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Highlight Review

Peptide-Assisted Intracellular Delivery of Biomacromolecules

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Abstract

Intracellular delivery of biomacromolecules offers opportunities for molecular and materials design, based on an understanding of their chemical properties and their modes of cellular responses. Peptides are advantageous as a delivery tool because of their ease in functional design and synthesis. This review highlights our approaches for the intracellular delivery of biomacromolecules using peptides with distinct modes of action.

Keywords: arginine-rich cell-penetrating peptide (CPP), attenuated cationic membrane-lytic peptide, macropinocytosis-inducing peptide

Introduction

Based on recent developments in peptide science and chemical biology, a variety of peptides and proteins have been designed with the potential to modulate cellular functions. These molecules could provide therapeutic impacts by modulating protein-protein interactions associated with illness.[1-4] However, biomacromolecules, including peptides and proteins, are generally not permeable through cell membranes (plasma membranes) due to their hydrophilicity and high molecular sizes. Thus, methods are needed to deliver such molecules into cells.

One delivery approach is the use of peptides with membrane permeation ability.[5] Simple conjugation or tight complex formation of such peptides (generalized as cell-penetrating peptides, CPPs) with peptides and proteins of interest has achieved efficient intracellular delivery.[6, 7] Because of its simplicity, numerous successful trials using this approach have been reported. A representative class of CPPs comprises those rich in arginine, which include HIV TAT[7] and oligoarginines.[6, 8] Detailed studies regarding methods of the cellular internalization of these peptides and their conjugates with cargo molecules provide chemical and biological insights that can be used to improve the design of CPP-based systems. Efficacy has been reported to decrease as protein size or molecular weight increases.[9] Therefore, we have targeted the development of more efficient approaches with different design concepts.

Methods of CPP internalization include direct penetration through the cell membrane and endocytosis (Figure 1).[10] Cargo molecules can exert their expected activity in the cell when directly delivered through cell membranes; this pathway is preferable to internalization through endocytosis. However, the translocation of large molecules through cell membranes may be accompanied by pore formation or rupture in membranes that enable molecule passage. These pores and ruptures may allow leakage of intracellular proteins and other cellular molecules to the extracellular space, thus causing serious damage. Use of the endocytic pathway may be more practical for delivery of macromolecules and nanoparticles. Endocytosis functions as a cellular mechanism for nutrient uptake and defense from pathogens.[11] This mechanism comprises pit formation on cell membranes, followed by invagination and membrane fusion to yield vesicular compartments (i.e., endosomes). The uptake molecules are delivered into cells while encapsulated in endosomes, which are then delivered to lysosomes (abundant in digestive enzymes) and degraded. Methods are needed to release endocytosed cargos from endosomes to the cytosol (i.e., endosomal escape).[5, 12] The major strategies currently employed to achieve this outcome include the use of materials for endosomal membrane destabilization, such as peptides and polymers.[5, 12] If these materials also perturb cell membranes, such perturbation should be accompanied by cell damage. Therefore, methods for selective perturbation of endosomal membranes over cell membranes have been developed. To switch perturbation efficacy between two membranes, the difference in pH between the endosome and extracellular pH is frequently employed.[13] Upon endosome formation, the V-ATPase on cell membranes is also incorporated into endosomal membranes.[14] V-ATPase is a proton pump, which transfers protons into endosomes, thus yielding endosomal luminal pH in the range of 5.0–5.5.[15, 16] A typical design of endosomolytic peptides with pH-dependent lytic activity is present in GALA.[13] This peptide has a potential amphiphilic structure with Glu residues as a pH-responsive moiety. At extracellular neutral pH, the side-chain carboxy group in Glu is dissociated and negatively charged. Because of charge-repulsion among Glu residues, this peptide does not form a helical structure in the extracellular medium, thus preventing interaction of the peptide with the membrane. When encapsulated in endosomes, the acidic pH leads to protonation of Glu, diminishing the charge-repulsion. This allows the peptide to interact with the membrane while forming an amphiphilic helical structure and perturbing the membrane, thus facilitating endosomal escape of cargo molecules.

