

PROTEIN MICROARRAYS FOR THE DIAGNOSIS OF ALLERGIC DISEASES: STATE-OF-THE-ART AND FUTURE DEVELOPMENT

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SUMMARY: In the emerging field of Proteomics, protein microarrays represent an elegant solution for the simultaneous analysis of the (i) abundance, (ii) function and (iii) interaction of proteins on a system-wide scale. The great power of microarray-based miniature solid-phase immunoassays lies in their potential to investigate in parallel large numbers of analyte pairs while employing only minute amounts of biological sample material (e.g., serum). This has inspired researchers to adopt this approach to the development of novel diagnostic tests with to date published examples in the fields of autoimmune diseases, allergy and cancer. 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.

INTRODUCTION

Microarrays

Following the first development of microarray technology in the early 1990s, miniaturised multiplex assays for the simultaneous analysis of gene-expression analysis and genotyping on a whole-genome scale have become state-of-the-art applications in Genomics-based research (Rhodes & Chinnaiyan, 2005; Syvanen, 2005). 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 (Fig. 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 (Fig. 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) (Fig. 1C). Each of these (usually) μm -sized spots is later employed in a miniaturized 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) (Fig. 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 [Stoll *et al.*, 2005]. Although the concept of miniaturized ligand-binding assays has been described almost 15 years ago [Ekins *et al.*, 1990], current technological solutions to develop protein microarrays have mostly remained in a conceptual state (Labaer & Ramachandran, 2005; Glokler & Angenendt, 2003; Zhu & Snyder, 2003). 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), the type and quality of surface functionalisation (Angenendt *et al.*, 2003) and the (partial) lack of technological solutions for high-quality deposition of large numbers of nl-droplets of biological solutions onto flat substrates in a microarrayed form.

Component Resolved Diagnosis (CRD) of allergy

Type-1 IgE-related allergy is a major health concern in the industrialised world (USA, Europe, and Japan). To date, 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

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of national health care budgets is spent for the diagnosis and treatment of allergy each year (Sennhauser *et al.*, 2005). IgE antibodies have been identified as central mediators of allergic diseases more than 40 years ago (Johansson *et al.*, 1968), and soon after, the first IVD tests for allergy became available based on the detection of IgE in the serum of allergic patients (Wide *et al.*, 1967). 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 the capturing of IgE antibodies in body fluids (Wide *et al.*, 1967). Considerable efforts have been spent by researchers and medical device manufacturers for the continuous improvement of the quality of the allergenic material applied in these tests (van Ree *et al.*, 2004). 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 [Kazemi-Shirazi *et al.*, 2002; Chapman *et al.*, 2000; Thomas & Smith, 1999]. To date, efforts are being made to use genetically engineered variants of the major disease-eliciting molecules to develop improved treatments for allergic patients (Niederberger *et al.*, 2005). 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 [Valenta *et al.*, 1999]. Eventually, this approach has been termed component resolved diagnosis (CRD), as it reduces

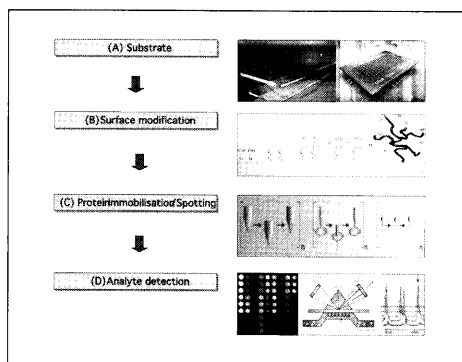


Figure 1: Protein microarray development. (A) Underlying the development of protein microarrays is the choice of a solid support for the stable immobilisation of bio-molecules. The most widely used material is flat glass slides (25x75 mm), whereas supports can also be made of plastic or silicon wafers. (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.

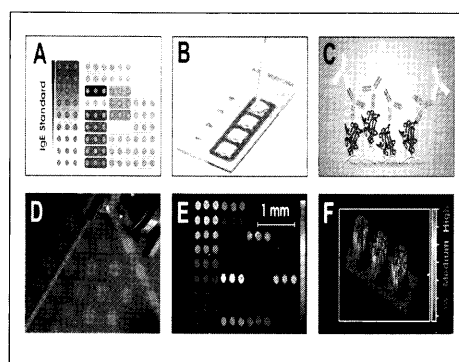


Figure 2: Allergen microarray design and assay procedure. (A) Schematic representation of the allergen microarray layout. Individual allergens of a single biological source are depicted as circles surrounded by a rectangle bearing the same colour. (B) Incubation of a 4-well chip with 4 individual serum samples (20 µl per serum). (C) Schematic representation of allergen microarray assay. Allergen molecules (blue) are covalently immobilized onto the chemically modified bio-chip surface to bind efficiently and in a biologically active manner. Allergens are recognized by serum IgE antibodies (red), which themselves are detected by a fluorescent 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).

