoxin A has allowed the cytosolic delivery of different proteins including GFP and monobodies (Mohammed et al., 2012; Schmit et al., 2019). However, there is still a need to understand which is the exact mechanism by which fused proteins are efficiently translocated from the ER to the cytosol and then properly refolded to avoid their degradation.

In an attempt to reduce the toxin segment needed for this process, a simpler approach that just includes the furin cleavable site (FCS) and the C-terminal signal tetrapeptide KDEL has been successfully used to induce the cytosolic delivery of CXCR4-targeted catalytic segment of exotoxin A and ricin toxin (Díaz et al., 2018; Sánchez-García et al., 2018). Of note, both constructions lacked the full translocation domain, but also, both toxins originally use the ERAD mechanism in their natural intoxication process (Inoue et al., 2011; Michalska and Wolf, 2015). Therefore, it is important to know if this simpler strategy can be transversally used for the lysosomal bypassing of other cargo proteins or is strictly restricted to specific toxins.

Taking all presented EE mechanisms into consideration, the stronger candidates to be potentially included in targeted delivery protein-based nanocarriers have been summarized in **Table 1.** Here, pore-forming peptides are presented as probably the less suitable candidates since their cell penetrating properties can strongly interfere with protein targeting. In consequence, just few engineered pH-sensitive analogs derived from antimicrobial peptides (Mellitin, Bombilitin V, LL-37) and especially the animal peptide Aurein 1.2 has raised interest for a targeted-delivery setting. Fusogenic peptides could represent a more sophisticated option since their pH-selective activity better restricts their fusogenic action to endosomes. Moreover, some of the candidates (DEN, preS2, B18) have already been validated in EGFR-targeted delivery approaches, and human-derived peptides have also been described (S19). Nevertheless, the precise mechanism by which peptides that promote membrane fusion are able to induce the transportation of proteins across the endosomal membrane is still unclear. Histidine-rich peptides are presented in this review as one of the most promising candidates for cell-targeted delivery settings. This is because the proton sponge effect is endosome-exclusive and although they display milder endosomolytic potency, they show no interference with extracellular protein targeting. Moreover, humanized candidates such as H5E (HEHEHEHEHE) have been also successfully developed(López-Laguna et al.,

2020). Finally, the use of particular fragments of toxin proteins for direct endosomal translocation (Diphteria toxin T Domain) or lysosomal by-passing (Exotoxin A T Domain, FCS + KDEL) has raised substantial interest but also important concerns at the same time. In this sense, they appear as attractive strategies because of their high protein specificity and general compatibility with protein targeting. However, their exact mechanism of action is yet to be revealed and in some cases their use may be also restricted to certain proteins or show partial interferences with targeting. Additionally, toxin domains are generally large segments within bacterial proteins (up to 21 kDa). Thus, their potential immunogenicity requires proper assessment if they are to be used in a clinical setting.

3. Analytical evaluation of the endosomal escape:

Appropriate tools for EE evaluation are important to properly determine the adequate performance of the EE elements incorporated to drug delivery platforms. In this sense, many methodologies, based on diverse protein properties, have been proposed to follow-up their intracellular fate. In general, all these strategies can be classified in two main groups that include basic and more simple assays performed using artificial membranes (*ex cellulo*) or complete and more complex assays performed in real cells (*in cellulo*).

3.1 Ex cellulo assays

Membrane models such as liposomes have been frequently used as a preliminary test for EE of peptides and protein domains. Although representing an easy model for membrane interaction studies, liposomes do not completely mimic endosomal membranes. In this sense, they lack protein content and lipid asymmetry, but more importantly, they also lack the cellular microenvironment. To improve their similarity, liposomes containing mixtures of zwitterionic 2-oleoyl-1-pamlitoyl-sn-glyecro-3-phosphocholine (POPC) and negatively charged phospholipids have been prepared (Akishiba et al., 2017; Madani et al., 2011). Among them, formulations that include the anionic lipid Bis(monoacylglycerol)phosphate (BMP) are frequent as working tools (Allen et al., 2019; Sudo et al., 2017; Yang et al., 2010) since this particular lipid is highly enriched in the intraluminal side components of late endosomes (Kobayashi et al., 2001). Related to that, pH is also an important parameter to be considered when trying to establish a liposome model, especially when testing EE proteins devoted to discriminate endosomes (acidic micro-environment) from extracellular membranes (neutral pHs). For that, liposomes are usually tested against the EE agent in a buffer at the selected pH. However, some authors have also elaborated a more sophisticated method where the EE molecule is encapsulated within a vesicle that is enriched with bacteriorhodopsin (Madani et al., 2011), a transmembrane protein that allows the control of intraluminal pH. Finally, other membrane models such as isolated endosomes (Ahmad et al., 2015; Evans et al., 2013; Liu et al., 2017; Prchla et al., 1995) or erythrocytes in hemolysis assay (Ahmad et al., 2015; Evans et al., 2013; Liu et al., 2017) are also usually used since they show higher similarity with the cellular environment.