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In this review, we introduce our research approach using designed peptides to deliver exogenous proteins and other biomacromolecules, as well as their methods of interaction with membranes and associated cellular responses.

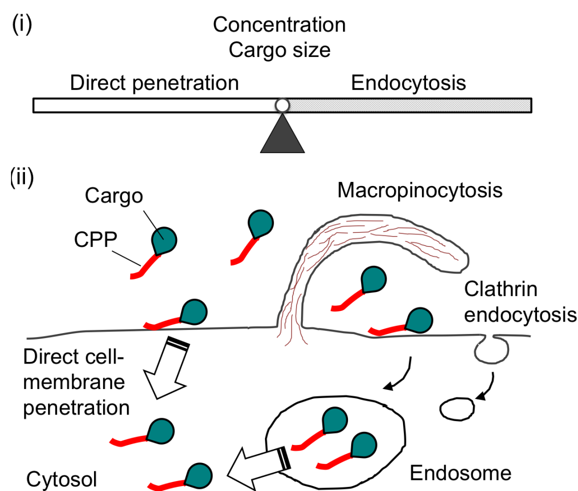

Figure 1. (i) Cellular internalization of arginine-rich cell-penetrating peptides (CPPs) is accomplished as a function of cell-surface concentration and physicochemical properties of each CPP/cargo conjugate, including cargo size. (ii) Direct cell-membrane penetration (left) is observed when relatively high CPP/cargo concentration (typically >10 μM) is employed and cargo size is relatively small (typically <10-20 kDa). In other situations, endocytosis including macropinocytosis (right) becomes the dominant internalization route.

Intracellular delivery using arginine-rich CPPs

Historically, establishment of the concept of CPPs can be traced back to reports by Frankel and Green in the late 1980s.[17, 18] Tat is a transcription regulator protein of HIV-1. External addition of the Tat protein and its fragments lead to initiation of viral transcription, suggesting the proteins enable membrane permeation. Note that the Tat protein is not involved in host cell infection. Fawell and coworkers demonstrated that conjugation with the Tat protein allowed exogenous proteins to be delivered into cells.[19] Vivès and coworkers reported the importance of a short arginine-rich, cationic segment corresponding to the RNA-binding segment (amino acids 48 60, denoted as the TAT peptide in this review) for membrane translocation.[20] Attachment of the TAT peptide segment was sufficient to cause cell permeation for bioactive peptides and proteins, indicating that the TAT segment is essential for cell permeability involving the HIV-1 Tat protein.[21, 22]

We became interested in the underlying mechanisms for membrane permeation by the TAT peptide. Considering that this peptide is rich in arginine residues, we prepared TAT analogs: nona-arginine (R9) and the D-amino acid version of TAT (D-TAT). Because the TAT segment corresponds to the RNA-binding segment of the Tat protein, we also prepared various RNA binding peptides that are rich in arginine.[23] Surprisingly, nearly all peptides could translocate into cells; efficacy was correlated with the numbers of arginine residues in these peptides. Rothbard, Wender, and coworkers also reported the importance of arginine, specifically the guanidine functional group in arginine, in achievement of membrane permeability.[24] They also showed the redundancy of peptide backbones, thus expanding the design possibility of intracellular delivery vectors.[8]

Initially, the TAT peptide was presumed to enter cells through cell membranes without the use of endocytic pathways, because of efficient internalization and accumulation into the nucleus even at 4 °C, a temperature at which endocytosis does not occur. Later studies, however, showed significant involvement of endocytosis in cellular uptake of the TAT peptide and its conjugates, while highlighting artifactual cellular localization caused by cell fixation during preparation for microscopic observation.[25] With respect to endocytic uptake, the involvement of actin-driven fluid-phase endocytosis (i.e., macropinocytosis) has been suggested as a major uptake pathway, together with the contribution of clathrin-mediated endocytosis and caveolar-mediated endocytosis.[26, 27] In contrast to the latter pathways initiated by means of pit formation on cell surfaces, macropinocytosis is typically induced by external stimuli, which lead to actin reorganization and cell membrane ruffling. Subsequent fusion of the ruffled membranes yields vesicular structures known as macropinosomes. In contrast to the sizes of the endosomes formed in clathrin-mediated endocytosis and caveolar-mediated endocytosis (~120 and ~80 nm, respectively[11]) by proteins assisting in formation of pit structures, there is no involvement of size-defining proteins in macropinocytosis. The sizes of macropinosomes are as large as 0.2–5 μm, which allows non-specific uptake of extracellular solute and cell-surface adsorbing molecules;[28] these compartments are therefore preferable for cellular uptake of various biomacromolecules and nano-particles.

