

Protein microarrays for the diagnosis of allergic diseases: State-of-the-art and future development

Protein-Biochips für die Diagnose allergischer Erkrankungen – Stand der Technik und zukünftige Entwicklungen

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Abstract

In the emerging field of *Functional Proteomics*, protein microarrays are considered to be one of the most promising tools for the simultaneous analysis of the a) abundance, b) function, and c) interaction of proteins on a system-wide scale. Resting on the technological grounds of widely used DNA biochips, the great power of microarray-based miniature solid-phase immunoassays lies in their potential to investigate in parallel large numbers of analyte pairs in a variety of biological samples. Consequently, this has fuelled aspirations that protein microarrays may serve as tools for the high-throughput functional investigation of complete proteomes and, moreover, that they will develop into promising candidates for innovative in-vitro diagnostic (IVD) applications. To date, published examples of protein microarrays for IVD purposes have included tests for allergy, autoimmune and infectious diseases. Here, we discuss recent advancements in the development of protein microarrays for the profiling of IgE antibodies in the diagnosis of Type 1-related allergic diseases.

Keywords: allergy; biochip; component resolved diagnosis; IgE; microarray, molecular diagnostics; recombinant allergen.

Zusammenfassung

Im ständig wachsenden Forschungsgebiet der funktionellen Proteomik zählen Protein-Biochips aus einer Reihe innovativer Methoden für die quantitative und funktionelle Bestimmung von Proteininteraktionen auf systemischer Ebene heute zu den aussichtsreichsten Kandidaten. Miniaturisierte Festphasen-Immunoassays, die analog

zur etablierten DNA-Chip-Technologie entwickelt wurden, entfalten ihr Potential in der hoch parallelen Untersuchung unterschiedlichster Liganden-Paare in biologischem Ausgangsmaterial. Es besteht die berechtigte Hoffnung, dass Protein-Biochips in Zukunft durch den hohen Probendurchsatz und die enorme Zahl der generierten Daten den Bereich der Proteomik revolutionieren werden. Des Weiteren sind sie viel versprechende Kandidaten für neue und innovative in-vitro-Diagnostika (IVD). In mehreren aktuellen Publikationen wurde veranschaulicht, dass Protein-Biochips für die Diagnose von allergischen, autoimmun oder infektiösen Erkrankungen wertvolle Beiträge leisten können. In diesem Artikel beleuchten wir neue Ansätze und Entwicklungen im Bereich der Diagnostik von IgE-vermittelten Typ 1-Allergien mit Hilfe von Allergen-Biochips.

Schlüsselwörter: Allergenchip; Allergie; IgE; Komponenten-basierte Diagnose; molekulare Diagnose; Microarray; rekombinante Allergene.

Introduction

Protein microarrays as promising tools in proteomics

DNA microarrays (or biochips) have become a widespread tool for genotyping studies and for the analysis of gene-expression states on a whole-genome level [1]. Since its introduction at the beginning of the 1990s, DNA microarray technology has improved rapidly, and DNA biochips are finally being adopted for genotyping purposes under routine diagnostic conditions [2]. Although DNA microarrays have been accepted as a reliable tool for conducting research in the fields of genomics and transcriptomics, it has been noted that the information about gene expression gained on the RNA level often correlates poorly with the abundance or function of the corresponding gene products [3]. Therefore, to gather reliable data about protein expression and to understand the complex network of protein interaction and functioning on a cellular level, the demand for large-scale and high-throughput methods has grown tremendously. Con-

