Review Article

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Different Peptide Cytosolic Delivery Methods.

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ABSTRACT

It was shown that the number of protein-based drugs has been increasing recently, in contrast to synthetic systems, drug delivery methods based on peptides possess numerous advantages because of their superior biocompatibility, biophysical and biochemical characteristics, and absence of toxicity. However, the absence of effective cytosolic delivery methods hinders the development of protein therapies against intracellular targets, where proteins can't directly flow through the cell membrane by passive diffusion because of their hydrophilic nature. As a result, delivery pathways that use endosomal escape after endocytic absorption have been investigated. As an alternative, delivery strategies that use modified peptides to promote endocytic uptake and endosomal escape or temporary permeabilization of cell membranes have been documented recently. This report emphasized the obstacles of peptide cytosolic delivery. Additionally, a brief overview of the tactics used to facilitate peptide-mediated cytosolic delivery of proteins was given. These tactics included drug delivery systems based on peptides, which are used to transport therapeutic substances like anti-cancer medications and drugs based on nucleic acids. These DDSs not only slow down the rate at which drugs degrade, but also guarantee the amount of the drug at the desired site and extend the t 1/2 of drugs in vivo. Lastly, the most popular in vitro methods for figuring out how protein cargo is distributed inside cells were described.

Keywords: Intracellular targets, Cell-penetrating peptides, Cytosolic protein delivery, internalization, endosomal escape

BACKGROUND

In the current medical era, macromolecules including peptides, proteins and nucleic acids are overtaking tiny molecules as the most effective medicines. These macromolecules must interact with their matching targets, which are frequently found inside the cell, to produce a therapeutic reaction. However, because of their size, they do not enter easily the cell membranes like many low molecular weight medications do. Rather, endocytosis usually takes them up into cells (Arafiles, Franke et al. 2023). By this mechanism, the majority of macromolecules are kept apart from their necessary location of action by being trapped inside membrane-bound endocytic vesicles. Therefore, an effective therapeutic delivery depends on overcoming endosomal entrapment. Peptides that penetrate the cells have become a hopeful delivery tool for improving cytosolic transport of biological treatments. According to reevaluation studies, endocytosis is the mechanism by which CPPs are taken up by cells, contrary to initial reports that claimed that they pass in cells by direct translocation through the plasma membrane. Certain CPPs are believed to enter the cytosol after endocytic absorption via encouraging endosomal membrane fusion and destabilization. Peptides are a type of biological molecules usually consisting of fewer than 50 amino acids linked together with peptide bond and have special qualities that make them appealing for application in biomedicine (Berillo, Yeskendir et al. 2021) . Their small size allows them to behave similarly to small molecule medications and have comparable transport characteristics. These biologically active biomolecules have the same functionalities as proteins because of their identical composition, where they have a number of uses, such as as medications, building pieces for more complex self-assembled structures, and ligands that target biomarkers expressed on diseased tissues. Because of their physical, chemical, and biological characteristics, peptides can bind and pass through a variety of barriers found in cells and tissues (Yazdi, Zarrintaj et al. 2020). Additionally, peptides are frequently less immunogenic, which adds to their benefits as carrier molecules or medicines. Peptides are diverse, just like proteins, and their sequences can be changed to produce a variety of chemical characteristics, including charge and hydrophobicity. Modification of these characteristics is particularly advantageous for promoting interactions

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with cell membranes and triggering reactions to various biological stimuli to support the intracellular transport of macromolecules and chemicals. As a result, peptides can be used as efficient carriers for drug to get past the intracellular and extracellular barriers found in different states of diseases. In this review, we give an overview of the latest developments in using peptides as drug delivery systems or carriers to move drugs across biological barriers, such as the GIT, the microenvironment of tumors, and the blood-brain barrier (Xu, Khan et al. 2019).

