Protein arrays: applications and implications in neuroscience

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PROTEIN ARRAYS: APPLICATIONS AND IMPLICATIONS IN NEUROSCIENCE

Summary. Introduction. *Genomics is bringing great advances into biomedical research that could help us to understand the physiopathology of diseases, and to find the features that differ disease from health. To understand the development of a given pathology, a global study is necessary that includes apart from the morphological and genomic study, analysis of protein interactions, the modifications that suffer their structure and even the changes in their concentration, as they are key molecules in the structure of the alive beings and in the execution of the biological processes.* Development. *With the purpose of studying these aspects different technological boardings have been developed, protein arrays among them. These incorporate different methodologies, many of them are appropriate to detect postranscriptional modifications, and other ones are optimized for protein quantification. In this review different methodological boardings and the possible applications of those arrays are discussed. This tool is adapted for the comparative study of protein expression profiles of different structures and metabolic situations. The aim is to consider protein arrays like a tool to study the proteome in neuroscience.* Conclusions. *After validating biomarkers of clinical relevance discovered using protein arrays, this technology is a promising step in the road toward the development of early diagnosis protocols. To be able to validate the results, technological advance must be highlighted and an international consensus must be reached in different methodological aspects. These aspects will include normalisation and quantification of the signal produced. [REV NEUROL 2007; 44: 285-90]*

Key words. *Antibody/protein array. Biomarker. Neuroscience. Protein analysis. Protein profile. Proteomic.*

INTRODUCTION

Once the human genome has been completely sequenced, we should be able to analyse the molecular bases of the majority of biological processes. Experimental data from diverse sources indicate a lack of correlation between the expression of messenger RNA and the corresponding transcription into proteins, since the activity of many of these is affected by post-transcriptional modifications [1] such as phosphorylation, glycosylation, acetylation and proteolysis [2-4].

Modifications in gene expression are the base of some diseases, as these alterations can lead to changes in protein synthesis. Recently, different methods –serial analysis of gene expression (SAGE) and c-DNA microrrays) have been used to study gene expression in several pathological processes (cancer, neuro-degenerative diseases, etc), making significant contributions to our understanding of the physiopathology of these processes [5].

Microarray technology was developed in the mid-eighties by Ekins et al [6]. The first application for which they were developed was immunodiagnosis [7], given its relevance at that time and the detection sensitivity demand. Therefore, the key concept of microarray technology is to provide high sensitivity, starting with a small number of molecules anchored to a solid support, and the same concept is applicable to protein arrays.

Since then, many publications have referred to this technology, its diverse applications and its impact on medicine. The presence of mutations, deletions and other genetic modifications are of great interest in the understanding and preventing disease, in the identification of risk groups and eventually in the design of new therapeutic strategies for specific diseases. The information contained in our genes is fundamental, but it is only influenced by a noxa when it is transcribed into proteins. Different circumstances can influence this process, hindering it or even preventing it, in which case the protein will not carry out its intended activity. As a result, although a genomic profile can help to predict an individual predisposition towards a particular disease, only knowledge and quantification of protein expression profiles can help us to understand the real participation of each molecule or group of associated molecules in a given disease. Such studies will be of incalculable help in making exact diagnoses, selecting treatment guidelines and establishing prognosis.

Protein arrays provide the necessary technology to study interactions between proteins, and also post-transcription modifications of a protein (phosphorylation, glycosylation, etc).

Interactions between proteins or between proteins and non-proteinaceous biological molecules play a crucial role in all cellular processes, both physiological and pathological. Consequently, the factors involved in these interactions are of special interest in the design of diagnosis and therapeutic strategies.

The importance of molecular characterisation has increased the need of high throughput quantification tools of proteins at low cost, using only a tiny sample [8], in order to complement the information provided by functional genomics.

Protein arrays have great possibilities for becoming a tool for the characterisation of protein extracts, just as c-DNA microarrays already are for oligonucleotides [9].

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DEVELOPMENT

One of the methods currently available for the study of protein expression is bi-dimensional electrophoresis [10,11] a costly technique in both time and money, that has limited reproducibility. Even using normalised polyacrylamide gels and combining this technique with mass spectrometry, it can only detect the more abundant proteins [12]. These methodological problems are encouraging increased efforts in the search for new technologies applicable to this field.