Spectrofluorimetry is one of the preferred technologies for the *ex cellulo* evaluation of pore-forming and fusogenic EE peptides (Martens et al., 2014; Selby et al., 2017). This methodology generally relies on the encapsulation of a quenched fluorophore within a

liposome, being then released upon the action of the EE promoter. Here we can distinguish between the use of self-quenched tracers such as calcein (Ahmad et al., 2015; Oude Blenke et al., 2017; Plaza-GA et al., 2019), sulforhodamine B (Burks et al., 2015) or carboxyfluorescein (Kakimoto et al., 2009) from the co-encapsulation of fluorophore/quencher pairs such as ANTS/DPX (8-Aminonaphthalene-1,3,6-trisulfonic acid disodium salt/p-xylene-bis-pyridinium bromide) (Akishiba et al., 2017; Yang et al., 2010) or HPTS/DPX (8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt/p-xylene-bis-pyridinium bromide) (Yang et al., 2010). In both cases, the release of encapsulated fluorophores and their subsequent dilution in the media results in a reduced quenching activity and consequent increase of fluorescence, which is easily detected in a spectrofluorometer. Although this methodology provides relevant data about the membrane integrity of liposomes and the permeability of small molecules, it does not necessarily predict their capacity to release larger molecules such as proteins (Smith et al., 2019). To overcome this limitation, alternative approaches for the direct detection of encapsulated proteins have also been proposed. In this regard, radiolabeled proteins (van Rossenberg et al., 2002) or fluorescent-labelled proteins (encapsulated with their quencher) (Madani et al., 2013) can be directly detected in the extra-liposomal media by agarose gel electrophoresis or spectrofluorimetry, respectively.

In general, experiments with membrane models represent a simple and efficient tool for the EE evaluation of fusogenic or pore-forming candidates. However, they show important limitations when dealing with more complex mechanisms such as the proton sponge effect, the direct translocation, or the retrograde transport, which require the cellular microenvironment and intracellular trafficking machinery. Because of this, several *in cellulo* evaluation methods have been developed.

***3.2 In cellulo* assays**

Two important considerations are needed when designing EE evaluation methods using living cells (*in cellulo*). First, a precise comprehension of the information that can be extracted from each particular technique is required. Second, different approaches must be usually combined in order to allow a precise and reliable interpretation of the obtained data (Selby et al., 2017).

3.2.1 Fluorescence measurement:

Fluorescence evaluation, generally based on flow cytometry or fluorescence microscopy, is the most common way to detect EE (Martens et al., 2014). In this sense, flow cytometry provides high-throughput and quantitative data about the total fluorescence intensity within cells. On the other hand, fluorescence microscopy and especially confocal laser microscopy provide qualitative data about the intracellular localization of this signal, also known as intracellular fluorescence pattern (IFP) (Salomone et al., 2012). Thus, a diffuse fluorescence signal within the cytoplasm would be indicative of a cytosolic distribution, while a dotted signal, would suggest an entrapped fluorophore (probably into endo-lysosomes). Therefore, the combination of both methodologies, flow cytometry and IFP analysis, represents a potent tool for EE evaluation.

