Review

Main methods and tools for peptide development based on protein-protein interactions (PPIs).

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Abstract: Protein-protein interactions (PPIs) regulate crucial physiological and pathological processes. PPIs are considered a class of biological targets almost infeasible for small molecules because the binding surfaces are usually large and shallow. Peptides are molecules able to bind to these drug targets; they can be used as modulators and mimic one of the interaction partners. This review details the advances in *in silico* peptide design and experimental approaches for the evaluation of PPI-based peptides.

Keywords: peptides; protein-protein interactions; in silico peptides design; in vitro peptides evaluation

1. Introduction

Within an organism, there are multiple protein connections that are crucial for cells. These cellular networks are known as protein interactome and can be defined as a set of physical and functional protein-protein interactions (PPIs). Protein interactome plays an essential role in different physiological and pathological processes such as signal transduction, cell proliferation, growth and apoptosis [1].

Around 650.000 PPIs have been estimated in the human body [2], being suitable for drug development. However, those interacting physically have been considered almost an "unviable" class of drug targets for small molecules, since the interfaces are often large and shallow making difficult the small molecules binding [3]. Exceptions of this rule has been reported such as small-molecule NMDAR/TRPM4 interaction interface inhibitors [4].

Peptides are short aminoacid sequences (<45 aa) having the ability to bind to molecular targets. Currently, more than 7000 natural peptides have been identified that play crucial roles in human physiology as oncological regulators, hormones, antimicrobials, growth factors, ion channel ligands among others [5–9]. They are promising ligands for modulation of PPIs [10–12]. Larger in size than small molecules, peptides are able to cover wider binding sites being ideal candidates for interacting with these therapeutic targets. As ligands, peptides have great therapeutic potential due to their high specificity and activity [13].

Peptides derived from specific protein domains can be used as modulators and mimic one of the participants in PPIs, disrupting the protein-protein binding [14]. A peptide could compete for binding at the interaction

site in solution, where they should have stronger bonds, enhancing binding stability [8]. They are known as competitive peptides.

This article presents a review of the advances in *in silico* peptide design and experimental approaches for the evaluation of PPI-based peptides.

2. Peptides as drugs

The use of peptides as therapeutic agents dates from the first half of the 20th century, since the isolation and first application of insulin, a polypeptide hormone. Insulin is utilized in patients with diabetes who do not produce sufficient amounts of the hormone to maintain normal blood glucose levels [15]. Since then, new techniques have been developed to generate new peptides targeting proteins associated diseases. In fact, there are currently about 400 peptide drugs in clinical development worldwide and more than 60 approved for medical use in USA, Europe and Japan [16]. The fundamental nature of peptides, as small parts of proteins, allows their *in vitro* synthesis. They mimic proteins to regulate specific cellular functions, enabling or blocking biochemical processes in the organism [17]. Latest research has shown the importance of peptides as promising immunotherapeutic agents for the treatment of malignant cancers [18,19].

Peptides as drugs have demonstrated great pharmacological potential for the development of competitive inhibitors that can destabilize the binding of two proteins and/or potentiate the action between them [13]. PPIs have proved their value as target candidates for clinical testing with an average of 20 clinical trials *per* year. In 2023, 119 approved peptides are reported in PepTherDia, a database that contains a complete profile of physicochemical characteristics and diagnostic and therapeutic peptides for human use [20].

However, today the therapeutic use of peptides has limitations. There are enzymes and biological processes capable of decreasing the half-time of peptides such as peptidases and excretory mechanisms. Also, digestive enzymes are capable of cleaving peptide bonds and the high polarity and molecular weight of peptides severely limits intestinal permeability [21]. Different approaches for the rational design of peptides based on PPIs have been studied. They have been shown that the susceptibility to proteolysis and stability of the active conformation could be improved by structural modifications. Two types of structural modifications can be performed: cyclization and modifications of the peptide backbone (See more in 3.3 section). In addition, aminoacid composition affects the physicochemical properties of peptides, such as solubility, stability and reactivity. The presence of hydrophilic aminoacids and charged groups in peptides generates hydrophilicity, which creates obstacles in permeability across biological membranes. Hence, several techniques are used to increase the cell permeability of the peptides while preserving their biological activity [22].

3. *In silico* tools for competitive peptides design

Advances in protein crystallography, the development of protein fragment libraries and the use of molecular simulation have enabled rational peptide recognition and design. *In silico* methods have been widely used in various aspects of different biological studies [23–25]. The advantage of computational methods over empirical methods is their low cost, high processing speed and the ease and reliability of PPI target selection using peptides. Through the use of different bioinformatics tools, it is possible to develop a methodology for the design, evaluation and improvement of competitive peptides for research and/or therapeutic purposes. Competitive peptide design could be divided into 3 main stages: (I) characterization of protein-protein interaction, (II) the design of peptide based on one of the interaction partners and (III) to validate the peptide interactions and evaluation of its activity.
In this section, different approaches for the construction of peptides based on protein-protein interactions through *in silico* methods will be discussed.

3.1. Characterization of protein-protein interactions

Structural information of protein-protein complexes is critical for the rational design of PPI modulators that may have therapeutic properties. Protein-protein complexes available in three-dimensional structure databases and the use of *in silico* methods can help to understand the essential PPIs at the atomic level [26]. The comprehension of these molecular mechanisms allows to predict, manipulate and design novel protein-peptide interactions with broad applications in biology, medicine and pharmaceutical sciences.
Peptides as modulators of PPIs can be subdivided into two different classes depending on the binding interface involved (Figure 1). PPI modulation can be accomplished by designing a peptide that binds to an orthosteric interface (a pocket located at the interface between the two proteins) or an allosteric pocket (a pocket distant from the interface of the interacting proteins) [27,28]. Orthosteric PPI modulators regulate protein contacts either as inhibitors (iPPI) to physically disrupt them and prevent the formation of protein complexes or as PPI stabilizers (sPPI), which work as "molecular glues" to connect two proteins promoting the function of the interaction participants [29,30]. In contrast, allosteric modulators mediate PPIs by binding to an allosteric pocket, inducing a conformational change of the binding proteins. The binding could result in the avoidance (iPPI) or enhancement (sPPI) of PPIs. Targeting allosteric pockets is attractive, as no large molecules are required to bind to these pockets in order to change the protein conformation, but this strategy remains a non-trivial task [26,30]. On the other hand, the orthosteric protein interface provides information about the interaction of the proteins involved that can be used to rationally design modulators mimicking PPIs [29].