For example, Gygi et al [13] have described a protein quantification process for complex mixtures denominated ICAT (isotope-coded affinity tags), using a method in which two samples are mixed with reagents with differing molecular weights, which when purified, are separated using affinity chromatography and analysed by using mass spectrometry to identify differentially-expressed proteins. Another method is the reverse-phase ProteinChip ® [14] in which different surfaces are used to absorb proteins which are analysed using SELDI (surface-enhanced laser desorption/ionization) mass spectrometry [15]. This combination of techniques is widely used to identify and characterize the proteins in a sample. Good results are obtained using this technique as regards sensitivity and reproducibility, although its ability to obtain a global protein profile of the samples is limited [16]. It is precisely for this purpose that protein arrays have been proposed [14,17], and they are also most useful in the study of interactions between proteins and of their enzymatic activity [18], both of which are high medical potential applications [19].

The initial trials revealed several difficulties in the generation of these arrays, due fundamentally to the biophysical and chemical properties inherent in proteins. These are very heterogeneous, due to post-transcriptional changes, and it is difficult to design a general detection system that can properly distinguish between different proteins. Moreover, many extraction and analysis techniques denature proteins, making their detection by protein arrays impossible. In the specific case of detecting antigen-antibody complexes using antibody arrays, the required bonding specificity is an important factor in detection.

Classification

MacBeath [20] distinguishes two types of applications for protein arrays, depending on the object of the study: quantitative proteomics and functional proteomics. In the first type, the objective is to carry out a complete protein analysis of the sample, studying the quantity, modifications, activity, localisation and interactions of the proteins in the sample. Present technology limits this method to the analysis of a couple of the parameters cited above. Different authors have given different names to the same method; while Kodadek [21] calls them 'protein detection arrays', in which the materials anchored onto the different surfaces are specific ligands for proteins in the complex samples being studied, such as cell lysates, Lueking et al [22] calls it 'discovery-oriented proteomics', since no specific result is anticipated, rather, one hopes to make discoveries in the results that are obtained.

The first trials using this method sought to reduce the laboriousness of biochemical and immunological techniques. Mendoza et al [23] designed an antibody array on a 96 well plate, with 144 elements in each well, for a conventional ELISA/sandwich procedure (Figure, a). Similar arrays have been used for different applications [24,25]. Other attempts were made to use filter membranes as anchor substrates, given their bonding capacity [5]; using this approach, Huang et al [26] designed a detection array for serum cytokines. It is no coincidence that the majority of these designs are aimed at the detection and quantification of cytokines; cytokines are released by cells, and their detection is far easier than detection of cytosolic proteins [20]. In fact, only five per cent of commercial antibodies tested by Nielsen et al [27] are valid for the study of cellular lysates based on arrays.

Included in quantitative proteomics there is a type of array called an 'antigen capture test' (Figure, b). This does not require the use of a secondary antibody [8]. These arrays are aimed at discovering new markers, rather than for quantitative analysis itself.

A third approach to array design, which consists of immobilising the sample itself on a solid substrate [16] is called 'direct testing' (Figure, c). The sample to be attached can be obtained from different sources, from serum or plasma, cerebrospinal fluid, cellular lysates, etc. This increases the specificity of the test, since the sample under study has not previously been manipulated.

The main aim of the second application, functional proteomics, is to define the function of each protein in the sample. In this type of array, each protein occupies a spot (anchor point) in order to study its activity in the natural state [21]. The only advantage of this application over quantitative proteomics is the possibility of studying interactions between proteins and non-proteinaceous types of molecule, such as nucleic acids, lipids and small organic compounds [18]. This has also been called 'functionality-oriented proteomics' [22].

Antibodies

Antibodies are the most appropriate molecules for discovering target proteins, since they possess the specificity required to identify a particular epitope. Given their characteristics, there are many factors that must be taken into account in order to achieve optimum recognition between the antigen and the antibody. Although antibodies recognise specific epitopes, the complementary bond with the target protein cannot be guaranteed. A particular epitope could mask another protein or bond to it non-specifically. The majority of antibodies are glucosylated or bonded to other molecules, and in any case, they have large surfaces for bonding to different molecules. This is why cross-reactions are found between different proteins in the sample [16]. In addition, the epitopes could be inaccessible when the antibody is anchored, or antigen-antibody recognition could be obstructed for steric reasons, due to the topography of the anchor. These factors complicate this type of analysis on a patient's serum.

Once the antibodies have been anchored, they do not maintain optimum activity for more than 8 months. Alternatives have been developed using recombinant antibodies that may resolve this problem [21]. These recognise and bond to specific proteins with high affinity. Moreover, since they are synthetic they may be more stable.

