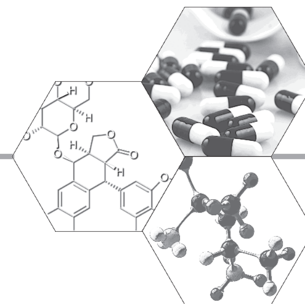

Using peptides to study protein–protein interactions

Protein–protein interactions (PPIs) govern all aspects of cell function and, as such, are a major target for research and therapeutic intervention. A major rate-limiting step in PPI research is the expression and purification of full-length proteins. The use of peptides to study PPIs significantly facilitates the structural and biophysical characterization of PPIs as well as the effort to develop drugs to control PPIs. Here we describe examples for the use of peptides to study PPI and some of the important experimental methods that are used in the field. Peptides have proved to be excellent tools to study PPIs and have been contributing both for understanding mechanisms of PPIs as well as for drug design for PPI modulation.

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Protein–protein interactions (PPIs) control the function of living cells. Signal transduction, cell cycle, proliferation and metabolism are just examples of the fundamental processes governed by PPI. Quantitative, functional and structural studies of PPI enable the understanding of these processes at the cellular and molecular levels and provide a basis for designing drugs that inhibit or stimulate PPIs. In recent years, the interest has been shifting from studying single PPIs to a systematic view on PPI networks and towards obtaining an interactome for organisms, comprised of multiple single PPIs [1,2].

Studying the biological role of a certain protein essentially involves the detection of partner proteins with which the protein interacts and exerts various biological functions. Several experimental methods were developed for discovering PPI by screening multiple proteins for interaction with a specific protein. High-throughput yeast two-hybrid [3] analyses identify direct binary PPIs [4]. MS combined with tandem affinity purification is another powerful method to detect and purify protein complexes [5,6]. Information about PPI is also obtained from DNA microarrays. Similarity of gene-expression profiles represents proteins that are present in stoichiometric amounts and are, hence, likely to interact. Genes of interacting proteins in yeast are more likely to be coexpressed compared with genes of noninteracting proteins [7]. Additionally, coevolution of gene expression better predicts PPI than coevolution of protein sequence [8]. Once a binary PPI is detected, the next step is a quantitative characterization of the interaction, including its structural, biochemical and biophysical parameters. These may include locating the binding sites on the surfaces of both proteins, detecting the residues that mediate the interaction and their differential thermodynamic contribution to the interaction, measuring the affinity of the interaction and studying the biological role of the interaction in cells. A major rate-limiting step in PPI studies is the expression and purification of the interacting proteins. For a full characterization, both proteins should be expressed and purified in the required amounts and their structure should be determined. Many proteins are insoluble or toxic to the expressing host and, thus, are very difficult to obtain in the relatively high concentrations required for structural and quantitative studies such as NMR, surface plasmon resonance, fluorescence anisotropy or isothermal titration calorimetry (ITC). The use of peptides to study PPIs introduces several substantial advantages:

- Peptides up to several dozens of residues long may be synthesized automatically and quite easily and, thus, small protein domains can be chemically synthesized, overcoming problems of expression and purification;
- Using peptides enables research to focus on the actual binding sites and precisely identifying the binding residues;
- Using peptides enables introduction of post-translational modifications (e.g., phosphorylation or acetylation), labels (e.g., biotin or fluorescein) or non-natural amino acids with 100% specificity;

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- Studying the interaction between a full-length protein and a short peptide derived from the protein's partner is technically easier than studying the interaction between two full-length proteins. To obtain the full interaction parameters, the interaction should be studied at both peptide and protein levels;
- Peptides offer a good model for binding studies of protein domains since, although they are usually unstructured in their unbound state, they undergo conformational complementarity upon protein binding to gain the native structure, as was shown for example for peptides derived from the BH4 domain of Bcl-2 [9] and from Bak [10].

Here we will review recent examples where peptides were used for characterization of PPI at both the structural and biophysical levels and examples where peptides were used for modulating PPI, such as agonists or antagonists. In some cases peptides served as the basis for further design of small molecules to target PPI.

Methods in peptide-based studies of PPIs

As biologically active substances, peptides are studied in the whole variety of research schemes in biology, biochemistry and biophysics. These range from the molecular level via the cellular level to the organism level, including, for example, theoretical modeling, biochemical and biophysical studies, molecular and cellular biology and animal models. In employing peptides to study PPIs, the frequently used methods are aimed at providing structural, quantitative and biophysical characterization of the biomolecular interactions. These methods include x-ray crystallography, circular dichroism (CD) spectroscopy and NMR spectroscopy for structural characterization, ELISA and other semiquantitative immunoasssys for binding detection, ITC and fluorescence anisotropy for obtaining the binding affinities and thermodynamic parameters and methods such as stopped flow and surface plasmon resonance to study the binding kinetics. The experimental methods that boosted the use of peptides to study PPI are related to peptide synthesis. These include the introduction of solid-phase chemical peptide synthesis, synthesis of stabilized peptides (e.g., by cyclization or hydrocarbon stapling) and the development of SPOT synthesis and peptide arrays, featuring the synthesis of numerous peptides on cellulose sheets and enabling simultaneous large-scale peptide interaction studies. Detailed descriptions of these methods are given below.

Peptide synthesis

The solid-phase peptide synthesis method (SPPS) was introduced in 1963 by Robert Bruce Merrifield [11], who was awarded the Nobel prize for his discovery. In SPPS, the peptide is synthesized on an insoluble resin from the C- to the N-terminus through cycles of amino acid protection, coupling and deprotection (for details see [12]).

The solid-phase method revolutionized the chemical synthesis of peptides. The effort needed for SPPS was estimated as approximately 50-fold less than a solution synthesis of the same peptide [12]. SPPS also overcame solubility problems by the attachment of the growing protected peptide to the resin support, which subsequently enabled the use of standardized protocols for the total chemical synthesis of peptides. This led to automation of peptide synthesis, which made synthetic peptides widely and routinely available for the scientific community. Advances in peptide chemistry led to the development of methodologies for chemical synthesis of proteins of up to 150 residues and more [13–15].

Peptide stabilization: peptide cyclization & hydrocarbon stapling

The main shortcoming of using linear peptides for therapeutic purposes is their wide conformational range and short lifetime. Thus, methods were developed to improve the stability of peptides and to capture their bioactive conformations. Two such important methods are the peptide cyclization and hydrocarbon stapling.