Peptides as vehicles for delivering therapeutics to the brain

In order to successfully transfer therapeutic agents into the brain, the blood-brain barrier functions as a transport and physiological barrier. Nearly 100% of macromolecules and 98% of small molecule drugs are unable to effectively pass the blood-brain barrier to treat brain disorders. Because of the three distinct characteristics of the blood-brain barrier :the compositions of vesicle of the brain capillary endothelium, the tight-tight junction complex among endothelial cells, and efflux transporters, drug delivery encounters very limited paracellular and cellular transport across the blood brain barrier and into the brain parenchyma (Ghosh, Peng et al. 2018). To address this challenging problem, great efforts have been made to improve drug delivery to the brain, especially via the transcellular channel. These days peptides have been discovered and designed to cross the blood brain barrier via a range of transit methods. Particularly, peptides have drawn attention as delivery systems for moving medicinal materials across the blood-brain barrier. Peptides actively move across the blood-brain barrier via particular, concentration-independent, and saturable routes. For instance, the transporter-mediated route is the primary means by which endogenous neuropeptides readily move between capillaries and brain tissue (Gonzalez-Cruz, Hernandez- Juarez et al. 2022). Transport of anionic and cationic medications, is made possible via a class of transporter proteins called organic anion and cation polypeptide transporters, which are found on the plasma membrane of the brain's capillary endothelial cells. Receptor-mediated transcytosis is the primary active mechanism that transports the vast majority of known BBB penetrating peptides (Liu, Fang et al. 2021).

Using peptides as vehicles to transport medications across the gastrointestinal (GI) tract

Although getting macromolecules like proteins and peptides into the gastrointestinal tract (GI) is still difficult, oral route is the most common method of administration of drugs. Because of their physicochemical characteristics, short t 1/2, quick clearance, and many biological obstacles in the GIT, poor oral bioavailability remains a significant issue even with the vast number of proteins and peptides that have lately been found. Greater hydrophilicity and larger molecular weight substances lead to poor drug absorption into the bloodstream, which lowers bioavailability and, in turn, limits the effectiveness of treatment (Luther, Jeon et al. 2021). Following oral administration, drugs and drug delivery systems face a number of physical, chemical, and biological obstacles on their way to the pharmacological target site of action, including mucosal barriers, limited intestinal epithelial penetration, and enzymatic breakdown in intestine and stomach (Goswami, Jeon et al. 2020). The secondary and tertiary structures of peptides can be impacted by low pH and enzyme exposure, which can result in peptide bond hydrolysis, aggregation and loss of function. Furthermore, the mucus layer of GI epithelium prevents medications and macromolecules from diffusing. Peptides have been created recently as carriers to improve the targeted delivery of drugs having poor gastrointestinal absorption, despite the promising improvements in employing them as treatments. Peptides aid in the transport and internalization of connected cargoes through the gastrointestinal mucosa and are selective ligands against cell targets because of their tiny size and complexity (Sánchez-Navarro 2021).

Peptides as vehicles for tumor penetration and targeting

The low penetration of clinically accessible anticancer medicines into tumors due to the use of passive drug delivery systems limits their therapeutic efficacy. Prior to reaching cells in the tumor, a therapeutic drug undergoing systemic delivery must pass via the three main transport pathways: vascular , transvascular and interstitial transport. Many therapeutic medications only reach 3-5 cell diameters in solid tumors, which reduces their effectiveness and promotes the emergence of drug resistance. This poor penetration is caused by the effect of enhanced permeability and retention, which is brought by the lymphatic system's malfunction and the tumor's leaky vasculature. On the other hand, the transport through the tumor extracellular matrix is weak due to the leaky vasculature of tumor, which produces increased pressure of interstitial fluid in the tumor microenvironment. This, in conjunction with low vascular flow, limits drug penetration by making diffusion the main mode of drug transport. The distribution of therapeutic agents into tumors may be improved by attaching a targeting molecule in the drug delivery vehicle attached to the target region in tumors precisely. This leads to increased therapeutic effect and fewer adverse reactions in the body (Yazdi, Zarrintaj et al. 2020). Peptide, or naturally occurring ligand of a receptor that is expressed selectively in tumors can all be considered targeting ligands. Peptides in particular are a desirable type of targeting ligands because they can more easily enter tissues and get to the target cells. They are generally non-immunogenic, tiny,

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and simple to synthesize. Peptides can be attached to various overexpressed targets or tumors such as :tumor antigens, fibrin deposits and integrins ,where the targeted receptors can be located either on cancer cells, tumor vessels or both. The use of peptides as targeting ligands against the blood for targeting of tumors has advanced significantly, though, as new research indicates that peptides may also help drugs and nanocarriers penetrate deeply into the tumor parenchyma for improved therapeutic efficacy (Xu, Khan et al. 2019).

