Quarterly Reviews of Biophysics

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Studies of cell-penetrating peptides by biophysical methods

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Review

Cite this article: Zorko M, Langel Ü (2022). Studies of cell-penetrating peptides by biophysical methods. *Quarterly Reviews of Biophysics* **55**, e3, 1–15. https://doi.org/10.1017/S0033583522000026

Received: 3 December 2021
Revised: 11 March 2022
Accepted: 15 March 2022

Key words:
Biophysical methods; cell-penetrating peptides; circular dichroism; electron microscopy; fluorescence; mass spectrometry; nuclear magnetic resonance; radioactivity

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Abstract

Biophysical studies have a very high impact on the understanding of internalization, molecular mechanisms, interactions, and localization of CPPs and CPP/cargo conjugates in live cells or *in vivo*. Biophysical studies are often first carried out in test-tube set-ups or *in vitro*, leading to the complicated *in vivo* systems. This review describes recent studies of CPP internalization, mechanisms, and localization. The multiple methods in these studies reveal different novel and important aspects and define the rules for CPP mechanisms, hopefully leading to their improved applicability to novel and safe therapies.

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Introduction

CPPs are potent carriers of a variety of biological cargoes into different cell types and also promising drug delivery systems (Langel, 2019). In this respect, they were intensively studied, in the last years, particularly for introducing anticancer molecules into tumors (Zorko *et al.*, 2022). Dozens of peptide-based drugs have been approved for clinical use (Zhang and Eiden, 2019), but among them, CPPs could not be found. Although CPPs have been known for more than 30 years, they have not yet entered clinical practice. Altogether, around 30 CPPs are involved in clinical trials, but only a few have entered Phase III; for oncological treatment, the study of which is very intensive in the last years, only one has reached Phase I clinical trials, and no CPP or CPP-drug construct has been approved by FDA for any disease so far (Falanga *et al.*, 2020). In this review, we present recent research summarizing studies of the composition of CPPs and their impact for carrying various cargoes into cells by biophysical methods, such as the use of fluorescence-based methods including confocal microscopy and flow cytometry, model membranes, circular dichroism (CD), nuclear magnetic resonance (NMR), and mass spectrometry (MS). The truth is that almost all methods used today in life sciences incorporate some degree of physical approach.

CPP

CPPs are short peptides of up to 40 amino acids. They can enter the cell and promote intracellular effects by themselves or by the delivery of bioactive cargoes. This definition is

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practically identical to its first appearance in the first book on CPPs (Langel, 2002) and many papers on CPPs about 20 years ago (cf. Zorko and Langel, 2005). Many different CPPs were found in the last 30 years. According to their structures and properties, the only real common feature of CPPs seems to be their peptide nature and their ability to internalize into cells together with the cargo that is otherwise not able to reach the cell interior. The name cell-penetrating peptide and its abbreviation CPP appeared in the scientific literature first in 1998 (Pooga *et al.*, 1998) and was soon generally accepted. In the literature, we can find other names for CPP, like membrane transduction peptide, Trojan horse peptide, and some others. CPPs generated from protein sequences are frequently named protein transduction domains, and peptides that interfere with processes in the cell interior by themselves are known as bioportides.

Horseradish peroxidase was the first functional protein to be carried into the cell in 1987 by using the trans-activator of transcription (Tat-protein) of the human AIDS virus (Frankel and Pabo, 1988). It was shown that for the transfer of the cargo into the cell, only 11 amino acids long domain of Tat-protein is needed (Green *et al.*, 1989), and the first CPP was born. From that time the number of CPPs increased enormously. The database of CPPs (https://webs.iiitd.edu.in/raghava/cppsite/) now (November 2021) contains almost 2000 entries with the following information: peptide sequence, nature of peptide, chemical modifications, experimental validation technique, structure of peptide, and type of cargo delivered. Analysis of the entries in this database revealed the following (Kalafatovic and Giralt, 2017): out of 1855 CPPs in the database, 95% of CPPs are linear and the rest 5% is cyclic; majority of the delivered cargo represent different fluorophores (54%), 15% nucleic acids, 9% proteins, 8% biotin, 7% nanoparticles, 4% peptides, and 3% other structures; majority of CPPs are composed of only standard L-amino acids (85%), 11% of CPPs consist of modified amino acids, 3% of D-amino acids only, and 2% are mixed.

Classification

Traditionally, CPPs are classified based on their physicochemical properties, origin of peptides, or other characteristic features. The physicochemical properties of CPPs arise from their basic structure. In this respect, CPPs are usually divided into three groups: cationic, amphipathic, and hydrophobic. Being composed of mostly positively charged amino acids, mainly Arg (R) and Lys (K), cationic CPPs are positively charged at physiological pH. Examples of cationic CPPs are Tat (GRKKRRQRRRPQ, Green *et al.*, 1989), and poly-arginine $(Arg)_{8-10}$ (cf. Arg_8 or R8 RRRRRRRR, Futaki, 2006). Amphipathic CPPs are composed of a combination of hydrophilic and hydrophobic amino acids where amphipathicity is due to the sequence or is achieved after folding of the CPP into an α-helix with a hydrophilic and a hydrophobic face: an example is MAP (KLALKLALKALKAALKLA, Oehlke *et al.*, 1998). Hydrophobic CPPs are made of hydrophobic amino acids but some hydrophilic amino acids must also be included, otherwise, they would be insoluble in body fluids and they would also be retained in the hydrophobic core of the lipid double-layer when passing through the cell membrane. An example is Pep-7, a synthetic hydrophobic α-helix (SDLWEMMMVSLACQY, Gao *et al.*, 2002). It should be mentioned that some anionic peptides were also shown to be able to penetrate into cells (cf. Neves-Coelho *et al.*, 2017).

According to the origin, CPPs are usually also divided into three groups: protein derived, chimeric, and synthetic. Protein-derived CPPs represent a part of the natural protein structure that can be transferred through the cell membrane. The oldest example is Tat. Chimeric CPPs have two different origins, a good example is transportan (abbreviated TP, GWTLNSAGYLLGKINLKALAKISIL, Pooga *et al.*, 1998), which is constructed from sequences of galanin and mastoparan. Synthetic CPPs are artificially designed, an example is MAP (see the structure above).

Another classification level, in particular, is clinically important. In this classification, CPPs are divided into cell-specific and non-cell-specific peptides. Cell-specific CPPs are able to deliver cargo into the specific cell, while non-cell-specific are not selective.

Because of the great number of very different CPPs, all levels of CPP classification are very broad and usually give overlapping results. To avoid this and to classify CPPs more precisely, taking into account as many properties as possible, a new approach was adopted. Classes of CPPs were grouped in pairs accordingly, to cover eight levels of classification. In each level, two classes of CPPs with the opposite properties were presented. This approach was recently published (Langel, 2019) and is not further elaborated here.

Other classifications of CPPs are also available. An interesting taxonomy of CPPs, based on the mechanisms of entry into cells is given by Berkeley Kauffman *et al.* (2015).

Cell internalization

There are two main routes of CPP with or without cargo by which they can be transferred into the cells and organelles: direct penetration or one of several endocytic mechanisms. However, detailed mechanisms of CPP internalization are still not clear. It seems that direct internalization is related to the destabilization of the membrane, which is particularly true for cationic CPPs that include lysine or arginine in the structure, for instance, poly-arginines (Futaki and Nakase, 2017; Murayama *et al.*, 2017). Destabilization of the cell membrane can be initiated by the accumulation of positively charged CPPs on a negatively charged membrane surface, by attracting water molecules that bind to charged amino acids of CPPs into the hydrophobic core of lipid bilayers (Grasso *et al.*, 2018), or by inducing transfer pores in the membrane (Islam *et al.*, 2018). Membrane curvature and the depletion of the amount of membrane cholesterol could further destabilize the lipid bilayer and help peptides to traverse the membrane. For penetratin, many years ago Prochiantz (1996) proposed that spontaneous direct translocation of the membrane proceeds by the formation of an inverted micelle. Later, Kawamoto *et al.* (2011) suggested that this mechanism could be applied by several cationic CPPs.

