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# Synthesis and Applications of Synthetic Peptides

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## Abstract

The synthesis and applications of the peptides are gaining increasing popularity as a result of the developments in biotechnology and bioengineering areas and for a number of research purposes including cancer diagnosis and treatment, antibiotic drug development, epitope mapping, production of antibodies, and vaccine design. The use of synthetic peptides approved by the health authorities for vaccine, for cancer, and in drug delivery systems is increasing with these developments. The aim of this book chapter is to review the recent developments in the use of peptides in the diagnosis of drug and vaccine systems and to present them to the reader with commercially available illustrations.

**Keywords:** synthetic peptide, solid-phase peptide synthesis (SSPS), peptide therapeutics, peptide vaccines, cell-penetrating peptides

## 1. Introduction

The aim of this chapter is to review some applications of synthetic peptides providing a brief knowledge about peptide synthesis. In the first part, information about the peptide synthesis was given in a very simple and readable format under the title of solid-phase peptide synthesis including a brief history, solid supports, linkers, protecting groups, and analysis method sections. Then the synthetic peptide vaccine application of peptides was reviewed. After that, the topic of nuclear imaging-guided peptidic drug targets and labeling techniques and recent developments in therapy was discussed. In the last part, information about cell-penetrating peptides that can be used as molecular carriers is mentioned with providing classification and cellular uptake mechanism of them.

## 2. Solid-phase peptide synthesis (SSPS)

The specific characters of peptides (high bioactivity, high specificity, and low toxicity) have made them attractive therapeutic agents. The synthesis of the peptides may provide sufficient material to enable further studies and to determine the structure-activity relationships or may provide discovery of new analogues with improved properties [1–7]. The peptides are able to synthesize in three methods: in a solution medium, on a solid support, or as a combination of the solid and the solution synthesis. Although peptide synthesis is often carried out by the solid-phase

method, the solution method was preferred by the pharmaceutical companies in the 1970s and 1980s [8]. In the solution medium synthesis method, except for the reversible protection of the N-amino group of the first amino acid or fragment, the orthogonal protection of carboxyl groups of the second amino acid or the fragment is needed. On the solid-phase method, the synthesis is carried out on a solid support, also called a resin. The peptide is separated from the resin after each amino acid in the peptide sequence is sequentially bound. In the solid-phase technique, the peptide that bounded to the insoluble resin is separated without any significant loss during the washing or filtration of the resin. All reactions are carried out in a single reaction vessel, and possible losses are prevented during processes such as exchange and transfer of reaction vessels [9]. Another one is hybrid synthesis which is the composition of the solution and SPPS methods. Herein, the peptide to be synthesized is obtained after the condensation from a solution of two or more suitable peptide sequences, which are obtained mainly by solid-phase synthesis [10].

The principle of peptide synthesis in the solid phase is quite simple. The peptide chain is attached to the stable solid phase. The peptide sequence remains bound on this resin for the duration of the synthesis. During the synthesis that includes deprotection, activation, and coupling steps, other soluble chemicals outside the resin beads are removed by filtration and washing. In the last stage, the desired product is separated from the solid phase by cleavage procedure. Purification and characterization procedures are carried out in the free solution of the desired product [11]. In SPPS, although there are two main strategies, the Boc approach requires the use of hard acids such as HF; thus the majority of synthetic peptides are nowadays synthesized by using Fmoc chemistry. The most important advantage of the Fmoc method compared to Boc strategy is that it does not require corrosive acids such as TFA during the synthesis cycle, and thus the synthesis can be automated. In this method, the N-terminal amine groups, the acid-labile side-chain protecting groups, and the linkers forming the protecting group of the C-terminal amino acid are protected by the base-labile Fmoc group [12, 13]. Solid support materials (resins), linkers, protecting groups, and cleavage cocktail solutions are general components of the solid-phase peptide synthesis.

*The resin* is a spherical polymer ball bearing the active groups to which the first amino acid (indicates the first amino acid at the C-terminus of the sequence) of the sequence to be synthesized can be attached. The solid supports used in the solid-phase peptide synthesis must be stable against mechanical mixing, various temperature conditions, and different types of solvents, must have a narrow range of resin bead sizes, and must have high swelling properties (should be able to swell up to 5–6 times in DCM, dimethylformamide (DMF), etc.); thus the reagents can reach the active regions of the resin. The solid support material is usually used in a size of about 100–200 mesh or 200–400 mesh, and the peptide chain is extended by the addition of amino acids on each other on the resin. The most common classes of resins are the classic polystyrene (PS) resins (cross-linked with 1% of divinylbenzene (DVB)), the PS-functionalized polyethylene glycol (PEG) resins, pure cross-linked PEG resins, and polystyrene cross-linked with 1,6-hexanediol diacrylate resin (PS-HDODA) [14–20]. The solid support used in the synthesis can be of two kinds depending on whether or not the initial amino acid of the sequence to be synthesized is bound to the resin. Accordingly, there are two types of resin, either pre-loaded or unloaded, but pre-loaded resin is generally preferred. The pre-loaded resins differ depending on whether they are lowly loaded or highly loaded. When the highly loaded resin is used, aggregation of the peptide sequences growing on the resin increases, and this will lead to the deletion of an amino acid which is an undesirable situation; therefore, the use of lowly loaded resins is common [19].