To avoid the problems of non-specificity and the scarcity of commercial antibodies that work properly in this method, the use of recombinant antibodies has become generalised. This is a most useful tool for this purpose, given the difficulty of obtaining monoclonal antibodies to identify the human proteome. The generation of identified recombinant antibodies using selection techniques in vitro [28] is an optimal tool for analysing the human proteome. The most common techniques for this purpose are the phage [29] or the ribosomal display [30]. These antibodies are highly specific (as has been observed in immuno-

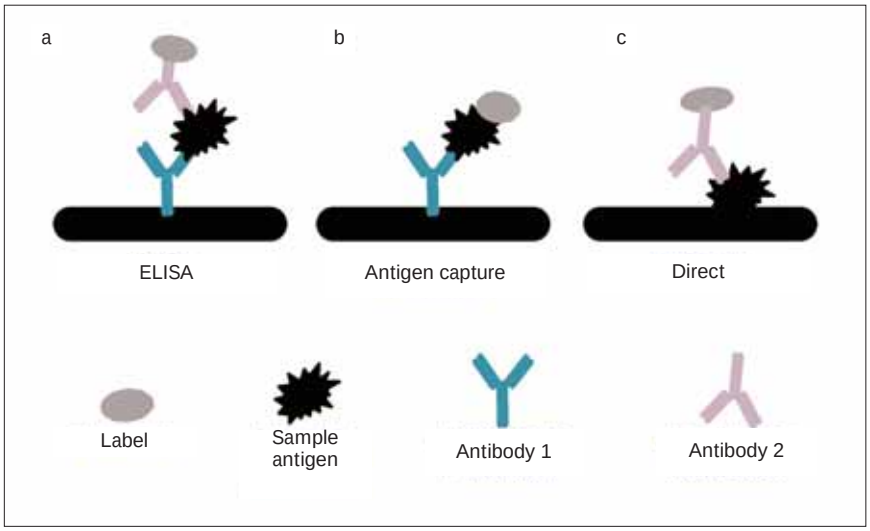

Figure. Types of protein arrays. a) ELISA-type tests: the primary antibodies are anchored onto a solid support, and specific antigens are detected in the mixture using a second marked (primary) antibody; b) Antigen capture test: a complex sample is conjugated with a chemical substance (biotin) or fluorochrome before being incubated with the array. Antigens in the sample bond specifically to the antibodies anchored to the solid substrate; c) Direct tests: the sample is anchored to the solid substrate and labeled antibodies bone specifically to the antigens.

histochemical studies) [31] and they also offer high sensitivity when quantifying a particular protein in a cellular lysate [32].

The phage display is normally used to mass-produce recombinant antibodies [19] and provides a new method for selecting the antigen-antibody interactions that could then be incorporated into an array. This is being exploited commercially by applying the functions of the phage using different technologies, such as *dyax* (http://www.dyax.com) and *morphosys* (http://www.morphosys.com), in which a massive amount of antibody fragments is produced, starting from large collections.

For antibody arrays, recombinant antibodies are ideal since they are derived from cell lines that are essentially immortal. The complete antibody can be anchored, or only a fragment may be anchored, such as Fab (fragments that contain the specific recognition region) or smaller, variable fragments (the scFvs) [17].

On the other hand, these recombinant antibodies do not incorporate the post-transcriptional modifications that are suffered by native proteins and which cause so much difficulty in the recognition of modified epitopes. Nam et al [33] and Qiu et al [34] have developed an array of natural proteins obtained by bi-dimensional liquid separation of lysed tissues or lysed cell lines. The fractions obtained are anchored onto a solid support in duplicate and with the corresponding controls. Once prepared, the arrays are exposed to serum from patients with diagnosed cancer (rectal cancers and adenocarcinomas) for the characterisation of tumour antigens that show reactivity with the antibodies anchored, and which will be subsequently identified by using mass spectrometry.

Substrates

One of the fundamental aspects when generating antibody arrays is the selection of a suitable surface on which to anchor the proteins, to avoid modifying their structure, and to obtain proper orientation and stable attachment. The accessibility, efficiency and specificity of the bond to the ligand or of the antigen-antibody recognition depend on this choice [9]. Proteins are chemically and structurally different from each other, and are far more complex and heterogeneous than DNA. They can easily lose their structure due to denaturalisation [35] and their activity is reduced when they have been immobilised [36]. Consequently, denaturalisation must be avoided, and the specific region of the antibody that recognises the antigen must be exposed on the surface. pH is a critical factor in the stability of the complex in this type of application.

The characteristics that an optimal [37] surface must possess are:

- Must not be self-fluorescent.
- Must limit non-specific bonds.
- Must possess a high ratio of anchoring surface/lysate volume.
- Must be inert.
- Must be compatible with the detection methods.