To enter into cells with or without cargo, most CPPs employ different mechanisms of endocytosis, including clathrin- and caveolin-mediated endocytosis, other receptor-dependent endocytosis, macropinocytosis, and possibly other endocytic routes (Doherty and McMahon, 2009). It seems that the most important route of internalization is micropinocytosis, particularly for cancer tissue (Yoo *et al.*, 2020). In all these mechanisms, active target cells and energy are required. In endocytic pathways, CPPs (and cargo) almost always end up in the endosomal vesicles which further mature into the lysosomes where they are usually degraded. Therefore, fast endosomal escape is a prerequisite for the effective delivery of cargo into the cell cytoplasm and in other cell organelles. Different strategies were developed to enhance the

https://doi.org/10.1017/S0033583522000026 Published online by Cambridge University Press

endosomal escape of CPP and cargo to achieve more effective delivery. A review of these strategies was recently published (Pei and Buyanova, 2019) but the use of some biophysical methods for the detection of endosomal escape is covered also in this review. It should be stressed here that in the delivery of cargo, many CPPs seem to be able to use both direct and endocytic routes of internalization. It was suggested for instance that Tat and penetratin can cross the membrane passively only at low concentrations while at higher concentrations, they use both direct and endocytic routes (Walrant *et al.*, 2017). It is interesting, that another group has obtained an entirely opposite result (Verdurmen *et al.*, 2017). Concerning the mechanism of CPPs, the details of this feature are not very well clarified.

CPP coupling to cargo molecules

Cargo molecules or complexes should enter cells in order to act effectively. Internalization of drugs, other molecules, and nanoparticles can be highly improved by coupling to CPP.

Cell-penetrating sequences in protein structures

Concerning proteins as molecules that should enter the cell, some can penetrate cells by themselves and are also called shuttling proteins. In some cases, cell penetration of proteins can be induced or enhanced by incorporating sequences that promote cell penetration into the structure of the protein. In addition to Tat protein, several other proteins with transducing capabilities have been identified, for instance, ANTP (Joliot *et al.*, 1991), HoxB4 (Amsellem *et al.*, 2003), the herpes simplex virus type 1 VP22 transcription factor (Elliott and O'Hare, 1997; Schwartze and Dowdy, 2000), OCT 4 acting as self-penetrating pluripotency reprogramming factor (Harreither *et al.*, 2014), homeodomain transcription factor engrailed-2, En-2 (Brunet *et al.*, 2005), Engrailed 1, EN1 (Beltran *et al.*, 2014), Knotted-1 (Tassetto *et al.*, 2005), Otx2 (Bernard and Prochiantz, 2016), Pax4 involved in vertebrate organogenesis (Lu *et al.*, 2007), the protein NeuroD/BETA2 (Chen *et al.*, 2006), protein-ligand complex neocarzinostatin, NCS (Moody *et al.*, 2013), omomycmini-protein, a Myc inhibitor (Massó-Valés and Soucek, 2020), human papillomavirus minor capsid protein L2 (Xie *et al.*, 2020*a*, 2020*b*), etc. Finding CPP sequences can be an issue of a reasonable guess according to the structure of known CPPs, or it can be generated by computer learning methods (Hälbrink *et al.*, 2005; de Oliviera *et al.*, 2021; Rives *et al.*, 2021). Successful incorporation of CPP sequence into proteins to promote effective cell penetration has been achieved in some cases. It is important not to disturb the structure and function of proteins by introducing cell penetrating sequences. Therefore, it is a usual practice to include cell penetrating sequences into the open loops (Chen and Pei, 2020) of proteins or as flanking CPP sequences at the C-terminal or N-terminal part of the proteins. When incorporated into protein loops, CPP sequences can induce cell penetration of proteins more effectively if they form cyclic structures. Genetic engineering is of great help to express such a construct in one step. In most cases, however, molecules that should act in the cells are coupled to CPP.

Approaches to couple cargo molecules to CPPs

Cargoes can be conjugated to CPPs in different ways, but essentially by covalent coupling or by forming a complex, using multiple weak interactions (Borrelli *et al.*, 2018). When covalent attachment of cargo to CPP is employed, cargo can be attached directly to CPP or via the suitable spacer where cargo is tethered to CPP through a cleavable bond, such as hydrazone or disulfide (El-Andaloussi *et al.*, 2007). It has long been known that cargo usually decreases the rate and efficiency of translocation which is dependent on the nature of the cargo and its dimension (Via *et al.*, 2020). However, in some cases, it was observed that penetration efficiency can be increased if CPP is designed to self-assemble into spherical micelles, thus locally increasing its local interface density (Weinberger *et al.*, 2017) or when the flexible spacer is replaced by a constrained cyclic spacer (Bhosle and Fernandes, 2017). The spacer can also contribute to the selectivity of penetration of the cargo in specific, for instance, cancer, tissue (Shi *et al.*, 2017*a*, 2017*b*). Selectivity or targeting is discussed in the next section in more detail. Oligonucleotides can also be covalently attached to CPP, either to the 3' or 5' site via a suitable linker, which can be cleavable or not – a recent review of these attachments of siRNA is available (Tai, 2019). When cargo is non-covalently conjugated to CPP, the conjugation is governed by multiple electrostatic and hydrophobic interactions between CPP and cargo. An interesting example is to couple modified negatively charged leucine zipper to CPP and modified positively charged leucine zipper to a cargo molecule, or vice versa; zippers can electrostatically bind to each other thus coupling CPP to the cargo (Kitamatsu *et al.*, 2021). Non-covalent coupling of CPP to the cargo usually results in the formation of complex nanoparticles – for review see McClorey and Banerjee (2018).

Recently, there has been extensive research aimed toward forming liposomes and nanoparticles as delivery vehicles, decorated with CPPs for enhanced penetration into the cell. Several reviews on this topic were published (cf. Shi *et al.*, 2017*a*, 2017*b*; Tayo, 2017; Gessner and Neundorf, 2020; Chiarpotti *et al.*, 2021). Although these constructs and CPPs, in general, have not been accepted for clinical use, their potential clinical application is under intensive testing and discussion. Three most recent reviews are enlisted here (Gomes Dos Reis and Traini, 2020; Xie *et al.*, 2020*a*, 2020*b*; Kurrikoff *et al.*, 2021). We briefly discuss the use of fluorescence and some other biophysical methods in characterizing these structures in the next sections. Finally, an important question after cargo delivery is where the cargo is really situated, particularly in which cell compartment can it be found. A number of methods have been developed to localize cargo in the tissue and cell. A recent review of these methods has been published by Xie *et al.* (2020*a*, 2020*b*).

Biophysical methods and CPPs

Fluorescence

Fluorescence is an extensively used approach to detect and explore CPPs. For this review, we have studied over 1200 papers dealing with CPPs that were published from 2013 to the present time and found over 450 papers in which fluorescence is the method of identification of CPPs. In the following, cell internalization of these peptides is discussed. Several review articles are available dealing with CPPs and fluorescence, we cite here only selected recent papers (Boisquérin *et al.*, 2015; Uusna *et al.*, 2015; Barba-Bon *et al.*, 2019; Seisel *et al.*, 2019; Deprey and Kritzer, 2020; Knox *et al.*, 2020; Sakamoto *et al.*, 2020).

