Review

Cell-penetrating peptides: breaking through to the other side

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Cell-penetrating peptides (CPPs) have been previously shown to be powerful transport vector tools for the intracellular delivery of a large variety of cargoes through the cell membrane. Intracellular delivery of plasmid DNA (pDNA), oligonucleotides, small interfering RNAs (siRNAs), proteins and peptides, contrast agents, drugs, as well as various nanoparticulate pharmaceutical carriers (e.g., liposomes, micelles) has been demonstrated both *in vitro* and *in vivo*. This review focuses on the peptide-based strategy for intracellular delivery of CPP-modified nanocarriers to deliver small molecule drugs or DNA. In addition, we discuss the rationales for the design of 'smart' pharmaceutical nanocarriers in which the cell-penetrating properties are hidden until triggered by exposure to appropriate environmental conditions (e.g., a particular pH, temperature, or enzyme level), applied local microwave, ultrasound, or radiofrequency radiation.

Moving across cell membranes

The inability of therapeutics to reach their designated cellular and intracellular target sites is one of the main obstacles for administering active molecules, particularly where cell membranes prevent proteins, peptides, and nanoparticulate drug carriers from entering cells in the absence of active transport. Targeting to specific intracellular organelles such as nuclei, mitochondria, and lysosomes could further expand the possibilities for drug delivery systems and the development of subcellular organelle-targeted therapy. In recent years, structural analysis and expression profiling of a variety of clinical disorders including tumors and cancer cell lines has identified candidate molecules that are altered in the malignant disease state [1], and many therapeutic targets have been found located within cells where pharmacologically active proteins, peptides, and other agents carry out cellular functions. Still, the bottlenecks along the path of converting molecular discoveries into substantial clinical endpoints are numerous and most compounds fail due to poor pharmacokinetics and the inability to deliver agents to the molecular targets within cells (Box 1).

Numerous pharmaceutical carriers, such as nanospheres, nanocapsules, liposomes, micelles, cell ghosts, lipoproteins, and polymers have been used widely over the past few decades to deliver a selection of therapeutic and diagnostic agents. Some of these carriers are nanosized and can be coated with polyethylene glycol (PEGylated) to form 'stealth' nanocarriers [2] that remain in the blood circulation long enough to passively accumulate in various pathological sites, such as tumors and infarcts, based on the cut-off size of their leaky vasculature. This phenomenon is defined as the enhanced permeability and retention (EPR) effect [3]. Because delivery of these carriers is based mostly on their passive accumulation in pathological regions, they cannot efficiently deliver their cargo to specific cells or to particular intracellular components.

The use of vector molecules such as antibodies, peptides, and certain sugar moieties to actively transport associated drugs or drug carriers into a targeted site (e.g., via receptor-mediated endocytosis) can more efficiently overcome the cell membrane barrier and deliver these carriers intracellularly. The primary drawback in the use of this endocytic pathway is that cell entry by this mechanism is often limited by insufficient escape from the endosomal compartment, restricted diffusion, degradation, or lack of nuclear uptake. This eventually leads to a majority of the carrier and its contents being trapped in endosomes, followed by lysosomal entry and enzymatic degradation. Since the first observation in 1965 [4] that histones and cationic polyamines stimulate albumin uptake by tumor cells, and after the discovery of natural polycationic cell-penetrating peptides (CPPs) from the HIV virus [5,6], these agents were found to have properties as macromolecule carriers and enhancers of cellular entry by different mechanisms. This has provided opportunities for the delivery of biologically active cargoes into various tissues, cells, and subcellular compartments.

In this review, we present an overview of CPP classifications, mechanisms, limitations, and their potential uses. We focus on surface modification of pharmaceutical nanocarriers for intracellular delivery using CPPs to control and improve nanocarrier properties. We also discuss recent developments in the usage of nanocarriers with 'hidden' CPP functions and their properties that can serve as potentially 'smart' delivery platforms.

Classes of CPPs

The proof-of-concept of protein transduction into cells was first described in 1988 in parallel by Frankel and Pabo [5] and Green and Loewenstein [6] who discovered that the transactivator of transcription (TAT) protein of HIV can cross cell membranes and be efficiently internalized by cells *in vitro*, resulting in transactivation of the viral promoter. In 1997, Vives *et al.* [7] used the same approach

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Keywords: cell-penetrating peptides; nanocarrier; drug delivery; transfection.

  http://dx.doi.org/10.1016/j.molmed.2012.04.012

Box 1. Clinical development of CPPs

Since the initial characterization of the protein transduction domain in 1988 [5,6], over 2000 papers have been published in this field. Over this period, numerous preclinical and clinical evaluations have been conducted or are underway, although no CPP or CPP conjugate has passed the FDA hurdle and reached the clinics.

