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Review

Overcoming the cellular barriers and beyond: Recent progress on cell penetrating peptide modified nanomedicine in combating physiological and pathological barriers

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ABSTRACT

The complex physiological and pathological conditions form barriers against efficient drug delivery. Cell penetrating peptides (CPPs), a class of short peptides which translocate drugs across cell membranes with various mechanisms, provide feasible solutions for efficient delivery of biologically active agents to circumvent biological barriers. After years of development, the function of CPPs is beyond cell penetrating. Multifunctional CPPs with bioactivity or active targeting capacity have been designed and successfully utilized in delivery of various cargoes against tumor, myocardial ischemia, ocular posterior segment disorders, etc. In this review, we summarize recent progress in CPP-functionalized nano-drug delivery systems to overcome the physiological and pathological barriers for the applications in cardiology, ophtalmology, mucus, neurology and cancer, etc. We also highlight the prospect of clinical translation of CPP-functionalized drug delivery systems in these areas.

1. Introduction

Facing the complex and heterologous physiological and pathological barriers *in vivo*, lack of efficient drug delivery is still a thorny problem which impedes effective therapeutic results. Nano-drug delivery systems have provided new opportunities in the field of biomedicine by changing the *in vivo* fate and pharmacokinetic properties of drugs, thereby enhancing targeted tissue or organ delivery and reducing side effects. Besides the physiological and pathological barriers, the hydrophobic bilayer that maintains the survival and physiological functions of cells, also presents a cellular barrier for preventing the influx of extracellular molecules, especially for nucleic acid or protein/peptide therapeutics that have to be

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internalized into the cytoplasm to exert their pharmacological functions. Nanoparticles (NPs) have demonstrated great potential in enhancing the cargo translocation across the cell membrane via different vesical translocation pathways. Nevertheless, the cellular internalization efficiency of nano-drug delivery systems is still limited.

During the past few decades cell penetrating peptides (CPPs) have emerged as powerful weapon and efficiently elevated the transport of the cargos through interaction with proteins on the cell membrane via different mechanisms. The combination of nano-drug delivery systems and CPPs may further strengthen the transcellular delivery of therapeutics. CPPs are a family of various peptides ranging from 5 to 30 amino acids that share the ability of passing through cell membranes via either energy-dependent or energy-independent pathways. In the 1990′s, the efficient transcellular internalization of the transactivator of transcription (TAT) protein of HIV-1 and the homeodomain of Antennapedia (a homeoprotein of Drosophila melanogaster) was reported by independent groups [1–3]. With the understanding of the cellular internalization capacity of these proteins, researchers were devoted to identifying the relation between sequence and the internalization capacity, as well as shortening the sequence [4–6]. After decades' of development, over 100 peptides have been designed or identified to efficiently transport therapeutic agents or biomacromolecules across eukaryotic and prokaryotic membranes, including cationic, cyclic, amphipathic and hydrophobic CPPs, which have been summarized in previous literatures [7–9]. The functions and mechanisms of CPPs have been thoroughly reviewed elsewhere [10–12].

CPPs have been identified as safe and effective delivery vehicles, and utilized in both *in vitro* and *in vivo* delivery of various cargos including peptides [13], proteins [14], small organic molecules [15], antisense oligonucleotides [16], fluorescent dyes [17] or small interfering RNAs [18]. For delivery of protein or peptides, CPPs can be directly conjugated to the C-terminus of protein or peptide. The hydrophobic interactions between CPPs and the protein/peptide cargos allow the formation of nanocomplex to facilitate the delivery of cargos. For nucleic acid delivery, electrostatic interaction is more commonly used to form nanocomplexes, promoting efficient cellular internalization of siRNA or DNA. Besides, covalent conjugation is another successful method to link siRNA with CPPs such as transportan and penetratin [19]. In addition, CPP-drug conjugates can be formulated with a variety of conjugation techniques including amid bond, bifunctional succinyl linker, disulfide linker or maleimide linker, etc. [15]. Direct conjugation of CPPs with therapeutic agents have significantly improved the solubility, stability and permeability.

The concept of modifying NPs with CPPs has emerged for decades and shows great potential in drug delivery to overcome physiological barriers, especially for the treatment or imaging of different pathologies such as inflammation [20], diabetes [21], ischemia [22], cancer [23], neurodegenerative diseases [24], exhibiting promising results. In the past decades CPP-aided drug delivery through non-invasive routes has emerged, including transdermal pathway, intranasal, intraocular, or oral delivery via gastrointestinal tract. These non-invasive routes are well tolerated by patients with high compliance. In this review, we mainly focus on features of novel CPPs and the conjugation of CPPs with nano-drug delivery systems. The applications of CPP-modified nanoparticles (CPP-NPs) in overcoming the physiological and pathological barriers have been summarized. We attempt to update and highlight the latest progressions in CPP-NPs and their advantages in overcoming these barriers in the field of biomedicine. We also aim to provide insights of the potential clinical applications of CPP-NPs and the state of art in the future perspective of clinical translation of CPPs.

2. Cellular internalization and endosome escape mechanisms of CPPs

Although CPPs have been developed for over 30 years, the exact cellular internalization mechanisms still remain to be fully elucidated. The cellular uptake efficiency of CPPs is affected by many factors including cell types, constitution of membranes, characters of cargo, incubation conditions (time and concentration), and CPPs themselves, etc. [25]. Generally, the internalization mechanisms can be categorized into direct penetration and energy-dependent endocytosis. Direct penetration is an energy-independent process and is associated with the interactions between positive charged CPPs and negatively charged molecules on cell membranes such as glycosaminoglycans, which involves formation of pores, inverted micelles and the "carpet model". Under the condition of low temperature and energy depletion, direct penetration usually occurs at a high CPP concentration. Notably, permanent or temporary destabilization of the membrane is needed for this energy-independent process. The active energy-dependent endocytosis mechanisms include micropinocytosis, clathrin-dependent, caveolin-mediated or lipid raft-mediated endocytosis. For some specific cell types (macrophages, neutrophils and monocytes), phagocytosis is involved in the internalization of large particles. In most cases, more than one pathway is involved in the internalization of CPPs. Besides, this process is strongly influenced by the cargos. TAT-protein conjugates are internalized via lipid raft, while TAT-flurophore utilizes clatherin-mediated endocytosis mechanism. Currently, the cellular internalization mechanisms of CPP-NPs need further exploration.

After endocytosis, endosome escape is necessary to avoid lysosomal degradation, which is also a limiting step for efficient drug delivery. One possible mechanism for endosome escape is the disruption of the endosomal membrane. The positively charged CPPs, such as TAT, are believed to interact with the negatively charged endosomal membrane and induce the rupture by forming pores on the membrane. Besides, forming ionic pairs between CPPs and endosomal membrane lipids may facilitate the endosome escape process. To overcome the limitation of endosomal compartment, several approaches can be considered. The use of fusogenic lipid such as dioleoylphosphatidyl ethanolamine (DOPE), which can shift from lamellar phase to inverted hexagonal phase in endosome, may enhance the endosome release

[26]. This strategy is especially feasible for CPP-modified liposomes containing DOPE. The incorporation of membrane disruptive peptides offers better solution for endosome escape, which will be discussed in the following chapter. Besides, utilizing the "proton sponge" effect of NPs containing cationic polyethylenimine (PEI) can enhance the efficiency of endosome escape.

3. Multifunctional CPPs

CPPs can be classified with different categories. Based on the amino acid constituents, CPPs can be grouped into cationic CPPs, amphipathic CPPs and hydrophobic CPPs, among which cationic CPPs are the most widely used. The mechanisms of how CPPs overcome the cell membrane barrier have been thoroughly analyzed and reviewed elsewhere [27]. As mentioned before, TAT (48–60) from HIV and poly-arginine sequences (R5-R9) are two frequently used examples. Some research groups showed the optimal number of arginine residues for efficient translocation is 8 [28,29]. Amphipathic CPPs contain both hydrophilic and hydrophobic amino acids, therefore second structures like α-helix or β-sheets are usually formed, which hold the potential to interact with cell membranes [30–32]. Recently, hydrophobic CPPs are identified by orthogonal high-throughput screening. More detailed information about classification and design strategies of CPPs have been thoroughly reviewed [33,34].

Despite the fact that CPPs have elevated the capacity of transcellular delivery and may alter the internalization pathways, due to the complex physiological conditions and multistage barriers existed *in vivo* delivery, CPPs still face the shortcomings of lacking specificity or endosome escape capacity. As a result, with the aim to enhance the delivery efficiency, multifunctional CPPs have been designed and combined with NPs. The majority of these novel multifunctional CPPs are derived from natural structures or the combination of different multivalent structures.

3.1. *Active targeting CPPs*

Although by rational design, the translocation efficiency and *in vivo* delivery stability can be improved, the lack of specificity still hinders the clinical translation of CPPs. Great efforts have been spent in improving the tissue targeting of CPPs. One strategy is to conjugate a targeting ligand moiety with CPPs. The targeting moiety can be a small molecule, peptide or protein. Kang et al. introduced various targeting peptides in a review article [35]. Integrin is highly expressed on tumor cells and serves as a frequently used target for tumor targeting. Several researches have designed novel CPPs by fusing cell penetrating moieties with integrin targeting ligand to enhance their specificity. In a previous study, RGD moiety, which has the ability to recognize and bind to $\alpha_v\beta_3$ on tumor cells, was conjugated with CPP R8 to yield enhanced tumor active targeting and penetrating. The results suggested that R8-RGD significantly achieved tumor active targeting *in vivo*, and elevated antitumor effects compared with R8 or RGD alone [36]. Further, a phenomenon that an exposed R/KXXR/K C-terminal sequence of peptide could recognize cell surface receptor neuropilin-1 (NRP-1) overexpressed on many tumor cells was first discovered by Erkki Ruoslahti in 2009 [37], which was termed "C-end Rule". Based on these, the sequence of RGD was reversed into dGR to meet the requirement of C-end Rule. And the resulting sequence exhibited triple functions including active targeting ability to both NRP-1 and $\alpha_v\beta_3$ as well as the cell penetrating capacity, which significantly elevated the tumor targeting of therapeutics [38].