BARRIERS OF PEPTIDE CYTOSOLIC DELIVERY

Efficent delivery to the cytosol is essential for numerous treatments, either due to the presence of the medications's receptor in the cytosol or because the intracellular organelle where they function must be moved through the cytosolic space. The varied physiochemical characteristics of therapeutics (ranging from macromolecules to small molecules; including both water-soluble and water-insoluble types) along with the distinct membrane-related and intracellular obstacles that these systems need to surmount for effective delivery and retention of therapeutics in the cytoplasm have resulted in the creation of multiple nanomaterials designed to facilitate efficient cytosolic delivery of therapeutics. This review highlights the importance of comprehending the molecular mechanisms involved in the intracellular transport of nanoparticles to create an effective cytosolic delivery mechanism and the barriers that hinders the peptide cytosolic delivery (Lee, Traver et al. 2024).

Membrane-Associated Barriers

Plasma Membrane

Cell membranes function as protectors and barriers. Due to their semi-permeability, some molecules are able to diffuse through the lipid bilayer, whereas others are not. Tiny hydrophobic molecules like oxygen and CO2 quickly cross membranes. While they travel at a slower pace, small molecules such as H2O and ethanol are also capable of passing across membranes. Nevertheless, cell walls block large molecules such as carbohydrates and amino acids, as well as highly charged molecules like ions, from diffusing. Specific transport proteins located within the membrane are essential for the passage of these molecules (Vedadghavami, Zhang et al. 2020). Transport proteins in membranes often utilize energy to facilitate movement and are both selective and specific for the molecules they transport. Furthermore, these proteins require additional energy to transport certain nutrients against the concentration gradient. The health and upkeep of cells rely on the capacity to maintain concentration gradients and occasionally move materials against these gradients. The cell can take in nutrients in larger amounts than present in the surroundings and, conversely, expel waste materials due to membrane barriers and transport proteins (Lee, Traver et al. 2024).

Endosomal Membrane

The endocytic pathway generally serves as the main absorption method for the intracellular transport of biological entities such as nucleic acids and peptides, encapsulating them within the endosome and lysosome. Only approximately 20% of human proteins associated with diseases are addressed by existing drugs. Most of these drugs are small compounds (MW<500) that can alter intracellular or extracellular targets but tend to be limited to proteins possessing profound binding grooves or cavities. Monoclonal antibodies and similar large molecular drugs (MW>5000) can attach to nearly any target, but they can only access extracellular targets because they are too large to cross cell membranes. This indicates that 80% of human proteins associated with diseases are currently undruggable, including several key targets in oncology. Two well-known protein families proteins that are defective or absent due to mutations and those involved in intracellular protein-protein interactions are known as "undruggable" targets. Peptides and complexes of protein-nucleic acid as CRISPR-Cas95 will likely be required to address these "undruggable" proteins. In theory, it is feasible to address disorders done by the loss of protein function by inserting the gene responsible for encoding that protein into the cells of the patient. CRISPR/Cas9 and other methods of gene editing might also be capable of permanently correcting certain genetic defects. Peptides recently have shown great efficacy in targeting external entities like PPIs; when inserted in the cell, they were shown to be effective against intracellular entities as well. To access the diseased tissue, these modalities require suitable delivery vectors as they are unable to penetrate the cell membrane by themselves (Liu, Cabral et al. 2024).

INTRACELLULAR BARRIERS

Lysosomal Degradation

Nanodrugs can surpass the pharmacokinetic limitations of traditional pharmacological agents due to their special biological and physicochemical characteristics. The five steps that a nanodrug given intravenously typically must undergo include circulation through the blood, concentration in the targeted region, deep penetration into tissues, uptaking by cells and the release of drug within the endosome. To overcome multiple intracellular obstacles during delivery, researchers have developed nanocarriers featuring diverse assembly designs. The ability to escape from endosomes/lysosomes is a crucial factor affecting delivery efficiency, as many drug delivery systems moves within the endosome–lysosome route after the uptake by cells . A delay in swiftly escaping from lysosomes often results in entrapment and

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potential breakdown, which may hinder the effective delivery of therapeutic drugs. Lysosomes has diverse degradative enzymes, such as phosphatases and nucleases. To effectively deliver nanodrugs for treating various conditions like cancer, neurodegenerative disorders, and infectious diseases, it is crucial to surpass the endosomal/lysosomal barrier. For example, addressing brain gliomas and disorders such as Alzheimer's disease can be challenging because the blood brain barrier may inhibit the access of nanodrugs to brain. Once internalized by endothelial cells through endocytosis, most nanocarriers are broken down by lysosomes; transcytosis cannot penetrate the blood-brain barrier. Therapeutic medications can be transported through the blood-brain barrier using endosomal/lysosomal barrier-disrupting methods. Likewise, techniques such as fusogenic or pH-sensitive nanoparticles can assist in the liberation of drugs from the lysosomal and endosomal regions of diseased cells, enhancing their efficacy in addressing hepatitis viral infections (Liu, Cabral et al. 2024) .