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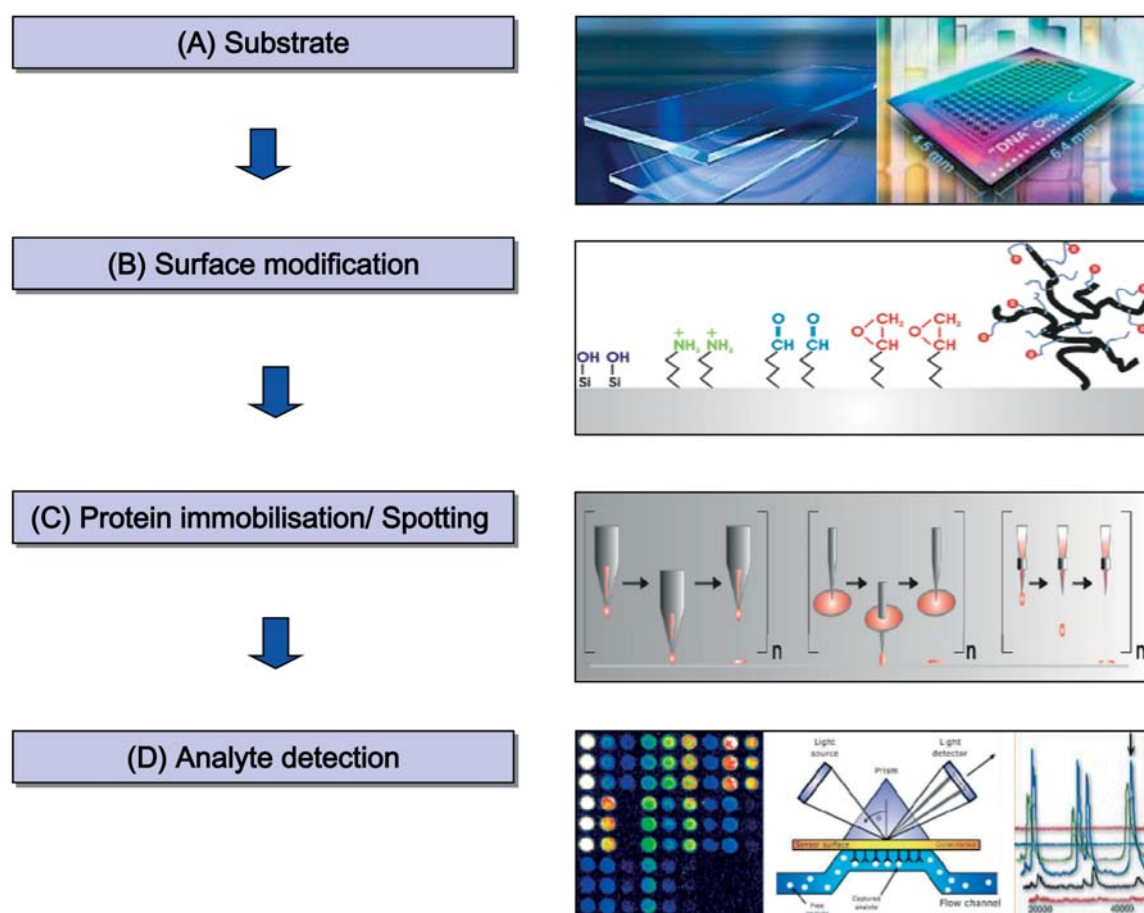


Figure 1 Protein microarray development. (A) Underlying the development of protein microarrays is the choice of a solid support for the stable immobilisation of bio-molecules. The most widely used material is flat glass slides (25×75 mm), whereas supports can also be made of plastic or silicon wafers. (B) Surface modification is usually accomplished by the application of chemical layers that may contain reactive groups for the covalent binding of target molecules (e.g., epoxysilanes). Alternatively, 3D-shaped structures, such as hydrogels or nitrocellulose-membranes, can be used to achieve a higher degree of protein binding and analyte accessibility. (C) Protein immobilisation occurs in a process called spotting, where n -volumes of protein-solution are dispensed on the microarray surface repeatedly in an automated (contact or non-contact) fashion ($1-n$). (D) The most widely used method for analyte detection is based on the measurement of analyte-specific fluorescence signals that have been produced in the course of a miniaturised solid-phase immunoassay. Alternative methods include RSL- (Resonance Light Scattering), SPR- (Surface Plasmon Resonance), or TOF- (Time-of-flight) based detection of the analytes of interest.

sequently, the concept of developing protein microarrays by adopting DNA biochip-based technologies has raised considerable interest among scientists in academia and industry [reviewed in 4].

