Cell-penetrating peptides for nanomedicine – how to choose the right peptide

Abstract: More than two decades ago, a group of peptides, now known as cell-penetrating peptides, sparked the hope that the ultimate carrier molecules have been found. The high expectations for these peptides, which are reflected in their bold name, led to many disappointments due to the controversial results their utilization entailed and nowadays even their effectiveness has been called into question. In this review, we discuss the uptake mechanism and application of cell penetrating peptides as mediators for organelle specific delivery of nanocarriers, pointing out the possibilities as well as strategies of their successful utilization. Additionally, we provide an overview of the conjugation techniques usually employed for the attachment of cell penetrating peptides to quantum dots, as well as silver and gold nanoparticles, and we address the various aspects that need to be considered for the successful implementation of cell penetrating peptides for organelle-specific delivery of nanoparticles into cells.

Keywords: cell penetrating peptide; drug delivery; gold nanoparticle; nanomedicine; polymeric nanoparticle; quantum dot.

Introduction

The plasma membrane and membranes of organelles are impermeable barriers to many biologically active molecules and carrier systems. More than two decades ago, Frankel and Pabo [1] as well as Green and Loewenstein [2] simultaneously reported the first peptide, which was able to cross the aforementioned barriers, the peptides exhibiting these properties, now known as cell-penetrating peptides (CPP) [3], “Trojan peptides” or protein transduction domains (PTD), sparked hope that the ultimate carrier molecules for the transport of small molecules and nanocarriers have been found. Since then, the literature has witnessed more than 4000 publications on CPPs and our understanding of their nature as well as the mechanism of uptake has been enhanced greatly. However, the more we know about these small peptides, the more questions arise. The high expectations of CPPs, which are reflected in their bold name, led to many disappointments and even their effectiveness has been called into question [4]. Several studies have reported their limited applicability in vivo, including the work of Caron et al. [5], who demonstrated that even subcutaneous and intra-arterial injections of CPP modified green fluorescent proteins (Tat-eGFP) led to only few stained fibers in the muscle periphery or those surrounding the blood vessels. On the other hand, CPPs offer advantages over other carrier systems for the therapeutic as well as...
diagnostic delivery of cargo. They have been employed for the delivery of a large variety of molecules and particles ranging from small drugs and fluorophores to proteins, polymer conjugates as well as nanoparticles, which can be further divided into several classes of inorganic nanomaterials differing in their nature as well as in their function. This review will highlight the recent applications of CPPs in the targeted delivery of their most prominent examples such as quantum dots as well as gold, silver and polymeric nanoparticles into mammalian cells in the context of organelle specificity. Additionally, we will give a short overview of the conjugation techniques usually utilized for the attachment of CPPs to these nanostructures, while leaving out the modification methods of polymeric nanoparticles due to the overwhelming number of available functional groups and the entailing possibilities for conjugation chemistry, which would be beyond the scope of this review. In order to present a more complete picture of the intracellular fate of the CPP-cargo conjugates, we will also discuss the mechanisms of peptide uptake and briefly introduce the different types of CPPs.

Types of CPPs

A large number of CPPs have been isolated and designed to facilitate intracellular delivery of therapeutic molecules. In the past, cell penetrating peptides were often classified as protein derived, model peptides or designed CPPs [6], however, the categorization in cationic, hydrophobic or amphipathic CPPs is becoming more common (see Table 1) [38]. Cationic CPPs were the first to be discovered starting with the HIV-1 derived Tat-peptide, which is by far the most intensely studied CPP [1]. Peptides belonging to this class often possess short arginine-, lysine- or histidine-rich amino acid sequences, which interact electrostatically with the negatively charged phosphates and sulfates on the plasma membrane that leads to receptor-independent internalization [39, 40]. The guanidinium groups of arginine are protonated at physiological conditions, whereas the amino groups of lysine and histidine are only partially protonated, making CPPs with an oligoarginine sequence the most effective in this type of carrier-cell interaction. In addition, it has been demonstrated that cationic CPPs require at least eight positive charges for efficient cellular uptake [41]. Hydrophobic peptides, on the other hand, are distinguished due to the hydrophobicity of the incorporated amino acids and their low net charge. Amphipathic CPPs possess lipophilic, as well as hydrophilic segments, of their sequence and can be further categorized in primary amphipathic CPPs (Pep-1), secondary amphipathic α-helical CPPs (hCT18-32), β-sheet amphipathic CPPs (VT5) and proline-rich amphipathic CPPs (Bac7) [38]. In this review, we will only highlight the CPPs most often applied for the delivery of nanomedicine, however, for a detailed and comprehensive description of all existing CPPs the reader is directed to the review by Milletti [38].

