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Investigation of stable and transient protein-protein interactions: past, present and future

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Abstract

This article presents an overview of the literature and a review of recent advances in the analysis of stable and transient protein-protein interactions (PPIs) with a focus on their function within cells, organs and organisms. The significance of post-translational modifications within the PPIs is also discussed. We focus on methods to study PPIs and methods of detecting PPIs, with particular emphasis on electrophoresis-based and mass spectrometry (MS)-based investigation of PPIs, including specific examples. The validation of PPIs is emphasized and the limitations of the current methods for studying stable and transient PPIs are discussed. Perspectives regarding PPIs, with focus on bioinformatics and transient PPIs are also provided.

Keywords

Protein-protein interactions; electrophoresis; mass spectrometry; proteomics; bioinformatics

1. Introduction: Overview of protein-protein interactions

Sequencing of the whole genome of many organisms has been a huge accomplishment with tremendous scientific impact. However, genomic information alone does not explain cellular functions. Proteins control and execute most cellular processes. Their properties are modified and controlled through interactions with their peers (protein-protein interactions, or PPI) or with other biomolecules. A PPI system is called the interactome [1, 2]. PPIs can be stable or transient, post-translationally modified (PTM) or not and can form homo- or hetero-protein complexes/PPIs.

PTMs can favor PPIs. PTMs are often reversible and therefore can act as molecular switches [3, 4]. Very often, PPIs require specific PTMs that recognize their binding partners. Protein phosphorylation is the most widely-studied modification. It can induce a protein to change its conformation and consequently its activity. Phosphorylation can function as a docking site for interacting biomolecules and therefore regulate the formation of complexes. It is

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important for activity of, for example, SH2 domains or the 14-3-3 protein family that can bind only to phosphorylated domains [5]. Phosphorylation of proteins by kinases is a central point of control and regulation of cellular functions, affecting one third of all protein and intercellular communication. Many signaling pathways are controlled by active/inactive proteins with their activity dependent on phosphorylation [6]. Other PTMs include acetylation and methylation (which provide binding motifs for chromatin-associated domains) [7], glycosylation (important for many ligand-receptor interactions) [8] or prenylation (which allows proteins to efficiently anchor into membranes and to form PPIs). Many prenylated proteins such as Rho, Rac or Ras are involved in basic cell mechanisms [9]. A summary of the roles of PPIs and the methods used to study PPIs are summarized in Figure 1.

2. Methods to study protein-protein interactions (PPIs)

For investigation of PPIs two main classical approaches are considered: genetic and biochemical. Initially, biochemical methods such as chemical cross-linking, combined fractionation during chromatography and co-immunoprecipitation were applied to explore PPIs. Later, high-throughput methods such as the yeast two-hybrid (Y2H) system, phage display and tandem affinity purification-mass spectrometry (TAP-MS) emerged as methods of choice for the investigation of PPIs in various organisms [10–13]. Also, microscopy techniques and computer or mathematical methods are currently being considered [10, 14, 15]. Genetic methods utilize the expression of a reporter gene upon interaction to demonstrate PPIs [16]. The Y2H system represents the genetic method of choice to unravel PPIs. In the last few years, several other genuine methods have been developed to analyze PPIs although classical methods and variants of these methods are still widely used by scientists. Computer-based methods are additionally very important methods for understanding PPIs, and are mostly used in combination with experimental methods to discriminate between true interactions and false positive results. Computer-based methods make use of available information on the structure of genes, proteins, and functional relationships between them as well as on protein functions [17]. The aim of this review is to describe traditional proteomics-based methods used for investigation of PPIs in detail. However, a brief review of recent and popular innovative techniques (genetic, biochemical, biophysical and chemical) will also be presented.

2.1. Historical method: two-hybrid system (2H)

The yeast two-hybrid system was first used by Fields and Song in 1989 [18, 19], as a method for screening libraries of proteins against a known bait-protein. The method has been extensively used in the past for the study of protein interactions [18–23]. Today, its range of application has extended to high-throughput, proteome-wide and sub-proteome-wide explorations of protein interaction networks [24]. The 2H system is a genetic method based on the interaction of two domains; a DNA-binding domain and an activation domain that can be brought together by the interaction of any two proteins. Therefore two systems need to be constructed: a DNA-binding domain fused to a protein of interest (A) and a transcription activation domain fused to a second protein of interest (B). Upon expression of these constructs, the A and B domains will lead to expression of a reporter gene if protein A and B interact. Otherwise, there will be no expression of reporter gene [25]. Common reporter proteins include β -lactamase, TEV protease, dihydrofolate reductase (DHFR), luciferase and fluorescent proteins such as GFP (green fluorescent protein) [26]. Common DNA-binding domains used include transcription factor GAL4 in *S. cerevisiae* or lexA repressor in *E. coli* and their respective activation domains GAL4 and protein B42 [27]. Historically, the system was developed in yeast, but it has been also applied successfully to mammalian cells. For instance, Y2H demonstrated interaction of the oncoprotein Ras with the oncogenic protein kinase Raf that plays a role in certain cancer types [28, 29]. One of the

advantages of this method lies in the fact that sites of interaction in the respective communicating proteins can be investigated through mutations of the gene encoding both proteins. Also, in the yeast two-hybrid system, PPIs takes place within the native environment of the cell. Since the invention of the yeast two-hybrid system, several variations of the original method have been reported. These variations were meant to address some serious drawbacks presented by the 2H method such as high false positive rate, the need to relocate cytoplasmic proteins into the nucleus where interaction takes place and the difficulty to investigate membrane proteins, toxic proteins and proteins with a strong cytoplasmic tropism. As a solution to these limitations, several methodical enhancements were undertaken. For example, the hSos/Ras system allows investigation of interaction in the yeast cytoplasm and does not depend on transcriptional activation [30]; the split-ubiquitin system where interactions is based on the reconstitution of an active ubiquitin and subsequent cleavage of a reporter protein [31] and the three-protein system which is based on the same principle as 2H but allows probing of interactions of more than two proteins [32], just to cite a few improvements.

2.2 Affinity and immunoaffinity-based protein-protein interactions

Another technique used in the investigation of PPIs in various model species is affinity purification (AP) in tandem with mass spectrometry (MS) [18, 21, 33–35] and thus called AP-MS. The method consists of tagging a bait protein with an affinity tag such as His-tag, flag-tag or Tandem Affinity Purification (TAP)-tag for expression *in vivo*. TAP-tag uses two tags. Upon interaction of the bait protein with its partners, the complex can be purified from the cell lysate by affinity or immunoaffinity purification followed by MS or MS/MS analysis for identification of the interacting components of the complex [36]. Therefore, its main advantages are multiple. First, the method allows for the exploration of PPIs under native physiological conditions. Further, the study of the dynamics of PPIs is possible under various conditions. Finally, the multi-component protein complex can be pulled down using this technique [33, 36–38]. Also among its disadvantages, one can cite a high false positive rate as in Y2H [37, 38].

In light of this fact, careful experimental design and controls are needed to distinguish false positive contaminants from true interacting components. A commonly used solution is tandem affinity purification mass spectrometry (TAP-MS). TAP-MS prevents isolation of proteins with affinity for the distal tag or the matrix. The technique was developed by Rigaut and colleagues in 1999 [12] as a technique for the purification of proteins expressed under natural conditions at physiological concentrations. In TAP, physiological expression of a gene encoding tag components and a target protein is undertaken. The mostly used tag in yeast is made of two immunoglobulin-G-binding fragments of *Staphylococcus aureus* protein A and sites sensitive to protease from tobacco mosaic virus and calmodulin-binding peptide (CBP). Affinity purification of the target protein complex occurs in two steps. First, protein A binds to IgG-Sepharose followed by cleaving of the complex by the protease. In the next step, CBP binds to calmodulin-Sepharose in the presence of calcium followed by elution of the complex with EDTA [17]. Coupled to MS, the method allows for rapid identification of proteins and their interactions [39]. To enhance the efficiency and specificity of the method, alternative tags like streptavidin-binding peptide instead of CBP and biotin for elution or tag based on G proteins that exhibit higher affinity to immunoglobulin G than protein A, can be used [40]. Very often, co-immunoprecipitation coupled with Western blotting or other detection methods is used to verify proteins identified by AP/TAP-MS. Therefore, it is also considered to be an integral part of AP/TAP-MS. Another possibility is the use of control proteins that are expressed with the same tag and at similar levels as the bait protein to select true interacting components. Finally, analysis of high-throughput data also helps to recognize false positive interactions [37].

AP-MS is often used to validate the results of Y2H since it has several limitations [41]. Amongst others, Y2H investigates the interaction between primarily two proteins rather than protein complexes. Also, in Y2H, the analysis is not performed in the natural environment where both proteins normally interact [42, 43]. Estimated error rates reported in the literature for TAP-MS, AP-MS and Y2H are ~ 15 %, ~ 50 % and ~ 45–80 %, respectively [44] and some of the steps that have been taken to reduce these error rates have been mentioned in this section as well as in the previous section reviewing the 2H method. By error rate, we mean the rate of false positive or false negative detections of protein-protein interactions determined based on validation of primary identifications.