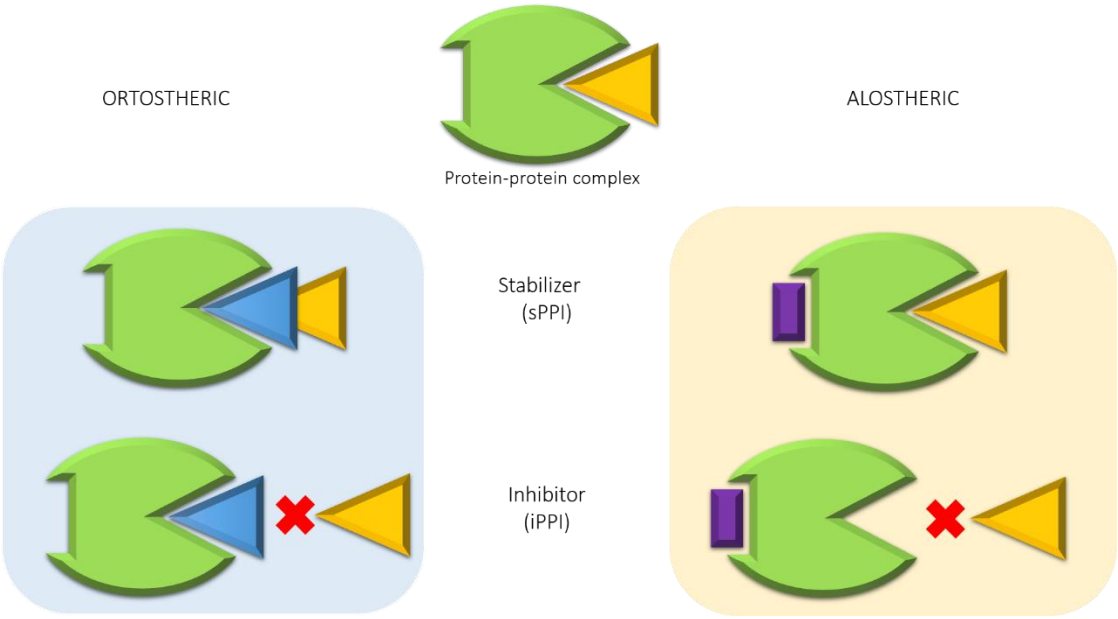

Figure 1. Orthosteric and allosteric mechanisms for stabilizer (sPPI) and inhibitor (iPPI) peptides. Proteins are colored in green and yellow, the allosteric modulator in purple, and the orthosteric modulator in blue.

Considering the knowledge about the type of modulation of PPIs and the proteins associated, different methodologies can be executed to study their physicochemical characteristics. Therefore, it's important to perform an updated bibliographic search to gather structural information about the protein interacting domains and to characterize them in greater detail. A good starting point is to search for the protein network associated with the molecular target in databases related to PPIs. Recognition of the interacting members is crucial, however, more information about the binding mechanism involved in the interaction is required. Different methods for the prediction of PPIs have been developed based on aminoacid sequence and/or three-dimensional structure. Table 1 lists different databases, servers and software related to protein-protein

networks, interaction prediction, allosteric pockets and others that may be useful. These methods can be a good approximation to the recognition of the most interacting regions in PPIs but may require more robust protocols for deep identification.

Table 1. Databases (*), servers (**) and meta-search engines (***) for the identification and characterization of protein-protein interactions (PPIs).

Database	Description	URL
ADAN Database*	Database containing protein-protein structural domain information. Contains 3505 entries with extensive structural and functional information available	http://adan-embl.ibmc.umh.es/
AlloPred**	AlloPred enables allosteric site prediction using perturbation of normal modes together with pocket descriptors in a machine learning approach that classifies pockets of a protein	http://www.sbg.bio.ic.ac.uk/allopred/
ANCHOR*	Database and tool that allows studies of binding pockets based on PPIs. ANCHOR includes a database of pre-calculated anchor residues for 31.000 PDB entries with at least two protein chains (no DNA/RNA chains)	http://structure.pitt.edu/anchor/
BioGRID*	It's a public database that archives and disseminates genetic and protein interaction data from model organisms and humans. BioGRID currently contains over 1.740.000 interactions curated from high-throughput datasets and refering studies derived from over 70.000 publications in the primary literature	https://thebiogrid.org/
comPPI*** (Compartmentalized protein-protein interaction database)	Open-source database with qualitative information for interactions, proteins and their localizations integrated from multiple databases for protein-protein interaction network analysis	https://comppi.linkgroup.hu/
DIP* (Database of Interacting Proteins)	Classifies experimentally determined protein-protein interactions. Combines information from diverse sources to create a single database, a coherent set of more than 11.000 protein-protein interactions	https://dip.doe-mbi.ucla.edu
HPRD* (The Human Protein Reference Database)	Centralized platform to visually represent and integrate information regarding to domain architecture, post-translational modifications, interaction networks and diseases association of each protein in the human proteome	http://www.hprd.org/
IntAct*	Open-source database system and analysis tools for molecular interaction data. Currently, there are 750.000 interactions collected	https://www.ebi.ac.uk/intact/

MatrixDB***	MatrixDB is an open-access database focused on the interactions between extracellular matrix proteins, proteoglycans and polysaccharides.	http://matrixdb.univ-lyon1.fr/
Mentha***	Meta-search engine that integrates protein interaction databases (MINT, IntAct, DIP, MatrixDB and BioGRID) and protein interaction information from published articles. Mentha generates each week a consistent interactome (graph) whit a reliability score for each protein interaction taking into account all the collected evidence	http://mentha.uniroma2.it/about.php
MINT* (Molecular INTeraction Database)	Database designed to store data of functional interactions between proteins. The library has more than 124.000 described interactions	https://mint.bio.uniroma2.it/
MIPS* (Mammalian Protein-Protein Interaction Database)	Collection of high-quality mammalian PPI data, manually compiled from the scientific literature	https://mips.helmholtz-muenchen.de/proj/ppi/
STRING*	STRING database systematically collects and integrates protein-protein interactions-both physical interactions as well as functional associations between proteins	https://string-db.org/
PASSer (Protein Allosteric Site Server)**	PASSer is a web server to predict and identify allosteric sites based on trained machine learning models	https://passer.smu.edu/
PINA v3***	Integrated platform for the construction, filtering, analysis, visualization and management of protein interaction networks. Integrates protein-protein interaction (PPI) data from public databases and builds a comprehensive protein interaction dataset	https://omics.bjcancer.org/pina/
PRO-Simat**	Simulation tool for the analysis of protein interaction networks, their dynamic change and pathway engineering. It provides GO enrichment, KEGG pathway analysis and network visualization from an integrated database of more than 8 million protein-protein interactions in 32 model organisms and the human proteome	https://prosimat.heinzelab.de/