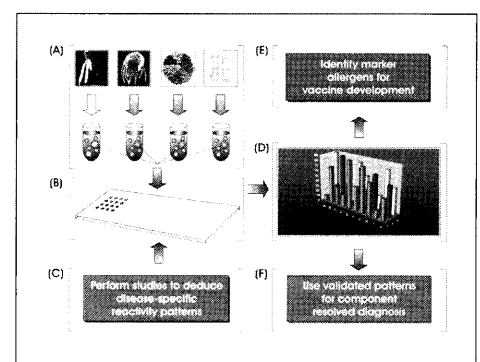


Figure 3: Allergen microarrays in the context of diagnosis and treatment. - Allergen molecules are prepared from the most relevant disease-eliciting natural sources (A) to create comprehensive epitope panels on allergen microarrays (B). These "universal microarrays" can be employed in large-scale clinical 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).

the complexity of natural extracts to a comparatively small number of the actual disease-eliciting molecules. However, using purified or recombinant allergen components imposes 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 (Schmid-Grendelmeier *et al.*, 2003). 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 the addition of fluorescence-labelled secondary antibodies (Deinhofer *et al.*, 2004). 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 x 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. Additionally, serially diluted purified human IgEs are spotted in concert with the allergens for the purpose of assay calibration and quality control (see Figure 2 A and E). Furthermore, each glass slide contains 4 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 whereas it permits the immobilisation of up to 150 individual allergens in a microarrayed form.

■ **Assay procedure and analysis:** Prior to the assay start, each slide containing 4 identical allergen microarrays is washed intensively for 120' to remove non-covalently bound allergens from the microarray-surface. Then, 20 µl of patient's serum is dispensed directly onto each reaction well. Following a 120' 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' (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) whereas a number of bioinformatic quality control procedures are applied [Harwanegg *et al.*, 2004]. By assaying a calibrator serum on each microarray slide containing pre-defined IgE quantities to a number of selected allergens being 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 4 identical allergen microarrays) can be accomplished in 5 hours. However, 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. 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 hundreds allergens in a single analytical step. Yet, the amount of biological material (e.g., patient serum) consumed

for a single investigation is considerably small compared to conventional immunoassays which is beneficial in cases where sample material is limited (e.g., in pediatric allergy testing). In addition, the emergence of printing machines that permit the production of a large number of microarrays from minute amounts of starting material (i.e., allergen solution) at high quality has led to a significant reduction in manufacturing costs. The latter is extremely important for the development of comprehensive allergen microarrays containing a large number of recombinant or purified natural molecules. Additionally, the availability of an increasing number of flexible substrates (e.g., plastic slides containing 10 or more reaction wells or microtitre-plate based microarrays) will lead to an increase in throughput of testing as well as to a further reduction of assay costs. In combination, these advancements are prerequisites for the realisation of large clinical studies as well as for the introduction of allergen microarray based tests into routine-diagnostic settings.

Future aspects

Most importantly, to create an allergy test of improved diagnostic value, allergen microarrays need to be validated 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 (Fig. 3A). An apparent solution to the currently existing lack in comprehensive allergen repertoires is the generation of mixed component/extract based allergen microarrays (Fig. 3B). "Universal" allergen microarrays containing the most relevant disease-eliciting sources for screening purposes may represent a primary means to determine regional, population- or disease-specific patterns of allergen-specific IgE reactivity (Fig. 3C). Utilising the IgE reactivity profiles obtained in the corresponding studies (Fig. 3D), novel marker allergens for diagnosis or vaccine development can be identified (Fig. 3E). In addition, customised allergen panels can be assembled to create (personalised) diagnostic tests of improved predictive value (Fig. 3F). The improvement of specific immunotherapy based on hypoallergenic forms of major allergens (e.g., Bet v 1) has been pursued now for several years. Efforts to translate these scientific successes into practical clinical applications has resulted in early proof-of-concept studies that demonstrate the use of genetically engineered allergens for the vaccination of allergic patients [Niederberger *et al.*, 2005]. Especially for this type of (upcoming) treatment, the availability of CRD tests will prove beneficial. Today, microarray-based identification of major and minor

allergen reactivities in a comprehensive panel of disease-eliciting molecules (e.g., derived from pollen, mites, moulds, animal dander, food) may be used to create individual formulations of extracts for improved specific immunotherapy. To further improve therapy of allergic diseases, allergen microarrays could be employed 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 multianalyte tests.

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BOOK'S REVIEW

Food allergies in children and adults

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■ The book:

➤ Over the past 25 years, the prevalence of food allergy has increased considerably. This disease is often the first step in the life of an allergic patient. If neglected, food allergy paves the way for multi-sensitization to environmental allergens, asthma, allergic rhinitis, serious anaphylactic accidents and even serious acute asthma.

➤ Screening for food allergies is based initially on the clinician's personal expertise. This book - after presenting basic information (definitions, mechanisms, epidemiology) - provides all the keys to effective screening: the frequency of allergens according to age, clinical pictures, which data to record during patient interviews, how to make a food enquiry, allergological testing. The authors give a detailed description of preventive and therapeutic strategies that can be practically applied.

Current practices of monitoring food allergy are documented. Future therapies are briefly discussed.

■ The public :

➤ A tool to help with the diagnosis and routine management of allergy, this book will be extremely useful to general practitioners who are increasingly concerned with this public health issue, and to allergists wishing to base their knowledge on precise and updated data. It will be a useful guide for professionals in the food industry.

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