2.4 Cross-linking and chemical modification

Recently, cross-linking methods have been successfully applied in the determination of PPIs. The study of membrane proteins with AP-MS has also been possible, partly due to advances made in the development of novel cross-linking methods [45]. Combined with immunoprecipitation and affinity tagging, the method has been extensively applied *in vivo* [46]. Compared to high-throughput techniques such as phage display or Y2H, cross-linking is a higher resolution structural method that enables the assignment of PPIs to specific regions or even amino acids. However, the binding strength of an interaction cannot be determined by cross-linking [47]. Another advantage of the method resides in the transformation of non-covalent interactions into permanent covalent interactions for further studies.

Two main groups of cross-linking reagents exist: homobifunctional and heterobifunctional. A homobifunctional cross-linker has two identical reactive groups connected by a spacer or linker. These reactive groups can react with amine-, sulfhydryl-, or photoreactive ends of biomolecules. In heterobifunctional cross-linking reagents, the two functional groups are different. The spacer allows additional topological information on interacting biomolecules. The most commonly-used cross-linking reagent is N-hydroxysuccinimide esters (NHS) [48], which forms stable amide bonds with primary amines at physiological pH and therefore can be used for *in vivo* labeling. The use of zero-length or small-sized cross-linkers has been touted as a way to gain structural constraint data.

Two of the most frequently applied reagents for cross-linking transient protein-protein interaction partners include formaldehyde and di-thiobis-succinimidyl-propionate (DSP). Cross-linking with formaldehyde functions by coupling to primary amines whereas DSP forms amide bonds with primary amines [22, 49, 50]. Lysines primarily constitute the amino acid most commonly used in cross-linking experiments since they are found in almost every protein in high numbers. Cysteines are another type of amino acids commonly used in cross-linking. Some commercially available cross-linkers are photoactive, i.e., they can be activated by light. Within this group, heterobifunctional benzophenones and aryl azides are commonly used through reaction with lysines or cysteines on proteins. The advantage of photoactivatable reagents lies in the possibility to control the cross-linking process through manipulation of the intensity/wavelength of the light source and the rapidity of the process. Nonetheless, the process often has a poor yield and generates many different products. Instead of using cross-linking reagents that generate a space, the use of zero-length cross-linkers has also been reported in the literature. For example, chelated Ni(II) or chelated Ru(II) can be employed for zero-length cross-linking by treatment with oxidants (e.g., magnesium monoperoxyphthalic acid) [51] or by photolysis in the presence of persulfate [52], respectively. In this case, tyrosine is the amino acid that is primarily cross-linked.

One of the key pieces information derived from the analysis of PPIs is the location of the site of interaction in the respective biomolecules. Interestingly, many protein interaction methods do not provide this important detail. However, recent advances in cross-linking

methods have improved the identification of cross-linked peptides and thus, the consideration of the technique for large-scale application. These advances pertain mainly to the decrease in sample and data complexity [53]. The decrease was achieved by enriching cross-linked peptides through modification of the spacer of the cross-linker (insertion of affinity tags [54], differential isotope label [55], chemically [56] and mass spectrometry cleavable bonds [57]). Isotopic signature patterns by incorporating ^{18}O or ^{15}N labeling [58], for example, allows one to identify more easily cross-linked peptides from the doublet peaks in the mass spectrum.

Further, chemical derivatization methods coupled with MS such as biotinylation [59–61] or acetylation [62, 63] have also been reported for the investigation of protein-ligand, protein-protein or protein-nucleic acid interactions. The principle is based on the differential chemical modification of specific residues in solution in the presence or absence of a ligand resulting in mass differences which can be analyzed by MS to determine interface sites. Other common methods that should be mentioned at this point are the hydrogen/deuterium (H/DX) exchange and the oxidative surface mapping. H/DX has been applied in the past in combination with MS (H/DX-MS) to map protein-ligand and protein-protein interfaces as shown in a study where this method was used to map the interface between MAP kinase phosphatase 3 (MKP3) and its physiological substrate ERK2 [64]. The principle of H/DX-MS is based on the presence of exchangeable hydrogens in proteins (usually polar side-chain hydrogens bound to the heteroatoms N, O and S; N- and C-terminal hydrogens; backbone amide hydrogens) [65], of which only backbone amide hydrogens play a role in H/DX. Given that the exchange kinetics of amide protons backbone are influenced by protein-protein interaction or ligand binding they thus can be considered for mapping protein binding interfaces [66]. Generally, the sample is incubated in D_2O to allow exchange of protons with solvent deuterium and in-exchange rates can be determined by MS. Detection and quantification of the level of deuterium incorporation is accomplished via proteolytic digestion at low pH and temperature to prevent hydrogen back-exchange. Oxidative surface mapping is from the principle similar to H-DX with the difference that it uses highly reactive hydroxyl radicals to oxidize the side-chains, therefore leading to covalent modification of amino acid side chains.

3. Methods to detect protein-protein interactions

3.1. Antibody-based methods

Antibody-based methods have been successfully employed to investigate protein-protein interactions in the past and are still being considered for high-throughput analyses since most of these methods have been perfectly automated. For example, protein array methods or protein chips (or antibody microarray) are increasingly considered for the detection of protein-protein interactions and generally for proteomic research [67, 68]. In these array methods, antibodies are often used as capture agents or detection molecules that carry fluorescence or chemiluminescence probes. For example, Bergsma and colleagues reported the detection of known as well as novel blood protein interactions such as the recently discovered interaction between the innate immune system c-reactive protein (CRP) and the inflammatory protein kininogen using antibody array interaction mapping (AAIM) [69]. Another standard antibody-based method is western blotting (WB), which probes the interaction of a tagged protein or an antibody-targeted biomolecule with another protein. Recently, a variant of WB called “Far WB” has been successfully used to investigate protein-protein interactions. The difference between WB and Far WB is that instead of incubating the membrane with an antibody solution as is done in WB, a probe of interest (very often a fusion protein) is employed. The probe is then detected with an antibody [70–72]. Similarly, the enzyme-linked immunosorbent assay (ELISA) allows probing of the interactions between two individual proteins by using pairs of capture and detection

antibodies in such a way that each antibody recognizes separate component of a protein complex [73]. In one report, Chowdhury and others demonstrated *in vitro* interaction of the sesbania mosaic virus movement protein with the viral protein genome linked (VPg) and a 10-kDa protein (P10) using ELISA [74]. Advantages of antibody-based methods include their suitability for largescale analyses, low sample volumes and concentrations, high detection sensitivity and their relative speed. The main disadvantage of these methods is the availability of highly specific antibodies and the expensive cost to produce these antibodies. Furthermore, the proteins being investigated are mostly either completely denatured or not properly folded.

3.1. Electrophoresis-based systems to investigate PPIs

The most frequently used gel-based protein fractionation method in a proteomics experiment is by far sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE separates proteins under reducing and denaturing conditions. SDS-PAGE also separates the proteins according to their molecular mass. However, variants of SDS-PAGE or hyphenated techniques that are combined with SDS-PAGE are also available and utilized. Two dimensional PAGE (2-DE or 2D-PAGE) is one example, where the proteins are first separated according to their isoelectric point by a method called isoelectric focusing (IEF) in the first dimension (1D) and according to their molecular mass in the second dimension (2D). Other examples of gel-based denaturing methods include SDS-PAGE in non-reducing conditions (SDS-PAGE-NR) or Tricine-PAGE, while examples of gel-based non-denaturing, native methods include Blue Native PAGE (BN-PAGE) and Colorless Native PAGE (CN-PAGE). All of these methods are compatible with mass spectrometry (MS)-based experiments where either matrix-assisted laser desorption ionization (MALDI) or electrospray ionization (ESI) can be used. Of these electrophoresis methods, we will focus here on those capable of analyzing protein-protein interactions such as electrophoretic mobility shift assay (EMSA), SDS-PAGE (NR), BN-PAGE, CN-PAGE, etc.

3.1.1 Electrophoretic mobility shift assay (EMSA)—The electrophoretic mobility shift assay, which is also called gel shift or gel retardation, was developed first by Fried and Crothers [75] and Garner and Revzin [76]. The method is mostly used to assess and visualize the binding of proteins to specific DNA regions. Typically, the DNA region of interest is a linear ds-DNA containing a response element (RE) for a transcription factor (TF), which can be radio-, fluoro-, or hapten-labeled. The labeled oligonucleotide is incubated with cell or nuclear extracts to probe for the formation of specific TF-RE complexes, which can be visualized on a non-denaturing PAGE since the motility of the complex and free DNA is based on both size and shape. In some EMSA, specific competitors (i.e., identical to labeled probe) or nonspecific competitors (i.e., unrelated to labeled DNA) are introduced in order to determine the specific complexes formed [77, 78].