Like their DNA biochip counterparts, protein microarrays are usually built on planar substrates, such as high-quality glass microscopy slides, silicon wafers or plastic devices (Figure 1A). For the immobilisation of proteins in a microarrayed fashion, the surface of the substrate is usually modified in order to bind protein compounds in a stable and biologically active manner. The latter can be accomplished by the chemical modification of the surface, or by the application of 3D-like functional layers, such as nitrocellulose-based or hydrogel-like structures (Figure 1B). Different types and numbers of capture molecules (e.g., native or recombinant proteins, antibodies, peptides or aptamer molecules) are

subsequently immobilised in a microarray fashion by robotic equipment to create individually addressable reaction sites (spots, features) (Figure 1C). Each of these (usually) μm -sized spots is later employed in a miniaturised ligand binding assay where each biological interaction can subsequently be monitored by applying specific detection antibodies (e.g., labelled with a fluorescence dye) combined with sensitive detection methods (e.g., fluorescence laser scanning microscopes) (Figure 1D). As a result, multi-spot fluorescence images are produced, and the individual signal intensities of each spot can be used for analyte quantification, to determine protein expression levels, or to monitor the function of specific enzyme-substrate pairs [5].

Although the concept of miniaturised ligand binding assays was described almost 15 years ago [6], current technological solutions to develop protein microarrays

have mostly remained in a conceptual state [2, 7, 8]. Among several reasons, this is due to the high level of complexity intrinsic to the antigens employed in miniaturised assays (e.g., size, charge, solubility, surface activity, 3D structure). Consequently, for protein microarray development, immobilised targets used to capture analytes of interest (e.g., proteins or antibodies) have to be selected carefully. Also, the conditions for spotting need to be adjusted in order to permit efficient immobilisation as well as to prevent negative effects like denaturation at the surface while retaining an optimal level of analyte accessibility. Furthermore, the assay conditions need to be optimised in order to achieve the necessary signal-to-noise ratio and to create a sensitive detection system. Notably, prior to routine use, the relevant assay parameters (such as sensitivity, specificity, reproducibility) must be validated in order to ensure accurate protein microarray-based results.

Type 1 allergic diseases

Type 1 IgE-related allergy is a major health concern in the industrialised world (USA, Europe, and Japan). Today, an estimated 25% of the population in these countries suffers from IgE-mediated atopic diseases (e.g., asthma, rhinitis, atopic dermatitis, conjunctivitis, or sinusitis). As a consequence, a considerable share of national health care budgets is spent on the diagnosis and treatment of allergy each year [9]. Type 1 allergy is initiated by the generation of IgE antibodies after the first introduction of an allergen (e.g., pollen) into the immune system of a patient. After a second allergen exposure, allergen-immune complexes formed on mast cells or basophils induce a central pathomechanism of allergy that culminates in the release of biological mediators (e.g., histamines or leukotrienes), eventually generating the well-known symptoms of allergy (e.g., hay fever, itching). IgE antibodies were identified as central mediators of allergic diseases more than 40 years ago [10], and soon after, the first IVD tests for allergy became available based on the detection of IgE in the serum of allergic patients [11]. For the development of these tests, allergenic material is typically immobilised onto a solid phase such as the well of a micro-plate, a nitrocellulose membrane, or the surface of a glass slide. Subsequently, the patient's serum is incubated in presence of the immobilised allergen(s), and specific IgE is adsorbed and retained at the surface. Bound IgE is then detected by a specific anti-IgE antibody, bearing a label (e.g., fluorescence or HRP) to generate quantitative results.

Component resolved diagnosis (CRD)

Since the first introduction of IVD tests for the determination of IgEs, crude or purified extracts produced from the allergy-eliciting sources (e.g., pollen, animal dander, mites, foods, moulds) have been used as agents for capturing IgE antibodies in body fluids [11]. Considerable

efforts have been made by researchers and medical device manufacturers to continuously improve the quality of the allergenic material applied in these tests [12]. Nevertheless, due to their intrinsic heterogeneity and the frequent instability of the individual components, biological extracts are difficult to standardise.

