PROTEIN MICROARRAY TECHNOLOGY FOR ANTIBODY DETECTION ASSOCIATED TO HUMAN PATHOLOGY

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ABSTRACT

Protein microarray is a proteomic technology that in recent years has gained momentum for high-throughput identification of biomarkers in various pathologies. It is based on the specific antigen-antibody reaction like any ELISA, occurred on a specific miniaturized support (chip or slide), but has the advantage of concomitant evaluation of tens to thousands of molecules in small samples with a highly specific recognition for the detection system. There are several classification criteria for protein microarrays, the most frequently used types being: 1. direct one, comprising standard – recombinant purified proteins that are spotted on the detection chip and the analytical one based on antibodies immobilized on the glass slide; 2. indirect one or reverse phase protein microarray that comprises total or fractionated cellular lysates spotted on the chip. The most popular type is antibody array exploited with success in various fundamental or recently for clinical applications.

Although fundamental research purposes prevail when it comes to array platforms, there is also a recent increasing trend in clinical research, diagnostics and even industry applications such as pharmacy or food. In our review we will give as examples of using protein microarray for antibodies detection in two human major diseases – HIV infection and autoimmune deregulations. In HIV infection this platform was highly used for immunoglobulins classes and sub-classes identification generated by the viral antigens in order to improve diagnostics and monitor therapy of this complex disease. In autoimmune diseases the platform identified an array of autoantibodies raised against cytokines, chemokines, growth factors, tissue/cell proteins.

Keywords: protein microarray, biomarkers, multiplexing, HIV, autoimmunity.

REZUMAT

Microarray de proteine reprezintă o tehnologie proteomică care a devenit în ultimii ani un instrument inovativ în identificarea de biomarkeri în patologii diverse. Tehnologia se bazează pe reacţia specifică antigen-anticorp similar tehnicii ELISA, care are loc pe un suport miniaturizat (chip sau lamă), dar prezintă avantajul de evaluare simultană a zeci până la mii de molecule într-un volum mic de probă biologică cu recunoaştere specifică în sistemul de detecţie. Există mai multe criterii de clasificare a tipurilor de microarray de proteine, însă cele mai uzuale sunt: 1. direct, cuprinzând subtipul standard cu proteine recombinate purificate fixate pe chip-ul de detecţie şi analitic cu anticorpi imobilizaţi pe suportul de detecţie; 2. indirect sau microarray inversat care cuprinde lizat celular total sau fracţionat imoblizat pe suport. Cel mai popular tip este array-ul cu anticorpi utilizat cu success în diferite aplicaţii de cercetare fundamentală şi chiar de cercetare clinică. Deşi destinat cu precădere cercetării fundamentale, există preocupări actuale pentru aplicaţiile array în cercetarea clinică, diagnostic sau chiar în industria farmaceutică şi alimentară. În acest review sunt prezentate câteva exemple de utilizare a microarray de proteine pentru detecţia de anticorpi în două patologii majore – infecţia cu virus HIV şi dereglările autoimune. În cazul infecţiilor cu HIV, aceste platforme sunt utilizate pentru identificarea claselor şi subclaselor de imunoglobuline generate de antigenele virale, în scopul imbunătăţirii diagnosticului şi monitorizării terapiei. Aplicaţiile în bolile autoimune permit identificarea unui panel extins de autoanticorpi faţă de citokine, chemokine, factori de creştere, proteine celulare/tisulare.

Cuvinte-cheie: protein microarray, biomarkeri, multiplexare, HIV, autoimunitate.

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INTRODUCTION

Protein microarray, as concept, is based on an earlier model - "ambient analyte immunoassay" - introduced in 1989 by Roger Ekins [1]. In 10 years this model was fruitfully changed into DNA microarray platform. These platforms were intended to determine mRNA expression for concomitant determination of thousands of genes. But, relatively soon it was shown that DNA microarrays were limited by the fact that mRNA profiles were not perfectly matching protein expression [2, 3, 4].

As the myriad of inter-connected proteins are the main substrate for all the tissues and cells processes, dedicated protein microarrays were designed to cover this complicated proteome machine and to pursue as a high-throughput tool in order to by-pass DNA microarrays limitations. Through these microarrays platforms, protein functionality and interconnection could be identified and analyzed.

The first type of protein microarray that was developed was based on specific antigen-antibody reaction, like any ELISA, and had the advantage of highly specific recognition for the detection system. Conventional immunoassays turned to the capability of the antibodies to be spotted onto arrays that enabled this newly developed technology to have multiple, parallel detection system for very small amount of biological sample [3, 5].