Intracellular Transport

Delivering the bioactive molecules into cells is crucial for acquiring therapeutic features since many drugs exert their therapeutic effects within targeted cells, like peptides and others. Nonetheless, due to their physicochemical properties , they encounter cellular obstacles such as : low drug-cell uptake, where the hydrophilic and negatively charged surface of cells exhibits a poor drug attraction , especially those that are also negatively charged and hydrophilic. Moreover, complimentary medications might interact with different cells, reducing their efficiency and causing negative adverse effects (possible off-target effects) (Yang, An et al. 2020). Experiencing difficulty entering cells is also an obstacle ,where some drugs (like nucleic acids) are unable to exit endosomes and will be degraded in lysosomes during the endocytosis process. Multidrug resistance (MDR) can restrict the application and efficacy of specific drugs, even though some (such as cisplatin) can enter cells (Lian and Ji 2020). Some drugs need to enter subcellular organelles to work effectively (for instance, plasmid DNA operates within the nucleus) which is an obstacle known as subcellular targeting. Solubility of drugs is considered an obstacle as well, where direct drug delivery is challenged by the reality that over 90% of chemical drugs (like camptothecin and CPT) exhibit poor solubility in aqueous solutions. Furthermore, certain medications have demonstrated significant toxicity, limiting their application , so encapsulating and safeguarding are essential to attain the intended biological outcome and deliver drugs safely to cells (Kawaguchi and Futaki 2024).

Cytosolic Environment

Peptides, proteins, and nucleic acids serve as examples of biological treatments that have demonstrated potential in addressing various genetic, immunological, and viral conditions. For a therapeutic response to occur, these macromolecules must engage with their conforming targets, which are found within the cell. Nonetheless, due to their size, they do not easily pass through cell membranes. Instead, endocytosis typically transports them into cells. Through this process, most macromolecules are separated from their essential sites of action by being enclosed within membrane-bound endocytic vesicles. For therapeutic delivery to succeed, it is essential to overcome endosomal entrapment. Nonetheless, endosomal escape remains a crucial rate-limiting factor in the intracellular transport of biological therapeutics and is an inefficient process (Teo, Rennick et al. 2021).

Nuclear Envelope (for nucleic acid-based drugs)

