Utilization of Innovative Chemical Compounds and Advanced Analytical-Chemical Techniques for the Molecular Analysis of Proteins in Disease Diagnosis

Editors

Shatha Abd Al-Jabbar
Department of Biology, College of Education for Pure Science, University of Kerbala, Iraq

Riyam Ghanem Hamed Kadhim
Department of Chemistry, College of Science, University of Babylon, Iraq

Mohmmed Hashim Hassan
Department of Chemistry College of science University of Karbala, Iraq

Sahar Ramzi Ahmed Abdel Latif
Department of Pathological Analysis, College of Applied Sciences, Samarra University Iraq

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Abstract

Molecular protein analysis requires a sensitive approach, due to the low abundance of individual proteins. Detecting proteins with precise chemical tagging has attracted additional interest because of the underlying principles. The same features that may provide an analytical advantage often make chemical tagging approaches also vulnerable to artifacts and bias during the analyses, especially in labelling-based workflows. Hence, these methods require validation by strict controls. Furthermore, following the initiation of protein-chemical interactions, the resulting chemical-protein systems can also be subjected to proper analytical protocols without any chemical manipulations in their analyses. Such a blind sample analysis setting is particularly desirable to be achieved in protein analysis with the smallest possible set of samples, as it can allow for the most direct route to identifying with the least chance for artefacts.

The protein corona of engineered nanoparticles can provide insight into the toxicological effects of these chemical entities. Upon entering biological systems, engineered nanoparticles associate with proteins, forming a protein corona. The specific properties of the nanoparticle, such as its size, surface chemistry, and polymer coating, define its interaction with proteins. These interactions affect the biodistribution, pharmacokinetics, and potential toxicity of the nanoparticles. Therefore, comprehending the nanoparticle-protein interaction is of paramount importance for the safe design of nanoparticles and their successful diagnostic-therapeutic applications *in vivo*.

Contents

Chapter - 1

Fundamentals of Protein Chemistry in Disease

The immense functional diversity of proteins arises from variation in their primary sequences and their dynamic response to the environment, resulting in a spectacular range of three-dimensional structures with different catalytic and binding sites. Proteins can be selectively or non-selectively involved in the stabilization of other proteins through chemical modifications. Such altered interactions are, nevertheless, usually transient and challenging to study. The formation and breakdown of such transient interface complexes are likely to be critical in cellular signaling and regulatory pathways, and yet probing the underlying interactions remains a major challenge for the drug discovery process.

Many properties of proteins are derived from these delicate chemical equilibria. Protein folding is therefore a highly complex and sophisticated process, especially in eukaryotes, where the sequential delivery of the different polypeptide chains to their final compartments is monitored by the presence of stabilizing cofactors and is assisted by chaperone proteins. The chemical structure of the 20 common amino acids determines the elaborate folds that proteins can adopt and account for their extraordinary function and stability. The oxidation state of the protein can lead to reduced catalytic or binding activity. Hence oxidative stress is also a major feature of disease related processes, and simple redox-related measurements can provide valuable diagnostic insights. Patience and practicality are required when establishing

commonly accepted protocols or values for protein analysis; these require careful but unglamorous work [1, 2, 3].

Protein structure, folding, and stability

The structural organization of proteins allows for the folding of a linear polypeptide chain into a unique three-dimensional conformation that is energetically favorable compared to all other possible structures. The sequence of a polypeptide chain determines its unique structure. Each protein possesses its own stabilizing interactions that dictate how it folds, and these interactions are very important for maintaining its integrity in changing environments. Knowledge of the stabilization and folding of proteins is essential for understanding its biological functions. Proteins must aggregate or denature to become biologically inactive. Folding of proteins not only determines activity, but aggregation or misfolding into certain structures also plays a major role in neurodegenerative diseases like Alzheimer's disease and Huntington's disease. The aggregates of amyloid proteins can be detected from various biological samples of the patients. Knowledge about protein folding and stability provides better crude methods for diagnostic purposes.

Protein structure must be analyzed before experimentation or its applications even in the diagnosis of diseases. The three-dimensional structure is responsible for its proper function. Disease conditions change the conformation, and folded but chemically altered structure leads to disease. Hence a detailed study of the forces is essential as they play a crucial role in diagnostics. Upon misfolding, proteins might take different conformations *in vivo*, and the nature of these conformations can affect protein structure and lead to disease. The structure of misfolded proteins can be used as biomarkers for disease [4, 5, 6, 7].

Post-translational modifications and functional diversity

Post-translational modifications expand the functional and

structural diversity of proteins beyond the 20 canonical amino acids. Reversible, covalent modifications regulate protein activity, solubility, stability, localization, and degradation, thereby affecting protein function in response to physiological stimuli. Proteins may also undergo irreversible modification. A wide range of chemical groups can be introduced to proteins, including acetyl, phosphate, methyl, ubiquitin, glycan, lipid, and nucleotide. The analytical sensitivity required for detection depends on the physiological concentration and turnover rate of the modified protein. Acetylation, phosphorylation, and glycosylation are the most studied PTMs. Other protein modifications associated with disease are underrepresented in current proteomics methods. Although antibodies can provide the required specificity, they are costly, are limited in availability, and can cross-react with similar but unrelated proteins. PTM analysis remains technically challenging and remains primarily the purview of proteomics specialists. Because protein levels vary across different diseases and at different stages of the same disease, population-wide PTM screening has not yet been achieved for all diseases with reliable correlation to diagnostics.

In biological systems, proteins are constantly undergoing various modifications, including the addition of chemical groups to specific amino acid side chains. Such post-translational modifications catalyzed by modifying enzymes alter the side chain's charge, size, structure, or hydrophobicity, thereby changing local chemical environment or reactivity and, ultimately, protein activity and stability. These changes can alter protein conformation and charge, affecting protein-protein, protein-ionic species, protein-lipid, and protein-small molecule interactions, thereby modulating the biological functions of proteins in a dynamic manner. Different cell types and tissues employ the same PTM in unique patterns, thus creating a functional code that governs UPS-related signaling [8, 9, 10, 11, 12].

Protein misfolding and aggregation in disease

Abnormal protein misfolding and consequent aggregation have been implicated in a variety of neurodegenerative and systemic disorders collectively termed protein misfolding diseases or proteinopathies [13]. Multiple specific mechanisms can lead to protein misfolding, including genetic mutations, changes in environmental parameters that affect folding or stability, and modification of the protein after synthesis [14]. Misfolded proteins possess non-native topologies and often expose free hydrophobic regions that promote aggregation [15]. Aggregation can generate large non-diffusible deposits that disrupt normal cellular processes and ultimately culminate in cell death. Given that the onset of protein misfolding and aggregation frequently occurs prior to the appearance of symptoms, they offer considerable diagnostic potential.

Protein biomarkers in clinical diagnostics

The distinct properties of proteins reveal their immense potential as clinical diagnostics. Clinical diagnostic biomarkers are indications of normal and pathological processes that are easy to measure and monitor. Protein biomarkers undergo changes that are generally associated with host response during the onset and progression of a disease. An ideal biomarker should have the capacity for disease detection, prognosis, or prediction of drug response; ideally, its levels should indicate the necessity for intervention or treatment. Biomarkers should be easy to measure, noninvasive or minimally invasive, and useful for population screening. Such proteins are often expressed in trace amounts, making their chemical enrichment necessary for analysis.

Biomarkers can be classified based on their source and their specificity and sensitivity. Tumor-associated antigens are proteins or glycoproteins with a broad distribution. Pathogen- or disease-specific markers are either unique for a specific disease

state or produced in specific diseases, like the beta-amyloid and tau proteins for the diagnosis of Alzheimer's disease. Tumor-specific markers are identified in cancerous tissues along with the associated processes. A biomarker can be a diagnostic tool only if it has a validated relationship with the disease, particularly for diagnosis, prognosis, drug selection, and risk prediction. Diagnostic biomarkers need multicenter studies with consistent results in independent sample groups [16, 17, 18, 19].

Chapter - 2

Chemical Properties of Proteins Relevant to Analysis

Chemical properties of proteins dictate the design of many analytical strategies. The analysis of these properties informs the suitability of methods and the interpretation of results. The structure of proteins explains their reactivity, charge, solubility, and electrochemical properties, which in turn determine their behavior during lysis, separation, and detection. Chemical modifications can provide further important information, including both direct quantification of the modified residue and indirect elucidation of a variety of other aspects.

In general, proteins are soluble, structurally stable, and charge-neutral under physiological conditions and so require denaturing conditions with extremes of pH or ion strength for effective separation by charge. Detection of proteins typically relies on signature spectroscopic or electrochemical properties, redox-active tags for electrochemical detection, or nanomaterial-enhanced biosensors. Redox-active probes can be integrated into sensor functionalization to amplify responses, and controls can be incorporated to demonstrate compatibility with native redox balance. However, DNA, RNA, and LPS share many of these properties and offer the challenge of false-positives in protein biosensors. Nanomaterial-enhanced biosensors can improve sensitivity, selectivity, and price. Point-of-care systems can facilitate mass testing by non-specialists, but redundancy in transport, storage, and instruction sets is necessary to ensure reliability and accuracy in challenging environments [20, 21, 22, 23].

Amino acid chemistry and side-chain reactivity

The chemical properties of amino acids are relevant to the use of reactivity- based analytical strategies, which include labeling, modification, and reaction with other molecules. Amino acids, the building blocks of proteins, are molecules possessing both amino and carboxyl groups. Although their amino and carboxyl groups are often involved in peptide bond formation and do not participate in other chemical reactions, the reactivity of their side chains is chemically diverse. The reactive groups incorporated into protein structures make proteins amenable to a variety of chemical reactions, including oxidation, alkylation, nitrosation, glycation, acetylation, phosphorylation, sulfonation, and glycosylation. The specific amino acid involved in these reactions provides information about the specificity of these transformations. The functional diversity of amino acids also allows the introduction of unique characteristics into proteins, such as an isoelectric point that deviates from that predicted based on the amino acid composition or redox activity. The readouts arising from these chemical transformations can be an indirect reflection of cellular components and processes and can be employed as tools for disease diagnosis.

Although the amino acid side chains in the protein structure introduce numerous new reaction sites, the carboxyl and amino groups of each amino acid are also responsible for a number of chemical processes. The carboxyl groups of aspartic acid and glutamic acid can form ester and amide linkages with alcohol or amine, respectively, while the amino group of lysine and the side chain of cysteine can undergo acylation and express redox behaviour, respectively. The presence of these potentially reactive chemical groups can be exploited to develop bioanalytical tools for protein analysis and diagnosis [24, 25, 26, 27].

Protein solubility and charge behavior

The solubility of proteins in water is governed primarily by the surface properties of proteins, arising from the chemical

nature of the amino acid residues constituting the protein. Surface formation of protein as well as protein-protein and protein-solvent interactions depends on the relative number of exposed nonpolar and polar groups. Solubility and charge, which is dependent on pH, also define the capability of the protein to be extracted from a specific source, for example, lipids in the retina that can only be lesioned with alkaline lysis or phosphoproteins from acid lysis. The study of protein solubility in different ionic strengths and pH also aids in defining optimal pH conditions for separation. Several proteins play important roles in diagnostic kit development. Thus, the study of such chemical properties acts as a reference for biological and physiological functions as well as their utility in biomedical diagnostic kits.

The ionic character of proteins enables their separation by charge and interaction with the ionic environment. At a pH far from the protein isoelectric point, the protein carries an opposite charge to the ionic moiety on the chromatographic resin, which ideally favours protein retention and separation; at the isoelectric point, the protein carries a neutral charge. Moreover, gradient elution with varying pH or ionic strength often provides efficient separation when using ion-exchange chromatography in two-dimensional or multidimensional systems for complex proteome analysis [28, 29, 30].

Redox chemistry of proteins

All biological processes depend on electronic or protonic transfer, and redox couples are central to energy-producing reactions. This generally determined the concept of "bioenergetics" in biological chemistry. The term also refers specifically to biochemical reaction schemes in which electron transfer is accomplished within biological systems. Protein and redox-active centers that aid in catalytic electron proceses are an essential aspect of bioenergetics, and they include related areas

such as oxygen transport, oxygen transfer, and the photoreduction of carbon dioxide with accompanying electron transfer. Formation of free radicals occurs through insertion of unpaired electrons in normally paired processes. Transmission of protons is such that redox free-energy diagrams must include three components: the change in the number of redox equivalents, the change in the number of protons, and the temperature. Direction of transmission depends on proton concentrations. Primary sensors that transmit electrons in naturally occurring biological sysems are redox centers. Their basic chemical nature includes porphyrins and quinones, minerals in the enzyme nitrogenase, and hemes.

Oxidative stress can negatively impact the structure and function of cellular biomolecules. Compounds designed for controlling the redox state of biomolecules and tracking their changes over time provide indirect information about oxidative stress. However, redox-active compounds also serve as means of monitoring specific proteins and protein states, including for the development of diagnostic assays. Reducing agents can directly alter disulfide bonds in proteins, but the common mechanistic pathway for chemical] oxidation is species-induced changes in proteins. Specific oxidative modifications made by hydrogen peroxide or reactive oxygen species to biomolecules; diagnostic assays based on them, especially those exploiting chemistry at cysteine residues; and direct detection of redox states (disulfides vs. thiols) or states (oxidized vs. reduced) of proteins using redox-active compounds—these represent redox-based monitoring strategy [31, 32, 33, 34].

Chemical stability and degradation pathways

Chemical instability and hydrolysis of proteins can obscure the true state of a sample and lead to inaccurate quantitation. Although such changes can occur in solution, even well-

preserved proteins in powder form are not immune to degradation. Substantial shifts in protein composition may occur over time, but aqueous formulations can remain stable for many years if conditions are ideal. Storage at -80 °C usually minimizes degradation but requires thawing prior to use. Chemical agitation using freeze-thaw cycles or vortexing during prolonged storage may intensify hydrolysis, affecting volatile compounds in proteins with high cysteine and methionine contents.

Sample processing can be accelerated by chemical modulation, but care must be taken to eliminate uninformative changes. Spiking isotope-labeled analogs can confirm the extent of the natural chemical process, while the addition of a different compound can neutralize the undesirable effect. Despite these controls, results should be interpreted cautiously, especially when discovering new phenomena. Chemical stability must always be considered to prevent surprises during analysis, and it is prudent to develop systems for other potential hazards. Anticipating sample degradation during natural history studies remains essential for avoiding misinterpretations [35, 36, 37, 38].

Chapter - 3

Innovative Chemical Compounds for Protein Targeting

All physiological changes in disease ultimately involve some form of alteration in protein profiles and properties. Chemical compounds that specifically target proteins and the underlying chemistry of chemical reactions are therefore of paramount importance in developing new analytical assays.

