Biomaterials and Biosensors

ANSER PRESS

Review Article

OPEN ACCESS

Overcoming the challenge: cell-penetrating peptides and membrane permeability

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ABSTRACT

Cell-penetrating peptides (CPPs) have emerged as a promising strategy for enhancing the membrane permeability of bioactive molecules, particularly in the treatment of central nervous system diseases. CPPs possess the ability to deliver a diverse array of bioactive molecules into cells using either covalent or non-covalent approaches, with a preference for non-covalent methods to preserve the biological activity of the transported molecules. By effectively traversing various physiological barriers, CPPs have exhibited significant potential in preclinical and clinical drug development. The discovery of CPPs represents a valuable solution to the challenge of limited membrane permeability of bioactive molecules and will continue to exert a crucial influence on the field of biomedical science.

KEYWORDS

Cell-penetrating peptide; Membrane; Central nervous system diseases; Bioactive molecules

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ISSN 2972-3418

Received June 15, 2023| Accepted June 28, 2023 | Available online June 29, 2023

1. Introduction

In the early 1980s, the United States Food and Drug Administration (FDA) approved recombinant human insulin as the first medical application of recombinant proteins for treating diabetes. Since then, bioactive molecules such as proteins, peptides, and nucleic acids have progressively found utility in various therapeutic areas. These molecules offer several advantages as drugs, including high activity, strong specificity, and good tolerance. However, their ability to penetrate cell membranes is often limited, hampering their therapeutic efficacy. Notably, for the treatment of central nervous system (CNS) diseases, bioactive molecules must cross the blood-brain barrier (BBB) to exert their effects on the brain. This process is particularly challenging as the BBB primarily consists of tightly arranged brain microvascular endothelial cells (BMVECs) that effectively prevent the entry of most small and large molecules from the bloodstream into the brain. Consequently, enhancing the membrane permeability of bioactive molecules has been a prominent research focus in biomedical science for many years.

Advancements in science have led to the discovery of certain proteins with cell-penetrating properties, offering hope for overcoming the challenges associated with bioactive molecule membrane permeability. In 1988, two independent research groups reported the ability of the trans-activator of transcription (TAT) protein from the HIV-1 virus to enter cells in vitro, which was subsequently confirmed. In 1991, the antennapedia homeodomain peptide, a homologous protein derived from fruit flies, was also found to enter cells. These discoveries prompted researchers to investigate the structure-activity relationships of these proteins, aiming to identify the shortest amino acid sequences with cell-penetrating ability. Derossi et al. later identified a shortened effective transmembrane sequence of the antennapedia homeodomain peptide, a 16-amino acid peptide known as penetratin. Vives et al. confirmed that the 13-amino acid sequence TAT48-60, derived from TAT, was primarily responsible for cellular uptake. Furthermore, Park et al. identified a further shortened effective transmembrane sequence of TAT, a 9-amino acid peptide known as TAT49-57.

Following the discovery of the membrane-penetrating properties of TAT and antennapedia homologous peptides, numerous peptides with membrane-penetrating abilities, collectively termed cell-penetrating peptides (CPPs), were identified. These CPPs typically consist of 5-30 amino acid residues and possess the capability to traverse tissue and cellular membranes through energy-dependent or energy-independent mechanisms without specific receptor interactions. Common CPPs, shown in Table 1, have found applications in preclinical or clinical drug development. These peptides have demonstrated the ability to transport various bioactive molecules into cells, including proteins, peptides, DNAs, siRNAs, and small-molecule drugs. The coupling of these molecules with CPPs can be achieved through covalent or non-covalent strategies. Covalent binding involves chemically linking the bioactive molecules to CPPs using chemical bonds or expressing CPP fusion proteins via cloning technology. However, this binding strategy may sometimes alter the biological activity of the transported molecules. In such cases, non-covalent strategies have shown clear advantages. Non-covalent complexes are primarily formed through electrostatic and/or hydrophobic interactions between molecules. For instance, negatively charged oligonucleotides can assemble with positively charged CPPs through electrostatic attraction to form stable complexes. Non-covalent strategies allow CPPs to protect bioactive substances from degradation by proteases or nucleases, thereby increasing the half-life of the active molecules in the body.

Table 1. Classifications of Common CPPs

CPPs	Sequence	Classification	Ref
TAT	RKKRRQRRR	cationic peptides	19
R8	RRRRRRRR	cationic peptides	20
DPV3	RKKRRRESRKKRRRES	cationic peptides	21
DPV6	GRPRESGKKRKRKRLKP	cationic peptides	22
Penetratin	RQIKIWFQNRRMKWKK	cationic peptides	23
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	amphipathic peptides	24
pVEC	LLIILRRRIRKQAHAHSK	amphipathic peptides	25
Pep-1	KETWWETWWTEWSQPKKKRKV	amphipathic peptides	26
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	amphipathic peptides	27
MAP	KLALKLALKALKAALKLA	amphipathic peptides	28
CADY	GLWRALWRLLRSLWRLLWRA	amphipathic peptides	29
C105Y	CSIPPEVKFNPFVYLI	hydrophobic peptides	30
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	hydrophobic peptides	31
Pep-7	SDLWEMMMVSLACQY	hydrophobic peptides	32

Since the discovery of CPPs, their utilization has significantly increased in both fundamental and translational research. They have proven to be highly effective tools for cell transfection and have facilitated drug delivery to challenging anatomical sites, thereby enhancing therapeutic outcomes. CPPs have been employed to transport various types of drugs across a wide range of diseases, including antibiotics, anti-inflammatory drugs, anti-tumor drugs, and neuroprotective drugs. Notably, CPP-based drugs have exhibited promising results in preclinical experiments for numerous diseases and have advanced into clinical trials. This progress highlights the potential of CPPs in treating human diseases and indicates a promising outlook for their application.

2. Classification

CPPs can be classified into three types based on their physicochemical properties: cationic peptides, amphipathic peptides, and hydrophobic peptides. Cationic CPPs are characterized by a high number of positive charges under physiological pH conditions, primarily derived from the guanidino group of arginine side chains and the amino group of lysine side chains. These positive charges facilitate electrostatic interactions with the cell membrane surface. TAT and oligoarginine are examples of cationic CPPs, both adopting a random-coil structure in hydrophobic and hydrophilic environments. Research has indicated that the number of arginines plays a crucial role in the transmembrane properties of cationic CPPs, with arginines contributing more to cellular uptake than lysines. The guanidino group of arginine side chains not only interacts with the cell membrane through charge interactions but also forms hydrogen bonds with certain membrane components, further enhancing binding and cellular uptake. Studies comparing TAT and oligoarginine (R9) have demonstrated that R9, with a higher number of arginines, exhibits superior cellular uptake ability. Moreover, studies on oligoarginine peptides of different lengths have shown that transmembrane ability increases with peptide chain length, albeit with a concomitant

increase in cytotoxicity. Notably, oligopeptides composed of 6-9 arginines exhibit optimal transmembrane properties with low cytotoxicity.

