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Revisited BIA-MS combination: Entire "on-a-chip" processing leading to the proteins identification at low femtomole to sub-femtomole levels

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ABSTRACT

We present the results of a study in which biomolecular interaction analysis (BIA, Biacore™ 2000) was combined with mass spectrometry (MS) using entire "on-a-chip" procedure. Most BIA-MS studies included an elution step of the analyte prior MS analysis. Here, we report a low-cost approach combining Biacore analysis with homemade chips and MS *in situ* identification onto the chips without elution step. First experiments have been made with rat serum albumin to determine the sensitivity and validation of the concept has been obtained with an antibody/antigen couple. Our "on-a-chip" procedure allowed complete analysis by MS/MS^2 of the biochip leading to protein identifications at low femtomole to sub-femtomole levels. Using this technique, identification of protein complexes were routinely obtained giving the opportunity to the "on-a-chip" processing to complete the BIA-MS approach in the discovery and analysis of protein complexes.

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1. Introduction

Many cutting-edge technologies are available in proteomics but the combination of two or more technologies will help overcome major drawbacks in analytical methods. Some of these combinations such as liquid chromatography (LC) with mass spectrometry (MS) giving LC–MS and LC–MS–MS approaches are used routinely today. Recently, new methods combining biosensors with MS have been explored. Out scope of passive surface which provided no information on the biological events that occur at the surface of the chip, the last decade has seen an increasing interest in intelligent surfaces mainly based on surface plasmon resonance (SPR) which allow monitoring of biorecognitions and captures in real time without any labeling (Nilsson et al., 1995; Nieba et al., 1997; Frederix et al., 2003; Grosjean et al., 2005). Few groups have developed the combination of SPR devices with MS leading to a new concept called BIA-MS for biomolecular interaction analysis-mass spectrometry (Natsume et al., 2000; Nedelkov and Nelson, 2000; Lopez et al., 2003; Catimel et al., 2005; Larsericsdotter et al., 2006; Bouffartigues et al., 2007). Briefly, this combination of instruments has led to better global proteomic analysis by combining qualitative (kinetics of interactions) and quantitative (amount of bound proteins) information provided by SPR with the structural features of the proteins thanks to MS. The first step of this analytical method provides intact proteins immobilized on a chip. The proteins can then be analyzed further using MS, either directly on the chip or after an elution step. The main approach deals with elution of bound materials, which slow down the sensitivity. In order to overcome major drawbacks of this elution way (rate of eluted protein of interest, dilution of the sample, loss of materials and incompatibility between the elution buffer used and the MS, reproducibility, etc.), very few studies have explored direct digestion of the immobilized proteins, which are *in situ* analyzed using MS after SPR experiments (Grote et al., 2005). From our knowledge, this approach has not been yet successfully explored and some drawbacks must be overcome as the conception and realization of low-cost SPR chips, the optimization of pre-treatments prior to MS analysis, the global sensitivity of this approach. So, if the combination of SPR with MS has great potential in functional proteomics, the association of two techniques still presents a challenge (Buijs and Franklin, 2005).

In this paper, we described an efficient "off-line" method leading to an optimize combination of SPR experiments with MS analysis on the same chip. Biological models are rat serum albumin (RSA) for the establishment of the procedure and sensitivity optimization and LAG-3 protein, a specific marker of human breast cancer and tuberculosis for validation of this approach for the characterization and identification of protein complexes (Triebel et al., 2006). Our developments consisted in the conception and the realization of homemade protein chips compatible with SPR analysis. Then we presented complete on-a-chip treatments prior to MS analysis with a homemade MALDI plate. Finally, all the proteins were completely

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identify by peptide mass fingerprints (PMF) and MS–MS analysis at the sub-femtomole to femtomole levels.

2. Materials and methods

2.1. Reagents

Rat serum albumine, DiThioThreitol (DTT) NH_4HCO_3, acetonitrile, TriFluoroAcetic acid (TFA) were obtained from Sigma–Aldrich. HCCA matrix was from Bruker Daltonics (Bremen, Germany). Absolute ethanol was from Carlo Erba. HBS buffer was purchased from Biacore (GE Healthcare).

Ultrapure water was produced with an Elga Instrument.

2.2. Design and fabrication of homemade chips

First, a 2-nm thick chromium (Cr) layer was deposited on a SiO_2 wafer (width: 13 mm; thickness: 0.17 mm from AGAR) with plasma sputtering technology to optimize the adherence of gold to the substrate. The 40 nm thick Au layer was deposited onto the top of the Cr layer using plasma-sputtering technology. The deposition time and the argon flow pressure were optimized to obtain the theoretical thickness. The deposition time for the Cr and Au layers were, respectively 3 and 22 s. For all depositions, the argon flow pressure and current were, respectively 7 μbar and 0.3 A. With these parameters, the deposition rates for the Au and Cr layers were, respectively 109 and 60 nm/min.

2.3. Chemical functionalization and SPR experiments

The homemade chips were chemically functionalized as follows:

For the RSA chip, a solution of octadecyl mercaptan (OM), 1 mM in an ethanol/water solution (4/1 by vol.), was sonicated for 20 min using Elma (power 90 W, frequency 50/60 Hz), at maximal power. The sensor chips were cleaned in baths of absolute ethanol then treated overnight and rinsed with ultra pure ethanol and water. Ultrapure water contact angles were measured with a goniometer system (DIGIDROP by GBX, France) with an accuracy of $\pm 2^{\circ}$. All measurements were performed in the ambient atmosphere at room temperature.