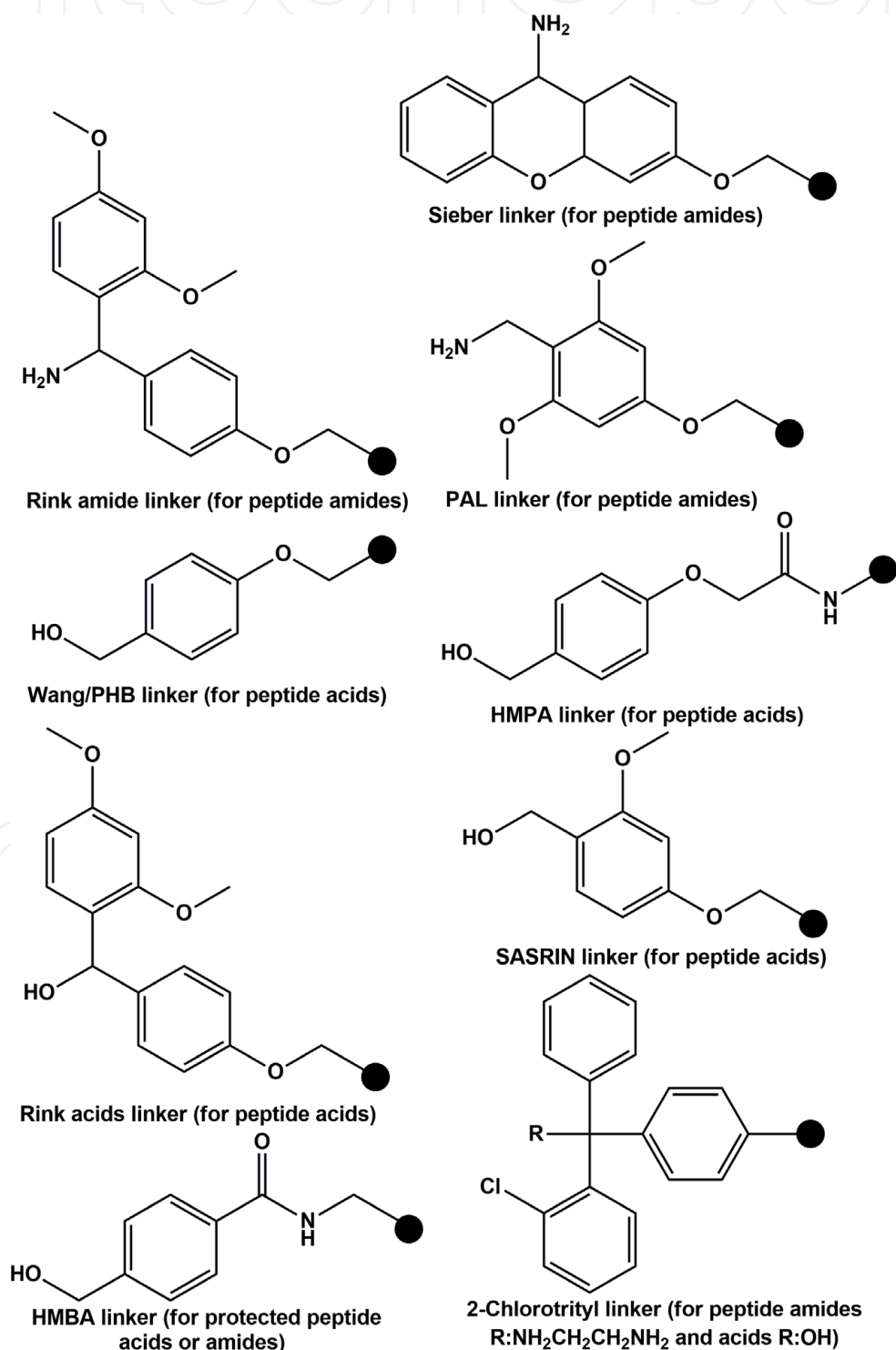
In order to bind the first amino acid to the resins, chemical structures containing functional group(s) called *linker* are attached. The number of these active groups on the resin (substitution) is important in calculating the theoretical yield of a peptide to be synthesized and in determining the amount of chemicals (deprotection, activation, coupling) required for the synthesis of a peptide. Depending on the functional group of the C-terminal end of the target peptide sequence, peptide acids or peptide amides can be synthesized by means of an ester or an amide bond, respectively. While the peptide acids are obtained using 4-benzyloxybenzyl alcohol (Wang), 2-(4-hydroxymethyl) phenoxyacetic acid (HMPA), superacid-sensitive resin (SASRIN), and 2-chlorotrityl linkers, the peptide amides rink amide, 4-methyl benzhydryl amide (MBHA), and Sieber and primary amide (PAL) linkers are required (**Figure 1**) [13]. After the cleavage procedure, C-terminus of the peptide sequence remains  $\text{—COOH}$  or  $\text{—NH}_2$  depending on the type of the linker on the resin beads [13, 19, 21, 22].

The reaction between the activated carboxylic group of one amino acid and the amino group of another amino acid is needed for a peptide linkage. In order to prevent side reactions from occurring, the remaining functional groups of each amino acid must be appropriately protected. For this purpose, many *protecting groups* are used to prevent the reaction of different types of functional groups. Because each amino acid contains different groups in its side chains, the protecting groups which prevent these groups from reacting vary according to the amino acid and used methodology (Fmoc or Boc chemistry). Some amino acids have not any functional groups to react in the side groups; only alpha-amino groups of these amino acids are protected by Fmoc or Boc [23–33].

*Cleavage* is one of the most important steps in solid-phase peptide synthesis. Exposing a resin to a cleavage cocktail is not an easy reaction; it brings a series of competitive reactions. Unless appropriate reagents and reaction conditions are selected in the cleavage process, the desired peptide can be irreversibly modified or damaged. Also, since the DMF used in the solid-phase peptide synthesis can inhibit TFA acidolysis, before starting the cleavage, the peptide-bound resin should be washed with DCM to remove all DMF. The goal of cleavage is to split the synthesized peptide sequence from the resin while also removing amino acid side-chain protecting groups. For this process, strong acids such as anhydrous HF, TFMSA, or TMSOTf are used in Boc chemistry, while cocktails containing TFA are preferred in the Fmoc process [12, 22, 34]. Additional substances such as EDT, phenol, and thioanisole called *scavengers* are put into the cleavage medium to prevent the cationic products occurring during the cleavage process to attack the amino acids that are having electron-rich side chains such as Trp, Tyr, and Met. Considering the amino acid types involved in the resin-linked peptide sequence synthesized by the solid-phase peptide synthesis method, it is decided to which type and how much scavenger add to the cleavage cocktail. For example, when there is one or more Trp in the peptide sequence, using EDT in the cleavage will substantially protect Trp against oxidation. Typically, the peptide-bound resin is treated using 95% TFA with gentle shaking for 1–3 h [12, 22, 34, 35].

The crude peptides synthesized by solid-phase peptide synthesis with the deprotection, activation, and coupling and cleavage steps are mostly analyzed on analytical HPLC using C18 columns at a concentration of 1 mg/mL with gradient elution method of water (0.1% TFA) and acetonitrile (0.1% TFA) as mobile phase. If purification of the synthesized peptide is required according to the application to be used, preparative HPLC is used. For the analysis, the peptide should be dissolved in a minimum volume of 0.1% TFA in water, and the acetonitrile and water gradient elution is adjusted according to the polarity of the peptide [12, 22, 35–37]. The molecular weight of the synthesized peptide is confirmed by mass spectroscopy. The most commonly used methods are LC-MS, MALDI-TOF, and LC-QTOF-MS. Systems

with such a combination of chromatography and spectroscopy are useful for the complete characterization of synthetic peptides. While chromatographic analyses allow us to have knowledge about the peptides' purity, the molecular weight of the peptides is determined by MS analyses. It is also found in some systems that determine the amino acid sequence of the peptide molecule like amino acid analyzer and LC-MS-MS. Information on structural properties of peptides can also be obtained by NMR and FTIR methods. With NMR and FTIR, the structures of the peptides are further elucidated via specific binding and functional groups [12, 35]. Moreover circular dichroism (CD) spectroscopy gives information about the conformation and secondary structures of polypeptides. This technique can be used to distinguish between random coil, alpha-helix, or beta-sheet structures [38, 39].