Furthermore, as this technology has evolved, it was endowed with higher sensitivity and improved reproducibility in the assays that were seeking quantitative results, based on a calibration curve, also like in ELISA. The fact that this technology has gained sensitiveness and overall performance led to its recognized validity when studying complex biological samples.

When antibody arrays were developed, another design of protein microarray was starting to be developed. This new protein microarray version immobilized purified proteins, not antibodies, on glass slides. Hence protein microarray classification was needed, so as to distinguish it from the antibody arrays that were called microarrays, this new type was denominated as functional arrays [6]. Dissimilar to antibody arrays, functional protein microarrays are designed spotting on the glass slide various proteins that append to a certain cell, group of cells, tissue and/or an entire organism. These functional microarrays are used for studies related to protein functions evaluation, interconnection in protein-protein binding, metabolic/biochemical action, relationship between a specific ligand and its receptor, between an enzyme and its substrate, immune protein triggered by an active response and so on [6, 7].

Reverse-phase arrays were another step in the history of protein microarray development where total protein from tissue/cell lysates or specifically fractionated tissue/cell lysates are spotted on the glass slides [7].

Protein microarray analysis has an increasingly use both for research purposes as well as for various biomedical applications, *including the niches ones* like evaluating markers of apoptosis activated by various therapies such as photodynamic therapy (PDT) [8], assessment of epigenetic milieu or transcriptional activity in treated cells [9]. Thus protein microarray is a proteomic tool that can deliver high-throughput data for revealing new therapeutical targets.

Protein microarray history has spanned the last two decades, the basic principle being identical with ELISA, but there are several advantages such as spotting in terms of miniaturization, multiplexing and large data obtained in an ELISA equivalent time. Briefly, biological samples of interest (e.g. serum, plasma etc.) are incubated on a slide containing immobilized antibodies, proteins or peptides. An antigen-antibody reaction occurs between an analyte from the tested sample and the corresponding antibody from slide followed by the detection step through various methods (e.g. fluorescence based detection). The slide is further scanned, followed by image acquisition, data processing and analysis. There are several classifications of this technique, but it could fall into two main categories: direct phase (e.g., antibody-, protein-, peptide array) and reverse or indirect phase where the sample of interest is spotted on a slide and the corresponding antibody is further added. The most popular

Table 1. Classification of protein microarrays types

Classification criteria	Type		Ref
Immobilized protein structure	**Direct**	Standard – recombinant purified proteins	[12, 13]
		Analytical - antibodies	[3]
	Indirect	Reverse phase protein microarray (total or fractionated cellular lysates)	[14]
Determined parameter	**Abundance**	Capture	[15]
		Indirect	[16]
	Functional	Protein In Situ Arrays	[17,18]
		In situ Puromycin Arrays	[19]
		Nucleic Acids Programmable Protein Array	[20,21]

type is antibody array exploited with success in various fundamental or even clinical applications.

The general principle of any protein microarray is presented in Fig. 1.

Among all these variants, the antibody array type is preferred in tumor research domain or in biomarkers discovery/quantification due to technique's high versatility and reproducibility [10]. The reverse phase array format could also be used for biomarker discovery because it is very specific but has the disadvantage of being more laborious. This type has lower standardization potential and multiplexing possibilities; in addition, there is a low accessibility for both detection labeled antibodies and specific protein antibodies [11], the format being prone to cross-reactivity because all the proteins harbored by complex samples are investigated at once. A comprehensive overview of the types of protein microarrays is presented in Table 1.

