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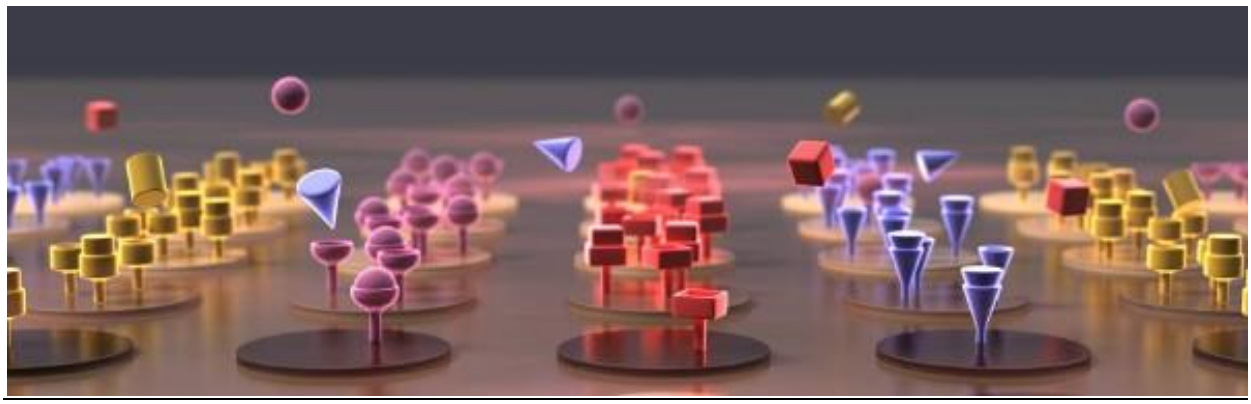

IBIS White Paper

Epitope mapping of protein digests

 # 8.130917

1. Abstract

In this white paper, a new method is presented for discovery and mapping of the immunogenic epitopes of auto-antigens. Antigens containing undetermined antigenic epitopes were subjected to tryptic digestion and then separated using strong cation exchange liquid chromatography. The resulting fractions were then used to create arrays on the surface of surface plasmon resonance (SPR) sensors dedicated for use as part of IBIS' MX96 System. The arrayed fractions were then exposed to a series of patient serum samples containing auto-antibodies or case controls; fractions containing the dominant immunogenic epitopes derived from the auto-antigen digests were then revealed by the resultant response signals.

In the example given, auto-antibodies contained within the sera of Rheumatoid Arthritis (RA) patients were used to identify dominant immunogenic epitopes. Rheumatoid Arthritis is a systemic autoimmune disease characterized by chronic inflammation of the synovial joints. In this white paper, the hypothesis that specific post-translational modifications in proteins may result in auto-immunity was tested. To investigate this possibility, proteins were synthetically citrullinated, digested and then artificially modified peptide-containing fractions were used to create arrays. The auto-antibody binding response generated using just minute quantities of finite patient sera samples showed that LysN- and trypsin-digested peptide fractions independently showed presence of auto-antigens. In particular, the Cit1 and Cit2 peptides and the protein could be a potential new biomarker for RA. Interestingly, there was significant variability between patients in terms of the epitope identity of the protein auto-antigen investigated.

In depth investigations of clinical features including the profiling anti-citrullinated protein autoantibodies of RA patients are now feasible and under further examination. This approach could be extended to investigate the role of citrullination or other post-translational modifications in auto-immune diseases.

2. Introduction

Autoimmune diseases are characterized by the presence of high-affinity autoantibodies directed against self-proteins, such as rheumatoid factor (RF) for rheumatoid arthritis, dsDNA for SLE and SS-A and SS-B for Sjogren's syndrome. Although at least some autoantibodies are known to be involved in cell and tissue damage, their mechanistic role in the pathogenesis of the disease is not fully known. Nevertheless, the specificity and pathogenicity of autoantibody responses highlight their potential as important tools for further mechanistic disease studies, but also for improved diagnosis, classification and prognosis. Miniaturized multiplex assays can deliver a fingerprint of a patient's autoantibody repertoire requiring only a limited amount (3 microliters only) of patient material.

Recently, it has been shown that the so-called citrulline residue, present in post-translationally modified proteins, is a very important antigenic determinant for RA disease and is implicated in the generation of autoantibodies. The peptidyl arginine deiminase (PAD) class of enzymes have been under general scrutiny of autoimmunity researchers: These are transcriptional co-regulators that catalyse the calcium dependent conversion of specific arginine residues, present for example in histone and other proteins, to citrulline. In diseases such as Multiple Sclerosis as well as RA, PAD enzymes seem to be de-regulated. For instance in RA, these enzymes become active when they exit the cell in regions of joint inflammation where they subsequently deiminate arginine residues present in proteins such as vimentin and fibrinogen. The level of PAD activity has been correlated with RA severity.