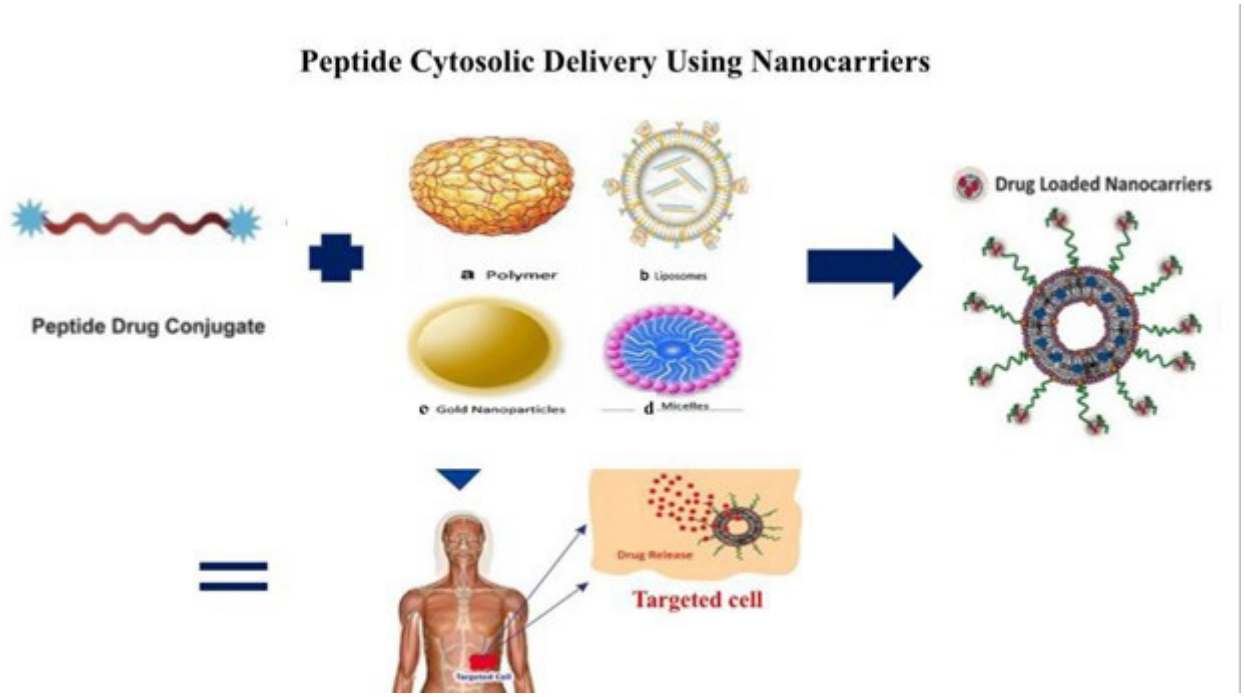
In eukaryotic cells, the nuclear envelope serves as a meticulously regulated membrane barrier that separates the nucleus from the cytoplasm. It contains several unique proteins that are associated with gene regulation and chromatin organization. The nuclear membrane poses a challenge for cell division despite allowing intricate levels of gene expression. The nucleus of metazoans must entirely break down during mitosis so the mitotic spindle can access the chromatin, requiring the nuclear compartment to be reformed at the end of each cell division (Kawaguchi and Futaki 2024).

DEVELOPMENTS IN THE CYTOSOLIC DELIVERY OF PEPTIDES

One essential technique for medical applications is the effecient delivery of proteins into cells. Recombinant proteins delivered intracellularly have quick-acting effects and have been effectively shown to be a promising treatment option for a variety of diseases. Since it can be difficult to move protein species across cellular membranes, almost the majority of protein therapies have been used to target extracellular targets. The application of these therapeutic techniques is significantly hampered by intracellular delivery, and developments in this area would significantly increase the usefulness of protein therapies. Membrane impermeability is largely to blame for the difficulties in delivering recombinant proteins intracellularly. Although endosomal uptake offers a way around this restriction, entrapment after endo-lysosomal degradation pathways poses an even bigger challenge for the majority of applications, (He, Xing et al. 2020). Cytosolic administration is necessary for both general intracellular activity and therapies aimed at particular organelles, such as the mitochondria (for anticancer medications), the nucleus (for antisense therapy or genome editing). Platforms with

the ability to deliver protein-based therapies cytosolically would offer novel approaches to the treatment of oxidative stress-related disorders , diabetes , inflammatory diseases, cancer , and neurodegenerative diseases. The creation of efficient protein delivery systems has been a major area of scientific interest due to the possible effects of intracellular protein therapies. The requirements to preserve activity and structure in many transport cargos have all contributed to the difficulty of their direct delivery into the cytosol or escaping from endosomal routes. Scientists have inserted desired proteins by delivering their corresponding mRNA or DNA in order to avoid the difficulties associated with protein transport into cells and enable the cell to manufacture the protein. Since viruses were shown to be able to deliver their genetic payload in order to infect the host cell, they serve as efficient vectors for the delivery of these nucleic acids. For the insertion of desired genes into cells, this method has been widely employed (Mai, Wimberley et al. 2024). Viral delivery techniques present serious problems for therapeutic applications, such as inherent toxicity that cast doubt on their viability as safe and transferable systems. However, methods that include the transfer of nucleic acids do not provide control on the amount of protein that the cell expresses or the duration of protein expression; instead, they usually produce various levels of expressions. This variability poses difficulties for basic biology research and medication dosage. Proteins can be delivered directly to cells, avoiding the problems with expression level and timing that come with nucleic acid delivery methods. But intracellular delivery has proven difficult for researchers, and this problem has yet to be resolved. It is difficult to transport directly to the cytosol , and endocytic uptake usually leads to significant sequestration and concurrent protease destruction, especially by cathepsins. The release of protein from the endosome has been the subject of significant effort (Wang, Zhang et al. 2024). Furthermore, a variety of strategies have centered on cytosolic administration directly. Despite their widespread use and established methods for introducing cytosolic proteins, physical techniques like electroporation have significant drawbacks when it comes to in vivo application. Techniques that make use of supramolecular interactions have emerged as important instruments for delivering desired proteins intracellularly, with noteworthy results shown in a range of applications. Proteins can be delivered via a variety of platforms, the most popular of which being polymeric systems, lipids, and nanoparticles. Recent methods for intracellular protein transport will be highlighted in this review, with an emphasis on supramolecular nano-assemblies used in this process (Tesauro, Accardo et al. 2019). Furthermore, molecular aspects of delivery as well as the uses, difficulties, and future prospects of intracellular protein therapies will be discussed.