To apply fluorescence methods to CPPs, they should be conjugated to suitable fluorophore molecules. This means that with

https://doi.org/10.1017/S0033583522000026 Published online by Cambridge University Press

fluorescence, we always deal with CPP-cargo constructs. Fluorophore moiety is usually coupled to CPP by a covalent bond (see above). As it is well known, cargo can influence the behavior of CPP in many ways, particularly when it is large – some examples are given in section 'Approaches to couple cargo molecules to CPP'. Fluorescence methods most often used are: fluorescence spectroscopy, fluorescence correlation spectroscopy (FCS), fluorescence microscopy, fluorescence confocal microscopy and confocal laser scanning microscopy, fluorescence-activated cell sorting (FACS), and, of course, fluorescent imaging. In all fluorescence methods, cells, vesicles, or other target structures are first incubated with CPP coupled to a fluorophore to allow its penetration into the cell. After a suitable time interval, the fluorophore in the target structure is excited by the absorption of light. When the fluorophore returns to the ground state, it emits light that is detected by the instrument. Each fluorophore emits light of a specific frequency. The intensity of the emitted light depends on the intensity of the excitation light and the efficiency of the fluorophore, depending on the molar extinction coefficient and quantum yield. However, light emission of the fluorophore can be modified by the fluorophore neighborhood and from these data, the interaction of the fluorophore with other molecules like proteins, peptides, or lipids can be deduced. In fluorescence spectroscopy, the bulk emitted light can be measured by a spectrofluorometer which is frequently equipped with a microplate reader to make scanning of the great number of samples faster. Pure fluorescence spectroscopy is today rarely used. By using a fluorescence microscope, the fluorescence is detected as a function of two or three-dimensional space, resolving in this way fluorescent objects in the target structure. A flow cytometer is used for the analysis of the fluorophore incorporated in the individual target cell when it flows in the liquid stream. All fluorescence-based methods have many variants and subclasses. It is a usual practice to combine these methods within fluorescence domain methods and also with other methods which do not exploit fluorescence. It seems that the best way to detect the internalization of the CPP-cargo construct into the cells and also into its target part (organelle) is the confirmation of the successful delivery of cargo by detecting the proper physiological effect of the cargo in the cell. Another approach is the co-localization of the emitted fluorescence light of the CPP delivered cargo with the light emitted or absorbed by specific target (molecule, organelle, cell type, tissue, or organ) markers. Also, potential cytotoxicity as a function of the construct concentration is checked for the safe delivery by CPP.

Due to the great number of studies that use fluorescence as a principal CPP detection method, only selected examples are presented, as follows. Some of the examples are gathered in Table 1 to enable a concise overview of these methods. The examples presented in the table do not overlap with those shown in the text.

Using fluorescence microscopy and flow cytometry, the precursor of interleukin 1 alpha equipped with nuclear localization sequence (NLS) and coupled to green fluorescence protein (GFP) was shown to have CPP abilities. It penetrates the nucleus of the Jurkat and HeLa cells delivering GFP. It was also shown that it is able to deliver proteins into the cells of different mice organs (spleen, liver, intestine) after intraperitoneal administration (Koo *et al.*, 2014). No cytotoxicity was observed even at 100 μM concentration of the construct. Using the same combination of fluorescence methods (Wu and Gehring, 2014) and applying low temperature, it was shown that complete Antennapedia homeodomain protein can preferentially enter the cells via macropinocytosis. By using a combination of fluorescence methods, He *et al.* (2013) first identified a series of novel CPPs which were selected using synthetic membranes and termed spontaneous membrane-translocating peptides (SMPTs). These peptides were able to deliver tetramethylrodamine (TAMRA) and Alexa Fluor 546 dye into the cytoplasm of different cells under conditions in which endocytosis was prevented. When injected into mice, SMTPs delivered TAMRA in many tissues where it could be detected even after 2 h after administration while SMTP unconjugated TAMRA was rapidly cleared. They speculated that SMTPs could deliver many other polar compounds into cells. Although the mechanism of internalization of the CPP prototype TAT, which was first discovered CPP, is not clarified, it is still intensively studied. Additionally, TAT serves as a model and reference CPP, and its activity as a CPP is frequently correlated with the activity of other CPPs (cf. Kim *et al.*, 2015). The cell-uptake of fluorescein isothiocyanate (FAM) labelled TAT was explored by various fluorescence methods, including fluorescence spectroscopy, flow cytometry, and confocal microscopy (Ben-Dov and Korenstein, 2015). It was found that the TAT-FAM construct can enter cells independently of endocytosis by dual entry routes with different energy dependences. CPPs like TAT are intrinsically cationic peptides composed of a large number of positively charged amino acids and are prone to interact with negatively charged biological structures. There is a need for less cationic CPPs to prolong their blood circulation time and availability. This problem was addressed by the use of membrane interacting proteins as the source of the potential membrane transferring peptides. It was found that from the sequence of annexin, a number of hydrophobic CPPs can be derived (Kim *et al.*, 2015) which were better cargo-delivery agents than the CPP prototype TAT, as shown by fluorescence flow cytometry and other methods. These peptides were very moderately cytotoxic, very stable in the serum, and showed no immunogenicity. An interesting method for CPP and cargo cell internalization was derived (Schmidt *et al.*, 2015). They first transfected cells with a plasmid encoding the non-fluorescent part of GFP (GFP1-10) and used a missing part of GFP as cargo conjugated to CPP. After delivery, a missing part of GFP by CPP in the cells, both parts of GFP fused, and the fluorescence of GFP was restored. They further improved the sensitivity of the method by modification of the peptide and more effective expression. A Japanese group has found a new class of CPP, polyhistidines (PHPs), that was inspired by the fact that many CPPs are cationic (Iwasaki *et al.*, 2015). PHPs were effective CPPs; uptake was improved with the increasing number of His residues up to 16. This most effective CPP was called H16. Cell transduction and the ability to carry cargoes into the cell were studied by attaching GFP to the peptide, obtaining a GFP-H16 construct. By confocal microscopy, it was shown that the construct accumulated in the human fibrosarcoma cells and is retained in fibrosarcoma tumors for more than 5 days. A new CPP was detected and purified from silkworm hemolymph (Park *et al.*, 2012), being thus the first CPP of insect origin. It was called 30Kc19 and was later produced from recombinant *Escherichia coli*. Fused with GFP and using immunofluorescence microscopy, it was shown that peptide can deliver cargoes *in vitro* and *in vivo* and that it is not toxic. A problem of quantitative analysis of CPP uptake into cells is a serious problem. Combining FACS with FCS overcomes this problem, enabling precise quantification of internalized CPP-cargo constructs (Rezgui *et al.*, 2016). It is carried out in three steps. First, cells are incubated with a CPP-cargo construct, then cells are sorted according to CPP uptake by FACS (Bonner *et al.*, 1972), and finally,

https://doi.org/10.1017/S0033583522000026 Published online by Cambridge University Press