Small molecules or antibodies can also be combined with CPPs to enhance the tissue specificity [39,40]. In our previous work, folic acid (FA) was co-modified on the surface of liposome with CPP dNP2 to target breast cancer and its brain metastasis. The BBB transmigration efficiency by the dual modified drug delivery system was significantly higher compared with CPP or FA alone [41,42]. For intracellular delivery of nucleic acid based-drugs such as plasmid DNA, the final destination is the nucleus. Nuclear localization sequence (NLS) can be combined with CPPs. A TAT-based endothelial cell targeting gene carrier was synthesized, which combined peptides and oligohistidines [43]. To simplify the structure of antibody, single-chain variable fragment (scFv) of antibody can be fused with CPPs. The fusion of BR2 (RAGLQFPVGRLLRRLLR), a 17-amino acid CPP based on the buforin II anticancer peptide with a single-chain variable fragment (scFv) was designed to target mutated KRAS in colon cancer cell model [40]. The strategies of active targeting CPPs are summarized in Table 1.

The second strategy is based on sequence screening procedures. Phage display is a common method to identify target tissues or cells and is well accepted and widely used in the screening of active targeting CPPs [44]. Zhou et al. identified a novel CPP, MT23 (LPKQKRRQRRRK) with high melanoma targeting and cell permeability based on phage display. After fusion with Apoptin, the obtained MT23-Apoptin successfully induced tumor apoptosis and suppressed tumor growth both *in vitro* and *in vivo* [45]. Compared with linear peptides, cyclic peptides represent better candidates for cellular permeation owing to the better metabolic stability. Yamaguchi et al. screened a cyclic heptapeptide DNPGNT in a Caco-2 monolayer and mouse intestinal epithelium model, which was capable of delivery macromolecular drugs with high transcellular permeability [46]. RKOpep (CPKSNNGVC) was discovered by Ferreira et al. by phage display, demonstrating that this peptide was able to target RKO colorectal cancer cells without interacting with normal cells with high affinity [47].

3.2. *Activatable CPPs*

Besides the previously mentioned active targeting CPPs, researchers have designed a series of smart CPPs that can be activated at the environment of target tissue *i.e.* the tumor microenvironment (TME). These stimulus sensitive smart CPPs present an alternative for elevating the specificity of CPPs. Since CPPs are lack of specificity, during blood circulation, CPPs are shielded and protected by polymers or organ targeting ligands, enabling a prolonged blood circulation. The targeting ligands or shielding polymers are conjugated on the surface of NPs via stimulus-sensitive bonds. Upon arrival at the target tissue, under local environment or

Table 1 – Examples of multifunctional CPPs.

Function		Sequence/Strategy	Application	Refs
Active targeting CPPs				
Conjugation or complexation	R8-RGD	RRRRRRRRRGD	Selective accumulation in glioma	[38]
	R8-dGR	RRRRRRRRdGR	Bind both $\alpha v\beta 3$ and neuropilin-1 receptors. Enhanced BBB transmigration and glioma targeting	[36]
	dNP2	KIKKVKKKGRKKIKKVKKKGRK	Co-modified with acid-cleavable FA to enhance BBB transmigration	[42]
	REDV-TAT-NLS-Hn	REDV-YGRKKRRQRRR-PKKKRKV-Hn	Complex with pZNF580 for enhanced transfection on HUVEC cells	[43]
	PF14:TG1 complexation	PF14:Stearyl-AGYLLGKLLOOLAAAALOOLL-NH_2 TG1: EEEEEEXTFFYGGSRGKRNNFKTEEY-NH_2	Improved specificity to U87 cells	[39]
	BR2	RAGLQFPVGRLLRRLLR	Specifically penetrates HCT 116 cancer cells without causing cytotoxicity	[40]
Sequence screening	MT23	LPKQKRRQRRRK	Melanoma targeting	[45]
	DNP	DNPGNT	Enhance permeability in mouse intestinal epithelium model	[46]
	RP-1	WHPWSYLWTQQA	Bind to $CD44^+$ gastric cancer cells with high affinity	[65]
	RKOpep	CPKSNNGVC	Colorectal cancer targeting peptide	[47]
	OSTP	PHLATLF	Target ovarian cancer cell membrane	[66]
Activatable CPPs				
pH activatable	TAT/PEG	pH-sensitive conjugation of PEG	Target certain pathological tissues or intracellular compartments	[49,50]
	TH	AGYLLGHINLHHLAHL(Aib)HHIL-NH_2	Substitute lysine into histine in TK peptide. Positively charged in TME	[54]
	TR	c(RGDfK)-AGYLLGHINLHHLAHL(Aib)HHIL-NH_2	Tandem peptide of RGD and TH peptide	[55]
Enzyme activatable CPPs	TAT/PEG	MMP-cleavable PEG	MMP-sensitive cleavage and ROS-induced DOX release	[56]
	Tri-block sequence: TAT-MMP-sensitive-zwitterionic antifouling sequence	CCVVGRKKRRQRRRPQGGPLGVEKEKEKEK	Extended systemic circulation and enhanced tumor tissue accumulation	[57]
	Fusion protein containing MMP substrate peptide	Low molecular weight protamine (VSRRRRRRGGRRRR)	Selective tumor imaging and intracellular protein delivery	[58]
	Oligoanionic-inhibitory domain shielding TAT via cathepsin B subsrate domain	EEEEEE-PGFK-CRRRQRRKKR	Tumor specific internalization of mesoporous silica-QDs nanoconjugates	[59]
	Polyanionic inhibitor peptide shielding polyarginine via PSA-cleavable peptide	DGGDGGDGGDGG-HSSKYQ-R8	Specifically expose CPP in PSA-positive prostate cancer	[60]
	Thrombin activated peptide	PPRSFL-R9	Thrombin sensitive peptide for *in vivo* imaging	[67]
Endosome escapable CPPs				
	TAT-EEDs	TAT-P6-GFWG, TAT-P6-GFWFG	Endosomal escape domains (EEDs) address the endosomal escape barrier.	[68]
	Pas2r12	FFLIG-FFLIG -RRRRRRRRRRRR	Enhance cytosolic delivery of cargo protein via caveolae-mediated endocytosis	[69]
	GALA3	LA EALA EAL EALA A	Derived from GALA, enhance endosome release efficiency	[70]
	L17E	IWLTALKFLGKHAAKHDAKQQLSKL	Preferential disruption of endosomal membranes and cellular uptake by macropinocytosis.	[61]
	CPP12–1	cyclo(F-D-F-L-3-benzothienylalanine-R-D-RR-D-RQ)	Derived from CPP12 by substitution of L-3-benzothienylalanine (Bta) for L-2-naphthylalanine (Nal), improve endosome escape	[71]

pathological conditions with internal stimulus (pH, enzymes, etc.) or external stimulus (temperatures, irradiation, etc.), the conjugating bond is broken, exposing the targeting ligand to facilitate cellular internalization of the cargo.

3.2.1. *pH activatable CPPs*

Utilizing the acidic microenvironment of tumor, various pH sensitive CPPs have been designed. Many chemical linkers (such as hydrazine or imine bonds) that are stable at neutral or alkaline environment can be hydrolyzed under acidic conditions [48]. Kale et al. designed a series of smart TAT-modified liposomes or micelles using pH sensitive sheddable PEG to shield TAT during blood circulation. Upon arrival at the TME, hydrazone bond broke and TAT was exposed to the tumor cells, resulting in elevated penetration of the liposomes or micelles into tumor cells and a more effective gene delivery [49,50]. Juang et al. reported CPP, neovasculature-targeting peptide and mitochondria-targeting peptide modified solid lipid nanoparticles, which was further coated with pH-sensitive PEG via imine bond. These NPs showed pH-responsive release and internalization in acidic colon cancer due to the break of imine bond, while remained biocompatible in blood circulation [51].

In addition, histidine is widely used to develop pH-responsive drug delivery systems due to the pH sensitivity of its imidazole group. Tu et al. changed the lysine and arginine residues with histidine in PTP-7 (FLGALFKALSKLL), L5 (PAWRKAFRWAWRMLKKAA) and citropin (GLFAVIKKVASVIGGL) to produce membrane-soluble peptides with pH-dependent cytotoxicity [52]. A dual-active targeting strategy using hyaluronic acid (HA) and a pH-responsive CPP (R6H4, rich in arginine and histidine) was reported to enhance tumor targeting efficiency of paclitaxel (PTX) and reduce toxicity in a mouse liver tumor model [53]. He et al. has designed a pH-responsive α-helical CPP TH peptide by substituting lysine in the original TK peptide with histidine. The imidazole ring in the histidine (pKa 6.5) can be protonated in the acidic TME and becomes positively charged to facilitate cellular internalization, while remains slightly negative charged during blood circulation. TH peptide efficiently mediates the transcellular delivery of its cargo both *in vitro* and *in vivo* [54]. To further endow TH peptide with tumor active targeting capacity, active targeting ligand peptide RGD (c(RGDfK)) was conjugated with TH to obtain TR peptide. RGD first mediated the active targeting by binding to integrin $\alpha_v\beta_3$ overexpressed on tumor cells. Upon arrival at TME, the peptide was protonated and became positively charged. TR peptide modified PTX-loaded liposomes exhibited better cellular internalization and therapeutic results compared with TH-, RGD- or PEG modified counterparts [55].

3.2.2. *Enzyme activatable CPPs*

Compared with normal cells, most tumors have unique features of enzyme expression. Matrix metalloproteinase (MMP) is one family of enzymes abundantly expressed in tumor tissues, which degrades the tumor matrix and facilitates tumor metastasis. By utilizing this feature, various MMP-activatable CPPs have been designed. One strategy is MMP-cleavable PEG shedding. Yoo et al. formulated a poly(L-methionine-block-L-lysine)-PLGLAG-PEG (MLMP) micelle, in which PLGLAG served as the substrate of MMP-2/9 and could be cut off in the TME. The exposure of TAT facilitated the targeted internalization of drug loaded micelles [56]. Wu et al. designed a tri-block peptide sequence using TAT as the inner fragment, MMP-9 substrate sequence GPLG as the cleavable linker and a zwitterionic peptide to shied nonspecific binding and stabilize the sequence, which was modified on the surface of gold NPs. TAT was covered to ensure a prolonged circulation time, and the subsequent exposure in the presence of MMP-9 enhanced the cell permeability of gold NPs [57]. MMP-activatable CPPs also enhance the specificity in tumor imaging. Taking advantage of the over-expressed MMP-2 enzyme, Sun et al. designed an MMP-2 activatable, cell permeable magnetic nanoprobe in which the fluorescence doner (mCherry fusion protein) and fluorescence quencher (a nickel ferrite NP) were conjugated with MMP-2 substrate peptide [58]. MMP-sensitive PEG cleavable strategy has endowed CPP-NPs with both stealth circulation and in time targeted cellular internalization, especially in tumor treatment. Besides, other enzymes highly expressed in tumors such as cathepsin B, prostate-specific antigen (PSA) and thrombin have been utilized to activate CPPs. Normally, polyanionic shield was conjugated to CPPs via the substrate sequence of these enzymes [59,60].