**Figure 1.**  
Some of the fundamental linkers used on the Fmoc-based SPSS [22].



### 3. Peptide vaccines

The increase in studies on peptide vaccines in recent years shows that peptide vaccines will be an important part of new-generation vaccine systems. Vaccines are indispensable in protecting human and community health in terms of reducing infectious diseases, disability, and deaths and, most importantly, eliminating and eradicating the long-term disease. The development of the vaccine, which began with the observations of Edward Jenner at the end of the eighteenth century, has significantly reduced the number of infections and diseases until now [40–42]. Traditional vaccines (live-attenuated or inactivated vaccines) have been widely used over the last hundred years to develop effective vaccines against many diseases. However, the use of this technology is a source of concern because of the problems encountered in the preparation and use of such vaccines. The disadvantages of classical vaccines such as the increased need for safety, high cost in the production of large-scale vaccine preparations, high genetic variability of pathogens, side effects, and risk of leak of the disease agent have necessitated research to develop new technological vaccines [43, 44]. The basis for new vaccine technologies is the production of high-purity subunit vaccines that contain a small part of the pathogen necessary to generate an immune response. Subunit vaccines can be composed of polysaccharides and proteins or their peptide fragments. Synthetic peptide vaccines consist of 20–30 amino acids containing the specific epitope of a corresponding antigen against various diseases. There is no limitation from cancer diseases to allergies in diseases targeted by peptide vaccines. The advantages of peptide vaccines have enabled peptides to be preferred in vaccine technology [45–48]. Although many peptide vaccine studies continue, there is no approved peptide vaccine for human use. According to the database of clinical studies maintained on ClinicalTrials.gov, in the search on the topic of peptide vaccine until mid-December 2018, 374 clinical trials in phase I, 272 clinical trials in phase II, 14 clinical trials in phase III, and 2 clinical trials in phase IV have been found [47, 49]. Furthermore, it is reported in the literature that a veterinary peptide vaccine against canine parvovirus, which is targeted against animal diseases, is effective [50]. Advantages involved in the choice of peptide-based vaccine can be summarized as follows: Peptide-based vaccines are more reliable than classical vaccines because they are produced using chemical synthetic approaches almost exclusively. It does not produce any unwanted immune response as it does not contain biological material. Also, this situation eliminates the risk of biological contamination. The rapid, easy, inexpensive, and high-purity synthesis of peptides by microwave-assisted solid-phase peptide synthesis methods is an important advantage for the use of peptides in vaccine systems. These synthesized peptide antigens can be fully characterized by analytical methods. “Cold chain” is not required generally during transport and storage of peptide vaccines. Peptide vaccines have high stability and are typically water-soluble. Allergic and/or reactogenic effects are avoided by the usage of peptide vaccines. Peptide vaccines can be customized using the peptide epitope of one or more antigens [46, 51, 52].

Despite all its advantages, there are some limitations of peptide-based vaccines. The peptides alone are generally weakly immunogenic, have poor release properties and are easily undergo enzymatic degradation. These disadvantages can be overcome using adjuvants and delivery systems. Adjuvants, derived from the Latin word “*adjuvare*,” which means “to help,” play a crucial role to the elicitation of a strong immune response to an antigen [49, 53–56]. The first step in the development of peptide vaccines is the identification of the structure of peptide epitopes which will provide effective and long-term immunity. After the synthesis and characterization of specific antigens to be determined, the appropriate adjuvant and delivery system selection is carried out [43, 46]. Currently, instead of classical adjuvants, the use of

new generation of more effective adjuvants is being studied. It is aimed to overcome the disadvantages of the traditional adjuvants with the development of modern adjuvants and delivery systems. Alum (insoluble salts of aluminum), which is a classical adjuvant, is a weak immune stimulant; its mechanisms of action are not fully elucidated and have a possibility of side effects. Emulsion adjuvants such as incomplete Freund's adjuvant (IFA), complete Freund's adjuvant (CFA), and lipid A are used; however, it is important to develop safer adjuvants due to their toxicity [46, 49, 57, 58]. Protein and synthetic polymer carriers play an important role in new-generation vaccine systems. The immunogenicity of peptide vaccines based on conjugation to protein and polymeric carriers is enhanced. Bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), human serum albumin (HSA), and ovalbumin (OVA) are the most preferred proteins in peptide-protein conjugates [51, 59, 60]. In recent years, peptide-polymer conjugations using synthetic polymeric carriers have gained importance. Polymers to be selected in peptide-polymer conjugates, should be biocompatible, be relatively easy to synthesize and be modifiable according to the recommended use. This situation is pointed out that such polymers are important carrier candidates for peptide vaccines [61–63].

Nanoparticles are investigated for their potential use as vaccine delivery systems. The use of nanoparticles in peptide-based vaccines may be useful in elimination of limitations with small peptide antigens. It can play a critical role in increasing the size of the antigen molecule, protecting the peptides from enzymatic degradation, and the delivery of the peptides to the targeted cells. Inorganic NPs, lipid-based NPs, polymeric NPs, and carbohydrates are common classes of nanoparticles. Especially polymeric NPs are of great interest for vaccination due to their biocompatibility and predictable degradation. In addition to natural polymers such as chitosan, dextran, and albumin, synthetic polymers such as polyesters, polyanhydrides, and polyamides can be mentioned. Mostly studied poly(lactic-co-glycolic acid) (PLGA) is a synthetic copolymer composed of lactide and glycolide and approved by the European Medicine Agency (EMA) and US Food and Drug Administration (FDA) [64]. This polymer, which is biodegradable and biocompatible, is suitable for vaccine and drug delivery systems in humans. In the usage of polymeric nanoparticles as delivery systems for peptide vaccines, the peptide may be covalently/non-covalently attached to the surface of the particles through functional groups, or the peptide may be loaded into the nanoparticles [65–69].

Peptide vaccines are considered to be critical in the production of effective, safe, inexpensive, and easy to produce vaccines by the abovementioned advantages and the development of new adjuvants.

#### **4. Utilizing of peptides as radio-theranostic agents**

This part of the chapter will introduce the chemistry behind the radiolabeling of peptide-based diagnostic and/or therapeutic pharmaceutical systems used for nuclear oncological imaging and present research related to drug discovery in various areas such as chemistry, bioengineering, nanoscience, and nuclear oncology [70]. Synthetic peptide-based drugs labeled by radiochemically different techniques are used in routine clinical applications for diagnosis and therapy of diseases. Peptides targeting the somatostatin receptor subtypes have been routinely applied for peptide receptor radionuclide therapy (PRRT) of neuroendocrine and prostate cancers. Tumor regression, lengthening survival, and increased quality of life were observed in the patients treated with  $^{177}\text{Lu}$ -/ $^{90}\text{Y}$ -labeled peptide conjugates [71]. This section provides an overview of various targeting and labeling techniques for receptor-targeted imaging.