For the induction of macropinocytosis by arginine-rich CPPs, membrane-associated proteoglycans on cell surfaces, including syndecans, play an important role.[29] Proteoglycans are composed of a membrane-associated protein modified with one or more glycosaminoglycan chains (i.e., repeated sulfated disaccharide units). Arginine-rich peptides have positive charges and are recruited to cell surfaces by interaction with negatively charged proteoglycans. It remains unclear whether proteoglycans are the direct receptor for induction of macropinocytosis or whether the interaction of proteoglycans can enhance interaction with some other receptors, as observed in the interaction of fibroblast growth factor with its receptor. However, macropinocytosis induction and cellular uptake of arginine-rich peptides are markedly suppressed in the absence of proteoglycans.[29] Accumulation of arginine-rich peptides on cell surfaces leads to activation of Rac-1 (a low-molecular-weight GTP protein), remodeling of cytoskeletal protein F-actin, and membrane ruffling.[27] Eventual fusion of ruffled membranes yields large endosomes known as macropinosomes. Macropinocytosis is considered non-specific fluid-phase endocytosis, which contributes to engulfment of cell-surface adsorbed molecules and solutes in extracellular fluid.[30] Therefore, cell-surface adsorbed arginine-rich peptides and their conjugates with bioactive molecules

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(cargos or payloads) are efficiently internalized into cells. Although such delivered cargos often exert the expected cellular activity, they must be released from endosomes into the cytosol; the exact methods of endosomal escape remain have not been fully established.

Although the involvement of endocytosis in cellular uptake of arginine-rich peptides and cargos has been confirmed, these components can also be internalized by direct translocation through cell membranes without the use of endocytosis.[31, 32] Direct cell-membrane penetration is achieved as a function of cell-surface peptide concentration and cargo size. Translocation of smaller cargos is achieved by using a higher peptide concentration, which can more easily penetrate membranes. The inside of a cell is known to have a lower voltage, compared with the external environment; the voltage difference (membrane potential) serves as a driving force for the influx of positively charged arginine-rich peptides into cells.[33] Considering the hydrophobic core in cell membranes, movement of positively charged and hydrophilic molecules (e.g., arginine-rich peptides and cargos) through cell membranes should be energetically unfavorable. Therefore, the high concentration cell-surface accumulation of arginine-rich peptides may allow pulse-like translocation through membranes, although methods of direct cell-membrane translocation have not been clarified in detail. Proteoglycans on the cell surface contribute to recruitment of arginine-rich peptides to the vicinity of the cell surface, thus increasing the local peptide concentration. Hydrophobic counter-anions in the membranes (e.g., pyrenebutyrate) may form a complex with arginine-rich peptides to neutralize positive charges and allow peptides to undergo easier membrane translocation.[34-36]