3.2.1.1 Independent fluorescent tracers:

The use of self-quenched tracers is an extended method for the evaluation of endosomal membrane integrity, as seen in *ex cellulo* approaches. In this sense, calcein is a widely used membrane-impermeable fluorophore that is loaded into endosomes by passive micropinocytosis (Smith et al., 2019). Calcein is weakly fluorescent inside the endosomes due to its self-quenching activity and low pH. Thus, when endosomes are disrupted, the release and consequent dilution of calcein into the cytosol results in an increase of fluorescence (Hu et al., 2007; Kim et al., 2018; J.-S. Kim et al., 2016; Salomone et al., 2012; Wong et al., 2015) (Figure 2A). In a similar approach, acridine orange (AO), forms red fluorescent dimers at high concentrations and acidic environment. Then, it shifts to green fluorescence when released into the cytosol (Kandil et al., 2019) (Figure 2B).

This kind of tracers have been proven very useful in the evaluation of endosomal permeability to small molecules but have shown some limitation when dealing with proteins. In this regard, an alternative approach takes advantage of the lysosomal degradation of DQ-ovalbumin, a high MW protein (45 KDa) conjugated with a self-quenched green fluorescent tracer. Here, the self-quenched tracer becomes highly fluorescent upon protein degradation in lysosomes (Quarta et al., 2017) (Figure 2C). Thus, in this innovative approach, the absence of fluorescence signal directly correlates with protein release.

3.2.1.2 Direct protein detection:

Not all the analytical strategies are based on the indirect detection of independent tracers. Some methods directly track the intracellular fate of proteins through their intrinsic fluorescence (Auger et al., 2015; Niikura et al., 2015; Sánchez-García et al., 2017; Sudo et al., 2017) or by labelling them with a fluorescent tracer (Ahmed et al., 2016; A. Kim et al., 2016; Mellert et al., 2012). Here, fluorescence microscopy is the preferred tool, allowing the qualitative evaluation of IFP (Figure 2D). Some protocols, however, have also tried to stablish a quantitative evaluation of IFP using microscopy. For that, they measure the median fluorescence value (Mellert et al., 2012) or the absolute fluorescence value at determined regions of interest (Basha et al., 2011). Going still further, Lee et al. reported an innovative approach based on a fluorescence resonance energy transfer (FRET) assay. For that, a green fluorescent tracer with capacity to perform FRET was attached to mCherry together with its quencher molecule, the last one attached by a disulfide bond. Thus, in case of EE, the disulfide bond is reduced into the cytosol and the quencher molecule released. In consequence, upon excitation of the green tracer, fluorescent energy is transferred to mCherry (FRET) and red fluorescence is detected into the cytosol (Lee et al., 2008) (Figure 2E).

3.2.1.3 Co-localization studies:

The use of fluorescent labels, usually highly hydrophobic, have raised some concerns when dealing with direct protein tracking since they can interfere with the EE process (Mellert et al., 2012). Similarly, the use of acidotropic dye molecules such as Lysotracker® or Lysoview®, which are widely used in organelle co-localization studies (Ahmed et al., 2016; Allen et al., 2019; J.-S. Kim et al., 2016; Lieser et al., 2022; Sakurai et al., 2011), might influence the acidification process of endosomes due to their weakly

basic nature (Selby et al., 2017). The use of such molecules can be avoided by the direct detection of lysosomal-associated membrane proteins such as LAMP-1 by immunostaining (Basha et al., 2011; J.-S. Kim et al., 2016; Wensley et al., 2020) or by its genetic fusion with a fluorescent protein (Allen et al., 2019; Wong et al., 2015). The lysosomal protein LAMP-2 (Huang et al., 2010) or the endosomal protein EEA1 (Huang et al., 2010; Wensley et al., 2020) have been also used in this regard. Altogether, although co-localization studies are mainly qualitative, some efforts have been done towards turning them quantitative (Bolte and Cordelières, 2006; Vercauteren et al., 2011).

3.2.1.4 Fluorescence accumulation pattern:

Flow cytometry is the quantitative partner of fluorescence microscopy. Here, the intracellular accumulation of fluorescent proteins, in combination with endosomal maturation inhibitors such as chloroquine, has served as a lysosomal degradation indicator. Thus, a larger increase of intracellular fluorescence in presence of chloroquine has been correlated with a lower ability of EE (López-Laguna et al., 2020; Sánchez-García et al., 2017; Serna et al., 2019). However, the use of chloroquine is still controversial since its precise role in EE is unclear yet (Hajimolaali et al., 2021; Wensley et al., 2020). Alternatively, other endosomal acidification blockers such as the v-type proton-pump ATPase inhibitor Bafilomycin A1 have been also applied in the same sense (Martens et al., 2014).