Peptide cyclization

This approach evolved both in nature [16–18] and in synthetic work. Cyclization imposes conformational constraints on the peptides. By stabilizing peptides, cyclization conveys higher binding affinity, selectivity and bioavailability and enables peptides to be used as lead compounds for drug design. Different chemical methods for peptide cyclization are reviewed elsewhere [19,20].

Conformational peptide libraries, in which all peptides have exactly the same amino acid sequence but different conformations, are used to identify the cyclization mode that will give the peptide its bioactive conformation [21–30].

■ Miniature proteins

The miniature proteins approach was developed by Schepartz *et al.* [31,32]. A miniature protein is a well folded but inactive protein fragment. Once a protein binding site is recognized, it may be grafted on the miniature protein and serve as a PPI modulator. In the next stage, chemical diversity may be introduced to generate libraries of miniature proteins for binding optimization. Miniature proteins provide better proteolytic stability over synthetic peptides. Selected examples for the use of miniature proteins include the apoptosis regulators Bcl-2 family [33,34] and the tumor suppressor p53 with its inhibitor MDM2 [35]. The miniature proteins concept is reviewed with more examples elsewhere [36].

■ Stapled helices

Helical peptides affect many PPIs, but native helical peptides usually display relatively low potency, instability and inefficient delivery to cells. To overcome these problems, methods were developed to stabilize helical peptides. Verdine and co-workers developed the hydrocarbon stapling method **(Figure 1)** [37,38]. Hydrocarbon-stapled peptides are short, single α-helices stabilized by hydrocarbon bridges at the (i + 4) or (i + 7) positions of the helix via the introduction of olefinic side chains at the α-carbon of non-natural amino acids employing metathesis chemistry. Another method for helix stabilization was developed by Arora and co-workers, where one of the main-chain hydrogen bonds is replaced with a covalent linkage, named covalent stapling [39].

■ Peptide arrays

SPOT synthesis was developed by Ronald Frank [40] and co-workers in 1990. SPOT synthesis technology enables the parallel synthesis of large numbers of peptides in small amounts on cellulose sheets. The SPOT method is based on SPPS and follows standard Fmoc chemistry. The cost per peptide is estimated as less than 1% of peptides synthesized conventionally on resin. This led to the development of peptide arrays – cellulose membranes on which multiple peptides are synthesized and screened for binding a target protein **(Figure 2)**. This interacting protein is scattered on the cellulose arrays, allowing it to interact with the synthesized peptides. Following washing of the unbound protein, the detection is mostly carried out by labeled probe methods, such as fluorescence, chemiluminescence, electrochemiluminescence or radioactivity detection [41].

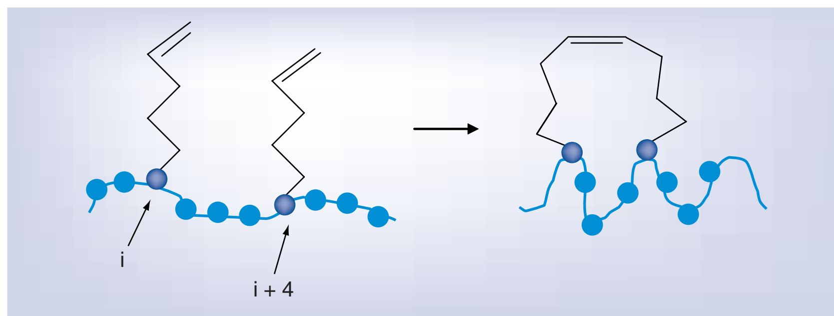

Figure 1. Hydrocarbon-stapled α-helices. Stabilized α-helices are formed by the hydrocarbon stapling of olefinic side chains at the α-carbon of non-natural amino acids in positions i, i + 4 [38].

In the context of using peptides to study PPIs, peptide arrays provide an efficient tool for the simultaneous detection and analysis of interactions between a full-length protein and numerous peptides. The peptides may be derived from known binding protein partners, allowing location of binding sites, but also from proteins that are not known as binders, thereby allowing identification of new PPIs and they may even originate from randomly generated sequences, usually in the process of identifying new modulators of PPI.

The large-scale synthesis offers large-scale analysis and is thus especially suited for screening purposes. On the other hand, the large number of different peptides synthesized on one cellulose sheet lead to varying yields, amounts and purity of peptides. As a consequence, results obtained by the SPOT method are not fully quantitative and are usually confirmed using standard peptide synthesis, purification and analysis. Nonetheless, the SPOT technology has been frequently reported to be highly reliable [42].

Examples for using peptides in the studies of PPI

The use of peptides in studying PPIs can be divided into the characterization or modulation of PPIs. Examples are given for the use of peptides in the structural and biophysical characterization of PPIs and for peptides that modulate PPIs, serving as agonists or antagonists.

■ Using peptides for the characterization of PPIs

Structural characterization of PPIs

Full 3D structure determination of protein–peptide complexes

One of the most important uses of peptides is to enable the full 3D structure determination of complexes involving PPIs by x-ray