Affinity ligands or agents specifically capturing proteins are some of the most useful chemical compounds for diagnostics. The invention of antibodies now allows the creation of affinity reagents with extremely high specificity and affinity to almost any protein. Small-molecule affinity ligands complementary to proteins with high specificity and affinity have also been developed for a few proteins. Chemical probes that use tagged small molecules to selectively label or modify proteins in cells and tissues are also becoming important for diagnosis. Chemical tags that covalently and stably attach detection groups to proteins and their derivatives serve as universal labeling reagents for diverse probes in the field of protein disease or diagnostic research. Smart or environment-responsive compounds that spontaneously react or change properties in the vicinity of diseased proteins or analytes have also attracted considerable attention. Such compounds not only increase specificity and reduce background signals, but also eliminate tedious sample preparation, thus greatly accelerating detection speed [39, 40, 41, 42].

Affinity ligands and selective binding agents

The described binding agents target proteins in a variety of

ways. An affinity ligand binds a protein with exceptionally high specificity and affinity, much better than antibodies or other naturally occurring proteins. Such ligands are frequently obtained using an *in vitro* selection technique termed SELEX. While tightly binding into the nanomolar range or lower, ill-defined ligand specificity may lead to off-target recognition. Small molecules capable of weakly binding their targets with binding constants in the low millimolar range can also be identified. These have tumor accumulation enabling protein detection in fluorescence imaging. More accessible than antibodies, selective protein binding agents are nevertheless maintained for a limited time; new compounds enabling sustained detection *in vivo* remain to be introduced.

Affinity compounds take advantage of the unique chemical characteristics of proteins or other biomacromolecules to selectively bind and identify them in complex systems. Affinities stronger than for antibodies enable selective recognition at lower concentrations, thus broadening the application area. Synthetic affinity ligands mainly include small molecules, peptides, and DNA molecules, which can be chemically synthesized and modified for specific labels. In addition, smart compounds can dynamically respond to microenvironments, such as pH and redox state, ultimately producing stabilized fluorescent signals. These properties offer unique advantages over chemical probes but remain unexplored for many proteins, giving ample room for novel designs. Affinity compound design focuses on ensuring selectivity, and the spatial and temporal stability of the probe-target binding interaction within the complex biological environment [43, 44, 45, 46].

Small-molecule probes for protein detection

Small-molecule protein probes serve as complementary detection tools for protein chemical modifications and can enable

the visualization and quantification of proteins in biological systems. These small organic ligands are often based on fluorophores or mass reporters, offering a wide range of chemical and detection-readout possibilities. The development of small-molecule probes for proteins has followed the trends of medicinal chemistry and medicinal probe development. As such, a small-molecule protein probe typically consists of a desirable scaffolding structure, a reactive handle for modification, and a suitable recognition element to improve target protein selectivity.

The first principle of designing protein-specific small-molecule probes is to incorporate specific recognition elements into the ligand system. The recognition elements should help to increase probe selectivity and avoid interference from the other abundant components present in proteomes. There are several common designs of protein-specific probes, such as peptide sequence-encoded probes that are responsible for protein-targeting and receptor-ligand interaction-based probes that target protein enzyme-inhibiting interactions. The second principle is to select a chemical scaffold that can undergo irreversible reactions toward protein functional groups upon certain conditions. In general, small-molecule protein probes with selective fluorescent or mass-detecting moieties are widely developed for specific mutations—e.g., detection of the different stages of cancer—these probes can also be based on non-fluorescent quenching strategies or applied with highly abundant proteins, such as albumin-related probes.

Chemical tags and labeling reagents

Chemical tagging of proteins serves two main functions: (i) a chemical modifier is covalently attached to a protein to yield derivatives less complex than the native material but more informative than artificial model systems; or (ii) an isotopic reporter is incorporated to enable the quantification of relative

levels of natural proteins present in different samples. are diverse; the needs imposed by a given system may largely determine the choice of tag or of methodology, with efficiency of labeling often being crucial. In parallel, the ability to probe abundance differences of proteins in respect of Affinity reagents, driven by diagnostic follow-up or control, underpins their use despite less instructive labeling.

Labeling reactions may target one individual protein (the enzymatic reaction need not be controlled for excess) or multiple proteins; in the latter case, precisely determining the extent of incorporation may be important to avoid quantitative errors. The complexity perceived from either approach is apparent, but interpretation is generally tractable when experimental design is comprehensive. For mass tag labeling, high sensitivity of LC-MS/MS methods allows for the differential quantification of low-abundance proteins in different samples, in both single- and multiple-labeling strategies. compartometric analysis of chemically encoded peptide ligands has potential to extend the approach into new analytical modalities [47, 48, 49, 50, 47, 48, 49, 50].

Smart compounds responsive to disease microenvironments

Smart compounds that respond to disease microenvironments represent an important category of novel chemical molecules designed to facilitate protein detection and analysis. Such compounds are typically unreactive until triggered by a specific stimulus, at which point they undergo a designated transformation. In the context of protein analysis, smart compounds can be activated by ambient chemical, physical, or biological changes generally associated with disease progression. These microenvironmental characteristics include elevated levels of reactive oxygen species, reactive nitrogen species, pH, temperature, or changes in glucose concentration. Following activation, the compounds can participate in conjugation or

labelling reactions with proteins, thereby enabling their detection.

The need for rapid and sensitive detection of inflammation-related cytokines has motivated the development of a pH-driven fluorescent probe containing an imprinting cavity for silica nanoparticle (SiO2 NP)-assisted cytokine detection. These cytokines, such as interleukin IL-6, IL-1β, and TNF-α, play crucial roles in the inflammatory response and pathogenesis of certain diseases. The probe enables instantaneous cavity-switching behaviour from emission quenched state to fluorescent turn-on mode through protonation/deprotonation, leading to the formation of silica-imprinted fluorescent nanosensors. These sensors offer excellent sensitivity and selectivity in detecting low-abundance cytokines in the acidic pH environment under inflammatory conditions. The imprinted NPs successfully mimic the biological function of the imprinting molecule for effective separation and accurate detection of cytokines.

Chapter - 4

Chemical Modification of Proteins for Analytical Purposes

For mass spectrometry and other sensitive analytical methods, proteins can be chemically modified before the experiment. Reactions that covalently add a modifying group usually proceed with relati-vely low success. However, they advantageously make these proteins amenable to new reactivity and detection/readout systems that the unlabeled proteins do not offer. Such labeling or modification can reduce the extent of chemical background, improve quantitation and increase selectivity by removing undesired endogenous proteins. For example, proteins can be tagged with stable isotope labels, enabling precise quantitation by mass spectrometry and allowing the analysis of low-abundance proteins through multiplexing. Chemical cross-linking followed by mass spectrometric identification can provide distance constraints in protein structures. Click chemistry—all fast reactions that proceed at mild conditions and that are modular, highly selective and bioorthogonal—has been widely adopted the past decades in all areas of biofunctionalism, including the labeling and detection of biomolecules.

In the reaction with 1,2-diketones or analogous compounds, chemical derivatization is a simple and high efficient means of preparing mixtures that are ready for aligning measures of 2D or multilabeled electrophoresis, MS and other methods. Such derivation profoundly reduces chemical noise and readily

permits much wider spanning of optical detection modes. Despite the merit of deriving proteins with 1,2-diketones, MS detection lacks a strong definitive basis because such chemical noise can still fold even higher than that of the chemical target system can handle [51, 52, 53, 54].

Covalent derivatization strategies

Covalent modification is a powerful strategy aimed at introducing novel chemical moieties into the protein of interest. These moieties can facilitate quantification through incorporation of isotopic labels (Chapter 4.2) or can simplify the analysis by reducing fragmentation of highly charged surfaces or the presence of post-translational modifications that introduce multiple polar groups. Covalent attachment of a second recognition element can also expand the set of chemical approaches used to examine a specific protein. Such tagging reactions, however, depend critically on the relative location of the introduced moiety and the unmodified regions that act as bacterial or yeast recognition surfaces.

Derivatization reactions can also introduce groups specifically reactive toward a second labeling reaction. A mutant form of the Cys-less HaloTag, in which the residue in the active site of the embedded hydrolaze was substituted by a serine to create a protein with a unique Cys, has been used to investigate S-thiodisulfide protein folding by labeling with an S-based probe and a FRET donor—acceptor pair. Quantitative measurements have then been obtained from the ratio of the fluorescence intensities of the two probes conjugated to the protein. Derivatization thus offers a powerful tool to introduce any desired chemical group for probe coupling, but care is required in the design and execution of such experiments [55, 56, 57, 58, 59].

Isotopic labeling and mass tagging

Global profiling of proteins in a given sample in a sensitive, accurate, and high-throughput manner is a crucial goal in

proteomics. Mass spectrometry-based proteomic analysis has emerged as a powerful approach but, because of the limited dynamic range of mass spectra, detecting low-abundance proteins can be a challenge. Accordingly, chemical strategies for improving sensitivity are warranted. Isotopic labeling or mass tagging of proteins can enable global, high-throughput, and accurate analysis of the proteome. The method can be employed for relative quantitation of multiple samples, is generally performed by tandem mass spectrometry, and enables the development of a high-resolution database. Protein samples do not need to be chromatographically separated prior to mass spectrometric analysis. Subsequent analysis can yield a large number of proteins in a single LC-MRM-MS run.

In the simplest form of isotope labeling, complex samples generated from different experimental conditions (e.g. diseased versus healthy tissue or different mutation) are separately labeled with different isotopes. The chemically identical isotopes differ only in mass and are therefore indistinguishable from each other except at the mass spectrometer. The tandem mass spectrum includes the same set of peptide peaks, each pair differing in mass. Proteins whose levels differ in the two conditions appear with different ratios of peak heights. However, often the number of samples in an experiment exceeds the number of available isotopes. This limitation can occur in metabolic labeling, which normally requires at least six stable isotopes, particularly heavy isotopes such as ^13C or ^15N, are limited in nature. In contrast, radioisotopes are abundant in nature and δ early mass-based tagging strategy introduced methods for multiplexed isotope labeling which involved the introduction of chemically distinct tags to different samples prior to combining them [60, 61, 62, 63].

Cross-linking reagents for structural analysis

Proteins display a vast range of biological functions, and their structure-function relationship is the basis of their use as

diagnostics. The spatial arrangement of the protein molecule is the result of non-covalent interactions and stabilizing disulfide bonds in the normal physiological state. The collection of spatial arrangements and the changes involved in going from one arrangement to another are defined as conformational dynamics. Analysis of proteins in specific experimental conditions exposes the conformations visited and the physicochemical interactions stabilizing these states, from which important biological information can be deduced. Mapping interresidual arrangement and interaction differencies—protein-protein interactions (PPIs) and protein-ligand interactions (PLIs)—are important for understanding function and designing drugs. These analysis could be performed with high accuracy when the local environment of reactive functional groups of a protein is probed, thus giving sequence-specific information.

Cross-linking (CL) is a convenient method to investigate the links between different protein structures. Chemical reagents that create covalent bonds in the vicinity of a reactive functional group can be utilized, and the distance of the two reactive groups can be properly chosen (1-5). The two or something greater is simplified to two lengths so that CLMS serves the purpose of investigating the link between two local structures. Key signatures of the majority of spatial models are the presence of Lys residues and involvement of regular secondary structural units, especially α-helices and β-sheets. The residues of intra-chain bonds are also of primary interest; specifically, the residues involved in the termination of α-helices and β-sheets, clusters of redox-active residues, and the presence of aromatic amino acids supplying important long-distance interactions. The constrained mechanisms for long-distance communications are established in some concentrated proteomes, and they should be eventually discovered in diverse other proteomes in normal and different disease conditions. Such investigations serve as a genuine pathway towards the elucidation of Protein-Protein Interaction

Networks and Protein-Ligands Interaction Networks, leading to targeted diagnostics [64, 65].

Click chemistry applications in proteomics

Click chemistry encompasses bioorthogonal, highly selective reactions that proceed rapidly under mild conditions. Numerous applications exist within proteomic research, including the preparation of azide- or alkyne-containing proteins that can engage subsequent chemical probes; however, the use of these reactions within protein analysis is hampered by the relatively low stability and availability of synthetic systems that incorporate both functionalities. Combining the copper(I)-catalyzed azide-alkyne cycloaddition to chemical probes with common protein modification strategies has been widely pursued in different subdisciplines of proteomics.

Different azide-containing monosaccharides have been incorporated into the glycan moieties of glycoproteins, allowing selective chemical tagging with cyclooctyne derivatives and subsequent detection using a nonbiochemical approach. Disease-driven changes in glycosylation patterns serve as the basis for these experimental designs; whether shifts in glycosylation pattern lead to changes in chemical selectivity remains an open question. Endogenously generated protein azide tags have been generated through the incorporation of the corresponding amino acid using an expanded genetic code. Sequential click labeling in the living organism was demonstrated; however, the efficiency of the labeling remained low. An alternative experimental strategy relies on selective chemical azide modification of the protein secretome.

These examples highlight the limitations of click chemistry methods in proteomics, namely, the low stability of the reagents under common experimental conditions and the unavailability of protein systems that simultaneously incorporate both reactive

groups. Nevertheless, the ease and versatility of click reactions continue to stimulate creative applications. Using previously described approaches that rely on the generation of either azide- or alkyne-containing protein systems, Levin and coworkers have incorporated the cyclooctyne moiety into the chemical toolbox for tag-and-modify applications [66, 67, 68, 69].

Chapter - 5

Sample Preparation and Protein Isolation Techniques

Technical requirements of chromatographic techniques determine the ways that protein samples are prepared prior → protein purification, whereas methods using redox, ‑ charge, and conductivity properties are variable or even optional. Chemical lysis methods that exploit redox or chemical activity of proteins and other biomolecules in living cells are relatively quick and simple, enabling both rapid-IsoTemp105 °C and cell-culture protocols. Expressed proteins can be purified with high-performance chemical affinity systems that recognize isolation domains or protein fold structures with high affinity and selectivity. Enzymatic digestion to peptide caches prior to identification and quantitation is common. Meticulously applied controls further minimize chemical interference, especially to ensure reliable protein concentration measurements that exploit fine-control techniques and variants of the colorimetric total-protein assay.

Techniques that utilize redox changes, charge or solubility effects for protein separation or concentration are explored in Chapter 6. Protein extraction strategies using chemical lysis agents are broadly considered. These agents facilitate control of the cellular environment and enhance extraction of proteins from cells in multicellular organisms using combinations of organic solvents that alter solubility properties. Reductive agents are important in these protocols as they maintain protein thio-

disulfide reduction, thus guaranteeing that proteins reflect the continuous redox balance of the host organism and preventing the formation of solvent-sensitive aggregates that central or systemic proteins may inadvertently assume [70, 71, 72, 73, 70, 71, 72, 73].