Uptake mechanism

In spite of the fact that CPPs have been employed in a wide range of applications for more than two decades and although several internalization models have been suggested (Figure 1), the exact uptake mechanism of the various CPPs still remains to be fully unveiled [42, 43]. Koppelhaus et al., for example, investigated Tat- and penetratin-mediated cellular uptake of peptide nucleic acid oligomers in five different cell lines (HeLa, SK-BR-3, IMR-90, H9 and U937) demonstrating poor or no uptake at all [44]. The authors concluded that the efficiency of the internalization largely depends on the composition of the membrane (membrane-bound components and lipids). Heparan sulfate proteoglycans (HSPGs), cell surface proteoglycans composed of a core protein and one or more heparan sulfate (HS) glycosaminoglycan (GAG) chains [45], have been recently shown to interact electrostatically with CPPs as initial binding sites, which increase the local concentration of CPP-cargo conjugates and as a consequence promote internalization [46–49]. In particular, arginine rich CPPs such as Tat, penetratin or oligoarginine, which possess a positive net charge at physiological conditions, show a high affinity towards HSPGs, one of the most highly negatively charged biopolymers [50]. Besides the HSPG-mediated cellular uptake other uptake mechanisms have also been described such as the clathrin-dependent, caveolae-mediated and the clathrin/caveolae-independent endocytosis (see Figure 1) [43]. Understanding the internalization route a CPP-cargo conjugate takes is an important step for successful organelle-specific targeting. The energy-independent route of direct translocation into the cytosol, for example, can circumvent the setbacks of vesicular trapping of cargo, as well as need of endosomal escape mechanisms, and therefore facilitates the targeting of cellular structures such as the nucleus or the mitochondria. However, depending on the employed CPP, the plasma membrane composition of the cell type, as well as on the characteristics of the cargo molecule, either endocytosis
or direct translocation can be the predominant uptake mechanism [51]. The intracellular fate of their cargo can also be influenced, for example, by equipping the carrier system with sorting peptides, hence exploiting the subtle differences of vesicle membrane composition of the respective uptake mechanisms [52]. Therefore, all of these parameters need to be taken into account when choosing a CPP and it is wrong to assume that a certain CPP, which has been shown to deliver therapeutic molecules in a definite way into selected cells, would be able to deliver the same molecule in the same manner in all of the respective structures.

Table 1: Examples for CPP-mediated transport of various cargo including the proposed uptake mechanism.