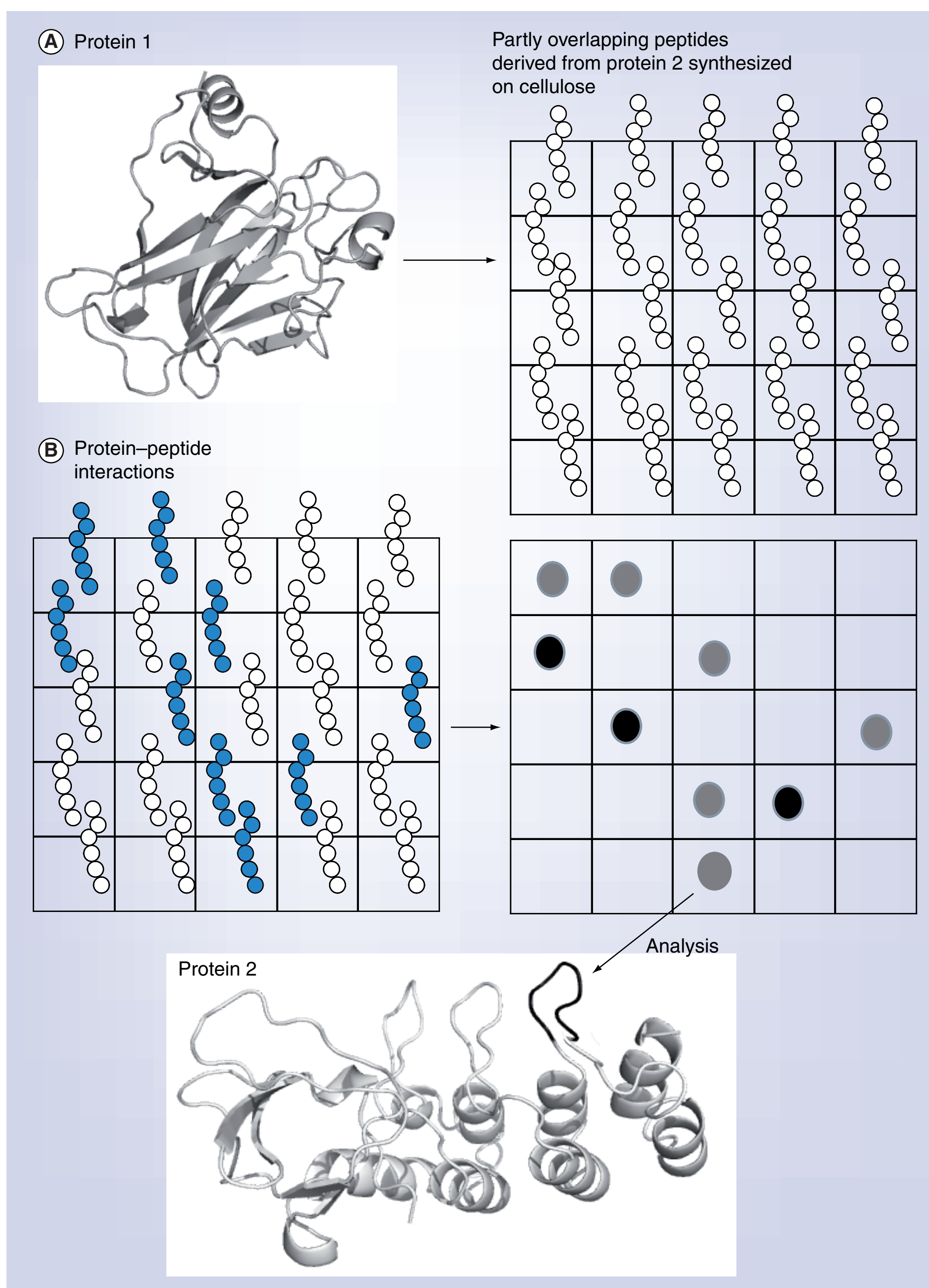

Figure 2. Peptide arrays. (A) Left: a protein of interest (protein 1) is scattered on an array of peptides that are synthesized on cellulose. Right: The peptide array is illustrated with the partly overlapping synthesized peptides derived from protein 2 shown as open circles. **(B)** Left: Protein 1 interacts with some of the peptides (interacting peptides are colored circles). Right: these interactions are detected, for example by chemiluminescence and analyzed to locate the site in protein 2 that binds protein 1. The binding site (marked in bold) is mapped on the 3D structure of protein 2.

crystallography or NMR, where one partner is represented by a peptide derived from it, saving the effort of producing of the full-length protein. Structures resolved at such an atomic resolution reveal the basis for the function of the PPI and provide the direction for designing molecules to modulate the interaction. The protein data bank holds numerous examples for protein–peptide complexes where one peptide is derived from and represents a larger parent protein. For example,

the pro- and anti-apoptotic Bcl-2 family members, which regulate programmed cell death [10], and the p53:MDM2 interaction, which regulates p53 function [43].

Detecting binding sites by peptide mapping

In the peptide-mapping approach, a full-length protein is tested for binding with multiple partly overlapping peptides derived from other proteins, usually proteins that are known or suspected to bind it. The peptides cover the sequence of the protein (or domain) known to mediate the interaction. Peptides for mapping may be obtained by systematic synthesis in the laboratory. For example, the binding sites of the core domain of p53 (p53CD) with its partner proteins Rad51, CTF2 and Pirh2 were discovered by peptide mapping using systematic peptide synthesis [44].

Peptide mapping is more efficient using peptide arrays that enable the simultaneous detection of interactions between a target protein and multiple peptides (see previous discussion) [41]. Peptide arrays typically contain partly overlapping peptides derived from the full-length sequence of the protein or protein domain of interest that are synthesized and screened for binding. Peptide arrays may also constitute peptides that do not originate from natural proteins such as combinatorial or computationally designed libraries [45]. The method is increasingly used for the discovery of PPI binding sites. For example, the two binding sites of the hypoxia-inducible factor-1α with p53CD were found using peptide array [46]. The binding specificity of the DnaK chaperone was studied by screening peptides derived from 37 biologically relevant proteins. The binding results have enabled the deduction of the sequence-binding motif of the various binding sites [47,48]. The interactions between DnaK and a model all-α-helical globin (apoMb) were explored by peptide scanning [49]. The binding specificity of the full-length DnaK chaperone was compared with that of its minimal substrate-binding domain, DnaK-β. Six specific chaperone-binding sites were identified on apoMb. The binding site locations were identical for the full-length chaperone and its substrate-binding domain, although the affinities differed [49]. Peptide array also enabled the analysis of binding specificity and extraction of a binding motif for the ribosome-associated chaperone trigger factor [50] and the SecB chaperone [51]. The interaction network of the pro-apoptotic protein ASPP2 was also explored by peptide arrays. The peptide binding results lead to the discovery of the binding sites of ASPP2 with the anti-apoptotic proteins from the Bcl-2 family [52] and with the transcription factor NF-κB [53]. Furthermore, for the Bcl-2 family, previously unknown PPIs were discovered using peptide arrays between ASPP2 and the anti-apoptotic Bcl-2 members Bcl-W and Bcl-X_L. Peptide arrays have also revealed an intramolecular interaction in ASPP2, between its proline-rich domain and its protein-binding region. The specific binding sites on both domains were located and enabled the elucidation of the intramolecular regulatory mechanism of ASPP2 [54].

Using peptide binding data for structural modeling of protein complexes

After identifying the binding sites using peptide mapping or peptide arrays, the binding information may be used for modeling the 3D organization of the complex between the full-length interacting proteins **(Figure 3)**. Protein docking algorithms suggest the 3D conformation of a complex, given the 3D coordinates of the participating proteins. Often, the near-native conformation is present in the list of suggested transformations but is not always highly ranked. Hence, and in particular in unbound docking, where the binding molecules are docked as determined in their free conformation, it is extremely helpful to provide additional biological information for more accurate ranking and filtering of the biologically valid docking models. For example, knowledge of hot-spot residues in one or both of the proteins is helpful in filtering relevant structural models. Another useful source of information is binding peptides. This information may be obtained by peptide array screening **(Figures 2 & 3)**. Following this approach, 3D structural models were built for complexes of the pro-apoptotic protein ASPP2 with Bcl-2 [52] and with NF-κB [53]. Peptide arrays combined with docking studies were also used to model the interaction between the structural protein titin with Src-homology 3 domains [55]. Peptide-binding data were also used to propose a 2D model for the organization of the p300–p53 tetramer interaction [56].

