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Proteins bioassay: recent scenario for new target validations

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Abstract- This article reviews different aspects of protein engineering technologies such as protein screening, NMR, EM & x-ray crystallography, In silico protein structure, protein cell free technologies, protein chips and arrays, custom peptide synthesis as well as nanoscale / microscale separation or purification methods briefly and where they are being used to solve protein queries.
Keywords- Boiassay, protein lysate microarray, Cell free technology, NMR, X-Ray cryastallography

Introduction

Consistently high quality protein production demands a need of robust purification process, especially when scaling up to large quantities. Purifying and recovering proteins is typically viewed as a time-consuming bottleneck in commercialization. Now a day's numbers of experimental methodologies are available for solving the three dimensional structure of protein interactions which are X-ray crystallography; Nuclear Magnetic Resonance (NMR) spectroscopy; electron microscopy and electron tomography. Rapid determination of protein folds also has become attractive in recent years as it can quickly yield structural information for proteins, providing evolutionary insights and clues to biochemical functions [1]. The X-ray structure technology provides a wealth of information on how to interpret past experiments and how to design new experiments that will provide information about the function of the protein, but does not reveal much information regarding the physical characteristics of the protein [2]. X- Ray crystallography and NMR are two methods which are basic and critical in the field of protein structure determination. The strengths and weaknesses of one of the two methods are of those kinds which supplement the holes and gaps in the other method to make it possible that different kind of important data for a structural question can be answered with the parallel or supplemental application of the two methods. But the output of experimentally determined protein structures by time-consuming and relatively expensive X-ray crystallography or NMR spectroscopy is lagging far behind the output of protein sequences. Identification of proteins of interest from a particular biological study requires the application of bioinformatics tools to process and prioritize the data. From a protein function standpoint, transfer of annotation from known proteins to a novel target is currently the only practical way to convert vast quantities of raw sequence data into meaningful information [3]. The goals and approaches of computational molecular design and protein sequence analysis provide tools for the rational mutagenesis and functional modification of proteins. These approaches are used for analysis of the three-dimensional structure of a protein to guide the selection of appropriate amino acid sequences to create desired properties or functions [4]. Another technology known as protein chips are disposable arrays of microwells in silicone elastomer sheets placed on top of microscope slides [5]. A protein microarray, sometimes referred to as a protein binding microarray, provides a multiplex approach to identify protein-protein interactions, to identify the substrates of protein kinases, to identify transcription factor protein-activation, or to identify the targets of biologically active small molecules. The array is a piece of glass on which different molecules of protein or specific DNA binding sequences (as capture probes for the proteins) have been affixed at separate locations in an ordered manner thus forming a microscopic array . Protein microarrays have the potential to dramatically increase the throughput of proteomic analysis and allow the high-throughput analysis of biochemical activities There are several types of protein chips, the most common being glass slide chips and nano-well arrays Glass slides for protein chips offer a low fluorescent background, superior handling properties, require small screening volumes and are suitable for automation [6]. Applications of protein chip experiments include identifying biomarkers for diseases, investigating protein-protein interactions, and testing for the presence of antibodies in a sample and also find applications in cancer research, medical diagnostics, homeland security and proteomics. Upcoming technology known as Cell free protein technologies are now becoming an increasingly attractive alternative to conventional in vivo expression systems, especially when parallel expression of multiple proteins is required.[7]. They reduce complexity, remove structural barriers, and do not require the maintenance of cell viability. But cell-free systems are limited by their inability to co-activate multiple biochemical networks in a single integrated platform.[8]. CFPS has now been converted from an expensive laboratory method into a potentially disruptive technology for the development and large-scale production of biopharmaceuticals and

vaccines. Also Nanoscale and microscale separation and purification methods are recently major topics of research for protein studies.

