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Chapter

Mass Spectrometry-Based Proteomics Study on *Candida* Infection of COVID-19 Patients to Discover New Antifungal Target

Debashis Dutta, SurendraManuri, Vivek Kumar Sidhu and Prashant Kumar

Abstract

The molecular foundation of fungal disease can now be better understood and treated because to advances in mass spectrometry (MS) based proteomics technology. Numerous disease-related biomarkers and potential new drug targets have been discovered over the course of the past 30 years of proteomics research, which examines dynamic protein expression, post-translational modifications, cellular and sub-cellular protein distribution, and protein–protein interactions. Although MS proteomics was of paramount importance to understanding the molecular progression involved in their differential expressions but was challenging under invasive and non-invasive growth conditions of *Candida*. species but was challenging especially due to the lack of diagnostic morphological features for early prediction. The long-term goal of this chapter is to identify the biomarkers relevant for early prediction and future target molecules for drug discovery and to determine proteins linked to fungal action, made the identification of alterations in fungal physiology and host-pathogen interactions between cells and antibiotics during COVID-19 infection therapy. Here, we also discussed the developments of proteomic-driven interactions between the host and the fungal pathogens, clinical application of spectrometry-based *Candida.* proteome identification diagnosis, and treatment with antibiotics. Proteomic approach advancements open new pathways for effective prevention and medication development for infectious diseases brought on by fungi.

Keywords: mass spectrometry, proteomics, candidiasis, biomarker, drug–target interactions prediction, COVID-19

1. Introduction

The use of proteomics based on mass spectrometry (MS) for diagnosing infectious diseases relies on the type of pathogens being examined, such as bacteria or viruses. Laser desorption/ionisation time-of-flight with matrix assistance (MALDI-ToF) MS has been developed to identify bacteria in clinical microbiology. It is a quick,

straightforward, and affordable method of identification. High-throughput bacterial species identification is made possible by specialised mass spectrometers, standardised sample preparation methods, and spectrum libraries that the relevant authorities have approved. Utilising MALDI-ToF to biotype Protein MS is used most effectively by MS in clinical laboratories [1–4]. The third most common source of infections in healthcare is bloodstream infections (BSIs) [5]. BSIs often result from the urinary tract, intestinal or community-acquired pneumonia infections, mainly caused by bacteria or fungi [5, 6]. Hospitalised individuals frequently have "candidemia, "or the blood-borne fungus *Candida*. *Candida albicans, Candida glabrata, Candida parapsilosis, Candida tropicalis*, and *Candida krusei* are the most commonly associated with candidemia [7].

In adults and children, *C. albicans* is the most prevalent fungus isolated from BSI and is linked to high mortality rates [8]. Recently, technologies based on genetic information and DNA sequencing have been utilised to find infections in the blood. Lipopolysaccharides for Gram-negative bacteria and galactomannan for fungus are two examples of serological techniques [8].*C. albicans* is the primary source of invasive candidiasis (**Figure 1**), associated with considerable morbidity and mortality in immunocompromised and severely ill patients [9, 10]. Several circumstances predispose IC. These linked to altering the host's microbiota (for instance, through prolonged use of broad-spectrum antibiotics), (ii) rupturing the cutaneous and gastrointestinal barriers (for example, through organ transplantation or the use of central venous catheters), and (iii) weakening the host's defences (for instance, through cancer, neutropenia, chemotherapy, or corticosteroid therapy) [10, 11].

Over the past two decades, mass spectrometry (MS)-based proteomics has significantly contributed to microbiology. A search of PubMed papers revealed a steady increase in contributions due to the application of MS-based proteomics in fungal biology, host-fungal interactions, and the creation of antifungal medicines. Based on high-resolution MS, proteomics is a powerful method for measuring and profiling proteins in cells, organs, and tissues [12, 13]. It offers in-depth knowledge of the dynamics of cellular interactions, modifications, and processes.