Table 1. Selected examples of use of fluorescence methods in CPP studies

CPP – cargo	Method	Achievement	Reference
Pip-peptides bound to morpholino oligomers (PMO)	Fluorescence spectroscopy and microscopy	Construct pass cardiomyocyte membrane by calveolae and clathrin-mediated endocytosis	Lehto *et al.* (2014)
Penetratin (TAT, polyR)-carboxyfluorescein (CF)	Fluorescence microscopy	Penetratin-CF was the most effective and less toxic penetrator to cornea	Liu *et al.* (2014)
stapled CPPs-sMTide-02 or sMTide-02A	Two-hybrid assay (fluorescence microscopy)	Construct enter nucleus and disrupt p53-Mdm2 and p53-Mdm4 interaction	Yurlova *et al.* (2014); Zolghadr *et al.* (2012)
Ypep-GFP and Ypep -nLuc	Life cell fluorescence microscopy	Mutated Ypep effectively and selectively transfers GFP and nLuc into prostatic cancer cells	DePorter *et al.* (2014)
Penetratin-KLA	Life cell fluorescence microscopy	Construct is cytotoxic to various cancer cells through the disruption of mitochondria	Alves *et al.* (2014)
dfTAT – EGFP	Life cell fluorescence microscopy	dfTAT (and cargo) is internalized via endocytosis, but dfTAT effectively disrupt endosomes and deliver cargo in the cytoplasm and nucleus	Erazo-Oliveras *et al.* (2014)
R8 – siRNA or anti-miRNA	Fluorescence quenching and imaging	Single stranded RNA was delivered in the cytosol escaping endosome in glioblastoma cells	Zhang *et al.* (2014)
Different CPPs with single AA modifications-fluorophores, proteins or quantum dots	Life cell fluorescence imaging and flow cytometry	Cargo affects cell delivery; fluorophore Alexa488 was most effective delivered by TP10, albumin by penetratin and quantum dots by octa Arg	Sayers *et al.* (2014)
CPPecp – fluorophores FITC or TMR	Flow cytometry and fluorescence microscopy	CPPecp is effective CPP with strong internalization ability. It is selective to the tumor cells that overexpress heparan	Chen *et al.* (2015)
(KF)3 K – PNA with anti acp gene sequence	Fluorescence microscopy	CPP (KF)3 K delivers anti acp PNA into plant cells and caused the growth inhibition of the plant pathogen bacteria	Patel *et al.* (2017)
CsA-FITC and CsA-PAD-FITC	Flow cytometry and confocal microscopy	CsA is neutral CPP with better delivery of cargo (PAD) potential as TAT	Gao *et al.* (2017)
R9 – AcPHF6	Fluorescence imaging	Amyloid peptide AcPHF6 is carried into the cells; toxic amyloid oligomers kill preferentially cancer cells	Veloria *et al.* (2017)
A2-17 – FAM	Fluorescence spectroscopy and confocal microscopy	A2-17 efficiently delivers cargo in SUV and CHO cells	Ohgita *et al.* (2019)
HL6 – FITC	Fluorescence spectroscopy and flow cytometry	HL6 can be coupled to different cargoes including QD and efficiently deliver cargo in A549 cells	Lee *et al.* (2019)
Dot1 l-different cargoes	Fluorescence spectroscopy and microscopy	Dot1 l can safely deliver different cargoes (nucleic acids, GFP, plasmids) into the cytosol of five cancer cell-lines	Geng *et al.* (2020)
R8 – curcumin	Fluorescence spectroscopy and confocal microscopy	Curcumin kills Gram-positive and Gram-negative bacterial cells. R8-curcumin is more effective than curcumin itself	Ratrey *et al.* (2020)
CPP sequence inserted in 3 proteins (EGFP, PTP1B, PNP)	Flow cytometry, live cell confocal microscopy	Insertion of 7 cationic AA in surface loop of proteins make them membrane permeable	Chen and Pei (2020)

AA, amino acid; A2-17, A2, 20 AA of N-terminal domain of human apolipoprotein E, truncated by 3 AA; A549, human bronchoalveolar carcinoma cells; AcPHF6, amyloid peptide from the sequence of apolipoprotein A1; anti acp PNA, peptide nucleic acid with the antisense sequence to block mRNA of the essential bacterial gene acp; CF, carboxyfluorescein; CHO, Chinese hamster ovary cells; CPPecp, NYRWRCKNQN, heparan sulfate binding CPP; CsA, cyclosporin A, cyclic neutral CPP; Curcumin, polyphenolic natural antimicrobial compound from plant Curcuma longa; dfTAT, tetramethylrhodamine-labeled dimer of TAT; Dot-1 l, 24 AA long CPP sequence from Dot1L protein; EGFP, GFP derived from *Aequorea victoria*; FAM, carboxyfluorescein; FITC, fluorescein isothiocyanate; GFP, green fluorescence protein; HL6, L6, bovine lactoferricin, prolonged by 5 Hys on both termini; (KF)3 K, cationic CPP, KFFKFFKFFK; Mdm-2 and Mdm4, regulatory binding partners of p53; nLuc, NanoLuc luciferase; p53, tumor suppressor; PAD, proapoptotic domain peptide (14 AA); Pip peptides, modified hexaArg-Penetratin conjugate; PNP, purine nucleoside phosphorylase; PTP1B, human protein tyrosine phosphatase 1B; QD, quantum dot; R8, octa Arg; R9, nona Arg; sMTide-02 and sMTide-02A, stapled CPPs; SUV, small unilamellar vesicles; TAT, CPP, GRKKRRQRRRPQ; TMR, tetramethylrhodamine; TP10, transportan truncated by 6 AA; Ypep, CPP, YTLGFKTSFNVQ.

cells are lysed and the internalized peptide is quantified by FCS. Before the experiment, CPPs must be labelled with a suitable label (authors used penetratin with some modifications and fluoroAlexa Fluor 488-succinimidylester for labelling). According to the fluorescence signal, FACS collects only intact cells with internalized constructs. In the cell lysate, a precise number of fluorescently labelled molecules in the sampling volume can be determined by FCS, thus measuring intracellular fluorophore concentrations in the nanomolar range. As claimed by the authors, 'The FACS-FCS approach provides a means for quantifying any

https://doi.org/10.1017/S0033583522000026 Published online by Cambridge University Press

intracellular biochemical entity, whether expressed in the cell or introduced exogenously and transported across the plasma membrane'. A French group has analyzed cell uptake of CPP-fluorescein conjugate by two methods, fluorescence spectroscopy and flow cytometry, and they correlated the obtained results with MS (MALDI-TOF) optimized internalization analysis (Illien *et al.*, 2016). They checked the ability of TAT, penetratin, and R9 CPPs to carry fluorescein into the Chinese Hamster Ovary CHO-K1 cells and obtained quantitatively and qualitatively consistent results with fluorescence spectrometry using cell lysate and MS. On the other hand, flow cytometry was less reliable due to several limitations because of fluorescence quenching; this was particularly true for the construct penetratin-fluorescein. By using doubly labelled fluorescent and biotinylated penetratin analogues, more consistent results were obtained. Fluorescence was used also to determine the role of chirality of amino acids in TAT dimer in the delivery of fluorescent cargo (tetramethylrhodamine) into HeLa, MCH58, and HDF cells (Najjar *et al.*, 2016). It was shown that all D-peptides were more stable and were detected in the cytosol for several days, but all L-peptides were no more present in the cell after several hours. The ability to deliver cargo into the cell was approximately the same with both enantiomers, but a more precise observation revealed that the L-peptide was better in penetrating the cell but was less prone to escape endosomes after being internalized by the endocytic process. For the use of fluorescence methods, CPPs should be labelled with a fluorophore. It has been shown that fluorophore attachment to CPP can induce different effects on the cellular distribution of CPP (with cargo) and affects cell viability (Birch *et al.*, 2017). It has been suggested that the selection of fluorophore can be critical for the results of delivery experiments. Birch *et al.* tested seven different fluorophores of various structures with penetratin and were able to draw the following general conclusions: fluorophore-penetratin conjugates exert strong structure-dependent reduction in viability of several mammalian cell types as compared to non-labelled penetratin; effects on membrane integrity, as well as intracellular distribution patterns, differed among the conjugates; neutral hydrophobic fluorophores or negatively charged fluorophores conferred less cytotoxicity as compared to the effect exerted by positively charged, hydrophobic fluorophores. To improve the detection of cell uptake of CPPs in the cytosol, two β-lactamase fluorogenic assays were developed (Ston *et al.*, 2018). In one assay, a complete functional enzyme β-lactamase is delivered into cells by CPP. Then cells are incubated with dye CCF2-AM, which rapidly enters the cells, and when in the cytosol, it is converted to CCF2 by endogenous esterases. CCF2 is a negatively charged fluorescence resonance energy transfer (FRET) incorporating substrate of β-lactamase and is retained in the cytosol. If this enzyme is delivered to the cytosol by CPP, it cleaves FRET. This results in the change of fluorescence from green to blue and the presence of β-lactamase in the cytosol is confirmed. The second assay is the variant of the first one based on the splitting of β-lactamase into two non-functional components. One component is expressed in the cell and another is carried into the cell by CPP. Both components are fused in the cell cytosol and the enzyme becomes functional.