During the last decades, the major IgE-binding proteins of the most prevalent allergy-causing biological sources have been characterised on a molecular level, and many of them have been produced as recombinant proteins [13–15]. To date, efforts have been made to use genetically engineered variants of the major disease-eliciting molecules to develop optimized treatments for allergic patients [16]. Also, it has been demonstrated that using allergen components can improve allergy diagnosis since this allows for greater standardisation than state-of-the-art methods [17]. Eventually, this approach has been termed component resolved diagnosis (CRD), as it reduces the complexity of natural extracts to a comparatively small number of actual disease-eliciting molecules. However, using purified or recombinant allergen components poses novel problems for the development of IVD allergy tests. When using individual molecules, the number of tests necessary to provide a comprehensive diagnosis for a patient can increase considerably, since for most natural allergen sources (e.g., a particular pollen or food), more than one component needs to be included into the test in order to identify the complete repertoire of disease-relevant epitopes. Also, while recombinant proteins are advantageous over biological extracts in terms of standardisation, some of these molecules may not display the same immunological reactivity as their natural counterparts [18]. As a consequence, for the development of CRD-based multi-analyte tests, the individual allergens need to be validated carefully in order to provide an accurate IVD application.

Protein microarrays for the profiling of allergen-specific antibodies

The principle of allergen microarrays

Protein microarrays employing a large number of allergen components display one elegant solution for the multiplex determination of allergen-specific IgE reactivity. In principle, allergen microarrays represent multi-analyte (indirect) solid-phase immunoassays for the capturing of antibodies in the serum of allergic patients and subsequent monitoring by adding fluorescence-labelled secondary antibodies [19]. A schematic overview of a representative allergen microarray layout is depicted in Figure 2. A panel of (recombinant and purified) allergen molecules is immobilized onto a chemically modified glass slide in an arrayed fashion (see Figure 2A). The dimensions (75×25 mm) of the microscopy glass slide are compatible with standard laboratory instrumentation for slide handling and microarray scanning. Each allergen is spotted in a triplicate form to assure high assay reliability.

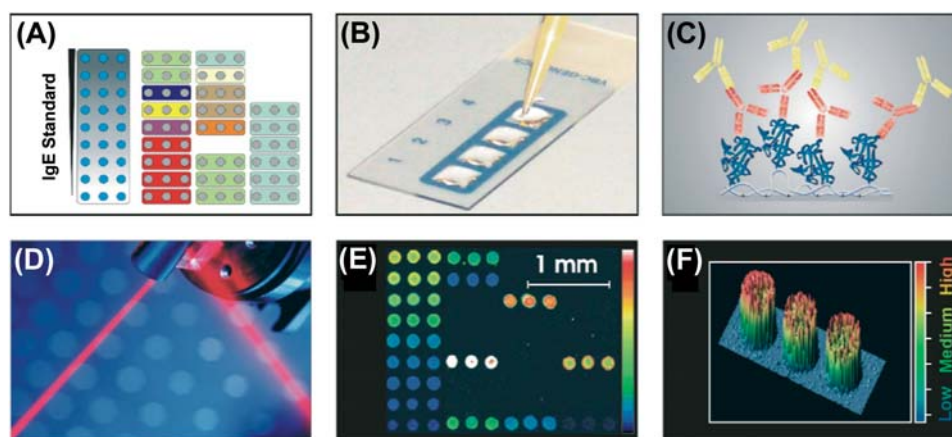


Figure 2 Allergen microarray design and assay procedure. (A) Schematic representation of the allergen microarray layout. Individual allergens of a single biological source are depicted as circles surrounded by a rectangle bearing the same colour. (B) Incubation of a 4-well chip with 4 individual serum samples (20 μ L per serum). (C) Schematic representation of allergen microarray assay. Allergen molecules (blue) are covalently immobilised onto the chemically modified biochip surface to bind efficiently and in a biologically active manner. Allergens are recognized by serum IgE antibodies (red), which themselves are detected by a fluorescently labelled anti-human IgE antibody (yellow). (D) Schematic representation of fluorescence laser scanning. (E) Allergen microarray false colour sample image. In the left part of the image, specific signals of fluorescence-labelled anti-human IgE antibodies bound to increasing concentrations of immobilised human IgE are shown. Fluorescence signals that correlate to the specific binding of labelled anti-human IgE to allergen triplicates are shown in the right part of the image. (F) Schematic representation of an allergen triplicate quantification and the corresponding classification range (low–intermediate–high).

bility. Additionally, serially diluted purified human IgEs are spotted in concert with the allergens for assay calibration and quality control (see Figure 2A and E). Furthermore, each glass slide contains four identical allergen arrays that allow for the testing of more than one serum or several serum dilutions on a single chip (see Figure 2B). Each of the allergen microarrays is surrounded by a thin layer of Teflon® in order to create individual reaction wells as well as to prevent sample overflow during the assay (Figure 2B). Basically, the existing layout contains 48 different allergen components, while it permits the immobilisation of up to 150 individual allergens in a microarrayed form.