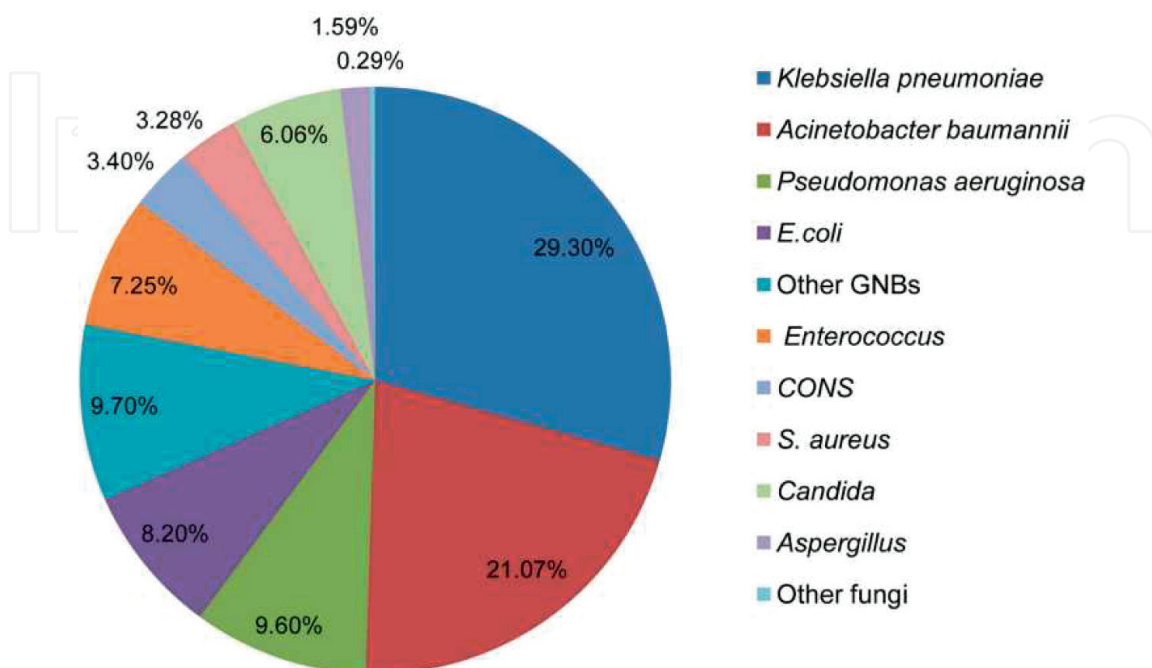

Figure 1.
Distribution of COVID-19 patient-isolated bacterial and fungal pathogens [9].

2. Antifungal resistance mechanisms

For instance, a quantitative proteomics study using iTRAQ labelling comparing haploid and diploid *C. albicans*, cultures looked for proteins linked to amphotericin B resistance, the "gold standard" antifungal drug [14]. The analysis also discovered 100 distinctively abundant proteins among the various fungal strains, focusing on those involved in the oxidative stress response, a key mechanism in Amphotericin B cytotoxicity. Alkyl hydroperoxide reductase 1 (ahp1) was crucial in determining antifungal susceptibility. This study revealed a unique antifungal resistance mechanism in *C. albicans*, linking Amphotericin B tolerance to ahp1 expression via preservation of persister cells' oxidative capability in biofilms. Profiling the proteome in response to drug treatment is another method for identifying antifungal resistance processes, as was recently shown in *Candida glabrata*. This study uses iTRAQ-MS from membrane-enriched samples to define a unique resistance mechanism for the azole antifungal Clotrimazole [15]. After clotrimazole treatment of *C. glabrata*, twelve proteins, including four multidrug resistance transporters—two previously characterised and linked to imidazole resistance—and two novel targets, were up regulated. By finding the novel targets (CgTpo1 2 and CgTpo1 1) by gene deletion, the mutant strains' greater susceptibility to various antifungals was discovered, highlighting the many functions played by these transporters in the survival of the fungus during treatment.

3. Principles of MS-based proteomics

Simple protein denaturation and solubilisation using a chaotropic agent, such as urea, to severe tissue destruction, such as probe sonication and boiling in a detergent are only a few sample preparation methods available for MS analysis (e.g., sodium dodecyl sulfate). Using sequence-specific proteases like trypsin or Lys-C, proteins are broken down into peptides for bottom-up and targeted research before being purified on C18 resin [16]. Specifically, metabolic (such as isotopically stable amino acid incorporation at the cellular level [17, 18] or chemical (such as the inclusion of mass tags or chemical derivatisation [19–21] or label-free [22] approaches are utilised for the absolute and relative quantification of proteins or peptides. Depending on size, mass, or charge, samples may be divided to streamline them further and promote excellent proteome coverage. The second MS scan (MS2) or MS/MS scan chooses and fragments peptides or ions for identification based on fragment masses after the first MS scan (MS1) records groups present at a particular instant. With the aid of LC-MS/MS, top-down proteomics analyses intact proteins to separate them based on size and peak isolation, as well as to identify and count various proteoforms. Bottom-up proteomics uses LC-MS/MS to objectively assess proteolytically digested proteins (peptides) to identify and quantify proteins in a sample. Targeted proteomics may identify, describe, and measure specific proteins and biomarkers using a predetermined set of peptides (isolation of parent ion by mass in Q1, collision of the ion in Q2, and mass filtering of synthesis in Q3).