For the immuno-chip, we used a self-assembled monolayer composed of a mixture of 11-mercapto-1-undecanol (11-MUOH) and 16-mercapto-1-hexadecanoic acid (16-MHA) (purchased from Sigma–Aldrich). The mixture of 11-MUOH/16-MHA (97/3 by mole) at 1 mM in absolute ethanol was sonicated 10 min using a sonicator Elma (power 90 W, frequency 50/60 Hz). LAG-3 protein is provided by Immutep SA (Châtenay Malabry, France) and is known as a prognostic indicator for survival of primary breast carcinomas. Monoclonal antibody A9H12 recognize specifically LAG-3 protein and dimeric LAG-3 recombinant protein used for this study. Their molecular masses are 160 kDa both.

Biacore experiments were performed with the Biacore™ 2000 apparatus at 25 °C with a flow rate comprise between 2 and 30 μl/min. According to the experiments, the running buffer was either ultrapure water (Rathburn), saline phosphate buffer (PBS, 100 mM at pH7.4 with NaCl 50 mM) or HBS buffer. The degree of protein immobilization and the level of interactions in the Biacore technology apparatus were plotted on a sensorgram (response unit (RU) versus time (s)). One thousand RU correspond to a shift in the resonance angle of 0.1°. Calibration of the apparatus gives a correlation between the shift in angle and the mass, ranging from 0.1 to 1 ng/mm^2, deposition on the surface of the biochip (Stenberg et al., 1991). After exposure to the analytical solution, the chips were then removed from the biosensor unit via undock procedure with empty flow cell command.

2.4. On-chip digestion

To identify the bound protein on the sensor chip, the proteins were reduced with DTT (10 min) and digested with trypsin. On-target tryptic digestion was performed by depositing 1 μl of 5 μg/ml trypsin (Trypsin Gold Mass Spectrometry Grade, PROMEGA), dissolved in 100 mM NH_4HCO_3, pH 8.2, onto selected sample spots. During digestion, water was added and the chip was kept at 37 °C for 10 min according to Terry's procedure (Terry et al., 2004). After digestion, the spots were dried and 1 μl saturated HCCA in 30% acetonitrile/0.1% TFA was added to each spot using a pipette for RSA analysis and the matrix was not saturated (3.5 mg/ml) in 50% acetonitrile/TFA 0.1% in the case of protein complexes. To calibrate the mass spectrometer, standard peptides (pepmix standard peptide from Bruker Daltonics) were deposited onto the sample spots of the chip. The global pre-treatment is no longer than 30 min, which warrants an entire BIA-MS study in few hours.

2.5. MALDI-TOF mass spectrometry

The chips were then introduced in a mass spectrometer MALDI-TOF (Ultraflex, Bruker Daltonics Gmhb, Bremen, Germany), using a homemade MALDI target. Ionization was performed in MS and MS/MS (PDS-LIFT technology) by irradiation of a nitrogen laser (337 nm) operating at 50 Hz. Data were acquired at a maximum accelerating potential of 25 kV in the positive and reflectron modes. The MALDI mass spectra were calibrated using the Peptide Calibration Standards from Bruker Daltonics.

The software packages Ultraflex version 3.0, Flex control, Flex Analysis and Biotools version 3.1 were used to record and analyze the mass spectra. The database search was performed with Mascot (Matrix Science, London, UK) in the NCBI database. Peptide tolerance was set at ±50 ppm in MS and ±0.4 Da in MS/MS. Methionine oxidation was accepted as a variable modification.

3. Results and discussion

3.1. Proof of concept of entire "on-a-chip" BIA-MS analysis of RSA at sub-femtomole/mm^2

Crude gold chips were chemically functionalized with OM in order to confer the same properties than the HPA sensorchip® (provided by Biacore). The self-assembled monolayer (SAM) process, obtained using a previously published procedure (Boireau et al., 2002), leads to the formation of a packed monolayer of OM, which confers a high degree of hydrophobicity to the chip (Fig. 1A). This was confirmed using contact angle measurements as summarized in Fig. 1B, the average angle formed by small water drops onto the SAM reached $107 \pm 2^{\circ}$, which is close to the value expected for such a substrate (Kawasaki et al., 2000). Hydrophobicity is the driving force, which allows the adsorption of a large number of biological compounds (proteins, nucleic acids, lipids, etc.). In our study, the OM layer was used as hydrophobic matrix to adsorb RSA. The adsorption process was followed in real time by SPR measurements. Briefly, the functionalized homemade chip was inserted into the Biacore apparatus: the response in water at 25 °C and 30 μl/min was measured. In order to prepare the surface for adsorption process, it was cleaned with a pulse of organic solvent, ethanol (50% in water), followed by a pulse of detergent, OG (40 mM). Following this procedure (i) various concentrations of RSA were injected onto the chip, (ii) the level of adsorption in real time was measured and (iii) the injection was stopped when the signal reached the expected values. The sensorgrams of the experiment are presented in Fig. 1C and D. At high concentrations of RSA (i.e. above

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