Automated cancer marker characterization in human plasma using SURface Plasmon Resonance in Array combined with Mass Spectrometry (SUPRA-MS)

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Abstract

The combination of Surface Plasmon Resonance technology with Mass Spectrometry becomes a key method for the characterization of targeted proteins in the fields of diagnosis and functional proteomics. We demonstrated in this work the ability of our SPRi-chip to capture targeted protein in biological fluids and in situ analyze by MS and MS/MS modes through automated procedure to go beyond classical immunoassays. Here, we established a proof of concept of SUPRA-MS for the detection, the identification and the characterization of a potential breast cancer marker.

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Keywords: Surface Plasmon Resonance; Mass Spectrometry; Immuno sensor; Biomarker; Proteomics; SUPRA-MS; biosensor; chip
1. Introduction

In the field of diagnosis of human diseases very few biomarkers have been validated during the last decade and the development of novel “omics” approaches could be decisive for their characterization in biological fluids and cellular extracts. Classical bioassays are based on antibodies pair to capture and detect targeted proteins but the specificity for the discrimination of protein isoforms or variants in complex media is limitations.

Mass spectrometry (MS) analysis appears as an evolved tool to characterize and identify proteins [1]. Prior MS, a preparative step, as fractionation or protein enrichment, is often required to perform analysis in complex media [2]. Liquid chromatography LC coupled to MS (LC-MS/MS-MS) approaches are used routinely [3-6] but there is a need for more selective and easy-to-use methods especially for clinical applications. A great challenge at the beginning of the 2000’s was the combination between bioassays and mass spectrometry called BIA-MS method for Biomolecular Interaction Analysis coupled with Mass Spectrometry [7-12]. BIA-MS can be realized in two different analytical pathways: MS analysis of eluted biomolecules from the biochip (the off-chip approach) or directly on arrays (the on-chip approach). The off-chip approach has shown intrinsic limitations as lacks of robustness and multiplexing that are limited its spreading in clinical proteomics. At the contrary, the on-chip analysis presents high potential in this prospect but only few studies have really explored its potential [13]. A major drawback was the inability to achieve in situ enzymatic digestion of the captured proteins thus reducing MS analysis only to whole proteins with problem of sensitivity for high mass proteins (>60 kDa). It was only recently that sensitive detections of bond analytes from ideal mixtures have been efficiently coupled with their unambiguous identifications by MS and MS/MS [14-15]. However, BIA-MS analysis will impact in this field once it could be efficient in complex biological fluids like immuno-MALDI-MS technology [16-19].

In this paper, we described, for the first time, a complete procedure of “on-a-chip” combination of Surface Plasmon Resonance imaging in Arrays experiments with Mass Spectrometry analysis entitled “SUPRA-MS”. Briefly, our developments consisted in the conception and the realization of home-made gold chips presenting a SPRi-array of 16 spots compatible with commercial SPRi apparatus. Real time and sensitive detection of a potential marker of human breast cancer, the LAG-3 protein [20], spiked in human plasma is performed without any labeling. Then, the SPRi-chip undergoes an automated protocol of in situ reduction step, enzymatic digestion and chemical matrix deposition prior mass spectrometry analysis. Finally, such treated proteins were characterized and identified by peptide mass fingerprints (PMF) and MS-MS analysis at the femtomole level.

2. Design of the biochip

2.1. Substrate

SPRi-Plex II instrument (Horiba Scientific/GenOptics, Chilly Mazarin, France) was used to monitor the antibody-antigen interactions on the biochip surface. This instrument is based on surface plasmon resonance imaging phenomenon and is composed of an optical bench, a CDD camera and a fluidic system. The whole process of SUPRA-MS analysis takes place on disposable and highly sensitive biochips, their production at low cost is a prerequisite. They are based on standard glass slides (borosilicate, optical index 1.51, 12.5 x 26 x 0.3 mm from agar). A DC magnetron sputtering from Plassys (pressure of 7 µbar of argon and an intensity of 0.3 A) at the technology center “MIMENTO” (Besançon, France) was used to deposit on the glass slide thin layers of chromium (Cr) and gold (Au).
The thickness of Cr and Au were respectively 2 nm and 48 nm. The thickness of the gold layer and roughness (RMS 0.55 nm) was controlled by Atomic Force Microscopy (AFM).