<table>
<thead>
<tr>
<th>Name</th>
<th>Amino acid sequence</th>
<th>Targeting</th>
<th>Cargo</th>
<th>Uptake mechanism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cationic</td>
<td>Oligoarginine</td>
<td>R8, R9, R10, R12</td>
<td>Cytosol</td>
<td>PEI600-CyD</td>
<td>A</td>
</tr>
<tr>
<td>Tat 49-57</td>
<td>RKKRRQRRR</td>
<td>Random distribution</td>
<td>Nucleus</td>
<td>Liposomes</td>
<td>C</td>
</tr>
<tr>
<td>Penetratin</td>
<td>RQIKIWFQNRRMKWKK</td>
<td>Cytosol, Endosomes</td>
<td>Liposomes</td>
<td>A</td>
<td>[10]</td>
</tr>
<tr>
<td>DPV3</td>
<td>RKKRRRESRKRRRR</td>
<td>Cytosol</td>
<td>Peroxidase, IgG</td>
<td>E</td>
<td>[11]</td>
</tr>
<tr>
<td>DPV10</td>
<td>SRRARRSPRHLG</td>
<td>Nucleus</td>
<td>Liposomes</td>
<td>B</td>
<td>[12]</td>
</tr>
<tr>
<td>DPV15</td>
<td>LRRERQSLRERRQ</td>
<td>Nucleus</td>
<td>Liposomes</td>
<td>B</td>
<td>[13]</td>
</tr>
<tr>
<td>NrTP</td>
<td>YKQCKKKGKGK</td>
<td>Nucleus</td>
<td>Fluorescent dyes</td>
<td>G</td>
<td>[14]</td>
</tr>
<tr>
<td>Hph-1</td>
<td>YARVRRGPR</td>
<td>Nucleus</td>
<td>Quantum dots</td>
<td>B</td>
<td>[15]</td>
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<tr>
<td>Hydrophobic</td>
<td>K-FGF</td>
<td>AAVLPVLLAAP</td>
<td>Nucleus</td>
<td>125Iodine</td>
<td>A</td>
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<tr>
<td>C105Y</td>
<td>CSIPPEVKFNKFVYL</td>
<td>Nucleus</td>
<td>DNA nanoparticles</td>
<td>A</td>
<td>[17]</td>
</tr>
<tr>
<td>Amphipathic</td>
<td>MAP</td>
<td>KKLAKLALKALKAALKA</td>
<td>N.a.</td>
<td>Dye</td>
<td>B and other</td>
</tr>
<tr>
<td>Cady</td>
<td>GLWRALWRLSRLWRLWR</td>
<td>Cytosol</td>
<td>siRNA</td>
<td>n.a.</td>
<td>[19]</td>
</tr>
<tr>
<td>Transportan</td>
<td>GWTLNSAGYLLGKINLAAALKKIL</td>
<td>Membrane structures</td>
<td>Biotinyl</td>
<td>A</td>
<td>[21]</td>
</tr>
<tr>
<td>ART (1-22)</td>
<td>MVVRFLVTIRRIACPPRVRV</td>
<td>Endosomes</td>
<td>–</td>
<td>G</td>
<td>[22]</td>
</tr>
<tr>
<td>SS-02</td>
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<td>Mitochondria</td>
<td>Antioxidant</td>
<td>A</td>
<td>[23]</td>
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<tr>
<td>MTS</td>
<td>FFrKFrFkFrKk</td>
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<td>Dye</td>
<td>A</td>
<td>[24]</td>
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<tr>
<td>NLS</td>
<td>CGGFSTSLRARKA</td>
<td>Nucleus</td>
<td>Quantum dots</td>
<td>A</td>
<td>[25]</td>
</tr>
<tr>
<td>NLS</td>
<td>CGGGPKKKRKVG</td>
<td>Nucleus</td>
<td>Gold nanoparticles</td>
<td>B</td>
<td>[26]</td>
</tr>
</tbody>
</table>

*Direct translocation, †Endocytosis, ‡Macropinocytosis, §Vesicular transport, ‡HSPG-mediated endocytosis, ‡HSPG-independent endocytosis, ‡Clathrin-mediated endocytosis, ‡Lipid raft-mediated endocytosis, ‡polyethylenimine-β-cyclodextrin nanoparticles, Fx=cyclohexylalanine, r=d-arginine.
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Table 1 and Figure 1 depict the complexity of this topic, since they also show that even systems which are similar in cargo type as well as the used CPP, can lead to different uptake mechanisms simply due to variations in shape and size of the cargo. An increase in size of the CPP-cargo conjugate, for example, diminishes the direct translocation and enhances the energy dependent endocytotic uptake [53]. Adsorptive transcytosis (AMT), a time- and energy-dependent endocytotic mechanism, enables molecules to cross the blood-brain barrier (BBB) [54]. Since AMT largely depends on electrostatic interaction between the cell-surface of the brain capillary network, which possesses a highly negative charge density, and the positive charge of the crossing molecule, cationic arginine rich CPPs can be exploited for the successful delivery of conjugated cargo molecules without affecting the biological activity [55]. However, the exact mechanism of AMT for the transport of CPP-cargo conjugates across the BBB still remains unknown, in spite of its proven efficiency [56].