4. Fungal pathogenesis by MS-based proteomics

The secretome and vesicles of the external environment, the complete proteome of the cellular compartment, and changed microbial states for survival could all be

molecular structures (i.e., biofilms and spores). Phosphorylation, glycosylation, and ubiquitination are a few of the most frequent post-translational modifications (PTMs) [8].

4.1 Cellular compartment

In addition to providing an overview of cellular regulatory mechanisms and signal transduction pathway alteration, the study of intracellular proteins also suggests connections between protein synthesis and disease. A subset of considerably elevated proteins focused on central carbon metabolism pathways (such as arabitol synthesis and glycerol formation) were found in *C. albicans* cells cultured under normal conditions (e.g., yeast potato dextrose (YPD) medium) and those under high osmolarity salt stress [23]. A closer look at this protein subset indicated strong selectivity for the accumulation of osmolytes during cellular stress adaptation during the osmotic stress response. This study highlights the significance of fungi adapting to shifting environmental conditions and illustrates how osmoregulation affects proteins and metabolic pathways. Proteomics can characterise the several growth states that thermodimorphic fungi like Paracoccidioides can alternate between [24]. Microbiological models of fungi are beneficial for the creation of MS-based proteomics methods. This was recently proven using *C. albicans* and enhanced stable isotope labelling in amino acid cell culture (SILAC). To properly quantify proteins in a sample, SILAC is a metabolic labelling technique that uses light- or heavy-labelled lysine and arginine. Traditionally, efficient labelling in SILAC requires auxotrophic mutants; however, Native SILAC (nSILAC), recently developed, offers excellent labelling throughout exponential cell growth without the need for auxotrophic strains [25]. Notably, strong proline conversion from heavy arginine causes issues with peak detection in *C. albicans*. To address this issue, the authors developed a computational strategy to modify the protein SILAC (heavy/light) ratios, which balanced the ratios of proline-containing to proline-free peptides.

4.2 Extracellular environment

The secretory apparatus and pathways also produce vesicles, which may affect the pathogenesis of fungi.

The extracellular vesicular fraction of *C. albicans* was proteomically profiled, and 75 proteins with highly varied biological activities were found to be exclusive to the vesicular fraction [26]. These 60% had a glycosylphosphatidylinositol (GPI)-anchor or a signal peptide, suggesting conventional secretion pathways. However, the other 40% lacked a signal peptide and most likely used other export mechanisms (e.g., vesicular pathways, non-classical secretion, or proteins capable of performing dual or multiple functions depending on cell localisation). Notably, the released immunogenic protein Bgl2 was present in the vesicular and supernatant fractions, and the isolated protein was examined as a possible vaccine against invasive candidiasis [26, 27].

4.2.1 Modifications and interactions

In order to build a list of targets, which is essential in understanding the function of proteins, including kinases and phosphatases, it is required to identify proteins that interact physically. In order to distinguish real interactors from

proteins that adhered non-specifically to the affinity matrix in *C. albicans*, SILAC labelling and affinity-purification MS (AP-MS) were used in conjunction with a substrate-trapping mutant of Cdc14, a crucial player in the regulation of mitosis and cell division [28]. One hundred twenty-six proteins were found to interact with Cdc14 due to the proteome study, of which 44% have a Cdc14 dephosphorylation motif and 80% are considered novel interactors. These proteins are necessary for DNA repair and cytokinesis during the cell cycle. This study provides an expanded list of Cdc14 interactors in *C. albicans*. It creates a reliable and quantitative mechanism for locating genuine kinase and phosphatase partners in a fungal system [8].