Chemical lysis and extraction methods

Chemical lysis methods use chemical reagents to accomplish lysis, solubilization, and extraction in a single step or a series of steps. Such methods offer advantages over physical or heat-induced lysis that are related to enhanced reliability, reproducibility, and health safety. However, as a consequence of their chemical nature, they may produce artifacts or chemical modifications that do not occur with other lysis strategies. To minimize these chemical artifacts, the choice of the chemical protocol for protein lysis, solubilization, and extraction must be carefully considered for each application, and control experiments must be performed in parallel. These can include the use of biological control systems based on human or animal biological media or fluids, incorporation of internal standards, and validation with alternative methodologies.

Protein extraction is generally understood as the separation of proteins from biological fluids or tissues with little or no change in their native structure and biological properties. The extraction process is often combined with solubilization because the solubility of the proteins in the media usually decreases below physiological levels. Chemical extraction and solubilization methods for proteins are mostly based on chaotropic reagents and detergents. At low concentrations, the former weaken protein-protein and protein-solvent hydrogen bonds, lowering both the electrophoretic mobility of the proteins and the viscosity of the system, whereas at high concentrations they denature the proteins. Detergents primarily act as solubilizers by lowering the

surface tension of the medium and forming micelles, although they may also weaken hydrogen bonding at low concentrations.

Protein purification using chemical affinity systems

Affinity chromatography uses chemical affinity systems to isolate target proteins, thus supporting subsequent detection, quantitation, and structural analysis. Various resins are engineered with distinct affinity ligands to achieve high selectivity for a broader group of proteins. In general, target binding relies on the specific interaction of a protein with its affinity ligand, while contaminations are eluted with a wash buffer. Elution is then performed by disrupting the affinity interaction, which can be achieved through different approaches.

Proteins may be purified using engineered or native chemical affinity systems. Engineered systems are designed to utilize affinity interactions between a protein of interest and a synthetic ligand. Target proteins are fused with an affinity tag that confers the ability to bind chemoselectively and reversibly to a resin derivatized with an affinity ligand. Following binding, contaminants are cleaned from the affinity column, and the captured proteins are eluted and subsequently released. To enable tug-of-war scenarios for target detection, a secondary ligand, often conjugated to a sensor surface, is moreover incorporated within the pendant unit of a chemical nanoprobes platform. The selectivity of chemical affinity systems is ultimately facilitated by the use of a native ligand that directly recognizes and interacts with the target protein, mimicking pathogen recognition in host-pathogen systems [74, 75, 76, 77].

Enzymatic digestion and peptide preparation

Peptide preparation typically follows sample preparation and protein digestion and strongly depends on the specific proteolytic enzyme employed. Chymotrypsin, widely recognized for its high specificity and activity, is generally used when identified

peptides may not be required for subsequent proteomic detection. Excess enzymes relative to proteins, overnight digestion at 37 °C, and addition of small nonsurfactant interfacial-active agents such as tweens, have a synergistic effects on digestion yield. Porcine trypsin is the main choice of protease for digestive preparation. Although a limiting step for many methods, the efficiency of its cleavage can be increased by using reduced and methylated proteins, different protease ratios, preactivation and long incubation times. Its slow reaction speed relative to chymotrypsin is alleviated by the addition of peptidase A and D.

Averagely, 10 folds more enzyme than protein mass is sufficient for tryptic digestion. However, the use of over-shot protease quantity can introduce chemical bias. Therefore, protease quantity should be optimized according to both choice of substrate and type of protease or combination for maximum efficiency. When peptides are just required for informatics validation of mass fingerprint results, in-gel digestion by trypsin with an enzyme/internal standard ratio of about 1:10 is frequently adopted. A method using tandem infection of pepsin with sequencing-grade trypsin or the addition of LysN has been proven for overcoming trypsin's caveat of cleavage before proline [78, 79, 80].

Minimizing chemical artifacts in analysis

Following the introduction of innovative chemical compounds, new analytical platforms, and directional progress in chemical proteomics, work outlined these advances regarding the diagnostic profiling of proteins in different diseases. Contributions from chemical science originated from three contexts: the introduction of new chemical compounds to facilitate protein analysis, integration of these into innovative analytical platforms, and application in the profiling of infectious, inflammatory, and neurodegenerative diseases.

Chemical-disease-proteomics, the translation of protein profiling for diagnostics or biological exploration, is a burgeoning field. It capitalizes on novel developments spanning proteomics profiling combined with annotation, establishing clinical relevance, and translation of these annotated profiles into patient-specific diagnostic platforms. Proteins linked to infectious diseases in preclinical contexts are particularly amenable given their strong immunogenicity and potential for point-of-care detection. Rapid screening technologies are vital, but accurate detection is paramount for complex diseases. A solid foundation in analytical science, particularly chemistry and analytical biosensing, is essential to manage the requisite balance. Roles of specific classes of proteins and their interactions with proteins from commensals or pathogens are key.

Chapter - 6

Chromatographic Techniques for Protein Analysis

Ion-exchange chromatography (IEC) separates proteins based on charge differences. Proteins pass through a resin column containing fixed chemical groups with cationic (for anions) or anionic (for cations) charge. When the analyte is within a suitable pH range for the selected ion-exchange group, electrostatic attraction occurs, while off-target species pass through rapidly. Charge differences result in different retention times; proteins elute by increasing ionic strength or pH. Proteins may carry multiple charges and can interact with multiple oppositely charged resin sites, with elution order determined by binding strength. Experimental conditions must be chosen carefully to avoid loss of resolution caused by protein aggregation or secondary structure changes. Different types of resins are available, selected according to expected net charge and desired separation range [81, 82, 83, 84].

Size-exclusion chromatography (SEC) operates on molecular size, using porous gels for resin. Large molecules cannot enter the pores and elute first, with smaller molecules arriving later. SEC does not change protein structure, making it useful for sample desaltation prior to mass spectrometry analysis. IEC and SEC can be used sequentially with buffer exchanges in between, removing the need to manufacture and functionalize special affinity resin. Multidimensional chromatographic systems combine different chromatographic methods to separate highly complex proteomes from diverse tissue and biological sources.

Ion-exchange chromatography

(IEC) separates biomolecules based on residual charge. Proteins are applied to a column containing charged resin, allowing electrostatic attraction to oppositely charged counterions bound to the functional groups on the resin. Charged centers on the proteins compete for binding with the ionic centers on the resin, and the ones with weaker associations are washed away. Selected conditions are then altered to promote elution of the bound proteins. IEC is commonly the first preparative step in proteomics.

A major consideration is which type of standard resin to use, concerning the ionic nature of charged functional groups in the target proteins. Anion-exchange columns are appropriate for proteins that acquire a net negative charge, whereas cation-exchange columns are used for positively charged proteins. A minor pH range change in the mobile phase can be employed to enhance separation. High-resolution IEC with low-throughput speed can also utilize mono-dimensional resins, in which different ionic functional groups are separately implanted. Chemical additives that promote protein solubility can be included in the mobile phase or in the column resin for proteins with aggregation concerns [81, 82, 85, 86].

Size-exclusion chromatography

Throughout the experimental procedure, proteins undergo continuous processes such as association and dissociation. Because of that complexity—intricate structures, functional diversity, and interaction with various ligands—analysis by direct separation is mostly impossible.. also referred to as gel filtration chromatography and they separate molecules according to their size and hydrodynamic volume in solution. Beads with a specific pore size are selected, allowing the smallest molecules to enter and exit the three-dimensional bead structure while

retaining larger molecules outside, thereby facilitating separation based on hydrodynamic volume. The mechanisms, separation factors, and possible combinations of these resins for further purification are considered. Size-excluded fractions can also be analyzed to determine a protein's native molecular mass.

Size-exclusion chromatography is based on the differential diffusion of solutes into pores of a gel-filled material. Size-exclusion materials contain a three-dimensional matrix of porous beads, the cross-linking density of which defines the size of the pores. Large molecules, which cannot enter the pores, coelute in the void volume of the column, whereas all smaller solutes diffuse into the beads and pass through the column more slowly. The mass of the solute is to some degree orthogonal to the gradient since globular protein shapes are retained in solution. Because of the narrow diffusion channel, there is a limit to the solute shape since elongated proteins take longer to diffuse through the channel, resulting in unproductive diffusion. High-molecular-mass solutes that are able to permeate the porosity of the bead structure exhibit higher retention since, although they diffuse rapidly into the beads, their molecular mass prevents them from diffusing out rapidly [87, 88, 89, 90].

Affinity chromatography using engineered ligands

Affinity chromatography exploits specific interactions between ligands and target proteins to achieve separation. The versality of affinity chromatography is owed to immobilization strategies that cover a wide range of chemistries and the availability of ligands that target a multitude of proteins. Ligands can be specific proteins (antibodies, receptors) or other biomolecules (e.g., nucleic acids) as well as small molecules. Protein affinity tags, short peptide sequences recognized by specific proteins, are routinely engineered into proteins and used for purification. When affinity tags are less selective, the

presence of vector-source proteins in the purification mixture must be considered. Personalized ligand generation is applicable to any protein of interest but demands effort for each application. Biosensor designs, using either antibodies or receptor proteins, allow flow-through analysis of unknown samples.

For screens on known microbial pathogens, libraries of secreted proteins can be scanned for nanomolar-affinity ligands, and protein-binding assays can reveal their exact target. Engineered *in vivo* labels target their respective proteins in different hosts, enabling super-resolution microscopy. Beyond simple recognition, various protein-ligand interactions can be harnessed: biotin-avidin, histidine binding, Rho-Roc, protein A-Ig, and other such pairs have received attention. High-throughput analysis of an affibody library identified nanomolar-affinity ligands for label-free MALDI-TOF detection. Multiple-choice aptamer libraries can likewise provide specific affinity probes. Other ligands, including chemical probes to selectively capture post-translationally modified proteins (e.g., glycoproteins), are also survival factors [91, 92, 93, 94].

Multidimensional chromatographic systems

Integrate multiple chromatographic techniques into a single platform. One-dimensional approaches using distinct separation modes can be conceptually combined with only moderate increase in experimental complexity, while two to four dimensions bring accompanying challenges. The rationale for proceeding can be high proteome complexity, combined sparing amounts of analyte, and the desire for more complete information from a proteomic dataset: absolute quantitation, changes in abundance, analysis of specific post-translational modifications, and information on protein-protein interactions.

In low- to semi-complexity proteomes, a peptide mixture can be separated by two or three orthogonal dimensions before

characterisation by mass spectrometry, the first dimension often employing chemical or isotopic labelling to facilitate relative quantitation. To shallow-inject a sample across a depth- or resolution-matched dimension for more complete proteome profiling, clean separations and a device, gel, or microsystem that is compatible with sensitive detection for all analytes of interest are prerequisites. For shotgun proteomics, a narrow mass range of one-dimensional separation that corresponds to the analysis window of the downstream mass spectrometer reduces the time required for separation and improves sensitivity [95, 96, 97, 98].

Chapter - 7

Mass Spectrometry in Molecular Protein Diagnosis

Mass spectrometry (MS) is a key tool for molecular diagnostics by enabling the non-targeted detection of disease-associated proteins. It determines analytes in the femtomolar to nanomolar range by means of gas-phase ion detection, which requires vaporization, ionization, and fragmentation of the protein issue. Ionization can rely on laser or electronelectron bombardment, matrix-assisted laser desorption/ionization (MALDI), electrospray ionization, or thermal desorption. In the related ion mobility spectrometry, ions travel along a drift tube before detection and provide additional structural information.

For the analysis of protein samples, peptide fingerprints combined with in-silico sequence analysis using an uncharacterized protein genome are typically employed for identification of proteins. Peptide samples can be assayed via quantitative proteomic techniques based on isotopic labeling, label-free approaches employing multiple reaction monitoring or parallel reaction monitoring, or PRM utilizing stable isotopes for chemical modification. Validation generally combines biochemical techniques and biological data before the resulting diagnostic assay moves toward clinical practice [99, 100, 101, 102].

Ionization techniques and chemical matrices

The selection of an appropriate ionization technique is one of the most important considerations in mass spectrometry (MS). Two decision points are required, firstly to choose between the diverse soft ionization methods or the high-energy electron

ionization processes that generate fragment ions, and then to select a specific method, e.g. electrospray ionization (ESI), laser ablation, laser desorption ionization (LDI), matrix-assisted LDI (MALDI), desorption chemical ionization, thermal desorption chemical ionization or secondary-ion mass spectrometry (SIMS). Ideally, the method must provide both sensitive detection at concentrations in the attomole-level range for biological samples and the formation of H-species or non-fragmenting ions that facilitate accurate mass measurements. In addition, depending on the analytes and the analytical purpose, analyses of low molecular weight substances (molecular mass < 2000 Da) or biomolecules (molecular mass > 2000 Da) may employ either soft ionization methods or a combination of the two classes of methods, respectively.

For MALDI MS of proteins, a suitable chemical matrix is essential. High-energy state molecules (molecular sizes in the range of a few hundreds Da), the solvents or co-matrix must display high vapor pressure or low boiling point so that they readily sublime or evaporate during sample preparation, establishing a vacuum condition for the MALDI source. Low volatile matrices facilitate co-crystallization with peptide samples. High temperature and sufficient vaporization are required to prevent unsaturated co-adsorption of molecular ions onto large molecules such as proteins at the very final stage. Considering all these chemical factors, a wide range of organic acids/esters/anfipaths, α-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (2,5-DHB) and 5-cyano-2,3-dihydroxybenzoic acid (5-Cy DHB) with additive agents, 3-hydroxypicolinic acid, 2-hyroxycinnamic acid, Au Nanoparticles, 2,4,5-trihydroxybenzate, adipic dihydrazide, α-methyl-2-pyridone, catechin, 2-acetylphenylboronic acid, (6,6, 7,7)tetrabromofluorescein, flavonoids and α-glucosylglyceride have been evaluated at both the matrix level and the MALDI MS

level of operation and accuracy, Mass spectra of naturally occurring glycomacromolecules or peptide-glycomacromolecular conjugates have been recorded and structurally assigned using 2,5-DHB as a matrix, 2-Acetylphenylboronic acid has been demonstrated as an appropriate matrix for the mass spectrometric analysis of glycoproteins and glycopeptides due to its affinity for boronic acids, and many novel matrices have also been developed and successively employed [103, 104, 105, 106].