CPP mediated delivery of nanoparticles

Cell-compartment targeting with CPPs

The most promising property of CPPs is the possibility to combine targeting and transduction properties [57–59]. CPPs can influence tissue localization of a conjugated molecule by altering organ distribution and retention. In the work of Kameyama et al., for example, the CPPs REV (TRQARNRNRWRERQR-GC), Tat (GRKKRRQRRRPQ-C), and ANP (Antennapedia (43–58), RQIKIWFQNRRMK-WKK-GC) were conjugated to the Fab unit of an antibody (antigen binding domain) and significant changes in the distribution as well as retention in organs, such as spleen, liver, adrenal gland and kidney, were observed [60]. Targeting at subcellular level is also possible, enabling the delivery of biologically active substances into different cellular compartments such as Golgi apparatus, mitochondria or nuclei (see Table 1). Organelle-specific
targeting can be achieved, for example, by utilizing signal peptides, which are a common tool used by cells for sorting and trafficking of newly translated proteins [61]. Nuclear localization sequences (NLSs), which consist of roughly 10 amino acids and possess a high cationic net charge as well as inherent cell-permeation properties [62], belong to the above-mentioned class of signal peptides. Nuclear localization and transfection efficiency, important properties for gene therapy, have been repeatedly demonstrated for NLS-mediated [59, 63–65] delivery of not only polymer or phage particle encapsulated DNA [66, 67], but also of gold nanoparticles [37] and proteins [68]. Signal peptides also enabled efficient targeting of mitochondria, which are of great interest for drug delivery, as mitochondria house the protein complexes of the electron transport chain [69], generate reactive oxygen species and are therefore essential for cell proliferation [70] and intracellular signaling [71, 72]. However, the naturally abundant signal sequences for mitochondrial targeting cannot be utilized for organelle specific delivery since they are only functional in the presence of the respective full-length protein [73]. Artificial signal sequences offer a way to circumvent this setback. Zhao et al. utilized tetrapeptide sequences such as SS-02 (Dmt-D-RFK-NH₂), SS-20 (Phe-D-RFK -NH₂), SS-31 (D-R-Dmt-KF-NH₂) in order to achieve localization of antioxidants in mitochondria as a means to arrest the progress of apoptosis by incorporating the unnatural amino acid 2,6-dimethyltyrosine (Dmt), a radical scavenger, into the sequences [34]. Horton et al. developed different peptide sequences (toxFxKFrYK, to-FrYKFrYK with F x =thiophene, to=Thiazole orange and r=d-arginine) containing cationic as well as lipophilic residues for mitochondrial targeting, which were additionally shown to possess cell-penetrating properties [35]. They demonstrated that these octameric sequences with varying degrees of lipophilicity and cationic charge led to localization in either mitochondria or the nucleus and cytosol. Kelley and coworkers further described a molecular charge dependent lipophilicity threshold, which governs organelle targeting. Subcellular localization in mitochondria and the nucleus was achieved by altering the sequence of the signal peptides and by conjugating the peptides to Thiazole orange. These organelle-targeting properties of peptides, which are also able to transport cargo across plasma membranes, have been repeatedly applied for drug delivery applications. The advantage of equipping a cargo molecule with both traits in only one step has also been capitalized upon in a variety of recent studies on nanoparticles, which will be introduced in the next section.