3.3. *Endosome escapable CPPs*

As mentioned in previous section, after cellular internalization, extracellular materials are encapsulated into vesicular compartments called endosome, which is another obstacle restraining efficient drug delivery. Ideally, CPP-NPs should facilitate the endosome escape of the cargo. One of the major mechanisms of endosome escape is the disruption of endosomal membrane. Derivatives or fusogenic peptides from viral protein are good source of endosome escapable CPPs. Some viral protein sequences undergo conformational changes upon pH changes in endosomal compartment during the infection process, forming pores in the membrane. Futaki et al. described an endosomolytic peptide L17E, which was a membrane-lytic amphiphilic peptide derived from spider venom peptide M-lycotoxin. L17E was protonated in the endosomal compartment and preferentially disrupted negatively-charged membranes within endosomes, while being nontoxic in neutral environment [61]. Supercharged peptides, such as dimers or trimers of TAT, also disrupt the membranes of late endosomes and release the cargo [62]. The efficiency of endosome escape is affected by the peptide sequence, especially the number of arginine in the sequence [63]. Besides, by changing the chirality of amino acids, the endosome release profile of CPPs may be improved. CPPs seem to be less sensitive to the change in chirality and conformation. Some researchers also find that altering the chirality in HIV-TAT fragments and oligoarginine peptides can directly release cargo into the cytosol and bypass the endosome pathways [64]. Notably, due to the toxicity of membrane disruption, the pore-forming activity should be limited inside the endosome. The impact on cell homeostasis is often overlooked. Using pH-dependent cleavage of shielding moieties may allow temporary endosomolytic activity of these CPPs. Therefore, the fusogenic activity should be

delicately designed and controlled when attempting these strategies.

4. Various NPs can be modified with CPPs to enhance delivery efficiency of drugs

Nanotechnology has been applied in diagnosis and treatment of disease for several decades. In 1965, the idea of nanotechnology was first introduced into drug delivery field by Bangham to explore the potential of liposome in therapy [72]. NPs as drug delivery systems have greatly advanced the drug delivery efficiency and reduced *in vivo* toxicity. The advantages include prolonged blood circulation, altered biodistribution and reduced accumulation in non-target organs, etc. Based on the materials used, NPs can be divided into organic NPs (including liposomes, polymeric micelles), inorganic NPs (including metal NPs or salt NPs) and hybrid NPs. Though NPs exhibit great potential in changing the biodistribution of therapeutic agents and enhancing the accumulation in target organ or tissue, the cellular internalization efficiency needs further improvement. Surface modification of NPs may further strengthen passive or active targeted delivery and the endocytosis of the cargos.

4.1. *Liposomes*

Liposomes are a class of nano-scale drug delivery systems that have biofilm similar structure and good biocompatibility, which have exhibited great potential in targeted drug delivery [73]. The modification of CPPs greatly enhances the transcellular delivery of therapeutic agents. The most common modification strategy is covalently conjugating CPPs with an amphiphilic co-polymer, such as PEG-DOPE or 1, 2-distearoyl-sn–glycero-3-phosphoethanolamine-PEG (DSPE-PEG), in which the hydrophobic segment can insert into the lipid layer of liposomes, forming stable particles. A series of functional CPP-modified liposomes of different formulations are developed for the delivery of chemotherapeutics, demonstrating enhanced drug delivery efficiency and antitumor efficacy [74,75].

4.2. *Polymeric micelles*

Other than liposome, polymeric micelles (PMs) are another class of the most studied nanocarriers in diagnosis and treatment of various diseases, which are generally composed of amphiphilic polymers that self-assemble into nanostructure when the concentration is above the critical micellar concentration (CMC), ranging between 20 and 200 nm in diameter.

To enhance the cellular internalization of therapeutic drugs, various attempts have been made to conjugate CPPs with PMs. CPPs including TAT or TME sensitive CPPs have been modified with polyethylene glycol-polylactic acid (PEG-PLA) to enhance drug delivery in tumor models [76,77]. CPP-modified micelles are also proven excellent non-viral gene delivery systems. Layek et al. designed PM using chitosan (CS) hydrophilic segment and linoleic acid as hydrophobic segment. CPP penetratin was conjugated to CS to facilitate the transcellular delivery of nucleic acid drugs, leading to 5-fold increase in cellular internalization and ~40-fold higher transfection in different cell lines compared with unmodified CS-Lin [78].

4.3. *Quantum dots*

Quantum dots (QDs), a class of NPs with unique properties such as bright and intensive fluorescence, reduced light scattering and low tissue absorption, were first used in biological imaging in 1998 [79]. CPPs can significantly enhance the cell permeation of QDs either in covalent or non-covalent linkages. Xu et al. covalently modified QDs with CPPs consisting of TAT and endosomolytic peptide HA2 to enhance the internalization into mammalian cells [80]. The most common conjugation is forming stable amide bonds with the primary amines on the surface of QDs using crosslinkers such as 1-(3-Dimethylaminopropyl)−3-ethylcarbodiimide hydrochloride (EDC). Besides, thiol-amine coupling is also commonly used in QDs modification. Sulfo-SMCC is a coupling linker that reacts with primary amine on QDs by N-hydroxysuccinimide (NHS) ester on one end and reacts with thiol moiety of cysteine-containing CPPs by maleimide group on the other end, forming thioether bond [81,82].

4.4. *Gold NPs*

Gold NPs emerge as potential therapeutic and diagnostic platform for their biocompatibility, small size that allows deep tissue penetration, and the easy modification with antibodies, peptides or drugs by gold-thiol conjugation. Nevertheless, gold NP-based therapy also faces challenges such as off-target effect and poor accumulation in target tissues. One possible solution is functionalization with CPPs. Fusion peptides including CRRRRRRRRGDS or CLPFFD-oligoarginine have been reported to enhance the targeting and cellular internalization of gold NPs [83,84].

4.5. *Mesoporous silicon nanoparticles (MSNs)*

Inorganic NPs have received great attention in the field of particulate drug delivery systems in recent years. Compared with organic drug delivery systems, such as liposomes and PM mentioned above, inorganic drug delivery systems have the advantage of good biocompatibility, facile preparation and easy surface modification etc.

MSNs have demonstrated great potential in drug delivery for several advantages such as large surface area, high drug loading capacity, good biocompatibility and biodegradability. CPPs can also be modified on MSNs by direct incubation or covalent linkage. Jussi Rytkönen et al. proposed CPP NF51-modified MSNs by simple vortex and incubation for efficient delivery of oligonucleotides, which promoted efficient endosomal release and transfection of Splice correcting oligonucleotides (SCOs) [85].

4.6. *Conjugation strategies*

The conjugation between CPPs and NPs can be based on covalent binding or non-covalent interactions (Fig. 1). Non-

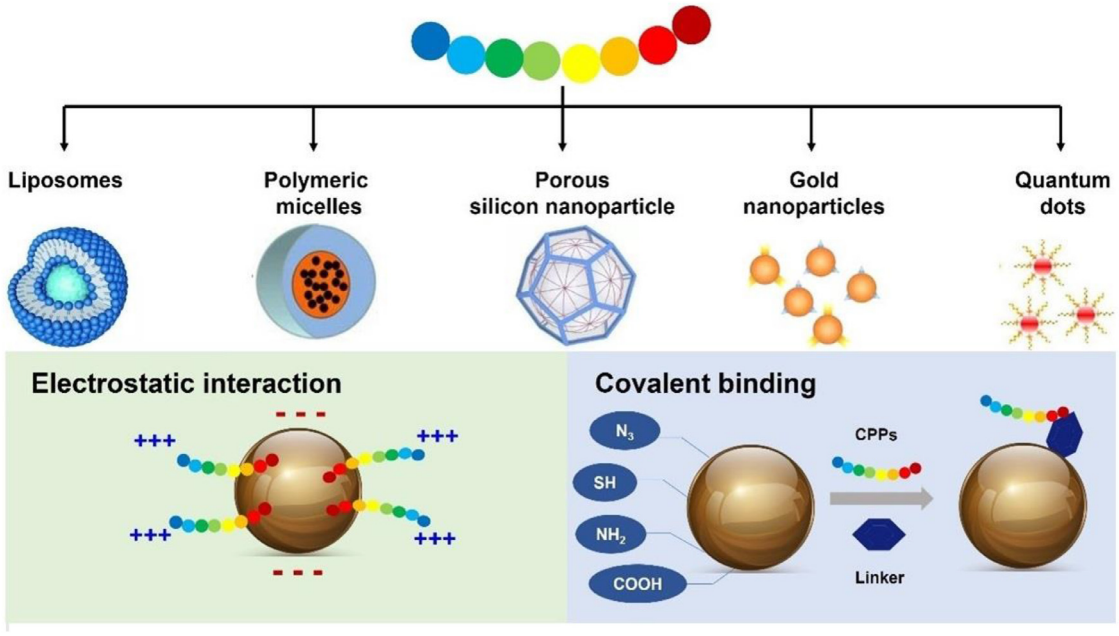

Fig. 1 – Multifunctional CPP-NPs as efficient drug delivery systems.

covalent binding through electrostatic interaction between CPPs and NPs holds the advantage of easy formulation. Nevertheless, the major drawback of non-covalent binding is the low stability of the complex, especially *in vivo* delivery. In comparison, covalent coupling of CPPs and NPs enhances the stability. The reactive groups include amino, carboxyl and sulfhydryl at the C- or N- terminus of CPPs. Usually, a linker is needed to facilitate the conjugation and adjust the optimal distance between CPPs and NPs. The common strategies include (1) Michael addition between sulfhydryl and maleimide; (2) disulfide exchange; (3) amidation reaction and (4) click chemistry between alkynyl and azide and other groups. The conjugation efficiency of CPPs strongly affects the stability and behaviors of CPP-NPs. Insufficient modification of CPPs compromises the internalization efficiency, while over modification may lead to unspecific interactions with tissues and cells, lowering the *in vivo* stability. To maximize the efficiency and stability, the conjugation strategy and modification ratio should be carefully designed and optimized.