Protein screening

Protein detection is a common yet time-intensive task in many laboratories. Conventional Y2H (yeast two hybrid) assays are not well suited for high-throughput screening of the protein interaction network ("interactome") due to several limitations, including labor-intensive agar plating method and colony selection processes associated with the use of nutrient selection markers, complicated reporter analysis, and incompatibility of the liquid handling robots [62]. Phage and ribosome display technologies have emerged as important tools in the high-throughput screening of protein pharmaceuticals. The implementation of such tools is the need to purify large numbers of proteins for screening. Several methods for small-scale, high-throughput protein purification have been evaluated of which the use of a 96-well-based method facilitates the purification and subsequent screening of large numbers of fusion proteins [9]. For large-scale screening of expressed proteins Microwave-assisted dot blotting is suggested as an effective way [10]. Microwell refolding has been calibrated for scale-up with an initial model protein, allowing researchers to implement and compare several assays for protein refolding, including turbidity, enzyme activity, and chromatographic methods, and assess their use for microwell-based experimentation [11]. Researchers developed a GFP-based, genome-wide screening method for the identification short-lived proteins [12]. GFP, the green fluorescent protein from the jellyfish *Aequorea victoria*, which has been widely used to monitor gene expression and protein localization. An efficient, high throughput compatible method based on photometric turbidity measurements that allows a gentle and controlled concentration of the protein solution while constantly analyzing the solution state is developed and also acts as a basis for the determination of process relevant solubility phase diagrams and analysis of kinetic effects during precipitation [61].

Bioassay and Discovery

In vitro bioassay is very useful in biomedical experiments as various protein involvements in both normal and aberrant physiology create a need for rapid, sensitive and methodologically simple assays to evaluate their activity from a variety of biological samples [13,64]. Previously alkaline phosphatase based assays, ELISA and luciferase based bioassays have been used to evaluate certain individual or total protein activity. A method of rat bio-assay for the evaluation of the nutritional quality of proteins and protein digests has been described in which the assay animals are subjected to a state of protein depletion, a condition created which simulates that present in human patients requiring dietary protein therapy [14]. The mouse bioassay is sensitive and robust and does not require specialized equipment. However, the mouse bioassay is slow and not practical in many settings, and it results in the death of animals too. So scientist describes an in vitro cleavage assay for SNAP-25 (synaptosome-associated proteins of 25 kDa) for measuring the protein activity such as toxin activity with the same sensitivity as that of the mouse bioassay [15]. Recent advances in the in vitro assay technologies includes protein misfolding cyclic amplification and immunoassays which have made it possible to detect infectious agents such as prions in the blood of animals and even of asymptomatic animals infected with prions [16]. Researches now focuses on the development of the various in vitro bioassay systems for the rapid amplification and detection of infectious agents (prions) that might be present in blood and tissues for blood transfusion and transplantation recipients. The combination of bioassay and phage display technologies is a powerful tool in the study of many uncharacterized proteins that conventional approaches fail to do [63].

NMR

Nuclear magnetic resonance is extremely useful for analyzing samples non-destructively.A combination of electrophoretic NMR and diffusion NMR experiments can measure the average charge carried by ions in solution. This average charge can be used as a quantitative indicator of ion pairing [17]. Stereo-array isotope labeling (SAIL), when combined with the fully automated NMR structure determination algorithm FLYA we can determine the three-dimensional structure of the protein from different sets of input NMR spectra [18]. A high-throughput proton (1H) nuclear magnetic resonance (NMR) metabonomics approach is used to characterize systemic metabolic phenotypes. NMR data can also be used to reveal associations between systemic metabolic phenotypes and the metabolic syndrome (n = 4407) [19]. Another method Isotope-aided multi-dimensional NMR technique enables observations of conformations and functions of proteins in living cells at the atomic level and also provide information on protein-ligand interactions and conformations [20]. High-resolution NMR is also used for investigation of membrane proteins and membrane-active peptides embedded into lipid-protein nanodiscs (LPNs) [21]. A new labeling scheme is introduced which facilitates the measurement of accurate chemical shifts of invisible, excited states of proteins by relaxation dispersion NMR spectroscopy.[22]. NMR enables structural studies of small proteins that are partially disordered, exist in multiple stable

conformations in solution, exhibit weak interactions with ligands , or often fail to crystallize readily [69]. Various expensive biological samples, such as nucleic acids, including RNA and DNA, or proteins, can be studied using nuclear magnetic resonance for weeks or months before using destructive biochemical experiments which makes nuclear magnetic resonance a good choice for analyzing dangerous samples. NMR technology is a highly complementary approach to X-ray crystallography method for protein structure determination [70,71]. Many proteins that provide good NMR spectra have not been successfully crystallized [45].