In contrast to TAT, the second CPP discovered, penetratin, contains a significant proportion of hydrophobic amino acids such as tryptophan, isoleucine, phenylalanine, and methionine, in addition to a large number of cationic amino acids. These hydrophobic amino acids are essential for the transmembrane properties of penetratin. CPPs that incorporate both cationic and hydrophobic amino acids are referred to as amphipathic CPPs. In amphipathic CPPs, the cationic amino acids facilitate binding to the negatively charged cell membrane surface, while the hydrophobic amino acids interact with the hydrophobic portion of the cell membrane to promote transmembrane transport. Amphipathic CPPs can be further classified into primary-structure amphipathic peptides and secondary-structure amphipathic peptides. Primary-structure amphipathic peptides, such as MPG and Pep-1, feature a hydrophobic domain at the N-terminus and a hydrophilic domain at the C-terminus. The hydrophobic domain of MPG is derived from the HIV-1 fusion protein gp41, while that of Pep-1 is a short peptide rich in tryptophan. The hydrophilic domain of both peptides is derived from the nuclear localization signal (NLS) of simian virus 40 (SV40). A linker region separates the hydrophobic and hydrophilic domains, enabling them to function independently. In hydrophilic environments, MPG adopts a random coil conformation, while in hydrophobic environments, it displays a β-fold structure. Pep-1 exhibits a weak α-helical structure in hydrophilic environments but forms a strong α-helical structure in hydrophobic environments.

Secondary-structure amphipathic CPPs, such as MAP and CADY, exhibit a random coil conformation in hydrophilic environments but can adopt a secondary structure in the cell membrane environment, with hydrophobic and hydrophilic amino acids positioned on opposite sides of the peptide chain. For example, MAP consists of a repeating lysine-leucine-alanine sequence, which, in the cell membrane environment, forms an α-helical conformation with hydrophilic lysine and hydrophobic leucine residues arranged on opposite sides of the peptide chain. Studies on MAP derivatives have demonstrated that α-helical peptides with a regular hydrophobic-hydrophilic interface exhibit excellent cell-penetrating ability.

Hydrophobic CPPs comprise mostly hydrophobic amino acids with a few cationic amino acids, and their transmembrane process primarily relies on hydrophobic interactions with the cell membrane. Examples of hydrophobic CPPs include C105Y and Pep-722. The hydrophobic interaction between CPPs and the cell membrane significantly influences their transmembrane performance. Studies have shown that replacing hydrophilic arginine or serine residues in the amphipathic CPP pVEC with hydrophobic alanine improves its transmembrane efficacy while replacing hydrophobic leucine and isoleucine residues with hydrophobic alanine reduces its transmembrane effectiveness. This highlights the importance of hydrophobic amino acids for the transmembrane ability of peptides. Consequently, hydrophobic aromatic amino acids such as tyrosine, tryptophan, and phenylalanine are often incorporated into the peptide sequence to promote hydrophobic interactions with the cell membrane and facilitate cellular uptake. However, introducing hydrophobic amino acids can decrease peptide solubility and increase cytotoxicity. Therefore, achieving a balance between cell-penetrating ability and cytotoxicity necessitates proper arrangement of the number of hydrophobic and cationic amino acids when designing CPPs.

3. Membrane translocation process and intracellular transport pathway

3.1. Integrated into the cell membrane surface

Prior to cellular uptake, CPPs undergo a binding process with the cell membrane. Except for a few CPPs that exhibit specific binding to cell membrane surface receptors, the binding of most CPPs to the cell membrane is non-specific and primarily driven by electrostatic forces between CPPs and the cell membrane. In the case of

healthy mammalian cells, CPPs predominantly bind to negatively charged glycosaminoglycans (GAGs) present on the cell membrane. GAGs are linear polysaccharides, including heparan sulfate (HS), dermatan sulfate (DS), chondroitin sulfate (CS), and keratan sulfate (KS). Among these, HS plays a crucial role in the binding process of CPPs to the cell membrane. HS interacts with various membrane proteins to form heparan sulfate proteoglycans (HSPGs), such as glypican and syndecan, which can activate intracellular signaling pathways and participate in diverse cellular uptake processes. Studies by Console and Fuchs have demonstrated that mutations in GAGs in CHO cells lead to a significant reduction in the cellular uptake efficiency of CPPs like penetratin, TAT, and oligoarginine.

In the case of tumor cells and bacterial cells, CPPs primarily bind to abundant negatively charged molecules on their cell membranes, such as phosphatidylserine (PS) on the surface of tumor cells and teichoic acid (TA) and lipopolysaccharide (LPS) on the surface of bacterial cells. Upon contact with the cell membrane, certain CPPs undergo conformational changes driven by hydrophobic interactions. For instance, amphipathic CPPs typically exhibit disordered coil structures in solution but adopt ordered structures like alpha helices or beta folds upon interaction with the cell membrane. Initially, when in contact with the cell membrane, CPPs align parallel to the cell membrane surface, with the polar side exposed to the hydrophilic lipid exterior and solvent surface, while the nonpolar side partially enters the hydrophobic lipid interior. As CPPs bind to the cell membrane surface, they aggregate and subsequently enter the cell through either a membrane-disrupting or non-membrane-disrupting mechanism.

3.2. Cellular uptake

3.2.1. Cellular uptake pathway

CPPs employ two main cellular uptake pathways: energy-dependent endocytic pathways and energy-independent direct transduction pathways. Endocytic pathways are non-destructive to the cell membrane, while some direct transduction pathways may cause permanent damage to it. The endocytic pathways encompass phagocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, non-clathrin and non-caveolin-mediated endocytosis, and macropinocytosis. Non-clathrin and non-caveolin-mediated endocytosis, such as Flotillin-mediated endocytosis, are primarily associated with the lipid raft structure on the cell membrane. Although this endocytic pathway has been studied to a lesser extent, its mechanism has not been fully elucidated and will not be discussed further here. Phagocytosis is exclusive to immune system cells, namely macrophages, neutrophils, and monocytes, which serve to clear viruses, bacteria, or infected cells. However, phagocytosis is not the primary endocytic pathway utilized by CPPs.

Direct transduction pathways consist of various models, including the membrane thinning model, inverted vesicle model, barrel-stave model, toroidal pore model, and carpet model. The membrane thinning and inverted vesicle models do not cause permanent damage to the cell membrane. On the other hand, the barrel-stave, toroidal pore, and carpet models may result in permanent damage to the cell membrane. These direct transduction pathways represent alternative routes for cellular uptake mediated by CPPs.