Peptide mass fingerprinting

Employs matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) to compare the peptide mass profile of an unknown sample to that of a database of predicted profiles. In this application, the analyte is an unknown protein readily amenable to in-gel digestion and purification prior to mass spectrometric analysis. An efficient workflow summarizing the identification procedure is as follows: (1) digest the protein with an appropriate protease; (2) acquire the peptide mass profile; (3) search the profile against the database; (4) select protein candidates from the list; (5) score the candidates on the basis of additional criteria; (6) if needed, repeat from step 2 using a different enzyme; (7) choose the most promising candidate or combine multiple candidates; (8) for validation, synthesize the selected candidate and compare to the experimental profile. The confidence in a match is gauged using a scoring system based on several aspects of the search. Other factors external to the sequence database can also influence the identification confidence, such as the quality of mass spectral measurements, the extent of sequence coverage, and any supporting experimental observations. An overlapping set of peptides should ideally be detected for different protein candidates, as should the nonapeptide fragment pair predicted by Peptide Mass. Finally, peptide mass fingerprinting can be used to identify bits of protein

that may not appear in the amino-acid sequence of the whole gene but that are in the protein [107, 108, 109, 110].

Quantitative proteomics using chemical labeling

Chemical labeling strategies for quantitating analytes by mass spectrometric methods have gained increasing popularity in proteomics. These strategies introduce distinctive isotopic signatures on the analytes using robust chemical chemistry approaches and subsequently allow quantitation by mass spectrometry. Chemical isotopic labeling for relative quantitation of analytes can be complemented with (1) multiplexed library-independent quantitation using a single labeling reaction, (2) sensitivity enhancement by the ultra-high stability of isotopically labeled but chemically stable reagent ions generation and detection, (3) reduced matrix impurity interference during analysis, (4) digital countable signatures for high-confidence identification, (5) indicator-free detection by stable isotopic labelling, and (6) unlimited multiplexing capability using widely available mass spectrometers.

Development of a chemical labeling strategy that enables digital countable, non-labeled, stable isotopic signatures at native chemical stability ratios for mass profiling and multiplexed quantitation of low-abundance analytes in complex biological systems has been reported. This strategy combines the concept of ion-trap assisted signal enhancement with indole-based chemosensory capability. By taking advantage of the high ion-trap stability of deuterium-labeled 1-oxido-2-(4-sulfobutyl)-3H-indol-3-ylium ions, rapid electrophilic library-independent ion-cycling labeling of any nucleophilic analytes followed by on-line capillary electrophoresis-tandem mass spectrometry allows both detection of low abundance ions below the level of natural abundance interference and library-independent quantitation at isotopically natural abundance ratios [111, 112, 113, 114].

Clinical applications of MS-based protein diagnostics

Recent MS-based developments have yielded clinically applicable assays targeting diverse disease-related analytes. A novel MAP-SERS immunoassay for the serotype-specific identification of dengue virus captures serum proteins from infected patients and employs Desulfoglucosinolates to probe the main serotypes. The sensitive detection of N-acetylglucosaminidases, together with a known Alkaline Phosphatase signature, facilitates diagnosis, severity evaluation, and prognosis. A stable-isotope-enabled proteomic platform with multiplexing capacity quantitatively profiles the effector and effector-associated proteomes of the Fusarium Avenaceum pathogen in infected oat plants. The high-resolution data yield distinct virulence profiles among isolates and detect novel effectors, including Avenacein. An SERS-based sensor comprising a cocktail of five labeled antibodies discriminates four distinct strains of Escherichia coli in food and clinical samples.

Progress toward MS-driven diagnostics of Down syndrome, Alzheimer's disease, myocardial infarction, schizophrenia, and infectious diseases is examined. The development of the public SpectraBank database and S/MAR minicircle technology opens novel diagnostic vistas. The detection of subpicomolar concentrations of heavy metals in clinical matrices is demonstrated using antibody-functionalized nanocomposite films and multiwalled carbon nanotubes. The use of quantum dots as labels in DNA biosensors for MS detection of pathogens is also illustrated [115, 116, 117, 118, 119].

Chapter - 8

Spectroscopic Techniques for Protein Characterization

1. Introduction

Spectroscopic techniques exploit the interaction of electromagnetic radiation with matter to gain information on electronic structure, molecular motion, bond vibrations, and configurational ensemble. Spectral peaks arise from transitions of molecular species between states of different energy. The type of radiation employed, the range of wavelengths scanned, and the physical basis of the interaction determine the specific type of analysis and information accessible from the spectral profile.

Ultraviolet-visible (UV-vis) and fluorescence spectroscopy measure absorption by pi- or n-electrons performing a transition to a higher energy level. Infrared and Raman spectroscopy measure infrared or Raman-active vibrational modes directly related to bond strength, length, and angles. Nuclear magnetic resonance (NMR) spectroscopy utilizes magnetic properties of nuclei of specific isotopes to determine structural and dynamic features in solution. Circular dichroism (CD) spectroscopy probes the preferential absorption of left- and right-handed circularly polarized light by the chiral centers in protein backbones and side chains to assess secondary structural content.

2. Ultraviolet-Visible and Fluorescence Spectroscopy

UV-vis spectra are recorded by scanning a selected spectral range (typically 200-500 nm) based on the absorbing groups

present (e.g., tryptophan, tyrosine, free or protein-bound cofactors). Protein concentration is typically determined by measuring absorbance at 280 nm (A280) using the Beer-Lambert law for solution thickness in centimeters and molar absorptivity in M-1 cm-1 obtained from known concentrations of an appropriate standard (e.g., a recombinant protein with a known extinction coefficient).

Fluorescence spectroscopy records emission spectra after excitation at a specific excitation wavelength. Sensitivity is further enhanced by using synchrotron light sources in fluorescence-excitation or emission-scanning mode; single-cell readouts become possible using microspot samples. Tryptophan residues are often mainly responsible for emission (with quantum yields ranging from 10-4 to 0.9). Excitation spectra reveal direct dependencies of concentration and proximity to other fluorophores or quenchers. Fluorescent cofactors such as NAD(P)H or FAD can also serve as reporters, with sensors designed for different measurements more sensitive in the specific wavelength range of their excitation maxima [120, 121, 122, 123].

UV-visible and fluorescence spectroscopy

UV-visible and fluorescence spectroscopy are widely used techniques for protein analysis. UV absorption spectra of proteins generally display a pronounced band between 180 and 230 nm, attributed mainly to peptide bond absorbance. The combination of these bands gives rise to a relatively broad minimum between 205 and 208 nm, while side-chain substituents of aromatic amino acids produce additional bands with molar absorptivity about 1000 times lower.

Absorbance at 280 nm, attributable to tyrosine and especially tryptophan residues, is generally employed for protein quantitation. Experimental conditions for these determinations

must be selected with care to avoid major artifacts. Protein samples containing disulfide-linked oligosaccharides can have their concentration determined at 295 nm with minimal interference. Fluorescence spectroscopy exploits the inherent fluorescence of tryptophan residues to provide extremely sensitive detection of native or chemically labeled proteins, and provides conformational information when recording the intrinsic fluorescence or quenching efficiency of tryptophan residues. The fluorescence quantum yield of proteins can be determined using a reference standard with known quantum yield, while an unquenched sample can be used for attenuation correction. Other fluorescent materials, such as dyes or fluorescent proteins, can also be used to label proteins for detection [124, 125, 126, 127].

Infrared and Raman spectroscopy

Are complementary vibrational techniques for characterizing structural properties of proteins. The intrinsic fingerprint regions for chemical and structural changes are 400-1500 cm-1 for infrared and roughly 50-1700 cm-1 for Raman spectroscopy. In proteins, the vibrations of the amide groups (C=O, N-H) are responsible for the IR absorption and Raman intensity variations of the backbone amide bonds. Quantitative differences in the intensities of these amide vibrations provide information about secondary structural features, in particular, the relative populations of α-helices, β-sheets, and unordered conformations. Vibrations of the side chains contribute mainly to the spectral region above 1500 cm-1. They are generally less useful for structure determination because, except for tyrosine, their intensities are much weaker than those of the amide vibrations and cannot be considered absorptions in the mathematical sense. Nevertheless, a few secondary structure characteristics can be retrieved from this region, such as proline content or variations in side chain envelope [128, 129, 130, 131].

Strong changes in the amide II region have also been attributed to the presence of a structure-destabilizing agent, although the peak positions of the α-helical and unordered components remain unchanged. Vibrational CD at low frequencies (<400 cm-1) and for the amide II band region also provides high-quality probe of the secondary structural content of the protein, while a combination of IV CD and near-Raman properties allows determination of the relative population of substrates and the pareatonic state. Raman is the preferred method of choice in physiologically relevant surroundings such as membrane-mimicking arrangements and native environments; indeed, it is the method of choice for folic acid and NADPH, while the analysis of foetal bovine serum in the gelatin state suggests the presence of small-distance interactions. All analyses converge toward the protein being present in the same conformational state as discussed in the etyconic/electronic comparison.

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopic studies offer valuable insights into protein structures and dynamics. By exploiting the magnetic properties of atomic nuclei, NMR identifies neighboring atoms at resolutions comparable to X-ray crystallography, thus resolving micro-solvent-protein interactions inaccessible to X-ray studies. Furthermore, NMR elucidates the dynamics of folded, unfolded, and denatured proteins in solution. However, capturing 3D structures using NMR remains challenging and generally requires isotopic labeling to maintain resonance sensitivity.

The magnetic fields used in NMR spectroscopy generally probe the hydrogen nuclei present in an aqueous protein sample. However, the low sensitivity caused by the unfavored natural abundance of isotope labeling imposes great limits on the

detection of a wider range of molecules and on the development of complex systems such as large protein complexes, transiently interacting species, and membrane proteins. These limits are progressively overcome by employing different magnetic nuclei that are more suitable for particular applications (e.g. 19F, 31P, 15N, 13C, 2H). While the theoretical principles remain the same, the experimentation and interpretation of results require deep expertise to unveil three-dimensional structures, molecular dynamics, metabolomics signals or detect mechanisms associated with disease progression and treatment [132, 112, 133, 134].

Circular dichroism for protein conformational analysis

Circular dichroism spectroscopy quantifies the differential absorption of left and right circularly polarized light due to molecular chirality. Protein secondary structures impart unique circular dichroism spectral signatures in the near-UV region (230-260 nm). Analysis of amide N-H transitions in the far-UV (185-230 nm) region provides quantitative estimates of α-helical and β-sheet content when adequate reference spectra are available. Core secondary structure motifs are also evident among deconvolution packages. Although circular dichroism is principally limited to secondary structural estimation, characteristic spectral features have been linked to local tertiary structure, prediction of experimentally unobserved structures, folding and unfolding transitions, and residual structural content in protein-destabilizing formulations.

There are several important caveats for circular dichroism measurements and structural analyses. Nonbiological chirality may be manifested through certain absorbance-based circular dichroism assessments, particularly for polynucleotide and polypeptide systems. The Soret band of heme-containing metal-conjugated proteins can contribute strong absorbance therein. β-Sheets, irregular regions, and aggregates introduce spectral noise,

complicating quantitative secondary structure estimates. Absorption saturation precludes analysis of samples exhibiting high concentrations and optical path lengths. A combination with UV-visible spectroscopy can assist with sample selection [135, 136, 137, 138].

Chapter - 9

Electrochemical and Biosensor-Based Protein Detection

Proteins constitute the primary actors in cellular functions. Studying protein expression levels and modifications can provide critical biomedical information. The redox behavior of protein constituents enables probing protein presence and concentration by electrochemical biosensors, including label-free sensors for disease detection. A classic category of biosensors for protein recognition employs a modified surfaces template that is capable of specifically capturing the target. The main challenges include decoration chemistry, signal enhancement, and high-performance biosensors suitable for point-of-care applications. Innovations in nanomaterial chemistry allow the preparation of nanocomposites that combine several functionalities beneficial for sensitive and selective protein detection. These materials, together with interdigitated array electrodes and advanced interfacing possibilities, broaden the applicability of electrochemical detection in the life sciences and clinical assays.

Direct electrochemical detection of proteins requires specific/sensitive electrode modification suitable for the analyte and delivers a response proportional to concentration. Enhancement of the electrochemical signal using electroactive nanomaterials can increase the detection limit. Nanoparticles such as gold, silver, platinum, or metal oxides possess large specific surface areas and often exhibit redox activity associated with their own ions, improving the detection of protein-labeling electroactive probes. These nanoparticles can also be combined

into other nanostructures or frameworks to achieve a synergistic effect and can be used with label-free electrochemical signals based on redox- or electrocatalysis-active moieties on the proteins [139, 140, 141, 142].

Electrochemical properties of proteins

Proteins possess a wide variety of electrochemical properties due to the distribution of charge in both the peptide backbone and the various functional groups along the protein sequence. The molecular weight of proteins confers them with very low solubility and chemical reactivity at electrodes unless special conditions are established. Nevertheless, the determination of the standard reduction potential of redox-active amino acids in proteins and their location in the protein sequence modulates the physiological redox process. Thus, a deeper understanding of the electrochemical behavior observed in proteins is desirable since they can then be employed as natural redox-active intermediates in the study and development of electrochemical sensors. Moreover, these natural redox-active intermediates can modulate the electron transfer of biomolecules involved in many physiological processes.

There are two main strategies in the design of electrochemical biosensors: the first is based on the interaction of proteins with the designed surface and focuses on the modulation of the signal, while the second employs redox-active proteins (or enzymes) within the sensing interface. In this sense, highly porous conducting polymeric electrodes can promote the entrapment of redox proteins or enzyme-active sites within their structure, creating an ideal environment for biocatalytic or electron transfer processes. In the case of the first strategy, Electropolymerization of films at the conducting surface modifies the physicochemical and electrochemical properties of the material, thus. enhancing its interaction with pollutants and analytes [143, 144, 145, 146, 147].

Chemical functionalization of sensor surfaces

Chemical modification of sensor surfaces enhances protein detection sensitivity and selectivity. Proteins are inherently electroactive and suitable candidates for electrochemical immunosensors. Current experimental conditions use pyrrole or thiol to modify the nanomaterial and increase affinity with the protein detection. Other effective chemistries for enhancing the signal are shown based on adsorption, covalent binding, and non-covalent interactions, which addition results in electrochemical signals easily detectable with high sensitivity.

Nanotechnology-assisted electrochemical sensors integrate macromolecular biological recognition and nanotechnology via electroactive nanoporous sensing materials, greatly improving their analytical performance and sensor application potential. Supporting nanoparticles amplify the electrical signal via increasing the electroactive area, enhancing the effective concentration of marker and/or producing an enzyme-like electrochemical catalysis effect. In addition, conducing polymer-nanoparticle composite also used in electrochemical sensors to integrate the advantages of both components [148, 149, 150, 151].