It is worth to emphasize that protein microarray could be customized in terms of

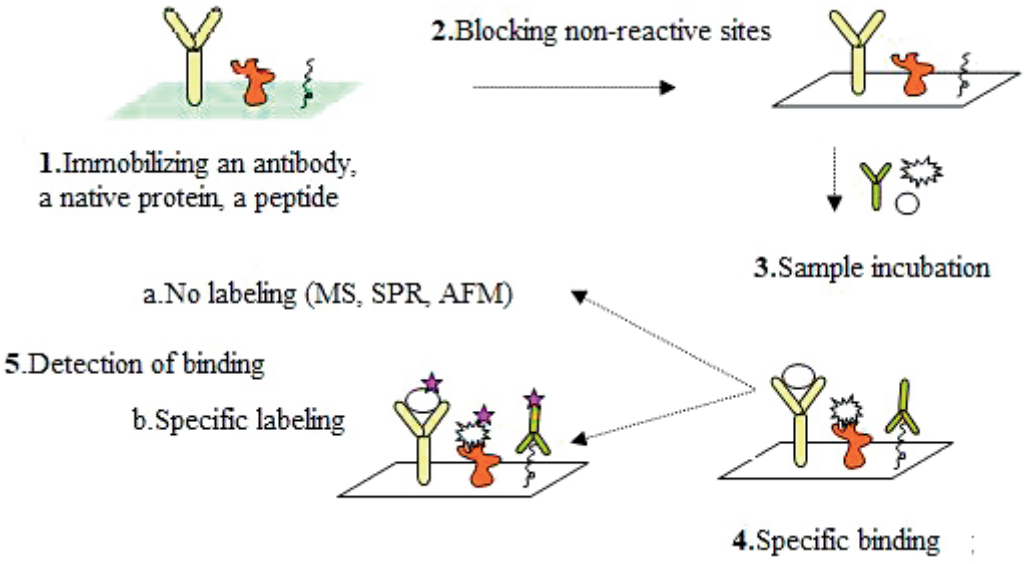

Fig. 1. The general outline of any protein microarray work-flow; 1. Immobilizing an agent *(only for customized protein microarray slides)*; 2. Blocking the non-reactive sites; 3. Sample incubation on specific slide; 4. The specific binding between an analyte from the sample and its corresponding antibody attached on the slide; 5. Detection step that can be without any labelling through e.g. MS (mass spectrometry), SPR (surface plasmon resonance), AFM (atomic force microscopy) (a) or with a fluorescent dye (b).

number and multiplicity of tested analytes one achieving new research and clinical benefits through this technology. Thus, although fundamental research purposes prevail when it comes to array platforms, there is also a recent increasing trend in clinical research, diagnostics and even industry applications such as pharmacy or food. In our review we will give as examples of using protein microarray platform for antibodies detection in two human major diseases – HIV infection and autoimmune deregulations.

1. Antibody screening using protein microarray platforms

High-throughput screening in antibody detection was used in several fields. Customizing an array in relation to clinical purpose confirms the flexibility of these platforms in assisting molecular management of the disease. For example, one of the first customized platforms was designed in order to monitor severe acute respiratory syndrome (SARS) infection by screening hundreds of sera based on reactivity against selected proteins from SARS coronavirus. Authors have reported that with this customized array, viral infection could be monitored for many months after infection [22]. Zhu *et al.* [22] developed this first viral protein microarray that consisted of all SARS corona-virus (SARS-CoV) proteins and proteins from five unrelated corona-viruses. This platform distinguished SARS-positive or SARS-negative sera with a 94% accuracy compared to ELISA method, but with a significantly reduced time span, a critical criterium for SARS rapidly expanding infection.

This type of microarray platform has been further updated to a serological assay for the specific detection of IgM and IgG antibodies against the S1 receptor-binding subunit of the spike protein of emerging human coronavirus hCoV-EMC and SARS-CoV as antigens [23].

As further elaborated, recent attempts were made using arrays platforms for antibody screening in autoimmune disease and discovering new insights in this complex field. Thus, novel antigen arrays have been developed in order to discover new autoantibody targets, providing analysis for hundreds of samples and of their reactivity pattern against thousands of antigens simultaneously [24].

Protein microarray is a technology in continuous evolution offering multiple possibilities in updating other proteomic techniques. Therefore the development of the "microwestern array" is a clear proof how traditional methods like Western blot can be linked to novel technology, thus significantly expanding the research technological arsenal [25]. Another import application used for screening was reported by Chen *et al.* [26] for biomarkers discovery in inflammatory bowel diseases (IBDs). In this study sera from healthy and IBD patients, both Crohn's disease (CD) and ulcerative colitis (UC) patients were screened using a complex proteome microarray. In the used platform, *E. coli* K12 microorganism was the target. The authors report 417 proteins as potential candidates, 169 proteins were identified in healthy controls as highly immunogenic, 186 proteins in CD, and just 19 highly immunogenic in UC patients. After applying bioinformatics analysis, this screening showed new biomarkers candidates for the diagnosis of CD versus healthy control and CD versus UC [27].

If the groups of screened patients are small the study encounters an issue that is denominated as the "overfitting problem". This issue means that even though a candidate biomarker is highly relevant in a small patient cohort, in a much larger cohort (over 100 individuals) its discrimination capacity of patients from healthy individuals can be disappointing. Hence validation of potential biomarkers in larger groups and in a double-blind mode is always wanted. Thus Song *et al.* [28] designed a two steps strategy for identification of biomarkers in autoimmune hepatitis (AIH). In the first step, a human protein microarray was designed where 5,136 purified proteins were spotted on poly-L-lysine-glass slides. On this microarray 11 candidate autoantigens were identified. In the second step another chip was designed with the 11 identified proteins and autoimmunogenic profiles from sera harvested from patients were performed. In this second step 44 AIH patients, 50 healthy controls, and

184 patients diagnosed with hepatitis B, hepatitis C, systemic lupus erythematosus, primary Sjogren's syndrome, rheumatoid arthritis, or primary biliary cirrhosis were used. When applying statistics on these data three antigens, RPS20, Alba-like and dUTPase, were found as AIH specific biomarkers, displaying good sensitivities: 47.5% for RPS20, 45.5% for Alba-like and 22.7% for dUTPase. In double-blind tests these potential candidate biomarkers were validated using additional AIH samples.