3.2.2. Influencing factors of cellular uptake pathways

The cellular uptake mechanism of CPPs remains a subject of debate, as different studies have reported varying mechanisms for structurally similar CPPs, and the same CPP may adopt different uptake mechanisms under different conditions. The mode of entry into cells by CPPs depends on factors such as CPP concentration, cell type and membrane properties, CPP structural properties, and CPP self-assembly. CPP concentration is a

primary determinant of the uptake mode. In most cases, at low concentrations, CPPs enter cells through endocytic pathways, while at high concentrations, they enter through direct transduction pathways. When it comes to the three models of membrane disruption by direct transduction (barrel-stave, toroidal, and carpet models), the CPP concentration on the cell membrane surface must exceed a specific threshold for CPP aggregation and insertion into the membrane. Thus, to exert their membrane-disrupting effect during cellular entry, CPPs need to reach a high concentration on the cell membrane surface. The CPP concentration on the cell membrane surface primarily relies on the interaction between CPPs and the cell membrane, which is greatly influenced by membrane properties.

In healthy mammalian cells, negatively charged lipid phosphatidylserine (PS) is predominantly distributed on the inner side of the cell membrane, while neutral lipids such as phosphatidylcholine (PC) and sphingomyelin (SM) are mainly distributed on the outer side of the cell membrane. However, tumor cells exhibit disrupted lipid distribution, with PS being widely distributed on the outer surface of the cell membrane. Additionally, various negatively charged molecules, including heparan sulfate, O-glycosylated mucin, sialylated gangliosides, and sialic acid residues, are overexpressed on the outer surface of tumor cell membranes, resulting in a high density of negative charges. Consequently, cationic CPPs are more likely to bind to the surface of tumor cells, leading to a relatively high CPP concentration on the tumor cell surface, facilitating their membrane-disrupting effect. Furthermore, the cholesterol content in tumor cell membranes is relatively low compared to healthy mammalian cell membranes. This lower cholesterol content contributes to increased fluidity and reduced stability of tumor cell membranes, making them more susceptible to CPP-mediated disruption. Moreover, the disordered surface of tumor cell membranes provides a larger contact area for CPPs, further increasing the CPP concentration on the tumor cell surface and facilitating their membrane-disrupting effect.

In addition to CPP concentration, the structural properties of CPPs themselves are crucial factors influencing cellular uptake. For instance, the cationic CPP TAT and the amphipathic CPP pVEC can both induce pore formation on the cell membrane at higher concentrations. However, TAT does not cause membrane damage during translocation, while pVEC-mediated translocation is accompanied by membrane rupture. The differences in pore formation between these two CPPs are related to the rate of pore opening and closing. CPPs with membrane-disrupting properties tend to form pores that remain open for longer durations, whereas CPPs without membrane-disrupting properties form transient pores that rapidly self-repair and close. The hydrophilicity/hydrophobicity and secondary structure of CPPs play significant roles in determining the rate of pore opening and closing. Cationic CPPs, lacking hydrophobic regions and the ability to form α-helical structures, rely on interactions with phosphate groups on the cell membrane surfaces to cause transient pore formation without permanent damage. In contrast, amphipathic CPPs, upon binding to the cell membrane surface, can adopt α-helical structures, allowing extensive hydrophobic interactions with membrane lipids, resulting in longer-lasting pore formation and subsequent disruption of the cell membrane structure. When CPPs carry macromolecules such as proteins or fluorescent dyes through membrane pores, these macromolecules can temporarily occupy the pores, preventing leakage of cellular contents.

In practical applications, CPPs sometimes form self-assemblies with cargo molecules and enter cells together. Self-assembly has been shown to reduce the toxicity and immunogenicity of its components by enclosing structural domains that may contribute to these effects. When CPPs participate in self-assembly, their peptide chain structures are not fully exposed, limiting their interaction with cell membranes and reducing their disruptive potential. Additionally, the entry pathway of CPP-containing self-assemblies primarily involves endocytosis, potentially due to changes in molecular weight. Self-assemblies with a molecular weight exceeding

2000 mainly enter cells through endocytosis. Moreover, the surface structure and charge density of self-assemblies can be controlled through rational design to mitigate their damaging effects on cell membranes.

In summary, CPPs typically enter cells through endocytosis at low concentrations and direct transduction pathways at high concentrations. All membrane-disrupting pathways require CPPs to surpass a concentration threshold on the cell membrane surface, and the enrichment of negatively charged molecules on the surface of tumor cells promotes CPP accumulation, surpassing the required threshold. Cationic CPPs generally enter cells via non-destructive pathways, while membrane-disrupting pathways typically involve amphiphilic peptides with hydrophobic domains and α-helical structures in the cell membrane environment. CPP-containing self-assemblies primarily enter cells through endocytosis. These insights provide valuable guidance for the design of CPPs with diverse applications, including membrane permeation and disruption.

3.3. Lysosome Escape

Once taken up by cells, cargo delivered by CPPs typically enters endosomes through one or more endocytic pathways. However, endosomes are not the final destination for these cargos, as they need to reach the cytoplasm to exert their effects. Substances remaining in endosomes are prone to degradation in the acidic endosomal environment or by hydrolytic enzymes, or they may be transported to lysosomes for degradation (Figure 1). Therefore, efficient escape from endosomes is a critical step in enhancing the cellular uptake efficiency of CPPs and their cargos that enter cells via endocytic pathways.

Research has revealed that certain cationic CPPs possess the ability to escape from endosomes, although the precise mechanisms of this escape are not yet fully understood. One potential mechanism involves the membranolytic properties of CPPs, where the cationic charge of the CPPs interacts with the anionic charge of phospholipids in the endosomal membrane. This interaction leads to the formation of membrane pores and the subsequent release of the cargo. Notably, TAT has demonstrated the capability to disrupt endosomal membranes through its interaction with negatively charged phospholipids within the endosomal membrane. Another mechanism involves the formation of ion pairs between CPPs and negatively charged phospholipids in the endosomal membrane, followed by their cleavage and penetration through the endosomal membrane. Oligoarginine has been shown to achieve endosomal escape through this mechanism. In addition to cationic CPPs, certain hydrophobic CPPs also possess endosomal escape ability. For example, melittin, a hydrophobic CPP with antibacterial and hemolytic activities, can induce endosomal escape through pore formation, employing mechanisms such as the carpet model, barrel-stave model, and toroidal pore model. However, the non-specific membrane-disrupting effect of melittin can lead to high cellular toxicity. To address this concern, researchers have explored various modifications of melittin to reduce its toxicity while preserving its endosomal escape capability. For instance, Meyer et al. covalently linked melittin modified with dimethyl maleic anhydride to polylysine, resulting in a gene delivery carrier with reduced toxicity and retained endosomal escape ability. While some CPPs can achieve endosomal escape on their own, their escape ability may decrease after loading cargo. In cases where bioactive substances require a specific concentration to exert cellular responses, the endosomal escape ability of CPPs alone may be insufficient to elicit the desired biological effects. Hence, various strategies have been employed to enhance the efficiency of endosomal escape in CPP-mediated delivery, including membrane fusion mechanisms, "proton sponge" mechanisms, photosensitive mechanisms, and others.