Figure 1. Cytosolic delivery of peptide to targeted cells using nanocarriers.



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APPROACHES FOR ENDOSOMAL UPTAKE

A distinct barrier between the internal and external environments is created by the cell membrane, which isolates the intracellular environment of the cell from the extracellular one . This inhibits the intracellular delivery of many compounds to small molecules that can diffuse across membranes. Endosomal absorption which is the primary cellular approach for navigation across the plasma membrane, allows cells to receive foreign species, including nutrients and signaling chemicals. Through engulfing mechanisms like phagocytosis and endocytosis, cells can enter foreign species, leading to endosomal release or degradation (Du, Liew et al. 2018). In order to encourage cellular uptake of therapeutic cargo, nanocarrier systems have been investigated. Nanomaterials are frequently modified to have a cationic charge since the phospholipid bilayer is generally anionic. This facilitates the electrostatic contact and uptake via endosomal methods. Nanoparticles are thought to use synthetic delivery vehicles or cell-penetrating peptides to facilitate direct membrane transfer. These methods are regarded as effecient mechanisms for navigation through the lipid bilayer, in contrast to endosomal absorption . Delivery, trapping and engulfment within the endosome is the most prevalent absorption process for the intracellular delivery. Endosomal entrapment is a significant obstacle to the delivery of medicines since this process frequently results in breakdown pathways (Vedadghavami, Zhang et al. 2020). Since proteins and other biomacromolecules cannot naturally leave the endosome, scientists have used a number of strategies to cause the trapped cargo to be released. One possible method of endosomal disruption is the pH-buffering, which is facilitated by artificial vectors with an increased buffering capacity. The input of H+ and subsequent counterions like Cl- in this method promotes osmotic pressure, which causes the endosomal membrane to rupture. The endosome's contents are then released into the cytoplasm. For intracellular protein delivery, direct membrane translocation is an alternate method to endosomal uptake. These methods overcome problems related to cargo degradation and restricted cytosolic release by completely ignoring the endosomal pathway, which generally improves delivery efficiency. Usually, translocation happens due to several interactions among the membrane composition and the protein or nanocarrier, such as electrostatic interactions and lipophilic/hydrophobic switches, which can cause transient membrane disruption. For effective membrane penetration in CPPs, arginine residues are frequently essential. Therefore, to encourage intracellular transport, guanidinium-functionalized delivery agents have been used. Depending on the material design, guanidinium-functional nano- scale vehicles can produce locally repartitioned membrane areas. Micellar holes are created in the plasma membrane as a result of this alteration in its composition, and this facilitates the direct trafficking of delivery payload. Recently, various of strategies and developments were used to deliver proteins intracellularly and eventually cytosolically. Supramolecular techniques have been particularly successful among these carrier technologies, mostly due to the utilization of nanomaterials such as lipids, polymers, and nanoparticles (Ferrie, Fuselier et al. 2024).

STRATEGIES FOR PEPTIDE CYTOSOLIC DELIVERY

Nanoparticles

Gold Nanoparticles

There are two primary approaches to using gold nano particles in peptide cytosolic delivery for cancer immunotherapy. First , gold nanoparticles can be used as vehicles for to enhance the efficacy and targeted distribution of immunotherapeutic medications. Through the increased permeability and retention (EPR) action, they can aid in drug enrichment in tumor tissues. Covalent or noncovalent interactions can be used to directly bind drugs to the surface of gold nanoparticles. Furthermore, gold nanoparticles' surface can be altered to make drug administration and encapsulation easier. By using particular modifiers, such as antibodies, aptamers, sugars, and other ligands that recognize tumor-associated indicators, targeted drug administration can be accomplished. Certain modifiers can regulate drug release by reacting to environmental inputs like pH or enzymes. Secondly, utilizing gold nanoparticles' effective photothermal conversion capacity, temperature variations can be induced for tumor ablation and in situ medication release. This method enhances the anti-tumor immune response by inducing innate or adaptive immune responses in addition to tumor immunogenic cell death (Amina and Guo 2020).