Homemade SPRI-chips present the same characteristics as the commercial chips in terms of sensitivity with around 69 %/°. However, the homemade chip was thinner than the commercial chip (300 μm against 500 μm for a commercial chip) which induces its deformation when deposited onto the prism for SPR analysis. In order to evaluate the mechanical stress effect on the homemade chip, the plasmon signal of the gold chip was measured (Fig. 1A). The deformation leads a loss of sensitivity, homogeneity and a significant baseline drifts (0.1 %/min) (Fig. 1B). To avoid this problem, we used an etched prism in its center (7 μm deep) provided by Horiba Scientific that reduces the mechanical stress on the slide in the SPRI-Plex apparatus. The optical coupling between the prism and the chip is obtained using oil with an optical index of 1.67. In this configuration, the gain in homogeneity is about 25 % (Fig. 1C) and the baseline drift, measured at 0.005 %, is very low.

2.2. Bio functionalization

In the field of biochip, small areas of analysis called spot are necessary at low, medium or high densities depending of the applications. The miniaturization of spots leading to micro-array format was historically developed for DNA chips and take benefit from the robustness of nucleic acids. However, the development of protein chips suffers from the fragility of proteic entities like receptors, enzymes or
antibodies mainly when they are based on spotting processes (discrete fluidics in comparison with lateral flow devices).

As a proof of concept, we developed a SPRi-chip composed of spots of antibody α-LAG3 (courteously provided by Immutepe SA) and spots control antibody α-RSA (purchase from Sigma-Aldrich). To achieve 16 spots in macro-array (about 1 mm in diameter) format on a surface of 64 mm², we have designed and realized a mechanical guide (Fig. 2A). It helps to guide the micropipette tip (Fig. 2B) allowing the accurate deposition of droplets of 300 nL according to a specific pattern (pitch: x = 2.3 ±0.1 mm, y = 2.1 ±0.1 mm) that will permit an automatic MS analysis of the chip. The mean diameter of obtained spots was about 1.48 mm ±0.12. The mechanical guide includes water reservoirs and a confined enclosure to keep hydrated antibody droplets. Ultrasonic activation of the droplets was used during incubation to lead internal agitation which improves the homogeneity of grafting inside spot as demonstrated with acoustic devices [21-22]. These parameters allowed homogeneous pattern of spots (Fig. 2C) without showing classical biases as coffee-ring effects and aggregation observed during classical spotting process.

![Fig. 2 (A) Mechanical guide to deposit 16 drops of 300 nL each in a matrix 4 x 4 in less than an 0.64 cm²; (B) Principle of the mechanical guide; (C) SPR image of the chip surface before spiked LAG3 protein injection showing four α-LAG3 IgGs spots (number 1-4) and two α-RSA IgGs spots (number 5 and 6); (D) graphic representation of the biomolecular architecture of the LAG3 biosensor.](image-url)
Prior spotting with the mechanical guide, the gold chip was chemically functionalized (Fig. 2D) by immersion over night in a solution composed of a mixture of 11-MUOH and 16-MHA (97/3 by mole, 1mM in absolute ethanol, from Sigma–Aldrich) which was previously sonicated for 10 min using an Elma sonicator (power: 90 W, frequency: 50/60 Hz). Such self assembly monolayer (SAM) is highly stable and useful for the grafting at the surface of a gold chip of nucleic acids, protein receptors and glycosylated proteins [23-25].

The functionalized gold chip was rinsed with ethanol and ultrapure water and dried. The activation of carboxylic groups was performed by covering the chip with an EDC Sulfo-NHS solution for 30 min then rinse with ultrapure water and air dried. N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased from Biacore (GE Healthcare, Sweden) and N-Hydroxysulfo succinimide (Sulfo) from Sigma–Aldrich.