In this application note, array-based SPR imaging is used to measure the presence of citrullination-dependent auto-antibodies in the serum of a cohort of Rheumatoid arthritis (RA) patients. In particular, human fibrinogen was artificially citrullinated by PAD enzymes and then digested with 3 different proteolytic enzymes (Trypsin, Chymotrypsin and LusN). The digests were fractioned using liquid chromatography, and aliquots used to create arrays for functional interaction studies. The identities of individual peptides revealed by binding of auto-antibodies present in various RA patient samples were definitively identified using mass spectrometry.

The results can be found in a publication [1]. Details of this study can be obtained from Prof. G. Pruijn Radboud University of Nijmegen, Netherlands. Email: G.Pruijn@ncmls.ru.nl

3. Materials and methods

The figure below illustrates the protocol used to create the array.

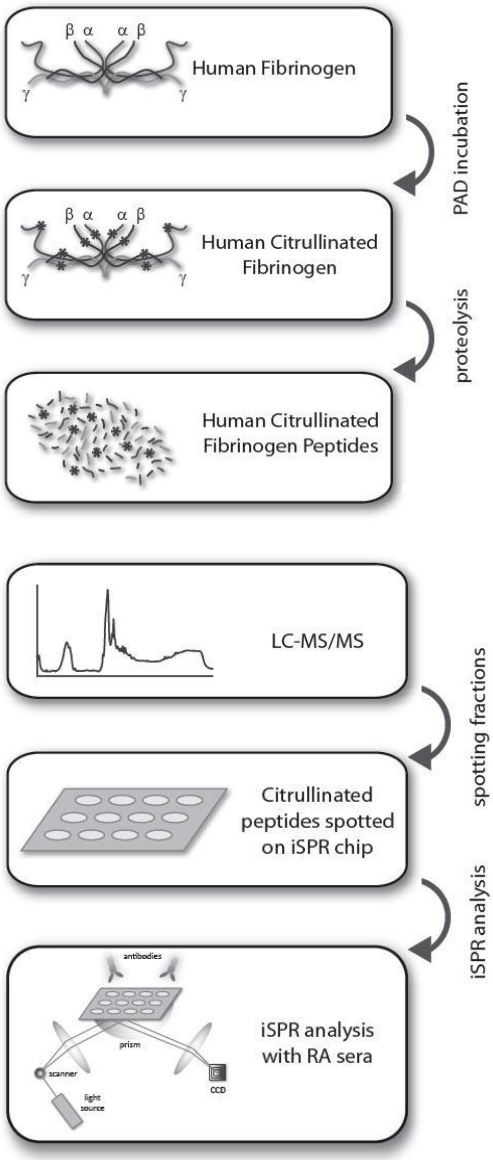

Figure 1. *Human fibrinogen was citrullinated and digested. The peptide digest was separated using LC-SCX and the fractions were deposited using CFM, to create an array on surface of an SPR sensor. RA -patient sera were injected and the responses, indicating the presence of auto-antibodies to peptides contained in the individual fractions, were investigated by imaging SPR.*

Standard protocols were employed to create a 4x6 feature array containing human IgG as well as two different linear citrullinated peptides (Cit1 and Cit2), their two corresponding control peptides (Arg1 and Arg2) each containing arginine instead of citrulline, and the fractionated LC-separated peptide digests. Miniscule quantities (1 nanoliter per feature) were consumed in coupling of constituent peptides using N-hydroxysuccinimide pre-activated polycarboxylate-coated gold sensor surfaces using the

non-contact spotting instrument Top-Spot (Biofluidix. Freiburg, Germany). In summary, a single micro-array contained in total 2 citrullinated petides, 2 arginine controls, 4 IgG spots and 16 digested citrullinated protein fractions. After measuring each patient serum the array was regenerated using an acid step to clean and prepare the sensor for the subsequent serum sample.

4. Results

Figure 3 shows a screenshot generated using the IBIS-SPRint1 software in which, the serum samples are listed – and can be selected/deselected for viewing on the left side, whilst the list on right side identifies the individual features (fractions and controls in this case) for which interaction data is displayed. In this way, each combination of sample and feature can be selected or deselected to make scrutiny of the data easier. Raw data for interactions between 32 samples, serially applied to the array, and 26 features including 2 reference ROI's are shown. This demonstrates the capacity of IBIS' MX96 System to generate >800 sensorgrams in a single fully automated overnight run.