Silica

Due in significant part to silica's adaptability, flexibility, and relatively acute toxicity, silica-based nanostructures are among the most often employed nanomaterials for delivery. Functional proteins can be delivered into cells by immobilizing them on the surface of hydrophobic nanoparticles of silica. In order to encapsulate proteins by hydrophobic contact, nanoparticle of silica were modified with a hydrophobic region (Zhang, Li et al. 2022) . The scientists confirmed cell death in breast cancer cells after delivering ribonuclease A and the antibody against these cells using silica nanoparticles (Yadav and Mohite 2020). Silica nanoparticles having mesopores that can encapsulate proteins and/or small molecules are known as mesoporous silica nanoparticles (MSNs)
. They are appealing carrier vehicles for protein delivery because of their adaptability, minimal intrinsic toxicity, and high loading capacity. Modified MSNs are used as a synergistic

co-delivery strategy to deliver superoxide dismutase and glutathione peroxidase (two antioxidant enzymes) to improve ROS scavenging. Moreover, MSNs can efficiently transport proteins, where mesoporous silica nanoparticles were covered with a lipid bilayer containing lipopeptides were used to promote fusion with the cell membrane to create 230 nm MSN-based nano-vehicles. After loading cytochrome C as a delivery payload, the core MSNs were encased in the bilayer. Complementary coiled-coil lipopeptides were pre-treated into the lipid bilayers and the cell membrane, resulting in effective cytochrome C delivery and consequent cell death. The fluorescence and absence of protein in nucleus suggested that the bulk of cytochrome C remained trapped in endosomes, even while the apoptosis suggested that some CytC was present in the cytoplasm (Liu, Cabral et al. 2024).

Polymers

Modifying polymeric carrier systems to incorporate moieties that support particular functionality, such as biological stability and cellular uptake, is a highly reproducible and scalable process. The cargo can be encapsulated in the polymeric carrier that can be engineered to interact directly with the protein therapy such as polymeric micelles. Drug delivery systems at the nanoscale known as copolymeric micelles are distinguished by their core-shell architecture. These systems are created when amphiphilic block copolymers in aqueous solutions self-assemble. Because they possess both hydrophobic and hydrophilic areas, amphiphilic molecules can function as surfactants and live independently in diluted aqueous solutions, lowering the surface tension at the interface between air and water (Wang, Zhang et al. 2024). Upon reaching the critical micelle concentration,the polymeric units aggregate to form micelles. The degree of adsorption at the interface increases in tandem with the concentration of the solution due to the addition of additional amphiphilic molecules. Copolymeric micelles can be utilized to deliver any near-infrared dye, DNA, small interfering (si)RNA, , paclitaxel, docetaxel and doxorubicin to a particular target. The efficient delivery of these chemicals is made possible by loading them into micelles (Varanko, Saha et al. 2020).

Lipid-Based Delivery

Mammalian cell membranes are lipid-based barriers by nature. Therefore, lipid-based approaches to intracellular protein delivery are beneficial not just because of their composition but also because they can provide encapsulation for the delivery cargo, which frequently avoids the requirement for cargo functionalization. Functionalized with these peptides, lipid-based nanoparticles can bypass the endo-lysosomal pathway and instead be absorbed by membrane fusion, increasing payload delivery. Additionally, they are not aimed at receptors that are exposed to the surface, rather they can be utilized to target cells or organs. Rather, they identify complementary peptide sequences, which makes it easier for cells to absorb them. Another important kind of lipid nanoparticle that combines the benefits of lipids and polymers for a range of biomedical applications is called a lipid polymer hybrid nanoparticle (LPN). LPNs have lipid/lipid-PEG shells as a coating for better in vivo circulation and polymer cores that carry medicinal ingredients.They are the perfect drug delivery vehicle because of their special structural makeup, which provides the best possible biocompatibility and physical stability. Nucleic acids and other medications have been effectively encapsulated in LPNs for longer-lasting release and increased stability. Additionally, adding functional groups to the polymer surface makes it easier to distribute drugs to particular tissues or cells (Wagner, Gran et al. 2018).