Entrance of viruses into cells is mediated by their capsid proteins. Therefore, virus capsid proteins are potential sources of new CPPs. It was observed (Yu *et al.*, 2018) that porcine circovirus type 2 has a single capsid protein Cap, which contains a nuclear localization signal sequence (NLS) incorporating also several positively charged amino acid residues. This makes this sequence a potential CPP. NLS of the virus was synthesized and was named NLS-A. Its ability to carry cargo into cells was tested using flow cytometry and confocal laser scanning microscopy. For this purpose, NLS-A-EGFP and NLS-A-FITC constructs were developed. As it was shown, NLS-A was rapidly transferred into cells via direct translocation of the peptide. That occurred after the accumulation of NLS-A on the cell membrane surface what resulted in the increased membrane permeability. After entering the cell, NLS-A was found to be evenly present in all components of the cell.

As explained in many review articles on CPPs, first, it was thought that CPPs can enter unselectively in all types of cells and all tissues. As drug delivery vehicles, it is desired for CPPs to be selective and to deliver cargo into specific types of cells or in specific organelles, particularly when they are designed to carry anticancer drugs into tumor cells (Zorko *et al.*, 2022). Here we present a design of CPPs targeting the cell nucleus and subnuclear region, as confirmed by confocal scanning system and flow cytometry (Gronewold *et al.*, 2016). Peptide N50 with the NLS and NrTP, a peptide with a nuclear targeting sequence, were coupled to the shortened CPP sC18, obtaining thus two novel peptides N50-sC18 and NrTP-sC18. It was shown that these two peptides function as efficient vectors for different cargo delivery into the nucleus and nucleoli. No toxicity of these peptides up to 100 μM concentration was observed. As it was shown, muscle ischemia can arise from mutations in the genes encoding dystrophin protein, possibly leading to different types of muscular dystrophy. In particular, mutations in the region that encodes dystrophin binding domains for nitric oxide synthase (nNOS) promote loss of nNOS from the sarcolemma, resulting in non-functional muscles. Some efforts were undertaken to reverse this pathological state of muscle by use of CPP and by control via fluorescence methods (Zhao *et al.*, 2019). They first identified the dystrophin sequence responsible for binding to nNOS and then constructed a peptide with this sequence (called R16/17) fused to GFP for fluorescence detection and different CPPs for cell internalization. Out of several CPPs tested, TAT showed the best internalization abilities. Construct TAT-R16/17-GFP successfully entered impaired muscle cells, partly restored muscle force, and improved muscle perfusion during contraction. Many times, in fighting diseases, this can be achieved by introducing antibodies into the cell or even into the specific cell compartment. This can be done by binding the antibody to CPP. However, to retain antibody functionality, it is important to know where the transporting peptide is attached to the antibody. This was first explored by genetically fusing five selected CPPs to different positions of IgG in order to transport IgG into the cytosol of the target cancer cells. Authors selected six positions in the structure of IgG for the attachment of CPP: N and C terminals of the light chain, N and C terminals of the heavy chain, and two positions inside the heavy chain, one before the -SS- bridges in the hinge and the other after the -SS- bridges. To report the exact position of the internalized antibody in the cell, GFP was attached to CPP in the IgG-CPP construct. It was shown that two CPPs, Pep-1 (Morris *et al.*, 2001) and PEPth (Arrouss *et al.*, 2013) attached into the heavy chain of the antibody before the hinge was able to transfer functional IgG into the cytosol of three types of colon cancer cells in concentrations around 10% of that outside of the cells. Recently, CPP promoted the delivery of antibodies into cells. This was also studied by other groups that monitored the process by fluorescence. Sauter *et al.* (2020) attached eight different tetrameric variants of CPP to the after-hinge part of the heavy chain of a monoclonal antibody,

https://doi.org/10.1017/S0033583522000026 Published online by Cambridge University Press

out of which TAT and penetratin were very effective transfer vehicles. Another study of the antibody-CPP construct was provided by Chong *et al.* (2021) who non-covalently bound IgG (against NF-κB) with domain Z (affibody to bind antibodies) decorated with dimeric or tetrameric LK-1 CPP (LK-1 is cationic CPP composed of 16 amino acids out of which 14 represent Arg and Lys). These constructs entered the cells using ATP-dependent endocytosis. Anti-NF-κB IgG was functional in the cytosol which was accessed after internalization and successful escape from endosomes. They also provided a cleavable link that released antibodies from CPP after reaching the cytosol. Finally, we present here a study of Jakka *et al.* (2019) in which they showed that a very small change of one hydrogen atom in Tyr-39 of GFP for the halogen (they used Cl, Br, or I) can facilitate the membrane transport of GFP in mammalian cells. They also claimed that such a strategy can be used to improve cell penetration of small molecules (including CPPs) into cells. This example is included because GFP is frequently used as a reporter molecule to detect the internalization of different CPP constructs into cells. Proteins can be made membrane-permeable also by incorporating a CPP sequence into their open loops (see Table 1 and section 'Cell-penetrating sequences in protein structure').

There is a huge number of different small particles like quantum dots, nanoparticles, co-polymer structures, liposomes, and micelles that are used or tested for drug delivery. Many of these structures are decorated with CPPs to enhance their cell penetration. Composition, size, cell-delivery potential, and other characteristics of these structures are determined with biophysical methods. However, this is a specific topic that deserves a separate presentation and will be here only briefly described. As for fluorescence use, quantum dots behave as fluorophores able to absorb and consequently emit light. It has been shown that by coupling CPPs to quantum dots, these structures can be efficiently carried into cells and can be detected by fluorescence microscopy (Farkhani *et al.*, 2016). Other small particles should be labelled with fluorophores to enable fluorescent detection and imaging. Liposomes are small closed membrane particles in which interior solutions can be loaded with different cargoes. Recently, liposomes were extensively used to deliver parts of DNA, RNA, or other structures into cells. They are able to deliver cargo, for instance, siRNA, by fusing with the cell membrane spontaneously (Alshehri *et al.*, 2018), but are much better cargo deliverers when decorated with CPP. For this purpose, different CPPs can be used, most frequently TAT (cf. Bartomeu Garcia *et al.*, 2017), polyarginines (cf. Wu *et al.*, 2018), and many others. Micelles have a hydrophobic interior and are usually used to deliver non-polar molecules, many times cytostatic. An example is the delivery of the cytostatic paclitaxel (PTX) into model breast cancer cells or organisms (Shuai *et al.*, 2020). Many times, CPP molecules polymerize or form larger spherical structures by non-covalent bonding when they are mixed with cargo molecules, particularly fragments of nucleic acids. These molecules are negatively charged and form electrostatic interactions with cationic CPPs, but other combinations are also known. An example is the roughly spherical complex between pDNA and fluorescein labelled nona-arginine which entered the cell and was internalized into the nucleus, as revealed by fluorescence imaging (Oba *et al.*, 2015). Nanoparticles are not very strictly defined and can represent very different small particles (smaller than 500 nm in diameter, but the definition is not precise). Therefore, some overlapping can be encountered particularly with small complexes described above. In spite of this, the term nanoparticle is widely used. A good example of this is WRAP-siRNA nanoparticles introduced by Deshayes *et al.* (2020). They developed amphipathic CPPs called WRAP that can form a complex with siRNA which they called nanoparticles. WRAP successfully delivered siRNA into the cells of several different cell lines mainly by direct cell membrane translocation directed by membrane destabilization, but also by endocytic pathways. Internalization was observed by fluorescence spectroscopy and microscopy. Golden, silver, and other metallic nanoparticles are also known and they were decorated by CPPs to enhance adsorbed drug delivery (Riveros *et al.*, 2020; Zucker and Boyes, 2020). An overview of nanoparticles behavior in *in vivo* systems is given in Lane *et al.* (2015); Chiarpotti *et al.* (2021); and Gessner and Neundorf (2020).