3.2. Competitive peptides design

The design of competitive peptides based on protein-protein interactions can be divided into three main scenarios (Figure 2), each with varying first steps and a common second step across these situations: (A) Using a protein-protein structure as a template: To generate the peptide based on one of the interacting proteins (Step 1). To produce *in silico* mutant versions of the peptide with a protein design algorithm while keeping the peptide backbone coordinates fixed (Step 2). (B) When the PPI binding site is known in both proteins of the complex but the structure of the complex is absent, the peptide can be modeled based on the

interacting protein structures. One of them will be docked in the binding site of the second before peptide modeling (Step 1) [29,30]. (C) Designing a peptide while knowing only the approximate putative PPI site on one or both proteins, e.g., based on evidence of related domains by homology.

The appropriate scenario will depend on the characteristics and available biological information of the system. For example, to successfully apply protein-protein backbone peptide design (scenario A), the three-dimensional structure of the PPI model is needed. In addition, the binding site between the two proteins must be characterized in order to identify the nature of the aminoacids and interactions (hydrogen bonds, salt bridges, etc.) that mediate the binding. The application of this approach can be seen in Zuo. et al. [31] who studied the interaction between GABAB and KCTD16 receptors using a peptide based on the major subunit of the GABAB receptor. The GABAB receptor is a G protein-coupled receptor involved in the activation of downstream signaling pathways. The KCTD16 protein is an auxiliary subunit that interacts with the GABAB receptor and disrupts the signaling kinetics of the receptor. The described peptide-protein interaction model can be used for the development of new ligands that can potentially modulate the association of GABAB and KCTD proteins. In situations where the interaction site is not known, approach C should be employed. However, it requires a large computational capacity to perform a proteome-wide assessment which is limiting in certain cases where the target has a large molecular size (e.g., ion channels). Molecular docking techniques can be considered attractive alternatives for the C scenario [29]. The use of molecular docking gives an approximation of the complex that would form between the peptide and the molecular target [33].

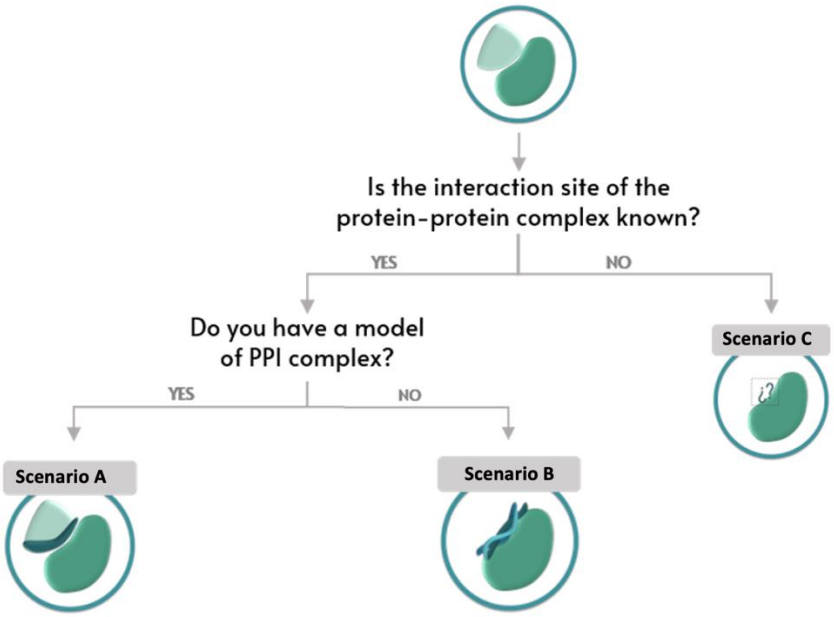

Figure 2. Flowchart of scenario selection for the analysis and design of PPI-based peptides.

A global approach combines the use of molecular target homology structures or models and molecular docking algorithms to design the PPI model and build peptides from the predicted interaction surface between both proteins. Molecular docking is one of the best-known computational methods for predicting binding interfaces between biological targets and molecules [34]. There are two main approaches to protein-protein or protein-peptide docking: global docking and local docking. Global docking methods perform a search for both the binding site and pose of the peptide. Whereas, local docking performs the search for the most favorable pose for the peptide at a known, user-defined binding site [35]. In the case of scenario C, global docking is required to recognize the protein-protein binding interface. From the obtained coupling model, further analysis can be performed to robustly analyze the interaction and recognize the site of highest interaction (see below). It is also possible to use local docking to dock one or several peptide ligands and

to recognize similar binding anchor segments to generate new peptides based on these stereochemical features to be applied in scenario B. In conclusion, molecular docking techniques can be a great tool to recognize: (A) the approximate binding site and/or (B) the most favorable pose for peptide ligands. Thus, the accuracy of docking depends on the input information about the binding site: the more accurate, it's better. The search algorithms mostly employed in docking calculations are Fast Fourier Transformation (FFT) correlation, geometric hashing, geometric recognition and genetic algorithms [33]. Table 2 shows characteristics and descriptions of the main protein-protein and peptide-protein docking tools and servers currently available.

The top ranked docking pose is usually selected as the most likely structure, similar to the native one, and is used to further investigate the protein-peptide interaction interface [36]. However, scoring functions are constructed to be fast and informative, they do not completely correlate with biochemical affinity values. Futhermore, docking scores are often not fully indicative for a most likely biologically relevant structure. In summary, the selection of a likely binding pose based on scoring functions can be imprecise [3].