Recently, an innovative flow cytometry methodology based on the pulse shape analysis of accumulated fluorescent signal has been proposed (Wensley et al., 2020). In this approach, when the fluorescence homogenously distributes in the cytosol (endosomal escape), the width of the pulse signal increases. In contrast, when the fluorescence is trapped into the endosomes (dotted pattern), the height of the pulse signal increases while the width decreases. This assay was found to be more sensitive than confocal microscopy and represents an elegant tool for EE quantification of fluorescent proteins.

3.2.1.5 Protein complementation assays:

Protein complementation assays are also very useful for EE evaluation in fluorescent proteins. In such assays, one region of the fluorescent protein is constitutively expressed in the cytosol of the target cell. Then, the other complementing region is extracellularly administered in form of a fusion protein that include the EE enhancer and the targeting moiety for cell internalization. Thus, in case of efficient EE, the full protein is re-assembled into the cytosol and restores its fluorescence (Figure 2F). In this regard, GFP-complementation has been the most explored strategy (Kim et al., 2018, 2015; Lönn et al., 2016).

3.2.2 Biological activity measurements:

Measuring the action of specific proteins that perform their biological activity into the cytosol or other subcellular organelles can be also an indirect way to evaluate their EE (Figure 3A). However, it is important to note that this process is highly dependent on the intracellular trafficking of particular cell lines or types, which can somehow narrow the transversal value of data interpretation (Deprey et al., 2019). Thus, the detection of apoptosis-associated markers is frequently used when studying the action of proteins

with cytotoxic activity such as targeted toxins or proapoptotic proteins (Díaz et al., 2018; Hetzel et al., 2008; Kiesgen et al., 2014; Wensley et al., 2020). Other approaches combine EE peptides with enzymes such as α-amylase (Auger et al., 2015), β-galactosidase (Maier et al., 2012), luciferase (Jiang et al., 2020) or β-lactamase (Garcia-Castillo et al., 2015) that show easily measurable enzymatic activities into the cytosol. Another more sophisticated method measures the cytosolic activity of Cre recombinase in a cell line that has been previously transfected with a plasmid susceptible of gene recombination. Thus, if EE takes place, Cre recombinase promotes the recombination event, which can then be measured through the expression and activity of the encoded reporter protein (Akishiba et al., 2017; Li et al., 2015; Wadia et al., 2004) . The use of antigens and the subsequent analysis of the immune response by assessing the production of IL-2 (Yuba et al., 2010) or MHC I presentation kinetics (Vasdekis et al., 2012) have also served as a proof of cytosolic delivery.

Finally, the EE of targeted proteins has been also evaluated by measuring the biological activity of a cargo nucleic acid in the target cell. However, the need of reaching the cell nucleus when transporting DNA introduces an additional barrier that significantly complicates the interpretation of data resulting from this approach (Selby et al., 2017). To avoid this problem, the delivery of cargo siRNAs for endogenous (e.g. syntaxin 5) (Dyer et al., 2015) or reporter (e.g. luciferase) (Hatakeyama et al., 2009; Sakurai et al., 2011) gene knockdown have been more extensively used, as they directly perform their action in the cytosol. Still, this methodology may present some important limitations such as RNA degradation or intracellular dissociation during the process.

3.2.3 Cellular fractionation:

Cellular fractionation and subsequent protein localization is a very interesting approach for EE evaluation. Although this methodology has mainly been explored for subcellular localization of nucleic acids using qPCR (Akita et al., 2004; Hama et al., 2006), its basis can also be applied to protein localization assays via fluorescence measurements or Western Blot immunodetection (Lee et al., 2010; Niikura et al., 2016) (Figure 3B). The major advantage of this particular approach relays in its suitability for *in vivo* EE determination, as cellular fractionation can also be performed from tissue lysates (Richardson et al., 2010). Nevertheless, the methodology is laborious and cross-contamination between different subcellular fractions must be prevented during the process (Lee et al., 2010).