5. Microbial competition demonstrating antifungal properties

Identifying proteins produced by each bacterium to ensure their survival through symbiosis, competition, or predation may be made possible by studying the interactions of microbial species coexisting in an environment. In support of the quickly expanding field of biocontrol agents, this strategy most recently resulted in the discovery of lugdunin, the first novel class of antibiotics discovered in more than 30 years [29]. In the nose, *Staphylococcus lugdunesis* competes with *Staphylococcus aureus* for lugdunin production. Researchers have discovered a connection between the activity of bacterial chitinase against fungal cell walls and the anti-pathogenic effects of *Bacillus safeness*, *Cryptococcus neoformans*, and *C. albicans* in the natural environment [30, 31]. The potential for new biocontrol agents can also be shown by comparing distinct fungal species. For instance, the mycoparasitic yeast *Saccharomycopsisschoeni* kills the growing multidrug-resistant *Corynebacterium auris* [32] despite the lack of clear explanations of the molecular mechanisms underlying this interaction. Here, quantitative live-cell microscopy assays and genomic, transcriptomic, and proteomic methods were combined to identify the genes and proteins overproduced by *S. schoenii* during its predation of model prey cells, *Saccharomyces cerevisiae* [33]. Proteome analysis of the interaction between *S. schoeni* and *S. cerevisiae* revealed an abundance of proteins related to catabolic processes and the regulation of sulphur metabolism. Predation, however, resulted in an enrichment of proteins related to cell walls. Additional investigation into predator–prey relationships indicated that aspartic protease overexpression was associated with predatory behaviour and that overall nutritional deficiencies were the main cause of predation [32]. When considered as a whole, this body of data offers a thorough and objective analysis of *S. schoeni's* predatory behaviours. It suggests *S. schoeni* yeasts as viable biocontrol agents as an alternative to the persistent misuse of antifungals, which encourages the emergence of resistant fungal strains [33].

6. Proteomics of host-fungal interactions using MS

The interaction between host and germ is essential for the early control and clearance of invasive microorganisms as well as the prognosis of the disease. Determining the conflicting functions of different biological systems from a global perspective is necessary to discover novel approaches to combat infection (i.e., taking into account both the host and microbial responses).

6.1 Host perspective

The host's immunological response, particular proteins or disease-fighting pathways, and potential treatment targets to boost the host's defence mechanisms can all be learned by profiling the impacts of fungal infection from the host's point of view. In *C. albicans*, a recent study found that protein abundance increased in correlation with reported transcript levels but that a decrease in transcript levels had no corresponding effect on protein abundance [21]. The relationship between reduced transcript levels and protein abundance depends on post-translational regulation and the intracellular stability of the protein (such as protein turnover rates) [27, 28].

6.2 Pathogen perspective

By characterising the host-pathogen relationship from the pathogen's point of view, it is feasible to identify new virulence factors, characterise new mechanisms of action for previously described fungal proteins, and discover anti-virulence strategies to avoid infection. According to an image-based high-throughput screening experiment to examine host-fungal interactions, a proteinS-acyltransferase (PFA4; involved in catalysing lipid modifications of proteins) influences fungal adherence and phagocytosis in human monocytic cells in *C. neoformans* [29]. Here, 72 Pfa4-specific host protein substrates were discovered using click chemistry, MS-based proteomics, and biorthogonal palmitoylome-profiling (metabolic labelling of fatty acids with a palmitic acid analogue containing an alkyne group).

6.3 Dual perspective

The development of MS technologies and bioinformatic platforms for integrated data processing has increased the accessibility of studying host-pathogen interactions. This is because it enables researchers to compare different points of view in a single experiment. In order to understand how *C. albicans* escapes from macrophages, dual proteome profiling is used [30]. This study discovered 1253 macrophage proteins, 227 *C. albicans* proteins, and 483 *C. albicans* proteins (5 showed differential regulations).

The scientists contrasted their mixed proteome analysis with conventional proteome analysis, which does not distinguish between different cell types but instead allows all the proteins in a sample set to be processed before being bioinformatically categorised as either fungal or mammalian. Traditional proteome analysis involves isolating and dividing cells according to their biological origin (for instance, fungal or mammalian), examining two databases that are particular to those two organisms.

7. Proteomics using MS to create new antifungal drugs

Antifungals face difficulties overcoming the close evolutionary link between eukaryotic fungal cells and the human host, in contrast to antibiotics that treat bacterial infections. Antifungal drugs must focus on the eukaryotic fungal cell while minimising cytotoxicity and injury to human biological processes [31, 32]. Currently,

polyenes, azoles, pyrimidine analogues, and echinocandins are the four antifungals frequently used in monotherapy or combination. Today, finding and creating new antifungal treatment methods to thwart the spread of fungal diseases is made possible by MS-based quantitative proteomics.