## 4.1 Radiolabeled peptide analogues

PRRT with radiolabeled peptide pharmaceuticals is a suitable and promising next-generation therapy method for inoperable patients with metastatic tumor. Radiolabeled peptide ligands are effective vectors for both detection and treatment of tumor cells overexpressing receptors specifically binding these ligands. Such radiopharmaceuticals, called as “theranostics,” can be administered for diagnosis besides treatment, depending on the type of radionuclide being bound to the peptide sequence. Especially somatostatin and prostate-specific membrane antigen (PSMA)-based peptide analogues have significantly influenced the staging and therapy of patients. Mentioned analogues are used in clinical routine as an integral part of the treatment [72].

### 4.1.1 Radionuclide therapy using somatostatin analogues

PRRT and radiolabeled somatostatin (SST) peptide analogues are highly effective treatment agents. Somatostatin is in the form of dominant but functionally less active 14 amino acids called SST-14 and SST-28 in the form of a larger and stronger 28 amino acids [73]. According to the structure-activity researches of SST-14, although the amino acid residues of Phe, Trp, Lys, and Thr which contain a  $\beta$ -turn are important for bioactivity, different amino acids may be replaced by the Phe and Thr amino acids for this bioactivity, whereas Trp and Lys residues are definitely required. Octreotide, lanreotide, vapreotide, and pasireotide, which are the 4SS analogue synthetic peptides, are currently used actively in routine clinical applications. The octreotide compound, which is the synthetic peptide compared to the natural SST, was found to be 19 times more effective in inhibition of growth hormone secretion [74]. The presence of the D-Phe at the N-terminal end and the L-Thr at the C-terminal end and the replacement of the L-Trp at position 8 with D-Trp ensure that the peptide is resistant to degradation [75]. The somatostatin receptor family contains five receptor subtypes, sst1–sst5. Most of the neuroendocrine tumors, especially subtype 2 (sst2), have a strong overexpression of sst. The clinical use of radiolabeled sst-targeting analogue [ $^{111}\text{In}$ -DTPA<sup>0</sup>]octreotide initiated the development of numerous somatostatin analogues. This analogue is used as a theranostic agent in positive metastatic tumors. Tumor regression and survival improvement can be provided with [ $^{90}\text{Y}$ -DOTA<sup>0</sup>,Tyr<sup>3</sup>]octreotide and [ $^{177}\text{Lu}$ -DOTA<sup>0</sup>,Tyr<sup>3</sup>]octreotate. Several phase-1 and phase-2 PRRT trials were carried out using [ $^{90}\text{Y}$ -DOTA<sup>0</sup>-Tyr<sup>3</sup>]octreotide. Tumor regression responses in the clinical studies with these agents in patients who suffered from GEP-NETs ranged from 9 to 33% [70]. As a result of the administration of the radiolabeled antagonist  $^{125}\text{I}$ -JR11 and agonist  $^{125}\text{I}$ -Tyr<sup>3</sup>-octreotide in different tumors, it was found that the antagonist binds to the SSTR2 region at a rate of 3.8–21.8-fold higher [76]. This significantly increased binding not only provides a more accurate localization in tumor and metastasis but also enables therapeutic interventions with radiolabeled SSTR antagonists to be more effective. Despite the low SSTR2 concentrations, tumors other than GEP-NETs and lung NETs have been targeted with SSTR2 antagonists; tumors of these types are breast cancer, medullary thyroid cancer, non-Hodgkin lymphomas, renal cell cancer, and small-cell lung cancer [76, 77].

### 4.1.2 Radionuclide therapy using PSMA analogues

PSMA has become an interesting target for the diagnosis and therapy of peptide receptors for small molecule ligands [78]. Since PSMA is often overexpressed in prostate cancer (PCa) cases, many different PSMA ligands are aimed



to be targeted by the same biological mechanism to diagnose and treat metastatic castration-resistant prostate cancer (mCRPC). Many studies proved the superiority of  $^{68}\text{Ga}$ -PSMA PET/CT (positron emission tomography/computed tomography) as compared to CT, MRI, or bone scan for determination of metastases for first staging at initial diagnosis [79, 80]. Imaging with PSMA in nuclear medicine substantially affected the detection and treatment of patients with prostate cancer. PSMA has been known to be promising and frequently preferred in advanced clinical studies because of providing preliminary information for different types of clinical conditions and detecting lesions with low PSA levels [81]. PET/CT data obtained using PSMA provides a noninvasive evaluation of PSMA expression and is used for restaging prostate cancer after radical prostatectomy when PSA level is low [82]. Also, it can be applied in endoradiotherapy because of the intracellular internalization feature of PSMA. A small molecule inhibitor of PSMA ((S)-2-(3-((S)-1-carboxy-5-(3-(4-[ $^{124}\text{I}$ ]iodophenyl)ureido)pentyl)ureido)pentanedioic acid; MIP-1095) has been administrated to evaluate potential therapeutic use of  $^{131}\text{I}$ -MIP-1095 in men with mCRPC [83]. Due to their molecular and specificity similarity,  $^{68}\text{Ga}$ -PSMA-11,  $^{68}\text{Ga}$ -PSMA-617, and  $^{68}\text{Ga}$ -PSMA-I&T are abbreviated as  $^{68}\text{Ga}$ -PSMA.  $^{68}\text{Ga}$ -PSMA-11 biodistribution was known to correspond well to cellular detection of PSMA expression.  $^{68}\text{Ga}$ -PSMA-617 and  $^{68}\text{Ga}$ -PSMA-I&T peptidic ligands demonstrated similar distribution and imaging properties [79].

#### 4.1.3 Radionuclide therapy using other peptide analogues

For clinical applications, it is preferred that the peptide analogues are metabolically stable. The natural structure of the peptides makes them sensitive to peptidase and rapidly breaks down in blood and other tissues, so their potential uses as radiopharmaceuticals are restricted. Strategies for stabilizing peptides include incorporating biologically insoluble peptide bonds, stabilized amino acid derivatives, and cyclization [70]. Although the stability is advantageous, it is necessary to keep the nuclide in the tumor area for a long period of time for a good target-to-nontarget ratios and to rapidly clear the nuclide from nontarget tissues and blood. Researches on the topic of peptide-based radiopharmaceuticals have mostly been focused on receptor agonists. However, in recent years, somatostatin and bombesin antagonist peptide analogues have also been best shown for receptor targeting [72].