3.2.4 Cytosolic enzyme modifications:

Several research groups have evaluated the EE of proteins by measuring specific modifications generated into the cytosol by endogenous (farnesyltransferase, de-ubiquitinase) (Falnes et al., 1995; Loison et al., 2005), or recombinant (β-galactosidase or Biotin ligase) (Chao and Raines, 2013; Verdurmen et al., 2017) enzymes. These modifications, that usually result in a change in the protein molecular weight, are easily detected by SDS-PAGE of Western Blot (Figure 3C). Although these approaches are versatile and compartment-specific, the addition of tags in the protein sequence may potentially affect their behavior or generate artifacts due to protein degradation (Serna et al., 2019).

644 3.2.5 Electron microscopy:

645 Visualization through electron microscopy has been proved useful for the localization of
646 intracellular inorganic particles, which showing high electrodensity, require no labelling
647 for detection (Martens et al., 2014). However, this methodology appears to be more
648 challenging when dealing with protein materials or reporter proteins, since their low
649 electrodensity prevents their easy distinction from other subcellular structures and
650 proteins. In this regard, the subcellular localization of specific proteins can be easily
651 detected in electron microscopy by immunogold approaches (gold-labeled protein-
652 specific antibodies) (Chanoca and Otegui, 2014; Guzmán et al., 2010; Peters et al., 2006,
653 1991)(Figure 3D).

654 Altogether, many different strategies have been developed for EE evaluation, being *in*
655 *cellulo* assays the ones providing the most accurate information about specific proteins
656 intracellular localization. However, an adequate combination of these approaches is still
657 recommended to overcome the main drawback presented in each method. In this
658 regard, different *in cellulo* methods have been summarized in **Table 2**, making emphasis
659 in their key advantages and major drawbacks. It is important to note that strategies
660 requiring protein labeling are the ones raising most important concerns regarding
661 targeted proteins, as the labeling could potentially interfere with protein targeting and
662 its subsequent intracellular trafficking.

663

664 **4. Future perspectives**

665 Poor or moderate EE is a main bottleneck in receptor-mediated drug delivery, especially
666 when the drug and/or the cargo are protein-based. The rapidly increasing number of
667 protein drugs, protein-based cell-targeting agents and protein materials used as carriers,
668 combined with the urgent need of receptor-targeted medicines make this limitation,
669 long time neglected, very evident. Then, while the catalogue of specific ligands or cell
670 surface receptors is increasing, the number of EE protein agents remains limited. More
671 importantly, their mechanisms of actions are diverse, controversial and in general poorly
672 understood. Therefore, the mere incorporation of any among of such agents to a protein
673 drug or carrier does not guarantee a significant increase in the cytoplasmic release of
674 active molecules. Importantly, the background cell penetrating activities of some of the
675 suspected EE peptides might interfere with the receptor-targeting agents present in the
676 protein-drug nanomedicine. Because of the relevance of specific cell-targeting,
677 especially in oncology but also in other applications of precision nanomedicines, the
678 potential loss of specificity should be a matter of attention when designing a
679 multifunctional construct with EE activities. In fact, the progressive elucidation of the EE
680 mechanics for specific peptides combined with a detailed design and tailoring of the
681 protein constructs is expected to provide highly selective drugs with relevant endosomal
682 escape abilities.

683 In this context, methodologies and approaches to finely analyze EE emerge as new and
684 extremely valuable tools, necessary for a proper functional design of drugs for
685 cytoplasmic activities. Despite the intrinsic complexity of the EE determination, since it
686 involves procedures that interfere with the cell biology, enormous advances in the field
687 are paving the way for a better comprehension of the diversity of EE mechanisms, and

for their functional and structural adaptation to specific drug delivery platforms. Effective nanoscale carriers that combine high receptor targeting and efficient endosomal escape are expected to be developed in the next years. This will allow a quantitative and significant jump in the effectiveness and performance of protein-based drugs. After 40 years of recombinant protein drug production, the design of a new generation of highly efficient biopharmaceuticals should finally fulfill the current, pushing and unpostponable demands of innovative medicine.