In its early days, EMSA was used to characterize polyribosomes on bacterial mRNA and to explore the interaction of 16S rRNA with small ribosomal subunit proteins [79, 80]. In a slightly varied form of EMSA denoted supershift assay, higher-order multi-complexes can be studied by adding a TF-specific antibody to the incubation mixture, thus resulting in the formation of DNA-TF-antibody complex that has an even more slower mobility in comparison to the free DNA (supershifted) [81]. Other novel forms include two- and three-dimensional EMSA (2D-EMSA, 3D-EMSA) [82]. In 2D-EMSA, samples are pre-fractionated via 2D-PAGE followed by EMSA. In other cases, EMSA can be performed first and then a denaturing PAGE is undertaken in the second dimension. Identification of TFs specific to the selected RE is determined by LC-MS/MS. The coupling of non-denaturing EMSA with 2-DE gave rise to 3D-EMSA. Besides monitoring binding, EMSA allows also the determination of binding affinities of, for example, a TF to several DNA sites or

different TFs to the same DNA fragment [83]. The binding affinity can be calculated by the ratio of complexed to free DNA at varied DNA concentrations using Scatchard analysis.

The main advantage of this method lies in the fact that it is not disturbed by matrix effects in that crude nuclear or whole cell extract can be analyzed without prior sample preparation steps. Further, its resolution power allows for the distinction of TF-RE complexes of different stoichiometries or conformation. However, it also presents several limitations. For example, it is not very specific as some TFs bind related or even unrelated DNA sequences. Also, identification of proteins in highly complex samples is difficult. Further, it requires prior knowledge of the RE sequence to be used [77]. To increase the specificity of the assay, excess of competitor DNAs such as poly (dI:dC) or poly (dA:dT) are included in the reaction to reduce non-specific and low-specificity protein-DNA interactions [84].

3.1.2. SDS-PAGE under non-reducing (NR) conditions or SDS-PAGE (NR) for investigation of covalently-linked PPIs

—SDS-PAGE is the most frequently used biochemical fractionation method for proteins. However, when SDS-PAGE is not compatible with the end goal of the experiment, methods that can be used to focus on only a set or subset of proteins within the proteome (sub-proteomes) can be built and used, based on a logical biochemical approach, and depending on the type of the sample to be analyzed [85–93]. Therefore, the use of the physico-chemical properties of proteins and their PTMs (that would allow one substitute for the inability to use SDS-PAGE and/or to complement it) may be helpful to achieve the end goal. One such option is using biochemical approaches that would allow one to analyze post-translationally modified sub-proteomes such as phosphoproteomics, secretomics [93], glycoproteomics, or disulfide proteomics [92, 94, 95]. Recent papers highlighting the use of biochemical approaches that would allow one to analyze artificially [96] and naturally post-translationally modified sub-proteomes with focus on analysis of plasma membranes [95], secretomes [93] or disulfide proteomics [92, 94] were recently published by our lab (Darie). In addition to the disulfide-linked, reversible PPIs, there are also additional covalent linkages for PPIs, but not reversible, such as proline-glutamine PPIs. One example includes vitelline envelope proteins [97].

3.1.3. Blue Native PAGE (BN-PAGE) as a method for investigation of protein complexes and PPIs

—BN-PAGE was developed by Schaeffer and von Jagow, and was used for isolation and characterization of the respiratory complexes from bovine mitochondria [98–100]. However, for a long time, this method was used primarily by plant biologists. Their focus was to identify the protein complexes and protein subunits of the protein complexes from the thylakoid membranes of chloroplasts, a very good starting material for BN-PAGE due to the high stability and high concentration of the protein complexes [86, 101]. These complexes were also easy to follow during the electrophoresis run simply because they contain chlorophyll and can be easily visualized and therefore, the separation can be stopped or be extended, allowing the scientist to tune runs, and to obtain the highest quality results. Years later, animal and bacterial biologists started to use BN-PAGE in their research, but the number of users is still far lower than it could or should be.

The principle of BN-PAGE is simple, yet powerful: the protein complexes from the sample will interact with Coomassie dye through hydrophobic interactions and, since the dye is negatively charged they will become negatively charged as well. Having the same negative charge, the protein complexes will migrate at a neutral running pH towards the anode, the same way SDS forces proteins to run towards anode in SDS-PAGE. The difference between SDS-based SDS-PAGE and Coomassie-based BN-PAGE is that SDS is a strong denaturing agent, while Coomassie dye does not affect the integrity of the native protein complexes. Therefore, due to the external charge induced by Coomassie dye, BN-PAGE separates

native protein complexes according to their molecular mass, with a mass ranging between 100–1500 (or even higher) kDa.

The amount of information that BN-PAGE gives is rather large: it can provide information about size of the complex and the subunit composition. BN-PAGE can also provide information about the stoichiometry of the subunits in a protein complex, assembly of the protein complexes into the supercomplexes or the relative abundance and stability of a subcomplex within a protein complex. Finally, the relative abundance of these protein complexes can also be determined.

A very important feature of BN-PAGE is its versatility. Depending on how it is used and handled, BN-PAGE can provide the researcher with a large amount of information that can be used to answer particular scientific questions. For example, BN-PAGE followed by destaining with water can reveal the protein pattern in the gel. Further electroblotting onto PVDF membrane by Western blotting (WB) and probing with antibodies against particular proteins can reveal the mass and oligomerization state of a protein complex that contains that protein. Denaturing and reducing the BN-PAGE gel lane and further separation in the second dimension by SDS-PAGE following Coomassie or Silver staining could reveal the subunit composition of a protein complex or of many protein complexes simultaneously. Furthermore, WB using antibodies against a protein that is part of a protein complex can also be used. Of particular importance is the compatibility of BN-PAGE in either 1D or 2D (Coomassie or silver stained or even WB) with MS, which allows one to identify the full, intact protein complexes when analyzed in 1D or the subunit composition of these complexes when analyzed by 2D. This compatibility is of particular significance in the proteomics field and it has been used more and more over the past ten years.

When one sample has to be analyzed, BN-PAGE may be used as described above, either in one dimension (1D) or two dimensions (2D) and then stained by Coomassie or silver or analyzed by WB. However, BN-PAGE may also be used to compare two different samples at any of the steps described above. When two samples have to be analyzed and compared with each other for protein content, protein complexes, or PPIs, BN-PAGE can be a good choice and can be used in either 1D or 2D. Furthermore, BN-PAGE can also be used to compare the PPIs for both formation of PPIs and implicit protein complexes, as well as dissociation of the PPIs or protein complexes. Examples include analyses of PPIs as a result of a stimulus such as heat, stress or ligand stimulation. A good example was shown in a recent study, in which phosphotyrosine-affinity immunopurified protein complexes that resulted upon ephrin (a growth factor) stimulation were analyzed by BN-PAGE and then the proteins were identified by LC-MS/MS [87]. Therefore, the differences that result upon stimulation can be identified in terms of both protein identification, as well as PPIs identification. In addition, the combination of BN-PAGE with MS can also reveal both structural and functional information [87, 102, 103]. Furthermore, perhaps one of the best applications for BN-PAGE is in combination with differential gel electrophoresis (DIGE) technology and MS [104, 105]. DIGE involves labeling of the proteins by Cy dyes, separation by 1D isoelectric focusing (IEF) and 2D SDS-PAGE. When coupled with MS, protein identification can be achieved. The main outcome is protein quantitation due to the use of the Cy dyes. In BN-PAGE, labeling of the proteins by Cy dyes can help to monitor a protein complex's subunit composition, subunit stoichiometry, and complex stoichiometry (when it forms super-complexes). However, although it is a very attractive method, the combination of BN-PAGE and DIGE is not used often because the method is expensive, the method is not very accurate (the labeling is not 100%), and data analysis is time-consuming and may lead to misinterpretation of the results.

BN-PAGE was or could be successfully applied in either general proteomics experiments or in particular applications that allow one to answer to a specific question. For example, BN-PAGE and MS were successfully used as tools for determination of the molecular masses and subunit composition of stable homo- and hetero-protein complexes. This has been demonstrated since its discovery in the analysis of protein complexes from mitochondrial membranes [98–100]. Here, the authors were able to separate protein complexes and reveal their subunit composition using BN-PAGE 1D and 2D and Coomassie/silver staining or WB. A calibration curve was created for BN-PAGE using individual protein complexes such as thyroglobulin, ferritin or aldolase. Since then, many researchers have used BN-PAGE to investigate the subunit composition and the molecular mass of known as well as unknown, uncharacterized protein complexes. An example of BN-PAGE (1D) followed by LC-MS/MS experiments is shown in Figure 2A, where proteins that were part of homo-protein complexes (Valosin-containing protein and ATP citrate lyase) or hetero-protein complexes (Proteasome and eukaryotic translation initiation factor 3 complex) were identified. Since BN-PAGE and LC-MS/MS allows identification of both subunit composition and molecular mass of a protein complex, this combination is particularly useful in identification of transient PPIs, or protein complexes that assemble from different subcomplexes under different conditions, with a different molecular mass.