\[ \text{I-LAG3 and I-RSA IgGs were prepared in sodium acetate buffer (10mM, pH 5.2 and 5.5 respectively). The spotting was performed through a homemade mechanical guide and the incubation time was 60 min into an ultrasonic water bath (Elma, frequency: 37 kHz, power: 30 %). Excess antibody solutions were removed and the biochips were rinsed with ultrapure water. A solution of Rat Serum Albumin (RSA, 40 \mu g/mL purchase form sigma Aldrich) was deposited over the entire surface of the chip as a blocking agent for the entire surface of the biochip. After 30 min incubation, the chip was rinsed with water and the remaining free ester reactive groups were deactivated by ethanolamine (HCl, pH 8.5, 1M from Biacore, GE Healthcare, Sweden) for 30 min and then rinsed with water and air dried.} \]

3. SPRi Analysis

Then, the biochip was inserted in the SPRi-Plex II instrument to perform experiments in a running buffer composed of Phosphate Buffered Saline, (10 mM at pH 7.4 with NaCl (138 mM), KCl (2.7 mM)) and tween 20 (0.05%) at 50\mu L/min. Three families of region of interest (ROIs) were selected to record the plasmon signal (Reflectivity Variation: %RV). The first two families of ROIs were selected inside the spots of I-LAG3 IgGs (I-LAG3 arrays) and on spots of I-RSA IgGs spot (control arrays). The third family was a selection of ROIs outside IgGs spots named control surface. ROIs had a diameter of 1mm located at the center of each spots where the MS analysis will take place.

A LAG3 protein injection was performed at 10 nM in total human plasma at 2.5% for 10 min at 20\mu L/min (Fig. 3A) then followed by an injection of detergent (Octyl-\beta-D-Glucopyranoside, Sigma-Aldrich) at 40 mM for 30s at 50 \mu L/min. The global procedure includes a pulse of detergent that allows better homogeneity of the responses all over the SPRi-array and slightly improves the signal to noise ratio. To remove salts from the biochip surface, an injection of ultrapure water was performed at 50 \mu L/min and the chip is extract from the apparatus at this stage. We note at the end of analysis 5.85 ±0.4 % of RV on I-LAG3 arrays, 0.42 % of RV on control arrays and only 0.27 % ±0.03 of RV on control surface. The antigen surface density was deduced from the SPRi measurement experimentally proved that the variation in reflectivity AR can be considered as a linear function of antigen density, since the thickness of the immobilized antigen layer is small compared to the evanescent wave penetration depth [26]. By applying the following conversion:

\[ \Gamma (\text{pg / mm}^2) = 184.21 * \Delta R(\%) \] (1)
α-LAG3 spots captured an average of around 1780 pg/mm² corresponding to 6.7 fmol/mm² of LAG3 protein per spot. The signal to noise ratio up to 20 shows the specificity of interaction. The differential image (Fig. 3B) shows the good inter & intra homogeneity of spots in bound proteins. The non-specific induced by plasma on α-LAG3 arrays was evaluated by injecting human plasma diluted 40 times without the protein of interest (Fig. 3C). We note 1 ±0.6% of RV spots on the α-LAG3 arrays and 0.26 ±0.14% of RV on control arrays. The quantity of non-specific on the biochip surface was 0.12 ±0.08% of RV corresponding to adsorption of 22 pg/mm² of plasmatic protein demonstrates that architecture of chemistry is perfectly adapted to work in complex media.

Fig. 3 (A) SPRi kinetic curves recorded from α-LAG3 arrays, control arrays and the chip surface upon LAG3 protein injection (10 nM) in human plasma (dilated 40 times), followed by detergent injection; (B) SPR image recorded from SPRi-chip after injection of LAG3 protein in human plasma and detergent injection; (C) Comparison of the results (average of three chips) after injection of LAG3 protein (10 nM) in PBS, human plasma (dilated 40 times) and LAG3 protein (10 nM) in human plasma (dilated 40 times).

4. MS Analysis

4.1. Prior treatment MS

Peptide mass fingerprinting is an analytical technique for protein identification in which MS is used to measure the masses of proteolytic peptides. The captured proteins are treated with the ImagePrepTM station (Bruker Daltonics) prior to MS analysis.