##### 4.1.3.1 GRP receptor-targeting peptides

Overexpression of gastrin-releasing peptide receptors (GRPR) is seen in many human tumors, including prostate and breast tumors. Bombesin (BN), a 14-amino acid peptide with high affinity to the GRP receptor, is used for GRP receptor-targeted tumor imaging and treatment.  $^{111}\text{In}$ - and  $^{99}\text{Tc}^{\text{m}}$ -labeled BN analogues have been improved for SPECT imaging of GRP receptors. After a while, a novel DTPA-bound BN analogue, [ $^{111}\text{In}$ -DTPA-ACMpip5, Tha6,  $\beta\text{Ala}$ 11, Tha13, Nle14] BN (5–14) (Cmp 3), was synthesized. Replacement of the DTPA chelator in the  $^{111}\text{In}$ -Cmp3 analogue with a DOTA makes the compound suitable for therapeutic use and diagnostic PET imaging. Another promising peptide-conjugated DOTA is  $^{177}\text{Lu}$ -AMBA (DOTA-BN (7–14)), which exhibits good tumor-background ratios. The  $^{177}\text{Lu}$ -AMBA is excreted through the kidneys, but kidney excretion cannot be reduced by co-lysine injection because there is not any lysine in the peptide structure. Nowadays, it is the first choice in the targeting of  $^{68}\text{Ga}$  GRP receptors as a positron emitter radionuclide which provides shorter half-life and in-house radio-labeling procedures as well as more accurate high-resolution PET images. Recently,

imaging studies have been studied to detect a GRPR and integrin  $\alpha v \beta 3$  dual targeting tracer  $^{68}\text{Ga}$ -BBN-RGD for PET/CT imaging of metastatic breast cancer [84]. Recent researches on BN analogues have focused on the DOTA chelating systems for multi-use options such as SPECT, PET, and PRRT [72].

#### *4.1.3.2 NT receptor-targeting peptides*

Neurotensin (NT) with 13 amino acids in its structure is expressed in the central nervous system, peripheral tissues, and gastrointestinal system [72]. Despite overexpression of neurotensin receptors in 75% of ductal pancreatic carcinomas, NT receptor expression of endocrine pancreatic tumors, pancreatitis, and normal pancreatic tissue is negative (somatostatin positive) [85]. In a clinical study using  $^{111}\text{In}$ -labeled DTPA and DOTA-conjugated NT analogues, a number of specific changes were made to unnatural amino acids, indicating that the C-terminus plays a key role in the binding and biological properties of the peptide, and there is not a critical change in receptor binding activity of the peptide [72]. Because of high renal involvement of  $^{111}\text{In}$ -NT analogues, PRRT is not considered appropriate with these analogues. With the discovery of three times more stable  $^{99}\text{Tc}^{\text{m}}$ -labeled NT (NT-XIX) analogue, a better tumor-to-kidney ratio, higher tumor involvement, and higher kidney excretion were achieved.  $^{99}\text{Tc}^{\text{m}}$ -Demotensin, which was a high-stability NT analogue, has a higher tumor-to-intestinal and tumor-to-liver ratios, but the most appropriate ratios for imaging pancreatic tumors were obtained with the  $^{111}\text{In}$ -labeled analogues [86].

#### *4.1.3.3 CCK2 receptor-targeting peptides*

It has been determined that in the majority of medullary thyroid carcinomas (MTCs), in a high rate of small-cell lung cancer patients, stromal ovarian cancers, astrocytomas, and some other tumor types have cholecystokinin-2 (CCK2) receptors. The CCK2 receptor-specific CCK peptide analogue was conjugated with DTPA for targeting this receptor [85]. The highest tumor uptake and too high renal involvement have been demonstrated at minigastrin analogues containing the CCK8 sequence. The addition of the histidine residues to the array almost reduces the kidney uptake by twofold. According to a study carried out in mice bearing the AR42J tumor, it was reported that the DOTA-conjugated HHEAYGWMDF peptide sequence exhibited the highest tumor-to-kidney ratio compared to all peptides studied [86]. Additionally,  $^{99}\text{Tc}$ -labeled N4-derived analogues of minigastrin have been synthesized [72]. Although studies with these radioligands are still in the initial stage for PRRT, they have significance features for the future.

#### *4.1.3.4 GLP-1 receptor-targeting peptides*

The glucagon-like peptide 1 (GLP-1) receptor which is overexpressed in human endocrine tumors, insulinomas, gastrinomas, and pheochromocytomas is a subtype of glucagon receptors used as a vehicle for in vivo tumor targeting. Because natural GLP-1 receptor agonists are metabolized quickly in the blood, a more stable and specific exendin peptide has been developed for use in the scintigraphic imaging of GLP-1 receptor-expressing tumors [72]. In the following years,  $^{111}\text{In}$ -DTPALys40-exendin-4 [72] and [Lys40 (Ahx-DTPA- $^{111}\text{In}$ )  $\text{NH}_2$ ] exendin-4 conjugates with high tumor-background ratios have been optimized with using animal models [87]. In a clinical study based on the development of GLP-1 receptor-targeting analogues, PET/CT imaging with the  $^{68}\text{Ga}$ -NOTA-exendin-4 peptide analogue has been reported to be a highly susceptible imaging technique for the detection of insulinoma localization [88].

#### 4.1.3.5 $\alpha\text{v}\beta 3$ integrin-targeting peptides

$\alpha\text{v}\beta 3$  integrins are a transmembrane protein that can be expressed in proliferative endothelial cells and overexpressed in newly formed blood vessels where tumors are fed. The arginine-glycine-aspartic acid (RGD) tripeptide is essential for the interaction of extracellular matrix proteins to  $\alpha\text{v}\beta 3$  receptors. The cyclic RGD analogue containing these amino acids has the highest binding affinity. Many radiolabeled DTPA and DOTA-RGD conjugates with  $^{111}\text{In}$ ,  $^{90}\text{Y}$ ,  $^{177}\text{Lu}$ ,  $^{68}\text{Ga}$ , and  $^{64}\text{Cu}$  which provide SPECT and PET imaging and PRRT have been discovered in recent years. Monomeric, dimeric, and tetrameric RGD peptides are bound to DOTA for developing receptor binding affinity and then radiolabeled with  $^{111}\text{In}$ . Although the monomeric and dimeric analogues have higher in vitro receptor affinity, the in vivo tumor uptake of the tetrameric analogue is higher. Also, it has been shown that multimeric RGD peptides are effective clinical molecules for in vivo determination of tumor angiogenesis in cancer patients [72].

## 4.2 Radiopeptides

In clinical practice, radiolabeled receptor ligands are used routinely for diagnostic imaging of overexpressed receptors and PRRT. Causes of clinical success of radiopeptidic receptor ligands are the following:

1. First, the presence of different radionuclides, having similar chemical properties, enables to label the same peptide with different radionuclides for different clinical purposes (SPECT, PET, PRRT).
2. Second, the influence of the high hydrophilic radiometal complex on peptide pharmacokinetics leads to rapid renal excretion and good target/background ratios.
3. Third, one-step in-house labeling methodology that facilitates the preparation of peptide radiopharmaceuticals in clinical routine [89].