BN-PAGE can also be used to monitor the PPIs of individual proteins or a small number of proteins, with the final goal of determining which part of the protein(s) is involved in PPIs. In a recent study, Darie et al. [106] investigated the purified vitelline envelope (VE) proteins from rainbow trout by BN-PAGE using various solubilization procedures and polymerization assays and determined that the VE beta and VE gamma proteins polymerize differently from each other, and when forced to co-polymerize, they behave differently from the VE beta and VE gamma homopolymers, even the end-product of polymerization for each VE beta, VE gamma or VE beta-gamma monomers is the same: a three dimensional polymeric structure. An example of similar work is presented in Figure 2B, in which individual VE proteins were incubated to polymerize and then analyzed by SDS-PAGE (Figure 2B left), BN-PAGE (Figure 2B middle) and electron microscopy (Figure 2B right).

3.1.4. Colorless Native PAGE (CN-PAGE) as a method for investigation of protein complexes and PPIs—CN-PAGE (Clear-Native PAGE or Colorless Native PAGE) is a variant of BN-PAGE and separates protein complexes and PPIs under native conditions. CN-PAGE was developed by the same group that developed BN-PAGE [98–100] and further modified by others [86]. Unlike BN-PAGE, where the protein complexes bind the Coomassie dye and therefore are separated according to their molecular mass, CN-PAGE does not use Coomassie dye (the sample and the cathode buffers do not contain the Coomassie dye). As a consequence, CN-PAGE does not separate protein complexes according to their molecular mass, due to the external charge induced by the Coomassie dye, but rather according to the internal charge of the protein complexes, which is given by the charge of the subunits of each complex. Therefore, although the separation in both BN-PAGE and CN-PAGE is similar (it takes place under native conditions), the principle of separation of BN-PAGE and CN-PAGE is very different: in BN-PAGE, the protein complexes can be separated according to their molecular mass, while this information cannot be obtained by CN-PAGE. Therefore, CN-PAGE can be regarded as a BN-PAGE incapable to determine the molecular mass of the protein complexes. As such, CN-PAGE is used for very specific applications [86, 99, 107, 108]. For example, when one needs to use fluorescence, e.g., where the Coomassie dye from BN-PAGE interferes with the fluorescence signal, CN-PAGE can be employed [104, 105, 109, 110]. Or when two or more protein complexes have identical molecular mass, as determined by BN-PAGE and WB or MS, but the subunit composition of these complexes is not known, CN-PAGE can be used to pre-purify the protein complexes with identical mass but different internal charge, while

BN-PAGE can be used as a 2D method to identify the protein complexes with identical mass, that were already fractionated according to their internal charge [86].

3.2. Biophysical characterization of PPIs

3.2.1. Analytical ultracentrifugation—Analytical ultracentrifugation (AUC) is one of the methods of choice for the investigation of macromolecular interactions under physiological conditions [25, 111]. The principle of analytical ultracentrifugation relies on the sedimentation of macromolecules in solution inside a sample cell when this is subjected to a high centrifugal force inside a rotor [112]. The first use of analytical ultracentrifugation dates back to the 1920s when Svedberg and co-workers used analytical ultracentrifugation to study gold particle size distributions. In 1926, Svedberg received the Nobel Prize for chemistry for his work on disperse systems, employing among other techniques analytical ultracentrifugation [113, 114]. During analytical ultracentrifugation, the sample is subjected to sedimentation equilibrium or sedimentation velocity experiments and the process is monitored by spectroscopic techniques (absorption, interference or fluorescence) depending on the type of sample present in the cell. The reported data are either molar mass (sedimentation equilibrium, SE) or size and shape (sedimentation velocity, SV) of the macromolecular assembly. Classical analytical ultracentrifugation uses absorbance at near-ultraviolet or visible wavelengths for detection of molecules. However, this detection method presents many limitations. First, many biomolecules have overlapping absorption spectra rendering the study of multiple molecules in a single sample difficult. Second, the absorbance of highly-concentrated components will shield the contribution of others [115]. To remedy these constraints, analytical ultracentrifuges with fluorescence optics were developed [116, 117]. Currently available instrumentation on the market are provided with a laser light source emitting at 488 nm, which is far from the UV-VIS range of most proteins and nucleic acids [112].

3.2.2 Fluorescence resonance-energy transfer (FRET)—Rapid technological advances in image acquisition and processing together with the development of genetically encoded fluorescent proteins have triggered multiple opportunities to probe PPIs in living cells. FRET (also known as Forster's inductive resonance transfer of electron excitation energy) is a biophysical method based on the transmission of non-radiative energy from an excited donor protein onto an acceptor protein. For FRET to occur, both donor and acceptor have to be in close proximity, i.e., interacting (stokes radius) and the emission wavelength of the donor has to correspond to the excitation range of the acceptor [118]. It has been defined as a molecular ruler [119] due to its exquisite dependence on molecular distance, which operates in the range of 1–10 nm. It can resolve molecular interactions and conformations with a spatial resolution exceeding the inherent diffraction limit of conventional optical microscopy [118]. It allows for real-time monitoring of PPIs *in vivo* [120]. Bioluminescent resonance-energy transfer (BRET) is a variant of FRET widely used, which in contrary to FRET does not require an external light source. BRET makes use of the enzyme *Renilla* luciferase, which produces emitted light that is compatible with YFP excitation [121].

Typical fluorophores used in FRET include green fluorescent protein (GFP) or variants thereof such as cyan (CFP) or yellow (YFP) fluorescent proteins. GPCRs, which constitute the biggest group of receptors targeted by available drugs and represent the largest family of mammalian genes, were studied in detail by both RET methods [122–124]. Both techniques (BRET and FRET) can be combined to form a new method called SRET (sequential BRET-FRET) that is useful for the identification of PPIs of three different proteins. For SRET, one of the interacting proteins coupled to luciferase oxidizes a *Renilla* luciferase substrate leading to excitation of the second protein by BRET followed by FRET-mediated excitation of the third protein [125]. Another variant of FRET also used for the investigation of GPCRs

at the surface of living cells is time-resolved FRET (TR-FRET). In TR-FRET, a long-lived emission fluorophore (europium cryptate) is employed to overcome the problem of photobleaching [124]. Apropos photobleaching, there is a FRET-like method that uses the process of photobleaching for the exploration of PPIs. FRAP (fluorescence-recovery after photobleaching) as it is called, enabled the probing of oligomerization of transmembrane-receptors in living cells [126].

3.3. Microscopy: electron microscopy, fluorescence microscopy & confocal microscopy

Microscopy techniques allow detection of protein-protein interactions at the level of the whole cell. They find applications in the quantitative assessment of concentration variations and intracellular localization of different proteins as well as qualitative exploration of PPIs [17]. With microscopy, visualization of sites of complex formation within the cell is possible with resolution up to 4–5 nm [127–129]. The average diameter of protein globule lies around 3–5 nm whereas that of macromolecular complexes is in the range of 10–100 nm. Many *in vitro* methods as well as Y2H do not investigate protein interactions in their native environment. In recent years, advances in imaging and software technologies as well as novel fluorescent probes have made the investigation of protein interactions *in vivo* very interesting with high quality. Microscopy techniques widely-used nowadays for detection of PPIs include electron microscopy, fluorescence microscopy and confocal microscopy.

Electron microscopy (EM) enables visualization at the nanoscale compared to the microscale possible with standard light microscopy techniques. There exist many types of EM: single-particle EM [130], electron tomography [129] and electron crystallography [131]. EM also generates 2D projections of 3D objects. EM was also used to investigate PPIs, such as polymerization patterns using antibodies against the protein of interest [106, 132] or just for investigative, qualitative analysis, shown in Figure 2B and discussed earlier. Fluorescence microscopy has been mostly applied for co-localization of two labeled proteins, for FRET measurements and protein-fragment complementation assays (PCA). Different parameters can be looked at with a fluorescence microscope, e.g. intensity, quenching, polarization, wavelength. With fluorescence microscopy techniques such as FRET and FLIM (fluorescence lifetime imaging microscopy), qualitative analysis of protein-protein interactions with investigation of dynamics of conformational changes occurring in proteins in space and time, and of amino acid residues involved in these interactions, are possible [133]. In FLIM, lifetime fluorescence of each point of a spatial image is taken for evaluation of interaction between proteins [134]. Laser scanning confocal microscopy is another microscopy technique which enables the visualization of intracellularly co-localized proteins *in vivo* and thus their interaction in their physiological environment [135]. Typically, the proteins of interest are over-expressed in a cell with different tags to allow their tracing using a primary antibody directed against the tags followed by a fluorescently labeled secondary antibody. Fluorophores are chosen in such a way that their emission spectra do not, under optimal conditions, overlap.

3.4. Mass spectrometry (MS) approaches for detection and characterization of stable PPIs

The application of MS for the *direct* measurement of protein complexes has a rich history dating back to the early 1990s shortly after the development of ESI and MALDI ionization methods [136, 137]. Various techniques can be used to separate and isolate protein complexes of interest, and mass spectrometry can be used to identify the interactors within a complex, as discussed in previous sections. For example, immunoaffinity pull-down experiments and other affinity-based tagging strategies (e.g., His-, FLAG-, TAP-tagging) can be coupled with SDS-PAGE separation and subsequent mass spectrometry measurements (via trypsin digestion of the proteins) for identification of the protein constituents. Alternatively, the SDS-PAGE separation can be by-passed and “shotgun”

proteomics with LC-tandem mass spectrometry is employed for protein identification. These strategies have been used to yield putative protein interaction networks, or “interactomes” (*vide supra*), that can yield up to thousands of putative protein interactors [138, 139].