Nanomaterial-enhanced biosensors

Nanomaterials improve biosensors by amplifying detection signals. Enhanced electronic, optical, mechanical, thermal, or magnetic properties enable sensor array miniaturization and sensitivity. Carbon-based materials allow facile chemical surface functionalization, improving analyte binding and electronic resonance transfer with signaling labels. Noble-metal nanomaterials function as carriers or amplifiers, facilitating strong binding and sensitive colorimetric detection. Hierarchically structured three-dimensional nanomaterials integrate multiple functions and improve binding kinetics, analyte accessibility, and signal production. Their performance,

reproducibility, and operational simplicity are positively correlated with size, dimension, and shape.

Detection using nanomaterials is ubiquitous in biosensors. Biosensors designed for actual-point clinical diagnostic applications face portability, cost, and user-friendliness challenges. Low-resource settings, without specialized infrastructure, experience language barriers and lack trained personnel. Point-of-care or point-of-need biosensors—easy to use, rapid, sensitive, and specific—facilitate effective decision making and disease management, especially infectious diseases. To ensure proper design, important factors include clinical considerations, validation of the target pathogen or biomarker, selection of the reported readout, suitable technology, incorporation of a user-friendly operation procedure, and translation into non-scientific language [152, 153, 154].

Point-of-care diagnostic biosensor systems

Point-of-care biosensors for protein detection allow a rapid determination of target analytes, ideally combining high sensitivity with portability and user-friendly operation. To achieve signal amplification in a relatively simple, inexpensive and rapid manner, nanomaterials have been employed. Nanoparticles possess strong nanoscopic effects and a high surface area, and thus different kinds of biological sensing molecules can be immobilized to enhance the detection of target proteins. The good electrochemical conductivity of these materials or the catalytic activity of labels appears to be the source of signal amplification for electrochemical sensors.

Signal amplification may also be realized by increasing the number of immobilized sensing probes or labels, as enabled by a larger-distance label setup. However, the complicated methods or large-scale operations involved in the synthesis of nanomaterials have raised reliability questions during the

experiments. Sensors that incorporate nanomaterials as substrate-modifying materials or signal-enhancing agents may benefit from the advantages of both nanomaterials and whole biosensing platforms. Among point-of-care diagnostic sensor systems, those based on electrochemistry, fluorescence, and colorimetry are most widely reported. In addition, surface plasmon resonance technology has also been applied in biosensing. Nevertheless, the potential of biosensing systems for clinical application depends not only on sensitivity and ease of use, but also on the accuracy, specificity, cost and speed of detection, as well as on regulatory approval for implementation in real clinical samples [155, 156, 157, 158, 159].

Chapter - 10

Nanotechnology and Chemical Nanoprobes

Nanoparticles interact with proteins by adsorption and corona formation, yet the effects of surface characteristics and protein structure on these processes remain poorly understood. Appropriately functionalized nanomaterials enable efficient and specific capture of proteins, supporting diagnostic applications. Signal amplification strategies based on label-free or nanomaterial-enhanced detection improve sensitivity. Finally, chemical nanoprobes for the specific diagnosis of infectious diseases are emerging, but clinical validation and point-of-care formats are rarely implemented.

Nanoparticle-protein interactions determine nanoparticle biodistribution, transport, and safety. *In vivo*, a protein corona forms around nanoparticles, regulating cellular interactions and providing a nano-bio interface. The effects of nanoparticle shape, size, charge, and material on protein adsorption and corona formation, as well as the effects of protein concentration and structure, are important for designing safe nanomaterials. Proper functionalization of nanoparticles facilitates chemical or covalent binding to proteins, enabling their efficient capture in solution. For instance, gold nanoparticles with covalently linked imidazolium salts efficiently capture a variety of proteins, including thrombin, lysozyme, and immunoglobin G (IgG), in a concentration-dependent manner. Triazine-phenyl-naphthyl-dicarboxylic acid coated with n-dodecanethiol preferentially captures α-chymotrypsin, lipase, and albumin of Amaranthus tricolor, Bhuj, and pumpkin sap [160, 161, 162, 163, 164].

Nanoparticle-protein interactions

Are critical for assessing the biosafety of nanomaterials and understanding the implications of nanomaterial use in biological contexts. Nanoparticles are generally administered in a biological fluid—the bloodstream or a cell culture medium—that contains proteins. High concentrations of proteins and other biomolecules can result in the formation of a protein corona, a layer of proteins that wraps around the nanoparticle surface and masks its original identity. The protein corona varies with the physicochemical characteristics of nanoparticles (e.g., size, charge) and the biological environment (e.g., type of biological fluid and temperature). Interactions can differ according to the nature of the protein involved; binding is not necessarily nonspecific, and there is evidence that proteins are adsorbed according to their structure and intended function in the body. Regardless, protein-nanoparticle binding can suppress the specific interaction of engineered nanomaterials with other proteins for which they were designed. Consequently, understanding protein adsorption onto nanomaterials is an essential aspect of nanoparticle application in medicine and as biosensors. Minimizing adsorption of proteins incompatible with the diagnostic process is thus a key consideration.

Some interactions between proteins and nanoparticles are beneficial, such as when functionalized nanomaterials are applied for protein capture. A nanoparticle-biomolecule-based sensor is constructed by immobilizing capture probes on a sensor surface and detecting analyte-target molecules through biodetection reactions. The sensitivity and stability of such sensors can be enhanced by using nanomaterials, including metal nanoparticles, carbon nanomaterials, and nanocomposites, capable of amplifying signal output. Nevertheless, biosensors may not match laboratory methods because Little sensor signal and nanoparticle-catalyzed signal amplification provide limited

dynamic ranges, suppress the detection limit, and do not guarantee high sensitivity. These problems can be alleviated by combining several strategies to form a point-of-care diagnostic biosensor system [165, 166, 167, 168, 169].

Functionalized nanomaterials for protein capture

Nanomaterials functionalized with chemical ligands that specifically bind to designated proteins can be used to capture targeted proteins from complex samples. The labeled, isolated proteins can then be detected using a variety of methods to monitor proteomic alterations associated with disease. Proteins are usually present in exceedingly low concentrations in biological fluids compared to other matrix components, so sensitive detection methods are necessary. Metal ions in nanomaterials can enhance detection signal through several mechanisms, offering improved sensitivity over conventional detection methods. As several types of metal ions can be incorporated, detection assays using multiple proteins can be performed simultaneously to interrogate high-dimensional disease diagnosis information. Such multifunctional metal nanomaterials have demonstrated high sensitivity and specificity for the quantification of SARS-CoV-2 proteins in serum samples, providing a potential diagnostic tool for COVID-19 and an intelligent approach for the detection of other respiratory viruses.

Functionalized silica nanoparticles with silica shell thicknesses optimized for the desired application enable facile capture, recognition, release, and concentration of high-abundance proteins using an enzyme/amino-acid immobilization strategy. Nanomaterial-based affinity systems that specifically target low-concentration proteins in body fluids have also been reported. Detection limits for various proteins markedly lower than in traditional methods can be achieved with these enhanced amplification strategies. Target proteins can be eluted from nanomaterials using mild conditions compatible with subsequent

detection and quantification by distinct methods, adding versatility to the approach. Such technologies can be integrated into point-of-care diagnostic platforms featuring direct capture and detection to facilitate rapid sample analysis and reporting [170, 171, 172, 173, 174].

Nano-enabled signal amplification strategies

Functionalized nanomaterials can also serve as catalyst scaffolds to enhance the sensitivity of biosensors. Catalysis-based strategies incorporate redox, precipitation, hydrolysis, or chemical reactions into the detection process, enabling the amplification of the analyte signal without the need for an additional tag. The catalytic signal is often transduced through electrochemical or optical means. For example, based on copper phosphide, Fe3O4, Au2S nanocomposites exhibiting peroxidase-mimicking activity were developed for the sensitive colorimetric detection of H2O2 and glucose. In these designs, immunoassays with ultrahigh sensitivity were realized by introducing a trace quantity of the catalytic nanocomposite as a signal reporter.

Nanomaterials can also act as tracers to amplify detection signals. For example, nanoparticle-enhanced desorption electrospray ionization-mass spectrometry (DESI-MS) was developed to detect and identify peptides from Brucella species. Colloidal gold and magnetite nanoparticles were used to enhance the detected ion yield, resulting in better detection limits and improved spectral resolution [175].

Diagnostic applications of protein nanoprobes

Nanotechnology has become an integral part of medicine. Its ability to manipulate materials at the level of atoms and molecules promises innovative strategies for early disease detection and therapy. Novel functionalized nanomaterials, capable of selecting proteins with diagnostic significance within biological fluids or tissues, are being developed. Their

implementation in a clinical context requires validation in real biological systems. The analysis of the protein corona formed around nanoparticles under *in vitro* and *in vivo* conditions can provide important information on biosafety. Other potential applications include imaging-assisted early diagnosis and treatment.

An ongoing challenge in development of nanomaterials is selection of the best protein targeting agents for a specific disease. Recent advances in nanotechnology have introduced innovative protein-compound diagnostic platforms. These systems combine interconnected nanoparticle-chemical compound interactions with the ability to perform "smart" detection in the presence of specific disease microenvironments. Transcription activator-like effectors (TALEs) programmed to specifically target genes of interest are being covalently attached. Expression of conjugated genes in living cells is evaluated using luminescent or fluorescent agents. Nanoprobes have also been proposed to enhance the detection of disease-related proteins in a field-effect manner.

The core of the nanomaterial is a modified 3-aminopropyltriethoxysilane-based silica particle, covalently bound with phenylboronic acid as the recognition and signal amplification site. Detection exploits the electrochemical response of gold nanoparticles, deposited on the surface of modified silica by a layer-by-layer approach, and the formation of a reducing environment, generated by the specific binding of 3,4-dihydroxyphenylalanine. Other strategies make use of the presence of a fluorescein-based energy donor on the nanomaterial, with redox ability, and a rare-earth metal-ion-doped luminescent nanocrystal serving as the energy acceptor. Pairs of phenolic compounds and lanthanide precursors are introduced into the particles, achieving specificity by different pairs and revealing potential for detection of cancer-relevant

protease activity. The a-aminobutyric-acid-triggered self-assembly of Au35 nanoparticles has been exploited to develop a turn-off sensor for -amino acid oxidase activity [176, 177, 178, 179, 176, 177, 178, 179, 180].

Chapter - 11

Computational and Chemometric Approaches

Molecular interactions between proteins and chemical compounds are key to designing new chemical agents for protein analysis and diagnostics. Accurate molecular modeling can provide more detailed structural insights than traditional analytical approaches and facilitate virtual screening of compound libraries. Validation of the developed models enables prediction of interaction strength and positioning for ligands to support focused compound design or testing. Predicting correlated interaction information across large numbers of protein samples can increase understanding of classical protein signatures for disease, as can applying relevant chemometric approaches to proteomic datasets.

Molecular modeling can be used to predict interaction information for any two molecules for which structural data are available and that bind one another. The current approach has been applied to map the interaction energy of specific protein residues with different small tags from labile TMTpro mass tags. Interaction energy matrices have also been predicted to provide insight into the redox state of the protein sample based on disulfide group formation or cleavage and to compute interaction energies of common noxious compounds with an enzyme library for testing or development of biosensor systems. Such predictions help reduce testing numbers by prioritizing compounds most likely to bind target proteins [181, 182, 183].

Molecular modeling of protein-compound interactions

Molecular docking predicts compound binding positions and affinities within target protein cavities. Based on X-ray or NMR protein structures, molecular-dynamics-simulated conformations, or homology models, suitable replicas may be selected. Compounds can be either rigid structures or flexible ligands with parametrized rotatable bonds. The most common docking suite is AutoDock, which uses a scoring function reflecting various energetic contributions. Following docking, poses are ranked by estimated binding free energy, enabling virtual screening of large compound libraries to identify promising hits for experimental evaluation.

Quantum mechanics can model protein and small-molecule interactions at the electronic level, yielding highly accurate energetics and geometries. Given the high computational demand, such calculations typically target a limited number of well-suited candidates. Computer-aided design based on these modeling principles can facilitate the generation of additional probes. Molecular dynamics can further justify and support the design of candidate compounds. Such strategies have contributed to the development of small molecules that inhibit or propagate protein-protein interactions and probes that specifically sense selective nucleophiles or oxidative environments. Molecular docking and quantum-mechanics-based interaction models have paved the way for new bisulfite-based fluorescence probes capable of detecting pathogens. Such modeling approaches add strong predictive power to chemical pathology studies.

Chemometric analysis of proteomic data

Chemometric techniques offer a powerful approach to transforming raw spectrometric data into interpretable knowledge. The implementation of chemometrics is driven by the aim of analyzing the distinctions between an experimental

group and a control group, where the background signal of the control group is subtracted from the experimental dataset obtained from the patient group. A distinctive set of features reflects the contributions to pathology by the experimental group of analytes, allowing protein characteristics to be connected to progress, activity, severity, or a diagnosis of a disease. The analytes may represent the terminal products of metabolism or be derived from the patient-microbe interplay in infectious diseases. Such interpretation and biological assignment of differentially expressed analytes are enabled by statistical analysis of these features using univariate or multivariate models.

The datapoints (spectrometric features) must be selected with respect to the context and objectives of the study, including the discrimination required and the groups under comparison. Procedures for selection of interesting variables and signatures, data normalization approaches, and appropriate univariate tests are thus critical for achieving significance and meaningful but not overstated correlations. In addition to helping reduce the complexity of high-dimensional proteomic databases, chemometric approaches can pinpoint the molecular size class of the selectively altered proteins, highlight unconsidered classes of underrepresented proteins, indicate the source of markers with low discriminatory capacity, or simply facilitate a clearer visual presentation of the data. Selected features of potential importance warrant particular attention, whenever proteomic data support biological conclusions [184, 185, 186, 187].

Machine learning in protein diagnostics

Machine learning has proliferated during recent years and permeated various domains, including protein diagnostics. A report leveraged the increasingly available dataset of protein or antibody-antigen interactions in order to analyze the link between protein sequence and structure, and the ability to predict their interaction affinity using machine-learning strategies on datasets

covering diverse antigen types. These results may be informative for the prediction of protein recognition over non-target protein or peptides, and ultimately protein detection or separation applications.

The main aim of a different study was to obtain a protein expression index that would be easily interpreted and correlated with seed quality in quinoa. Proteomes from quinoa seedlings under osmotic stress were assessed via label-free quantitative proteomics and a set of integral cellular function-related proteins was selected. The classification of these proteins in metabolic pathways, based on either the seed-storage function or the abiotic-stress-response function, was employed as features to train machine-learning classifiers, leading to a reliable proteomic signature-related seed vigour index [188, 189, 190, 191].

Integration of computational chemistry with analytical data

Computational chemistry supports diverse analytical modalities. The modeling of molecular docking and/or molecular dynamics studies is especially common, and results help interpret disease-relevant compounds or biological macromolecules. Proteomic research also routinely involves the identification of possible interacting partners through peptide or protein matching against an existing proteomic database. Integrating extra-analytical data to complement the experimental results would seem logical.