These techniques involve identifying the processes underlying antifungal resistance, identifying microbial interactions with antifungal qualities, and identifying fresh possibilities for developing antifungal medicines, vaccine candidates, and therapeutic repurposing.

8. New antifungal developments, vaccine design, and drug repurposing

The creative use of clinically approved drugs alone or in conjunction with other substances to fight infection is made possible by drug repurposing approaches. This strategy may lessen the amount or length of time that antifungals must be administered, which lowers the chance that fungus will acquire resistance. Recently, a novel therapeutic repurposing strategy was proposed to interfere with proteostasis utilising an FDA-approved anti-cancer drug to treat cryptococcosis caused by *C. Neoformans* [34].*C. neoformans* is controlled by the cAMP/PKA signal transduction pathway, and quantitative proteomics investigation of this organism revealed protein clustering associated with translation and the ubiquitin-proteasome pathway. The ubiquitin-proteasome pathway, PKA activity, and protein degradation in neurological illnesses are connected. Further investigation of proteasome function using the inhibitor bortezomib revealed an impact on capsule creation and pathogenicity [35]. This study establishes synergistic drug studies combining bortezomib with commonly used antifungals to treat cryptococcal infection (such as fluconazole and amphotericin B). Designing vaccinations that can trigger protective immune responses against infection is an alternative strategy for halting the global spread of fungal illness. Th1-type CD4+ T cell-mediated immunity is essential for the host response because Th1 cytokines promote the recruitment of lymphocytes, phagocytes, and delayed-type hypersensitivity responses [36]. Proteomics and immunological techniques were used to identify three cytoplasmic proteins that could trigger a Th1 response and four immunogenic cell wall-associated proteins in *C. neoformans,* pointing to potential new vaccine candidates [37]. In another study, 13 species of medically necessary fungi were subjected to quantitative proteome analysis to create a pan-fungal or broad-spectrum vaccination to guard against infection by various fungal species [38].

These included Crf1, Ecm22, and EglC, 1,3-glucanosyltransferases (Gel1–4, Bgt1, and their homologues), Gel1, and Crf1. Gel1 and Crf1 had previously been identified as promising vaccination candidates, validating the use of proteomics in discovering potential candidates. These proteins were discovered to be prevalent in a wide variety of fungus species and to be unrelated to human proteins. Last but not least, it was found that two recently developed antifungal prototypes, thiosemicarbazide (TSC) and a camphene derivative of TSC (TSC-C), had advantageous medical properties, including the capacity to inhibit *P. lutzii* growth [39]; however, the targets of these antifungals have not yet been established. Here, the compounds interacting with TSC and TSC-C in *Paracoccidioidesbrasiliensis* were discovered using a chemoproteomics technique. After being immobilised on resin, the substances were treated with cell extracts [40]. Integrating multi-OMICs datasets (such as the transcriptome and proteome) revealed numerous targets of the medicines' activity,

including mitochondrial membrane damage, cell cycle arrest, and metabolic process inhibition. The findings of this study showed that TSC and TSC-C had no adverse effects on mammals while still having antifungal effectiveness against *Paracoccidioides brasiliensis*.

9. Antifungal-resistant Candida strains

Approximately 7% of all *Candida* blood samples evaluated at the CDC have fluconazole resistance.

Even though *Candida albicans*, one of the *Candida* species, is the main culprit behind severe *Candida* infections. Other species, particularly *Candida Auris, Candida glabrata*, and *Candida parapsilosis*, exhibit resistance the most frequently. It is especially alarming because echinocandins, a different type of antifungal medication, are becoming resistant. It appears that echinocandin resistance is rising, particularly in the species *Candida glabrata*. According to CDC monitoring data, the antifungal fluconazole is already highly resistant to *C. glabrata*, and this resistance has been broadly stable over the previous 20 years. Echinocandin resistance could significantly reduce the range of treatments available to individuals with *C. Glabrata* induced candidiasis. Echinocandin is the chosen treatment for *C. Glabrata* [41].