Less complex samples containing only a few stable protein-protein complexes can be separated and sized by liquid-based separation methods such as size exclusion chromatography (SEC); MS can be used to identify the interacting proteins. The intact molecular mass of the individual proteins can be measured to yield preliminary information regarding identity and the presence of PTMs. Similarly, non-denaturing gel-based methods such as native PAGE and BN-PAGE are often coupled with MS-based protein identification. Less common, but technically feasible, is the measurement of the intact mass of the proteins after native gel separation formats. An earlier report used non-denaturing isoelectric focusing (IEF) to separate protein complexes, with direct MALDI-MS measurement of the proteins still embedded in the gels [140]. The protein complexes migrated within the gel as intact complexes, and the MALDI-MS yielded the masses of the individual proteins from the complexes. The combination of BN-PAGE and direct laser desorption-MS was reported recently, yielding measurement of proteins from complexes greater than 900 kDa [141].

3.4.1. Direct measurement of protein complexes by native ESI-MS—The direct measurement of the masses of the protein complexes is possible with mass spectrometry. Although proteins interact through relatively weak non-covalent forces, they can remain intact in solution and in the gas phase during the MS measurement, especially with ESI [136, 137]. Protein complexes can be stabilized by covalent cross-linking chemistries for subsequent measurement using MALDI-MS [142–144]. The mass measurement accuracy is not especially high because of the uncertainties introduced by the cross-linking, but the measurement accuracies are typically sufficient to discern stoichiometries (e.g., aggregation size) for simple complexes. More accurate measurements are made by ESI-MS, where accurate masses of the individual denatured protein components followed by measurement of the non-denatured protein complexes can yield stoichiometries for heterocomplexes and even the presence of small molecule ligands (e.g., cofactors, metals, etc).

The application of ESI-MS and ESI-ion mobility spectrometry (IMS) for characterizing the size and structure of large protein assemblies, up to intact viruses, has matured during the past decade [145]. The ability of ESI to maintain the integrity of weakly-bound protein complexes during the transition from solution to the gas phase is the key enabling feature of the method. Protein complexes that exist in their intact form in solution can be transferred to their gas phase molecular state as the intact complex. This by itself is not direct proof that the *structure* of the gas phase complex is *identical* to the solution phase complex. In fact, this question is highly debated within the mass spectrometry community currently. However, it is clear that most studies report the fidelity of the mass spectrometry-derived stoichiometry, i.e., the size of the measured complex is consistent with the solution phase complex. Figure 3 shows a simple example of how ESI-MS is applied to measure the sizes of protein complexes. Rieske and Rieske-type proteins contain a 2Fe-2S cluster ligated by cysteines and histidine residues. Rieske-type proteins can be part of dioxygenase and other detoxification systems. Non-heme iron-containing dioxygenases in soil bacteria are critical to the microbial degradation of persistent and toxic aromatic and aliphatic compounds in the environment. The ESI mass spectrum of carbazole 1,9-dioxygenase (CarDO) from a pH 3 solution containing 50% acetonitrile shows multiple charging to over 59+ charges with a measured mass of 44.7 kDa, consistent with the mass of the denatured single protein chain (Figure 3A) [146]. Raising the solution pH to near 7 and eliminating the organic solvent shifts the peaks to higher mass-to-charge ratio (m/z) and reduces the measured charge states to a maximum of 28+ (Figure 3B). Also, the molecular mass has increased to 134.8 kDa that is consistent with the expected trimer complex and each monomer bound to one 2Fe-2S

cluster and one iron ion. Furthermore, the ESI mass spectrum of the related naphthalene dioxygenase (NDO) from a pH 6.8 solution shows a mass of 218.6 kDa for a $\alpha_3\beta_3$ complex (where $\alpha = 49.6$ kDa and $\beta = 22.8$ kDa) and one 2Fe-2S cluster and one iron ion is bound to each subunit (Figure 3C).

Non-covalent complexes of molecular machines, even exceeding 1 MDa are amenable to ESI-MS. Complexes such as the 700–800 kDa 20S proteasome have been measured in their intact state [147]. Robinson's group was the first to demonstrate the measurement of the 2.3 MDa 70S ribosomes (from *Thermus thermophilus*) [148]. Heck's lab has studied the assembly of intact hepatitis B virus (HBV) 3–4 MDa viral capsids (Figure 4) [149]. Moreover, by disassembling large complexes in solution, models for how complexes are assembled, e.g., its topology can be constructed. Robinson's study on the translation initiation factor eIF3 showed it to be composed of 13 unique protein subunits with a total mass of 794 kDa (measured 798 kDa) [150]. Dissociation of the gas phase complex using tandem mass spectrometry methods (MS/MS) provided information on the relative position of some of the "outer" subunits. Additional experiments to perturb polar and ionic interactions within the complex by recording ESI mass spectra from different ionic strength solutions (up to 500 mM ammonium acetate) yielded several stable subcomplexes. The topological model that was composed from this data fit well with the published electron microscopy (EM) data. Such reports reinforce the applicability of ESI-MS for studying stable, soluble protein complexes. The measured mass and stoichiometry information from the small sample size used by ESI-MS are difficult to obtain using other biophysical techniques.

3.4.2. Tandem mass spectrometry of protein complexes—Methods and applications to exploit the dissociation of the gas phase non-covalent protein complexes are being developed. This "top-down" approach could have unique advantages over other methods for characterizing protein complexes. Just as tandem MS is commonly used in "bottom-up" MS-based proteomics to identify and quantify proteins (via the analysis of tryptic peptides, typically), top-down MS shows potential for protein identification and PTM elucidation. Top-down MS of protein *complexes* can be used to assess protein assembly topology (*vide supra*). Collisionally activated dissociation (CAD) of protein complexes usually results in the release of one or a few subunits that are located on the peripheral areas of the complex topology. A phenomenon called asymmetric charge distribution usually results; the leaving subunit(s) retains proportionally more charge than its mass would suggest. For example, a 100 kDa homotetrameric complex with 25+ charges may dissociate into a monomer subunit with 13+ charges and the remaining trimer retaining 12+ charges, rather than the departing monomer with 25% of the mass of the complex leaving with 25% of the initial charge, or 6–7+ charges. Often CAD mass spectra are not consistent with the expected subcomplex composition, e.g., a complex composed of a dimer of dimers (i.e., tetramer) may dissociate into a monomer and a trimer. Surface induced dissociation, SID, appears to generate tandem mass spectra more consistent with the expected assembled subcomplexes [151].

The electron-based dissociation methods, such as electron capture dissociation (ECD) and electron transfer dissociation (ETD), have been exploited to provide positional information for small ligand binding onto proteins. Because it has been hypothesized that ECD does not disrupt weaker non-covalent bonds, the interactions between a ligand and a protein should be retained upon ECD. Our lab (Loo) has demonstrated this utility of ECD to determine the site of polyamine ligand binding to α -synuclein, a protein likely involved in Parkinson's disease [152]. Other reports of ECD-MS for determining the site of ligand binding have resulted after this initial publication [153, 154]. ECD tandem mass spectrometry has been used to address larger protein complexes. Gross' group showed that ECD of large tetrameric

complexes yields product ions from cleavage of protein backbone bonds found on the outer surface of the complex [155]. This area of research is still in its infancy, and it is hoped that advanced methodologies can yield protein-protein contact information directly without resorting to cross-linking chemistries.

3.4.3. New experimental developments for native protein ESI-MS—There are several other areas of active research designed to improve the applicability of direct MS for studying stable protein-protein interactions. Unlike the MS analysis of peptides and smaller biomolecules, on-line chromatography or another form of on-line sample separation has not been well exploited for the analysis of intact protein complexes. Non-denaturing liquid-based separations are challenging, but examples using capillary isoelectric focusing (cIEF) electrophoresis [156] and size exclusion chromatography of protein complexes on-line with ESI-MS have been reported with moderate sensitivity and mass range [157, 158].

A slightly different ESI-like format for presenting protein complexes to the mass spectrometer was reported by Chen and Loo [159]. Liquid sample desorption electrospray ionization (DESI) is a variant of the DESI technique developed by Cooks' group [160]. With DESI, a dried analyte mounted on a solid target is desorbed and ionized by a spray of multiply charged solvent droplets electrosprayed at the analyte target, and the resulting droplets carrying the analyte molecules are introduced into the mass spectrometer for their measurement. A liquid droplet containing the analyte replaces the dried analyte in the liquid sample DESI platform; this variation of DESI improves the apparent molecular mass range for protein analysis. Proteins as large as 150 kDa antibodies were measured by liquid sample DESI. Moreover, stable protein complexes can be measured by liquid sample DESI, and the platform allows a convenient way to test different solvent systems for desorption/ionization of the complex and to add dopants that may act as reactive agents (by changing the solvent composition of the DESI spray and adding the dopants into the DESI spray).