Assay procedure and analysis

Prior to the start of the assay, each slide containing four identical allergen microarrays is washed intensively for 120 min to remove non-covalently bound allergens from the microarray-surface. Then, 20 μ L of the patient's serum is dispensed directly onto each reaction well. Following a 120-min incubation and a short washing step, binding of allergen-specific IgEs is monitored by the addition of a fluorescently labelled anti-human IgE antibody for 60 min (Figure 2C). After a second washing step, the chip is analysed by scanning in a (confocal) fluorescence laser scanner. Figure 2E displays a false colour image produced after a typical allergen microarray assay.

To calculate (semi-) quantitative IgE results, the fluorescence image has to be analysed by customised software packages. In short, the fluorescence intensity (FI) values of the individual spots are recorded (Figure 2F), while a number of bioinformatic quality control proce-

dures are applied [20]. By assaying a calibrator serum on each microarray slide containing pre-defined IgE quantities to a number of selected allergens which are part of the microarray layout, the FI values determined in the “unknown” samples can be interpolated by linear regression analysis. Eventually, for each individual component, the specific IgE concentrations are determined and these values are further assigned to four distinct classes (negative, low, medium, and high) that correlate to RAST classes 0, 1–2, 3–4, and 5–6, respectively.

In principle, processing a single slide (containing four identical allergen microarrays) can be accomplished in five hours. However, by using conventional microarray incubation chambers, up to 50 slides (equalling 150 assays or several thousand individual allergen-specific IgE determinations) can be carried out by an experienced user in a single day. By using appropriate microarray scanning equipment and customised software packages for image analysis, several hundred experiments can be processed automatically.

The benefits of microarray-based allergy diagnosis

In contrast to conventional allergy diagnosis, allergen microarrays are multi-analyte tests that permit the simultaneous investigation of up to several hundred allergens in a single analytical step. Yet, the amount of biological material (e.g., patient serum) consumed for a single investigation is considerably smaller compared to conventional immunoassays. Moreover, the use of different