In proteome analyses, such complementary data could enhance the discussion or validation of a biological hypothesis using resulting potential biomarker candidates. Selected dimensions from other disciplines are suggested for use—biochemical, clinical, or human- related—and chemometry & machine-learning approaches may help consider multidimension inputs simultaneously [192, 193, 194, 195].

Chapter - 12

Proteomic Profiling in Disease Diagnosis

Global strategies identify systematic alterations, providing potential diagnostic signatures. Discovery-oriented research combines proteomics and clinical data analysis, revealing novel markers whose systemic variation informs disease status across diverse conditions. Targeted approaches assess focus markers, such as Alzheimer's-associated proteins and tumor-associated proteins.

Disease-linked proteomic profiles also derive from independent studies. Statistical analyses clarify differential expression and biological significance, while annotated validation cohorts support clinical relevance. Enrichment strategies address low-abundance proteome components, enhancing detection of specific markers, such as circulating neuronal proteins in neurodegenerative disease and exosomal tumor markers. The resulting profiles have diagnostic value, particularly when placed in the patient context [196, 197, 198, 199].

Global proteome analysis strategies

Proteomic profiling strategies broadly fall into two categories - discovery and candidate approaches. Discovery profiling typically uses highly sensitive methods amenable to global exploration, resulting in large data sets across numerous patients. Statistically significant protein expression level alterations may subsequently be tested for biological relevance and the information applied to a smaller, more targeted proteomic analysis in a focused patient cohort. Such a strategy enables both

the sensitive detection of low-abundance disease-associated proteins (Section 12.3) and the identification of potentially relevant protein expression level changes that cannot yet converge into disease-associated markers due to patient heterogeneity. The practical use of many known and putative markers for diagnostic purposes remains difficult, inconsistent, or utterly non-informative. In contrast, candidate approaches select a limited set of proteins based on functional involvement in the disease process, availability of specific antibodies, or prior establishment as putative disease biomarkers.

A common application of candidate proteome analyses is the detection of post-translational modifications (Chapter 1.2) associated with cancer. Such markers may serve as indicators of the malignant status of cancer in a patient or be associated with disease activity. Ideally, assays detecting these modifications are specific for cancer at all stages. For example, AD7c-NH2, a 13-residue peptide amide and neurologic biomarker candidate derived from the modification process of tau protein, has been detected in human cerebrospinal fluid (CSF) using a direct assay with 18F-labeled AD7c-NH2 and in serum samples from Alzheimer's disease patients with the use of a gold nanoparticle-multisandwich assay. Quantitative mass spectrometry-based proteomics have also been employed for the analysis of dysregulation of glycosylation, sialylation, and phosphorylation on specific proteins in cancerous tissues [200, 201, 202, 203].

Differential protein expression in disease states

Monitoring changes in protein levels across a wide variety of diseases has been a popular research strategy. Numerous proteomic studies identified differentially expressed proteins in a variety of diseases and their diseased states using disparate experimental conditions. Global proteomic profiling of cerebrospinal fluid differences between Alzheimer's disease

patients and controls led to the identification of 17 proteins, including calmodulin and pentraxin 3, that increased with the disease and were associated with synaptic injuries. Notably, chronic traumatic encephalopathy is caused by repeated head trauma, but brain injury occurs in other dementias; differential expression of the 28 proteins in these disorders could help establish whether chronic traumatic encephalopathy and neurocognitive dysfunction are present in football players.

In primary progressive aphasia, a rare dementia syndrome leads to the loss of the ability to generate and comprehend speech; silencing of serpinB2, CCL2, and pro-collagen-α3(VI) is thought to influence synapse formation and integration. Distinct CSF proteomic patterns were observed in sporadic and familial frontotemporal dementia cases. The sensorimotor cortex of amyotrophic lateral sclerosis patients showed a downregulation of the neuroprotective αB-crystallin and an upregulation of proteasome and cytoskeletal proteins as well as eukaryotic translation initiation factors associated with neuroinflammation. A targeted mass spectrometry approach also detected nine previously validated neurogranin, brain-derived neurotrophic factor, and C-C motif chemokine network proteins that accurately separated multiple sclerosis patients from healthy controls [204, 205, 206, 207].

Chemical enrichment of low-abundance proteins

Detecting low-abundance proteins remains challenging despite the sensitivity of modern proteomic techniques. While many proteomic studies focus on proteins belonging to the most abundant families, chemical approaches are increasingly being used to enrich low-abundance species prior to analysis. Lossless sample preparation methods that limit chemical interference can produce cleaner protein structures, leading to more reliable identification. Statistical methods for validating differential

proteomic expression are essential, as are experimental designs that ensure sufficient replicates and monitor variance on a biological-relevant scale.

Several recent investigations have demonstrated such chemical enrichment strategies. Addition of isotopically labeled ligand proteins such as cationic afamin enhances the detection of protein synthesis in mainly eukaryotic viruses by peptide mass fingerprinting of total proteins—helpfully enriching the low-abundance signals without degrading the abundant populations from the mass-spectrum fingerprinting analysis. Flag-tagged peptides introduce a similar orthogonal affinity-layer for multiplexing approach, where the Signal Peptide-peptide binding protein pair effectively represent photoreceptors in the Notational Plankton of Lake Merced and Monterey Bay by normalizing the biological variance and error detection [208, 209, 210, 211, 208, 209, 210, 211].

Clinical interpretation of proteomic profiles

Proteomic profiles in disease diagnostics must be interpreted in the context of the specific patient being tested. Large publicly available databases provide thousands of differentially expressed genes and proteins in a variety of diseases or conditions at different stages. However, given the large number of proteins expressed in any specific disease, together with the redundancy in some pathways, not all differentially expressed proteins are relevant for any specific patient. In addition, their upregulation or downregulation can give different and even contradictory information on the patient status. Validation experiments focused on a limited number of proteins and applying sensitive analytical methods have proved their interest in many areas of clinical diagnostics.

Proteomic profiling is therefore often combined with other information, such as clinical findings, radiological data and the

analysis of other biological fluids, for decision-making support. The regulatory agencies responsible for drug approval increasingly demand the development of companion diagnostics for new therapeutic solutions that selects those patients who are most likely to benefit from the treatment. These considerations underline the importance of developing affinity reagents for many of the proteins indicated by clinical proteomic studies, even those indicated as not being altered in concentration in the various analyses. They can form part of larger assays enabling the evaluation of pathways of biological activities active in all conditions or can be merely coincidental findings.

Chapter - 13

Chemical Approaches to Protein-Protein Interactions

Chemically based strategies enable the study of difficult-to-analyze protein-protein interactions in complex biological environments, including living organisms. Chemical probes illuminate the binding interfaces of interacting proteins, providing insights into their roles in life processes and levels of druggability. Cross-linking mass spectrometry captures the fleeting connections in dynamic complexes, generating comprehensive interaction maps with high spatial resolution. Chemical stabilisation of transient interactions facilitates dedicated studies of pathological associations, such as those involving regulatory proteins and toxins.

Significantly, the experimental exploration of protein interaction networks has begun to yield clinically useful tools. Multicomponent assays detect specific aberrations within networks and are rapidly emerging for neurodegenerative, infectious, and other inflammatory diseases. Such examples open the door for similar strategies targeting diagnostic hotspots in other diseases [212, 213, 214, 215].

Mapping interaction interfaces using chemical probes

Chemical probes—often affinity reagents—can provide information on protein-protein interactions at a fine-spatial scale. Such probes usually harbor two functional units that allow for an affinity tag to subsequently be introduced and conjugated either onto the probe during synthesis or following its interaction with

the target protein. The locations of conjugated tags reveal the interaction interface of the protein under study, which can attest to its biological function or, with some caution, be used to infer druggability. Furthermore, mapping can also be accomplished using functionalized azide or alkyne-bearing labels that react with compatible cycloaddition reaction partners in the vicinity of the protein surface exposed after complex dissociation. In this approach, an affinity unit is not necessarily required to steer the reaction to the protein of interest but can speed up the reaction with low-abundance proteins or nanomaterials.

Protein-protein interactions are known to play fundamental roles in a variety of biological processes, including signal transduction, regulation of metabolic activity, the maintenance of genomic stability, and cell cycle control. In addition, recent studies have highlighted the association of aberrant protein-protein interactions with certain diseases, opening a new avenue for disease diagnosis, treatment, and drug development based on the modulation of such interactions. Chemical probes that can tag particular protein interfaces and subsequent chemical analyses have extensively provided insights into these aspects [216, 217, 218, 219, 216, 217, 218, 219].

Cross-linking mass spectrometry

(XL-MS) pairs the application of cross-linking reagents with mass-spectrometry to elucidate static and dynamic protein-protein interactions. Cross-linking reagents are chosen to react with residues at or near the protein-protein interaction surface; interaction footprints can be detected either indirectly by an increase in the mass of the complexed proteins or by the formation of labelled cross-linking derivatives. The patterns formed by multiple intermolecular reaction events can be resolved by mass spectrometry and serve to indicate which of the multiple potential partners interact with each protein.

XL-MS is increasingly used to map the interaction interfaces of protein-protein complexes for which no high-resolution structural data are available or as an orthogonal analytical means to support other computational interaction-mapping procedures such as the generation of protein-fragile complexes. Enzyme-mediated cross-linking of transient protein-protein interactions has been demonstrated using the Klenow fragment of DNA polymerase I. Reagents that enable capture and subsequent analysis of unstable or short-lived interacting protein complexes have been developed.

Chemical stabilization of transient complexes

Transient macromolecular complexes fulfill myriad cellular functions, but their study proves challenging due to inherently short half-lives. An approach that exploits covalent cross-linking of structural components present in the same supramolecular cluster at a specific moment in time serves to structurally stabilize such complexes. Directed towards cellular systems, this strategy tracks, captures, and characterizes transient structures, thereby advancing an understanding of the fundamental biochemical and molecular bases for physiological and pathological processes and supporting the identification of potential targets for pharmacological intervention. At the diagnostic level, the methodology is particularly valuable for defining the driving forces that govern host-pathogen interactions.

The strategy has been applied to the immunostimulatory activity of the Herpes Simplex Virus protein UL39 and to the biochemical characterization of pre-immune human Vγ2Vδ2 T cell receptors. The S-phase checkpoint response has also been studied by showing that the p24 protein family associates with Chk1 in cyclic-dependent fashion in human cells and promoting its cross-linking with a chemical probe during S-phase activation

of the checkpoint. Moreover, the transient nature of the interaction between p21 protein and proliferating cell nuclear antigen has been addressed with a similar tagging and cross-linking strategy, enabling the capture of the expected protein complex and its characterization by mass spectrometry. Such a chemical capture strategy should aid the validation of multiple potential protein-protein interactors identified by high-throughput proteomics approaches [220, 221, 222, 223].

Disease-related protein interaction networks

A conceptual dialogue between Zhang *et al*. and Kim discusses chemical features of enzymes employed in tumorous tissues or during mammalian infection. A map of such interaction partners is rapidly emerging, sometimes with validated links to diagnostic strategies. Disease-perturbed interaction networks can aid diagnosis and guide therapeutic intervention. These concepts have been described in a recent review.

Chemically constructed maps of the interaction partners of disease-associated proteins may support diagnostic discovery as well as therapeutic strategy development. The direct proteins that respond to the infection process, or under pathological conditions such as cancer, pregnancy, or receiving treatment, can distinguish the status of the body. These may include both "non-self" proteins (such as those of the pathogen or drug) and "non-native" proteins (such as certain proteins of the human body) involved in host-pathogen interactions. The proteomes of various samples under these conditions have been established. Protocols for developing the chemical probes used to capture these proteins, and for providing structural biological information, have also been rapidly developed. A good number of probes have achieved successful shifts from the test tube to the patient bedside, realizing rapid analytical detection or therapeutic applications [224, 225, 226, 227].

Chapter - 14

Innovative Diagnostic Platforms and Devices

Novel diagnostic platforms and devices based on chemical methods rapidly analyze proteins and provide accurate results, drawing growing attention as new directions in diagnostic research. These systems can integrate several processes, either completely or partially automatically, and have been scaled down to a lab-on-a-chip format. Microfluidic devices, utilizing a closed system, may possess high throughput and require a small volume of samples. The demand for commercially available diagnostic products has led to the adoption of combined systems able to rapidly analyze proteins from various biological samples in real time. The workflows involved should involve suitable clinical and analytical validation.

Chemical detection of proteins lies at the core of protein-based diagnostic analysis. Nanoparticles have been designed to undergo color changes in response to specific protein targets by chemical modifications of their surface, allowing naked-eye detection. Proteins present in inflammatory diseases are also common targets for fabricating rapid diagnostic prototype systems. These systems can use colorimetric or electrochemical sensors and may or may not use nanomaterials [228, 160, 229, 230, 231].

Lab-on-a-chip chemical systems

Microfabrication technologies have enabled the integration of multiple chemical-functional modules on a single chip. Such lab-on-a-chip systems exploit miniaturization advantages, such as reduced reagent consumption, shorter reaction time, improved

signal-to-noise ratio, and easier automation, and offer additional application-specific advantages depending on their design. For example, microfluidic devices designed for protein analysis combine viscoelasticity, electric field, and hydrodynamic forces for accelerated, controlled separation and detection of various analytes. The rapid processing of a whole blood sample using such devices sustains a concentrated electroosmotic flow that cannot be achieved with a single pump. A lab-on-a-disc system also performed whole blood analysis by spinning a disc and briefly stopping the rotation. Elution of a colored marker from a source well and detection by a smartphone camera provided a practical antibacterial evaluation method.

Lab-on-a-chip systems are custom made for each specific application, with design considerations and performance metrics delicately tailored toward the targeted functionalities. Consequently, results from different lab-on-a-chip systems are necessarily difficult to compare. Despite these limitations, multiple lab-on-a-chip chemical systems have achieved important research breakthroughs and promises for clinical use, especially point-of-care diagnosis [232, 233, 234, 235, 236].

Microfluidic protein analysis devices

Microfluidic systems facilitate high-throughput biological analyses while mimicking physiological conditions. Incorporating chemical functions into lab-on-a-chip platforms enhances their diagnostic utility. Combined with parallelism, microfluidic operations minimize sample consumption and processing time. Merging multiple analytical components into single devices improves functional integrity, simplifies workflows, and reduces overall costs. Integrated protein analysis devices can include microreaction arrays, immunoreaction devices, protease arrays, enrichment compartments, and detection modules. Such devices support applications in

proteomic profiling, food safety, infectious disease detection, and toxicological analysis [237, 238, 239, 240].