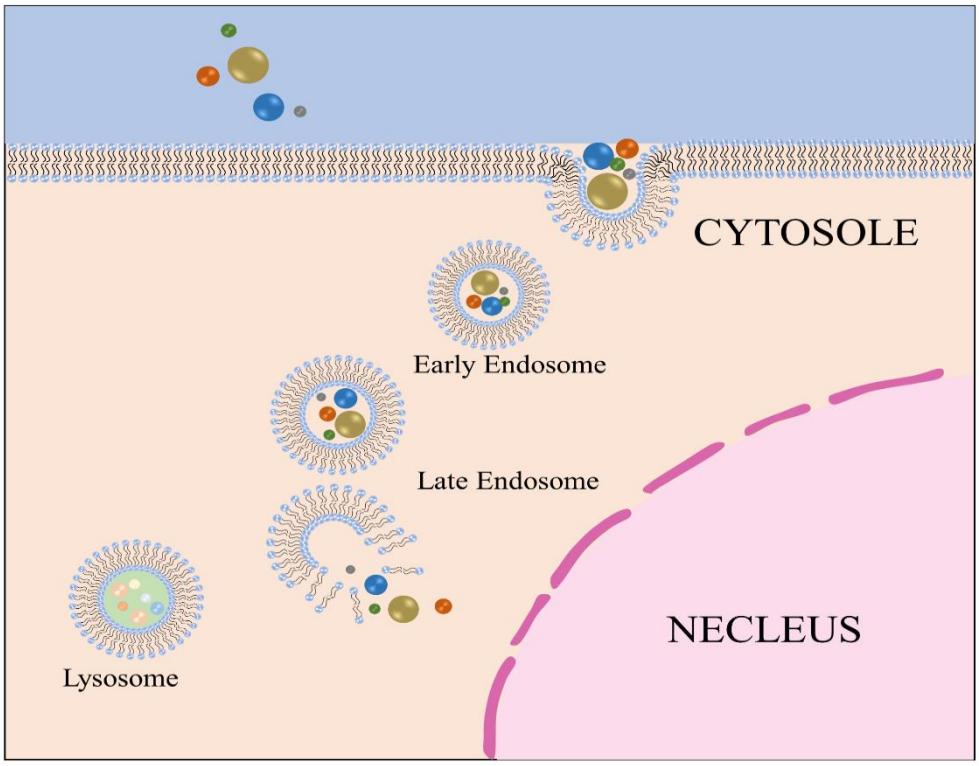

Figure 1. Intracellular transport process of CPPs after entering the cell through endocytosis pathway.

4. Application examples

4.1. Gene delivery

With the advancement of molecular biology and genomics, a multitude of disease-related genes have been discovered. Delivering modified or engineered disease-related genes to human cells for intracellular targeting has emerged as a potential therapeutic approach for genetic and acquired diseases such as cancer and viral infections. In gene therapy, not only conventional DNA but also siRNA and oligonucleotides have been employed as therapeutic agents. However, these nucleic acid molecules carry a large negative charge and are relatively large in size, posing challenges to their efficient cellular uptake and susceptibility to degradation by endogenous nucleases. Consequently, developing safe and effective gene delivery vehicles is crucial for successful gene therapy.

Currently, gene delivery vehicles can be categorized into two main types: viral and non-viral carriers. Although viral carriers exhibit higher delivery efficiency, their safety profile is a concern, leading to increased interest in non-viral carriers such as liposomes, polymers, and peptide-based carriers. Figure 2 illustrates the in vivo delivery process of non-viral carriers. Among these carriers, peptide-based systems have gained significant attention due to their advantageous properties, including good biocompatibility, facile synthesis and modification, and high loading capacity.

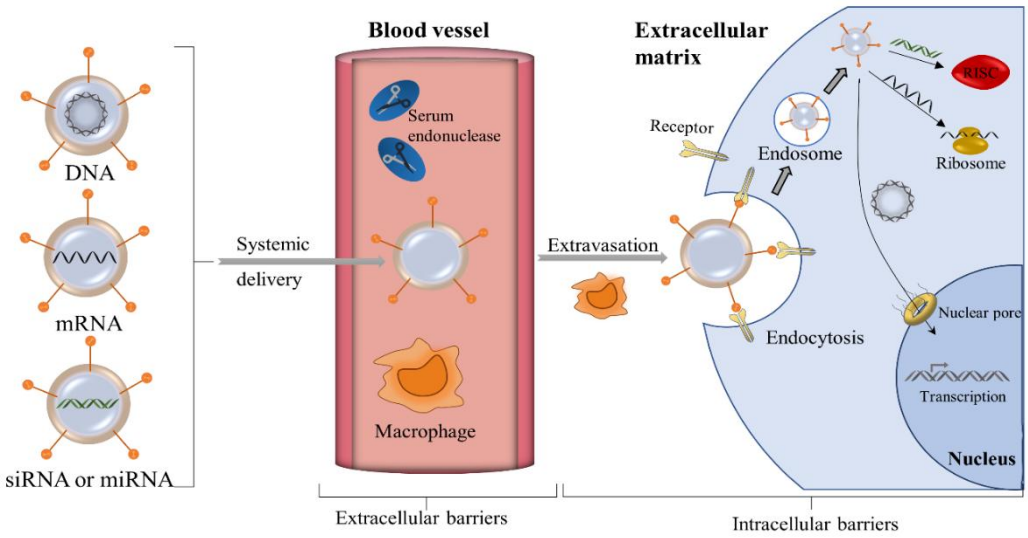

Figure 2. *In vivo* gene delivery process of non-viral vectors.

An ideal gene delivery system should possess several key functions. Firstly, it should efficiently condense and protect genes, with the carrier/gene complex size controlled within the range of 10-300 nm. Secondly, the system should maintain stability from the administration site to the target site. Thirdly, it should effectively reach the target cells or tissues and enter them. Lastly, if endocytosis is the mode of cellular entry, the system should enable escape from the endosomes and facilitate nuclear entry.