The radioiodine which is used for radiolabeling of biologically active molecules is frequently preferred in PET imaging ( $^{124}\text{I}$ ), SPECT imaging ( $^{123}/^{125}\text{I}$ ), treatment of different cancer types ( $^{131}\text{I}$ ), and biodistribution and kinetic investigation of novel peptide radiopharmaceuticals ( $^{125}\text{I}$ ). Direct radioiodination is based on the substitution of an aromatic proton with  $^*\text{I}^+$  (electrophilic radioiodide) and is successful only in electron-rich aromatic systems including activating substituents such as  $-\text{OH}$ ,  $-\text{NH}_2$ ,  $-\text{OR}$ ,  $-\text{NHCOR}$ , or  $-\text{SR}$ . For in situ production of  $^*\text{I}^+$ , chloramine-T (sodium tosylchloroamide) and iodogen (1, 3, 4, 6-tetrachloro-3a, 6a-diphenylglycoluril) are utilized. Chloramine-T is added to the reaction medium with sodium metabisulfite to terminate the labeling reaction and prevent oxidative damage. Oxidative enzymes (lactoperoxidase) are used for substitution of peptide sequences that have high sensitivity to oxidation. Tyr- and also His-containing peptides are readily radioiodinated in buffers such as phosphate buffered saline (PBS) or Tris (hydroxymethyl)-aminomethane (TRIS) at pH 7–8. Pre-radioiodination should be carried out by prosthetic groups. Selective prosthetic group conjugation is provided to the thiols with the help of pre-radioiodinated maleimides, and pre-labeling of the corresponding peptide is carried out by the use of stannylated vinyl alkylating agents. In this way, tissue deiodinase and unfavorable structural conditions for radioiodination can be overcome [90].



Complex biomolecules such as peptides or proteins cannot be directly labeled with a highly basic  $[^{18}\text{F}]$  fluoride by nucleophilic substitution and cannot tolerate labeling conditions. Activated aromatic precursors ( $\text{NO}_2$ ,  $\text{CN}$ ,  $\text{Cl}$ , etc.) are substituents bound to the leaving group in the ortho- or para-position. Many receptor binding peptides such as octreotide, bombesin, neurotensin, and RGD analogues have been labeled using  $[^{18}\text{F}]$ FP-NP(4-nitrophenyl-2- $[^{18}\text{F}]$ fluoropropionate) or  $[^{18}\text{F}]$ SFB(N-succinimidyl-4- $[^{18}\text{F}]$ fluorobenzoate) [91]. Chemoselective strategies provide a one-step prosthetic group labeling reaction by unprotected precursors. Reaction of a  $^{18}\text{F}$ -labeled aldehyde with aminoxy- or hydrazino-functionalized peptides so-called click chemistry has recently found most popular application [89]. A suitable chelating agent is required for the radio metallization of the peptides. When a chelating agent is conjugated with a receptor binding peptide, it can affect both the binding affinity of the peptidic ligand and peptide pharmacokinetics [89]. Radiolabeling of peptides with the oxo-technetium ions ( $\text{TcO}_2^+$ ,  $\text{TcO}^+$ ) is carried out by using peptide-bound tetradentate  $^{99}\text{Tc}^{\text{m}}$  chelators ( $\text{N}_3\text{S}$  or  $\text{N}_4$  scaffold). The peptides are coordinated with donor groups such as amine, carboxylate, or hydroxyl of the HYNIC (hydrazinonicotinic acid) chelator. In order to initiate the labeling reaction, a generator eluate ( $^{99}\text{Tc}^{\text{m}}$ -saline) should be added into a vial containing all the mixtures. For the labeling with  $^{99}\text{Tc}^{\text{m}}$ , the  $[\text{}^{99}\text{Tc}^{\text{m}}(\text{CO})_3(\text{H}_2\text{O})_3]^+$ ; disodium boronocarbonate,  $\text{Na}_2[\text{HBCO}_2]$  (which also serves as an in situ CO source); and stannous ion,  $\text{SnCl}_2$  (reducing agent) are used [89].  $^{68}\text{Ga}$  labeling reaction is initiated by eluting the  $^{68}\text{Ge}/^{68}\text{Ga}$  generator using hydrochloric acid ( $\text{HCl}$ ) (0.05–0.60 M). Approximately 2 mL of the eluent is transferred to the reaction vial. Reaction vial containing a mixture of the lyophilized DOTA-peptide conjugate and sodium acetate buffer with the eluent is heated for 15 minutes at  $100^\circ\text{C}$  (25 min at  $90\text{--}95^\circ\text{C}$ ). The intermediate product is pushed through an extraction cartridge. The final product is analyzed to determine the labeling efficacy and purity [92].

## 5. Cell-penetrating peptides as molecular carries

The selective permeability and hydrophobic profile of the cellular membranes provide strict control of the molecular changes between the cytosol and the extracellular environment [93–95]. Generally, peptides are selective and effective signaling molecules which bind to specific cell surface receptors that are involved in physiological mechanisms such as peptides, hormones, neurotransmitters, growth factors, G-cell receptors (GPCRs), and ion channel ligands [96]. This characteristic of the peptides mentioned above and their attractive pharmacological profile represent a new starting point in the redesign and in-cell recruitment of molecules for therapeutic purposes [93–97]. Prior to the discovery of cell-penetrating peptides (CPPs), various methods have been used for cellular uptake of therapeutic agents and drugs, such as microinjection, electroporation, and liposome- and viral-based vectors, but these have disadvantages such as restricted bioavailability, low productivity, high toxicity, and low specificity [95]. After all these developments, in the late 1980s, a group of short peptides, such as the protein translocation site, membrane translocation sequence, Trojan peptide, or most commonly CPP, which serve as cellular uptake and delivery vectors of large molecules for therapeutic purposes, were identified [94, 96].

### 5.1 Definition and classification of CPP

CPPs are mostly defined as the short (containing less than 40 amino acid residues) partially hydrophobic and/or polybasic natural and synthetic peptides [94, 97]. With the discovery of CPPs, it has emerged as a new tool that allows cell membrane



translocation without significant membrane damage and at low concentrations without using any chiral receptors. Due to the penetrating properties of these effective cell-penetrating peptides, it opened new opportunities for medical treatment and delivery of therapeutic agents across cells *in vivo*. Furthermore, and even more importantly, interdisciplinary studies show that CPPs are able to assist in internalizing covalently or electrostatically conjugated bioactive cargos such as nucleic acids, polymers, liposomes, nanoparticles, and low-molecular-weight drugs, with dose-dependent efficiency, cheapness, degradability inside cells, and low toxicity [94, 95, 97].