Table 1. EE agents with potential interest in targeted delivery approaches

	Amino acid sequence	Origin	Specificity mechanism	Cis delivery tested	Targeted delivery tested	Refs
Pore-forming peptides						
Mellitin analogue	CGIGAVLEVLTTGLPALISWIEEEEQQ	Animal*	CC at acidic pH	No	No	(Ahmad et al., 2015)
Bombollitin V analogue	CINVLGILGLLGEALSEL	Animal*	CC at acidic pH	No	No	(Ahmad et al., 2015)
LL-37 analogue	CLLGDFFEESEEEIGEEFEEIVQEIEDFLE NLVPETES	Human*	CC at acidic pH	No	No	(Ahmad et al., 2015)
LZEP	CLELLEELLELLEELLELLEEL	Synthetic	CC at acidic pH	No	No	(Ahmad et al., 2021)
Aurein 1.2	GLFDIIKKIAESF	Animal	Affinity for AL	Yes	Yes	(Li et al., 2015; Lieser et al., 2022)
Fusogenic peptides						
INF-7	GLFEAIEGFIENGWEGMIWDYG	Synthetic	CC at acidic pH	Yes	Yes	(Lin et al., 2014; Liu et al., 2017; Sung et al., 2013)
GALA3	LAEALAEALEALAA	Synthetic	CC at acidic pH	Yes	No	(Li et al., 2020)
Pre-S2 domain	PLSSIFSRIGDP	Viral	Unclear	Yes	Yes	(Fuchs et al., 2007; Oess and Hildt, 2000)
DEN	MVDRGWGNGCGLFGKGGIV	Viral	CC at acidic pH + affinity for AL	Yes	Yes	(Kiesgen et al., 2014)
B18	PYDLGLLLRHLRHHSNLLANI	Animal	Unclear	Yes	Yes	(Niikura et al., 2016, 2015)
S19	PFVIGAGVLGALGTGIGGI	Human	Affinity for LE membranes	Yes	No	(Sudo et al., 2017)
Histidine-rich peptides						
Polyhistidine tags	HHHHHH HHHHHHHH HHHHHHHHHH	Synthetic	Protonation at acidic pH	Yes	Yes	(Dhankher et al., 2021)
Humanized histidine tags	HAAHAH HTHTHTHTH HEHEHEHEH	Human*	Protonation at acidic pH	Yes	Yes	(López-Laguna et al., 2020)

ppTG21	GLFHALLHLLHSLWHLLLHA	Synthetic	Protonation at acidic pH	No	Yes	(Rouet and Christ, 2019)
Toxins-derived peptides						
Diphtheria toxin T domain	**GNRVRRSV**GSSLSCINLDWDVIRDKTK TKIESLKEHGPIKNKMSESPNKTVSEEK AKQYLEEFHQTALEHPELSELKTVTGT NPVFAGANYAAWAVNVAQVIDSETA DNLEKTTAALSILPGIGSVMGIADGAV HHNTEEIVAQSIALSSLMVAQAIPLVG ELVDIGFAAYNFVESIINLFQVVHNSYN RPAYSPGHKT	Bacterial	CC at acidic pH	Yes	Yes	(Gilyazova et al., 2006)
Diphtheria toxin C domain C-ter region + T domain	GQDAMYEYMAQACA**GNRVRRSV**GS SLSCINLDWDVIRDKTKTKIESLKEHGPI KNKMSESPNKTVSEEKAKQYLEEFHQT ALEHPELSELKTVTGTNPVFAGANYAA WAVNVAQVIDSETADNLEKTTAALSIL PGIGSVMGIADGAVHHNTEEIVAQSIA LSSLMVAQAIPLVGELVDIGFAAYNFV ESIINLFQVVHNSYNRPAYSPGHKT	Bacterial	CC at acidic pH	Yes	No	(Aullo et al., 1993)
Exotoxin A T domain	GGSLAALTAHQACHLPLETFT**RHRQP RGWEQL**EQCGYPVQRLVALYLAARLS WNQVDQVIRNALASPGSGGDLGEAIR EQPEQARLALTLAAAESERFVRQGTG NDEAGAANADVVSLTCPVAAGECAG PADSGDALLERNYPTGAEFLGDGGDV SFSTR	Bacterial	ERAD pathway	Yes	Yes	(Mohammed et al., 2012; Schmit et al., 2019)
Exotoxin A FCS + KDEL	**GNRVRRSV** + cargo + C-terminal KDEL	Bacterial	ERAD pathway	Yes	Yes	(Díaz et al., 2018)