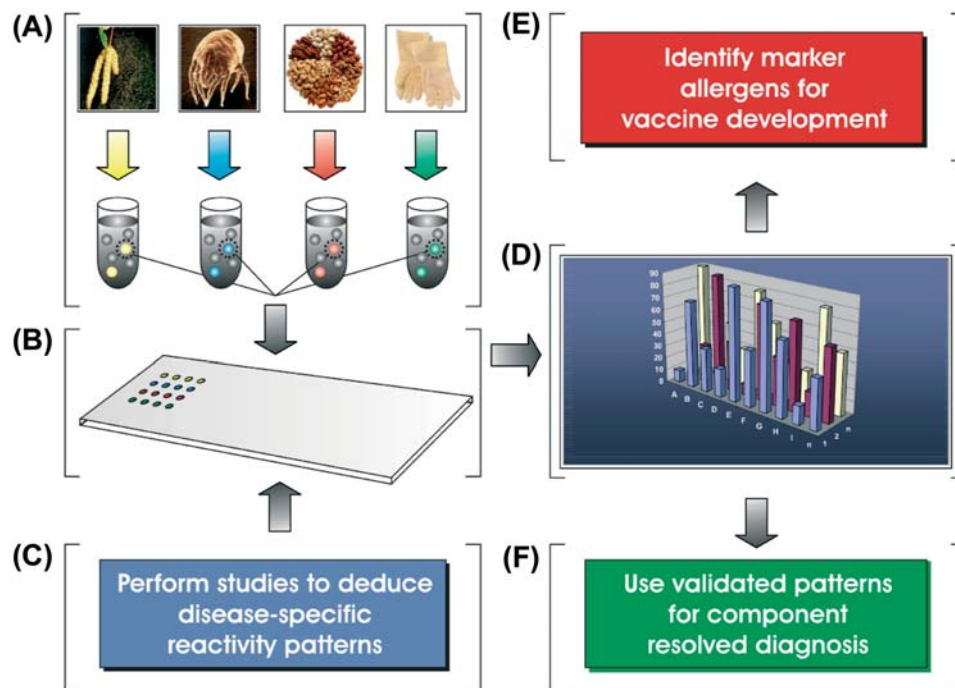


Figure 3 Allergen microarrays in the context of diagnosis and treatment. Allergen molecules are prepared from the most relevant disease-eliciting natural sources (A) to create comprehensive epitope panels on allergen microarrays (B). These “universal microarrays” can be employed in large-scale clinical studies to determine disease-, population-, or region-specific IgE reactivity patterns (D). Results obtained from these investigations may be used to identify novel potential marker allergens for disease development (E) or to use validated allergen panels as diagnostic signatures for improved component resolved diagnosis of allergy (F).

fluorescence labels in combination with individual specific detection antibodies permits the measurement of several analytical parameters (e.g., allergen-specific IgEs and IgGs) in a single assay [21, 22]. For the development of CRD-based tests, the expandability of the microarray format permits to employ a panel of allergen molecules that comprehensively represents the epitope repertoire of the respective natural sources. The first reported allergen microarray used crude allergen extracts and an isothermal signal amplification technique to achieve a sufficient degree of assay sensitivity on functionalised glass slides [23, 24], while Kim et al. produced crude allergen extract microarrays on nitrocellulose membrane slides [25]. In 2002, Hiller et al. adapted the concept of component-resolved diagnosis to the microarray format and used 96 purified recombinant allergens to determine specific IgE antibodies from human serum [26]. Subsequently, allergen microarrays have been used to determine allergen-specific antibody reactivity in a number of published examples showing that sensitivity and specificity of these novel test systems are comparable to state-of-the-art technologies [19, 25, 27].

Future development

Most importantly, to create an allergy test of improved diagnostic value, allergen microarrays need to be vali-

dated carefully against the complex background of well-defined clinical samples. In order to do so, it will be important to create allergen microarray panels that contain IgE binding epitope repertoires similar or identical to those occurring in the natural disease-eliciting sources (Figure 3A). As long as not all of the relevant allergen molecules are available in sufficient number and quality, a combined approach of microarrays containing selected extracts plus available components may prove most sufficient to achieve a reliable diagnostic tool (Figure 3B). “Universal” allergen microarrays containing the most relevant disease-eliciting sources for screening purposes may represent a primary means to determine region-, population- or disease-specific patterns of allergen-specific IgE reactivity (Figure 3C). Based on the IgE reactivity profiles obtained in the corresponding studies (Figure 3D), novel marker allergens for vaccine development can be identified (Figure 3E) and customised allergen panels can be assembled to create (personalised) diagnostic tests of improved predictive value (Figure 3F). The improvement of specific immunotherapy based on hypoallergenic forms of major allergens (e.g., Bet v 1) has now been pursued for several years and has already resulted in proof-of-concept studies that demonstrate the successful use of genetically engineered allergens for the vaccination of allergic patients [16]. Especially for this type of treatment, the availability of CRD tests employing the same type of molecules may prove beneficial. How-

ever, component-based immunotherapy is still in an early stage of clinical validation, and the first recombinant allergens for therapy are expected in several years only. Nevertheless, microarray-based allergy testing to identify patterns of major and minor allergen reactivities within a comprehensive portfolio of disease-eliciting sources (e.g., pollen, mites, moulds, animal dander, food) may be used to create individual formulations of extracts for specific immunotherapy already today. Subsequently, allergen microarrays could be employed in a "pharmacoproteomics" approach to monitor the efficacy of specific immunotherapy (e.g., by simultaneously measuring the up- or down-regulation of allergen-specific IgE/IgG antibodies in the course of the therapy) or to unveil potential side-effects during the treatment (e.g., by identifying the possible appearance of novel allergen sensitisations). In conclusion, the potential of allergen microarrays for the improvement of allergy diagnosis and treatment will be fully exploited when comprehensive allergen (component) panels have been validated in carefully designed clinical studies and antibody (IgE/IgG) reactivity patterns have been identified that improve the diagnostic and predictive power of these multi-analyte tests.

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