5. CPP-NPs as powerful tool to overcome physiological barriers

During the last few decades various NPs have demonstrated great potential in targeted drug delivery, especially in the field of cancer therapy. The cationic charge of CPPs is considered an important parameter to enhance the penetration of cargos in the TME. Various CPP-modified nanomedicines have been designed to enhance antitumor efficacy, especially for deep penetration of tumors with dense stroma and poor vascularization. Nevertheless, although NPs may elevate drug accumulation in targeted organ, facing the complex physiological barriers *in vivo*, such as the blood-brain barrier (BBB), ocular barrier, dermal barrier, etc., the delivery efficiency is still unsatisfactory. For instance, the existence of gastrointestinal barrier severely hampers the oral delivery efficiency, especially for peptide/protein drugs. Brain targeting is extremely difficult due to the presence of blood-brain barrier, which precludes almost all macromolecules and 98% of small molecular drugs (Fig. 2). The combination of CPPs and nano-drug delivery systems offers feasible solutions, which has already been utilized in overcoming these barriers successfully, demonstrating encouraging results (Table 2). In this session, we will focus on CPP-NPs in circumventing different physiological barriers, discuss the potentials of using CPP-NPs to treat various diseases.

5.1. *CPP-NPs for myocardial delivery*

Cardiovascular diseases (CVDs), including myocardial infarction, heart failure, coronary artery disease and peripheral vascular disease, are the first cause of mortality in the world and account for almost one third of deaths. With the rising of the risk factors such as diabetes and obesity, the incidence is expected to increase, which severely affects the life quality of patients [86,87]. Currently, the therapies for CVD include heart transplantation, coronary artery bypass surgery, ventricular assistant devices and pharmacological interventions. Among these strategies, pharmaceutical intervention is less invasive and cost-effective, and more acceptable by patients, offering new avenue for the treatment of CVDs for the controlled biodistribution, reduced toxicity and excretion from the body.

In chronic inflammation, injured myocardial cells and ischemic tissues, enhanced permeability and retention (EPR) effect is also observed [88]. In the case of CVD, nanoparticles will reach the myocardium within 20–25 min after intravenous injection. Nevertheless, some barriers remain and the

Table 2 – Strategies of CPP-NPs for overcoming various physiological barriers.

Barriers	CPP-NPs	Sequence	Functions	Refs.
Myocardial delivery	PCM modified liposome	WLSEAGPVVTVRALRGTGSW	Prolong residence and accumulation in heart	[91,92]
	Hph1–Hph1–dsRBD/siRNA complex	Hph1: YARVRRRGPRR	Enhance gene silencing effects	[140]
	Lipoplexes modified with TAT and antimyosin antibody 2G4	RKKRRQRRR	Increase accumulation in the ischemic rat myocardium	[93]
	R7W-MP modified CaP NP	DQRPDREAPRS	Target the Cavb2 cytosolic subunit of the L-type calcium channel (LTCC), improve cardiac contractility in pathological conditions	[94]
	TPSi NP	RKKRRQRRR	Increase both the cell survival rate and the delivery precision of stem cell transplantation	[95]
Ocular barrier	POD-PEG NP	$GGGG(ARKKAAKA)_4$	Target cell surface sialic acid. Reduce cell apoptosis and enhance the thickness of outer nuclear layer	[141]
	POD-SS-PEG/DNA nanocomplex	GGG[ARKKAAKA]4	Deliver FLT1 plasmid and achieve a 50% reduction in choroidal neovascularization in AMD model	[142]
	POD conjugatedQD	GGG[ARKKAAKA]4	Enter neural retina and localize to retinal pigment epithelium	[143]
	Penetratin fused HDL mutants	RQIKIWFQNRRMKWKK	Enhance the delivery efficiency to the posterior segment of the eye	[144]
	RFP plasmid /PAMAM/Penetratin	RQIKIWFQNRRMKWKK	Improve cellular uptake and transfection	[102]
	cRGD/penetratin-PEG-PAMAM	RQIKIWFQNRRMKWKK	Deliver cargoes to the cornea and retina with a prolonged retention	
	RGD/TAT-PEG-PLGA	RKKRRQRRR	Increase uptake in the choroid-retina	[105]
Mucosal barrier	Penetratin- mesostructured silica NP	RQIKIWFQNRRMKWKK	Enhance cellular transport and promote mucus penetration	[109]
	R8-PLA NP	RRRRRRRR	Enhance the bioavailability of liraglutide	[111]
	R12/PEG/OVA NP	RRRRRRRRRRR	Oral vaccination against H. pylori	[112]
	Alginate/TAT/plasmid nanogel	RKKRRQRRR	Oral vaccination against melanoma	[113]
	TAT/PEG derivative APS/adenovirus nanocomplex	RKKRRQRRR	Prevent entrapment in mucus layer in the vaginal tract. Promote transduction of adenovirus vector	[114]
	TAT-PEG-PCL nanomicelle	RKKRRQRRR	Target delivery to glioma via nasal cavity	[145]
Blood-brain barrier	R8-liposome; R8/Tf-liposome	RRRRRRRR	Enhance delivery across BBB	[121,122]
	R8-RGD tandem peptide-liposome	RRRRRRRRRGD	Target integrin $\alpha v\beta 3$ and facilitates BBB penetration.	[36]
	T7-AT7-PEG-PEI/pVAXI-En nanocomplex	RKKRRQRRRCATWLPPR	Enhance BBB traversing with high binding affinity to VEGFR-2 and NRP-1	[146]
	T7/TAT-dual modified liposome	RKKRRQRRR	Transport across BBB and target glioma.	[147]
	RGV peptide modified poly(mannitol-co-PEI) /siBACE1	YTIWMPENPRPGTPCDIFTNSRGKRASNG	Enhance AD treatment by binding to nicotinic acetylcholine receptors on BBB	[148]
	^{D}CDX-liposome (D-peptide ligand of nAChRs)	DGDRDEDIDRDTDGDRDADEDRDWDSDEDKDF	Cross BBB via lipid raft/caveolae endocytic pathway	[149]
Skin barrier	MEL/TAT-MEL/PEO-b-PCL gel-like nanovehicle	YGRKKRRQRRR	Enhance cellular uptake and transdermal delivery	[132]
	APTstat3–9R	HGFQWPGSWTWENGKWTWKGAYQFLKGGGGSRRRRRRRRR	Efficient skin penetration	[136]
	TAT-nanostructured lipid carriers (TAT-NLCs) and TAT-lipid–polymer hybrid NPs (TAT-LPNs)	YGRKKRRQRRR	Improve skin permeation and therapeutic effects.	[150]
	R11-NLC	RRRRRRRRRRR	Enhanced cellular internalization and skin permeation	[151]

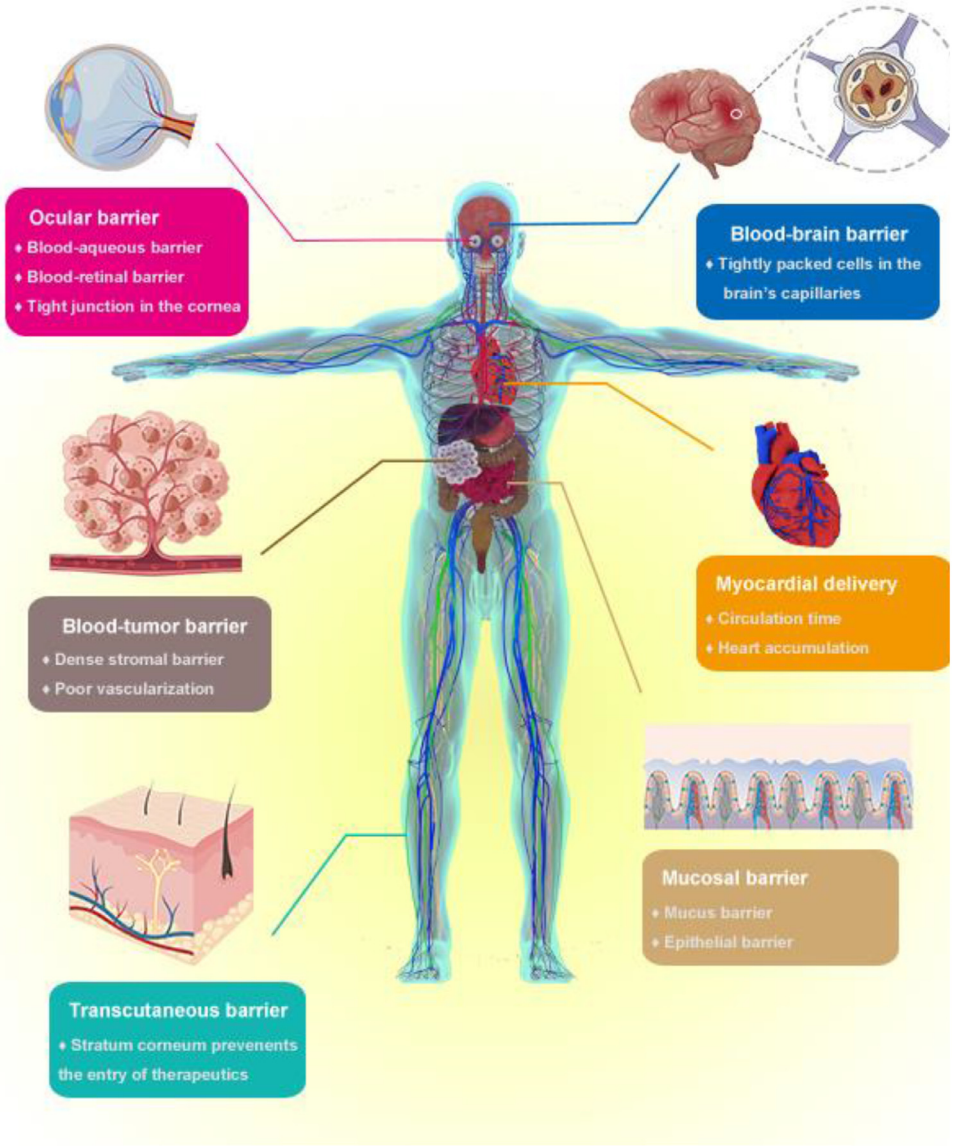

Fig. 2 – Physiological and pathological barriers facing efficient drug delivery.

efficiency of this targeting approach is still controversial [89,90]. First, during blood circulation, NPs need to be stable to withstand the sheer forces of blood flow and the pressure in the heart. Besides, limited cell permeability also restrains the transcellular delivery of therapeutic drugs.