The first CPP clinical trial was a cyclosporine–polyarginine conjugate [75] for the topical treatment of psoriasis (PsorBan® by CellGate Inc.). This oligoarginine chimeric transporter enabled full penetration of cyclosporine into cells throughout the epidermis and dermis of human skin. It entered Phase II clinical trials (2003), but was ultimately discontinued. Revance Therapeutics, Inc. have completed a Phase II clinical trial (RT-001) using a TATp cell-penetrating-based platform technology (TransMTS™), which enables topical delivery of botulinum toxin across the skin. AZX-100 (Capstone Therapeutics) is a cell-permeant peptide that mimics heat shock protein (HSP20) and bypasses the signaling pathways, leading to smooth muscle relaxation after topical application. It has been evaluated in Phase II trials for prevention of dermal/keloid scarring. KAI Pharmaceuticals is now evaluating in Phase I/II protein kinase Cδ inhibitor-$TAT_{(47–57)}$ conjugates for myocardial infarction, pain, and cytoprotection/ischemia (KIA-9803, KIA-1678, and KIA-1455, respectively). Avi Biopharma is currently working on an *in vivo* steric block for splicing correction using 6-aminohexanoic acid-spaced oligoarginine [$(R\text{-}Ahx\text{-}R)_4$] [76]. Their previous aortocoronary bypass therapeutic applications [antisense peptide–morpholino oligomer (PMO) conjugate, AVI-5126] [77] for restenosis prevention reached Phase II trials, but was eventually terminated. Another clinical trial involving a CPP–PMO conjugate for Duchenne muscular dystrophy treatment is in preclinical development (AVI-5038). A Phase I clinical trial for an HIV vaccine based on HIV-1 TAT and V2-deleted Env proteins is currently being conducted by Istituto Superiore di Sanita and Novartis (ISS P-002). Their clinical trial results on safety could be of great interest for the future use of TATp in delivery applications. Traversa, Inc. has developed siRNA delivery technology (PTD–DRBD) comprising multiple TAT peptide transduction domains (PTDs) linked to a double-stranded RNA binding domain (DRBD) [78]. Diatos has developed the agent, DTS-108, for cancer treatment. It is a prodrug of SN38, the active metabolite of the anticancer drug irinotecan, conjugated with the peptide, DPV1047, based on the Diatos Vectocell® technology platform [79]. *DTS-108* is currently ready to begin Phase I clinical trials in Europe by Diatos and its partner, Drais Pharmaceuticals.

to study truncated versions of TAT and identified a minimal sequence that enabled cell entry. A few years later, the 16 amino acid peptide penetratin (pAntp), derived from the amphiphilic Drosophila Antennapedia homeodomain, was discovered [8], followed by several other proteins and peptides that displayed translocation activity. These include VP22 [9], transportan [10], model amphipathic peptide (MAP) [11], signal sequence-based peptides [12], and synthetic arginine-enriched sequences [13]. Over the past 20 years, more than 100 peptidic sequences (varying from 5 to 40 amino acids in length) have been described that are capable of internalization into mammalian, plant, and bacterial cells to mediate the transport of a variety of biologically active molecules, cargos, and drug delivery vectors [14–16].

CPPs can be divided into subgroups defined by their origin or sequence characteristics (Table 1). Most known CPPs are not cell type or tissue specific, and most rely primarily on the positively charged sequences of amino acids at physiological pH (primarily arginine and lysine) and electrostatic interactions with negatively charged cell-surface glycoproteins (before internalization). The guanidine head group of arginine can form hydrogen bonds with the negatively charged phosphates and sulfates on the cell surface membrane and might lead to internalization with cell surfaces under conditions of physiological pH. The amino acid lysine has the same net positive charge as arginine, but does not contain the guanidine head group, and as a consequence is less effective at penetrating the plasma membrane when acting alone. The number and order of amino acids in the peptide sequence, mostly arginines, is critical for determining the transduction properties of the CPP, as described by Lindgren and Langel [14]. Examples of this low amphipathic peptide class include penetratin [8], TATp [17], and polyarginines [13]. The second CPP class comprises peptides with a high degree of amphipathicity, where the charge contribution originates primarily from lysine residues. Examples include MAP [10], transportan [11], and Pep-1 [18]. Pep-1 was also the first commercial peptide carrier for the non-covalent delivery of proteins into cells (Chariot™ Protein Delivery Reagent, Active Motif, USA). In the third class, the charged and hydrophobic residues are separated lengthwise on the chain, as amplified by the vascular endothelial-cadherin (pVEC) and MPG peptides.

In addition to this 'bulky' CPP classification, additional CPP subgroups should be mentioned. Proline-rich and polyproline amphipathic sequences include the sweet arrow peptide (SAP), which is a sequence with 50% proline content in addition to three arginine residues that is derived from a storage protein in maize. Antimicrobial peptides (AMPs) such as LL-37, S413-PV, and Buforin 2 damage bacterial membranes during cell entry and possess microbicidal properties. These antimicrobial peptides share molecular similarities with CPPs, are cationic, and may serve as potential structures for future drug delivery systems [19]. Another subgroup of CPPs is based on bipartite peptides, containing two or more of the listed motifs. Its origin is chimeric and it includes several CPPs described above (transportan, pVEC, MAP, Pep-1, and oligoarginine). Although CPPs can rapidly internalize across cell membranes, there is some evidence that peptide

Table 1. Examples of cell-penetrating peptides, their origins, structures, and mechanisms

Cell-penetrating peptide	Origin	Structure	Proposed mechanism
$TAT_{(48–60)}$	HIV-1 transcriptional activator	Random coil/PPII helix	Direct penetration, pore formation
Penetratin $(pAntp)_{(43–58)}$	Antennapedia *Drosophila melanogaster*	Amphipathic, α-helical/β-sheet (higher concentration)	Direct penetration, endocytosis
Polyarginines	Model peptide (chimeric)	Random coil, α-helical	Direct penetration, endocytosis
pVEC	Murine vascular endothelial cadherin	Amphipathic, β-sheet	Direct penetration, transporter-mediated
Pep-1	Chimeric	Amphipathic, α-helical	Direct penetration, pore formation
Transportan	Galanin-mastoparan (chimeric)	Amphipathic, α-helical	Endocytosis, direct penetration
MAP	Model amphipathic peptides (chimeric)	Amphipathic, α-helical	Multiple mechanisms

classes have different behaviors, especially concerning endocytotic uptake. The CPP properties, attached cargo, concentration, and cell type all significantly affect the mechanism of their cell internalization.