As a result, these methods are often complemented with molecular dynamics simulations (MDs) and free energy calculations (MM-GB(PB)SA) that can drastically speed up the process and decrease the computational cost of the entire therapeutic peptide design process. This approach is more accurate as it can be used in conjunction with experimental data to help distinguish false positive docking poses from native poses [24]. Molecular dynamics simulations are critical to determine how the subunits of the studied protein-protein complex interact or to understand how the peptide and its receptor bind through a trajectory. The MDs of the protein-protein and peptide-protein complexes can drive the biomolecular complex to an energetically stable conformations, which is more physically and biologically relevant for intermolecular interactions. Finally, generalized Born surface area molecular mechanics calculations (MM-GBSA) and Poisson-Boltzmann surface area molecular mechanics calculations (MM-PBSA) are probably the most widely used methods for making free energy predictions due to their high accuracy compared to most molecular docking score functions and require less computational power than alchemical free energy methods [37,38]. In the study "Improving Protein-Peptide Docking Results via Pose-Clustering and Rescoring with a Combined Knowledge-Based and MM-GBSA Scoring Function" it was shown that post docking re-evaluation with a combined knowledge-based and MM-GBSA scoring function improved binding mode prediction performance and RMSD correlation compared to the original docking method [39]. In addition, MM-GBSA and MM-PBSA calculations allow estimation of the contribution of specific residues by free energy decomposition analysis to identify the dominant interactions in the binding of a protein-ligand complex [40,41]. Among the most commonly used MDs software are NAMD [42], Desmond [43], AMBER [44], GROMACS [45] and LAMMPS [46]. It is important to note that the selection of a suitable software will depend on the structural characteristics of the studied complex. In addition, other calculations can be performed to analyze the stability of the protein-peptide complex along the MDs such as root mean square deviation (RMSD), root mean square fluctuation (RMSF) and principal component analysis (PCA).

Table 2. Tools and servers available to perform protein-protein and peptide-protein docking.

Type	Tool	Algorythm	URL
Protein-protein docking	3D-Garden	MCA	http://www.sbg.bio.ic.ac.uk/~3dgarden/
	ATTRACT	NMA	http://www.attract.ph.tum.de/services/ATTRACT/attract.html
	Autodock	GA	http://autodock.scripps.edu/
	ClusPro	FFT	https://cluspro.org/login.php
	FireDock	MC	https://bioinfo3d.cs.tau.ac.il/FireDock/

	FiberDock	NMA	https://bioinfo3d.cs.tau.ac.il/FiberDock/
	FroDock	FFT	http://frodock.chaconlab.org/
	HADDOCK	SA	https://wenmr.science.uu.nl/haddock2.4/
	Hex	SPFC	http://hexserver.loria.fr/
	InterEVDock v2	FFT	https://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::InterEvDock2
	LZerD	GH	https://kiharalab.org/proteindocking/lzerd.php
	LightDock	GSO	https://lightdock.org/
	MegaDOCK 4.0	FFT	http://www.bi.cs.titech.ac.jp/megadock/
	Piper	FFT	https://www.schrodinger.com/products/piper
	pyDock	FFT	https://life.bsc.es/pid/pydock/
	SmoothDock	FFT	http://smoothdock.ccbb.pitt.edu/
	UDock	FFT	http://udock.fr/
Protein-peptide docking	AnchorDock	MC	N/A
	CABSDock	MC	http://biocomp.chem.uw.edu.pl/CABSdock
	ClusPro PeptiDock	FFT	https://cluspro.bu.edu/
	Dinc 2.0	IA	http://dinc.kavrakilab.org/
	FlexPepDock	MC	http://flexpepdock.furmanlab.cs.huji.ac.il/
	GalaxyPepDock	TB	http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=DOCK
	HPEPDOCK	TB	http://huanglab.phys.hust.edu.cn/hpepdock/
	pepATTRACT	MDB	https://bioserv.rpbs.univ-paris-diderot.fr/services/pepATTRACT/
	PepCrawler	RRTA	http://bioinfo3d.cs.tau.ac.il/PepCrawler/
	Pep-Fold 3	HMM	https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/
	PIPERFlexPepDock	FFT	http://piperfpd.furmanlab.cs.huji.ac.il/
	Rosetta FlexPepDock	MDB	https://new.rosettacommons.org/docs/latest/application_documentation/docking/flex-pep-dock
	SurflexDock	MC	https://htpsurflexdock.biocomp.uenf.br/

FFT: Fast Fourier Transform; GA: Genetic Algorithm; GH: Geometric Hashing; GSO: Glowworm Swarm Optimization; HMM: Hidden Markov Model; IA: Incremental Algorithm; MC: Monte Carlo; MCA: Marching Cubes Algorithm; MDB: Molecular dynamics based; NMA: Normal-mode Analysis; RRTA: Rapidly exploring random tree algorythm; SA: Simulated Annealing; SPFC: Spherical Polar Fourier Correlations; TB: Template based

3.3. In silico peptide optimization

In silico methods for peptide optimization have several advantages, such as low cost, reduced operational time and avoidance of the ethical issues of empirical analysis. MDs analyses can facilitate and indicate which PPI regions are most favorable for peptide design based on the stereochemical characteristics of the binding site. In addition, more rigorous calculations can be performed for the identification of the most important residues for interaction (hotspots) [47,48]. Hotspot recognition is essential for the peptide refinement stage in order to recognize those aminoacids that do not contribute to the binding interface and that can be modified to generate new variants of the peptide. In addition to residue-based energy decomposition via MMGB(PB)SA to detect hotspots, methods such as site-directed mutation, alanine scanning and sequence analysis can be used to look for the most conserved aminoacids within the protein family associated with the molecular targets. There are several alternatives to increase the binding energy

and/or physiological stability of the peptide in the organism based on hotspot information; among the most used are modification and cyclization methods (backbone modifications or cyclic linkers).

The first approach is based on performing modifications to the peptide structure to enhance its activity and/or facilitate cell permeability. Among the strategies employed are variation of the stereochemistry, modification of the sidechains or addition/substitution of D-aminoacids forming hybrid peptides (containing D and L aminoacids) or D-peptides (containing only D aminoacids) (Figure 3A). Other modification is the displacement of the sidechains towards nitrogen atoms (peptoids) (Figure 3B). On the other hand, β-peptides are formed by β-aminoacids that differ from common residues because the amino group is attached to the beta carbon (Figure 3C). AApeptides are based on the chiral peptide nucleic acid (PNA) backbone. The nucleic acid bases are replaced by aminoacid side chains. They can have at least two subclasses, α-AApeptides and γ-AApeptides, based on the positions of their chiral aminoacid side chains along the peptide backbone (Figure 3D) [49].