Recently, our group proposed that the loosening of lipid packing promotes direct membrane translocation of arginine-rich CPPs.[37] Curvature induction has been proposed as a translocation mechanism for arginine-rich peptides.[38-43] Alteration of the lipid phase on curvature induction, which yields a transient mesh-like porous structure formation in membranes (e.g., formation of the inverted micellar cubic phase), was hypothesized to allow permeation of the peptides through cell membranes.[39, 42, 43] Our group reported the marked promotion of membrane translocation of arginine-rich peptides in the presence of a curvature-inducing peptide (EpN18) derived from epsin-1.[38] EpN18 is the N-terminal amphiphilic peptide of epsin-1; this segment is involved in the induction of membrane curvature at the initiation of clathrin-coated pit formation, by means of its insertion into the cytoplasmic leaflet of cell membranes.[44] Furthermore, associated loosening of lipid packing has been proposed in curved membranes. The higher membrane curvature is accompanied by a greater packing defect; thus, curvature-sensing proteins, including those with membrane-binding α-helical segments called the Amphipathic Lipid Packing Sensor (ALPS) motif, can distinguish target intracellular vesicles of hydrophobic faces bearing amphiphilic peptides of different extents.[45] We assumed that, if the EpN18 also induces curvature and associated lipid packing loosening, the hydrophobic membrane cores would be more exposed to cell surfaces. The guanidino function of arginine-rich peptides has been proposed to form ion-pairs and hydrogen bonding with the lipid-head phosphates in cell membranes.[6] However, for membrane translocation of arginine-rich peptides,[34-36] the peptide backbone should become immersed in the hydrophobic core of the membrane, enabling passage to the other side. Exposure of the hydrophobic core or acyl chains of phospholipids should contribute to the interaction of the peptide backbone to facilitate this translocation. To support our hypothesis, we confirmed the induction of lipid packing loosening by the EpN18 peptide using the membrane polarity sensing dye di-4-ANEPPDHQ.[37] Additionally, pyrenebutyrate, a hydrophobic counteranion that can facilitate the membrane translocation of arginine-rich peptides, has been found to induce lipid packing loosening.[37, 46] Therefore, lipid packing is an important factor in facilitating the membrane translocation of arginine-rich peptides. Figure 2 illustrates the methods of translocation of arginine-rich peptides in curved membranes, implying final transient pore formation in these membranes.

In contrast to a simple ligand-receptor interaction, arginine-rich peptides can interact with a variety of molecules on cell surfaces; the modes of interaction are a function of the physicochemical properties (e.g., charge, hydrophobicity, and size) of arginine-rich peptides together with the cargos, peptide concentrations, numbers and properties of counter molecules in cells, and time-dependent cellular responses evoked by the peptides. It is thus difficult to evaluate the methods of translocation through a single mechanism; multiple factors must be considered. Furthermore, arginine-rich peptides can employ a variety of methods for different targets and in different conditions; thus, they can attain high cellular uptake efficacy either via endocytosis or via direct membrane translocation.

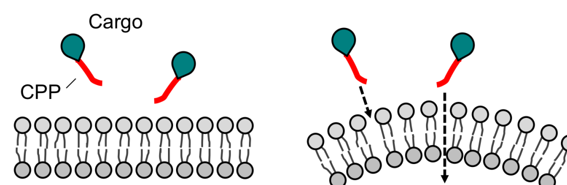

Figure 2. Curved membranes (right) have a higher packing defect, allowing easier permeation of arginine-rich CPPs through lipid core in membrane.

Intracellular delivery using attenuated cationic membrane-lytic peptides

Although arginine-rich peptides are effective as an intracellular delivery tool, this approach is inadequate for delivery of large molecules (e.g., antibodies). Arginine-rich peptides can penetrate cells; however, their ability to rupture the membrane is lower than that of membrane lytic peptides bearing a cationic amphiphilic helical structure (e.g., bee venom melittin).[47] This property is favorable to arginine-rich peptides, because it is accompanied by low cytotoxicity when applied to cells. However, when the peptides and cargos are taken up into cells via endocytosis, the efficacy of endosomal escape remains insufficient. Only a small proportion of arginine-rich peptides and cargos are presumed to escape from endosomes; the remaining peptides and cargos are retained in endosomes without exerting their bioactivities.[47-50] Therefore, development of complementary approaches to promote the endosomal escape of endocytosed cargos should be beneficial

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for achievement of efficient delivery and the required intracellular concentration for activity.