704

705 * Modified from the original sequence
706 CC: Conformational Change.
707 LE: Late Endosomes
708 AL: Anionic Lipids
709 Furin cleavable sites (FCS) have been highlighted in bold

710 **Table 2. *In celullo* methods for EE evaluation**

Category	Subcategory	Technique	Key advantages	Major drawbacks	Ref
Fluorescence measurements Detection by fluorescence microscopy (mostly qualitative) and/or flow cytometry (mostly quantitative)	Require the use of tracers	**Calcein leakage assay**	Easy pH-specific	Possibility of false positives due to membrane destabilization Not useful for translocations	(Kim et al., 2018; J.-S. Kim et al., 2016; Salomone et al., 2012; Wong et al., 2015)
		Acridine orange assay	Easy pH-specific	Tracer may affect endosomal maturation process Possibility of false positives due to membrane destabilization Not useful for translocations	(Kandil et al., 2019)
		DQ-ovalbumin assay	Measures degradation Measures protein escape	Labelling requirement Not useful for translocations	(Quarta et al., 2017)
		Co-localization studies with tracers	pH-specific Useful for all mechanisms	Tracers may affect endosomal maturation process High-levels of background Difficult to quantify	(Ahmed et al., 2016; Allen et al., 2019; J.-S. Kim et al., 2016; Sakurai et al., 2011; Selby et al., 2017)
	Requires the use of inherent fluorescent proteins or fluorophore-conjugated proteins	**Co-localization studies in stable cell lines**	pH-specific Useful for all mechanisms	Requires stable cell line expressing exogenous gene Difficult to quantify	(Allen et al., 2019; Huang et al., 2010; J.-S. Kim et al., 2016; Wensley et al., 2020)
		Cytosolic specific FRET	Measures protein escape Cytosolic specific Useful for all mechanisms	Double labelling requirement Possibility of false positives due to spontaneous reduction of S-S bond Possibility of false negatives due to spontaneous unlabelling	(Lee et al., 2008)
		Fluorescence accumulation pattern	Easy Useful for all mechanisms	Possibility of false negatives due to fluorophore dilution Controversy in the use of some agents	(López-Laguna et al., 2020; Sánchez-García et al., 2017; Serna et al., 2019)
		Pulse-shape analysis	Easy Useful for all mechanisms Do not rely on the use of any additional compound Higher sensibility than confocal microscopy	More data is required Endosomal distribution in used cell line affects the sensiblity	(Wensley et al., 2020)
		Complementation assay	Useful for all mechanisms Do not rely on the use of any additional compound No labelling	Requires stable cell line expressing exogenous gene Subunits must find each other in the cytosol	(Kim et al., 2018, 2015; Lönn et al., 2016)
Biological activity measurements	Measure the activity of proteins	**Cell viability assay**	Easy Applicable to therapeutic candidates Useful for all mechanisms No labelling High sensibility Compartment-specific	Possibility of false negatives due to subsequent protein trafficking Possibility of false negatives due to the effect of endosomal escape promoter to the protein	(Díaz et al., 2018; Hetzel et al., 2008; Kiesgen et al., 2014; Wensley et al., 2020)