The second approach is based on giving rigidity to the active conformation of a peptide. Cyclization is a type of peptide modification that consists of creating a "loop" structure within the peptide sequence by means of a "linker". In addition to their artificial synthesis, it is also possible to extract cyclic peptides from nature. Hepcidin is a hormone that plays an important role in the regulation of iron homeostasis. The structure of hepcidin consists of 25 residues and four disulfide bonds necessary for its proper folding and activity. The authors developed a new strategy for the synthesis of human hepcidin using a cysteine protecting group (S-(4,4'-dimethylsulfinylbenzhydryl)) that allows the regioselective generation of the peptide disulfide bonds [50]. Many naturally occurring pharmacologically active cyclic peptides have a head-to-tail configuration, conferring resistance to hydrolysis by exopeptidases due to the absence of N- and C-termini [51]. This type of modification is usually done through an amide bond between the C-terminal carboxyl and the N-terminal amine in a head-to-tail conformation or by promoting a disulfide bond by mutating one of the residues of interest to cysteine. Cyclized peptides are formed besides head-to-tail (Figure 3E), through head/tail to side-chain (Figure 3F), backbone to backbone (Figure 3G) and side-chain-to-side-chain (Figure 3H) cyclization reactions [52]. In head-to-tail cyclization or also known as *backbone* cyclization the C-terminal acid or N-terminal amine polar groups are removed to reduce the impact of cell membrane permeability and limiting degradation [53]. Side-chain to side-chain linkages are distinguished by the introduction of covalent bridges knowns as “staples”[53,54]. In backbone-to-backbone different configurations can be used such as backbone binding through mutation by proline [55], by insertion of two cysteines generating a disulphide bond [56] and by using linkers to generate other configurations, such as proline-cysteine bonding generated by alkylation of the cysteine side chain with benzyl bromide based electrophilic linkers [57]. The use of cyclic peptides has shown greater cell permeability and stability than linear peptides [58,59]. Cyclization facilitates intramolecular hydrogen bonding within the “ring” structure, reducing the ability to form hydrogen bonds external to the molecule; this decreases the polarity and increases the membrane permeability of the peptide compared to its acyclic precursors [60].

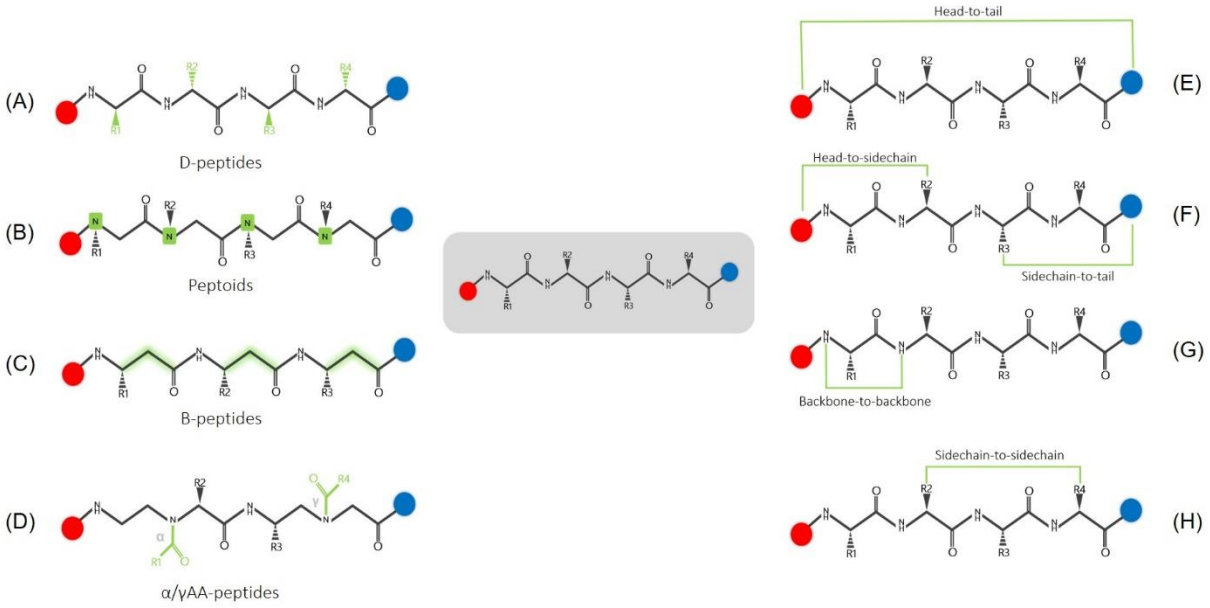

Figure 3. Structural modifications for peptides. Backbone modification (A-D) and Cyclization (E-F). The original peptide is in the gray box. The modifications/cyclization are shown in green.

Peptide cyclization can be carried out using various linkages, among the most common types of links are disulfide and amid bonds. Disulfide bonds are the most common used to cyclize peptides. They are formed by the oxidation of two cysteine residues, resulting in a covalent bond between the two sulfur atoms. Disulfide bonds are a common covalent bond found in proteins and other naturally occurring compounds that confer structural stability. Thus, the specific distances suitable for two cysteines to take alpha-helical (i, i+7)[61] and β-sheet conformations were already known [62]. However, disulfide bonds are intrinsically unstable in a reducing environment. Amide bonds are a covalent bond that forms between the carboxyl group of one residue and an amine functional group of another aminoacid. Traditional amide bond formation can be challenging for head-to-tail cyclization of peptides of less than seven residues, as cyclodimerization and C-terminal epimerization can occur [51]. Furthermore, particular care must be taken at the retrosynthetic planning stage since aminoacids that have esteric hindrances to the generation of the amide bond may reduce yield [63]. New types of artificial linkages have been developed with new techniques to generate peptide cyclization. When choosing the type of linkage to use in the design of in silico peptides, it is important to recognize the advantages and disadvantages in terms of synthesis, bioavailability, solubility, toxicity, among others. Tools and servers for rational peptide design are listed in Table 3. Finally, it should be kept in mind that any change in the chemical structure of the peptide is likely to have an im-pact on its biological activity, so it should be evaluated simultaneously or subsequently perform wet experiments to estimate the physicochemical properties of the molecule.

Table 3. Useful software and servers for peptide design.