Electron Microscopy

The imaging methods employed should provide unique information about the object under study and the combined correlative data if used should be more informative than that obtained by any of the imaging methods alone [65]. Most commonly fluorescence and electron microscopy are combined in correlative microscopy. A technology is used to quantify the chemical composition of atomic columns using high angle annular dark field (HAADF) scanning transmission electron microscopy (STEM) images and this method is based on a quantification of the total intensity of the scattered electrons for the individual atomic columns using statistical parameter estimation theory [23]. For some difficult-to-fix samples and for optimal preservation of ultrastructure in samples which are larger than a few micrometers, high-pressure freezing was the method of specimen preparation for electron microscopy. Light microscopic observations are completely sample dependent, but the choice of high-pressure freezer depends on the speed required to capture (freeze) the biological event of interest [24]. Studies suggested the use of ILEM (integrated laser electron microscopy) for identification of early morphological markers of apoptosis and their further analysis at high resolution by TEM (transmission electron microscopy) [25]. Local crystal structure analysis has been done based on annular dark-field (ADF) imaging in scanning transmission electron microscopy (STEM) [26]. The characterization of nonmaterial's with complex three-dimensional (3D) geometries can be carried out using scanning transmission electron microscope (STEM) or transmission electron microscope (TEM) [27] Also SEM assesses the effect of drugs on crystal growth morphology and tissue histology [68]. Phase contrast electron microscopy can be effectively used not only for weak phase objects but also for strong phase objects in biology. For weak phase objects phase contrast utilizes phase plates suitable for high-contrast observation [28]. The Fluorescence microscopy allow to study processes in living cells with specific labels and stains that follow the movement of labeled proteins and changes within cellular compartments but does not provide sufficient resolution to define the ultra structure of intracellular organelles [66]. The cryo-EM maps that are generated from protein studies can be used as viable models for MR(molecular replacement) solution of X-ray crystal structures [67]. The main disadvantage of Electron microscopes is that expensive to build and maintain and samples largely have to be viewed in vacuum.

X- Ray Crystallography

X-ray crystallography technique is an indispensable tool for studying the structure of proteins and other macromolecules as the importance of proteins continues to grow, in fields of biochemistry and biophysics to pharmaceutical development and biotechnology. Principles of Protein X-ray Crystallography provide the theoretical background necessary to understand how the structure of proteins is determined at atomic resolution. When an X-ray beam bombards a crystalline lattice in a given orientation, the beam is scattered in a definite manner and characterized by the atomic structure of the lattice. This technology can provide us with the 3-D structure of a protein from which we can often predict the protein's molecular function (of its biochemical and biophysical roles)," and these structural predictions provide us with a good lead towards identifying the protein's cellular function and how it is networking with other proteins." A major step toward the protein structure determination by nuclear magnetic resonance (NMR) spectroscopy is the assignment between multidimensional NMR signals that provide through-bond and through-space inter-atomic correlations [29]. This principle when incorporated into conventional three-dimensional (3D) heteronuclear NMR experiments, allows the retrieval of additional frequency correlation information at high resolution. This approach gives increased accuracy and size of protein structures determined by NMR.eg .Recent study has been carried out to screen the FOL (fragments of life) library against leukotriene A4 hydrolase (LTA4H) by X-ray crystallography [30]. Also X-ray crystallographic data could greatly assist the design and development of various inhibitors [32]. For e.g., The renin-angiotensin-aldosterone system (RAS) cascade is a major target for the clinical management of hypertension but the development of renin inhibitors has proven to be problematic because of poor bioavailability and complex synthesis. This enzyme is a promising target for the development of novel treatments for hypertension so x-ray crystallography solves this problem by greatly assisting in the production of rennin inhibitors.