Cellular uptake mechanisms of CPPs

Although the mechanism of CPP accumulation in the cytoplasm is not fully understood, it seems clear that two types of intracellular uptake coexist, but differ dramatically in terms of the efficiency of accumulation, and therefore, in possible applications. In addition to the CPP electrostatic interactions and hydrogen bonding that are responsible for the direct transduction of small molecules through the lipid bilayer [20,21], energy-dependent macropinocytosis is a primary endocytotic pathway responsible for CPP-mediated intracellular delivery of large molecules and nanoparticulates and their subsequent enhanced release from endosomes into the cell cytoplasm [22] (Figure 1). It is now evident that various CPPs and CPP–cargo conjugates can enter cells using different (single or multiple) endocytotic mechanisms [23] and can end up in different subcellular compartments. The term endocytosis relates in this case to the pinocytosis process, which can be classified into four pathways: macropinocytosis, clathrin-mediated endocytosis, caveolae/lipid raft-mediated endocytosis, and clathrin/caveolae-independent endocytosis [24]. The variety of pathways correlates with the high variability of chemical and physical properties of the transducing peptide sequences, their concentration, the biophysical characteristics of the cargo, and the cell type-dependent composition of the plasma membrane, which is a barrier for every type of delivery platform [25].

The entry route of individual CPPs and of CPPs-conjugated to small, low molecular weight cargoes such as peptides (fewer than 50 amino acids) is a controversial issue. Although transduction mechanism, electrostatic interactions, and hydrogen bonding were reported for these small peptides, several groups also reported that uptake of CPPs including TATp, oligoarginines, and penetratin did not differ from internalization of high molecular weight cargoes fused to CPPs and delivered via endocytosis [26]. It was also shown that Antp, nona-arginine, and the TAT peptide simultaneously used three endocytic pathways: macropinocytosis, clathrin-mediated endocytosis, and caveolae/lipid raft-mediated endocytosis [23]. It was further suggested that the endocytic uptake mechanism for CPPs strongly depends on its attached cargo [27]. For example, TATp uses lipid raft-mediated endocytosis when conjugated to a protein [28] and clathrin-dependent endocytosis when conjugated to a fluorophore [29]. When high molecular weight cargos (larger than 30 000 Da), such as a nanocarrier–large peptide–CPP conjugate, are delivered intracellularly through the endocytic pathways, the arrival and storage of the internalized CPP species and its cargo in endosomes or lysosomes may be for extended periods of time, thus reducing bioavailability and activity. If the target of the delivered bioactive molecule is located outside endocytic vesicles (e.g., nucleus, mitochondrion), the cargo