Mapping of conformational changes

The binding of a peptide to a full-length protein may also be used to characterize conformational changes that occur on one partner participating in a PPI. For example, NMR of binding peptides was used to characterize the conformational changes in MDM2 upon binding

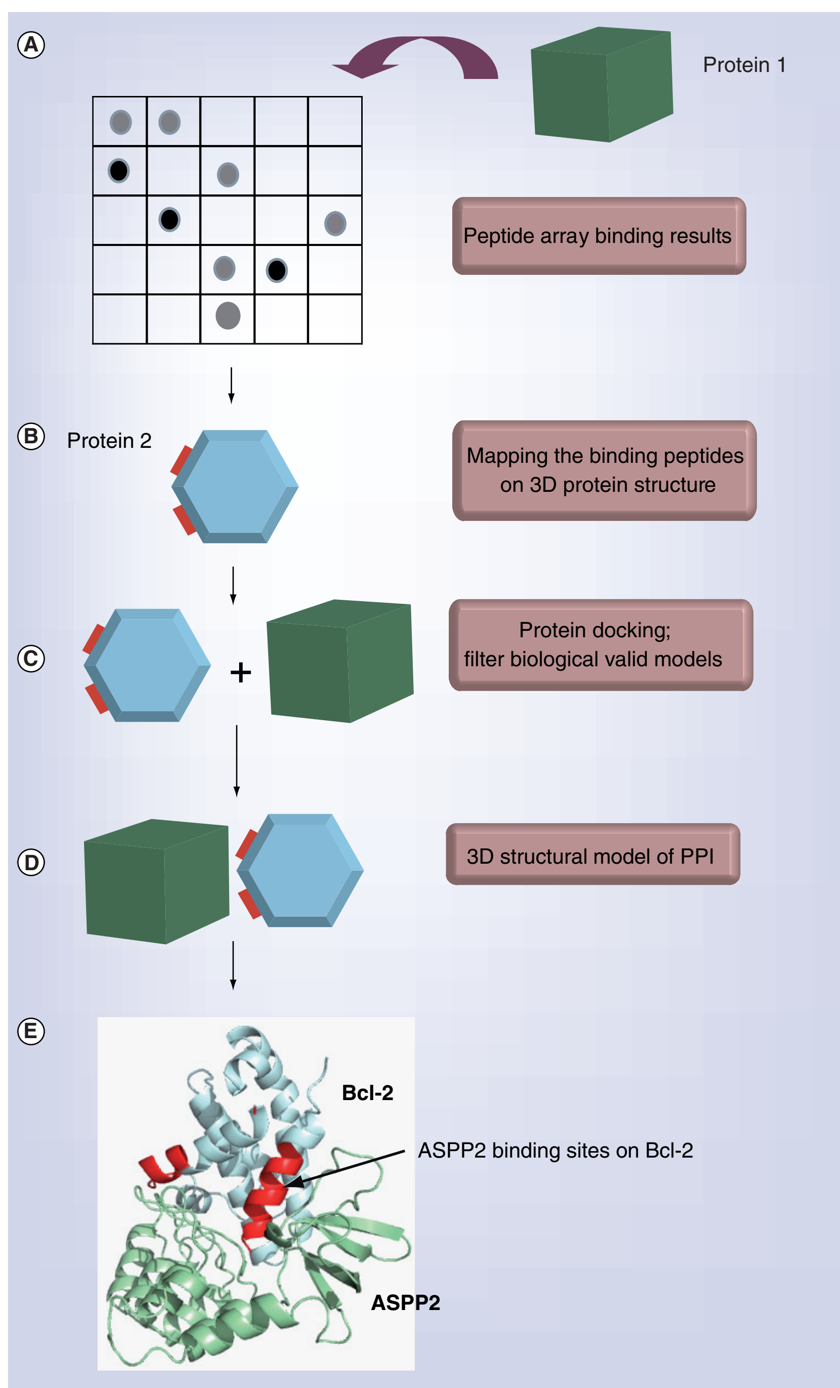

Figure 3. Use of peptide binding data for structural modeling of protein–protein interactions. (A) Proteins 1 and 2 participate in a PPI. Protein 1 (depicted as a cube) is scattered on a peptide array that contains peptides derived from protein 2. **(B)** The interacting peptides from protein 2 (depicted as a hexagon) are deduced. The peptide binding data are mapped onto the surface of protein 2 and provide the approximate binding site, depicted as stripes on the side. **(C)** Proteins 1 and 2 are subjected to computational protein docking prediction, where the approximate binding sites are used for better ranking and filtering of suggested binding orientations of the two proteins. **(D)** An experimentally supported 3D structure model for the PPI complex is built. **(E)** Example for the use of peptide binding data for structural modeling between the pro-apoptotic protein ASPP2 and the anti-apoptotic protein Bcl-2 [52]. ASPP2 binding peptides on Bcl-2 are marked.
PPI: Protein–protein interaction.

peptides derived from the N-terminal domain of p53 [57,58]. Another method for characterizing conformational changes upon PPIs using peptides is to apply molecular dynamics simulations, detailed in the next paragraph.

Studying PPIs via simulation of protein–peptide binding

Molecular dynamics simulations are a useful tool for studying biological systems. They enable knowledge to be gained regarding dynamic processes of a system that is usually unavailable by other methods. The speed and efficiency of a simulation depends on the size of the system studied. Hence, as in PPI simulations, reducing the size by studying only a peptide from one of the interacting proteins may enable more efficient simulations.