**Delivery of nanoparticles using CPPs**

**Quantum dots**

Fluorescent markers are a useful tool for studying living cells due to their ability to illuminate intracellular or exogenous molecules and are often applied to observe and investigate various dynamic cellular processes [74]. Quantum dots (QDs) are nanocrystals with a core diameter of 1–6 nm. They can be comprised of varying elements (groups II to IV or III to V), but CdSe and CdTe are the most commonly used in life science [75]. The use of non-biodegradable heavy metals for clinical applications, however, requires careful consideration of their biodistribution and long term toxicity [76]. It is therefore important to ensure rapid renal clearance of QDs by utilizing to a small final hydrodynamic radius (<5.5 nm) and formulations with completely nontoxic as well as biodegradable coating-components [77, 78]. These coatings are also usually used to improve the solubility in aqueous media and as a means to equip quantum dots with functional groups, which in turn are necessary for the subsequent conjugation of bioactive molecules (Figure 3A). QDs are often depicted as excellent candidates for fluorescent labeling especially for prolonged observations, since they possess not only resistance to photobleaching, high quantum yields, as well as tunable photoexcitation [79], but also sharply defined emission peaks [80]. However, they are not able to cross plasma membranes unaided, since, as a result of common synthetic procedures, QDs are water-insoluble, which makes the utilization of either direct injection into cells or the modification of their surface a necessity [81, 82]. The approach of using of positively as well as negatively charged coatings for improved water solubility can be expanded by the conjugation of bioactive molecules, such as CPPs, in order to influence the uptake mechanism and intracellular targeting [83, 84]. Xue et al., for instance, conjugated Tat peptide to thiol capped CdTe QDs (2–4 nm in diameter) and compared their effectiveness in intracellular delivery to unmodified CdTe QDs in human hepatocellular carcinoma (QGY) cells and human breast cancer (MCF7) cells by means of confocal laser scanning microscopy. The authors were able to demonstrate that the conjugation of Tat enhanced intracellular delivery in both cell lines [85]. The same holds true for the PEG-encapsulated CdSe-ZnS QDs, which were functionalized with covalently bound Tat in order to achieve efficient uptake into mesenchymal stem cells [86]. In a study by Liu et al. CPPs did not need to be covalently associated to QDs to achieve efficient cellular internalization. This non-covalent attachment of histidine- and arginine-rich peptides to carboxylated QDs allowed for direct membrane translocation and diminished the setback.
of endosomal, as well as lysosomal trapping [87]. Equipping QDs with cysteine-terminated NLS peptides, such as the adenovirus-derived CGGFSTSLRARKA, the SV40 large T antigen-derived CGGPKKRRKVKGG or the HIV-1 Tat protein-derived CGGRKKRRQRRRA by means of the heterobifunctional linker (Succinimidyl-4-[N-maleimidomethyl] cyclohexane-1-carboxylate) led to efficient cellular internalization. This modification also allowed active targeting of the nucleus in spite of the increased size of the particles (14.1–18 nm in diameter) [36]. We should mention that the cellular uptake can be influenced not only by the conjugation of CPPs, but also by the number of QD-associated CPPs and the cell-type, which has been confirmed by Delehanty et al. [88] The functionalization of QDs with CPPs can facilitate their transport across the plasma membrane, and thus enable these particles to cross the blood-brain barrier (BBB). Tat-modified QDs, for example, were successfully used by Santra et al. to fluorescently label brain tissue of rodents (SD adult rats, Sprague-Dawley rats) [89].

**Silver and gold nanoparticles**

Silver nanoparticles possess a broad spectrum of valuable optical, electrical, thermal and antibacterial properties. A great wealth of information regarding their biocompatibility has been collected, as silver has been applied for medical purposes since the Middle Ages, and silver nanoparticles are still being applied as a component for antimicrobial coatings of textiles, wound dressings and other biomedical devices. The cytotoxicity of silver nanoparticles has been reported to result from their size and the solvatization of the particles in a codependent manner [90]. Liu et al. were able to further enhance the biocidal effect of silver nanoparticles towards bacteria by coating the particles with CPPs such as Tat by the DMF reduction method, which indicates that the surface modification is of greater importance for the biocidal properties than the size and shape of the nanoparticles [91]. Liu et al. used a modification method, which utilizes the thiol functionality of cysteine terminated peptides, in order to equip silver nanoparticles (8 nm in diameter) with the Tat peptide and provided a proof of concept for the size-exclusion mechanism of multidrug-resistant (MDR) tumor cells. These modified nanoparticles demonstrated not only efficient cell-uptake, but also showed antitumor activity in MDR, as well as non-resistant tumor cells, while exhibiting reduced adverse toxicity in vivo [14].