Endosomal escape is a central issue in the design of intracellular drug delivery systems and numerous approaches have been developed.[5, 12] The most popular approach is the selective rupturing of endosomal membranes without damage to cell membranes, which requires alternative mechanisms of perturbation. The pH in endosomes is reduced during the endocytic process.[5, 12] While the pH of the environment outside of cells is approximately neutral, the pH in late endosomes and lysosomes is presumed to be 4.5-5.5. Numerous pH-dependent lytic peptides and polymers have been developed for selective perturbation of endosomal and lysosomal membranes, relative to cell membranes[5, 12, 51] (Figure 3). Hydrophobic peptides and polymers bearing a carboxy group are often used for this switch.[13, 51] The carboxy group exists in its dissociated, negatively charged form at neutral pH; it becomes protonated and loses charge at acidic pH. In this context, we designed an intracellular delivery peptide named L17E, which was originally derived from the cationic membrane lytic peptide, M-lycotoxin.[52] A hydrophobic amino acid, leucine (Leu), is substituted with glutamic acid (Glu) containing a carboxy functional group in its side chain. M-lycotoxin shows high lytic activity against cell membranes, accompanied by high cytotoxicity.[53] A single Leu-to-Glu substitution of M-lycotoxin yielded greater than a 30-fold reduction of lytic activity on cell surfaces. The design concept of L17E was that endosomal pH-driven Glu-protonation would restore the lytic activity of M-lycotoxin accompanied by little damage to cell membranes. The addition of L17E with cargo proteins, including antibodies, into culture media achieved cytosolic delivery with high efficacy as expected. The feasibility of targeting to specific cellular proteins and modulation of signal transduction was exemplified using monoclonal antibodies (IgGs). This approach enables intracellular delivery of large macromolecules without the need for conjugation with L17E.

Although the originally intended mechanism of L17E was to facilitate endosomal escape, subsequent studies regarding the methods of achieving delivery suggested that the majority of antibodies achieve cytosolic translocation within 5 min after administration.[54] A detailed study demonstrated that L17E induces membrane ruffling and may permeabilize the ruffled membrane. Notably, L17E does not have this permeabilization ability in the absence of the ruffling states. Although L17E should have a perturbation ability with respect to endosomal membranes, the major action stage should be in membrane ruffled states and in the early fluid stage of endocytosis. Further analyses are needed, because these methods of membrane permeabilization are more dynamic than the simple pore/rupture formation mechanism involving lytic peptides; these methods suggest a new category of membrane permeabilization by peptides (Figure 4). The attenuated cationic membrane-lytic peptides may be employed as CPPs. One study on this topic used analogs of membrane lytic peptides, δ-hemolysin, and chrysophsin-1.[55] The attenuation of their membrane lytic activity by placement of multiple Glu residues allowed intracellular delivery of bioactive peptides in conjugation with these analogs. The positioning of Glu also contributed to improved delivery activity.[56] Efforts are ongoing in our laboratory to develop further intracellular delivery systems using attenuated cationic membrane-lytic peptides, by means of Glu inclusion in the peptide segments.

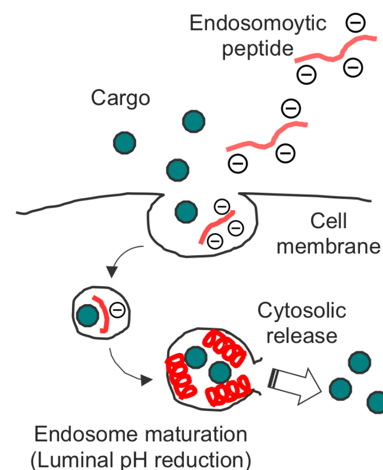

Figure 3. Design example of pH-dependent endosomolytic peptides. In extracellular media, repulsion of negative charges from embedded Glu prevents peptide from forming amphiphilic helical structures, ensuring peptides remain innocuous to cell membranes. Reduction of pH inside endosomes leads to protonation of Glu residues, which allows peptides to form an amphiphilic helical structure and perturb endosomal membranes, thereby facilitating cytosolic release (i.e., endosomal escape) of cargo molecules.