Database	Description	URL
Bioware	Several tools for short linear motif discovery (2-12 residues) and peptide characterization, as well as related analysis of proteomic data.	http://bioware.ucd.ie/~compass/biowareweb/
CPPpred	Server for cell-penetrating peptide prediction based on a novel N-to-1 neural network. CPPpred aims to determine for a set of peptides the probability that each peptide is cell-penetrating. Peptides can be simply entered into the text box, and will be ranked according to their CPPpred score.	http://distilldeep.ucd.ie/CPPpred/
Cons-PPISP (consensus Protein-Protein Interaction Site Predictor)	It uses a consensus neural network method to predict protein-protein interaction sites. Through the server, the position-specific score matrix (PSSM), solvent accessibilities and spatial neighbors of each input protein residue can be obtained in PDB format.	https://pipe.rcc.fsu.edu/ppisp.html
CPPSite 2.0	Updated and easy-to-use database that provides a variety of information on CPPs and contains 1855 entries. This database provides complete information on experimentally tested CPPs and prediction of their secondary and tertiary structures to realize their structure-function relationship.	https://webs.iiitd.edu.in/raghava/cppsite/
finDr	A novel web server for the computational identification and optimization of D-peptide ligands to any protein structure. finDr provides a low cost and easy-to-use alternative for the identification of D-peptide ligands against protein targets of choice without size limitation.	https://findr.biologie.uni-freiburg.de/
mCSM-PPI	It allows prediction of the effects of nonsense mutations on protein-protein affinity. The method uses an optimized graph-based signature approach to better assess the molecular mechanism of mutation by modeling the effects of variations in the non-covalent interaction network between residues using graph kernels, evolutionary information, networks and energy term.	http://biosig.unimelb.edu.au/mcsm_ppi2/
MDockPeP Server	It predicts the structures of protein-peptide complexes from protein structure and peptide sequence. The prediction process consists of three steps: (1) Modeling of peptide conformers; (2) Global and flexible sampling of protein-peptide binding modes; (3) Scoring and ranking of the sampled binding modes.	https://zougrouptoolkit.missouri.edu/mdockpep/
neXtProt	The peptide uniqueness checker. It allows scientists to define which peptides can be used to validate the existence of human proteins, i.e. uniquely mapping against multiplicity of human protein sequences taking	https://www.nextprot.org/tools/peptide-uniqueness-checker

	into account isobaric substitutions, alternative splicing and single amino acid variants.	
PARCE-1	Protocol for Amino acid Refinement through Computational Evolution that implements an advanced and promising method for the design of peptides and proteins. The protocol performs a random mutation in the binder sequence, then samples the bound conformations using MD simulations, and evaluates PPIs from multiple scoring	https://github.com/PARCE-project/PARCE-1
PCPIP (Protein Complex Prediction by Interface Properties)	It uses a classification scheme based on support vector machines. The server allows 2 inputs: individual and batch. In the individual option, a protein-protein complex (homo- or heterodimer) can be loaded in PDB format to identify whether the interaction exists and if so, whether they resemble interfaces extracted from native proteins.	http://www.hpppi.iicb.res.in/pcpip/info.php
PEPFOLD3	Uses a hidden Markov model-derived structural alphabet for *de novo* modeling of 3D conformations of peptides of linear and disulphide bonded cyclic peptides with 9-36 amino acids using benchmarked peptides	https://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::PEP-FOLD3
PeptideCutter	Predicts potential cleavage sites cleaved by proteases or chemicals in a given protein sequence. Returns the query sequence with the potential cleavage sites mapped onto it and/or a table of cleavage site positions.	https://web.expasy.org/peptide_cutter/
PeptideMass	PeptideMass cleaves a protein sequence from the UniProt knowledge base (Swiss-Prot and TrEMBL) or a protein sequence entered by the user with a chosen enzyme, and calculates the masses of the generated peptides.	https://web.expasy.org/peptide_mass/
PeptideMine	Web server developed to identify and analyze peptides suitable for protein-peptide binding studies. Integrated GO annotations, protein-protein interaction data, peptide-domain mapping, domain-domain interaction data and various peptide-related feature calculations provide a reliable approach.	http://caps.ncbs.res.in/peptidemine/
PROVEAN (Protein Variation Effect Analyzer)	Available as a server and as an executable. Predicts whether an amino acid substitution, insertion and deletion has an impact on the biological function of a protein. Uses a score-based method.	http://provean.jcvi.org/index.php
Signa1P	The server predicts the presence of signal peptides and the location of their cleavage sites in proteins from Archaea, Gram-positive and Gram-negative Bacteria and Eukarya. In Bacteria and Archaea.	https://services.healthtech.dtu.dk/services/SignalP-5.0/

Other methodologies suggest the addition of membrane permeability elements such as Cell-Penetrating Peptides (CPPs). CPPs are peptides with an extension of 5 to 30 residues that can be used as protein mimics and cargo transport vectors [64]. CPPs can be classified into: (I) cationic peptides, positively charged peptides that interact with the negatively charged cell membrane and facilitate the uptake of other molecules

into the cell. The crucial role of positively charged residues (such as lysine and arginine) of CPPs in the adsorption of macromolecules has been demonstrated [65,66]. Polyarginines are a well-studied group of CPPs. In 2007, Tünnemann *et al*. demonstrated that a minimum of eight arginine residues improves cell membrane penetration; a higher number would increase penetration efficiency [67]. Among the most studied CPPs are TAT (GRKKKRRQRRR) and penetratin (RQIKIWFQNRRMKWK). (II) amphipathic peptides (or amphiphilic) that contain in their structure alternate regions of polar (hydrophilic) aminoacids and non-polar (hydrophobic). Amphipathic CPPs are the most numerous subclasses of CPPs, often divided into two groups: primary and secondary amphipathic peptides based on their sequence, length and association with lipids [68]. In primary amphipathic CPP its two regions are distributed next to each other in the primary sequence. The secondary amphipathic CPPs form functional hydrophilic and hydrophobic regions after folding into α-helical and β-sheet-like structures [69]. The charge of these molecules can be positive, neutral or negative.

In addition to emulating proteins to bind to a molecular target and mimic its biological function, CPPs can be used to transport different molecular cargoes into the cell [70]. CPPs have great potential to enhance cellular uptake of various molecular cargoes into the cell through the mechanism of endocytosis [64,71,72]. There are two approaches for the adhesion of molecular cargo and CPPs: (I) Binding the CPPs vector and the cargo by non-covalent bonds such as electrostatic interactions. An example is the amphipathic peptide transporters MPG and Pep-1 which bind their molecular charges without crosslinking or chemical alterations [73–76]. (II) Use covalent bonds to join the two molecules. This method is the most widely used and has shown positive results with common delivery vectors such as penetratin, TAT and Poly-Args polypeptides [74,77,78].