Screening for infectious disease used protein microarray 10 years ago. Pathogen protein microarrays were used to screen serological profile responses after infection with bacteria and viruses [29, 30, 31, 32].

In these studies, besides the high-throughput in screening new biomarkers, some limitations occurred. Thus these arrays were designed using proteins translated in *E. coli* lysates without any purification so these proteins were contaminated with *E. coli* proteins, reducing the platform's sensitivity. In order to overcome this limitation blocking agents using *E. coli* lysates were used [29, 30, 31, 32].

Another domain for protein microarrays used in screening was to develop a protein microarray platform that can present both natural and denatured proteins for antibody biomarker discovery in order to optimize plasma screening protocols to improve detection. Thus authors report a covalent capture chemistry for protein microarray platform using HaloTag fusion proteins and ligand. The *in vitro* transcription translation system (IVTT) used HeLa cell lysate in order to enhance protein yield. The lysates of *Escherichia coli* were used in the blocking buffer to reduce nonspecific background as priorly mentioned. These newly designed protein microarrays were tested with plasma samples and further autoantibody responses were actually quantified. Blocking with the lysates extracted from *E. coli* reduced significantly the background signals, improving expression with IVTT and the antibody signals. The authors have shown that this flexible protein microarray platform permits the display of natural and denatured proteins offering distinct autoantibody profiles. Through this type of platform the discovery of disease-specific antibodies can improve clinical diagnostics [33].

2. Antibodies detection using protein microarrays in HIV infection

The most frequently use of protein microarray technology in human infection was reported in HIV disease. Developing human recombinant monoclonal antibodies (mAbs) has endowed this technology with high throughput characteristics that highlighted the specificity and functional activity of anti-HIV antibodies. Historically, tens of thousands of antibodies have been generated and further screened in assays, so that antibodies related to cross-strain neutralization and passive protection in primates were identified. High-throughput screening for mAbs classification and their antigen targets was reported. Dotsey *et al*. have analyzed a new microarray chip that comprised recombinant proteins gp140, gp120, gp41 of HIV-1 and various membrane proximal external region peptides. The protein microarray that analyzed 11 HIV-1 envelope-specific mAbs showed that there are diverse affinities and specificities for them. All the obtained data from the protein microarray analysis were significantly correlated with the data obtained from ELISA measurements. When investigating plasma samples from HIV-1 infected subjects with this protein microarray authors report polyclonal immune responses. Moreover different binding patterns were identified and different reactivity toward spotted proteins. Using this technology the authors point out that the specificity of the humoral response can be shown and that in HIV infection there is actually a high diversity of the antibodies generated to HIV infection [34].

Another team that developed a protein microarray platform designed a chip comprising a huge array of proteins and peptides of the HIV-1 clade C proteome, this being the HIV-1 subtype that covers the majority of infections throughout the world. In this recent study it was demonstrated using an HIV-dedicated microarray that simultaneous, sensitive and specific discovery of antibody responses (major immunoglobulin classes IgG, IgA, IgM, IgE

and subclasses IgG1-4) in small serum samples towards a large number of HIV antigens and peptides can be performed. In addition, this newly developed chip can be used in the course of antibody monitoring during disease clinical evolution and treatment. Also this platform can be further developed for studying immune mechanisms developed as an antibody could respond to the multitude of HIV antigens and epitopes in infected patients in order to further improve diagnosis, monitor treatment [35] and of vaccine trials like the already established HIV Vaccine Trials Network [36].

Besides identifying the classes and sub-classes of immunoglobulins raised by HIV infection, as the disease progression is complex, the analysis techniques should focus on examining clusterings of responses. Storey *et al.*, in order to identify clusters of antibody responses, assessed by protein microarray HIV-1-specific antibody responses associated with disease progression using Principal components analysis (PCA) in a cohort design. Interestingly, antibody responses associated with viral load. Thus before treatment, higher antibody responses against transmembrane glycoprotein (TM) and reverse transcriptase (RT) associated with higher viral loads, at the same time as antibodies against the surface glycoprotein (SU), capsid (CA), matrix (MA), and integrase (IN) proteins were associated with lower viral loads. During the first year higher antibody responses associated with lower decreases in CD4+ T lymphocyte count and reduced disease progression. The authors report that PCA and protein microarray analyses showed a collection of HIV-specific antibody responses associated with reduced disease progression. This technology is still to be explored when evaluating multifaceted host-disease interactions, such as HIV co-infections [37].