There are various classifications that rely on CPPs' qualities. A categorization based on the origin of the peptide is one of the classifications. The first subgroup is also called protein transduction domains (PTDs); they are including protein-derived peptides such as TAT and penetratin. The second subgroup peptides called chimeric peptides may occur two or more motifs from other peptides. And the last subgroup in this category is synthetic peptides such as the polyarginine family [94, 98]. In addition, CPPs are categorized according to peptide sequences and binding abilities to the lipids, including non-amphipathic (naCPPs), primary amphipathic (paCPPs), and secondary amphipathic (saCPPs) CPPs. R9 and TAT (48–60) are examples of naCPPs that have a high cationic amino acid ingredient, and they are bound to the cell membrane with a high consideration of anionic lipids. The second subgroup of paCPPs such as transportan or TP10 have hydrophobic and hydrophilic residues straight from their primary structure. The third subgroup in this categorization is saCPPs such as penetratin, pVEC, and M918. Their alpha-helix or beta-sheet patterns compose amphipathic properties [94].

In summary, *in vitro* cell and membrane modeling studies have shown that paCPPs are more toxic than naCPP and saCPPs.

## 5.2 Cellular uptake mechanism of CPP

### 5.2.1 Direct penetration

Direct penetration of CPPs eventuates along the membrane lipid bilayers without the contribution of receptors in low temperatures via energy-independent mechanism [93, 99]. There are three different types for the internalization pathway of CPPs by direct penetration [93].

#### 5.2.1.1 Barrel-stave model

In the barrel-stave model, a different number of peptides are placed in the inside part of the membrane to give a hoop appearance. Each of these peptide molecules that provide the formation of membrane channels is likened to the boards that make up the barrel. Due to the appearance of the membrane, this model is called as the barrel-stave model. The barrel-stave model is carried out by an accumulation of peptides consisting of cationic peptides in the amphipathic helix structure. In this model, the hydrophilic sides of the peptides are contacted with the solvent, and the hydrophobic sides of the peptide come into contact with the lipid bilayer of the membrane, resulting in channels or pores in the membrane. The critical step in the barrel-stave model is that the peptides recognize each other while they are attached to the membrane. The peptide molecules then interact with the lipid molecules present in the membrane, and the peptides pass to the hydrophobic side of the membrane. By adding new monomers or small oligomers to the resulting structure, the width of the pores increases. The peptides act in this way even at very low concentrations once they penetrate the phospholipid membrane in the target cell [93].

#### 5.2.1.2 Carpet-like model

According to the carpet-like model, firstly an electrostatic interaction occurs between the phospholipid groups on the negatively charged cell membrane bilayer and the positively charged peptide monomers. The hydrophilic sides of the peptide monomers are bound to the surface of the target cell in such a way that they overlap the phospholipid groups in the membrane and cover the cell as a carpet. Once the peptides in this state have reached a sufficient concentration, the peptide molecules rotate, reorganizing the hydrophobic portion of the membrane, causing a similar effect to the detergents and resulting in digestion of the membrane. On the contrary to the model of the barrel-stave model in the carpet model, the positively charged peptides cannot penetrate the hydrophobic parts of the membrane but instead are in a group with the hydrophilic parts facing one another. According to this mechanism, CPPs can be present in various secondary structures, sizes, and linear or cyclic form [93].

#### 5.2.1.3 Toroidal model

In toroidal model, after the helical peptides are parallel to the membrane, the polar groups of the membrane are replaced by the hydrophobic amino acid groups of the peptides. This leads to the formation of cleft that is vertical to the membrane in the hydrophobic part of the membrane, extending along the peptide and lipid groups. The difference of the toroidal model and the barrel-stave model is that the peptides are present vertically in the lipid membrane and always form the pores in combination with the lipid groups [93, 100].

Direct penetration pathway results in an irreversible membrane destabilization and can cause artifact distribution in cells [93, 99]. Therefore, it has been shown that CPP-cargo complexes can be taken into the cell by a mechanism known as endocytosis [93, 101].

#### 5.2.2 Endocytosis

Endocytosis is a natural process that occurs in all cells via energy-dependent mechanism. Different types of pinocytic pathways have been identified, since they vary according to the access of different CPPs and conjugated cargo complexes of CPPs [96].

##### 5.2.2.1 Macropinocytosis

Macropinocytosis, which is related to the folding of the outer surface of the plasma membrane, is a nonspecific uptake of the extracellular compounds. It starts with an invasion of a membrane promoted by actinic cytoskeleton elements to create a pocket and large endocytic vesicles containing different types of cargoes, and it finishes by the occurrence of vesicles called macropinosomes. Macropinocytosis has critical importance in the uptake of TAT and polyarginine [93, 102]. TAT uptake happens when using macropinocytosis without the need for GATs or sialic acids, while plasma membrane-active proteins must be present [103]. Passive transport does not have actin remodeling, so passive transport is recommended for CPP penetration instead of macropinocytosis [104].

##### 5.2.2.2 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis is the specific uptake of CPPs that have been shown to have significance in the uptake of penetratine, TAT peptide, and other

CPPs after inhibition by hyperosmolar media [96]. The mechanism starts with the creation of a vesicle that is covered by a crystal on the interior surface of the cell membrane. After the coating of clathrin proteins by vesicles, it combines with an early endosome which moves via microtubules from the cell into the nucleus. Macromolecules carried on late endosome are fused with vesicles of the Golgi apparatus, which include pioneer of lysosomal hydrolase. After combining, lysosomal hydrolase enzymes are activated, and the late endosome turns into active lysosome. In the lysosome, the endocytosis substance is decomposed [105].

#### 5.2.2.3 Caveolin-mediated endocytosis

Caveolin-mediated endocytosis which starts with a flask-shaped pit in a cave-like membrane is a specific uptake of extracellular molecules. The CPP with its cargo is bound to the membrane, trapped in caveolae which are linked to actinic cytoskeletal elements, and then a protein tyrosine phosphorylation and actin depolymerization are supported. Dynamin is the type of a GTPase liable for endocytosis in the eukaryotic cells that define other actin polymerization on the patch. Finally, cargo-loaded pouches are released inside the cytosol [106]. The various endocytic paths mentioned above have disadvantages such as low specificity, high dependence on the cell line used, and decreased cell viability [96, 107].

#### 5.2.2.4 Escape from endosomes

Endocytosis of CPPs happens in two stages as endocytic entry and endosomal escape. Endosomal escape is a major step to prevent disruption of the cargo in the lysosomes and to allow the cargo to reach the extra-endosomal aim and to achieve its biological activity. Several models have been proposed to prevent endosomal escapes of CPPs. For instance, as a result of the electrical interaction between the negative charges of the endosomal membrane and the positively charged parts of the CPPs, the membrane that triggers the release of the vesicle content leads to stiffing and rupture. According to the other example, reduced pH develops the capability of CPPs to attach to the endosomal membrane and then raises intracellular circulation; as a result of which, the increment of endocytic vesicles may ensure better escape from the endosomal membrane [93, 95].