The surfaces that have been used can be classified into two groups; according to if they are, covered or not by a gel, such as glass or plastic, normally subjected to a chemical treatment (addition of polylysine or aldehyde groups) in order to favour adhesion of the proteins [38].

Normally, protein expression studies use PVDF or nitrocellulose membranes. After protein arrays were introduced, new requirements have appeared in this respect. Angenendt et al [38] carried out an analysis of antibody anchoring on 11 different surfaces, studying different storage conditions, comparing their detection limits, and detecting inter- and intra-matricial variations. The results indicated great uniformity in the signal that was detected and in the reproducibility of the method. Polyacrylamide surfaces gave the greatest performance in terms of detection threshold, possessing high sensitivity, and for this reason their use is recommended in experiments in which only small quantities of sample are available. It was also observed that although the method used is the same as in normal immune tests, the majority of antibodies that work well in ELISA tests do not work in antibody arrays. This makes prior validation of the antibodies necessary on the chosen substrate as a function of the experimental design, i.e., the objectives of the study, the quantity of sample material available, the type of array to be used, etc.

Detection

The methodology used in the arrays for marking and detection depend on the format and type of array used (Figure), and on the substrate used. The detection system used for c-DNA microarrays (fluorescence) should not be used with the same protocol for protein arrays, although it has been used at times, especially in the early development phases of this tool. Given the heterogeneity of proteins, some fluoresce more than others, and this affects the sensitivity and specificity of the method, making a calibration curve necessary for each antigen-antibody complex. The problems with chemical marking of the proteins is due to the modifications in their surface structure, which can even prevent the antigen-antibody bonding [21].

The normal detection systems can be divided into two groups:

- *Direct methods:* in these, the antigen-antibody pair is marked

using a chromogen or fluorochrome that is compatible with the substrate used [37].
- *Indirect methods:* which are based on amplifying the signal.

The use of chromogens produces a permanent signal that is easily visible for quantitative analysis. One precaution that must be taken into account when using this method is not to use organic solvents in the protein extraction protocol, since the precipitates of some of the most common chromogens are soluble in alcohol. The intensity of the signal when this type of method is used varies, depending on the protocol used.

The application of fluorochromes is based on the high-energy photons absorption from an external light source, which excites the electrons and leads to light emission at a different wave length to the incident light. [37]. Fluorochromes suffer from the same limitations mentioned above, and in addition, the ability to fluoresce wanes over time.

The detection system based on radioisotopes is not widely used, due to the long exposure times required and the problems of bio-security inherent to this technique.

The most common indirect technique is based on chemoluminescence, the same technique that is used in the Western blot method, in which oxidation of the substrate produces a prolonged emission of light that is captured by a photographic plate or a camera [16,37].

Applications

The possibility of obtaining the protein profile of a disease or even different stages of the same disease is of great interest, and is applicable to immunodiagnosis, biotechnology and biomedicine. Within the field of neuroscience, we find that neuro-degenerative diseases are generally protein pathologies, in which one or several proteins are lacking or accumulate, or their normal metabolic pathways are altered in some way. Moreover, in growth alterations or uncontrolled growth, the cellular signalling routes, the cytokines or the adhesion molecules are substantially modified. But we should not only expect greater understanding of the difference between pathological and healthy states, we should also expect this methodology to play a significant role in the proteome catalogue project. From a clinical perspective, it will be of use in early diagnosis, in population screening to detect risk factors, and in the identification of new therapeutic targets.

The major clinical application of antibody arrays at the present moment is the detection of auto-immune antibodies [39]. This is a very important diagnosis method when the clinical symptoms are heterogeneous, or in the initial phases of a disease [19] Another application is allergen arrays [22].

One of the strategies proposed for analysis of the immune response in the case of cancer [33] consists in solubilising proteins from a cell line (in this case, rectal adenocarcinoma) to obtain 1760 fractions that are anchored onto an array and incubated with serum from patients who have been diagnosed with cancer. Those fractions that show high reactivity are analysed and identified using mass spectrometry. Little work has been carried out using this technique in neuro-oncology. Nielsen et al [27] have used an antibody array to study the expression of tyrosine kinase receptors in a line of tumour cells, and our group [40] has searched for differential protein expression profiles that could identify degrees of malignity in gliomas.