The measurement of very large protein complexes by native ESI-MS can be hindered by the mass-to-charge ratio (m/z) limit of the mass spectrometer. Although ESI generates multiply charged molecules, the number of charges retained by very large complexes is not linearly proportional to molecular mass. Rather, charge appears to follow the surface area of a complex. Heck's ESI mass spectrum of a 4 MDa viral capsid shows charging of 160+ charges measured at m/z 25,000 (Figure 4) [149]. Even the mass spectrum of naphthalene dioxygenase complex shown in Figure 3 shows 27+ charged molecules measured above m/z 8000, beyond the range of many commonly-used ESI mass spectrometers. This deficiency can be somewhat alleviated by the addition of a "supercharging" reagent to the protein analyte solution. Supercharging reagents, such as meta-nitrobenzyl alcohol (*m*-NBA), first shown by Williams' group for denatured proteins [161] and our lab [162] for non-denatured protein solutions, and sulfolane [163] can increase the ESI charging to a moderate extent. This is demonstrated for the 93 kDa enolase protein dimer in Figure 5 using the liquid sample DESI method. Supercharging of protein complexes also aid the tandem MS-based characterization because dissociation efficiency is generally increased with higher analyte charge. Yin and Loo showed that more product ions were measured by CAD and ECD of supercharged adenylate kinase bound noncovalently to ATP and carbonic anhydrase bound to zinc compared to the analysis of lower charged precursors [164].

Most direct studies of stable protein complexes using native MS techniques have focused on protein(s) that have been overexpressed and/or enriched to quantities that are much less than the requirements for most high resolution structural experiments (e.g., NMR, x-ray crystallography), but the levels are higher than most routine biochemical experiments. The development of purification strategies to isolate *in vivo* protein complexes present at low cellular abundance to levels sufficient for MS analysis is required. Immunoaffinity and

affinity tagging approaches are commonly used to identify the interactors involved in protein complexes, but they leave the proteins denatured for the analysis and stoichiometry information is lost (*vide supra*). The application of tandem affinity purification tags (TAP tags) can be sufficiently gentle that the isolated protein complexes expressed at physiological relevant levels retain functional activity and native stoichiometry can be probed by MS. Both the Robinson and Heck labs used the TAP tag approach to isolate ca. 400 kDa nuclear exosome complexes from *Saccharomyces cerevisiae* and measure their stoichiometries by native MS [165–167]. More of such experiments to obtain direct information on the architecture of physiologically relevant and heterogeneous cellular complexes should be reported in the future.

Despite constituting 30% of the total genome, membrane proteins are under-represented in many proteome profiles. Membrane-bound proteins, anchored within the cell's lipid bilayer, regulate the influx and efflux of molecules and information. They regulate intracellular vesicular transport, control membrane lipid composition, and organize and maintain the shape of organelles and the cell itself [168]. Membrane proteins are the targets of a large number of pharmacologically active substances and are responsible, in part, for their uptake, metabolism, and clearance. Membrane proteins represents 60% of the known existing and future drug targets [169].

Because of their hydrophobic nature, membrane proteins are difficult to work with, resulting in their under-representation in the Protein Data Bank. Their heterogeneity and low abundance further contribute to their under-representation in proteome studies. To overcome these limitations, various strategies have been applied in their enrichment, solubilization, and separation. The landmark paper by Barrera *et al.* established that the direct measurement of membrane-embedded protein complexes from solution can be addressed by native MS [170]. By protecting a membrane protein complex within a n-dodecyl- β -D-maltoside micelle and then gently stripping the detergent molecule away in the mass spectrometer, subunit stoichiometry and ligand-binding properties of membrane complexes can be determined directly [171]. A follow-up study showed impressively that ATPases/synthases from *Thermus thermophilus* and *Enterococcus hirae* composed of 26 subunits and 9 different proteins can be maintained intact with membrane and solution subunit interactions preserved during the MS measurement [172]. Lipids bound within the membrane rotors were measured and the regulatory effects of nucleotide binding on both ATP hydrolysis and proton translocation were revealed.

3.5. Other approaches for detection of stable and transient protein-protein interactions

3.5.1. Size exclusion chromatography—Size exclusion chromatography (SEC) is a separation method that is based on molecular hydrodynamic volume (defined by the Stokes radius) or size. It was applied first by Lathe and Ruthven for the separation of sugars, amino acids and proteins [173]. Generally, dependent on the solid phase which is usually a porous polymer beads with pores of different sizes, molecules travel at different speed rates through the chromatographic bed and are thus separated. SEC has been successfully used for the study of protein aggregation. For example, the method was used to determine the size and size distribution of aggregates of the amyloidogenic protein amyloid- β (A β) which is thought to be the cause of Alzheimer's disease [174]. Commonly, SEC is utilized in conjunction with another method for the detection of PPIs since SEC as a stand-alone method presents several limitations. One limitation is the fact that the elution time depends not only on the molecular weight of the protein or protein complex, but also on its shape. Further, modification of proteins such as glycosylation or any interaction of the protein with the column matrix might lead to erroneous elution time. Therefore, SEC can be combined to other analytical methods. For example, SEC was coupled to on-light scattering, absorbance

and refractive index detectors for the study of proteins and their interactions [175]. Using the latter approach, Wen et al. [175] studied the stoichiometry of the complex made of the human TNF- α trimer (tumor necrosis factor alpha) and the extracellular domain of the TNF type I receptor (sTNFR) and could confirm a molar ratio of three sTNFR to one TNF- α trimer that was prepared. In another study, coupling of SEC to inductively coupled plasma mass spectrometry (ICP-MS) allowed the determination of binding kinetics of metallodrugs to albumin and transferrin [176]. SEC has also been shown to correlate with other methods used to assess protein-protein interaction such as Surface Plasmon Resonance (SPR) or analytical ultracentrifugation [177, 178]. Moreover, it is even possible to identify sites of interactions in proteins or protein complexes using SEC as demonstrated by Tachiki et al. [179]. Using SEC, the interaction site for the MutS-MutS complex was located to the B-domain (MutS is a mismatched DNA recognition protein from *Thermus thermophilus* HB8).

3.5.2. Bioinformatic and computational approaches—Bioinformatics currently plays an important role in many disciplines such as genomics, drug design and proteomics. Its application to PPIs is a response to the increased and more complex amount of data resulting from high-throughput biological experiments. Investigation of PPIs by means of bioinformatics allows verification or confirmation of experimental results to avoid false positive and/or false negative conclusions. It can serve to assess the validity of possible interacting partners, given the enormous number of possible interactions within a cell. There are already many tools and algorithms for prediction of protein-protein interactions. Moreover, these tools have been implemented such that identification of interface residues and assignment of protein functions to genes are possible. Broadly, investigation of PPIs through bioinformatics first involves the retrieval of the amino acid sequences of the proteins of interest (e.g., from NCBI). Then, 3D-models are generated with adequate programs (e.g., Accelrys DS). Next, the model is optimized and evaluated with regard to energy minimization and finally validated. Protein-protein interactions or complex formation can be computed using several algorithms such as HEX6.3 [180]. For the estimation of the interaction, existing models take different paths. Previous models were based on the recognition of specific residue motifs [181]. Others focus instead on genomic sequence analysis [182]. Further, primary structure and associated physicochemical properties are also parameters that can be considered for the prediction of PPIs [183]. New domain-based protein interaction predictions have been advanced as another alternative [50, 184]. However, the “gold standard” for prediction of interaction is the docking method, which is computationally very challenging and thus very unlikely to be applied for high-throughput purposes. With docking, it is possible to figure out if two proteins interact and also how the interaction takes place. Today, there are quite a number of databases of protein-protein interaction data such as biomolecular interaction network database or BIND [185], database of interacting proteins or DIP [186], general repository for interaction datasets or GRID [187], saccharomyces genome database or SGD [188] and human protein reference database or HPRD [189].

4. Conclusions

The most important and commonly-used approaches for identification of PPIs, with particular focus on their investigation by MS, have been reviewed. In the past, most protein studies have focused on the (single) structure level or PTM level, but recently there has been a consistent increase in the number of publications where proteins were studied at the PPI level. As new tools become available and become more sensitive and more applicable, more protein studies will focus on protein interaction partners, bringing the science of proteins from the primary, secondary and tertiary structural level to the quaternary level. Upon completion of the draft of the human genome, Francis Collins and the US National Human Genome Research Institute defined a Grand Challenge to elucidate the organization of

genetic and protein networks and to establish how they contribute to cellular and organismal phenotypes. “Defining these systems and determining their properties and interactions is crucial to understanding how biological systems function...A complete interaction map of the proteins in a cell, and their cellular locations, will serve as an atlas for the biological and medical explorations of cellular metabolism [190].” Daring to analyze transient PPIs gives us confidence that the full interactome, the key to biochemical processes, is about to be revealed.