The anthrax toxin of the bacterium *Bacillus anthracis* consists of three distinct proteins, one of which is the anthrax lethal factor (LF). LF cleaves most isoforms of the family of MAPK kinases (MEKs/MKKs) close to their amino termini, resulting in the inhibition of one or more signaling pathways. Docking and molecular dynamics calculations were employed to examine the LF–MEK/MKK interaction along the catalytic channel by simulating the LF protein and peptides from MEK/MKK [59]. In another study, the different affinity between LF and various types of MEKs was addressed by simulation and it was found that peptides that form a β-sheet with the LF are better recognized and cleaved [60]. Turjanski *et al.* studied the mechanism of recognition and phosphorylation by MAPKs. These events are poorly understood, owing to the lack of complex structures of MAPKs with their bound targets in the active site. The interaction between MAPK extracellular activated protein kinase and a target peptide was analyzed by a quantum/molecular mechanics approach and provided a detailed description of the molecular events involved in the phosphorylation reaction catalyzed by MAPK, highlighting the importance of specific proline and lysine residues at the active site [61].

Latzer *et al.* used associative memory Hamiltonian to simulate the nuclear localization signal (NLS)-containing regions of NF-κB p50 and p65, in the free and bound states with IκBα and IκBβ. The simulation demonstrated the gain of a bent helical structure of the NLS-containing peptide upon interaction, providing insights into the mechanism of NF-κB

inhibition by IκB [62]. Similar behavior of the NF-κB NLS was observed in the simulation of the interaction between NF-κB p65 and the protein-binding region of ASPP2 [53].

Using peptides for biophysical characterization of PPIs

Protein–peptide interactions are also studied for biophysical characterization of PPIs, such as quantifying the affinity, kinetics and thermodynamics of the interaction. For example, the interactions between p53CD and peptides from three different proteins were studied and it was found that p53CD binds these peptides, as well as additional proteins, using a promiscuous binding site with a strong electrostatic component [44]. p53-derived peptides that bind MDM2 were used for kinetic and thermodynamic characterization of the p53–MDM2 interaction [57].

The spike (S) protein of SARS-coronavirus mediates viral entry into host cells. It contains two heptad repeat regions, denoted HRN and HRC. The effect of various modifications to create HRC analogs was biophysically characterized. Increased hydrophobicity, helical propensity, electrostatic interactions and stabilization of the α-helical conformation were tested. It was found that stabilization of the α-helical conformation best correlated with the binding affinity [63].

Aptamers were rationally designed by inserting peptides into a solvent-exposed loop on thioredoxin. The aptamers were designed to interact with the protein's elongation initiation factor 4E and MDM2, and binding was validated by competitive fluorescence anisotropy experiments. The dissociation constant (K_d) was measured by ITC and compared with the free linear peptide. Thermodynamic analysis suggested that an increase in the binding affinity of an aptamer over a free peptide is dependent on the increase in the favorable enthalpy of binding, which is ideally caused by stapling of the peptide or by additional interactions between the aptamer protein and its target [64]. The interaction between the anti-apoptotic Bcl-X_L and the BH3 agonist peptide BID was quantified by fluorescence correlation spectroscopy and it was found that the interaction is significantly enhanced in the presence of membranes [65].

The differences in binding affinity to ASPP2 between homologous anti-apoptotic members of the Bcl-2 family were studied by biophysical methods [52]. The binding constants between ASPP2 and ASPP2-binding peptides derived from the different Bcl-2 members were measured and the binding affinity was found to correlate with the charge of the peptide sequences: the more positively charged the binding site, the tighter the binding to the negatively charged ASPP2. The role of specific charged residues was confirmed by mutation analysis [52].

Coinfection of hepatitis G virus (GBV-C/HGV) with HIV has been associated with slower progression of the illness and a higher survival rate of patients once AIDS has developed. The structure and interactions between the fusion peptide of HIV-1, gp41(1 23) and synthetic peptide sequences of the E2 envelope protein of GBV-C/HGV was studied using biophysical techniques. The results show that a certain E2 sequence (AA 269–286) interacts with the target fusion peptide of HIV-1 and modifies its conformation [66].

Complexes between Src-homology 3 domains and proline-rich target peptides can have lifetimes in the order of milliseconds, making them too short-lived for kinetic characterization by conventional methods. NMR dynamics experiments are thus better suited to studying such rapid binding equilibria and can additionally provide information on partly bound intermediate states. NMR, together with ITC, was used to characterize the interaction of the Src-homology 3 domain from the Fyn tyrosine kinase with a 12-residue proline-rich peptide at temperatures between 10 and 50°C. NMR data at all temperatures were consistent with an effectively two-state binding reaction. ITC data at all temperatures also followed a simple two-state interaction model. Both the magnitudes and temperature dependence of k_{on} values were consistent with a diffusion-limited association mechanism. The combination of NMR and ITC revealed how the Fyn tyrosine kinase is activated by binding to proline-rich targets and may be used for characterizing transient protein–ligand interactions [67].

In summary, peptides have proved to be powerful tools for structural and biophysical characterization of PPIs. In characterizing protein–peptide interactions, the aim is both to study the PPI of full-length proteins and locate the binding sites, but also to determine whether certain peptides may be further used as lead compounds to control the PPI as agonists or antagonists, as detailed later.

■ Modulation of PPI

As PPIs govern cellular function and might be impaired in pathology, PPIs are therapeutic targets. There is an interest in either inhibiting or augmenting PPIs, depending on the specific biological system studied. Peptides may affect PPIs either as agonists or antagonists **(Figure 4)**, making them attractive tools in lead detection for drug discovery.

Peptides that modulate PPIs may be rationally developed based on a binding site within a known protein that participates in a certain PPI [68], or from the screening of peptide sequences that do not originate from natural proteins [45]. After an active lead peptide is identified, it is usually subjected to further modifications to increase its potency by stabilization or improvement of specific desired chemical properties for better clinical effect [68]. This is usually required in the shift to *in vivo* and clinical systems, since linear peptides are unstable, susceptible to proteolysis and have limited ability to penetrate cells. Based on the structure–activity relationship, nonpeptidic molecules or peptidomimetics may be designed and tested for any effects on PPIs.