Recently, peptides with tumor cell penetrating properties termed "tumor-homing peptides" have been reported. Tumour-homing CPPs are oligopeptides that consist of 30 or fewer aminoacids and can effectively and specifically bind to tumour cells. Multiple tumor-homing peptides have been developed (Table 4) that could be applied as non-invasive carriers *in vivo* for the detection and treatment of different oncological diseases. One of the most common methods for identifying tumour-homing CPPs is biopanning, using a peptide library based on *in vivo* phage display technology [70,79]. In this technique, the target cell type is exposed to a large combinatorial library of phages with modified envelopes that can carry peptides of different lengths and structures. This makes possible to discriminate which phages-associated peptides can bind or be internalized by tumor cells or normal cells. The strength of this method is that cell-specific peptides can be isolated without the prerequisite of knowing a surface biomarker [80,81].

Table 4. Tumor-homing peptides recently reported.

Peptide	Sequence	Target Molecule	Cancers	Reference
Angiopep2	TFFYGGSRGKRNNFKTEEY	LRP1	Glioma/Glioblastoma	[82]
CREKA	CREKA	Fibrin-fibronectin complex	Triple negative breast cancer	[83]
DWVAP	WVAP (D-amino acids)	GRP78	Glioblastoma	[84]
EETI 2.5F	GCPRPRGDNPPLTCKQDSDCLAGCVCGPNGFCG	α/β Integrin	Various lineage tumors	[85]

iNGR	-	CD13/NRP-1	Triple negative breast cancer	[86]
iRGD	CRGDK/RGPD/EC	αvβ3 integrin/NRP-1	Prostate cancer, breast cancer, lung cancer	[87]
Lin TT1	AKRGARSTA	p32	Murine breast cancer	[88]
LN-1	CTGTPARQC	Unidentified	Prostate cancer	[89]
LyP-1	CGNKRTRGC	p32	Breast cancer,	[90]
PL1	PPRRGLIKLKTS	Fibronectin/tenascin-C	Glioblastoma, prostate cancer	[91]
PL3	AGRGRLVR	Tenascin-C	Glioblastoma, prostate cancer	[92]
UNO	CSPGAKVRC	CD206	Breast cancer, glioblastoma, metastatic melanoma, peritoneal carcinomas	[93]

Once the peptide has been designed and optimized, using the techniques mentioned above, it is necessary to produce it in large quantities to evaluate the biological activity *in vitro* or *in vivo*. Considering that PPIs are peptides, it is more complicated to use traditional molecular biology techniques for protein expression, for example using cell-free gene expression systems such as Escherichia coli [94]. For the synthesis of smaller proteins or peptides, chemical techniques are used, mainly solid phase peptide synthesis (SPPS) and solution phase synthesis (SPS). In both methods, the process involves the directed and selective formation of a peptide bond. Contrasting ribosome protein synthesis, synthetic peptides are connected in the C to N direction, where the C-terminus of the aminoacid is attached to a resin. In summary, the free N-terminal amine group is coupled to a single N-protected aminoacid. This new aminoacid is then deprotected, revealing a new N-terminal amine group to which another aminoacid may be attached, thus the peptide increases in size [95].

SPS was the first method used for peptide synthesis. During SPS, the peptide chain elongation can be carried out by the segment condensation method. The segment condensation technique uses short fragments of a particular peptide previously synthesized and then are coupled together to form a longer peptide. The prime advantage of SPS for peptide synthesis is for large-scale synthesis of short peptides because SPPS can be expensive. Examples of peptides synthetized using SPS are Biphalin, an opioid agonist, and Oxytocin, a hormone used to facilitate childbirth [96,97]. However, a disadvantage of SPS is the long reaction time [95,98].

In contrast to SPS, the SPPS use a resin as a support to anchor the peptide growing chain. SPPS use t-Boc and Fmoc chemistry methods to produce synthetic peptides. However, t-Boc SPPS is used only for specific

applications being the Fmoc now used as the main way to synthesize peptides because it needs fewer corrosive reagents and can be automatized [99]. During SPPS synthesis, after the peptide formation, the protection group of the aminoacid must be removed, and the resin washed preceding the addition of the next aminoacid. This process is repeated until the peptide is fully accomplished, then the peptide is cleaved from the resin [100]. However, the synthesis of peptides sometimes can be challenging, for example, long or hydrophobic peptides, the introduction of unconventional aminoacid or cyclization. To address these limitations several strategies have been developed such as native chemical ligation (NCL), click chemistry or microwave assisted SPPS [98,101–104]. The broad application of peptides for biomedical purposes has promoted advances in synthetic peptide methods, researchers focused on improving the different techniques are rising trying to develop easier and faster techniques to produce synthetic peptides to fulfil the pharmaceutical industry demand [98,105–107]

4. Experimental analysis for the evaluation of peptides in vitro

Protein-protein interactions (PPIs) are critical in numerous biological processes, including signal transduction, metabolic regulation, and gene expression [108]. Understanding these interactions is essential for elucidating the molecular mechanisms underlying these processes and for identifying potential therapeutic targets. This section provides an overview of the widely used methodologies employed for assessing protein-protein interactions, including co-immunoprecipitation (Co-IP), yeast two-hybrid (Y2H), affinity purification-mass spectrometry (AP-MS), proximity-based labeling (PL), Förster resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET). Each method has its advantages and limitations, and the method of choice depends on the specific experimental requirements. Often multi-methodological approaches are necessary to determine PPIs reliability. In this sense, protein-protein interaction is usually determined using both imaging-based and biochemical-based approaches in a complementary manner. This section summarizes the main methodologies used to evaluate, determine and characterize protein-protein interaction.

4.1 Co-immunoprecipitation

Protein-protein interaction has been canonically evaluated by cell fractionation followed by affinity purification methods such as co-immunoprecipitation (Co-IP). This chromatography-based method is a widely used laboratory technique for studying protein-protein interactions in biological systems [109]. This technique allows the identification of interacting proteins and therefore helps reveal the components of protein complexes. Co-IP involves the selective precipitation of a target protein and its interacting partners using specific antibodies [110]. Despite these being shown to be an excellent approach for strong and stable PPIs, this is unsuitable for weak and transient PPIs interaction. In this sense, using crosslinkers, such as sulfur-NHS-ester and EDC crosslinkers, has been shown to stabilize those interactions [111] in order to overcome that limitation.