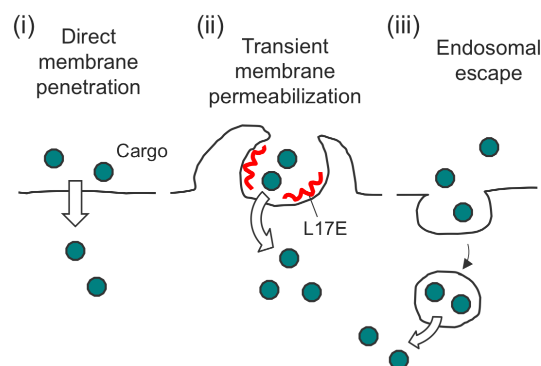

Figure 4. Possible mechanism of intracellular delivery of biomacromolecules (including IgGs) by L17E (ii). L17E induces membrane ruffling, which is accompanied by lipid packing loosening, leading to transient membrane permeabilization by L17E through ruffled membranes or at very early stages of macropinocytosis. Because permeabilization is observed only when membrane ruffling is induced and without the need for endosomal maturation, the method is conceptually distinct from (i) direct membrane penetration (including simple pore/rupture formation in cell membranes) and (iii) use of endocytosis followed by endosomal escape.

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Stimulated cellular uptake by the induction of macropinocytosis

Successful delivery through the endocytic pathway relies on methodologies that facilitate endosomal escape and an approach that yields efficient endosomal entrapment of molecules to be delivered into cells. Targeting cell surface receptors, followed by receptor-mediated endocytosis, is a major approach.[57] Although successful delivery to specific cells using this approach has been reported, the availability of different approaches should be beneficial to the delivery design. As described above, macropinocytosis is described as the extracellularly induced, actin-dependent, bulk uptake of extracellular fluid and materials, accompanied by large endosomes (i.e., macropinosomes) with diameters of >1 µm. The massive uptake and non-specific nature of macropinocytosis may yield flexibility in allowing efficient endocytic uptake of materials with various physicochemical properties.[58] Therefore, approaches using potent macropinocytosis-inducing peptides are attractive if employed with additional stimulation of endosomal escape of cargos taken up into the cells.[59]

We achieved this objective using a peptide derived from the N-terminal segment of stromal-cell-derived factor 1α (SDF-1α), in conjugation with an artificial membrane-lytic peptide LK15.[60] During our efforts to identify the potential cell surface receptor to stimulate cellular uptake of oligoarginines, a chemokine receptor CXCR4 (=CXC chemokine receptor 4) was found to induce macropinocytosis by stimulation with dodeca-arginine (R12) and with SDF-1α a ligand of CXCR4.[61] SDF-1α is a 93-residue protein, in which 17 N-terminal residues are responsible for its CXCR4 binding.[62, 63] The following study demonstrated that the N-terminal 17- and 21-residue peptides were also able to induce macropinocytosis.[60] Nearly 75% higher cellular uptake of extracellular fluid was achieved in the presence of the 21-residue peptide (known as SN21: KPVSLSYRCPCRFFESHVARA-amide), compared to absence of this peptide. Because SN21 did not exhibit marked endosomolytic activity, we prepared a hybrid peptide of SN21 with a de novo designed, cationic membrane lytic peptide LK15 (KLLKLLLKLLLKLLK)[64] in which two glycines served as a linker to connect these segments (SN21-LK15: KPVSLSYRCPCRFFESHVARA-GG-KLLKLLLKLLLKLLK-amide). Although LK15 demonstrated considerable cell lysis activity, its conjugation with SN21 resulted in a considerable reduction in cytotoxicity activity. Considering that LK15 has been employed for nucleic acid delivery, we examined the applicability of SN21-LK15 to nucleic acid and protein delivery into cells (Figure 5).

Evaluation of the conjugation effect of SN21 with LK15 on siRNA delivery via knockdown of the firefly luciferase reporter gene showed that SN21-LK15 reduced luciferase activity comparable to that of lipofectamine 2000 (LF2000), one of the most frequently employed commercially available transfection agents for DNA and RNA. For the endosomolytic segment, possible use of other cationic lytic peptides was also demonstrated by means of a bee-venom derived peptide, melittin.