3. Antibodies detection using protein microarrays in autoimmunity

In autoimmune diseases there is a huge array of autoantibodies raised by the deregulated immune system against cytokines, chemokines, growth factors, tissue/cell proteins and this entire array can develop the inflammatory processes associated to autoimmune disease. A proper detection or diagnosis of autoimmune condition demand an up-to-date methodology and appropriate biomarkers to accurately depict in a non-invasive mode specific biomolecules (antibodies or autoantibodies) associated with diseases onset, course or outcome. Hence, protein microarrays are an omic tool suitable in finding a powerful biomarker, by detecting those markers (e.g. antibodies) statistically correlated with disease state in different biological media, especially easy to use for serum samples [6].

Systemic lupus erythematosus (SLE) represents a severe autoimmune disease affecting connective tissue. The need for accessible biomarkers in SLE management could be successfully resolved by serum proteomics which would reflect with accuracy the SLE disease grade, diagnosis or monitoring. However this endeavor is still a challenge for SLE and autoimmunity. In this light, recombinant antibody arrays are assuring high-throughput proteomic profiling in serum combining specificity, sensitivity and miniaturization. Specific antibodies are orderly arranged on a solid support where biological sample is further added followed by specifically binding of proteins to immobilized antibodies. The binding is detected, quantified and then converted into a protein expression map that will decode the molecular composition of the analyzed sample. This antibody array type allows serum biomarkers analysis for SLE, improving diagnosis, classification, and disease prognosis [38].

Another attempt to evaluate serum biomarkers from autoimmune and immunodeficiency patients has been represented by a protein microarray on a nitrocellulose-support comprising human cytokines, chemokines, and other specific proteins. Serum profiling with this array platform reveals that SLE subjects show elevated IgG autoantibody to B cell-activating factor (BAFF) associated with SLE. This BAFF reactivity was correlated with certain severity disease SLE factors such as IFN-α influence. Thus, protein microarrays could detect autoantibody reactivity to serum factors and as

a consequence BAFF-reactive autoantibodies may be related with an elevated inflammatory milieu within SLE [39].

Microarrays concept is in continuous evolution thus it was recently shown that protein arrays with huge antigen content are very effective tools for exploration plasma autoimmune repertoire. Such arrays platform accommodating around 21,000 antigens can be used to profile plasma samples from secondary progressive multiple sclerosis patients in a fast and effective manner, thus allowing a high-performing plasma analysis for potential autoantibody targets detection in various autoimmune conditions [40]. Other efforts are directed towards developing microarray formats starting from previous immunoassay concepts, thus a microarray-based immunoassay for the detection of autoantibodies against Ro protein was recently developed. Hence, a microarray-based fluorescence system with immobilized Ro(+)-*E. coli* proteins has detected anti-Ro autoantibodies in SLE patient serum with high specificity, selectivity and enhanced efficacy [41]. Another endeavor for autoimmune context is related to selecting and placing clinically relevant antigens in a microarray format. Thus, a microarray for the detection of antiphospholipid antibodies was developed and used for analyzing 35 antiphospholipid syndrome (APS) patients, 6 lupus erythematosus patients and 24 healthy subjects. The array surface modification combined with proteinogenic as well as phospholipid-derived antigens composition (e.g. β2-glycoprotein I, prothrombin, cardiolipin) were allowed up to 20 successive tests on one chip, with good sensitivity (0.800-0.929) and specificity (0.733 - 0.969), reliant on the particular antigen. This optimized microarray platform detects four different APS significant antibodies with a sensitivity and specificity comparable to current ELISA [42].

4. Conclusions

Protein microarrays are continuously gaining landscape among omics involvement both in fundamental and, more important, in clinical applications. Identification and even quantitation of multiple analytes in a variety of biological samples and pathologies make protein arrays a reliable tool in assisting disease management. Moreover, other important research fields such as pharmaceutical area or food industry advance from arrays technology potential. However, the costs associated with this tremendous technology especially in case of functional protein microarrays, are still high and thus consumption of a large number of protein microarrays platforms may not be practical to ensure its applicability in a cost-effective way. But the accurate principle learned from ELISA extrapolated in multiple biomolecules detection entitled protein arrays to pave the way of omics in crucial life sciences domains.

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