In recent years, researchers have integrated peptides with various functionalities to create multifunctional peptide-based gene delivery carriers that achieve efficient gene delivery in vivo with low toxicity and immunogenicity. These multifunctional peptide segments primarily include membrane-penetrating functional segments, endosome escape functional segments, and targeting functional segments. Membrane-penetrating functional segments typically consist of cationic cell-penetrating peptides (CPPs) that carry positive charges. CPPs not only facilitate the binding and condensation of genes through electrostatic attraction but also mediate their translocation across the cell membrane. Endosome escapes functional segments aid peptide/gene complexes that enter cells via endocytosis in escaping from the endosomes. Targeting functional segments assists in directing the peptide/gene complexes to specific cells or tissues, thereby enhancing delivery specificity. In practical applications, peptide carriers are often covalently linked with polyethylene glycol (PEG) to reduce their adsorption to serum proteins, increase blood stability, and prolong their half-life in vivo by reducing renal clearance rates.

During gene delivery, the cell membrane serves as the primary barrier for entering nucleic acids into cells. Cationic or amphipathic CPPs have shown the ability to facilitate the penetration of nucleic acids through the cell membrane without significant cytotoxicity. Consequently, they are commonly employed as membrane-penetrating functional segments in non-viral gene delivery vehicles. Poly-L-lysine (PLL) derivatives, which are biodegradable, were among the earliest identified cationic CPPs with non-specific cell-penetrating ability. PLL can form charged nanoparticles with adjustable charge density through interactions with nucleic acids, with molecular weights ranging from a few thousand to nearly one million Daltons. Studies have demonstrated that compared to the commercial lipid-based transfection reagent SuperFect®, PLL exhibits higher gene transfection efficiency both in vitro and in vivo, while maintaining superior safety profiles. Star-shaped PLL/pDNA complexes have been incorporated into collagen scaffolds, successfully transfecting autologous host cells in vivo, showcasing their potential for tissue engineering applications. PEGylated PLL/pDNA complexes have been utilized for lung gene

therapy by transfecting airway epithelial cells. PEGylation diminishes non-specific interactions of PLL within the in vivo system, thereby improving delivery efficiency and safety. Similarly, PEGylated PLL/siRNA complexes have been employed in gene therapy for liver cancer, demonstrating potent anti-tumor effects by targeting angiogenesis. In addition to PLL, oligoarginine is another widely utilized CPP in gene-delivery vehicles. Compared to PLL, oligoarginine exhibits enhanced cell-penetrating ability and achieves higher gene delivery efficiency in vivo, as supported by multiple studies.

Kim et al. successfully delivered the beta-galactosidase gene to mouse skin tissue using the R15 peptide, resulting in high levels of gene expression. Another study employed a targeted, oxidation-sensitive oligo-arginine (rsPOLA) to deliver plasmid DNA (pDNA) to mice, effectively reducing atherosclerotic inflammation. The intracellular mechanism of action involves the reduction of disulfide bonds in rsPOLA within mouse endothelial cells, leading to the disintegration of the peptide/pDNA complex and subsequent release of pDNA (Figure 3). Compared to the control group, the experimental group exhibited a 30% decrease in the average lesion area of the aortic sinus. Several in vivo studies have confirmed the efficacy of reducing oligo-D-arginine (rPOA) as a non-viral gene delivery vehicle for the treatment of hypoxic-ischemic brain injury, spinal tumors, ischemic heart disease, and lung diseases. In certain cases, CPPs containing both arginine and lysine have demonstrated enhanced membrane permeability and higher gene transfection efficiency. For instance, Rossenberg et al. obtained a series of derivative peptides by replacing the lysine portion of the CPP YKAK8WK with arginine and applied them to gene delivery. The results indicated that the peptide/DNA complex containing both arginine and lysine exhibited increased resistance to plasma-induced breakdown and achieved higher transfection efficiency. Johnson et al. designed and synthesized a novel CPP, GGG (ARKKAAKA)4, and utilized it for gene delivery in ocular tissues. The complex formed between GGG (ARKKAAKA)4 and siRNA efficiently crossed the cell membranes of retinal and corneal tissue sites both in vitro and in vivo.

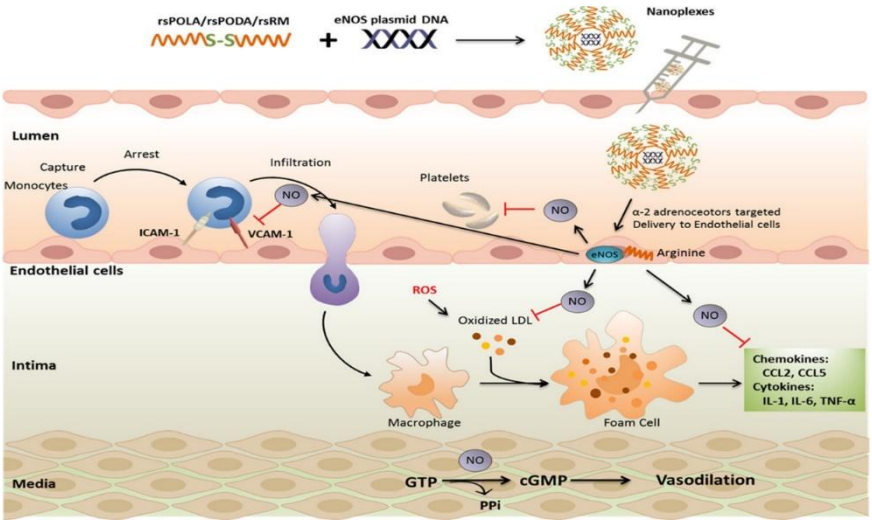

Figure 3. *In vivo* transport pathway and mechanism of rs POLA/pDNA complex[120]. Copyright 2017 Elsevier B.V.

It is noteworthy that highly branched macromolecules such as dendritic and star-shaped polymers have demonstrated uniformity and monodispersity, making them suitable non-viral carriers for gene therapy. Various branched macromolecules containing arginine or lysine have exhibited excellent performance in gene delivery. For instance, dendritic peptides rich in arginine delivered pDNA with six-fold higher transfection efficiency than PEI/DNA complexes and exhibited good tolerability in a breast tumor model. In another study, 14 cationic peptides, including linear peptides, lipid peptides, and dendritic peptides, were employed to deliver siRNA for

treating allergic lung inflammation in mice. Among these cationic peptides, a dendritic peptide rich in arginine (LTP) demonstrated superior delivery efficiency and therapeutic effects. Similarly, a comparison of modified linear and branched CPPs R9 revealed that the complex formed by branched CPP B-mR9 with siRNA exhibited enhanced serum stability, tumor site accumulation, and gene silencing effects compared to the linear CPP L-mR9. In vivo distribution experiments confirmed that the B-mR9/siRNA complex accumulated effectively at the tumor site, retaining fluorescent signal intensity even after 48 hours of administration, whereas the simple fluorescent dye dispersed throughout the body within 1 hour of administration and showed minimal fluorescent signal after 24 hours. Similar advantages were observed with a dendritic PLL/siRNA complex, which significantly inhibited tumor growth in vivo.