SN21-LK15 can also be used for intracellular protein delivery including immunoglobulin G (IgG), Cre recombinase,[50, 65] and an artificial transcription regulator protein with a transcription activator-like effector (TALE) motif.[66] Notably, SN21-LK15 achieved a comparable efficacy of intracellular delivery using lower amounts of proteins, compared with L17E, suggesting the higher ability of SN21-LK15 for delivery. The enhanced cellular uptake through induction of macropinocytosis and stimulation of endosomal escape by membrane lytic peptides may synergistically serve to enhance the efficacy of cytosolic delivery of bioactive molecules. This may be due to the use of a cationic amphiphilic segment (e.g., LK15 or melittin) that can easily form complexes with nucleic acids and recruit them into endosomes (macropinosomes) prior to their release into cytosol, an activity that may not integrate well with the use of oligoarginines and L17E.

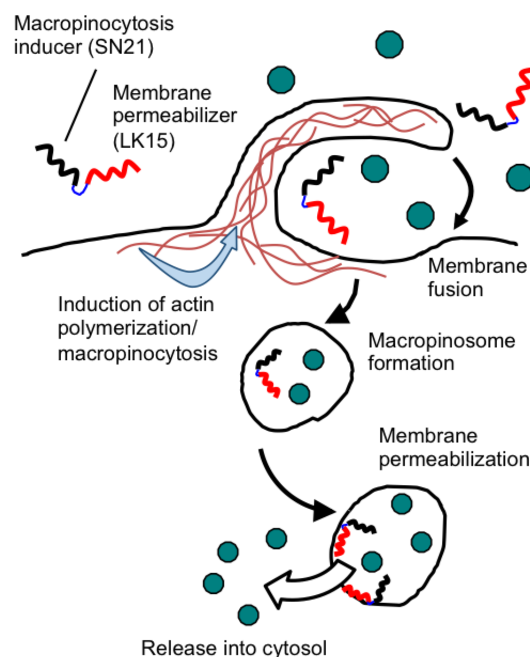

Figure 5. Combined delivery via stimulated endocytic uptake by induction of macropinocytosis (e.g. by SN21) and release from endosomes (e.g., by LK15).

Conclusions

Here, we introduced three of our peptide-based approaches to deliver exogenous biomacromolecules into cells: arginine-rich CPPs, attenuated cationic membrane-lytic peptides, and hybrids of macropinocytosis-inducing peptides and membrane lytic peptides. These approaches employ different modes of cell interaction and membrane permeation. Understanding the methods of internalization allows selection of the best methods of delivery, depending on the purpose and physicochemical properties of cargo molecules. Recently, delivery molecules and materials with unique modes of cellular internalization have been reported.,[67-76] suggesting various possibilities to allow facile intracellular delivery. These include cyclic oligochalcogenides,[67] cell-penetrating poly(disulfide)s and

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related disulfide-containing systems,[68-72] polymeric-protein nanocomposites,[73] cell-penetrating peptides bearing endosome destabilizing agents,[74] bioreversibly esterified proteins,[75] and functionalized dextrans.[76] These unique delivery systems may also lead to a more profound understanding of the structures and dynamics of biological membranes, which should have considerable impacts on biomedical applications and on basic studies on cell biology.

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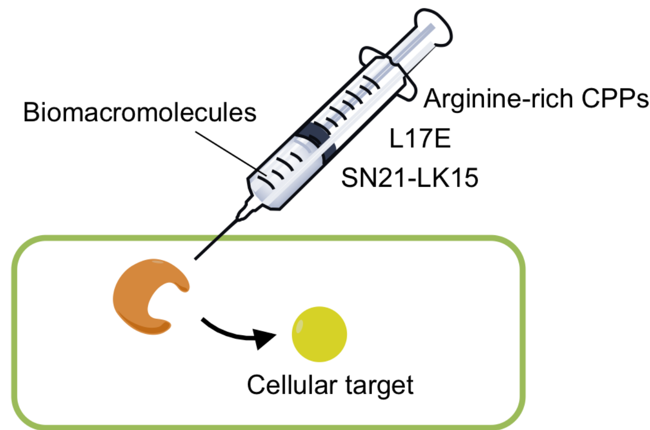

Peptides are advantageous as a delivery tool because of the ease of their functional design and synthesis – this review highlights approaches for intracellular delivery of biomacromolecules using peptides with distinct modes of action.

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