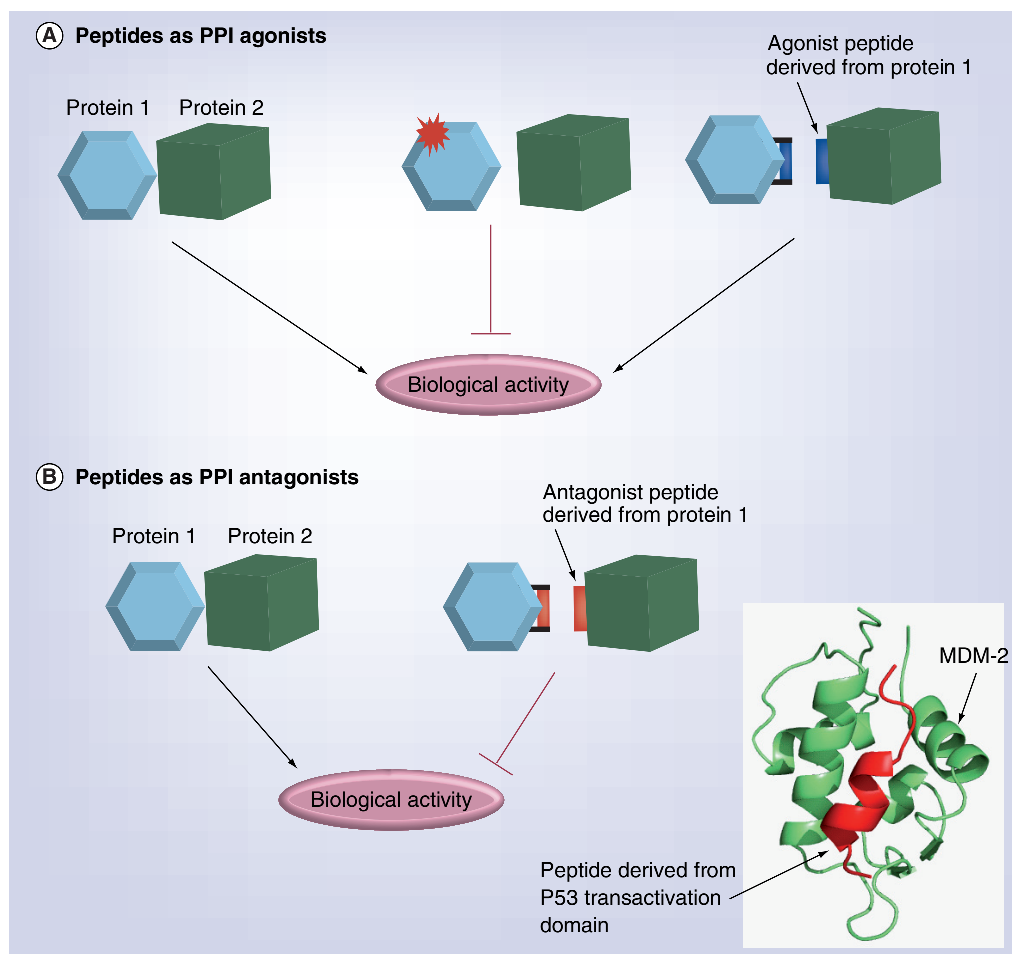

Figure 4. Peptides as protein–protein interaction agonists or antagonists. (A) Protein 1 (hexagon) and protein 2 (cube) participate in a desired PPI. Protein 1 is less functional or lacking due to, for example, mutations or low expression. Protein 1 may be replaced by an agonist peptide derived from it (depicted as a stripe on the side), which interacts with protein 2 and by this the original bioactivity of the PPI is restored. **(B)** Protein 1 (hexagon) and protein 2 (cube) participate in an undesired PPI. An antagonist peptide (depicted as a stripe on the side), derived from protein 1, may compete for the binding site on protein 2 and by this it can prevent the interaction between the two full length proteins and inhibit the original bioactivity. Right: an example of an antagonist peptide derived from the transactivation domain of p53 that inhibits the p53–MDM2 interaction [43]. Peptidic modulators of PPI are demonstrated here as proteinomimetics but can also be obtained from random library screening.
PPI: Protein–protein interaction.

Peptides that activate PPIs or serve as protein agonists

Peptides that act as replacements for one partner in PPI are analogous to peptide agonists. Discovery of such peptides usually starts from rational design based on known interaction partners but may also be obtained by screening or computational prediction [45].

Bcl-2 family proteins are critical controllers of apoptosis regulation. The PPI between Bcl-2 members dictates the cell apoptosis fate and is mediated through the death domain α-helical BH3 segment [38]. Walensky *et al.* developed a hydrocarbon-stapled BH3 helix that activated apoptosis *in vivo*. The stapled helix bound multi-domain Bcl-2 family members with increased affinity and activated the apoptosis pathway in leukemia cells [38].

Activation of the formyl-peptide receptor-like 1 pathway is important in the therapy of inflammatory diseases. Agonism at FPRL1 has a beneficial effect in animal models of acute and chronic inflammatory diseases. A 21-amino acid peptide agonist for FPRL1 that also activates FPRL2 was discovered using a computational platform designed to predict novel G-protein-coupled receptor peptide agonists cleaved from secreted proteins by convertase proteolysis. *In vivo*, the agonist peptide displayed anti-inflammatory activity [45].

Leptin, a hormone produced by adipose tissue, regulates energy balance in the hypothalamus and is involved in fertility, immune response and carcinogenesis. Drugs activating or inhibiting the leptin receptor (ObR) are continually being developed. The ability of various leptin fragments to stimulate the growth of ObR-positive and ObR-negative cells was studied. One of the effective peptide analogs featured non-natural amino acids at terminal positions to decrease proteolysis and a blood–brain barrier penetration-enhancing carbohydrate moiety. This peptide proved to be full agonist to ObR and was suggested as a potential lead in leptin-deficient diseases [69].

Selective activation of the neuropeptide Y2 receptor to suppress appetite is a promising approach to obesity management. A peptide agonist corresponding to residues 13–36 of human peptide YY and a nonpeptidic moiety was described [70]. The peptide elicited a dose-dependent reduction in food intake and bodyweight in mice and rats. [70].

In all the cases described, short peptides efficiently replaced full-length proteins (or a longer peptide, in the case of peptide YY) in their biological activity. This is a promising approach in various biological conditions where one protein partner is present in insufficient amounts or when its function is impaired.

Peptides as protein antagonists or PPI inhibitors

Peptides may act as inhibitors of PPIs. Undesired PPIs may be targeted by peptides via competitive or noncompetitive mechanisms. For example, blocking the MDM2–p53 interaction to reactivate the p53 function is a promising anticancer therapeutic strategy. The successful development of peptide (as well as small-molecule) inhibitors of the p53–MDM2 interaction is reviewed in Murray *et al.* [71]. Two recently determined structures demonstrate the structural basis for peptide-based inhibition of this biomedically important PPI [43,72].