Protein Chips and Array

Protein chip technology is essential for high-throughput functional proteomics [33]. Protein microarrays are now becoming very popular due to their possible future applications in the study of nucleic acid-protein, protein-protein, ligand-receptor, drug-protein target, and enzyme-substrate interactions. Monoclonal antibodies have been the prime choice as protein capture agents for the majority of protein chips developed to date. New technologies for the production of protein capture agents are more amenable to automation than traditional monoclonal antibody production and therefore carry the promise for industrialization. A new method for the generation of protein microarrays on coated glass slides with a high sensitivity for antibody screening and serum profiling has been developed. Combining cDNA expression libraries and robot technology, 2,413 different human proteins have been expressed, purified and spotted onto glass chips. A modified fabrication process of protein chips is developed which uses self-assembled monolayer in the fabrication of protein chips [34]. Researches utilized high-throughput protein chips to profile twenty-nine inflammatory cytokines around failed total joint replacements study [35]. In another approach a novel protein tags consisting of five tandem cysteine repeats (Cys-tag) at termini of proteins have been constructed. This gives highly sensitive immunodetection method due to the strong covalent binding of the Cys-tag to the substrate combined with efficient exposure of the protein to the surrounding solution. Thus, the Cys-tag should be useful for developing a novel protein printing method for protein chips that requires very low amounts of protein and can be used for high-performance analysis of protein-ligand interactions [36]. Protein expression profiling chips with distinct spots of immobilized protein capture agents allows the simultaneous measurement of hundreds to thousands of proteins from one sample [37]. With analysis of large sample numbers, identification of disease associated proteins to generate novel diagnostic markers may be possible. Scientists developed tiling array technology, which has been previously used for ChIP-chip assays [57] and transcriptome analyses [58], to protein-interaction analysis along with an in vitro display technique for the first time. This new method can be used to detect targets expressed at extremely low levels and can also be used in combination with other display technologies, such as phage display and ribosome display [59]. Recently printing expression ready plasmid DNAs onto slides can be converted into protein arrays on-demand which serves dual purposes as they not only direct the synthesis of the protein of interest but also serve to capture the newly synthesized proteins through a high affinity DNA-protein interaction. In this way all DNA microarray can be converted to a protein microarray on-demand without any need for additional capture reagent [60]. LMA (protein lysate microarray) because of its sensitivity, reproducibility, and high-throughput quality make it a potentially powerful technology for verification of the presence and quantification of specific protein markers in clinical samples where the sample amounts are limiting [38]. Protein chip technology is useful for high-throughput screening of protein biochemical activity with only small amounts of protein requirement [39].

Protein structures *in silico*

In the field of protein design there is major shift recently towards the use of unbiased in silico methods. These methods focuses on structural modeling of candidate protein sequences to asses the probability of particular sequence taking the desired protein structure [40]. The optimization based technology and its general tools has allowed widely different protein types to be designed and stabilized. New bioinformatics tools provide more sophisticated methods to transfer functional annotation, integrating sequence, family profile and structural search methodology [41]. In one of the approach a two stage evaluation procedure was developed, one of which the sequence - based method assessed the conservation of protein interface patches used in the original in silico prediction method, both in terms of position within the primary sequence, and in terms of sequence conservation. And another one involved in a high-throughput structure-based docking evaluation procedure assessing the soundness of three dimensional models produced for the putative interactions [42]. Rational engineering methods are now applied with reasonable success to optimize physicochemical characteristics of proteins such as antibodies. Recently scientists proposed a structure-based rational method to accelerate the development of affinity-matured antibody constructs with enhanced potential for therapeutic use [43]. The convergence of low-cost, high-speed computers, a tremendous increase in protein structure information, and a growing understanding of the forces that control protein structure has resulted in dramatic advances in the ability to control protein function and structure and led to create the first truly artificial proteins [44]. Stochastic sequence alignment methods defines a posterior distribution of possible multiple alignments. They highlight the most likely alignment, and they can give posterior probabilities for each alignment column. Such alignment posterior probabilities correlate with the reliability of secondary structure predictions, though the strength of the correlation is different for different protocols [45]. Although

computational algorithms have been developed for calculating everything from the dynamics of a protein to its binding specificity, only limited information is available on the ability of these methods to give accurate results when used with a particular X-ray structure [2].