10. Mass spectrometry in COVID-19 infection

Immunological assays or RT-qPCR are the two most extensively used methods for following disease progression and diagnosing COVID-19. The former is a quick and inexpensive procedure but is limited for early diagnosis because the immune response is still building. However, the outcomes depend on several variables, including appropriate sample techniques and premium extraction kits [9, 11]. Early in the viral infection, SARS-CoV-2 is found in high concentrations in saliva [34]. According to a recent study, salivary glands are known to serve as a viral reservoir; as a result, this biofluid can be employed as a source for COVID-19 diagnosis and prognosis [35]. MALDI-MS and machine learning algorithms have been used in many protocols due to their relative affordability and speed, including for the diagnosis and prognosis of several types of cancer, the identification of fungi and bacteria [42, 43], detection of fungi and bacteria that are resistant to treatment, and diagnosis and prognosis of COVID-19.Tandem mass spectrometry (LC-MS/MS) is a technique that combines numerous mass spectrometers with liquid chromatography to provide a thorough analysis as well as the separation capabilities of liquid chromatography. It is a technique for conclusively detecting SARS-CoV-2 in human samples. Comparing LC–MS/MS to techniques like RT-PCR and quick testing has shown great sensitivity and specificity while reducing the possibility of false positives [32, 33].

11. Identification of proteomics in COVID-19 treatment

Several COVID-19 therapeutics has been identified by MS-based proteomics; the development of these therapies relies on identifying SARS-CoV-2 replication machinery. In order to identify therapeutic targets, Gordon et al. investigated the ligands of

SARS-CoV-2 interacting proteins [24]. Two successful therapeutic classes were identified after discovering 69 such compounds: agonists for the Sigma receptors and protein synthesis inhibitors (including ternatin-4 and zotatifin) (such as hydroxychloroquine and haloperidol). It was found that both classes inhibited SARS-CoV-2 reproduction over 8 hours by lowering the viral nucleoprotein. The identification of COVID-19 therapeutics has also been aided by discovering case severity-related protein biomarkers and host-virus protein interactions. Suvarna et al. used label-free quantitative MS to differentiate 38 proteins with different expression levels between mild and severe COVID-19 cases, and they found abnormalities in several cellular functions. In cases of severe COVID-19, proteins like alpha-2-macroglobulin and fibrinogen gamma chain are raised for the processes of blood coagulation and inflammation, respectively. Serpin Family A Member 3 is another protein upregulated for these activities [25]. Medical experts rely on medical history, symptoms, physical examinations, and laboratory tests to diagnose invasive candidiasis. Most often, invasive candidiasis testing entails sending a blood sample or sample from the affected body site to a lab to see if it will grow *Candida* in a culture. Due to its high contagiousness, SARS-CoV2 is only processed in specialised research facilities, which presents significant challenges for systems-level molecular analyses. In our developing world, finding more efficient solutions is a challenge. SARS-CoV2 treatment, as well as point-of-care testing (PoCT), may, in the future, play a far more significant role in this.

12. Conclusion

The primary therapeutic targets of today are proteins, which are important in the modern drug design process. Pathogens are especially interested in host proteins as targets that less likely to experience an obstruction-causing mutation to the therapy and create resistance. Development of therapeutic target usually requires several processes, including the building medication formulations based on a certain drug's structure target, confirmation of therapeutic effectiveness, testing for toxicity, followed by a clinical trial. High throughput MS based proteomics systems will provide a far more comprehensive, a thorough and focused approach to studying an infection and demonstrating the intricate actions that take place inside an infected cell while mitigating for a technique's shortcomings when used alone. Posttranslational modification such as phosphorylation, specific changes include acetylation and ubiquitination can change a certain protein target's activity which control of extensive signalling networks and pathways inside cells. In order to find new antifungal medications and host susceptibility factors, a thorough knowledge of the molecular mechanisms underlying viral infection continues to be a significant problem. It is anticipated that systems-level modelling and the merging of the genomic and proteomic fields with existing wet lab techniques would lead to new developments in the field. Here, we made an effort to skim through the significance and advantages of combining two potent proteomic approaches in order to better understand the molecular interactions primarily by taking a broad view of cellular signalling networks.

To sum up, advances in technology, platform development, and protein chemistry have significantly advanced the field of proteomics studies over the past thirty years. The identification of the molecular signatures of diseases based on protein pathways and signalling cascades offers enormous potential and utility for disease diagnosis and therapy development, combined with the use of microarrays and bioinformatics tool sets, with other omics based approach.

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