In addition to cationic CPPs, certain amphipathic CPPs have also been utilized for gene delivery. For example, the amphipathic CPP TP10 modified with a stearoyl group formed a complex with pDNA that achieved efficient gene transfection in mice without significant toxicity or immunogenicity. Andaloussi et al. introduced trifluoromethylquinoline and fatty acids into TP10 to obtain the CPP PepFect6 (PF6), which possesses endosome-escaping functionality. PF6/siRNA nano complexes successfully achieved gene knockout in liver cells after intravenous injection into mice. As a reverse peptide of the CPP CADY-K, the amphipathic CPP RICK can deliver siRNA into cells through direct transduction. In vivo experiments demonstrated that PEGylated RICK and siRNA complexes exhibited higher efficiency in gene knockout targeting cell cycle proteins than control peptides, highlighting their potential in cancer therapy. Rittner et al. designed and synthesized two alpha-helical amphipathic CPPs, ppTG1, and ppTG20, which facilitated pDNA delivery. Compared to the control peptide, intravenously injected ppTG1/pDNA and ppTG20/pDNA complexes displayed higher lung gene transfection efficiency in mice. Liu et al. physically mixed the amphipathic CPP PVBLG-8 with the random coiled anionic peptide poly (lysine) (PLG) for siRNA delivery. The introduction of PLG enhanced the stability of the peptide/siRNA nano complex, and the alpha-helical conformation of PVBLG-8 facilitated its penetration through the cell membrane and endosome membrane. The nano complex successfully overcame multiple biological barriers, demonstrating good tumor targeting and cell penetration in a mouse xenograft tumor model. Similarly, the alpha-helical structure of the amphipathic CPP PPABLG played a crucial role in its penetration through the cell and endosome membranes. The PPABLG/TNF-αsiRNA nano complex achieved gene knockout of TNF-α in mice after systemic administration, exhibiting significant anti-inflammatory effects and promising potential as an anti-inflammatory drug. Most non-viral gene delivery vehicles are taken up by cells through endocytosis and are subsequently enveloped in endosomes. Therefore, endosome escape is a crucial step in achieving efficient gene delivery. Histidine, as an effective mediator for endosome escape, can disrupt the endosome membrane through the "proton sponge effect" and is commonly employed as a gene delivery carrier for peptides. The histidine-rich peptide LAH4-L1 displayed superior gene silencing efficiency when combined with siRNA. The histidine component of LAH4-L1 can absorb protons in an acidic environment, leading to the rupture of the endosome membrane and subsequent endosome escape. The introduction of histidine enabled PEG-modified PLL to exhibit higher gene transfection efficiency, with the delivered siRNA successfully reducing the expression of endogenous vascular endothelial growth factor in mice. Compared to non-histidine-modified PEG-PLL, histidine-modified PEG-PLL displayed a higher tumor growth inhibition rate in HepG2 tumor-bearing mice, attributed to histidine-mediated endosome escape. H3K4b, a nano-complex composed of a poly-peptide and siRNA, contains a lysine core and four branches composed of histidine and lysine repeat sequences, while the siRNA targets TGF-β1 and COX-2. In vivo experiments demonstrated that H3K4b effectively reduced the volume of human proliferative scar tissue transplants. The peptide SHRss, which combines arginine, histidine, and stearoyl groups, exhibited high cellular uptake and strong endosome escape ability as a gene delivery carrier. In vivo experiments revealed that the SHRss2/Cy5-siRNA nano-complex exhibited significantly higher average fluorescence intensity in tumor

tissue than free Cy5-siRNA after intravenous injection into mice. Moreover, the complex successfully suppressed luciferase activity in mouse tumor sites, indicating its potent gene-silencing ability. Similarly, a reducible oligoarginine/polyhistidine/stearoyl complex served as a co-delivery carrier for microRNA and the anticancer drug doxorubicin in the treatment of non-androgen-dependent prostate cancer.

While CPPs facilitate the cellular uptake of gene therapy drugs, their cellular penetration process is generally non-specific. This can lead to "off-target effects" in which the drugs enter non-target cells or tissues, resulting in unnecessary gene expression and potential side effects142. To address this issue and increase the selectivity of gene delivery vectors, researchers have incorporated specific ligands into peptide-based gene delivery systems. These ligands can recognize and bind to corresponding receptors on the target cell surface, enabling targeted delivery of gene therapy drugs to specific cells or tissues. Common ligands include antibodies, proteins, peptides, and small molecules.

For instance, a gene delivery vector composed of the monoclonal antibody H22 and the CPP R9 has been developed, achieving gene delivery of siRNA to monocytes both in vitro and in vivo. The monoclonal antibody H22 specifically binds to the CD64 receptor on the surface of monocytes. In another study, researchers screened peptides Y and E through phage display technology, which can target respiratory epithelial cells, and modified them on the surface of polymer nanoparticles. The modified nanoparticles successfully achieved efficient gene silencing in respiratory epithelial cells.

The peptide RGD, composed of arginine, glycine, and aspartic acid, can target overexpressed integrin receptors on the surface of tumor cells and endothelial cells. Therefore, RGD is often used as a tumor tissue targeting segment in gene delivery vectors, which can be in the form of linear peptides or cyclic peptides. For example, researchers modified the peptide PR_b containing the RGD segment on the surface of liposomes and used it for gene delivery to colorectal cancer cells. The modified liposomes showed significantly enhanced gene transfection efficiency compared to PEI and unmodified liposomes. Folic acid, a small molecular ligand, can specifically target folate receptors overexpressed on the surface of tumor cells. Researchers modified folic acid molecules on the surface of silicon dioxide nanoparticles and used them to deliver siRNA to breast cancer cells, resulting in increased cell uptake and silencing efficiency compared to unmodified nanoparticles.

In CNS gene delivery, specific ligands that can recognize BBB surface receptors and facilitate receptor-mediated endocytosis are widely used. The BBB mainly consists of tightly arranged brain microvascular endothelial cells (BMVECs). Common ligands targeting the BBB include RVG29, Angiopep-2, and TfR, which can recognize overexpressed acetylcholine receptors, LRP1 receptors, and TfR on the surface of BMVECs, respectively150. RVG29, derived from the rabies virus glycoprotein, is a peptide composed of 29 amino acid residues that can continue to target neuronal cells after crossing the BBB. Delivery of siRNA modified with RVG29-linked R9 successfully reduced the expression of endogenous SOD-1 enzyme in the mouse brain and prolonged the survival time of mice infected with Japanese encephalitis virus151. Similarly, the peptide Angiopep-2 can continue to target glioma cells after crossing the BBB. Researchers modified Angiopep-2 on the surface of polymers and used it to deliver relevant plasmid DNA to glioma cells in the mouse brain, leading to significant apoptosis of glioma cells and prolonged survival time152,153. Additionally, gene delivery vectors modified with transferrin (Tf) can selectively enter glioma cells by crossing the BBB. Researchers modified Tf on the surface of dendritic polymers and successfully delivered gene therapy complexes into the mouse brain, resulting in higher gene expression compared to the group without Tfn modification.