Gold nanoparticles have unique optical and electronic properties, which can also be tuned by size, shape and surface chemistry, making them highly useful for high technology applications. However, these non-biodegradable but biocompatible particles are also exceptional therapeutic tools in biological and medical applications, such as the delivery of drug molecules and bioactive agents, due to their large surface area and their ability to selectively incorporate recognition molecules such as peptides or proteins [92, 93]. The advantageous properties of gold nanoparticles lead to numerous studies investigating their toxicity and metabolization in various model organisms. The findings indicated low toxicity and efficient renal clearance, if the hydrodynamic radius of the said particles did not exceed 5–6 nm [94, 95]. The toxicity of gold nanoparticles also largely depends on the surface modifications [96]. Therefore, similarly to their silver counterparts, gold nanoparticles require not only fine-tuned radii but also functionalization with engineered coating, which promote targeting and hydrophilicity, in order to realize their full potential in drug delivery applications [52]. The conjugation of CPPs for improved internalization and specific organelle targeting is a prime example for further improvement of their innate properties, as unmodified nanoparticles cannot cross the plasma membrane unaided prohibiting targeted localization in the nuclei or other organelles [97]. NLS peptides have been largely utilized to achieve this aim. Feldheim and colleagues described the delivery of bovine serum albumin (BSA) coated gold nanoparticles into the nuclei of HepG2 cells by means of conjugating these particles with a diameter of 20 nm to NLS of different viruses [37]. The attachment of Tat is another method to achieve efficient cellular internalization and nuclear localization of nanomedicine. This approach has been used by de la Fuente et al., who conjugated Tat to tiopronin protected gold nanoparticles of adequate size (2.8 nm in diameter) to pass through the nuclear pores of primary human fibroblast cells (hTERT-BJ1) [15]. In this context it is important to mention that Krpetic et al. demonstrated Tat-modified gold nanoparticles (14 nm in diameter) to possess not only enhanced internalization into HeLa cells, but also a peculiar distribution pattern: The aforementioned modified nanoparticles were initially found in the cytosol, the nucleus and mitochondria, appearing to cross intracellular membranes freely. Later, they were densely packed within vesicles, from which they were subsequently released by membrane rupture or direct transfer across the membrane [98]. Therefore, the cargo was found in various cell compartments indiscriminately making organelle specific targeting impossible. Avoiding unselective cytotoxicity originating from the cargo, however, is a necessity for drug delivery purposes. Dekiwadia et al. achieved the desired specificity by equipping gold nanoparticles with a combination of CPPs and lysosomal sorting peptides for...
the treatment of storage diseases by delivering replacement enzymes into lysosomes [52]. Combining ionic and covalent formulation approaches, Conde et al. functionalized gold nanoparticles with a diameter of 14 nm with PEG chains, arginine-glycine-aspartic (RGD) targeting peptide as well as Tat. The resulting particles showed excellent biocompatibility, low cytotoxicity and good chemical stability when tested in Hela cells, freshwater polyps (Hydra vulgaris) and mice (C57BL/6j) [97].

In a recent study Oh et al. investigated the internalization and intracellular fate of Tat functionalized gold nanoparticles with varying diameters ranging from 2.4 to 89 nm. The authors we able to demonstrate that although the conjugated CPPs were largely responsible for the cellular uptake, the final cellular target was influenced by the diameter of the nanoparticles. Small nanoparticles (2.4 nm) localized preferably in the nuclei, intermediate particles (5.5–8.2 nm) were partially found in the cytosol and particles with a diameter exceeding 16 nm were not internalized in spite of the functionalization with CPPs [99]. Ryan et al. studied BSA coated gold nanoparticles with a diameter of 15 nm, which were equipped with modified SV40 large T antigen-derived peptides (rhodamine-Cys-Gly-Gly-Gly-Pro-Lys-Lys-Arg-Lys-Val-Gly-Gly-OH) and they were able to demonstrate that intracellular entry depends not only on the size but also on the number of CPPs positioned on the particle’s surface [100]. We should also note here that additional factors such as the cell type, nature and origin (e.g., adenovirus, SV40, or HIV-1) of the CPP, incubation time and the temperature during the experiment, which were not discussed in this review, can also strongly influence the efficiency of internalization of a CPP-nanomedicine conjugate [101].