Modifications with CPPs have offered feasible solutions. Various strategies have been developed to enhance drug delivery efficiency in CVDs. Direct conjugation of CPPs with therapeutic proteins or nucleic acid have opened the window for enhance CVD treatments. Modification of CPPs with NPs further combined the merits of CPPs with particulate drug delivery systems. Wang et al. developed a dual-peptide modified liposome and investigated the heart delivery efficiency. One of the peptides is PCM (WLSEAGPVVTVRALRGTGSW), a 20 amino acid peptide that binds to primary cardiomyocytes. The other is TAT, which enhance the transcellular delivery of the loaded cargo. The main components of these liposomes are soybean phospholipids, cholesterol and modified with DSPE-PEG, forming a nanostructure of ~ 100 nm and negative potential. The dual-peptide modification significantly enhanced the targeting and internalization in cardiomyocytes, exhibiting great potential in CVDs treatments [91]. The same group further modified this system with red cell membrane (RCM) to prolong the mean residence time (MRT) and increase the accumulation in the heart. First, the polypeptide-modified liposomes were prepared by mixing TAT and PCM with overnight stirring. Next, RCM isolated from mice blood was adhered to the lipid bilayer with the help of mechanical force. RCM-modified liposomes showed the highest cellular uptake and intracellular distribution on myocardial cells. After intravenous injection, RCM-modified liposomes significantly enhanced drug accumulation in the heart and prolonged the circulation time, representing an effective myocardium targeted drug delivery system [92].

Similarly, CPP-modified drug delivery systems also facilitate the delivery of gene therapy. Ko and colleagues designed a dual-targeted lipoplex for delivery of plasmid DNA

into myocardial ischemia (MI). A targeting moiety anti-myosin monoclonal antibody (mAb 2G4) has been co-modified on liposome with TAT peptide, which is able to recognize and bind ischemic cells when intracellular myosin is exposed and cross cell membrane barrier. The dual-modified lipoplexes showed enhanced transfection on rat hypoxic cardiomyocytes and four-fold higher expression of reporter gene. After *in vivo* administration in an ischemia mouse model, dual-modified lipoplex demonstrated elevated accumulation in the ischemia area in the heart, consequently leading to enhance expression of reporter gene. This system represents a potent candidate for the delivery of therapeutic gene into MI area [93].

Miragoli et al. designed a unique noninvasive inhalation formulation for therapeutic peptide delivery into heart. They proposed a biocompatible negatively charged calcium phosphate NPs (CaPs) loaded with a cell-penetrating mimetic peptide (R7W-MP, DQRPDREAPRS). The CaPs-R7W NPs were expected to be translocated from the pulmonary to the heart via bloodstream, followed by internalization in cardiomyocytes. *In vivo* biodistribution studies proved the hypothesis and showed that the NPs could be accumulated in the myocardium. In the rat model of diabetic cardiomyopathy, the echocardiography results demonstrated that after administration of CaPs-R7W NPs, the heart contractility and function was recovered. This strategy highlights the noninvasive route mediated by nano-drug delivery systems for treatments of CVDs [94].

Stem cells therapy, which is capable of stimulating cardiac regeneration potential, has exhibited great potential in regeneration of injured heart. To elevate the therapeutic efficiency and accuracy, Qi et al. utilized cell penetrating peptide TAT to enhance the cellular internalization of Wnt3a protein loaded porous silicon nanoparticles (PSi NPs). The formulated TAT-PSi NPs (TPSi NPs) demonstrated prolonged Wnt3a protein release and protected the labeled mesenchymal stem cells (MSCs) in myocardial infarction therapy. The intracellular aggregation of TPSi NP amplified the ultrasound signal and enabled precision stem cells transplantation into myocardium, which demonstrated great potential for clinical translation (Fig. 3) [95]. The researches provide evidence that CPPs significantly promoted drug delivery efficiency by enhancing the permeability across the cell membrane barrier, offering an alternative therapeutic strategies for treatment of CVDs. Currently, the exact mechanisms of myocardial delivery remain to be unveiled. Most strategies reply on passive accumulation of NPs through the blood circulation system. Active targeting strategies are expected in the treatment of CVDs.

5.2. *CPP-NPs for overcoming the ocular barriers*

The incidence of eye disease is rising these years due to the increase of aging population. The eye can be divided into an anterior segment (including anterior chamber, cornea, conjunctiva, iris, and lens) and a posterior segment (including posterior sclera, choroid, retina, and vitreous). For the treatment of the anterior segment diseases (cornea neovascularization, conjunctivitis, keratitis, dry eye disease, etc.), topical application is usually applied. Drugs need to pass through the cornea, a tri-layer tissue composed of the outer epithelium, the central connective tissue and the endothelium [96]. For the posterior segment diseases (diabetic retinopathy, age-related macular degeneration (AMD), retinitis, eye tumors, etc.), due to the presence of blood-retinal barrier, efficient delivery to the posterior segment remains a major challenge, especially for hydrophilic and high molecular weight (MW) drugs. Therefore, intravitreal injection is currently the most effective choice for the drug administration of the retina [97,98].

CPP-decorated NPs represent a class of potent drug delivery systems to overcome the barriers in ocular delivery. Direct conjugation or complexation of CPPs with proteins, antibodies, nucleic acid drugs or fluorescent probes in ocular delivery has been thoroughly reviewed elsewhere, therefore here we mainly focus on CPP-modified nano-drug delivery systems. Peptide for ocular delivery (POD, GGG[ARKKAAKA]4; 3.5 kDa) was modified on PEG-polylactic-co-glycolic acid (PEG-PLGA) NPs to increase the penetration across corneal epithelium of flurbiprofen [99]. Both *in vitro* and *in vivo* test showed that PDO–NPs were well tolerated. In an ocular inflammation model, POD-NPs significantly enhanced the anti-inflammation efficacy of flurbiprofen. Similar system has been used in the treatment of inflammation in the anterior and posterior segment of the eye. Garcia et al. modified PEG-PLGA NPs with different CPPs (TAT, penetratin and antimicrobial peptide G2) and encapsulated fluorometholone (FMT). Stronger fluorescence signals were observed in rhodamine labelled TAT-NPs and G2-NPs in both anterior and posterior segment of the eye, resulting in enhanced anti-inflammatory results. In comparison, penetratin-NPs and non-modified PEG-PLGA NPs showed higher presence in the posterior than the anterior segment of the eye [100]. Another ocular targeting CPP, Corneal Targeting Sequence 1 (CorTS 1), has been developed to target anterior ocular tissues by modifying leucine rich repeat (LRR). CorTS 1 interacts with small leucine rich proteoglycans and collagen in the corneal stroma, and enhances tissue penetration and accumulation of the cargo in the stromal region of cornea [101].

Cationic CPPs can be complexed with anionic nucleic acid drugs and form NPs through electrostatic interactions. Penetratin has been complexed with a reporter gene red fluorescent protein plasmid (pRFP) to promote its delivery to the posterior segment of the eye [102]. When the weight ratio of penetratin to plasmid reached 20:1, the cationic NP with a size of $\sim$ 420 nm significantly increased the transfection of reporter gene on both corneal and conjunctival cell lines. Moreover, the authors used cationic dendrimer polyamidoamine (PAMAM) and further formulated penetratin-PAMAM pRFP with a diameter of $\sim$155 nm. After topical instillation, the *in vivo* biodistribution results showed an enhanced distribution in the posterior segment of penetratin-PAMAM pRFP compared with penetratin-pRFP complex [102]. A similar research has described an ocular delivery system for antisense oligonucleotides using PAMAM, hyaluronic acid and penetratin, aiming for efficient posterior segment delivery. Though the *in vitro* results showed only limited enhancement in cellular uptake, an obvious higher accumulation in mice model was observed, proving that penetratin was able to pass across the epithelia and tissues. Besides, topical delivery of

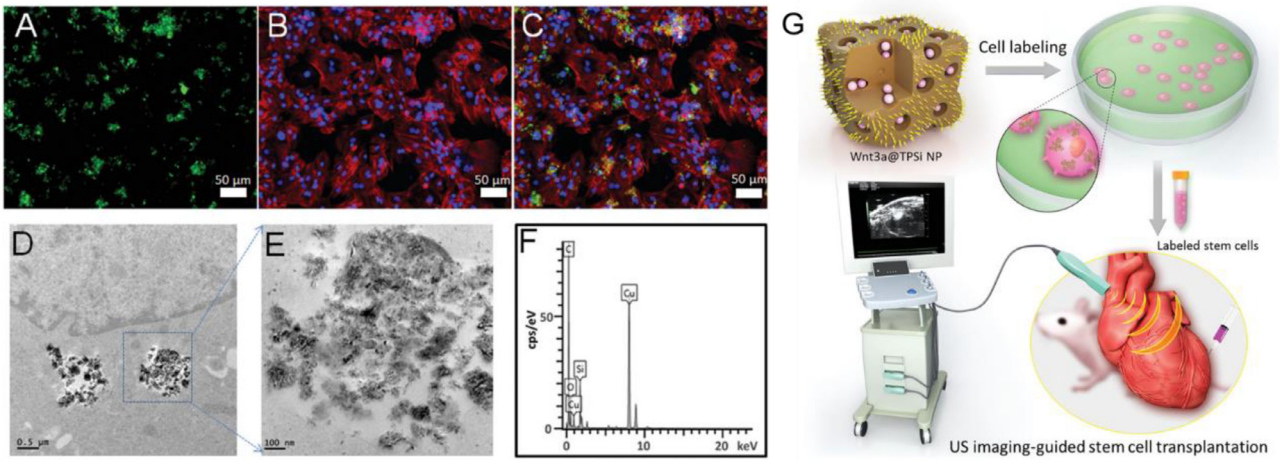

Fig. 3 – Fluorescence microscopy images of TPSi NP-labeled MSCs. (A) TPSi NPs with green fluorescence, (B) merged image of 4′,6-diamidino-2-phenylindole-stained nuclei and tetramethylrhodamine isothiocyanate–phalloidin-stained F-actin, and (C) merged image of TPSi NPs, nuclei, and F-actin. (D,E) TEM images of TPSi NPs within MSCs. (F) EDS spectra of the intracellular region containing porous NPs. (G) Schematic illustration of stem cells labeled with Wnt3a protein–loaded TPSi NPs injected intramyocardially into nude mice under US imaging guidance [95].