Radioactivity

Instead of fluorophores, CPPs can be labelled for visualization in cells also with other markers. One approach is radioactive labelling. In a typical experiment, cells are first incubated with labelled CPP. Afterwards, cells are washed with a solution that contains a suitable diluted acid to remove the labelled CPP which is adsorbed on the outer side of the membrane. Then radioactivity is determined by the radioactivity counter and the amount of internalized CPP is calculated. If the incubation of the cells is terminated after different time intervals, also, the kinetics of the CPP internalization can be followed. Different radioactive nuclides have been used for labelling CPPs and are presented in the following examples:

One of the first examples of radioactively labelled CPP is TP labelled with ^{125}I (Pooga *et al.*, 1998). The amount of internalized TP into Bowes cells could be determined as a function of time and in this way also, the kinetics of the labelled TP internalization was followed. A drawback of this method is the inability to discriminate between intact radioactive CPP and its radioactive products after the cleavage in the cell. A more recent example is using ^{125}I to label four cationic CPPs TAT, octa- and nona-arginines, and MAP, and follow their internalization into CHO cells (Zaro *et al.*, 2009). Another group of proteins labelled with ^{125}I involved in the metabolism of arginine can be fused to CPPs to observe the internalization of the construct in HeLa and breast cancer cells (Yeh *et al.*, 2016). The first of these two studies revealed that MAP was able to penetrate the nuclear membrane while TAT and poly-arginines were mainly retained in the cytosol. It should be stressed here that labelling with ^{125}I requires the presence of Tyr in the peptide structure; if this is not the case, the sequence of the peptide might be prolonged with Tyr, but its putative effect on the CPP behavior should be tested separately. In the study undertaken by Zaro *et al.* (2009), this was done with poly-arginines. Other nuclides recently used in radioactivity studies following CPP internalization were ^{68}Ga (Gronewold *et al.*, 2016), and ^{64}Cu (Bergmann *et al.*, 2017; Henry *et al.*, 2020). Recently, a review was published (Gharibkandi *et al.*, 2020) which does not deal specifically with CPPs but is generally aimed at the radioactive labelling of peptides and shows additional nuclides that could be used for the radioactive labelling of CPPs: ^{99}mTc, ^{111}In, and ^{177}Lu.

Mass spectrometry

To test molecules using MS, they must first be ionized or coupled to an ionic marker. An ionic molecule or its fragments pass through an electromagnetic field where they are separated according to the mass/charge ratio which is then revealed by a mass

https://doi.org/10.1017/S0033583522000026 Published online by Cambridge University Press

spectrum. In the field of CPPs, this is mainly used to confirm the proper synthesis of peptides – the calculated mass of the peptide is compared to the mass determined by a mass spectrometer. Ionization of the tested compounds can be achieved in the instrument by application of a high temperature, by an electric field, or by a beam of electrons, ions, or photons. Many biological molecules are very sensitive and should not be affected by high energy impact. For these, MALDI-TOF spectrometry is more suitable. MALDI means matrix-assisted laser desorption/ionization, and TOF is a time-of-flight mass analyzer. In this instrument, the tested molecule is adsorbed in the target plate of the instrument with matrix molecules that are heated by a laser beam. After this, the matrix molecules are energized and carry tested molecules through the electromagnetic field; during this process, the tested molecules are ionized, usually into one positively charged ion. Also, in this process, the tested molecules are much more softly treated than in the classical mass spectrometer; therefore, MALDI-TOF is appropriate for biological materials. Mass spectrometric methods are considered qualitative, but they can be used also for the quantitative determination of cell-internalized CPPs if those are labelled by isotopes, and particularly when the method is combined with chromatography, usually with high-pressure liquid chromatography. One of the first studies of CPP with MS was cellular uptake of pVEC and its all-D analogue (Elmquist and Langel, 2003). As it was shown by the MALDI-TOF approach, both peptides were internalized in aortic endothelial cells and murine fibroblasts, but only the all-D analogue remained intact in the cells while the all-L analogue was enzymatically decomposed. Later, several studies revealed that all-D analogues of CPPs remain stable in cells for a long time (cf. Youngblood *et al.*, 2007). MALDI-TOF was used (Burlina *et al.*, 2005) to determine cell internalization of different biotinylated CPPs labelled with stable isotopes (isotope was used as an internal standard). They could also follow the CPP degradation. More recently, MALDI MS was used to observe proper targeting of the brain by nanostructures composed of CPP TAT and P42 peptides (peptide derived from human Huntingtin protein) after per mucosal administration (Arribat *et al.*, 2014). Selenium labelled penetratin was studied by electrospray MS. The study revealed massive degradation of the construct after internalization into HeLa cells (Möller *et al.*, 2014). By a similar approach, the degradation of TP10 CPP was also studied, showing that for the stability of the peptide Lys in the C-terminal part of the peptide is very important and also for the penetration into cells (Xue *et al.*, 2015). MALDI-TOF was used also for the observation of the inhibition of histone demethylase by TAT conjugated demethylase inhibitor that was able to penetrate the nucleus (Dorosz *et al.*, 2017).

Electron microscopy

Electron microscopy has been used for a very long time to study biological structures and processes (cf. Scott and Parker, 1939). In principle, the electron microscope functions like any other microscope, only that instead of photons, it uses electrons. A beam of electrons is generated, focused by electron magnetic lenses, and accelerated by high voltage. The targeted sample material must be very thin and electrons pass through the sample where the electrons are scattered. According to the density of the different parts of the sample, the corresponding picture of the sample is obtained which can be refined by ocular electronic lenses. Two basic types of instruments with many variations are in use, transmission electron microscope (TEM) and scanning electron microscope (SEM). In TEM, electrons pass through the sample and the obtained picture can reveal the inner structure of the sample, for instance, the cell. In SEM, electrons will not pass through the sample but will induce a secondary beam of electrons that arises from the surface of the sample. In this way, a precise image of the surface is obtained. Both TEM and SEM types of electron microscopy are in use for studying CPPs.