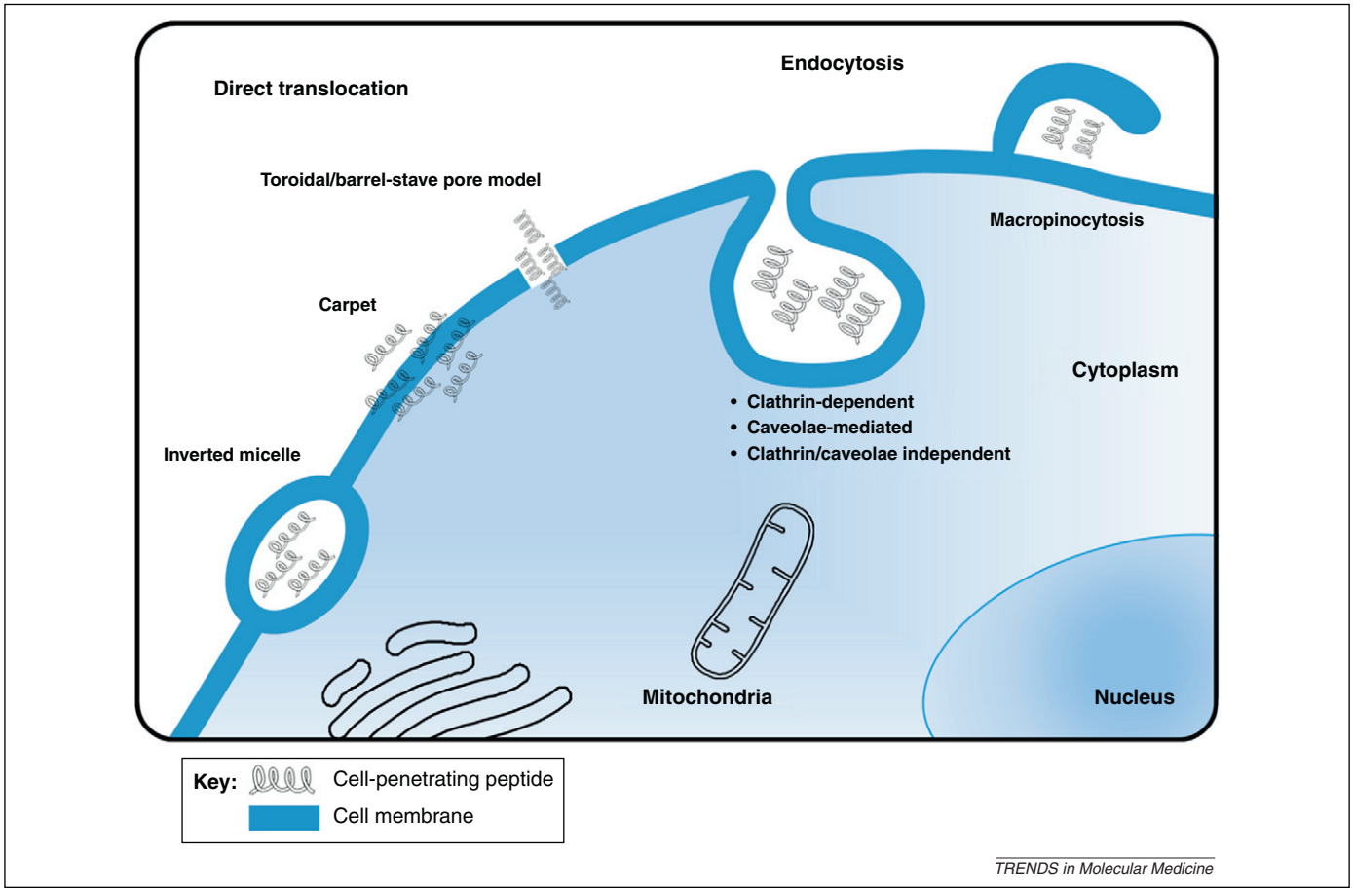

Figure 1. Intracellular pathways of cell entry for cell-penetrating peptides (CPPs).

must escape from endosomal vesicles before trafficking back to the plasma membrane for recycling or fusion with lysosomes. TATp-mediated intracellular delivery of large molecules and nanoparticles can proceed via energy-dependent macropinocytosis with subsequent enhanced escape from the endosome into the cytoplasm [22]. Another study suggested that TAT fusion proteins enter cells via the endosomal pathway, circumvent lysosomal degradation, and then sequester in the periphery of the nucleus [30]. Furthermore, polymers with a buffering capacity between pH 5.2 and pH 7.0 can be attached to the surface of a carrier to mediate their endosomal escape through the 'proton sponge effect', where the proton-absorbing polymer induces osmotic swelling and subsequent rupturing of the endosome [31].

CPP-modified nanocarriers for intracellular delivery

Although the mechanisms underlying the cellular uptake of CPPs and their conjugates remain highly debated, these peptides have been successfully used to mediate the intracellular delivery of a variety of molecules of pharmacological interest in different cell types and have the potential to improve intracellular delivery of a large arsenal of biologically active agents [32–34]. The major advantage of CPPs is their ability to transport cargo to intracellular compartments of the cell (e.g., mitochondria, lysosome, nucleus, and cytoplasm). Because endosomal escape might be needed to effectively deliver these cargos to their target, fusogenic lipids, membrane-disruptive peptides, membrane-disruptive polymers, and lysosomotropic agents have been used to enhance cytosolic delivery of CPP-attached cargos [35]. CPPs have also been conjugated to cargos with a large number of different sizes and efficiently transport both *in vitro* and *in vivo* peptides, proteins, antibodies, nucleic acids (oligonucleotides, cDNA, RNA, siRNA), fluorochromes, nanoparticles, lipid-based formulations, viruses, quantum dots, contrast agents for magnetic resonance imaging, and drugs, [33,34,36,37] (Figure 2).

By covalent or noncovalent attachment of CPPs to a cargo, an effective distribution of molecules of interest into cells can be achieved. Extensive data regarding the intracellular delivery of single CPPs or CPPs conjugated with low molecular weight agents have been reviewed in detail elsewhere [14,37,38].

Nanoparticles have been increasingly studied because a variety of bioactive molecules useful as diagnostic or therapeutic tools can be grafted to them. Their inability to cross the lipid membranes of cells, however, can greatly limit their use both *in vitro* and *in vivo*. Therefore, nanoparticles are often used in a complex with CPPs to circumvent this cell-penetrating difficulty (Figure 2).

Lipid-based nanocarriers

The use of liposomes as drug carriers of therapeutic agents has been extensively investigated [39,40]. Liposomes are