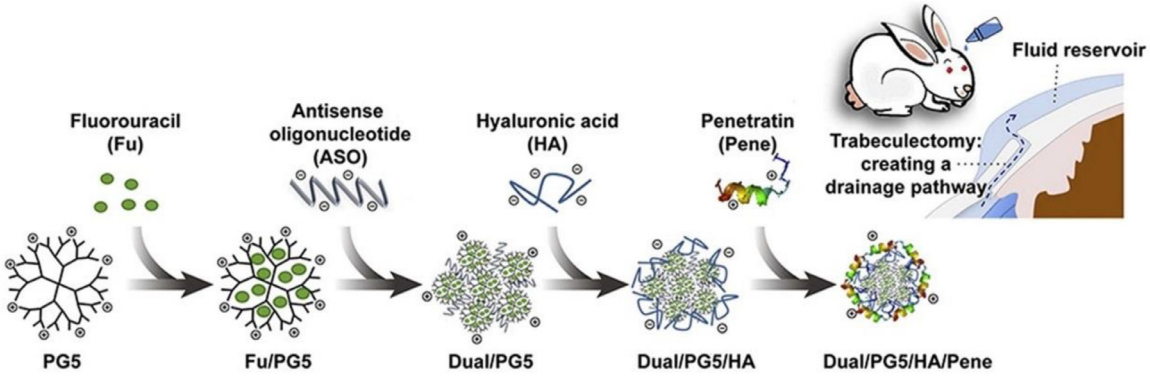

Fig. 4 – Schematic diagram showing the preparation process of Dual/PG5/HA/Pene (PG5: PAMAM-G5-NH_2) [103].

PAMAM/penetratin/oligonucleotide complex also showed significant inhibition of tumor growth in an orthotopic intraocular tumor mice model, exhibiting enhanced delivery efficiency (Fig. 4) [103].

Efforts are also spent to target retina by topical instillation. Suda et al. fused CPPs with high-density lipoproteins (HDLs) to formulate nano-drug delivery systems [104]. Human apolipoprotein (apoA-I) mutants fused with cationic CPPs (TAT or penetratin) were prepared and then further formulated into HDL with phospholipids to load either with a fluorescent probe or a tyrosine kinase inhibitor. Thirty minutes after instillation, CPP-modified HDLs was capable of increasing the distribution of fluorescent in the retina, while non-modified NPs showed little fluorescent in target tissues. Compared with TAT-modified HDLs, penetratin-modified ones showed higher delivery efficiency, probably owing to the internalization mechanism of penetratin. Moreover, the preclinical studies on a mice model of laser-induced CNV demonstrated reduced neovascularization after treatment with pazopanib-loaded penetratin-HDLs, which was superior to that of a commercial cyclodextrin-based pazopanib formulation (Captisol®) [104].

Active targeting CPPs have shown enhanced drug delivery efficiency in ocular disease treatment. Chu et al. modified PEG-PLGA NPs with RGD (arginine-glycine-aspartic acid) and TAT [105]. The internalization experiment on umbilical vein endothelial cells (UVECs) suggested that dual-modification with RGD and TAT showed higher cellular uptake compared with single peptide modified NPs. While in transport assay, only TAT modified NPs promoted the transport delivery since no integrin $\alpha_v\beta_3$ was expressed on these cells. In another study, Yang et al. modified pegylated PAMAM with cyclic RGD and penetratin for the posterior segment targeting of the eye. Integrin $\alpha_v\beta_3$ is overexpressed in the process of neovascularization. To efficiently target choroidal neovascularization (CNV), RGD/penetratin-PEG-PAMAM with average size of 15–20 nm was formulated. Cellular uptake of RGD modification significantly enhanced the affinity with integrin $\alpha_v\beta$, while penetratin improved the *in vitro* penetration of the delivery system. The *in vivo* ocular biodistribution studies showed that NPs modified with penetratin or co-modified with RGD and penetratin were able to delivery cargoes to the cornea and retina with a prolonged

retention [106]. CPP-NPs combine the merits of both sides and significantly enhance the delivery efficiency to the anterior and posterior segment of the eye. Co-modification of targeting moieties may further elevate the targeting efficiency.

5.3. *CPP-NPs for overcoming mucosal barriers*

Parenteral administration is the most well-tolerated drug administration route due to the noninvasiveness and the convenience of self-administration. Mucus administration consists oral, intranasal, vaginal and ocular applications. Oral delivery is the most common administration route for the large area of the gastrointestinal (GI) tract. Mucosal surfaces including GI tract, lung airways, reproductive tracts and eyes etc., are covered by adhesive gel, forming a protective layer against foreign pathogens by trapping them and hindering their access to the epithelium. However, the mucus layer also prevents the particulate drug delivery systems, trapping them in the mucus layer and quickly removing them from the mucosal tissue [107,108]. Besides, the tight epithelium beneath the mucus layer forms another physical obstacle for drug delivery, preventing the intake of pathogens and drugs. As a consequence, the mucosal layer clearance and low permeability caused by epithelium form barriers against efficient drug delivery.

Aiming at overcoming the mucus barrier and enhancing the penetration, drug delivery systems need to avoid mucin adhesion and steric inhibition by the fibers. Justin Hanse et al. have provided evidence that pegylation can enhance the diffusion and permeability in mucus layer. Furthermore, CPPs exhibit high efficiency in overcoming the mucosal and epithelia barriers. TAT, penetratin and polyarginine are commonly used to modify drug delivery systems and greatly elevate the transmembrane delivery of the loaded cargo. Tan et al. formulated CPP and PEG modified mesostructured silica NPs for the delivery of therapeutic protein and peptide. The final NPs were assembled by electrostatic interaction between CPP (penetratin)-loaded NPs (CPP-NPs) and TPP-loaded NPs (TPP-NPs). The mechanism study suggested that CPPs enhanced cellular transport and exocytosis by 8.45-fold, while PEG modification neutralized the electric charge and promoted the mucus penetration by 5.09-fold. Using recombinant growth hormone (RGH) as a model protein, the formulated NPs enhanced the *in vitro* and *in vivo* pharmacodynamics by 5.41 and 4.91-fold, respectively, representing great potential in oral delivery of peptide and proteins [109]. In another study, an oral peptide and protein delivery system was formulated by biodegradable polymer. Different CPPs (TAT, penetratin and R8) were modified on PLGA NPs in combination with a secretion peptide with N-terminal stearylation (Sec). The *in vitro* permeability test showed that Sec co-modified with penetratin exhibited enhanced permeation in Caco-2 model compared with only penetratin modified NPs. In comparison, Sec did not further promote the permeability of R8- and TAT-modified NPs. Using insulin as a model drug, the *in vivo* experiments indicated that the bioavailability of insulin was 1.71-fold higher in Sec-penetratin NPs compared with penetratin-NPs, demonstrating enhanced hypoglycemic effects [110]. Similarly, Uhl et al. modified polylactic acid (PLA) NPs with R8 for oral delivery of liraglutide, elevating the bioavailability of liraglutide by 4–5 times [111].

CPPs also played essential roles in oral vaccine delivery. Zhang et al. reported self-assembling NPs with hydrophilic and slightly negative surface and enhanced mucus penetrating ability. Anionic protein antigen was first complexed with cationic CPP poly arginine (R12) to form NPs of ~160 nm. To further enhance the mucus penetrating capacity, wheat-like anionic PEG derivative was coated on the surface of NPs via electrostatic interaction. The encapsulation of PEG elevated the permeation in mucus by 4-fold. During this process, the PEG derivative dissociated gradually from the NPs and exposed CPPs to facilitate transepithelial transport. After oral administration, the nano vaccine induced potent humoral and cellular immunity against antigen H. pylori, and the immune responses effectively protected mice from H. pylori challenge. The CPP- and PEG-modified NPs overcome the mucus barrier as well as the epithelial barrier and elicit antigens-specific immune responses (Fig. 5) [112]. The oral delivery of DNA vaccine can also be boosted by CPPs. Shen and co-workers constructed an alginate-TAT-DNA nanogel, which significantly increased the stability of DNA vaccine and enhanced its mucus penetration as well as transepithelial delivery. This nanogel also induced maturation of bone-marrow derived dendritic cells and activated $CD4^+$ and $CD8^+$ T cells and elevated expression of cytokines, which further led to 42.5% tumor inhibition in mice model [113].

Besides oral route, CPPs also promote drug and vaccine delivery efficiency of the intranasal and intravaginal routes, which are also major portals of pathogen infection. For intravaginal vaccination, the mucus barrier also exists. Ji et al. designed polymeric nanocomplex for intravaginal delivery of adenovirus (Ad) vector vaccine encoding HIV antigen gag protein. Anionic PEG derivative, TAT and Ad was complexed to form nanoscale particles. In this system, the hydrophilic anionic PEG derivative prevented the entrapment in the mucus layer, while TAT promoted the cellular internalization and transduction of Ad. After intravaginal vaccination, potent antigen-specific mucosal and systemic immune responses were induced [114]. Compared with GI tract and reproductive tract, the nasal cavity has a much thinner mucus layer, therefore, the epithelial barrier is the main obstacle. CPPs such as penetratin and poly arginine also facilitate intranasal vaccination, which can be co-administered with antigen or conjugated with polymers [115–117]. CPP-NPs overcome the mucosal barrier, enhance the transmembrane penetration of antigens, and substantially elevate the potency of mucosal vaccination, representing powerful weapons in combating mucus barriers. Considering the different physiological conditions in different mucosal sites, the design of delivery system should be adapted to the unique features of different site.

5.4. *CPP-NPs for overcoming blood-brain barriers*

Central nerve system (CNS) disorders including glioma, Pakinson's, Alzheimer's, epilepsy, etc., are facing an increase in incidence and unsatisfactory prognosis, causing severe physical and economic burdens for patients. Blood-brain barrier (BBB), forming tight junctions between adjacent