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Abbreviations

SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
BN-PAGE	blue native PAGE
CN-PAGE	colorless native PAGE
M_w	molecular weight
WB	western blotting
MS	mass spectrometry
LC-MS/MS	liquid chromatography mass spectrometry
MALDI-MS	matrix assisted laser desorption ionization mass spectrometry
m/z	mass/charge
CID	collision-induced dissociation
ATP	adenosine triphosphate
RNA	ribonucleic acid
PTMs	post-translational modifications
PPIs	protein-protein interactions
Y2H	yeast two-hybrid
TAP-MS	tandem affinity purification-MS
FRET	fluorescence resonance energy transfer
AP-MS	affinity purification-MS
EMSA	electrophoretic mobility shift assay
DNA	deoxyribonucleic acid
NR	non-reducing
R	reducing

IgG	Immunoglobulin G
AUC	analytical ultracentrifugation
SEC	size exclusion chromatography
IEF	isoelectric focusing
EM	electron microscope
CAD	collision-activated dissociation
ECD	electron capture dissociation
ETD	electron transfer dissociation
DESI	desorption-ESI
TNF-α	tumor necrosis factor alpha
ICP-MS	inductively coupled plasma-MS
IP	immunoprecipitation
IMS-MS	ion mobility spectrometer-MS
ELISA	enzyme-linked immunosorbent assay

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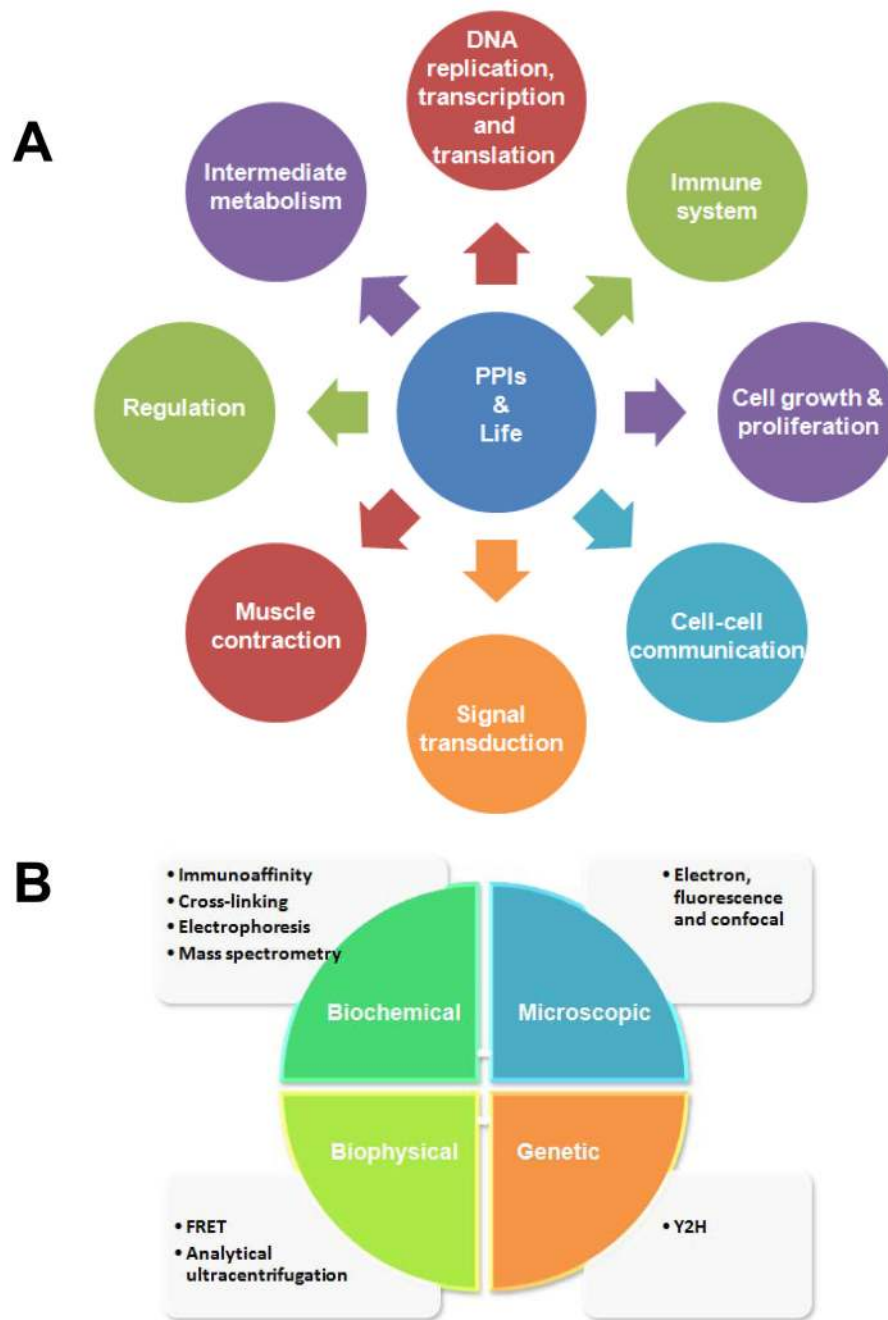


Figure 1. Roles of protein-protein interactions (PPIs) in organisms and methods for their investigation

(A) PPIs play an important role in every cellular process and thus in life as well. It is fair to say that there will be no life without the communication of proteins with each other. (B) Methods commonly used for the investigation of PPIs can be classified into four main groups: Biochemical, genetic, biophysical and microscopic. Of all these groups, biochemical techniques are the one most described in the literature and also most applied in experiments.

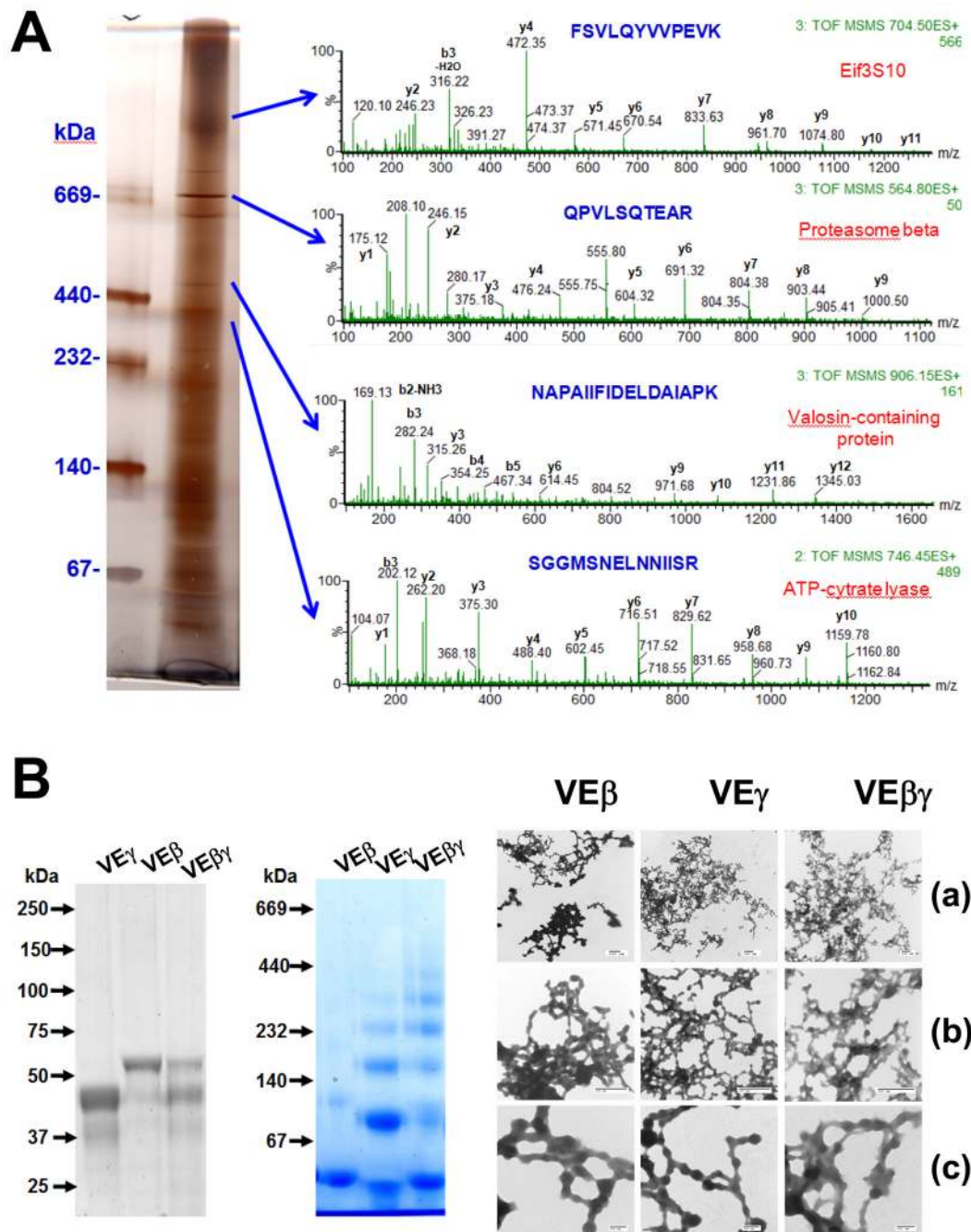


Figure 2. Identification of PPIs by BN-PAGE and LC-MS/MS (A) and by BN-PAGE and EM (B) (A) The cell lysate of NG108 neuroblastoma x glioma cells was separated by BN-PAGE and then silver-stained. The gel bands corresponding to protein complexes were excised and digested by trypsin and further analyzed by LC-MS/MS. The MS/MS of peptides that were part of Eif3S10, Proteasome beta, Valosin-containing protein and ATP citrate lyase are shown. The sequences of the peptides are indicated for each spectrum. The approximate position in the gel for the proteins mentioned is shown. These proteins are parts of multisubunit-protein complexes (Eif3S10 is part of an 800 kDa complex and Proteasome beta is part of a 700 kDa complex) or homo-protein complexes (Valosin-containing protein is a 540 kDa homohexamer and ATP citrate lyase is a 480 kDa homotetramer). The Mw