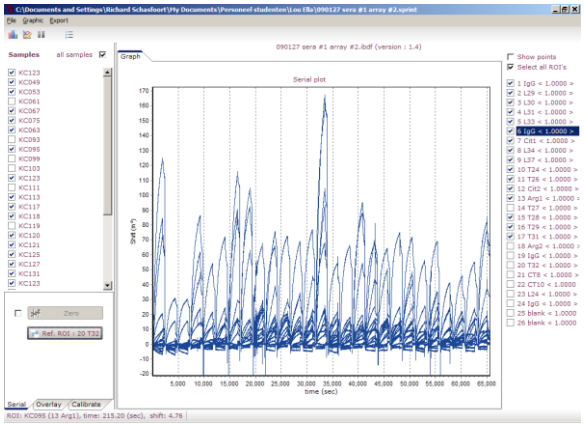

Figure 3. *A serial plot of RA patient sera interactions. Miniscule volumes of serum (3 µl, diluted 1/50) in PBS Tween 0.05% buffer were injected over 180 seconds. Interaction was pursed for 1800 seconds followed by 600 seconds dissociation. After each serum injection, the sensor was regenerated using glycine HCl pH 2.0. The various responses obtained from patient sera in this from this single overnight run can be observed.*

Using the selection/de-selection feature of the IBIS-SPRint software, sample or features can be independently selected for easier analysis of the binding of auto-antibodies to modified peptides in the various digest fractions. Furthermore, the user can define "reference" features for subtracting common mode effects as bulk refractive index shifts. In the following figures the fingerprint of RA patients is shown. Further results have been obtained and are to be found in reference [1].

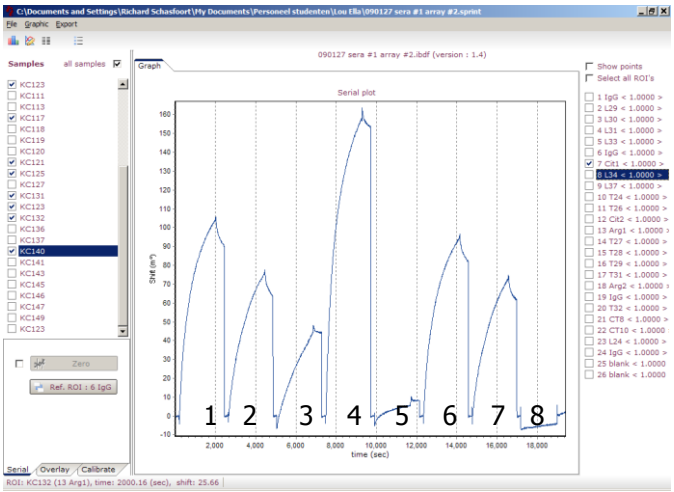

Figure 4. *Responses of 8 RA patient sera to the feature corresponding to Cit1. Patient 5 and 8 show very low (non specific) responses.*

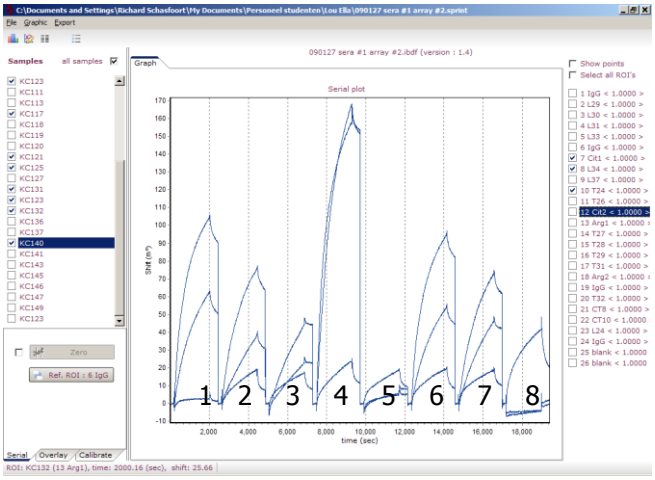

Figure 6. *Responses of 8 RA patient sera to the peptides contained in features corresponding to Cit1 (control), and fractions L34 and T24. Patient 8 has a typical response to fraction T24.*