4.2 Yeast two-hybrid

The yeast two-hybrid (Y2H) system is another widely used genetic method for identifying and characterizing protein-protein interactions [112]. This technique relies on reconstitution of a functional transcription factor when two proteins of interest interact. The system consists of two fusion proteins: one containing the DNA-binding domain (BD) and the other containing a transcription factor's activation domain (AD). If the two proteins of interest interact, the BD and AD come into proximity, activating the transcription of a reporter gene. A variation of this methods allows high-throughput screening of protein interactions using yeast libraries [113]. In this way, this method has the potential to reveal a complete interactome of a protein of interest.

4.3 Föster resonance energy transfer-based assays

Alternative methodologies used for PPIs validation are based on the Föster resonance energy transfer phenomena. Fluorescent and bioluminescence reactions have been used as alternative approaches. Fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) are two widely used methods for studying protein-protein interactions (PPIs) in living cells. Both techniques are based on the transfer of energy between two fluorophores, with FRET utilizing fluorescent proteins and BRET using bioluminescent proteins.

At first, FRET occurs when the excited state energy of a donor fluorophore is transferred non-radiatively to an acceptor fluorophore in close proximity, resulting in emission from the acceptor fluorophore. The efficiency of FRET is inversely proportional to the distance between the donor and acceptor fluorophores, making it a highly sensitive technique for measuring molecular interactions in the spatial distance of nanometer (<10 nm) [114].

In contrast, BRET relies on the transfer of energy from a bioluminescent protein, such as Renilla luciferase or NanoLuc luciferase, to a fluorescent protein, such as GFP or YFP, via resonance energy transfer [115]. Unlike FRET, BRET is a luminescent phenomenon and therefore does not require excitation by an external light source. This allows for non-invasive and real-time PPI monitoring in living cells [116].

Both FRET and BRET have been widely used to study PPIs in various cellular contexts, including signal transduction pathways, protein trafficking, and protein-protein interactions involved in disease pathways [116]. In recent years, improvements in the design and sensitivity of both FRET and BRET sensors have enabled more accurate and quantitative measurement of PPIs in living cells [117,118].

Overall, both FRET and BRET are useful tools for investigating the dynamics and regulation of PPIs in living cells. Their unique properties make them complementary techniques for measuring protein-protein interactions in different biological contexts, and they continue to be widely used in both basic and translational research.

4.4 Split-fluorescent protein assays

Similarly to FRET and BRET assays, the use of complementation of split-fluorescent proteins allows the determination of PPIs in living cells. This method has been probed successfully on multiple protein-protein interactions using bi (biFC) [119] or tri-molecular (triFC) fluorescent complementation [120]. Both approaches are based on the principle of complementation, which occurs when two or three fragments, respectively of a protein are brought into close proximity, allowing them to reconstitute a functional fluorescent protein. In biFC, the protein of interest is fused to two non-fluorescent fragments of a fluorescent protein, such as yellow fluorescent protein (YFP) or green fluorescent protein (GFP). The fragments are typically the N-terminus and C-terminus of the fluorescent protein. The biFC and triFC assays have several advantages over other methods for detecting PPIs, including their ability to detect weak or transient interactions and their compatibility with live-cell imaging [121]. Additionally, biFC can be used to study the subcellular localization of protein complexes [121].

One potential limitation of the complementation of split-fluorescent proteins is that the fusion of the fluorescent protein fragments to the proteins of interest may interfere with their normal function. However, this can be minimized by careful selection of fusion sites and controls [122][122].

4.5 Mass spectrometry-based approaches

High-throughput methodologies have allowed the identification and characterization of protein-protein networks (popularly known as interactomes). In this context, there are multiple mass-spectrometry approaches to determine those interaction networks. Affinity purification coupled mass-spectrometry (AP-MS) is a widely used method for the identification of protein complexes and the determination of protein-

protein interactions. In this method, a protein of interest is expressed as a fusion protein with an affinity tag (e.g., FLAG or His-tag) or by assessing Co-IP. The protein complex is then purified using affinity chromatography, and the interacting partners are identified using mass spectrometry. AP-MS provides high specificity, but its sensitivity may be limited by the abundance of proteins and the strength of the interactions [123]. On the other hand, for weak and transient PPIs interaction, using crosslinkers in the sample preparation has been shown useful to overcome that limitation [124].

Since the past decade, proximity-based labeling (PL-MS) has emerged as a novel methodology to improve the identification of weak and transient PPIs. This approach relies on using genetically encoded enzymes, known as proximity labeling enzymes, such as APEX or BioID, which are fused to a protein of interest.

A specific small molecule or light activates the proximity labeling enzyme and could then covalently attach biotin to proteins that are in proximity [125,126]. Biotin is a small molecule that might be easily isolated and identified using mass spectrometry. By identifying the biotinylated proteins, it might be determined the proteins that are near the protein of interest, which might provide valuable information about protein-protein interactions in the cell.

The PL-MS technique has several advantages over traditional protein-protein interaction assays. This can be used in live cells, allowing the study of interactions in their natural environment [127]. Despite the described methodologies being high-throughput and powerful tools, the results often require interaction validation by other methodologies, such as the aforementioned.

5. Final Considerations

The use of protein-protein interactions as precursors of suppressor or stabilizing peptides allows us to mediate protein networks involved in different pathologies. In addition, the use of *in silico* techniques can facilitate the development and cost of these molecules. Undoubtedly, the addition of computational methods to traditional methodologies allowed a breakthrough in the development of new therapies. New developments should focus on the rational design of peptides with the goal that they can be manipulated and thoroughly modified to increase their activity and/or cell permeability. The development of customized peptide databases based on PPIs to assess interaction sites and mold peptides based on binding pocket characteristics could be successful customization strategies that would allow comprehensive interaction mapping [128]. Also, the breakthrough of machine learning and artificial intelligence methods could be a useful application for peptide design, allowing the characterization of the binding site based on an algorithm that predicts the most favorable residues for the design of a peptide based on the interaction pocket.

These approaches could contribute to a better performance and optimization of the usual methods used in structural bioinformatics; which in turn, increases the possibility of generating novel *in silico* compounds with better performance to be evaluated in *in vitro* assays. Finally, we expected that this review can contribute as a quick guide of considerations to evaluate for the design of PPI-based peptides.

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