markers are indicated. (B) Isolated VE proteins were allowed to polymerize and then they were analyzed by SDS-PAGE (left), BN-PAGE (middle) and EM (right). Under denaturing and reducing conditions, VE proteins resolve in SDS-PAGE as monomeric proteins, but under native conditions, they polymerize into higher and higher polymeric structures. Polymerization pattern of VE gamma is different from the polymerization pattern of VE beta and each of them polymerizes different from the mixture of them. Magnification is x 15,000 (a), x 60,000 (b) and x 150,000 (c). (Figure adapted from reference 92 with permission from the publisher.)

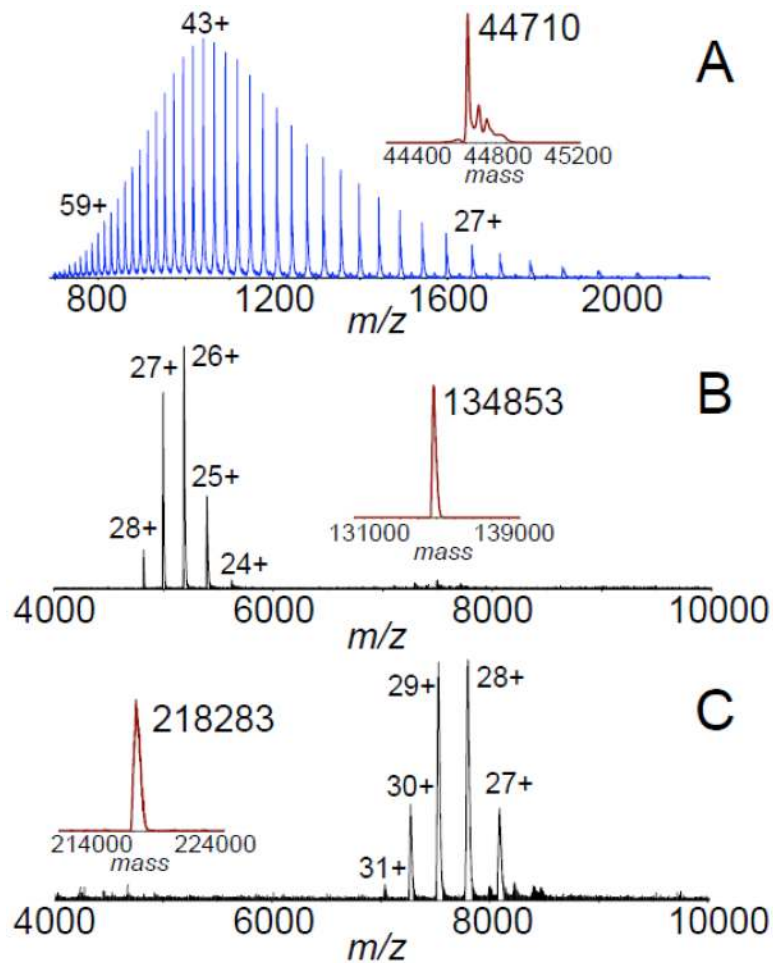
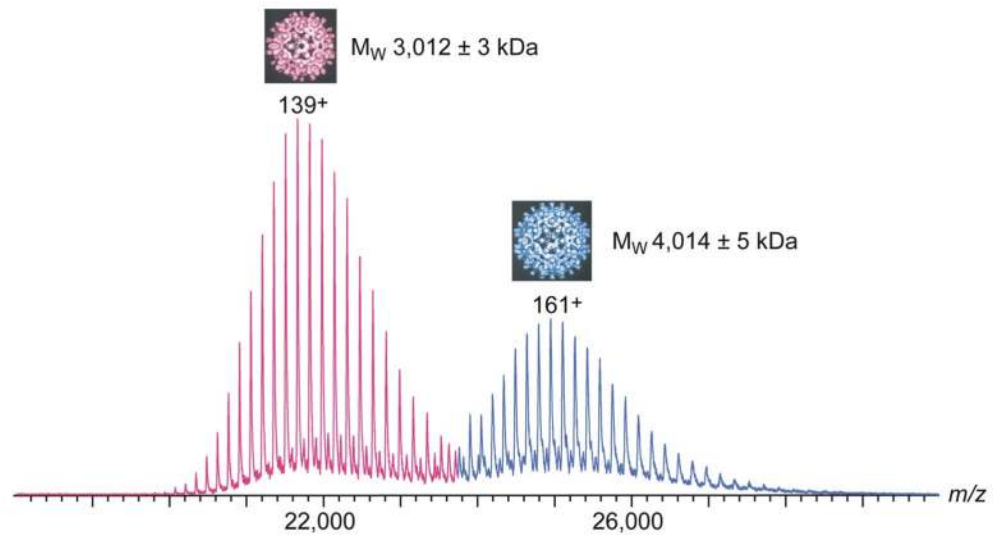
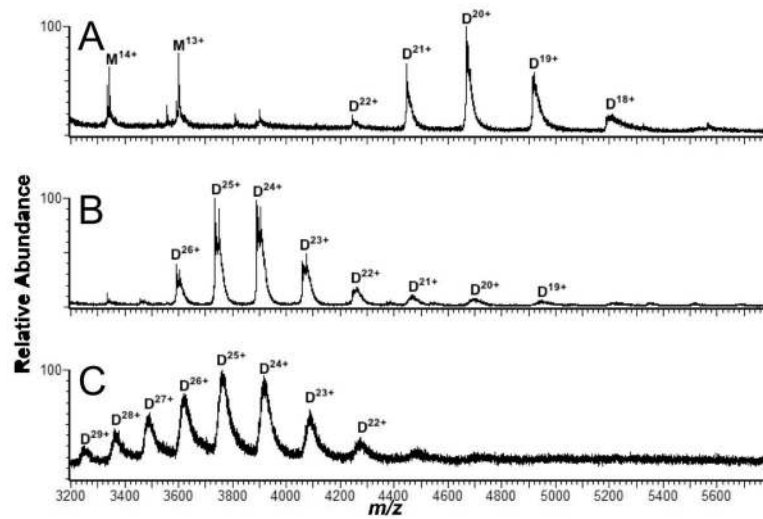


Figure 3. Investigation of PPIs by ESI-MS

ESI mass spectra of carbazole 1,9-dioxygenase (CarDO) (A) from a denaturing pH 3 solution containing 50% (v/v) acetonitrile showing the mass of the denatured 44.7 kDa polypeptide, and (B) from a 20 mM aqueous ammonium acetate, pH 6.8 solution showing the mass of the intact 134.8 kDa trimer complex (with each monomer bound to one 2Fe-2S cluster and one iron ion). The ESI mass spectrum of naphthalene dioxygenase (NDO) (C) from a pH 6.8 solution shows a mass of 218.6 kDa for a $\alpha_3\beta_3$ complex with one 2Fe-2S cluster and one iron ion bound to each subunit. The inset figures show the zero charge deconvolution mass spectrum. (Figure adapted from reference 146 with permission from the publisher.)

**Figure 4. ESI-MS of native PPIs**

Native ESI mass spectrum of intact HBV viral capsids from capsid particles reconstituted *in vitro* from truncated cp149 capsid monomers in 200 mM aqueous ammonium acetate, pH 6.8. The distribution of peaks around m/z 22,000 and 25,000 represent the $T=3$ and $T=4$ capsids, with a measured mass of 3,012 and 4,014 kDa, respectively. (Figure adapted from reference 149 with permission from the publisher.)

**Figure 5. Investigation of PPIs using DESI**

Liquid sample DESI mass spectra of enolase (10 μ M in water) with the DESI solvent composed of (A) 20 mM NH₄OAc (aq), (B) 1:1 ACN/H₂O with 1% FA, and (C) 1:1 ACN/H₂O with 1% FA and 40 mM *m*-NBA supercharging reagent. (Figure reprinted from reference 159 with permission from the publisher.)