This tool is used to treat biological tissues (histological samples) before mass spectrometry imaging analysis [27]. Using a porous metal membrane in vibration, it is possible to spray chemical solutions onto a target surface in a nitrogen atmosphere. To avoid spot-to-spot cross contamination, the duration and the frequency of the spray were controlled leading to small droplets (~20 μm). Three different steps were applied: a reduction step (TCEP 10 mM in 0.1 M NH4HCO3, Sigma Aldrich) followed by a tryptic digestion step (Trypsin 30 ng/μL, Gold Mass Spectrometry Grade, PROMEGA) both at 37°C and the
matrix deposition step (HCCA, in 50/50 v/v water/acetonitrile with 0.25%TFA) at room temperature. Adequate layers were applied to ensure homogeneous coverage at each step.

4.2. MS ans MSMS analysis

The biochip was placed into the UltrafleXtreme™ MALDI-TOF (Bruker Daltonics) using a homemade target designed for SPRi-chips. The automatic spectra acquisition was possible by setting in the acquisition FlexControl software (Bruker Daltonics) the coordinates of spots defined by the mechanical guide.

The acquisition of spectrum was performed using UltrafleXtreme™ MALDI (with a smartbeam-II laser) in MS and MS/MS reflectron mode. MS automatic measurements were performed using an AutoXecute method, an acceleration voltage of 25 kV and a pulsed ion extraction of 80 ns has been applied and each mass spectrum results from an average of 4000 laser shots in random mode around the center of each spot. The MS/MS measurements were performed in semi-automatic mode, with the source acceleration voltage set at 7.5 kV and a pulsed ion extraction of 60 ns. Each mass spectrum obtained, resulted from an average of 2000 laser shots in the parent mode and 4000 laser shots in the fragment mode.

A local Mascot server (Mascot version 2.2.01; Matrix Science) and Swiss-Prot TrEMBL database were used for protein identification based on MS or MS/MS spectra with the following parameters: Mammals, trypsin digestion and one missed cleavage site. The mass tolerance in the MS mode was set at 50 ppm. Mass tolerances of fragments in the MS/MS mode were set at 30 ppm.

Thanks to the biochemical automated treatments through the ImagePrepTM station (Bruker Daltonics), MS analysis generated peptide mass spectra on a range from 900 to 4000 m/z which showed the efficiency of the automated and collective procedure. The identification of LAG3 by PMF was obtained with, 13 peptides matched with LAG3 protein, a significant Mascot score of 101 and was validated by MS/MS analysis (Fig. 4A, insert) on the peptide m/z 1422.69 with a significant mascot score (25.26). The number of peptide detected and the sequence coverage (SC = 37%, data not shown) fit with the results of classical MS analysis after in-gel digestion. The results of the SPRi-chip analysis were summarized on the Figure 4B showing 100% of identification of LAG3 protein on each α-LAG3 spots. None of the LAG3 peptides were detected on α-RSA spots nor on the surface which increases the specificity of the analysis. Moreover, the low non-specific information on the surface has allowed the identification of the RSA protein with 16 peptides matched and a significant mascot score of 160.

The robustness of the SUPRA-MS platform has been demonstrated on 3 SPRi-chips (including 40 spots) leading to the capture of 4.87 ±1.49 fmol/mm² of LAG3 protein and the detection of 13 ±0.5 peptide per spot contributing to 100% identification of the target protein with a significant mascot score of 87.9 ±2.4.
**5. Conclusion**

Here, we established a proof of concept for the detection, the identification and the characterization of a potential breast cancer marker, LAG3 protein, spiked in human plasma. We validated total “on-chip” procedure based on biosensing using SPR and identification by MS in biological fluids with high robustness. Reliability of the biochip establishment procedures, the high S/N ratio of the biodetection and the stability of the automated procedures prior and during the MS analysis gave confidence on the emergence of new analytical solution entitled SUPRA-MS. In addition to being a new diagnostic tool in clinical study based on known biomarkers, SUPRA-MS could open a promising way to the discrimination of variant proteins involved in numerous human diseases.
Acknowledgment

The authors thank other members of our laboratory for help and fruitful discussions. The authors would like to thank Dr. Frédéric Triebel (from Immutep SA) for providing A9H12/LAG3 model and the technological “MIMENTO platform” (Besançon, France). We are grateful to Bruker Daltonics, especially Y. Hebert for his technological support. This study was supported by grants from HORIBA-Scientific, the ministry of Health and Research, Conseil Regional de Bourgogne and the Franche-Comté University.

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