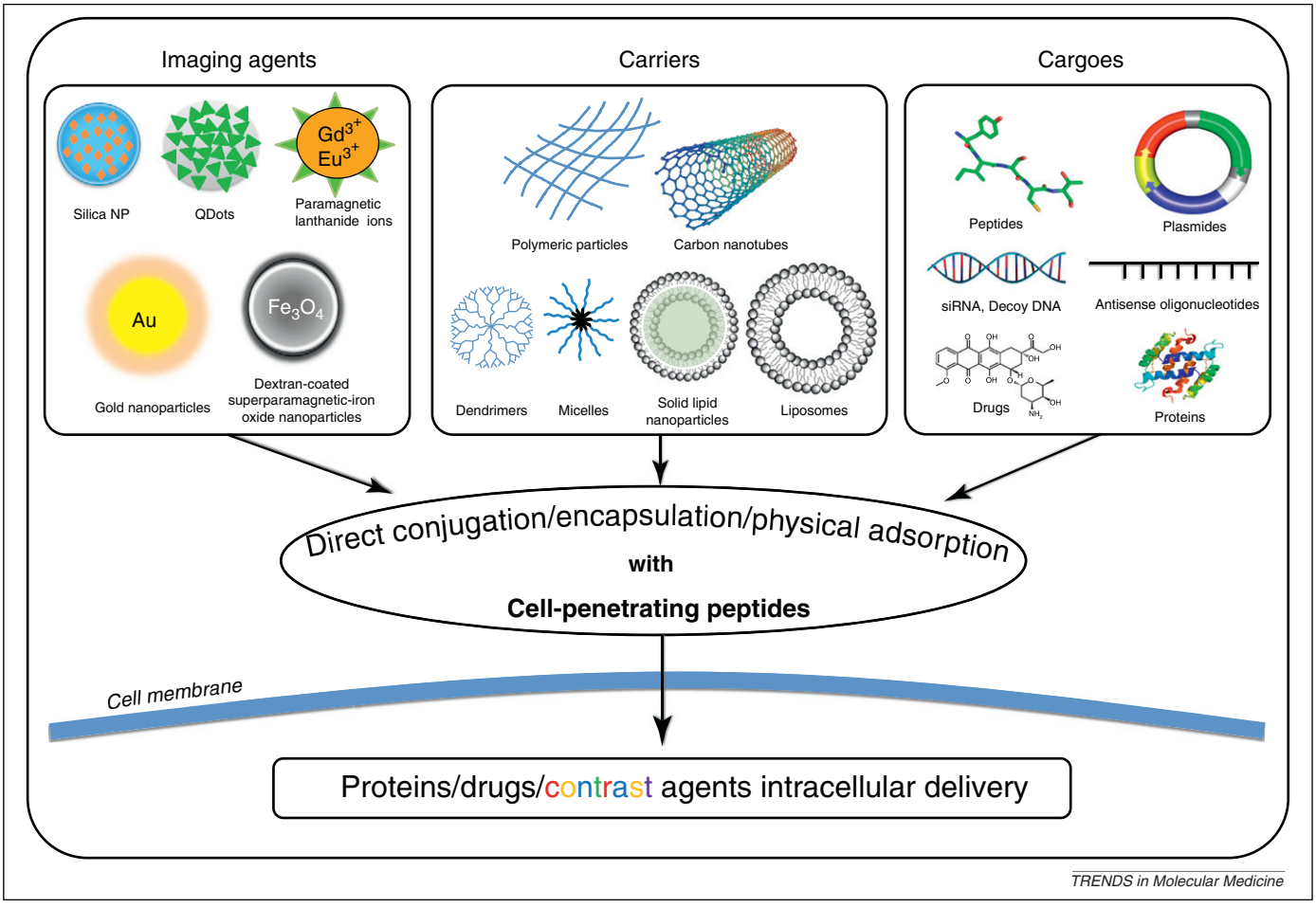

Figure 2. Applications of Cell-penetrating peptides (CPPs) as molecular delivery vehicles for a variety of drugs, nucleic acids, proteins, therapeutics, and imaging agents.

artificial phospholipid vesicles that vary in size from 50 nm to 1000 nm and that can be loaded with a variety of agents. Ligand attachment to polyethylene glycol (PEG) grafted, long-circulating liposomes at the polymer terminus or on the carrier surface can target these carriers and delivery of their cargo to sites of interest [40–43]. It has been shown that 200 nm liposomes can be delivered intracellularly with a TAT peptide attached to the liposomal surface [44]. In a kinetic–efficacy study, both TATp- and penetratin-modified liposomes had enhanced cellular translocation *in vitro* that correlated with the number of peptides attached to the liposomal surface, the peptide sequence, the origin of the cells, and incubation time [45]. CPP-modified liposomes have also been used for gene delivery; TATp–lipoplexes enhanced delivery of the plasmid pEGFP-N1 into U-87 MG tumor cells *in vitro* [46,47]. Intracranial injections of TATp–lipoplexes selectively enhanced delivery of pEGFP-N1 to tumor cells and their subsequent transfection compared with plain plasmid-loaded lipoplexes. Kale *et al.* [48,49] formulated a PEGylated liposomal delivery system for the plasmid pGFP with TATp conjugated to the surface of the particles along with long, pH-sensitive PEG blocks to act as a peptide shield. The liposomes that reached tumor sites (aided by the EPR effect) lost their PEG coating in the low pH tumor environment, exposing the underlying TAT peptides, which then mediated transport into the tumor cells. A triple functional liposomal carrier has also been designed, with its membrane decorated with the anticancer 2C5 monoclonal antibody, TAT peptide, and a 'shielding' pH-sensitive PEG block [43]. In addition to liposomes, Rudolph *et al.* [50] optimized *in vitro* and *in vivo* gene delivery of solid lipid nanoparticle (SLN)-based gene vectors by incorporating a dimeric HIV-1 TAT peptide (TAT_2) into the SLN gene vectors. In a skin model, TATp modification of nanostructure lipid carriers (NLCs) showed a significant increase in celecoxib skin permeation in all skin layers compared with control formulations [51].