Using TEM, one can explore biological membranes, organelles, nanoparticles, supramolecular complexes, and even bigger macromolecules, but the resolution does not permit direct visualization of a single CPP. For this, CPP must be labelled with an electron-dense tag like gold, iron oxide, and silica nanoparticles or are visible when coupled to big proteins (Padari *et al.*, 2005; Säälik *et al.*, 2009; Margus *et al.*, 2013). Alternatively, a label can also be put on cargo. This has been used with oligonucleotide cargoes that were usually labelled with colloidal gold particles or quantum dots (Arukuusk *et al.*, 2013; Margus *et al.*, 2015). Very rarely were TEM observations used as the single detection method, but was frequently combined with other biophysical and biochemical methods. Such an example is also the study of extracellular vesicles that were decorated by different arginine-containing CPPs (from 4 to 16 Arg) which make internalization possible (Nakase *et al.*, 2017). TEM was here used to explore (together with some other methods) the shape, dimensions, and consistency of vesicles, while internalization was followed by fluorescence. As it was found, internalization of vesicles was dependent on the amount of Arg residues in CPPs and peptides with 16 Arg residues as the most effective uptake vehicle. Similarly, TEM was used for the characterization of the morphology and size of liposomes conjugated to CPP Pep-1, but internalization studies were undertaken by fluorescence (Jiao *et al.*, 2017). On the other part, crotamine conjugated gold nanoparticles additionally decorated with polyethylene glycol were internalized in the HeLa cells which was followed by TEM (Karpel *et al.*, 2018). A variant of TEM, cryo-TEM was used to follow penetratin conjugated liposomes crossing the blood–brain barrier, accumulated *in vivo* in the rat brain (Tremmel *et al.*, 2016).

The SEM was also recently used for studying CPP mechanisms, usually in combination with other methods. An example is liposome treated with transferrin and CPP (penetratin and Tat) with encapsulating cytostatic doxorubicin that was able to penetrate the blood–brain barrier and deliver doxorubicin *in vitro* and *in vivo* (Sharma *et al.*, 2016). A combination of fluorescence microscopy and SEM revealed the modified membrane and morphology of the cells of five harmful microorganisms after interaction with CPP P-7; however, P-7 did not lyse the cells (Li *et al.*, 2014). The antibacterial effect of this CPP was also the result of its interaction with microorganism DNA after uptake into the microorganism cytoplasm. A peptide designed to have cell penetration and antimicrobial properties was designed and tested with *Escherichia coli* (Alaybeyoglu *et al.*, 2017). The antimicrobial effect of the peptide was monitored with fluorescence and SEM. Using TEM, SEM, and other methods, the effect of the modified peptide CADY-K (all-D retro sequence), forming nanoparticles that were able to deliver siRNA into cells, was studied (Vaissiére *et al.*, 2017). It was proven that the peptide has good antibacterial properties also because it is an effective CPP.

Circular dichroism

CD is a method that is based on differences of absorption of left- and right-handed circularly polarized light by chiral molecules. Regarding biomolecules, it is very useful for the determination

https://doi.org/10.1017/S0033583522000026 Published online by Cambridge University Press

of the secondary structure of peptides and proteins, but nucleic acids can also be analyzed. In proteins and peptides, light is mainly absorbed by the π-electrons of the atoms involved in peptide bonds. Each type of the secondary structure of peptides and proteins shows different spectra in the interval between 190 and 250 nm. The result is usually expressed in a relative proportion of α-helix, β-structure, and a random coil of the tested protein or peptide. Changes in the environment of the tested molecule are reflected in changes in the secondary structure and can be regularly monitored by CD spectrometers. Factors that affect CD spectrum are temperature, composition of the solution (solvent polarity, presence of ions, their composition, and concentration, etc.), and specific binding to different structures (receptors, lipids, and in the case of CPP different cargoes). Therefore, the conditions in which measurement is undertaken should be carefully monitored. A review of the physical principles of CD spectroscopy has been recently published (Andrews and Tretton, 2020).

It is almost a routine to determine the secondary structure of every new invented CPP, in relation to cargo delivery (Vasconcelos *et al.*, 2014). CD spectra are usually determined in water solution and in solutions containing phospholipid vesicles with the aim to find differences that would help to explain the mechanism by which CPP passes the lipid bilayer. It seems that no specific secondary structure exists that would be common to all CPPs and would ensure passage through the membrane; uptake into the cell was obtained with peptides that adopted an α-helical, β-sheet, or a random coil structure. In spite of this, it is sometimes suggested that peptides with some specific structure, most often α-helical structure, have some advantage in passing the lipid bilayer. This arises from the early determination of the secondary structure of the first known CPPs. Even before short TAT peptide was declared as a CPP, different parts of its parent protein has been inspected for the secondary structure in a vacuum and it was found that the fragment Tat(38–60), in particular, is able to adopt the α-helical structure and a shorter part of it, Tat (38–45) forms an amphipathic helix (Loret *et al.*, 1991). Other parts of Tat protein (Tat(65–81), Tat(32–48) and Tat(32–72)) were shown to be very flexible and can adjust their conformation to be able to bind to specific binding partners (Chen and Frankel, 1995; Metzger *et al.*, 1996). Another early confirmation study was done with penetratin and its analogues, for which, particularly for pAntp (43–58), it was shown that it can form an amphipathic helical structure, but it could also form aggregates in a 2(7) ribbon conformation (Berlose *et al.*, 1996). Finally, CD studies revealed that transportan has random coil secondary structure in water and can adopt up to 60% helical structure in SDS micelles. This was confirmed with transportan and penetratin that when being transferred from water to a solution containing different phospholipid vesicles, these two CPPs can be up to 60% helical (Magzoub *et al.*, 2001).

Many recent studies of the secondary structure of CPPs were focused on arginine-rich CPPs. By one such study (Patil *et al.*, 2014) was shown that arginine-reach CPPs $(R\text{-}X\text{-}R)_4$ with constrained amino acids in position X are improved cellular transporters because of the constraints introduced by the insertion of the proline-derived spacer. It was also shown that spacers keep peptides in a helical conformation and cell penetration ability is strongly influenced by the combination of hydrophobicity and flexibility of the peptide, which after internalization could be found in the cytoplasm. Another group has explored seven arginine-rich CPPs which were analogues of RW9 (RRWWRRWRR) by replacing Trp with Phe, measuring CD spectra, and assessing their penetration ability (Jobin *et al.*, 2015). In contact with the membrane, all peptides were structured, mainly in a helical conformation. This means that the replacement of Trp with Phe did not have much influence on the propensity of the peptides to be in a helical form, but it drastically reduced uptake into the cells and made the interaction of CPP with the membrane stronger. In another very recent study (Ohgita *et al.*, 2020), arginine-rich CPP was fused to a polyproline, which introduced a more stable helical structure and better uptake into CHO cells. A study of the artificial cationic CPP KL4 was undertaken (Qiu *et al.*, 2020) in which hydrophobic amino acid residues of leucine were replaced by alanine or valine in order to obtain a more soluble peptide. It has been observed by CD that this replacement shifted the secondary structure of the peptide from predominantly helical to a more disordered structure and also decreased the peptide's ability for internalization. All these studies fuel the opinion that the helical or at least ordered structure of cationic CPPs enhanced their ability to penetrate cells. The biological application of polypeptides with ordered secondary structures is discussed in more detail in the review (Ge *et al.*, 2020). These and similar observations raised the idea to stabilize the helix structure in CPPs to improve cargo delivery into cells. This was achieved mainly by introducing stapled CPPs in which the helical structure is fixed by inserting a staple spacer in the peptide structure which would decrease peptide flexibility and keep the peptide in the helical form. An early attempt of more stable helical CPPs was made by making seven types of peptides with three L-Arg and up to six L-Leu (Kato *et al.*, 2014) in which 1-aminocyclopentane-1-carboxylic acid was introduced. This stabilized the helical structure of the peptide and also improved the internalization potential of the peptides, however, without stapling. The best cargo deliverers were peptides composed of an equal number of Arg, Leu, and 1-aminocyclopentane-1-carboxylic acid; this peptide was retained in 310/α-helical conformation. Another strategy to obtain helical CPPs is to synthesize topologically constrained amphipathic peptides that are unordered in solution but form a very stable α-helix when in contact with the membrane (Jerath *et al.*, 2020). These peptides are also analyzed by CD and with complementary methods. They are effective deliverers of methotrexate in cancer cells and are much less toxic to normal cells. Real staple CPPs were described in the study by Tian *et al.* (2017) who synthesized a series of stapled peptides with different types of cross-links and comparatively studied their secondary structure, stability, and internalization potential. All synthesized peptides were able to enter the cells; however, their potential to deliver cargo into the cell was better correlated with their hydrophobicity than with their ability to form a stable helix. In the study of Hyun *et al.* (2018) a number of stapled CPPs were synthesized. All were stapled and contained various amounts of Lys and Leu residues. One of these peptides (stEK) was a deliverer of siRNA in nanomolar concentration and was further modified by replacing several Lys residues with His (it was named LKH-stEK). LKH-stEK was internalized via endocytosis but was able to escape from endosomes and successfully delivered siRNA into the cytosol. At least two review articles were recently published, dealing with stabilized and stapled peptides (Fominaya *et al.*, 2015; Li *et al.*, 2020).