Polymeric nanoparticles

Polymeric nanoparticles can be defined as structures with a diameter of <1 μm and are prepared from either natural or synthetic polymers. Natural polymers, such as polysaccharides, are suited for drug delivery applications due to their low toxicity and bioavailability [102]. However, they exhibit batch-to-batch differences and vary in purity making biocompatible and biodegradable synthetic polymers, such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA) or their copolymers [poly(lactide-co-glycolide) (PLGA)] the preferred choice [103]. Due to the large variety of applicable polymers and the resulting possibilities of design a wide range of cargo such as hydrophilic and hydrophobic drugs, proteins as well as vaccines can be delivered to diverse areas of the body for sustained periods of time. This performance, however, is only possible due to the numerous synthesis protocols and surface modification strategies [103]. The conjugation of CPPs improves the utility of polymeric nanocarriers, since these particles can neither cross the BBB unaided nor are they able to penetrate plasma membranes efficiently. Various examples for the successful utilization of CPPs for the enhanced delivery of polymeric nanoparticles exist, such as the Penetratin-functionalized PLA-PEG nanoparticles, which demonstrated successful delivery into brain cells. In comparison to conjugates with arginine-rich CPPs, this conjugate was also shown to possess reduced non-target tissue accumulation [23]. Jiang et al. equipped polyethylenimine-ß-cyclodextrin nanoparticles (PEI600-CyD) with oligoarginine (R8) as well as folic acid targeting ligands in order to improve the efficiency of gene delivery into rat glioma cells (C6 cells) [7]. Another example for the successful application of CPPs for polymeric nanoparticle delivery is the work of Jabbari et al., who exploited the hydrophilic properties of the V6K2 peptide (VVVVVVKK), which was conjugated to low-molecular-weight polylactide, in order to facilitate encapsulation of doxorubicin and paclitaxel as well as cellular uptake by 4T1 murine breast carcinoma cells [104]. Similarly to the other introduced nanosized materials, which have been described in this review, not only the surface modification but also the shape and size of polymeric nanoparticles can impact the cellular internalization. Zhang et al. investigated Tat modified spherical micelles (11 nm diameter) as well as short (20 nm diameter, 180 nm length) and long (30 nm diameter, 970 nm length) cylindrical micelles, which consisted of poly(acrylic acid)-polystyrol block copolymers, in order to underpin this statement [105]. They were able to demonstrate that the internalization efficiency of spherical micelles into cells is dependent on the CPP loading. The Tat-functionalization of short as well as long cylindrical micelles, on the other hand, did not improve the uptake efficiency. This effect was observed for low as well as high peptide loading and can only be attributed to the differences in size and shape.

Conjugation approaches

The advantages of CPP modified nanoparticles in regard to biological compatibility, targeting and cellular uptake of different cargo have been pointed out in previous sections. However, since the amount of bound cell penetrating peptides can affect the internalization properties and cellular distribution of the cargo, making conjugation efficiency an important factor in carrier design, it is also
important to give insight into the existing functionalization methods of nanoparticles.

The surface modification of gold nanoparticles is largely based on the work of Whitesides and Nuzzo [106, 107], who investigated the formation of self-assembled monolayers of molecules on planar gold. Bioactive molecules, such as CPPs, can be conjugated to the surface of gold nanoparticles by means of various passivating agents and linkers. The multifunctional passivating agents possess conjugation chemistry-facilitating functional groups as well as anchoring groups, such as thiols [108], dithiols [109], dithiocarbamates [110], amines [92], phosphines [111], carboxylates [92], and isothiocyanates [92], which enable the said molecules to coat the surface of gold nanoparticles. The choice of the anchor functionality depends on the desired stability of the established bond between the gold nanoparticle and conjugated molecule. The weak interaction between amine or carboxylate anchor groups and the gold surface, for example, leads to continuous release of the cargo, which is undeniably advantageous for gold nanoparticle mediated drug delivery [112]. Thiol-based anchors, on the other hand, ensure stability and even cysteine terminated peptides were successfully used in order to coat gold nanoparticles [98, 113]. Thioloates, which do not suffer from oxidative desorption in the same manner as dithiols, remain adsorbed to the gold surface for 35 days at physiological conditions and are therefore most commonly applied for the attachment of CPPs [114]. While the hydrophobic entrapment and

![Figure 2](image-url)

Figure 2: Functionalization of gold nanoparticles by (A) covalent coupling of bioactive molecules using carbodiimide, maleimide or click chemistry, (B) electrostatic adsorption and (C) direct functionalization of ligand-free gold nanoparticles.
charge-pairing (Figure 2B) play only a minor role for the attachment of CPPs to gold nanoparticles, classic coupling reagents, such as 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) or N-Hydroxysuccinimide (NHS) esters, on the other hand, are a useful tool in order to establish the desired stable chemical bond [15, 97, 100]. The same can be achieved by means of Michael addition or click-chemistry (Figure 2A). Peptides are commonly

![Diagram of quantum dot functionalization](image)

**Figure 3:** Functionalization of quantum dots (A) Composition of a functionalized QD (B) Partial and complete ligand exchange (C) Silanization.
conjugated to gold nanoparticles in this fashion and a variety of commercially available heterobifunctional polymers, as well as linkers with terminal maleimides, N-hydroxysuccinimide activated carboxylic groups or azide functional groups are available for the functionalization of gold nanoparticles with CPPs [115–117]. In some cases it is also possible to utilize bovine serum albumin (BSA) as the reducing agent in order to synthesize BSA-coated gold nanoparticles, which can be used as a scaffold for the attachment of CPPs [37, 118].