4.2. Anti-tumor

CPPs (cell-penetrating peptides) have gained significant attention for their ability to efficiently penetrate cell membranes, making them valuable tools for delivering anticancer drugs, including nucleic acid drugs, small

molecule drugs, peptides, and proteins, into tumor cells. These drugs typically lack membrane-penetrating capabilities on their own and rely on CPPs to enter the target cells. Once inside the tumor cells, these drugs can act on specific intracellular targets such as DNA, proteases, and mitochondria, inducing apoptosis and effectively eliminating the tumor cells. Peptide and protein drugs have emerged as promising candidates for cancer therapy due to their low toxicity, minimal resistance development, ease of synthesis, and modification. Practical applications of these drugs have shown promising results. For instance, Araujo et al. developed a chimeric peptide, pep5-TAT, which combined the tumor-inhibiting peptide pep5 with the TAT peptide. Pep5-TAT exhibited significantly enhanced membrane-penetrating abilities compared to pep5 alone. In vitro studies demonstrated the inhibitory effects of pep5-TAT on various tumor cells, as illustrated in Figure 4. Moreover, in vivo administration of pep5-TAT reduced the volume of rat brain C6 glioma by 50%.

In many human cancers, tumor-suppressor proteins like p53, p16, and SMAC lose their ability to induce apoptosis due to genetic mutations. To address this issue, researchers have explored strategies to restore the function of these proteins by delivering full-length proteins or key peptide sequences into tumor cells. Snyder et al. developed a chimeric peptide called Trojan p53, which incorporated the C-terminal sequence of the p53 protein with the TAT peptide. Intraperitoneal injection of Trojan p53 in mice with advanced peritoneal lymphoma significantly extended their average lifespan, demonstrating its therapeutic potential. Hosotani et al. synthesized a chimeric peptide known as Trojan p16, consisting of a disulfide-bridged compound comprising 20 amino acid residues from the p16 protein and penetratin. The anticancer effects of Trojan p16 were evaluated in a mouse pancreatic tumor model, revealing significant inhibition of tumor growth and increased survival rates in treated mice. Fulda et al. developed SMAC-TATp, a chimeric peptide composed of TAT and a seven-amino acid sequence from the N-terminus of the mitochondrial protein SMAC. SMAC plays a crucial role in regulating the apoptosis process by inhibiting apoptotic protein inhibitors upon release from mitochondria. In vitro experiments demonstrated that while SMAC-TATp alone did not directly kill tumor cells, it sensitized them to external apoptotic stimuli, thereby enhancing the effectiveness of other apoptosis-inducing drugs in killing tumor cells. Notably, in a U87 human glioma mouse xenograft model, combined administration of SMAC-TATp and TRAIL significantly suppressed tumor growth. Overall, CPPs have proven to be valuable in facilitating the intracellular delivery of various anticancer drugs, including nucleic acids, small molecules, peptides, and proteins. The combination of CPPs with peptide and protein drugs holds promise for developing effective and targeted therapies with reduced toxicity and enhanced therapeutic outcomes.

Protease drugs, known for their repeatable action, exhibit a stronger therapeutic effect compared to general peptides and protein drugs, making them promising candidates for tumor therapy. An emerging approach involves delivering highly toxic proteases into tumor cells to exert repeatable anti-tumor effects. Gelonin, a plant-derived toxin belonging to the class of ribosome-inactivating proteins (RIPs), can inhibit protein translation within cells with a half-inhibitory concentration (IC50) at picomolar levels. However, gelonin itself has negligible anti-tumor activity due to its lack of cell-penetrating ability, making it challenging to enter tumor cells and exert its effects. To overcome the cell membrane barrier, Park et al. developed conjugates of gelonin with cell-penetrating peptides (CPPs) such as TAT or LMWP. In vitro experiments demonstrated that both TAT-gelonin and LMWP-gelonin exhibited potent anti-tumor activity. In a CT26 colon cancer mouse model, both CPP-gelonin conjugates completely inhibited tumor growth at a dosage of 100 μg. In addition to gelonin, researchers have also combined caspase-3, asparaginase, and the pro-apoptotic peptide KLA (KLAKLAKKLAKLAK) with CPPs for anti-tumor therapy. In rat ascites models, a mouse acute lymphoblastic leukemia model and a mouse lung cancer model,

CPP-caspase-3, CPP-asparaginase, and CPP-KLA significantly improved the survival rate of mice, respectively. In addition to delivering anti-tumor drugs, CPPs can directly act on tumor cell membranes, exerting anti-tumor effects through membrane disruption. These CPPs, also known as membrane-disruptive peptides (MDPs), possess membrane-disruptive properties. MDPs target tumors based on charge interactions between peptides and the negatively charged surface of tumor cell membranes. Upon contact, the peptides adopt secondary structures like alpha-helices or beta-folds, enabling them to disrupt the integrity of tumor cell membranes, leading to cell death. MDPs are often derived from antimicrobial peptides (AMPs), which also possess cell-penetrating properties. Due to the overlap in characteristics, there is no clear boundary between the two categories.

The membrane-disruptive mechanism of MDPs is illustrated in Figure 4. Examples of MDPs include SC182, a branched cationic CPP derived from the C-terminal domain of the antimicrobial protein CAP18. SC182 transforms into an alpha-helical structure in the cell membrane environment, selectively entering and killing tumor cells through its membrane-disruptive effect. MPI-1, a synthetic analog of the AMP polybia-MPI, selectively binds to the surface of human prostate cancer and liver cancer cells, leading to rupture and death of cancer cells. MPI-1 damages the integrity of tumor cell membranes, and its anti-tumor activity is attributed to its amphipathic alpha-helical structure and the negative charge density on the surface of tumor cells. The parent peptide of MPI-1, polybia-MPI, has also shown selective inhibition of prostate cancer and bladder cancer cell proliferation, likely associated with the overexpression of phosphatidylserine (PS) on the surface of cancer cells. Gomesin, a cationic AMP derived from the hemocytes of the spider Acanthoscurria gomesiana, exhibits anti-tumor activity by killing melanoma cells, breast cancer cells, and colon cancer cells in vitro, as well as inhibiting the growth of subcutaneous melanoma in mice. The anti-tumor mechanism of Gomesin involves its beta-fold structure, electrostatic attraction, and hydrophobic interactions, which contribute to the formation of pores on the surface of cell membranes.