Polymeric nanocarriers

CPP-modified micelles can provide a tool for enhanced intracellular delivery of a large arsenal of poorly soluble biologically active agents. Increased interaction of TATp-modified micelles with cancer cells compared with unmodified nanocarriers has been demonstrated, resulting in a significant increase of the *in vitro* and *in vivo* cytotoxicity [52]. Jain and coworkers [53] encapsulated quantum dots into TATp-modified PEG-phosphatidylethanolamine (PEG-PE) micelles, which were taken up by mouse endothelial cells *in vitro* and also allowed tracking of these labeled cells to tumor endothelium. Furthermore, Kanazawa *et al.* [54] used TATp to promote direct brain delivery of polymeric micelles via intranasal administration. Juliano [55] described the use of TATp–dendrimer–oligonucleotide complexes. In addition, polyamidoamine (PAMAM) dendrimers and TAT peptide were conjugated to bacterial magnetic nanoparticles (BMPs) to construct a transmembrane targeted siRNA delivery system for gene therapy of brain tumors [56]. Recently, Jiang *et al.* [57] coupled octa-arginine and folic acid with gene vectors composed of PEGylated polyethylenimine (PEI) (0.6 kDa)–β-cyclodextrin to form an efficient nanovector for gene delivery. The Kissel group [58] investigated TAT-derived and arginine-rich sequences, as well as a model amphiphilic peptide, with respect to transfection efficiency of PEI in A549, Calu-3 cells and in mice after intratracheal administration. In addition, nanoparticles have been generated by complexes of pDNA with TAT-modified chitosan that were shown effective for transfecting pDNA compared with controls [59].

Inorganic nanocarriers

The pioneering findings of Weissleder and coworkers [30,60] described a 100-fold higher efficiency of intracellular magnetic labeling of lymphocytes cells using dextran-coated superparamagnetic iron oxide nanoparticles (SPIONs) (~40 nm diameter), conjugated with $TATp_{(48–57)}$, compared with nonmodified particles. Another group developed a PEG-modified phospholipid micelle coating strategy to functionalize SPIONs for magnetic resonance imaging (MRI) [61]. Santra *et al.* [62] described the design of a TAT peptide-conjugated fluorescent nanoparticle probe, based on a water-in-oil (w/o) microemulsion synthesis of a 70 nm sized monodisperse TATp–FITC–silica nanoparticle complex. It labeled human lung adenocarcinoma (A549) cells *in vitro* and *in vivo* efficiently crossed the blood–brain barrier in rat brains. Penetratin-conjugated gold nanoparticles were taken up by 100% of coincubated endothelial cell line GM7373 within 2 h [63]. Medintz and colleagues investigated the cellular uptake and fate of TATp-conjugated PEGylated gold nanoparticles with a size range of 2.4 nm to 89 nm [64]. Whereas *in vitro* nuclear localization was observed for the 2.4 nm nanoparticles, intermediate 5.5 nm and 8.2 nm particles were only partially delivered into the cytoplasm. The 16 nm and larger gold nanoparticles did not enter the cells and either localized at the cellular periphery or aggregated extracellularly.

CPP-modified stimulus-responsive and 'smart' nanocarriers

Although of considerable clinical potential, CPPs also have a few important drawbacks and limitations. First, they have the undesirable characteristic of nonspecificity and can enter any cell they come in contact with. This lack of selectivity affects the risk of drug-induced toxic effect on normal tissues. Secondly, the stability *in vivo* of these peptides is at risk until they reach their target. These peptides can be enzymatically cleaved by plasma enzymes and thus need to be sterically protected [65,66]. The use of a protease-resistant D-form of the peptides instead of the naturally occurring L-amino acid form is a prominent strategy for CPP clinical usage [67,68]. In addition to the conformation solution, another approach has been suggested [42,69,70], which proposed that CPPs be incorporated into 'smart' nanocarrier delivery platforms. Thus, during the first phase of nanocarrier delivery, the nonspecific CPP function is sterically protected ('shielded') by a polymer or targeting antibody. Upon accumulation in the target, the protective moiety attached to the surface of the carrier via a stimulus-sensitive bond will detach under local environmental conditions to reveal the CPP and effect targeted delivery (Box 2). Examples for local environmental conditions typical to cancer, infarcts, and inflammation

Box 2. Stimulus-sensitive pharmaceutical nanocarriers

Desirable pharmaceutical nanocarriers should have controlled release of their cargo through a timed mechanism, as opposed to an alternative burst release. The use of stimulus-sensitive pharmaceutical nanocarriers to achieve release in an 'on-demand' manner is of great interest for improved efficacy with lower doses and fewer off-target effects. Ultimately, a nanocarrier should have several characteristics and stages of action:

- Long circulation capabilities with a PEG-coating on its surface.
- Specific organ accumulation by passive (EPR effect) or active (monoclonal antibodies, specific peptides, etc.) targeting.
- Intracellular delivery capabilities (e.g., using CPPs).
- Effective payload delivery (drug, DNA, siRNA) to a specific intracellular compartment (e.g., nucleus, mitochondria) and successful endosomal escape.

