

Protein chips for biological assays and biosensors

Introduction

In the past few years, protein chips have become a powerful tool for diagnostics and analysis of protein functions and protein-protein interactions. This technology allows fast, easy and parallel detection of multiple addressable elements from a minimal amount of sample in a single experiment. It has been applied to analyse protein and protein-protein interaction as well as antibody-antigen interaction, enzyme-substrate and enzyme-inhibitor binding.¹⁻⁹

Assay systems based on chip technology are currently applied for the identification, quantification and functional analysis of proteins. Protein chip technology is of major interest for proteomic research as well as for diagnostic applications since this miniaturized and parallelized assay systems can be used for the identification of biomarkers and the validation of potential target molecules. However, such technology shows some problems in term of protein stability. These problems are due to the protein structure and functionality. Protein-protein interaction takes place by different means such as electrostatic forces, hydrogen bonds and/or weak hydrophobic Van der Waals interactions.⁸ Moreover, protein interaction does not only depend on their primary structure due to the amino acid sequence, but also on the tertiary structure. For these reasons, an improvement in the efficiency of protein immobilization on the slide surface, in terms of functional conservation and of reproducibility of the amount of immobilized protein is needed.

Proteins can be immobilized onto substrates in a non-covalent interaction by adsorption on glass slides coated with poly-L-lysine,¹⁰ agarose,¹¹ polyacrylamide, hydrogels,¹² with membranes made with polystyrene, poly-vinylidene fluoride (PVDF), thin film nitrocellulose, hydrophobic or hydrophilic surfaces,¹³ or in a covalent manner with functionalized glass surface, such as epoxy group-functionalized slides.¹⁴ A drawback of random covalent immobilization is a partial block of the active sites of the proteins, which become less accessible to the interacting molecules. The covalent binding enhances the stability of immobilized proteins but could be causing changes in the protein conformation after immobilization. Structural modification of the protein can result from the immobilization through interaction between the support and the protein, or from changes in the protein microenvironment. However, an oriented immobilization could provide better spatial accessibilities of active binding sites than non-oriented immobilization. Therefore, the greatest difficulty in the realization of a protein chip is the preservation of functionality of the immobilized proteins.

Initially, the first studies using protein chips were focused on antibodies for antigen screens.¹⁵⁻¹⁷ Glycerol is added to enhance the binding and stability of proteins: this effect has been related to a prolonged hydration of proteins.¹³

In our hand, proteins remained hydrated for longer time during the incubation step, probably because glycerol evaporates slowly. Kunitz-type protease inhibitors, as well as other proteins tested, were diluted at different concentration (0.1, 0.2, and 0.4mg/ml) in NaHCO₃ buffer (0.1M, pH 9) with glycerol (40% w/v), and spotted in duplicate, to find the optimum dilution for each type of assay needs. Usually the first two dilutions were found effective.

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Protein diversity on the array surface is also a cause of variability of results in protein chip studies. Several groups studied protease-protease inhibitor interaction, using multiple dilutions of the probe, to evaluate binding independently of the amount and density of protein bound in each spot.³

Another application of protein chips is the activity-based detection of enzymes on a microarray, such as the screening of protease specificity of inhibitors provided with a fluorescent signal. In this approach, commercial enzymes were immobilized onto epoxy-activated slides and incubated with labelled inhibitors or detected using anti-protease inhibitor antibodies: the formation of inhibitor-bound enzyme complexes was detected by a fluorescence-based microarray scanner.^{4,5,9,18-20} The screening of both enzyme activity and specificity in a chip format has been also successfully performed. A series of peptide substrates conjugated with fluorogenic coumarin bound to aldehyde-activated surfaces²¹ were used to screen enzymatic cleavage and release of coumarin and to find substrate specificity of proteases. In a similar study, different proteases and phosphatases were spotted onto N-hydroxysuccinimide glass slides coated with substrate-modified coumarin to screen for their activities.²²

Using protein chip technology, we used a protein chip system for detection of insect proteases, known major allergens in food and environment, using recombinant potato Kunitz-type protease inhibitors, (KPI) immobilized onto a glass slide, as capture probes for mite proteases.^{2,5,9,18-20} Kunitz-type inhibitors²³⁻²⁵ from wild and cultivated potato, belonging to three homology groups A, B and C, were expressed in *E. coli* and purified. KPI-A group includes inhibitors of aspartic proteinases such as cathepsin D. The group KPI-B contains inhibitors of serine proteinases from chymotrypsin clan, as trypsin, chymotrypsin, elastase and cathepsin B. The KPI group C includes proteins that inhibit bacterial subtilisins and plant cysteine proteinases as well as some non-proteinase enzymes such as invertase and α -amylase. The mite allergens detection system included two protease inhibitors of group A (with anti-cathepsin D and anti-trypsin activity), two of group B (with anti-trypsin and anti-chymotrypsin activity) and two of group C (with anti-cathepsin B activity), in addition to the soybean Bowman-Birk inhibitor. The protein chips were useful in identification of the presence of proteases from *Aleuroglyphus ovatus*, *Tyrophagus putrescentiae*, *Acarus siro*, for mites, and from *Tribolium castaneum* for red flour beetles contamination in environmental samples.