Cell free protein technologies

Cell-free transcription and translation systems provides an open, controllable environment for production of correctly folded, soluble proteins and allows the rapid generation of proteins from DNA without the need for cloning [7]. Limitations of cell-based expression systems for protein production can be circumvented by the use of cell-free translation systems [31]. Novel design and exploitation, powerful cell-free technologies of ribosome display and protein in situ arrays technologies can be combined for rapid detection of protein interactions. They offer lower costs to facilitate production of protein therapeutics and biochemical's that are difficult to make in vivo because of their toxicity, complexity, or unusual cofactor requirements [47]. Improvements of cell-free reaction mixtures, including new ways for efficient energy generation, have an additional impact on progress in cell-free protein synthesis technology [48]. The primary energy source ATP is regenerated from the secondary energy source through substrate phosphorylation in CFPS(cell free protein synthesis). Distinct from common secondary energy sources (e.g., phosphoenolpyruvate - PEP, glucose-6-phosphate), maltodextrin was used for energizing CFPS through substrate phosphorylation and the glycolytic pathway [49] Rapid production of solubilized and functional membrane protein involves simultaneous cell-free expression of an apolipoprotein and a membrane protein in the presence of lipids, leading to the self-assembly of membrane protein-containing nanolipoprotein particles [50]. CFPS can now be practiced at any scale using conventional bioprocess equipments and allows the generation of functional proteins that are difficult to produce by in vivo expression systems [51]. Continuous cell-free translation systems with perpetual supply of consumable substrates and removal of reaction products made the process of in vitro synthesis of individual proteins sustainable and productive [48]. Limitations in amino acid supply are recognized as a substantial problem in cell-free protein synthesis technology [46].

Microscale and nanoscale separation and purification

Miniaturizing protein purification processes at the microliter scale (microscale) holds the promise of accelerating process development by enabling multi-parallel experimentation and automation [52]. A small-scale version of line immunoelectrophoresis in combination with immunoprecipitate excision is developed as a rapid and convenient technique to purify proteins on a micro scale in various studies. The method also allows a simultaneous purification of several different protein antigens from the same sample, so it can be used as an alternative method to other procedures in the purification of proteins on a micro scale [53]. The cell disruption technique in combination with microscale chromatographic methods for protein purification enables to develop a strategy for the rapid process development of intracellularly expressed proteins.

Custom peptide synthesis

Chemical synthesis provides custom-made peptides in small quantities, but production approaches based upon systems such as transgenic organisms might be more cost-effective for large-scale peptide production so self-assembling peptide-based biomaterials are being developed for use as 3D tissue engineering scaffolds and for therapeutic drug-release applications [54]. Previously the discovery and development of a class of self-assembling peptides made of only natural amino acids and can undergo spontaneous assembly into well-ordered nanofibers and scaffolds. These peptides have been used for the various studies of cell attachment, survival and proliferation [55]. Designed peptide nanofiber scaffolds offer several advantages which include 1) easy design using the known, biologically active motifs, 2) commercially custom-synthesized with mature solid phase peptide synthesis technology 3) selection of an extensive repertoire of biological active motifs detected in some extracellular matrix components and cell secreted peptides or proteins. Specific microparticles that are addressed to the polymer-coated microchip surface in a well defined pattern releases preactivated amino acids upon melting, and thus allow combinatorial synthesis of high-complexity peptide arrays directly on the chip surface [56].

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