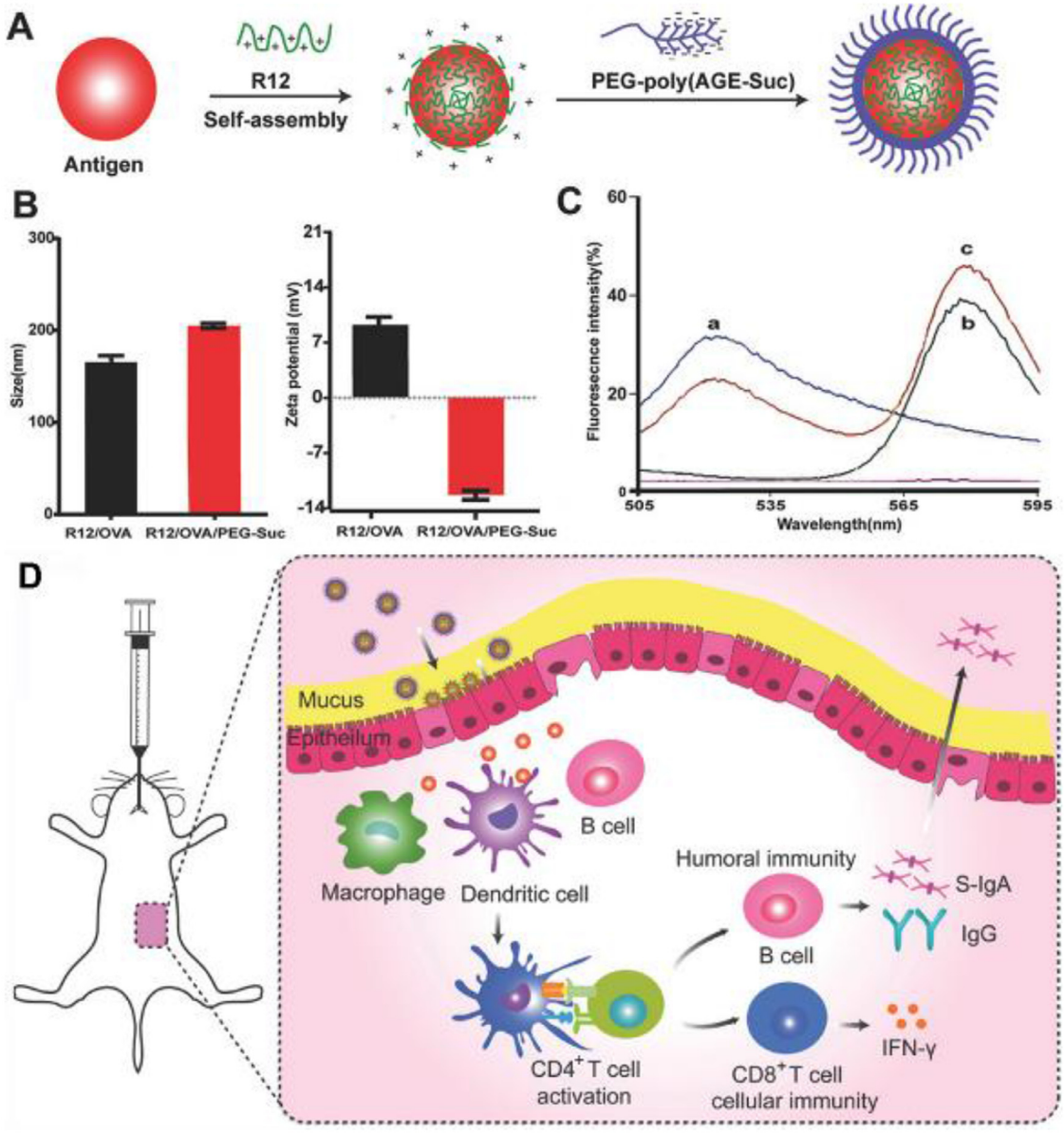

Fig. 5 – (A) Schematic diagram of the formation of ternary NPs. (B) Particle size and zeta potential of R12/OVA and R12/OVA/PEG-Suc NPs. (C) Emission spectrum of free FITC-labeled OVA (curve a), TRITC-labeled PEG-Suc polymers (curve b) and R12/FITC–OVA/TRITC-PEG-Suc NPs (curve c) at excitation of 440 nm. (D) Schematic illustration of the process of the ternary NPs permeation across intestinal mucus layer and epithelium barrier [112].

endothelial cells, prevents the access of infectious pathogens to brain cells and allows the entry of nutrients for metabolism. However, BBB also prevents the brain targeted delivery, resulting in extremely low accumulation in CNS. Overcoming the barrier of BBB has become a hot issue in brain targeted drug delivery [118]. Therapeutic drug delivery systems require facile design to enable transmigration across the BBB. NPs can transmigrate across BBB via receptor mediated transcytosis, adsorptive transcytosis, transport proteins mediated transcytosis or paracellular pathways [119,120].

CPP-NPs not only take the advantage of the size and shape of particulate delivery system, but also increase the permeability across cell membranes, demonstrating great potential in transmigration across BBB. Yuan et al. formulated CPP R8-modified cationic liposomes for glioma targeted delivery of DOX [121]. The formulated R8-Lips showed size of ~100 nm. The modification of R8 increased the cellular uptake in U87-MG cells by 8.6-fold compared with unmodified counterparts, and consequently led to reduced cell viability. The biodistribution results suggested that R8-Lips increased the brain accumulation of the payload by 2.4-fold than PEG-Lips [121]. In another research, to further strengthen the targeting efficiency to the glioma, Wang et al. co-modified DOX-loaded liposomes with CPP R8 and transferrin (Tf), which was used as a targeting ligand to bind transferrin

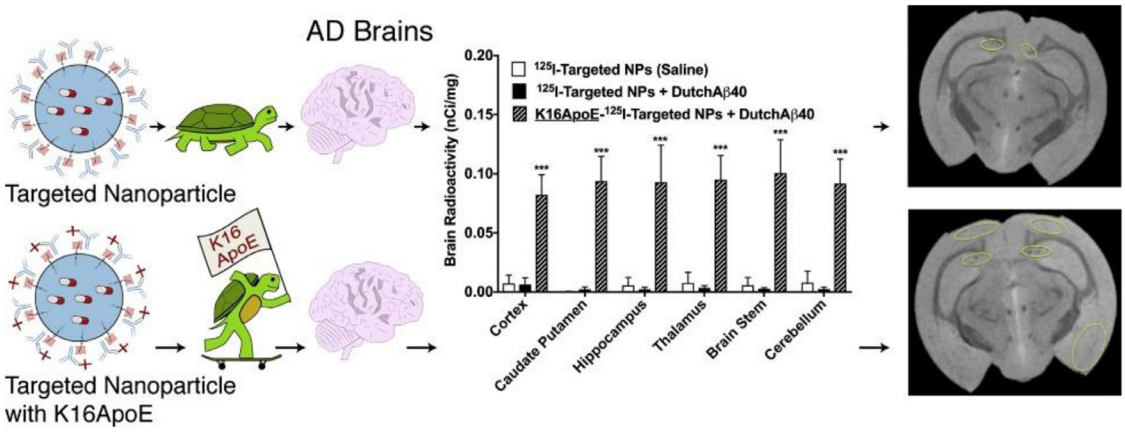

Fig. 6 – Coating NPs with K16ApoE enhanced their BBB uptake in AD treatment [126].

receptors overexpressed in both glioma cells and brain microvascular endothelial cell. R8 and Tf were modified on the surface of liposomes by post-insertion. The cellular uptake experiment conducted on U87 cells and GL261 cells indicated that the modification of Tf significantly enhanced cellular internalization. *In vivo* antitumor therapy further proved the BBB transmigration ability of R8/Tf co-modified liposomes [122]. For enhanced glioma targeting, Chaix et al. developed porous silicon nanorods modified with Neuro Filament Light (NFL) subunit derived tubulin binding site peptide (NFL-TBS). The nanoplatform preferentially targeted glioma cells and permeated cellular membranes effectively, demonstrating great potential for the treatment and imaging of glioma [123].

Alzheimer's disease (AD) is a neurodegenerative disorder in the brain featured by amyloid beta accumulation in the cerebral vasculature and intracellular neurofibrillary tangles [124]. Several drugs including donepezil, memantine have been proved by FDA, however, the therapeutic efficiency is limited by BBB. Various strategies have been developed to overcome the BBB barrier to treat AD. CPPs and therapeutic protein fusion protein can enhance the BBB penetration in AD model mice [125]. Ahlshwede et al. designed an anti-amyloid antibody fragment (IgG 4.1) conjugated PLGA NPs encapsulating curcumin to enhance the targeted treatment of AD (Fig. 6). To further enhance the transmigration across BBB, a cationic peptide originated from the apolipoprotein E (ApoE) peptide, was absorbed onto the surface of PLGA NPs. The formulated NPs demonstrated excellent targeting to the vasculo-tropic Dutch Aβ40 [126]. Pegylated gold nanostars (AuNSs) have modified with penetratin peptide and ruthenium complex (Ru@Pen@PEG-AuNSs), which function as both luminescent probes and drug delivery systems. CPP penetratin elevated the delivery across the BBB and overcame the barrier, leading to enhanced neuroprotection against cellular toxicity induced by Aβ, representing a promising AD therapy [127]. Samaridou et al. developed R8 modified nanocomplexes for direct nose-to-brain (N-to-B) delivery of miRNA against neurological disorder. CPP R8 was conjugated with lauric acid and complexed with RNA via electrostatic interaction. Then the cationic nanocomplexes were further enveloped with protective polymers such as hyaluronic acid (HA) or PEG-PLA, aiming to enhance the stability and delivery efficiency. The R8-modified nanocomplexes demonstrated great potential in overcoming the BBB via N-to-B route for the treatment of Alzheimer's disease [128]. BBB is considered as the most challenging barrier in treating CNS disorders. The combination of CPPs and active targeting strategies such as receptor mediated transcytosis, or transport proteins mediated transcytosis hold the potential to overcome this barrier.

5.5. *CPP-NPs in overcoming transdermal barriers*

Transdermal delivery is a non-invasive and on-demand delivery system of therapeutic genes and drugs, which has several advantages compared with the oral administration route. Efficient transdermal drug delivery is also facing great challenges. The outermost layer of skin is the stratum corneum (10–20 μm thick), forming a brick structure and representing the major barrier against transdermal delivery. The underneath viable epidermis this (50–100 μm) layer is an avascular structure. Then the dermis structure (1–2 mm) is rich in capillary, which is favorable for systemic absorption [129]. Only a limited number of hydrophilic small molecules (less than a few hundred Dalton) are amenable to transdermal delivery. Recent developments of drug delivery systems broaden the range of transdermal drugs. To further overcome the stratum corneum barrier and enhance skin permeability, permeability enhancers are used in transdermal delivery systems [130].

CPPs enhance skin permeability of a variety of cargos, including peptides, proteins and small molecule drugs. Direct conjugation between CPP and cargos is a feasible way to enhance the skin permeability [131]. Besides, CPP-modified particulate drug delivery systems also hold great potential in transdermal delivery. Park et al. formulated CPP-patchy deformable polymeric NPs with amphiphilic poly(ethylene oxide)-block-poly(ε-caprolactone) (PEO-b-PCL), mannosylerythritol lipid (MEL), and TAT-linked MEL. The modification of TAT significantly enhanced cellular uptake. The mechanism study revealed that the internalization was mediated by macropinocytosis and caveolae-/lipid raft-

mediated endocytosis. More importantly, TAT elevated the *in vivo* penetration, which proved the efficacy of TAT-patchy deformable nanovehicles [132]. TAT-modified liposomes have been utilized to treat human skin fibroblasts (HSF) via transdermal administration. Salvianolic acid B (SAB) was encapsulated in TAT-modified liposomes (TAT-Lips/SAB) by pH gradient reverse-phase evaporation, which showed good transdermal efficiency (17.21%) and retention (44.39 μg/cm^2 ± 6.87). TAT-Lips/SAB suppressed the proliferation, migration and invasion of HSF cells, offering a promising transdermal strategy [133].