Previously, we used a multi-protease chip in characterisation of new KPIs cloned from wild varieties in the *Solanum* genus.⁹ The Kunitz-type inhibitors were chosen as model to study the interaction protease-protease inhibitors on a glass slide and to set up a protein chip protocol since the inhibitory activity against specific proteases was at first assayed by biochemical tests. The proteases were chosen on the results of protease inhibition specificity using colorimetric assays, and on protease binding using the protein chip format. Subsequently, we developed a protease chip containing 11 different proteases, to perform high-throughput screening of protease inhibitors induced during tuber infection by *Aspergillus carbonarius*.^{2,5,9,18–20} and used KPI-B1 signal intensity as a standard to compare and estimate the level of protease inhibitors expression in response to 24 and 48 hr of fungal infection. As a result, we were able to show differential binding of newly induced ficin inhibitors and differently expressed bromelain inhibitors at later stages of infection.

In comparison to the performance of ELISA method, the resolution of the protein chip for mite allergens was very good even with lower amounts of sample used, and sensibility was increased of 1 order of magnitude in respect to dot blot membrane hybridization (500ng in protein chips, compared to 5µg of proteins in dot blot). Considering 5µg/ml as the sensibility limit of in dot blot detection, and assuming 100µl the volume used during dot blot concentration (in ELISA the micro wells contain similar reaction volumes), it is estimated that the total protein used was equal or higher than 0.5µg. In protein chips, the protein concentration and the total amount coincide, since we load 1µl in 20µl hybridisation buffer. Protein chips allow reduction of hybridisation volumes, with the concentration of the probe on a small surface, so that the total protein amount was limited to less than 10nl, using Spot Array robotic deposition, compared to a larger variability of volumes, 0.7–0.1µl, obtained using the manual Micro Caster spotting system. This resulted in a high reduction of volumes and reagents, since 0.5µg/100µl for ELISA and Dot Blot contain volumes of 100µl, corresponding to 0.5µg of total proteins. Furthermore, the slides shown were printed manually so the use of robotically spotted slides can further decrease the detection limit, by reducing the background signals. This difference can be explained with the higher background caused by antibody binding to membranes, whereas the glass slide showed lower signal-to-noise ratios.

Detection and differentiation of protease allergens was made possible using the protease inhibitor chips. In our hands, the results of this protein chip showed unique protease profiles for each of the species studied. Mite proteases are considered a threat because they can trigger allergy through their interaction with cells of the immune system which are activated by the protease activity on their receptors.²⁶

A system based on protein chips, able to recognise and detect low levels of proteases or protease inhibitors, could be very useful in food safety applications, such as identification of food pathogens.^{2,5,6,9,18–20} in analysis of agricultural biomarkers,^{2,5,9,18–20} as well as in studies of plant proteins and enzymes. Recently a very new field is the race arm involving plants and their pathogens; an array of proteases are needed by plants to set up an immunity response, and several pathogen effectors are targeting them, as protease inhibitors specific to one protease, or inhibitors with broader specificity.^{24,27,28} Therefore, new high-throughput assays have been set up, for screening of hydrolase activity. Activity-based protease profiling (ABPP) is the technique mostly used, enabling to differentiate enzyme activities, their tissue specificity, and roles in various physiological and pathological states. Specific assays for enzyme activity have been developed by means

of click chemistry and biotin-tag fluorescent probes featuring an electrophilic trap. The method is based on chemical probes that react with the catalytic site of different enzymes in an activity-dependent manner. Labelled proteins are detected on 2-D polyacrylamide gels and identified by MALDI-TOF mass analysis.^{9,10,14,15,29} Within these studies, subfamily-specific fluorescent probes for cysteine proteases displaying dynamic protease activities have been validated and exploited for further applications. It is possible to miniaturize the assays of enzymes and proteins on a chip, to screen the presence of proteases and their inhibitors and also to differentiate their specific binding and interactions.³⁰ It is envisaged that novel applications of proteins on chip or array format will continue to attract scientist for the possibility to perform assays in series and in high-throughput format.

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Conflict of interest

The author declares no conflict of interest.

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