Peptide-based inhibition was applied for several PPIs of HIV-1. The activity of the HIV-1 integrase protein (HIV-IN) was inhibited by peptides derived from the viral reverse transcriptase enzyme [73]. Peptides that mimic two NLS of two viral proteins, one from the matrix protein [25] and one from the Tat arginine-rich motif were designed and synthesized [24]. In both cases, the peptides served as inhibitors of the corresponding HIV proteins NLS. The proteinomimetics derived from the matrix protein NLS inhibited nuclear import of NLS-BSA and HIV-1 replication [25]. One of the peptides that mimicked the Tat arginine-rich motif proved to be a lead compound that could potentially inhibit the HIV-1 lifecycle by a dual mechanism of nuclear import inhibition and of RNA binding [24]. Dimerization inhibitors of HIV-1 protease were also designed and synthesized based on truncated, crosslinked interfacial peptides of HIV-1 protease [74]. Hepatitis C virus nucleocapsid assembly requires dimerization of the core protein, an essential step in the formation of the virus particle. Core-derived peptides inhibited the dimerization and the assembly of new virions [75]. Moellering *et al.* designed and synthesized a stapled peptide that prevents the assembly of the NOTCH transcription factor complex. Treatment of leukemic cells with the peptide results in genome-wide suppression of NOTCH-activated genes [76].

The protein gp130 is a transmembrane protein that acts as the signal transducing receptor subunit for IL-6 type cytokines, including viral IL-6, which is encoded by the Kaposi's sarcoma-associated herpes virus. Viral IL-6 has

been shown to mimic human IL-6 functions. A range of assembled peptides that mimic the sequentially discontinuous binding site of gp130 for viral IL-6 was designed and synthesized based on the crystal structure of the complex of gp130 with the viral IL6. These peptides inhibited the interaction of gp130 with viral IL-6 and the stimulation of viral IL-6-induced cell proliferation [77].

Peptides that shift the protein equilibrium

Shifting the quarternary equilibrium or oligomerization state

Peptides may shift the oligomerization equilibrium of proteins. Such peptides were termed 'shiftides' (**Figure 5**) [78]. A similar effect is observed for some small molecules that can affect the oligomerization state of proteins such as porphobilinogen synthase [79–80]. The shiftides approach was demonstrated for the inhibition of the HIV-1-IN protein by using peptides derived from its cellular-binding protein, LEDGF/p75. The LEDGF/p75-derived peptide inhibited IN activity by a noncompetitive mechanism; rather, the peptides inhibited the DNA binding of IN by shifting the IN oligomerization equilibrium from the active dimer toward the inactive tetramer [78]. Another study described two peptides derived from the HIV-1 Rev protein or selected from a combinatorial library that interact with IN and inhibit its activity *in vitro* and in cells by a shiftide mechanism [83–85]. A peptide derived from the tail of the motor protein nonmuscle myosin II was found to shift the oligomeric equilibrium of nonmuscle myosin II-C towards filament assembly [86].

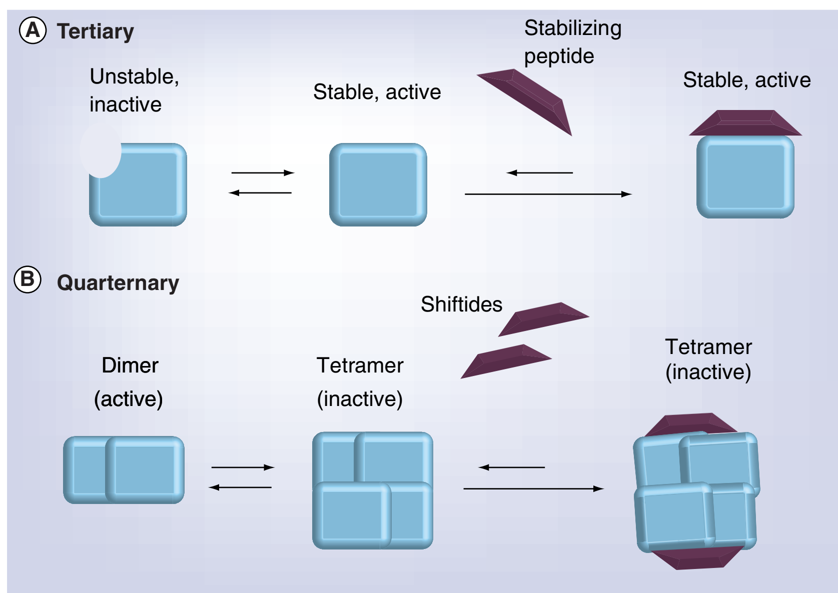

Figure 5. Peptides that shift the equilibrium of protein conformation or oligomerization. (A) Shifting the equilibrium between alternative conformations of a single domain. Left: Chemical chaperones: shifting the conformational equilibrium of proteins. When a protein is in equilibrium between a native, active conformation and a destabilized, inactive conformation, adding a stabilizing peptide that binds preferentially to the native conformation will stabilize it, shifting equilibrium towards it. **(B)** Shiftides: peptides that shift the oligomerization state of proteins. Left: equilibrium between two oligomerization states, such as an active dimer of undesired biological activity and an inactive tetramer. Upon adding shiftides that bind preferentially to the inactive tetramer, the inactive tetramer is stabilized and the equilibrium is shifted toward the tetramer form; the protein is then inhibited.

Shifting the tertiary structure equilibrium

The proper formation and function of a PPI may be compromised due to structural instability of one of the protein partners, typically caused by mutations or environmental conditions. Local or global instability may be rescued by molecules that bind and stabilize the native protein, thereby enabling it to exert its function. Such molecules are termed 'chemical chaperones', since they act to retain the native folding of proteins (**Figure 5**) [87]. For example, a peptide that binds and stabilizes p53CD was rationally designed based on the crystal structure of the complex between ASPP2 and p53 [88]. The peptide raised the melting temperatures of both the wild-type and R249S oncogenic mutant of the p53CD. The peptide also restored specific DNA binding activity to a highly destabilized mutant I195T [88]. The same peptide also rescued the destabilized R249S mutant of p53CD [89].

Peptides that were derived from the chaperones gp96 and clusterin, and that stabilized them, served as antagonists for their PPIs and, hence, exerted anti-inflammatory and anticancer effects for gp96 and clusterin, respectively [90].

Peptidomimetics

A peptidomimetic is a molecule designed to mimic a peptide. Typically the peptide is already identified as a bio-active lead compound. Chemically, a peptidomimetic is a nonprotein molecule. Peptidomimetics are designed to overcome problems such as susceptibility for proteolysis and poor bioavailability. During development of peptidomimetics, other properties such as receptor selectivity or potency are often improved [91].