TECHNIQUES FOR ASSESSING THE DISTRIBUTION OF CYTOSOLIC PEPTIDES IN VITRO

When developing cytosolic delivery methods, one of the main limitations is the lack of required methods to identify cytosolic localization. Current reviews have examined a wide range of techniques for assessing cytosolic access in general. The following tests are employed to assess peptide-dependent protein delivery.

Assays of Internalization

The mechanism of internalization may be ascertained by specific probes, proteins with an identified mechanism of uptake or inhibitory experiments with chemical compounds. However, it's crucial to take into account how these inhibitors impact additional endocytic pathways. Because differences in temperature, serum content, time, or imaging settings might produce radically divergent results, it is imperative to control experimental conditions. Co-localization and inhibition, are examples of internalization investigations that are frequently analyzed using flow cytometry or confocal microscopy. Flow cytometry is the suggested method for quantification, while fluorescence imaging provides an indication of the location of the fluorescent construct in the cell. The major rewards of flow cytometry are its ease of use and simplicity in terms of experimental setup. However, it doesn't make it possible to evaluate the cellular localization of a payload. A combination of the two tactics is usually the recommended course of action. Quantitative confocal microscopy analysis has recently attracted attention despite its significant complexity. It takes a lot of time because it calls for high-content photographs and specially created algorithms. Furthermore, distortions from the dense punctuation pattern of cytoplasmic trapping molecules may make it difficult to assess more diffuse cytosolic molecules (Ruseska and Zimmer 2020).

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Fluorescence correlation spectroscopy

The fluorescence intensity fluctuations of individual fluorescent chemicals within a tiny focus volume are the basis for FCS. Important physical properties, such as the fluorescent molecule's size, concentration, and diffusion coefficient, can be determined by analyzing these changes. Theoretically, this method can analyze any substance, small molecule, peptide, or protein as it could be precisely altered using a single fluorophore. The concentration of the target fluorescent chemical in the cytosol can be ascertained thanks to FCS's accurate and direct measurements. Furthermore, FCS can be utilized to identify cargo deterioration because the diffusion coefficient is proportional to molecule size. The primary disadvantage of this method is its intricacy, as it requires intricate data analysis that may be impacted by aggregates or degradation products. Furthermore, this method takes a lot of time and calls for highly skilled workers. The cytosolic entry of various peptides has been evaluated using this potent approach in conjunction with flow cytometry (Ruseska and Zimmer 2020).

Functional enzymes as cargoes for assessing of cytosolic access

Utilizing the substrate (CCF2-AM), this functional assay relies on the delivery of cell-penetrating peptide modified b-lactamase. Cells can pass through this esterified complex, which is converted intracellularly into a positively charged counterpart that is confined in the cytoplasm. CCF2 can be used as a tool to assess a peptide's capacity to deliver b-lactamase to the cytosol since b- lactamase hydrolyzes it, changing its green fluorescence to blue (Sánchez-Navarro 2021).

CONCLUSION

The field of peptide-based cytosolic delivery is one that is developing quickly and has the potential to completely transform treatment approaches, especially for diseases with intracellular targets. Peptides are used in drug delivery systems (DDSs) to transport a variety of therapeutic agents, including proteins, nucleic acids, and anticancer medications, due to their exceptional biocompatibility and adaptability. Cytosolic delivery is promising, but it faces significant obstacles like membrane impermeability, endosomal trapping, and lysosome degradation. Effective transport to target cells and tissues is now possible thanks to strategies including endosomal escape mechanisms, surface-functionalized nanoparticles, and cell-penetrating peptides (CPPs). Recent developments in peptide DDSs, such as stimuli-responsive systems and improved nanoparticle architectures, have improved drug stability, bioavailability, and targeted delivery. Additional understanding of intracellular transport pathways has been made possible by methods for assessing cytosolic delivery, including as enzyme-functional assays, flow cytometry, and fluorescence microscopy. These discoveries demonstrate how peptide-mediated cytosolic administration may be used to target intracellular targets that are now "undruggable" and enhance the therapeutic effectiveness of biomacromolecules. Still, there are obstacles to overcome, especially in improving delivery effectiveness and minimizing off-target consequences. For peptides to reach their full clinical potential in intracellular treatment, more study into molecular pathways and specialized delivery systems is essential.

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