As regards other fields of neuroscience, neuro-degenerative diseases have been studied using protein analysis techniques, and there have been a few relevant reviews along these lines in the last 5 years. Among the neuro-degenerative diseases, and given its prevalence, Alzheimer's disease has centred the interest of investigators both in the field of proteomics and in several other fields. Ho et al [41] have used protein arrays (PowerBlot Proteomic, BD Transduction Laboratories, Lexington, KY, USA) that apply the Western blot method on a large scale to analyse neural plasticity in this disease, to complement data from c-DNA microarrays concerning post-transcriptional changes. Another approach is that used by Vercauteren et al [42] which considers the applicability of bidimensional electrophoresis analysis to the study of neuro-degenerative diseases and the construction of the brain's proteome map. These studies analyse the differential protein expression in different areas of the brain post-mortem [43] or in cerebrospinal fluid [44], where they define new biological markers. Of particular value are protein expression profiles of tau and beta-amyloid proteins in this fluid, to distinguish this disease from other neurological diseases [45]. Since cerebrospinal fluid reflects the proteins present in the brain, both in normal conditions and in pathological conditions, it is of evident interest in the application of the methodology discussed to these diseases.

Studies by Butterfield et al [46] are aimed specifically at the identification of oxidised proteins in Alzheimer's disease. To detect and analyse derivatives of β-amiyloid as potential biological markers for this disease, Xiao et al [47] postulate SELDI TOF-MS technology as the ideal platform. This technology consists of a protein array, a mass-spectrometry study and a computer program to analyse the data. This method is also referred to by Zabel et al [48,49] in the study of protein alterations related to Huntington's disease, both in animal models and in humans, in order to establish differential expression with respect to tissues from people free of the disease.

Proteomics has sparked great interest among many neuroscientists, leading to initiatives to achieve the characterisation of the proteome of the human brain. At a European level, the European Human Proteome Project has been created (http://www.hbpp.org), with the aim of characterising the protein profile of a healthy brain and gain deeper knowledge of the more prevalent neuro-degenerative diseases such as Alzheimer's and Parkinson's disease.

CONCLUSIONS

Once the human genome has been sequenced, the challenge in biomedicine is to assign medical significance to the results obtained from bio-technological advances, in order to translate them into improvements in early diagnosis and subsequent treatment of patients. Studies to differentiate between the normal and the pathological state should not only be focussed from a morphological point of view, but also by applying genomics and proteomics. We must discover how to interpret these changes in proteins, and how the confluence of the parameters that are affected leads to disease, in order to respond to these diseases. To do this, protein profiles of complex mixtures have to be obtained. The greatest limitation on arrays is still their low resolution and the considerable depth involved, these being very important factors in the detection and quantification of proteins. These problems cause serious difficulties in their application to studies in which only small quantities of sample material are available. In addition, in order to validate the results, international consensus must be reached on the methodology as regards normalisation and quantification of these tools.

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MATRICES DE PROTEÍNAS: APLICACIONES E IMPLICACIONES EN NEUROCIENCIA

Resumen. Introducción. *La genómica está aportando numerosas claves que ayudan a comprender la fisiopatología de las enfermedades y a conocer mejor las diferencias respecto al estado de salud. Para entender el desarrollo de una patología es necesario que un estudio incluya, además de un análisis morfológico y genómico, el análisis de las proteínas y sus interacciones, de los cambios en su concentración y de las modificaciones en su estructura, dado que son moléculas clave en la estructura de los seres vivos y en la ejecución de los procesos biológicos.* Desarrollo. *Con la finalidad de estudiar los aspectos mencionados, se han desarrollado diferentes abordajes tecnológicos entre los cuales se encuentran las matrices de proteínas. Estas matrices incorporan diferentes metodologías que permiten detectar modificaciones postranscripcionales o la cuantificación proteica. En esta revisión se discuten los diferentes abordajes metodológicos y las posibles aplicaciones de dichas matrices. Esta herramienta es adecuada para el estudio comparativo de perfiles de expresión proteica de diferentes estructuras y situaciones metabólicas. El objetivo es considerar el uso de las matrices de proteínas como herramienta de estudio del proteoma en neurociencia.* Conclusiones. *Tras la validación de biomarcadores de interés clínico, esta tecnología es un paso prometedor en el camino hacia el desarrollo de protocolos de diagnóstico precoz. Para ello, no sólo hay que avanzar en el desarrollo tecnológico, sino que también se debe llegar a consensos internacionales en diversos aspectos metodológicos, como la normalización y cuantificación de la señal obtenida, para poder validar los resultados. [REV NEUROL 2007; 44: 285-90]*

Palabras clave. *Análisis proteico. Biomarcador. Matriz de anticuerpos/proteínas. Neurociencia. Perfil proteico. Proteómica.*