Peptidomimetics is the main source for small-molecule inhibitors of PPIs. The field of small molecules that inhibit PPIs was reviewed by Arkin and Wells [92], Wells and McClendon [93] and Fry [94]. Peptidomimetics also serve as PPI

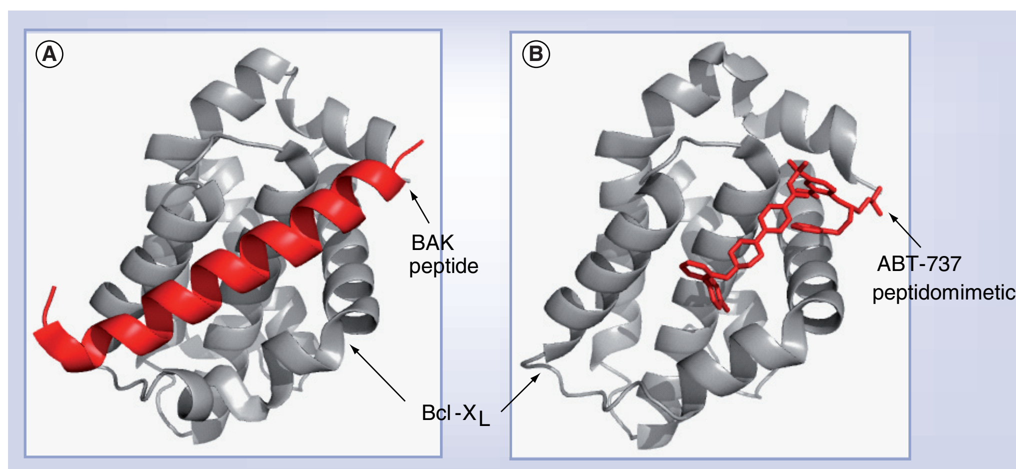

Figure 6. Peptidomimetics: design of peptide-based protein–protein interaction modulation. (A) The structure of the anti-apoptotic protein Bcl-X_L of the Bcl-2 family, in complex with its peptidic modulator, the BH3 peptide from the pro-apoptotic Bak protein (PDB id: 1bzw, [95]). **(B)** The structure of Bcl-X_L with the peptidomimetic small molecule ABT-737 (PDB id: 1yxj, [96]).

agonists, for example the BH3 peptidomimetics that mimic the BH3 peptide derived from Bcl-2 proapoptotic members that inhibit the anti-apoptotic Bcl-2 family members **(Figure 6)** [95,96].

A main challenge in developing small-molecule modulators of PPIs is the relative flatness of the interface and lack of well-defined binding pockets. This may be overcome by using the subset of hotspot residues that represent only a small subset of the interface that contributes most of the free energy for binding [92,93]. Another compensating observation is the existence of flexibility at the interface that enables proteins to accommodate small molecules in a way that is not observed in the binding of proteins or peptides. Such binding sites are also termed 'encrypted' binding sites [93].

Peptidomimetics led to the successful development of small-molecule modulators for some of the most clinically important PPI. Selected examples are p53:MDM2 [71], smMLCK:calmodulin [97], Smac:BIR [98] and Bak BH3:Bcl-2/Bcl-X_L [99]. More examples are reviewed elsewhere [36].

Future perspective on the use of peptides to study PPIs

We expect that the interest in PPIs will continue to grow, along with projects aimed at obtaining the interactomes for various biosystems and organisms. As a result, the need for further structural and biophysical characterization of PPIs will also continue to grow and peptides will continue to serve as an attractive tool for such studies. The amount of available high-resolution experimentally solved structures and structural models is expected to increase as a result of improvements in structure-determination technologies as well as computational prediction algorithms. This will provide more starting points for developing proteinomimetics and peptidomimetics and will facilitate drug design towards modulating PPIs.

Improvements in chemical peptide synthesis will allow the total chemical synthesis of longer peptides, protein domains and proteins [12]. Chemical modifications and the use of non-natural amino acids will improve peptide stability and their potential to serve as lead compounds for therapeutic intervention in PPIs. This should also improve the advantage of peptides over small molecules, as in principle they are more adjusted to accommodating the natural binding sites of PPIs [93].

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Executive summary

- Protein–protein interactions (PPIs) control the function of living cells. Quantitative, functional and structural studies of PPIs enables understanding of these processes at the cellular and molecular levels and provide a basis for designing drugs that inhibit or stimulate such PPIs.
- A major rate-limiting step in PPI studies is the expression and purification of the interacting proteins. For a full characterization, both proteins should be expressed and purified in the required amounts and their structure should be determined.
- The use of peptides to study PPIs introduces several substantial advantages:
  - Peptides up to a length of several dozens of residues can be synthesized quite easily;
  - Using peptides enables the actual binding sites and the binding residues to be precisely identified;
  - Using peptides enables the introduction of post-translational modifications or non-natural amino acids with 100% specificity.
- Peptides offer a good model for binding studies of protein domains since, although they are usually unstructured in their unbound state, they undergo induced fit upon protein binding to gain the native structure.
- As biologically active substances, peptides are studied across a variety of research schemes in biology, biochemistry and biophysics. In using peptides to study PPIs, the frequently used methods are aimed at providing structural, quantitative and biophysical characterization of biomolecular interactions. The most important technical advances in the field enable the chemical synthesis of peptides in the laboratory, at small or large scale and also enable stabilization of the peptides and chemical modifications.
- The frequently used methods in peptide-based studies of PPIs include x-ray crystallography, circular dichroism spectroscopy and NMR for structural characterization, ELISA and other semiquantitative immunoasssys for binding detection, isothermal titration calorimetry and fluorescence anisotropy for obtaining the binding affinities and thermodynamic parameters and methods such as stopped flow and surface plasmon resonance to study the binding kinetics.
- Peptides have been used for the characterization of PPIs at the structural level: mapping binding sites and conformational changes and complex structural modeling. At the biophysical level, peptides have been used for obtaining parameters of binding affinity and kinetics.
- Peptides have been used to control PPI as agonists (restore the desired impaired PPIs) or antagonists (inhibit undesired PPIs). The biological effect may take place via competitive or noncompetitive mechanisms.
- Peptidomimetics is the process of obtaining nonprotein molecules mimicking the structure of peptides, optimized for therapeutic applications.
- With the growing interest in PPIs as therapeutic targets, we expect that peptides will continue to contribute to the field. Use of peptides in PPI research will benefit from improvements in chemical synthesis of longer peptides and the ability to introduce chemical modifications that improve the stability and bioavailability of peptides.

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