Physiological microenvironments often limit concentrations of target analytes, particularly low-abundance proteins and potential biomarkers. These constraints make the creation of biosensor devices that accommodate microfluidic sample handling functions especially attractive. Coupling sample treatment to ultrasensitive detection methods expands the range of analytes detectible in pathogen testing. Generating false-positive results remains a challenge for pathogen test devices, since contamination with the action products of biological defenses possess considerably shorter half-lives than the interaction partners.

Automated chemical-analytical platforms

Commercially available clinical chemical-analytical systems comprise a range of individual electrical sensors designed to quantitatively detect and analyze specific analytes, leveraging various technologies (e.g., electrochemical) and specific detection reagents (e.g., molecular recognition compounds).. developed for reliable and reproducible analysis of specific analytical samples while minimizing manual interventions, integrate the functions of these individual clinical systems into a unit capable of sequentially treating and analyzing multiple samples using their inherent automatic sampling system. Such platforms have been applied, for example, to virus detection — protein-based methods using ELISA principles, and employed in studies involving the detection of IgM, IgA, and IgG against SARS-CoV-2 in blood.

Jiang and colleagues proposed a prototype based on low-cost sensors with the performance characteristics of conventional PCR processes. A disposable nucleic acid functionalized-lyophilized cyanine dye probe docked onto an Au-eletrode

surface enabled an isothermal amplification and detection system. A low-cost electrochemical system connected to a smartphone was utilized to readout data. The smart-phone validated the information directly and the platform worked in a lab-on-a-chip manner [241, 242, 243, 244].

Clinical validation of diagnostic technologies

For proper integration into clinical practice, novel chemical-diagnostic technologies must undergo rigorous testing. The accepted approach entails validating candidate assays in biological specimens collected from implicated patient groups, such as tissues, blood, or urine. These samples are typically anonymized to protect patient confidentiality before translational testing. For example, a yeast two-hybrid screen may unveil novel pathogen-host protein interactions with potential diagnostic applications. To advance this finding towards clinical implementation, a targeted approach using patient-derived samples is warranted. Candidate targets must first undergo validation in appropriate animal models (e.g., mouse, rat, cat, dog), evaluated for sensitivity and specificity, and finally tested within human cohorts.

Moreover, regulatory procedures must be considered before commercial release. The U.S. Food and Drug Administration (FDA) categorizes newly developed clinical tests into four groups based on assay type and use, with documentation required for each stage. Classification criteria and required documentation differ across countries, including Australia, Europe, and Japan, so researchers must strategically incorporate these steps into their experimental design to achieve successful commercialization [245, 246, 247, 248].

Chapter - 15

Protein Analysis in Cancer Diagnosis

Cancer-associated alterations in the proteome can potentially serve as specific disease biomarkers. Cancer remains one of the leading causes of death worldwide, primarily due to late-stage diagnosis. Tumor protein markers, due to their apparent linkage to the malignant process, offer a potential solution to this significant medical need. Though their expression is altered in different cancers, most markers lack sufficient sensitivity and specificity for population-wide screening. Nevertheless, they are still valuable in the clinical setting for aiding diagnosis, determining prognosis, monitoring treatment response, and detecting recurrence.

Chemical methods capable of directly detecting cancer-associated protein chemical modifications hold a unique position as diagnostic tools because they provide information above and beyond mere expression levels. Such chemical methods, together with signature proteomic differences at cancer stages, have the potential to reveal more informative attributes of cancer than conventional protein markers. Genetic alteration analysis, especially as applied in next-generation sequencing, has become a major driver of clinical oncology. The future may lie in a composite index of proteomic patterns, enabling translational potential from the laboratory bench to clinics [249, 250, 251, 252].

Tumor-specific protein biomarkers

A panel of tumor-specific protein biomarkers identified through proteomic methods may facilitate the diagnosis of multiple types of cancer. Expressed in the tumor but not in

adjacent healthy tissue, these proteins are ideally suited for the detection of primary malignancies and their metastases. A number of these biomarkers are secreted into bodily fluids, enabling non-invasive testing. However, individual sensitivity and specificity often remain inadequate for accurate diagnosis. Approaches focusing on specific oncogenic protein modifications or combining multiple markers for a particular tumor type may improve reliability. Furthermore, proteins or proteomic signatures correlated with disease progression hold prognostic value or can guide the selection of targeted therapies.

The rapidly expanding arsenal of tumor-suppressor and oncogenic proteins has also opened up new avenues for cancer diagnosis. At present, none of these proteins can serve as universal markers in cancer diagnosis due to limited sensitivity and specificity. For the majority of human cancers, tumor-specific proteins only have been observed individually or in small groups. In a few cases, a set of protein markers expressed in one type of tumor has been used for the diagnosis of another type of malignancy through multiplex detection. Thus, the identification of tumor-specific proteins remains an attractive target in cancer research, particularly for the discovery of new biomarkers applicable to multiple types of cancer [18, 253, 254, 18, 255, 253, 254].

Chemical detection of oncogenic protein modifications

Cancer cells undergo profound metabolic rewiring, resulting in the accumulation of protein post-translational modifications characteristic of these malignancies. These aberrant modifications have emerged as promising cancer biomarkers, particularly for early detection, cancer onset confirmation, and prognostic profiling. Detection platforms targeting well-characterized tumor-associated modifications such as glycosylations, palmitoylations, myristoylations, acetylations, methylations, and phosphorylations have been described. However, protein cancers remain elusive because it is often

difficult to determine the acquisition of new functions using generic protein modification—for example, the phosphorylation of important oncogenes by diagnostic protein kinases. Focusing on pharmacological targeting, profiling, and chemical detection using curated small recognition elements (SREs), quantification remains a major challenge, particularly in the case of pan cancer biomarkers.

Polydeoxyribonucleic acid polysaccharides generate a hydroxyl dominance structure that appears surface-activated like a chemical sensor, thereby decoupling the requirement for catalytic reaction. The calcification response of mycobacterium tuberculosis on the S□SDRE hybrid produces a crystal engineered catalyst for selective detection using densitometry.

Proteomic signatures in cancer progression

Advancing cancer diagnosis may benefit from protein expression profiling of tumor secretomes, as illustrated by two examples. The concentration of 20 secreted proteins was measured in breast cancer plasma, revealing markedly increased levels of RAP1B, THBS1, and TNFRSF12A and decreased CD55 and PROS1 in cancer compared to healthy controls. For the malignant group, TNFRSF12A was positively correlated with breast cancer size, while PROS1 was inversely related to cancer stage. The upregulation of the other four proteins was also indicative of malignancy. Multiplex detection of secreted proteins further indicated the potential of profiling tumor-derived proteins for clinical management. A second study showed that neuroblastoma-secreted proteins could stimulate disease-associated molecular alterations in neuronal cells. The protein expression profiles of TNF-α, IL-1β, IL-6, and IL-8 were measured in the cell supernatant, and concentrations of TSG-6, MCP-1/CCL2, CXCL10/IP-10, and IL-20 were evaluated in the cell lysate. A 624-gene expression analysis of SH-SY5Y cells

treated with conditioned medium from IMR-32 cells revealed that GNDF was upregulated, while genes in various pathways (angiogenesis, apoptosis, calcium signaling, cytokine-cytokine receptor interaction, TNF signaling, and the Toll-like receptor pathway) were inhibited [256, 257, 258, 259].

Collectively, proteomic signatures in cancer progression support diagnosis, monitoring, and treatment, although the clinical utility still requires validation.

Personalized oncology based on protein profiling

An individual's tumor may differ from the original tissue and subsequently change over time due to acquired mutations and adaptations that enable metastasis and/or facilitate therapy evasion. Personalized age-appropriate selections of systemic treatments to effectively disrupt or prevent recurrence of cancer relies on characterizing the tumor's hallmarks. These manifestations can involve aberrant cancer-related proteins that commonly respond to only a single therapeutic modality, such as chemotherapy, radiotherapy, or targeted therapy. As achieved for numerous other diseases, such responses can be directed by protein profiles rather than conventional selection of the most common tumor type.

As proteins underpin tumorigenesis, profiling their expression provides a reliable basis for personalized screening to inform patients whether available therapeutic options will respond or not, thereby eliminating unnecessary toxicity. Info-communications technology can also facilitate the widespread accessibility of screening in elderly populations, particularly for malignancies with exceptionally high mortality rates and no effective diagnostic programs, such as lung cancer. Cancer profiling based on proteins can additionally assist with choosing the most effective therapy and with monitoring progress and selecting additional treatment strategies [260, 261, 262, 255].

Chapter - 16

Protein Analysis in Neurodegenerative Diseases

Molecular mechanisms causing neurodegenerative diseases are complex but connect to misfolding and insoluble aggregation of specific proteins. Protein-based biomarkers can assist in diagnosis or prognosis and help direct research toward effective treatments. Detection of diagnostic biomarkers, like amyloid-β peptides or tau proteins in biological fluids, offers sensitive and relatively straightforward early diagnostic possibilities. Other known candidates may appear in the serum or cerebrospinal fluid over time, while their prognostic value remains less established. Further development of innovative tools for protein-based neurodiagnostics is needed to allow practical application in a clinical context.

Neurodegenerative diseases involve a gradual decline in brain and cognitive functions, ultimately leading to death. Alzheimer's disease (AD) is one such disorder for which the molecular mechanisms are not fully understood. However, it is accepted that the brains of AD patients contain large numbers of specific proteins that misfold and aggregate into insoluble fibrils. Detection of such aggregates in biofluids is a clear possible approach to early diagnosis. In addition to patients currently diagnosed with the disease, analysis of asymptomatic individuals with suspected but non-accessible brain lesions could improve understanding of the disorder, thereby facilitating treatment development. Specimens of circulating blood and cerebrospinal fluid are mainly employed, and proteomic tools have been

proposed for these applications, enabling monitoring of candidate markers that may assist in diagnostic, prognostic, or treatment-selection decision-making processes.

Other neurodegenerative diseases, including Parkinson's and Huntington's diseases, exhibit specific misfolded or aggregated proteins that may serve as markers. As the causes of all these illnesses remain poorly understood, the identification of candidate proteins involved in the neuropathological process in humans could provide new avenues for research and diagnosis. Multiple detection platforms, including advanced technologies beyond mass spectrometry, are therefore needed, along with innovative drug discovery pipelines. In addition to misfolded proteins, soluble forms of proteins thought to be involved in disease neurotoxicity may be analyzed, and secreted factors from human neurons cultivated *in vitro* provide a way to establish and validate diagnostic classifiers [263, 264, 265, 266].

Misfolded proteins and aggregation chemistry

Many proteins in the human body are sensitive to conformation changes. Changes under pathological conditions could result in misfolding and aggregation consisting of intermolecular β-sheets that create oligomers or fibrils. Alongside amyloid disorders (Alzheimer's, Parkinson's, Huntington's, type II diabetes) different neurodegenerative diseases are associated with deposition of misfolded species: tauopathy, prion diseases (Creutzfeldt-Jakob disease), frontotemporal dementia (Tau-associated frontotemporal degeneration), age-related dementia with tau deposits, amongst others. Misfolding and aggregation are driven by different mechanisms associated with ageing, oxidative stress, mutations, dysregulation of cellular processes, etc. Increasing evidence shows that these proteins, capable of inducing other proteins to misfold, act as infectious agent. Changes in protein conformation

therefore produce potential biomarkers useful for diagnosing neurological disorders. Detection of certain neurodegeneration-related proteins in biological samples can also provide further information about disease progression or activity.

Sensitivity to conformational change is a challenge for analytical chemistry, especially when developing diagnostic tests for amyloid or tauopathy associated with neurodegeneration. Producing antibodies that specifically discriminate the disease-aggregated form from the physiological monomeric one is difficult. In particular diagnostic potentials are provided by assays able to follow the temporal evolution of three situations during disease development: a) increase of the abnormal protein during accumulation phase; b) decrease in the transition phase; c) re-increase or stability of the normal form in the clearance phase. The potential of these approaches relies on the temporal monitoring of the content or presence of a certain target in patients or biological models, particularly in accessible biofluids as plasma or urine [267, 268, 269, 270, 271].

Chemical detection of amyloid and tau proteins

Amyloid plaques and neurofibrillary tangles containing tau proteins are widely regarded as the main pathological hallmarks of Alzheimer's disease (AD). Their detection is clinically significant for diagnosing AD as well as other neurodegenerative disorders characterized by tau deposition, such as frontotemporal dementia (FTD). Consequently, considerable efforts have focused on developing ultrasensitive and specific diagnostic approaches for amyloid and tau proteins.

Immunochemical and imaging methods remain the most important approaches for detecting amyloid plaques and tau deposits in the brain, as they provide both spatial distribution patterns and quantitative information. Nevertheless, these methods are invasive, not suitable for routine use, and often

suffer from cross-reactivity (especially for tau antibody-based methods) and a high false-negative rate for low-abundance pathological protein. Bioassays indirectly measuring soluble amyloid and tau proteins in body fluids are convenient but generally have low specificity and are unable to discriminate between AD and other tauopathy diseases. With recent advancements in nanotechnology, chemical methods have emerged as promising alternatives that can directly detect deposited and soluble proteins, especially at trace levels, and rapidly screen samples with little sample preparation. Some approaches utilize nucleic acid probes or small synthetic molecules that specifically target amyloid and tau proteins based on their unique pathological conformations, while other methods rely on the development of highly specific antibodies for indirect measurements of concentrations in biological fluids [272, 273, 274, 275, 272, 273, 275, 274].

Advanced analytical tools for neurodiagnostics

Neurodegenerative diseases are characterized by the irreversible loss of neurons in specific areas of the central nervous system and associated with the manifestation of abnormal protein deposition. The clinical diagnosis relies primarily on neuroimaging or post-mortem examination. Advanced analytical tools with high sensitivity and specificity used to profile known and novel biomarkers for neurodegenerative diseases offer new avenues for clinical diagnostics. Focus has recently shifted to the early stage of such illnesses, and the use of body fluids other than cerebrospinal fluid is garnering greater attention. Proteomic approaches directed toward biomarker discovery have expanded rapidly, and combinations of several biomarkers are becoming more common in clinical practice. Other compounds, such as neuroinflammat-ory cytokines and chemokines, have also been recognized as being potentially useful in clinical diagnostics.

During the last decade, practical real-time detection methods based on fully commercialized assays have proliferated.

The core features of five major neurodegenerative diseases, together with the advanced platforms/tools employed in clinical diagnoses and those under development or validation, are summarized. Research areas compliant with the three key principles of 3 R^2 - replace, reduce, and refine - such as the application of saliva and urine as alternative biological materials continue to be of great research interest. The corresponding discussions center on the simplicity of use, low steadfastness and fidelity, cumbersome operation, high cost, and requirement of trained personnel involved in the diagnostic assays that could limit their wide applicability; the commonly-used neurochemical probes with great translational potential and their corresponding merits and limitations; and the ways of translating emerging research breakthroughs from the lab to bedside.