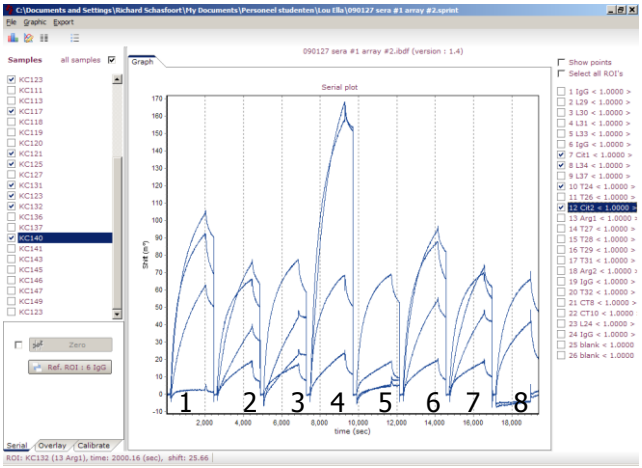

Figure 7. *Responses of 8 RA patient sera to features corresponding to Cit1, L34, T24 and Cit2. All patients have high responses to Cit2, which indicate the presence of anti-citrulline auto-antibodies. However the overall profiles of each of these 8 RA patients is totally different.*

5. Discussion

Autoimmune diseases are characterized by the prevalence of auto-antibodies. Though many auto-antigens have been identified and characterized, to date, most of the assays that have been developed to detect autoantibodies to these antigens are ELISA-based, allowing only separate analyses for each type of autoantibodies, which is laborious, time-consuming. Recent observations revealing the wide range of varying specificities of auto-antibodies for their citrullinated proteins in RA patient sera emphasize the need for multiplex analysis systems [1].

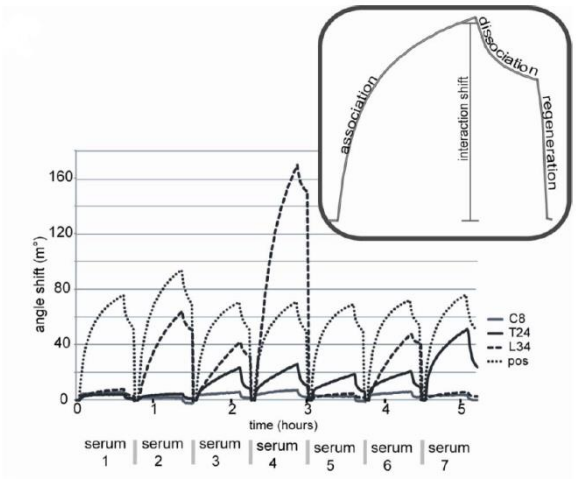

Figure 8 *Analysis of autoantibody reactivity in 3 µl patient sera, extracted from reference [1]. The reactivity of seven RA sera with four different features on the array was determined by iSPR monitoring. Sensorgrams show the reactivity of the sera to citrullinated peptides. The features contained either citrullinated fibrinogen fractions (C8, L34 or T24) or a positive control peptide (pos). In the sensorgrams the angle shifts in millidegrees (m°) are plotted as a function of time.*

In most of the studies which monitor multi-analyte protein-protein interactions, a secondary antibody conjugate has been required to visualize bound antibodies; this has inherent pitfalls (e.g. isotypic and allotypic variation). Here, however, we show that SPR imaging of protein/peptide arrays provides a method that allows a one-step multi-analyte detection of auto-antibodies in patient sera, which does not require additional reagents to visualize antibody-binding. The ability to measure in a fully automated manner with routine liquid-handling procedures increases reproducibility and, once an experiment is started, it can continue unattended. The ligand-containing sensor chips can be efficiently regenerated and re-used again at least 50 times, adding to increased inter-assay reproducibility. The lowest antibody concentration that could be measured was 0.5 pM. The real-time comparison of interactions with different auto-antigen preparations, simultaneously, from a single clinical sample is a great asset particularly when assessing finite samples from longitudinal clinical studies. The many referencing possibilities in a array which avoids channel to channel variations seen with other label-free technologies will reveal and compensate for artefacts typically seen in biomolecular interaction analysis.

Additional features of the IBIS MX96 System that are described elswhere, that are relevant for downstream analyses, include its ability to automate the elution and recovery of captured auto-antibodies. This offers the possibility to maximise, even further, the scientific investigations possible with rare clinical material.

6. Conclusions

Important features and benefits of the IBIS MX96 iSPR instrument are demonstrated which opens up new possibilities for researching into the nature of auto-immune diseases, potentially revealing their mechanisms but also potentially contributing the identification and development of biomarkers that could predict the onset of disease and monitor the impact of treatment regimens on the progression and severity in a number of conditions where citrullination and other protein modifications may be implicated.

The results presented in this application note are supportive of the fact that the IBIS MX96 System is the versatile, tiny sample consuming, robust work-horse of choice to expand the discovery horizon within biomolecular interaction laboratories and proteomics facilities.

7. References

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