Quantum dots (QD) possess different physicochemical properties than the noble metal nanoparticles and therefore require an alternative surface conjugation approach. Several methods have been developed for the design of water-soluble QDs by using polymers, thiols (Figure 3B) and silanization (Figure 3C) [119, 120]. Such coatings are not only required to improve the water solubility but also to equip the QD with functional groups, which in turn are needed for further modification and conjugation chemistry (Figure 3B). Tiopronin coated CdS QDs, for example, were functionalized with Tat peptide by using a ligand exchange method (Figure 3A) in order to achieve nuclear targeting in hTERT-BJ1 human fibroblasts [13]. Similar coating approaches have been used by Wei et al., by functionalizing CdSe-ZnS QDs with Tat for cell staining. However, the authors utilized not only the ligand exchange method but also the covalent conjugation of the cell-penetrating peptide to silan or polyacrylate coated QDs. The different coating approaches that were employed led to Tat-functionalized QDs with different sizes (6–25 nm in diameter) and surface charge (+8 to +35 mV), which in turn influenced the cellular uptake and subcellular targeting specificity. The particles with a diameter of 6 nm and a surface charge of +8 mV were taken up poorly but mainly showed perinuclear localization. Increasing their size and surface charge improved cellular uptake while diminishing their targeting specificity. Wei et al. were also able to demonstrate that nanoparticles with a diameter exceeding 13 nm were primarily taken up by endosomal pathways [121]. Another efficient conjugation technique was employed by Lagerholm et al., who utilized biotinylated oligoarginine in order to equip commercially available streptavidin-coated QDs with cell-penetrating properties for multicolour staining of mammalian cells [embryonic mouse fibroblasts (Swiss 3T3), human endothelial cells (HeLa), and human osteoblast-like cells (MG63)]. These modified QDs, however, were reported to be internalized solely by endocytosis [8]. The interaction of biotin and streptavidin was also exploited by Mok et al. for the preparation of PEGylated QDs, which could be specifically dePEGylated in the presence of the matrix metalloprotease-2 enzyme for targeted cellular uptake [26]. This effect was achieved due to the immobilization of biotin-PEG conjugates onto streptavidin-coated QDs by utilizing peptide-linkers, which were a substrate for the peptidase. In addition, the biotinylated CPP Hph-1 (YARVRRGPRR) was immobilized onto the surface of the PEGylated QD as well, in order to enhance the cellular uptake after dePEGylation. The introduced variety of modification methods for quantum dots and noble metal nanoparticles provide a basic set of necessary possibilities to optimize the conjugation efficiency of CPPs onto nanoparticles, which is crucial for customizing not only cellular distribution, but also uptake efficiency.

Conclusions

In this review we discussed the uptake mechanisms and the viability of CPPs as mediators for organelle specific delivery of nanoparticles, pointing out the possibilities and strategies of their successful utilization. Such functionalization of carrier-systems with CPPs, however, has become a controversial topic. Ever since their discovery, CPPs have been investigated as versatile components for drug delivery systems and although a large number of studies claim nearly unrestricted cellular internalization, researchers face the hurdles of CPP-mediated cellular access, which are not only restricted to metabolic degradation. The properties of the cargo as well as the varying composition of the membrane of cell lines and cellular differentiation can significantly influence the efficiency of cellular uptake. When considering these findings, it is unrealistic to expect that all CPP-conjugated cargo can be delivered across every type of membrane just because of their bold name. Here, we have shown the various aspects that need to be considered for the successful implementation of CPPs for organelle-specific delivery of nanoparticles into cells. We believe that a methodical approach to their study as well as guidelines for their selection, which consider all aspects of CPP-mediated delivery, need to be introduced in the future. In spite of the mentioned obstacles, the wealth of inspiring ideas and the long list of successful applications of CPPs has given rise to an ever-growing field of studies with more than 1150 scientific publications just in 2014, which corresponds to a rise of 32% in comparison to the number registered in 2009 and the trend is still rising. Therefore, the modification of cargo with CPPs will remain an effective strategy to improve the efficiency of the transport across plasma membranes, especially considering their organelle-targeting properties.
References


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