### 5.3 Factors affecting the mechanism of cellular uptake

#### 5.3.1 Role of glycosaminoglycans

Initially the CPPs and the cell membrane interact via electrostatic linkage to proteoglycans called as glycosaminoglycans (GAGs) such as chondroitin sulfate B. Since these glycoproteins are present all over the cell membrane, they make the cell membrane negative charges, and they compose a platform that connects the CPPs or CPP/cargo binding to the cellular matrix. Recent studies have shown that GAGs are less critical for CPP uptake than the previous opinions [93, 103, 108].

#### 5.3.2 Peptide secondary structure

The secondary structure of amphipathic and tachycardic peptides, the CPP conformation, and the length of the CPP sequence are very related to cellular uptake mechanisms.

### 5.3.3 Role of arginine-rich residues

Structure-activity relationship studies show how important it is to identify the single residues in the CPP structure. When the CPPs are rich in arginine (especially for its guanidinium group), they can compose hydrogen bonds with polar lipid groups. The presence of arginines in the CPP has been related with a better uptake efficiency. In addition, replacement or wiping of arginines may reduce the cellular uptake [93, 104, 106].

### 5.3.4 Role of chirality

Although L amino acid and D amino acid peptides have similar effects against heparin, cell binding affinities of CPPs containing with amino acids in the L amino acid peptides are higher than D amino acid peptides. On the other hand, studies show that using of the D amino acid peptides in terms of increasing cell sensitivity and decreasing enzyme degradation gives better results [93, 101].

### 5.3.5 Role of concentration

It has been understood from the result of thermodynamic analyses that primary and secondary amphipathic CPPs can pass directly through the cell membrane at low micromolar concentrations, but non-amphipathic CPPs often use endocytosis even at low concentrations [109]. The concentration threshold of direct penetration depends on the type of CPPs, cargo, and cell lines [93].

### 5.3.6 Role of cargos

The existence of the cargo could affect the CPP uptake pathway. In addition, dimension and binding methodology are also shown by CPP translocation mechanism.

## 5.4 Molecular detection of CPPs' cellular uptake

### 5.4.1 In cellular assay

The cellular researches largely propose to follow the CPP and/or the cargo uptake or to elicit the molecular mechanisms of the internalization. As a direct method, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has been improved, and quantitatively determination of the amount of intact CPPs in the cells or in the cellular membranes can be provided [110]. Electron microscopy and Raman spectroscopy methods are also other biophysical methods used on cells [111].

### 5.4.2 Fluorescence-based assays

The covalent binding of a peptide to the fluorophore and the measurement of the fluorescence (fluorimetry) of the treated cells are the most common methods used to detect both CPP uptake and localization. In this approach, while confocal microscopy provides location information, the probes in the living cells allow indirect quantification of the peptides. This method cannot show the molecular integrity of internalized entities, so it cannot prove whether the peptide is still attached to the fluorescent probe in the cells. Furthermore, fluorescence quenching can cause



false-positive outcomes to detect the involvement in intracellular parts and transitory binding areas [94, 96, 111]. Also, CPPs are known as cationic peptides, and they tend to link to the external of the cell membrane and may consequently lead to false-positive results [96]. Fluorescence-activated cell sorting (FACS) is another fluorescence approach used for quantitative measurement uptake of labeled CPPs. The cellular uptake of CPPs is measured by sorting cells which relied on fluorescence intensity by a cell sorter equipment [94]. The advantage of using the aforementioned fluorescence-based protocols in the confocal microscopy process is that it allows distinguishing between internalized and extracellular peptides [94, 96].

#### 5.4.3 Functional assays

These experiments, which are really suitable for biotechnological and therapeutic applications, can be carried out to determine the biological responses of linked molecules or cargos and to assess their mechanisms of uptake [1, 94]. These systems are based on internalizing antisense oligonucleotides (ONs) and upregulation of luciferase gene expression [96, 112]. One of the functional assays is the splicing redirection assay. Due to elucidation of the mechanism of CPP-based cell internalization, cell lines are transfected by molecular methods with a plasmid carrying a luciferase gene and having their abnormal splice junction. Technically, the pre-mRNA of luciferase is correctly working, producing the expression of the protein whose activity can be utilized by luminescence when the abnormal joint site is blocked by antisense ONs [94, 96, 112]. Another system takes advantage of Cre-mediated recombination of an improved green fluorescent protein reporter gene causing EGFP expression [94, 96]. Since abnormal splicing of the luciferase pre-mRNA inhibits the translation of luciferase, if this junction is masked with antisense ON, translation of luciferase will result in the expression of activity of the protein, which can be assessed by luminescence. For the investigation of CPP-based transfer of different ONs, their internalization systems have been studied with transfected HeLa pLuc 705 cells [96, 112].

#### 5.4.4 MALDI-TOF-MS assay

This approach is based on the quantification of internalization of peptides in living cells [111]. Furthermore, the quantity of both plasma membrane-bound and internalized peptides is accessible. In this method, an internal standard is needed for the ionization and the detection in MALDI which extremely depends on the structure of the molecule. Information about both the amount and molecular status (degraded or intact) of the internalized peptide can be obtained by using this method. The disadvantage of this method is that no data about the intracellular localization of the peptide can be obtained. Nevertheless this method can get a knowledge about mechanisms of internalization (endocytosis versus direct translocation) and compare the relevant internalization affinities of CPP [96].

#### 5.4.5 Membrane modeling assay

The assay often prefers to simulate the interaction, internalization, and direct translocation of CPPs through the inert plasma membrane [94, 96]. Membrane models are used in other techniques such as  $^{31}\text{P}$ -NMR, small-angle X-ray scattering, calcein or fluorescein-entrapped liposome leakage, and differential scanning calorimetry. These methods are not based on labeling of the CPP sequences with fluorescence agents [1]. Various researches have been performed by using giant or large unilamellar vesicles (GUV and LUV) to examine the translocation qualities of CPPs [96].

Unfortunately, the precise mechanism of CPP internalization is still not fully elucidated. CPPs are internalized into the cells by direct or endosomal mechanisms, and these uptake mechanisms have been demonstrated by various molecular techniques. Though many recent studies have provided strong data for translocation of CPPs into cells, a better physical definition of these entry mechanisms in cells by molecular techniques is still an open challenge for the future.

## 6. Conclusion

Consequently, this chapter provides a brief manual for anyone in the fields of solid-phase peptide synthesis, peptide vaccines, peptide theranostics, and cell-penetrating peptides. While the solid-phase peptide synthesis is the most convenient way to synthesize the peptides, the need for peptides with longer chain structure and peptides including unnatural amino acids has emerged with the identification of the pathological and physiological functions of the peptides through improvements in the molecular biology, bioengineering, and medical imaging areas. Synthetic chemistry will overcome these challenges, and peptides produced for different purposes, such as drugs, vaccines, and therapeutics, will be the solution for incurable diseases such as lots of epidemic disease and some cancer types with available materials and methods. It has been foreseen that the above-mentioned researches can be investigated within the peptide science and technology departments to be established academically in the next decade.

## Conflict of interest

The authors declare no conflict of interest.

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