A variety of materials with sensitive responses to cell environmental stimulus conditions, such as pH, temperature, MMPs, and redox [glutathione (GSH) inside cancer cells] or external stimuli, such as ultrasound, radiofrequency heating, magnetic field, and light, have been introduced in the past few years and used for the design of 'smart' pharmaceutical nanocarriers (Figures 3 and 4). The ability to 'switch-on' a desired function or expose a 'shielded' functionalized agent on the surface of the carrier has been described and has paved the way for the design of several multifunctional stimulus-sensitive delivery systems [41,43,49,69,74,80–82]. A doxorubicin–CPP-containing multifunctional long-circulating liposomal system has recently been described [43] (Figure I), where a nucleosome-specific monoclonal antibody, 2C5, was attached to the surface of the carrier via a long $PEG_{3.4k}$ spacer. The liposomal surface was decorated with TATp moieties, conjugated with short PEG_{1k}–phosphatidylethanolamine (PEG_{1k}–PE) derivatives. The nonspecific cell-penetrating function of TATp moieties was shielded by a protecting polymer, a hydrazone pH-sensitive degradable bond between longer PEG_{2k} and PE. This multifunctional nanocarrier has demonstrated high specific binding with antibody nucleosome substrates, where brief exposure to low pH values lead to hydrazone hydrolysis, TATp moiety exposure, and intracellular delivery of the contents of the carrier resulting in higher levels of cytotoxicity. Other internal and external stimulus-sensitive bonds can be combined in the design of tumor-specific drug or gene delivery systems using CPPs.

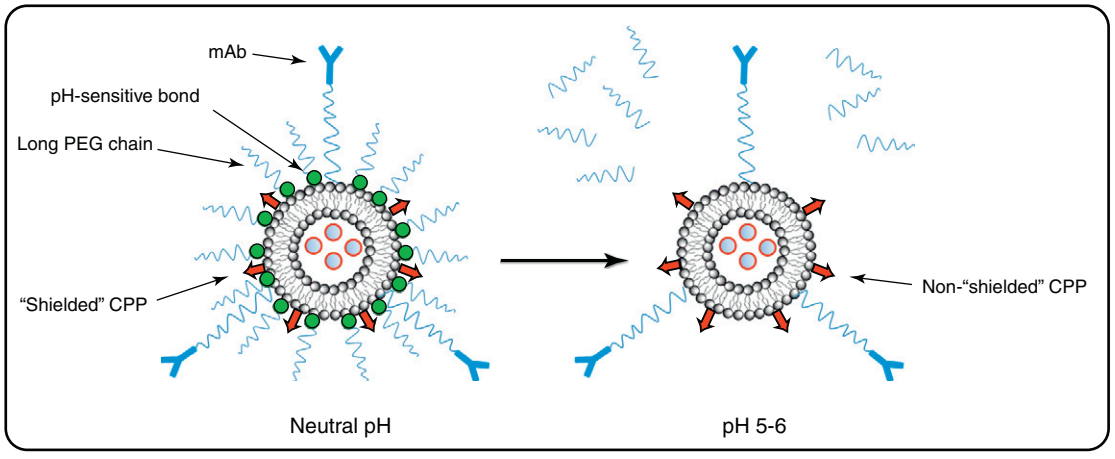

Figure I. Schematic of a ''smart'' nanocarrier with hidden CPP.

are lower pH, higher temperature, and the presence of matrix metalloproteinases (MMPs). External triggers such as heat, radiation, ultrasound, radiofrequencies, and magnetic fields can also be used. Selected bioconjugates used to control the release and shielding of CPPs are depicted in Figures 3 and 4.

A multifunctional stimulus-sensitive liposomal delivery system was recently introduced [43] that incorporated a cancer-specific 2C5 monoclonal antibody, a TAT peptide conjugated to short PEG, and a degradable pH-sensitive hydrazone bond between a long-shielding PEG chain and phosphatidylethanolamine. All of the polymers were attached to the surface of the liposomal formulation, Doxil® (doxorubicin HCl-containing PEGylated liposomes). This multifunctional carrier promoted enhanced cytotoxicity and carrier internalization, compared with the unmodified commercial Doxil®, in four cancer cell lines when the formulation was pre-exposed to a lower pH, typical of solid tumors and endosomes [43]. Nguyen *et al.* [71] examined *in vivo* visualization of MMP activity, by MRI and fluorescence techniques, of dendrimeric nanoparticles coated with 'nonactive' Cy5/gadolinium-labeled CPPs. This labeled CPP was coupled via a MMP cleavable linker to a neutralizing peptide. Upon exposure to proteases, one of the characteristics of tumor tissue, the MMP-sensitive linker was cleaved, dissociating the inhibitory peptide and allowing the CPP to bind and enter tumor cells. These nanoparticles had a 4- to 15-fold higher uptake in tumors than the uncleaved CPP formulation. Omata *et al.* [72] explored the effect of bubble liposomes (BLs) and ultrasound exposure on the gene transfection efficiency of TATp-modified PEG liposomes. They found that TATp–PEG liposomes were efficiently internalized into cells and the transfection efficiency of these liposomes was enhanced approximately 30-fold when BLs or ultrasound exposure were used. Harashima and coworkers developed a multifunctional envelope-type nanodevice (MEND) for gene delivery to tumors, based on carrier PEGylation and the EPR effect [73]. This carrier consists of a condensed DNA core and a surrounding lipid envelope. MEND with octa-arginine on the envelope had 1000-fold higher transfection activity than a DNA/poly-L-lysine/lipid complex. To circumvent the decreased cellular uptake and low endosomal escape associated with PEGylation, specific ligands, cleavable PEG systems, and