Besides, some novel CPPs have been developed and applied in transdermal delivery. Gautam et al. designed a novel CPP IMT-P8 and explored its transdermal potential. After fusion with reporter protein GFP, the obtained IMT-P8-GFP showed significantly higher cellular uptake than TAT-GFP in Hela cells. KLA, a proapoptotic peptide, was further fused with it to elevate the therapeutic effects. The results indicated IMT-P8-KLA successfully located in mitochondria and caused cell apoptosis. After topical application, IMT-P8 could overcome the barrier of the stratum corneum and penetrate into the viable epidermis and the hair follicles [134]. Tian et al. also designed and screened a series of novel CPPs based on a cyclic cationic CPP (ACSSKKSKHCG). The results indicated that the transdermal permeation of DLCC-2 (cyclopeptide, KWSSKKSKHCG-NH_2) was comparable to polyarginine and could serve as transdermal delivery enhancer [135]. In another study, a fusion peptide containing CPP arginine-9 and STAT3 inhibiting peptide was designed for the treatment of in psoriatic skin inflammation. Further complexation of APTstat3–9R with lipid formulation demonstrated enhanced skin penetration and psoriatic skin inflammation inhibition [136]. Compared with permeability enhancer which may be toxic to skin, the utilization of CPP-NPs provides safer and more efficient delivery strategy. CPP-NPs can also be applied in combination with some topical devices, such as microneedles, implants or hydrogels, to achieve prolonged drug retention.

5.6. *CPP-NPs in enhancing tumor targeted delivery*

Cancer is the second leading cause of death worldwide. NPs have merged as potent drug delivery systems for cancer treatment. The combination with CPPs further elevates the delivery efficiency of nanomedicines. Detailed progress has been reviewed elsewhere [137,138], so here we summarize the key advantages of CPP-NPs. Efficiency tumor targeted delivery faces the challenges of (1) targeting efficiency, (2) prolonged retention, (3) deep penetration [139]. Due to the special features of TME such as low pH and enzyme expression, activatable CPP-NPs have unique advantages, as described in Section 3. In addition, due to the EPR effect, NPs are able to prolong the retention of therapeutics in tumor. The high interstitial pressure of tumor presents a major obstacle for deep penetration. Designing small NPs or size-shrinkable NPs offer feasible solutions. Besides, the combination of CPPs further promotes deep penetration of nanomedicine. These strategies combining CPPs and NPs elevate the delivery efficiency of various steps in tumor tarting, which is a premise for enhanced therapeutic results, including overcoming the drug resistance or inhibiting recurrence.

6. Future perspective and conclusion

NPs are a class of drug delivery systems with a diameter range of several nanometers to several hundred nanometers, which can passively accumulate in organs or tumors by taking advantage of the blocking and retention of different sized-NPs. More importantly, NPs can alter the solubility and *in vivo* distribution of the cargos. CPPs also facilitate the intracellular transportation including targeted delivery to mitochondria, endoplasmic reticulum (ER), Golgi apparatus and nucleus. Further understanding the internalization mechanisms of CPPs will benefit the design of specific targeting CPPs and elevate the therapeutic results. Modification of NPs with CPPs combines the merits of both sides, further enhancing the stability and transmigration across the physiological and pathological barriers.

Though CPP-modified NPs have demonstrated promising results in overcoming various physiological and pathological barriers, many challenges still exist, which hampers the clinical translation of CPP-NPs. Some combinational therapeutic strategies of CPPs and chemo- or immune-therapeutics have been tested clinically (Table 3), including TAT-conjugated PKC inhibitor for treatment of myocardial infarction (phase II, NCT00785954), TAT-D-JNKI-1 treating acute inner ear hearing loss (phase III,), azurin-derived CPP P28 in against recurrent or progressive central nervous system tumors (phase I, NCS745104), and activatable CPP conjugated with Cy5 or Cy7 for tumor imaging (phase I, NCT02391194). Unfortunately, no CPP-modified nanomedicine has entered clinical trials. The *in vivo* stability of CPPs should be improved. Though *in vitro* studies prove the cell permeability of CPPs, rapid blood clearance and easy degradation before reaching the target site hinder the *in vivo* application. Some CPPs also have immunogenicity problems due to the polypeptide property, which may cause side effects and compromise the therapeutic effects of the cargo.

The *in vivo* stability and immunogenicity of CPPs have been improved through conjugation with NPs, nevertheless, the exact pharmacokinetics of CPP-NPs have not been elucidated yet. Recent studies have focused on the interactions between protein corona and NPs [158,159]. The materials, size, shape and surface chemistry all affect the formation of protein corona. As exogenous materials, CPP-NPs inevitably interact with serum proteins, which may influence the blood circulation and distribution of the cargos. The cationic CPPs are supposed to absorb a large number of serum proteins, while the shielding strategies using PEG or other materials significantly reduce the binding of serum proteins and endow CPP-NPs with “stealth” effects. Nevertheless, the specific interactions between serum protein and CPP-NPs, such as the category and quantity of proteins absorbed need to be identified. By elucidating these mechanisms, more precise targeting to specific organs may be achieved through rational design absorption of a certain kind of serum protein.

Another factor hindering the clinical translation of CPP is the cytotoxicity, which should not be neglected. The safety of CPPs is deeply corelated with the immunogenicity, pharmacokinetics and clearance. The cytotoxicity of CPP-NPs not only comes from the toxicity of CPPs, but also from the

Table 3 – Examples of CPP-drug conjugates in clinical trials.

CPP	Cargo	Dosage form	Application	Phase	Trial ID	Refs.
TAT	c-Jun N-terminal Kinase (JNK) inhibitor	A biocompatible hyaluronic acid gel for intratympanic administration a	Acute Unilateral Sudden Deafness	Phase III	NCT02561091	[152]
TAT	dextrogyre configuratedprotease-resistant peptide	sub-conjunctival injection	postoperative intraocular inflammation	Phase III	NCT02508337	[153]
P28	glutathione-S-transferase	subcutaneous injections	mild Crohn's disease	Phase II	NCT02281916	[154]
P28	Non-HDM2-mediated peptide inhibitor of p53	Intravenous injection	CNS malignancies	Phase I	NSC745104	[155]
P28	P28	Intravenous injection	Refractory Solid Tumors	Phase I	NCT00914914	ClinicalTrials.gov
highly charged oligopeptide of human origin	SN38	Intravenous injection	Tumor	Phase I	NA	[156]
Activatable CPP	Cy5 and Cy7	Intravenous injection	Tumor imaging	Phase I	NCT02391194	[157]

dosage of cargo and the conjugation strategies. Besides, the cell types, administration routes and frequency also matter. Some amphiphilic CPPs with antiviral activities are toxic due to the potential pore-forming effect, which triggers the influx of calcium ions and elevates the intracellular calcium concentration. High numbers of hydrophobic amino acids usually lead to elevated toxicity. Optimizing the sequence of CPPs, using shielding strategies and formulating NPs with biocompatible materials may hold the key to reducing the toxicity. Optimizing the density of CPPs on NP is also vital for achieving the balance of delivery efficiency and cytotoxicity.

One major concern of CPPs is lack of specificity, which is responsible for the cytotoxicity of CPPs since almost all types of cells can internalize CPPs and CPP-modified NPs. To further enhance the specificity of CPPs, various modifications are utilized to formulate activatable CPPs, especially in tumor targeted delivery. Based on the special features of TME including low pH, high enzyme expression and hypoxia, local activatable CPPs are developed, which remain intact during blood circulation and expose upon arrival at tumor tissue. Endowing CPPs with bioactive functions such as antitumor activity in cancer treatment is also a promising approach. Nevertheless, for diseases other than cancer, the internal stimuli still remain to be discovered. With the understanding of mechanisms of different diseases, the rational design of novel and multifunctional CPPs would accelerate the development of efficient drug delivery. Moreover, the coupling of CPPs to NPs is also a critical procedure. The coupling mechanism remains further exploration. It is a trend that the preparation of NPs becomes more and more complex. During the formulation of CPP-NPs, the activity of CPPs may be compromised. Therefore, development of simple method (*i.e.* "one-pot" synthesis) is needed. Although some efforts have been made to enhance the targeting efficiency and specificity of CPPs, such as the target site activatable CPPs, this process makes the production of CPPs more complex which is unfavorable for industrial production.

Moreover, the endo-lysosome degradation of CPPs and the cargos also hampers the delivery efficiency. It is believed that drugs need to escape from endo-lysosome before exerting therapeutic effects. Engineering and optimization of CPPs to endow them with endosomal escape ability may further enhance the delivery efficiency of CPP-modified NPs. To achieve this goal, direct conjugation with endosome escape sequence to form a fusogenic peptide. Besides, to discover and develop novel CPPs with enhanced efficiency and specificity, several approaches can be followed. Rational design based on the chemical and physiochemical properties of amino acid, such as the charge, chirality, hydrophilic/hydrophobic and aromatic content, and the interactions between different amino acids. Trial and error are also needed to testify the design. In this process, researchers should be familiar with the properties of a peptide, and the possible modifications. Phage display is a potent technology for screening and isolating target peptides and CPPs, which allows mass screening through a large number of candidates. With the development of genomic sequencing, enormous information about protein and amino acid coding sequences are unveiled. In silico approaches offer cheaper, faster largescale screening of new CPPs.

The current review summarizes the application of CPP-modified nano-drug delivery systems for overcoming various physiological and pathological barriers. CPPs exert great advantages in transmembrane delivery of therapeutic molecules. The internalization mechanisms may vary depending on the sequence of CPPs and type of NPs. Facing the complex *in vivo* conditions and barriers of different diseases, including the blood-brain barrier, ocular posterior barrier,

transdermal barrier, etc., CPPs offer effective solutions. Notably, in the current stage, it is hard to summarize the specific features of CPPs in overcoming certain physiological or pathological barriers, and some CPPs have been proven available for overcoming various barriers, *i.e.* TAT and R8-based CPPs have been applied in overcoming various barriers.

Though currently no CPP-NPs has moved to clinical stage, the combination between CPPs and NPs may synergistically overcome the *in vivo* barriers of drug delivery. The next challenge may lie in how to control the manipulation of CPP-NPs more specifically, such as controlling the shape, size, morphology and modification ratio, etc. The understanding of the kinetics of *in vivo* delivery and endocytosis will also benefit the application of CPP-NPs in overcoming physiological and pathological barriers.

Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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