		Enzymatic assays	Applicable to therapeutic candidates Useful for all mechanisms No labelling Compartment-specific	Possibility of false negatives due to subsequent protein trafficking Possibility of false negatives due to the effect of endosomal escape promoter to the protein	(Auger et al., 2015; Garcia-Castillo et al., 2015; Jiang et al., 2020; Maier et al., 2012)
		Immunologic assays	Applicable to therapeutic candidates Useful for all mechanisms No labelling Compartment-specific	Possibility of false negatives due to subsequent protein trafficking	(Vasdekis et al., 2012; Yuba et al., 2010)
	Measure the activity of nucleic acids	**Gene silenciation assays**	Applicable to therapeutic candidates Cytosolic specific Useful for all mechanisms	Possibility of false negatives due to subsequent cargo trafficking RNA labelling requirement For silencing exogenous genes, it requires stable cell line expressing exogenous gene	(Dyer et al., 2015; Hatakeyama et al., 2009; Sakurai et al., 2011)
Cellular fractionation	Measure protein	**Fluorescence measurements or Western Blot**	Suitable for *in vivo* assays No labelling Useful for all mechanisms	Labor-intensiveness Risk of contamination between subcellular locations Macromolecules may alter vesicle density	(Lee et al., 2010; Niikura et al., 2016)
	Measure nucleic acids	**qPCR**	Suitable for in vivo assays Useful for all mechanisms	Labor-intensiveness Risk of contamination between subcellular locations Macromolecules may alter vesicle density Labelling requirement	(Akita et al., 2004; Hama et al., 2006)
Cytosolic enzyme modifications	Require expression of exogenous enzymes	**Beta-galactosidase and biotin ligase assay**	Cytosolic specific Useful for all mechanisms	Requires stable cell line expressing exogenous gene Labelling requirement	(Chao and Raines, 2013; Verdurmen et al., 2017)
	Do not require the expression of exogenous enzymes	**Farnseylation assay**	Cytosolic specific Useful for all mechanisms	Requires subsequent cellular treatment CaaX motif C-ter might promote protein cytosolic degradation	(Falnes et al., 1995)
		De-ubiquitination assay	Cytosolic specific Useful for all mechanisms	Requires subsequent cellular treatment Labelling requirement	(Loison et al., 2005)
Electron microscopy	**Immuno-TEM**		Compartment-specific Direct visualization Useful for all mechanisms	Cell fixation requirement Labelling requirement	(Chanoca and Otegui, 2014; Guzmán et al., 2010; Peters et al., 2006, 1991)

711

Figure Captions:

Figure 1. Endosomal escape mechanisms. A. Pore-forming peptides can create channels in endosomes membranes following the barrel-stave (i), carpet (ii) or toroidal (iii) models. B. Viral fusogenic peptides (in blue) drive the fusion between viral and endosomal membranes due to a pH-dependent conformation change that induce their insertion into the endosomal membranes. C. Histidine-rich peptides in proteins (in red) become protonated at acidic pH. This fact promotes the entrance of protons (in yellow) in the endosomal lumen, which is followed by an entrance of chloride ions (in green) to compensate charges. The consequent increase in the osmotic pressure ends up in endosome lysis and in the release of its content to the cytosol (proton sponge effect). D. Direct protein translocation from the endosome lumen to the cytosol mediated by a toxin translocation domain (in yellow). A pH-mediated conformational rearrangement of the translocation domain initiates this process at late-endosomes. E. Lysosomal bypassing of proteins (in blue) following the ERAD recycling pathway. Specific toxin translocation domains drive the cargo protein to the endoplasmic reticulum, by which they are retrogradely transported to the cytosol. E: Endosome, L: Lysosome, ER: Endoplasmatic Reticulum.

Figure 2. Fluorescence based methods: A. Endosomal escape evaluation via self-quenched fluorescent tracers such as calcein. Endosomal disruption dilutes the fluorophore into the cytoplasm ending its quenching and triggering an increase in green fluorescence. B. Acridine orange is used similarly to calcein to detect endosomal escape. However, in acidic endosomes it assumes the form of red-fluorescent dimers, allowing a clear differentiation of endosomal entrapment or escape situations. C. Use of DQ-ovoalbumin, a self-quenched fluorogenic substrate for proteases that emits a strong fluorescent signal upon degradation to small labelled peptides inside the lysosome. D. Use of labelled or intrinsically fluorescent proteins to directly evaluate their endosomal fate via fluorescence microscopy. E. Use of a quenched FRET-dependent fluorescent protein that emits red fluorescence upon release into the cytosol from the endosomes. Reduction of the disulfide-bound quencher allows the green fluorescent moiety to excite the red fluorophore. F. Protein complementation assay where the endosomal escape peptide is fused to the complementing domain of a cytosol-expressed truncated fluorescent protein. When endosomal escape occurs, the truncated protein in the cytosol is complimented, restoring its fluorescence emission.

Figure 3. Other analytical methods: A. Overview of the main detection systems to evaluate endosomal escape based on biological functions or the alteration of these. B. Subcellular fractionation and subsequent antigenic or fluorescence detection in order to elucidate the fate of a given protein candidate with an endosomal escape domain. C. Endosomal escape is evaluated via determination of the candidate protein molecular weight. The studied protein is susceptible to cytosolic modifications that can increase or decrease its mass. D. Use of electron microscopy combined with gold immunolabeling to explore the subcellular location of the protein of interest.

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