Cycling CPPs are another type of CPPs characterized by enhanced stability and improved delivery potential. Models for these peptides are several natural cyclic peptides that are present in organisms and show remarkable stability. One reason for their stability is the fact that they do not have N- and

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C-terminals and thus are not substrates of exopeptidases. Cycles can be introduced into the peptides by different approaches. One, rather simple approach, is to use Cys residues to make a disulfide junction. An example of this approach is the study of D'Souza *et al.* (2014). They tested two ultra-stable disulfide-rich peptides, trypsin inhibitors from plants (MCoTI-II and SFTI-1). They are both good cargo deliverers but this ability can be further improved in MCoTI-II by introducing a higher number of positive charges into their structure, while mutation of SFTI-1 was not effective. A similar approach was used by Oba *et al.* (2017). They inserted two cyclic α,α-disubstituted α-amino acids into an L-Arg nonapeptide (R9 peptide). Then the stapled peptide was provided by the incorporation of a staple into the side chain of the unstapled peptide. The modified peptide formed a stable helical structure with an enhanced uptake into the cell. Another group has coupled TAT to GFP in two ways (Nischan *et al.*, 2015). One obtained construct was linear and the other was made cyclic by using azido-functionalized TAT and alkyne functionalized GFP. The cyclic construct delivered GFP into the cytosol and nucleus, but the linear construct was not detected – it was probably degraded in endosomes.

Nuclear magnetic resonance

NMR spectroscopy is a powerful spectroscopic method that uses the magnetic properties of atomic nuclei. These behave like small magnets that can absorb energy from the surrounding magnetic field provided by the strong magnets of the NMR equipment. The absorbed magnetic energy, which can be measured, depends on the close environment of the atom, particularly on the magnetic properties of atomic nuclei in the vicinity. On this ground, distances between atomic nuclei of the sample can be assessed and the structure of the sample molecule can be determined. For NMR spectroscopy, the most valuable are atoms with an uneven number of protons and neutrons in the nucleus, such as ^{1}H, ^{19}F, and isotopes, such as ^{13}C, ^{15}N, ^{31}P, etc., because they have the best magnetic properties. NMR can provide important information on the structure of a biomolecule, such as proteins, nucleic acids, carbohydrates, and lipids. Besides, it can also reveal the interactions between them. In the field of CPPs, most NMR studies were dealing with the interaction between CPPs and the lipid bilayer. An important advantage of the method is that it can provide the structure of biomolecules in solution, but its drawback is that it can be used only for the structure determination of relatively small molecules (up to 50.000 Daltons, even if the NMR active isotopes are incorporated in sample molecules).

The first NMR studies were undertaken with fragments of Tat protein even before part of this protein was shown to be TAT CPP. The potential of the 25 amino acids long fragment of Tat protein to form α-helical structure was revealed by CD and NMR (Mujeeb *et al.*, 1994) and the interaction of Tat(32–72) with RNA was shown (Metzger *et al.*, 1997). Later, it was shown that TAT CPP induces perturbations in anionic lipid (Ziegler *et al.*, 2003). Penetratin was studied in a nonpolar environment and its behavior toward lipids was addressed. In SDS micelles, penetratin adopts a helical structure (Lindberg and Gräslund, 2001) but also in phospholipid bicelles where it is located near the surface; its less polar analogue behaved very similarly (Lindberg *et al.*, 2003). It was also shown that penetratin is not able to cross an artificial phospholipid membrane (Drin *et al.*, 2001) and that its structure incorporates a bent helical structure (Czajlik *et al.*, 2002) that disappears in a more polar environment. Helical structure and ability to penetrate the cells are not correlated (Letoha *et al.*, 2003). Penetratin was also able to disturb a lipid membrane and induce it to be negatively curved (Lamaziere *et al.*, 2008). A number of CPPs were explored in SDS micelles and the phospholipid environment and it was shown that for Pep-1, some of its modifications, R9, Engrailed-2, HoxA-13, and Knotted-1 were able to form stable helices in this environment (Weller *et al.*, 2005; Balayssac *et al.*, 2006).

There are several other examples of exploring the interaction of CPPs with lipids in membrane bilayers, vesicles, or micelles, practically all showing the induction of the helical structure of CPPs in contact with lipids, but they are not presented here. More recent experiments are as follows. Protein Epsin-1 and its derived 18 amino acids long peptide are able to induce positive curvature in the membrane spots where clathrin-coated pits arise. This positive curvature of the membrane is the spot that is found by polyarginine CPPs and enhances their penetration into the cell (Pujals *et al.*, 2014). For BP100, a short 11 amino acid long peptide, it was shown that it obtained α-helical structure interacting with different artificial, prokaryotic, or eukaryotic membranes and induced substantial thinning of the membrane, which was shown to be a prerequisite for penetration into cells (Misiewiz *et al.*, 2015). A group produced the peptide EETI-II, a model Cys-knot peptide (Gao *et al.*, 2016), and showed by NMR its penetration into cells but peptide was retained in the endosome and subsequently degraded. The cell penetration was enhanced by co-incubation of the cell with CPPs which also altered its route in the cell since EETI-II was now found in the cell structures. NMR also revealed the altered intracellular trafficking of the peptide after endocytic internalization. Analogues of penetratin were synthesized using ^{19}F modified amino acids and their penetration was studied by NMR (Christensen *et al.*, 2019). Introducing ^{19}F into the peptide makes possible a more precise following of the fate of the peptide in the cell in comparison to ^{1}H-NMR because of a much better resolution.

Conclusions

The design and improvement of therapeutic CPPs are of great impact in situations where FDA approvals have not been achieved yet. Future development of CPP-based drugs greatly depends on approaches leading to the understanding of CPP mechanisms of (tissue, cellular) uptake. This review summarizes the recent biophysical studies of different aspects on the subject, including the interaction of peptides with interaction partners such as phospholipid membranes and proteins in plasma membrane and circulation as well as in cells.

Characterization of the kinetics of the multistep network of interactions in CPP internalization and CPP-cargo conjugates reveals multiple steps in these complicated interaction networks. Knowing and following the intracellular signaling pathways by biophysical methods demonstrates the endosomal escape of CPP-aided delivery. Promising nucleic acid-based therapies based on CPP-vectorized oligonucleotides (antisense, siRNA, miRNA, and plasmids) and CPP/oligonucleotide nanocomplexes require additional biophysical studies. Intracellular protein–protein or protein–DNA/RNA interactions have been addressed by short protein mimics or proteins using advanced biophysical studies.

The described visualization of CPPs certainly belongs to the biophysical set of CPP research with high impact in the characterization of novel CPP-based therapeutics.

https://doi.org/10.1017/S0033583522000026 Published online by Cambridge University Press

Conflict of interest. None.

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