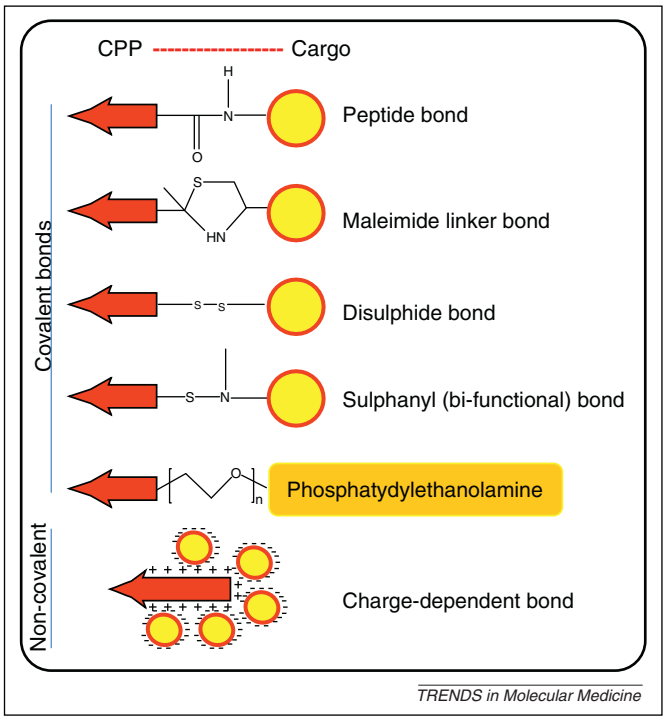

Figure 3. Covalent and noncovalent cargo linkages to cell-penetrating peptides (CPPs).

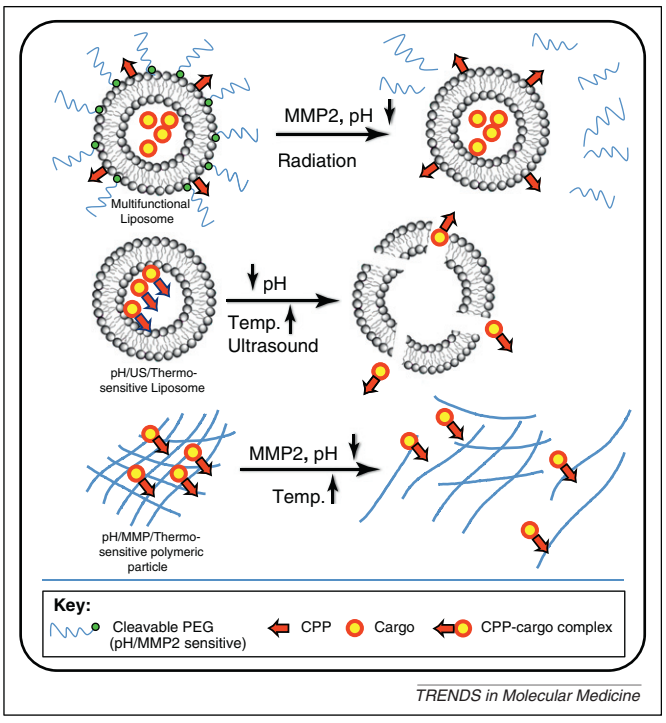

Figure 4. Selected strategies for the development of cell/environment-selective cell-penetrating peptide (CPP)-modified multifunctional carrier delivery following internal (e.g., pH, enzyme levels) and/or external (e.g., temperature, radiation, ultrasound) triggered release. Abbreviations: US, ultrasound; MMP, matrix metalloproteinase; PEG, polyethylene glycol.

endosomal fusogenic/disruptive peptides were used to improve the multifunctional gene delivery carrier. Kuai *et al.* [74] recently used a cleavable PEG system based on a cysteine (Cys)-cleavable PEG_{5000} bond to shield the TAT peptide conjugate. Optical imaging with this cleavable system showed higher tumor accumulation and much lower liver distribution compared with TAT liposomes.

Concluding remarks and future perspectives

CPPs have been shown to assist intracellular delivery of a variety of biomolecules both *in vitro* and *in vivo*. The absence of cell specificity of CPPs along with their susceptibility to proteolytic cleavage under physiological conditions has lead to the design of so-called 'smart' delivery platforms, based on the physiological or microenvironmental features peculiar to the targeted tissue or cell type. An external local trigger can also be used to enhance a carrier's cargo release. CPP-modified nanocarriers should be designed in such a way so that during the first phase of their delivery, surface CPP moieties are sterically shielded. After reaching their target (passively or actively), CPPs should be exposed under unique local conditions to enhance a penetration of the carrier through the cell membrane followed by intracellular delivery of its bioactive cargo. Cancer cells metabolism and additional pathological conditions provide a unique cellular environment that can be used as triggers for CPP exposure to then effect intracellular drug delivery. The fact that delivery platform technologies have matured into potentially useful CPP-based therapeutics and delivery agents provides optimism for a wide range of therapeutic applications which should eventually pave the way for their combined usefulness in the clinic.

Acknowledgments

This work was supported by National Institutes of Health grants RO1 CA121838 and RO1 CA128486 to V.P. Torchilin. W.C. Hartner is gratefully acknowledged for his help during preparation of this manuscript.

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