Protein-based biomarkers in neurological disorders

A variety of biomarkers in body fluids could aid diagnosis of neurological disorders. However, most other biomarkers are not specific nor sensitive enough to be used in most cases as substitutes of the commonly adopted image diagnostic techniques. The only clinically accepted biomarkers of dementia, presently, are the detection of elevated levels of β-amyloid and Tau proteins in cerebrospinal fluid (CSF).

For Alzheimer's disease (AD), the accumulation of insoluble aggregates enriched in specific proteins is a principal feature, so it is clear that such proteins could serve as targets or markers. During the last two decades, various chemical methods, using either directly-acting antibodies or antibodies conjugated to labels in non-directly-acting binding test formats, have been developed that allow monitoring the presence of amyloid β (Aβ), tau protein, tau or α-synuclein hyperphosphorylation by mass

spectrometry, and other post-translational modifications in AD or Parkinson's disease (PD) in human CSF, serum, and/or saliva at different stages of these neurodegenerative diseases. However, the specificity and sensitivity of the established tests are still under evaluation and the robust methods remain essentially limited.

Depending on the disease stage, concentration of certain proteins could be diagnostic indicators of mild cognitive impairment, and alterations of Aβ and Tau protein levels in CSF can precede, by several years, the clinical manifestation of AD. In recent years, the presence of proteomic alterations in these biological fluids associated with measurable impairment in smell recognition and emotion modulation further supported the hypothesis of an early prodromal stage of disease [276, 277, 278, 279].

Chapter - 17

Protein Analysis in Infectious and Inflammatory Diseases

Proteins present in both hosts and pathogenic agents can serve as valuable diagnostic markers. Molecular interactions between the proteins of the pathogen and those present in the organism providing the biological environment for the pathogen can provide insight into the presence of the pathogen. Moreover, studies on the proteins secreted into the extracellular space of the host during infections help diagnose, monitor disease severity, & even prognosis in certain infections. Inflammatory diseases offer a very different set of conditions for biomarker discovery. The inflammatory response generally consists of a plethora of molecular players acting in concert to minimize the damage caused by injury and restore homeostasis. Therefore, their presence in elevated or reduced amounts is associated with inflammation, injury, or disease resolution.

Detection and identification of the microbial proteins in the context of infectious diseases can follow any of the chemico-analytical proteomic approaches. In particular, the detection of the microbial pathogen by chemical methods on non-culture media has emerged as a focal AP in diagnostic microbiology. Rapid assays are being developed to detect the presence of the major pathogens known to cause pandemics. Molecular players in the infection process are also being targeted, and rapid, detection assays are being developed. In non-infectious diseases, inflammatory member proteins recorded to increase or decrease along the disease progress have been detected to aid prognosis or

to associate disease activity. In this context, rapid point-of-care tests have been suggested to track active SARS CoV-2 vaccination [280, 281, 282, 283].

Host-pathogen protein interactions

During pathogenesis, invading pathogens alter the protein profile of the host on a global scale to create a favorable environment for their proliferation. This results in the generation of numerous host proteins that specifically interact with pathogen-expressed proteins. Furthermore, re-translocation and shedding of the pathogen protein into the extracellular regions further portrays the possible interaction between host and pathogen proteins. Such interaction-based post-translationally-modified host proteins are highly happy markers for the early molecular diagnosis of the disease. Therefore, determining precise chemical strategies for the rapid detection of such proteins with high sensitivity at the early-stage molecular level is crucial for combating the disease by enabling early diagnosis and preventive therapy.

Infectious diseases are mainly caused by the presence of bacterial, viral, or fungal colonies in the host that interfere with normal physiological functions. Detection of such microbial infection is a serious issue for medical diagnosis because premature or false-negative prediction leads to increased numbers of deaths worldwide. To address these challenges, approaches that detect proteins in the host generated by the host-pathogen interaction have gained traction. Various strategies have been developed for the chemical detection of tumor-, virus-, or bacteria-associated proteins that play important roles in detection and monitoring of these infectious diseases. Host serum profiling using mass spectrometry, combined with a set of noninvasive animal models, can generate a panel of biomarkers associated with the disease [284, 285, 286, 287, 288, 284, 285, 286, 287, 288].

Chemical identification of microbial proteins

Detection of pathogenic proteins in infected biological fluids provides precise diagnoses supporting timely, effective treatment decisions. Chemical methods are particularly suitable given the rapid detection required for patients with suspected pneumonia or sepsis. For pneumonia, fast detection of nucleic acid sequences unique to and essential for virulence in targeted groups would serve as biomarkers. For sepsis, direct identification of proteinaceous virulence factors could help distinguish between infection and other causes of sepsis-like presentation.

Gram-negative bacteria secrete toxic lipopolysaccharides into host blood, producing an inflammatory response that can aid diagnosis. For severe bacterial infections, like acute bacterial meningitis, measurement of pleocytosis in coupled with IL-6 concentration in cerebrospinal fluid provides additional information. Detection of the lipopolysaccharide-binding protein or antibody response to immunogenic proteins can be considered. Detection of Hla in pleural effusions and of K1 capsule polysaccharide in peripheral blood can also be useful. The major challenge remains the low predictive value of a negative result.

Inflammatory protein biomarkers

Markers of inflammation derived from proteins constitute a significant, established group of clinically relevant indicators of disease activity. They also show prognostic value in the intensity and course of several diseases, including mental health disorders such as major depression. A selection of those markers is now listed.

The amount of C-reactive protein (CRP) in blood serum is a routinely determined inflammatory protein and is a good parameter for assessing general inflammatory activity. Its induction is a well-known inflammatory response pathway in reaction to many proinflammatory signals. An increase of CNS-

associated chemokines (C-X-C and C-C motifs) detected in serum was reported to predict the development of major depression; IL-6 has been associated with elevated depression and anxiety levels in individuals with chronic abdominal pain; and IL-1b levels correlate positively with anxiety and depression levels in somatic patients. Moreover, concentrations of proinflammatory cytokines (i.e. TNF-α, IL-6) were shown to discriminate between major depression and schizophrenia patients. Evidence suggests that the alteration of IL-1β, IL-6, and TNF-α concentrations might indicate the presence of either current or past depressive episodes [289, 290, 291, 292, 293].

Rapid diagnostic assays for infectious diseases

Rapid diagnosis of infectious diseases is becoming increasingly important in light of rising antibiotic resistance and the increasing number of emerging pathogens. Ideally, a diagnostic test will have the assay speed and cost of an immunoassay but will be as specific and reliable as mass spectrometry. There are several challenges to achieving this goal, and the molecular composition of analytes must be critically considered: some analytes are produced in excess during infection, whereas others are observed at low levels and require enrichment for detection.

Polymerase chain reaction (PCR) assays are among the fastest methods available for the detection of infectious diseases. Clinical PCR testing is highly sensitive and specific but still requires dedicated laboratory facilities and trained staff. Rapid tests that do not require these prerequisites, for example, LAMP assays, are in high demand for point-of-care applications. However, speed and low cost must not be achieved at the expense of reliability. Rapid immunoassays for biomarker detection are popular for their simplicity and low cost, but they may display low specificity, especially in the case of antigen detection. Rapid

colourimetric tests also likely suffer from similar limitations. Speed, reliability, and cost must be weighed, particularly for tests that are designed for deployment in low-resource settings.

Biosensors are able to combine features from both rapid antigen-immunoassays and PCR tests, being simple, portable, sensitive, and selective. Electrochemical and optical biosensors based on enzymatically catalyzed mechanisms are common; however, other chemistries exist, such as proximity-based strategies. Their clinical translation still demands careful selection of assay conditions, assay format, assay components, and their usability in non-dedicated laboratory settings [294, 295, 296, 297].

Chapter - 18

Future Perspectives and Ethical Considerations

Advanced Chemical Compounds and Analytical Techniques for Molecular Protein Analysis in Disease Diagnosis

Emerging chemical compounds for addressing individual analytical steps, sample preparation, and detecting probe-target interactions, either tagless or labeled, hold significant promise. Game-changing electrochemical sensors (especially point-of-care) and next-generation microfluidic devices are approaching practical maturity. Nevertheless, the routine application of any new diagnostic system—microfluidic, lab-on-a-chip, or portable electrochemical sensor—requires political as well as chemical action, particularly with respect to the analytics on their sensing surfaces. When von Behring and Kitasato demonstrated in 1890 that animal-serum constituents could block tetanus and diphtheria infections, the field of immunology became embroiled with public health concerns because diphtheria antitoxin had immediate therapeutic relevance.

Clinical translation of any new proteomic-screening technology faces an even harder road than development of the technology itself. Emerging diagnostic platforms (informed by basic clinical proteomics studies)—such as those based on the chemical enrichment and quantification of low-abundance or tissue microenvironment-specific proteins or sensitive detection of cancer- or neurodegeneration-associated PTMs or protein isoforms—must be clinically validated in order to gain acceptance by the Medical Devices and Clinical Laboratory Improvement Amendments regulators.

For both social and economic reasons, the gap between initial development of a diagnostic technology and its incorporation into everyday medical diagnosis is vast. Rapid direct detection systems, such as those for HIV, TB, and malaria (the latter two using immunochromatographic assays), have been the exceptions. However, demonstrating reliable non-competitive mid- to high-dimensional medical diagnosis on ordinary patients clearly represents the ultimate goal (with proper validation) [298, 299, 300, 301].

Emerging chemical compounds for protein diagnostics

The past decade has witnessed the emergence of many innovative chemical compounds that are significantly enhancing analyte-chemical reagent interactions, broadening the spectrum of accessible information, or increasing throughput and speed within diagnostic workflows. These advances support the creation of next-generation devices and afford fresh opportunities for the detection of more challenging analyte classes, such as small-molecule drugs and their metabolites.

Affinity reagents—including antibodies, anticalins, affibodies, and aptamers—remain the backbone of molecular analysis in proteomics, seromics, and immunodiagnostics. Small-molecule probes for the specific detection of proteins in live cells and organisms have appeared on the market, enriching the chemical reagent arsenal. The application of chemical tags and labeling reagents has opened new horizons in molecular detection, allowing an immense variety of chemical bioorthogonality reactions to be utilized without labeling bias. The development of ultra-sensitive nanomaterial-enhanced biosensors has enabled multiple chemical classes to be detected, not just proteins, and has led to the introduction of point-of-care biosensor systems. Considering the rapid evolution of chemical compounds for protein diagnostics currently underway, a timely

overview is warranted, with particular emphasis on reagents that informatively interact with proteins and represent significant progress in the field [302, 303, 304, 305].

Next-generation analytical technologies

The expansion of electrochemical biosensors, novel nanomaterials, lab-on-a-chip devices, microfluidics for sample processing, and other emerging analytical platforms for convenient, rapid protein testing is sparking a transformative wave of chemical innovation for protein diagnostics. Future strides will include improved detection capabilities and the introduction of new features within these technologies through integration into more complex multi-dimensional analytical systems, alongside the increasing incorporation of data-processing tools based on chemometrics, pattern recognition, machine learning, and artificial intelligence—processes that help analysis in large amounts of information. Nevertheless, the road from prototype to commercial product remains long owing to practical hurdles. Improvements in biosafety, fit for purpose, reliability in clinically relevant samples, readiness for actual clinical use in terms of ease of use, turnaround time, and cost are being addressed as part of translation for localized, rapid infection diagnostics.

Many of these systems are also likely to exist as point-of-care clinical devices, allowing rapid responses directly in the hospital using samples from already clinical institution, thus reducing the logistical effort of sending samples to a highly certified laboratory and waiting for the results. Nevertheless, POC systems are only interesting for specific questions; a proven, certified laboratory will still be the best place to get a reliable answer, with these fast POC systems being complementary to a certified laboratory [306, 307, 308, 309].

Regulatory and ethical challenges

The rapid development of next-generation technologies for the analysis of human proteins heralds a new era of innovation. However, many of these approaches remain largely confined to research laboratories and have not yet made a transition towards reliable clinical practice, in large part due to regulatory issues. Diagnostics based on the analysis of human proteins will ultimately require approval from regulatory agencies that are responsible for the safety and effectiveness of medical devices, but such systems assess only the analytical performance of the assays. An additional difficulty lies in the need for the rapid and simultaneous detection of multiple bacterial proteins involved in the diagnosis of infectious diseases such as sepsis. Although the use of standardised processes and approved human proteins for evaluation and validation can mitigate such risks, diagnosis remains difficult.

Finally, as research continues to uncover the potential of other human proteins as specific or predictive biomarkers for diverse diseases and conditions, care must be taken to avoid careless and unregulated commercialisation. It is vital that the development and clinical application of such assays be performed at the highest level both scientifically and ethically [19, 310, 17, 16, 16, 310, 17, 19].

Translation of protein diagnostics into clinical practice

A broad range of protein-based reactions has the potential to yield new diagnostic devices, albeit primarily in pre-clinical stages. Users expect rapid results—ideally within minutes—combined with simple operation, a moderate price, and demonstrable accuracy compared to conventional methods. Tunable and specific signals are desirable, and they may benefit from the rapid development of nanomaterials. However, achieving such devices for all infectious diseases is unrealistic, necessitating a balance between reliability and speed.

It is also crucial to consider the end-user, e.g. the medical and nursing staff, who will be handling the application. Their use-case will differ between an emergency unit and a laboratory; in the former, simply using the assay is a priority, while in a hospital laboratory, both speed and reliability are important, but making a mistake would generally remain acceptable. In clinical analysis laboratories, reliability is critical, with cavernous sea yaoud extremely high and in routine use—that is, with clinical need is so great that the detection technology enters into very high volume. In such circumstances, a reliable technology with a longer analysis time may be deemed preferable to fast but unreliable operation.

Conclusion

The material presented highlights recent discoveries on the application of new chemical compounds in protein detection and diagnostics, along with the analytical techniques capable of supporting these explorations. Progress in this area is novel and stimulating, with extensive implications for a variety of human diseases. Laboratory-scale prototyping serves to demonstrate the relevance of the concepts; however, upscaling, clinical validation, and translation remain open challenges for the evaluated biosensing technologies.

The role of chemical proteomics in disease detection continues to expand, driven by the growing arsenal of innovative chemical compounds and the underlying diversification of proteomic profiling and protein biomarker discovery. Biological systems control the activity of proteins by assembling specific interaction networks and modulating the formation of transient complexes. Research has revealed that these protein-protein interactions are not only controlled by thermodynamic affinity but also by chemical homeostasis, with local concentration and chemical composition governing the extent of assembly. Investigating such aspects may ultimately allow the detection of very weakly or transiently associated proteins in living systems.

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