Another example is the peptide SVS-1, which adopts a β-folded conformation upon contact with tumor cell membranes, leading to membrane disruption through pore formation mechanisms. SVS-1 demonstrates significant cytotoxicity against lung cancer cells, epidermal cancer cells, and breast cancer cells while exhibiting low toxicity towards healthy cells such as human umbilical vein endothelial cells and red blood cells. Similarly, peptides including epinecidin-1, dermaseptin B2, NK-18, ChMAP-28, magainin2, A9K, and temporin-1CEa induce tumor cell death by disrupting tumor cell membranes. Magainin2, A9K, and temporin-1CEa possess a dual anti-tumor mode, as they not only disrupt tumor cell membranes and induce cell death but also target intracellular components, promoting cell apoptosis once they enter the cells. The combination of magainin2 and penetratin has yielded the peptide MG2A, which exhibits an IC50 value in tumor cells at the micromolar level. Mechanistic studies have revealed that MG2A not only causes cell membrane lysis but also induces caspase-dependent cell apoptosis. A9K, as a short-chain amphipathic peptide, possesses both surfactant-like membrane-disrupting activity and the ability to induce cell apoptosis by disrupting mitochondrial function. A9K selectively inhibits the growth of various tumor cells, exhibiting high protease stability and low immunogenicity. Similarly, temporin-1CEa exerts its anti-tumor effect through membrane disruption and disruption of mitochondrial function upon entering the cell. It is worth noting that the expression of negatively charged molecules on the surface of tumor cells can influence the interaction between CPPs and tumor cell membranes. As a result, the same CPPs may employ different tumor-killing mechanisms against various types of tumor cells, and specific CPPs may exhibit preferential killing abilities against certain types of tumor cells.

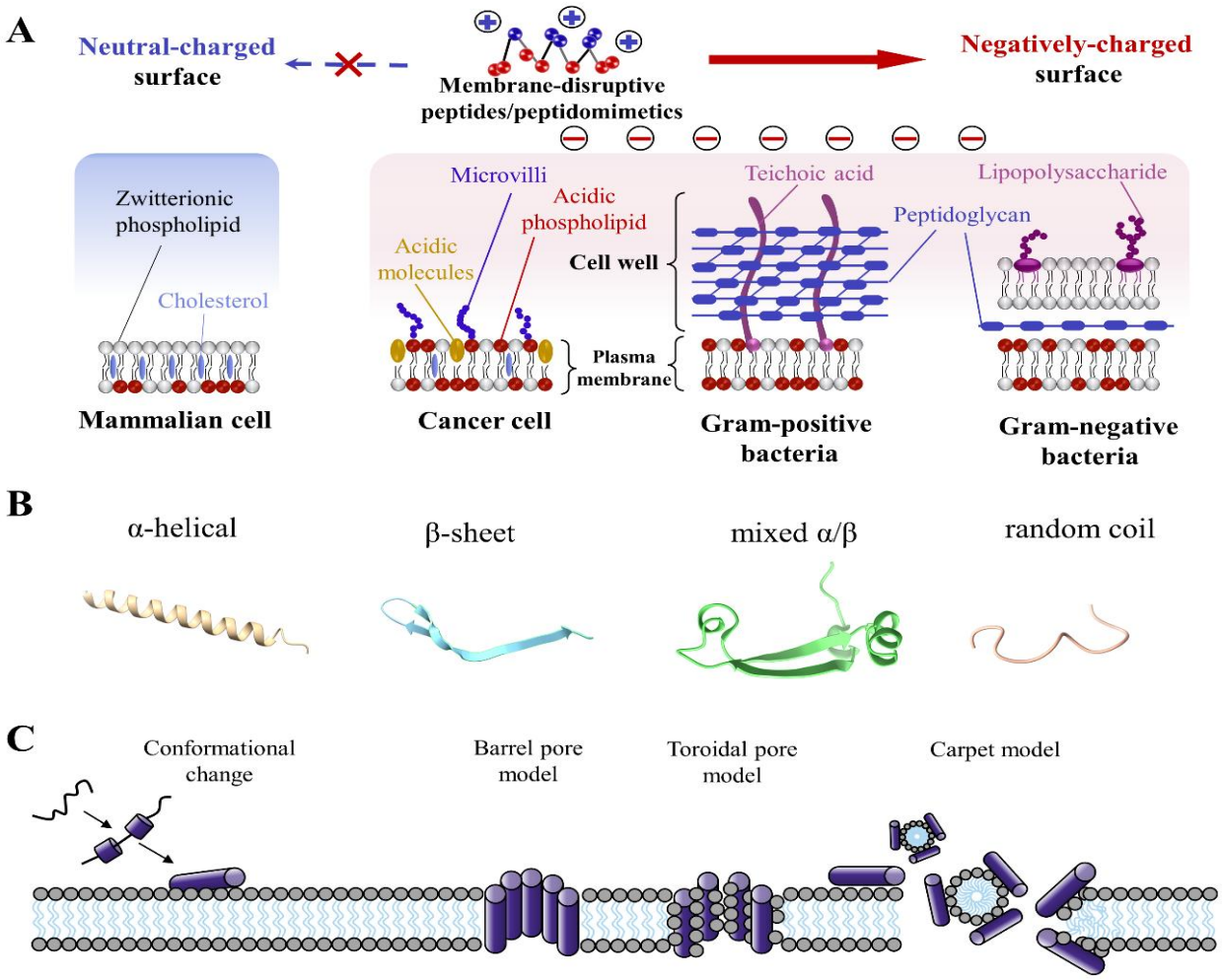

Figure 4. Membrane-disrupting mechanism of MDPs.

5. Conclusion

This review focuses on the significance of cell-penetrating peptides (CPPs) in overcoming the challenge of poor membrane permeability of bioactive molecules. The ability of CPPs to facilitate the delivery of therapeutic agents across physiological barriers offers a promising solution in drug development. CPPs employ covalent or non-covalent strategies to transport diverse bioactive molecules into cells, with non-covalent approaches preferred to preserve molecular activity. Extensive research has demonstrated the potential of CPPs in both preclinical and clinical settings, highlighting their role in enhancing the efficacy of bioactive molecules by improving their membrane penetration capabilities. The emergence of CPPs represents a significant advancement in biomedical science with broad implications for drug delivery.

Conflict of interest

All the authors claim that the manuscript is completely original. The authors also declare no conflict of interest.

Author contributions

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Yuan Gu; Writing–original draft: Long Wu; Writing–review & editing: Yasir Hameed, Mohsen Nabi-Afjadi.

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