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Application Note

Rapid coupling of Surface Plasmon Resonance (SPR and SPRi) and ProteinChip™ based mass spectrometry for the identification of proteins in nucleoprotein interactions

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The coupling approaches of SPR to LC-MS and ProteinChip™-based mass spectrometry (SELDI) as a means of identifying proteins captured on DNA surfaces has been compared. The potential to allow multiple, quantitative analysis of macromolecular interactions followed by rapid mass spectrometry identification of retained material has been studied on the bacterial nucleoid protein H-NS. A number of H-NS responsive promoters have been shown to contain regions of intrinsic DNA curvature located either upstream or downstream of the transcription start point. A working model for H-NS binding has been proposed invoking differential binding to relatively high and low-affinity sites. Consequently, differential H-NS binding to immobilized DNA containing high and low-affinity sites has been studied by SPRi and, then recovered and identified by mass spectrometry.

Materials and methods

1) Biotinylated DNA probes produced by PCR

For H-NS:
- 5A6A (223-bp fragment with 75-bp curved sequence)
- 1A (220-bp fragment non-curved sequence)
- proU (372-bp fragment containing 200-bp region known to bind H-NS).

For IHF:
- 205-bp fragment of the paca236.

2) Functionalization of SPRi-Biochip™ with extravidin

The gold surface of SPRi-Biochip™ was treated with 11-mercaptoundecanoic acid (MUA) coupled to a layer of poly(ethylenimine) to which extravidin was covalently attached.

3) Immobilization of biotinylated DNA probes

Biotinylated DNA probes were spotted through the biotin–extravidin interaction. The singly 5'-end labeled DNA fragments containing the proU, 5A6A and 1A sequences were immobilized on extravidin functionalized SPRi-Biochip™ by direct spotting of 10µg/ml solutions of each DNA fragment.

4) SPRi experiments

Spotted SPRi-Biochips™ were inserted in the SPRi–Plex™ apparatus and washed by a short (10µL) injection of 1M NaCl followed by continuous flow (50µL/min) with running buffer. Various concentrations of protein were injected across the surface. The surface was regenerated with an injection of 10µL of NaCl (1M) which removed any bound H-NS. H-NS (250 nM) was then reinjected at 50µL/min and after 2 min into the dissociation phase the flow was stopped, the surface removed from the apparatus and air dried.

5) iProtein recovery from the SPRi–Plex™ and SELDI measurements

Each spot was treated by the addition of 1 µL of NaCl (1 M). The spots were allowed to sit for 30s then the solutions recovered and spotted onto a ProteinChip™ H50 hydrophobic surface and left to incubate for 1h in a humid chamber to avoid evaporation. Excess liquid was removed from each of the spots which were then washed by the 2-fold addition of 1µL of distilled water. The spots were then dried, co-crystallized with 1µL of a 20% solution of matrix and read in a SELDI ProteinChip™ reader.

These same samples have been studied using the Biacore 2000® instrument and protein recovery has been analysed on LC-MS instrument (data not shown).

Results and discussion

1) Relative comparison of binding using the Biacore and SPRi–Plex™ approaches

Two SPR technologies have been compared. The first involves a Biacore 2000® based approach where a solution flows through a microfluidic interface across four dextran coated gold surface on a biosensor chip. The second used the SPRi-Plex™ in which a microcuvette, in contact with a sensor surface, is used to introduce macromolecules by continuous flow to ligands immobilized on the sensor surface.

Three different DNA fragments containing the proU, 5A6A and 1A sequences have been spotted to pre-treated extravidin SPRi-Biochip™. At time t=0s, a solution of H-NS (250nM) was injected at 50µL/min through the microcuvette and an image was recorded every second during the association and dissociation phases (Fig. 1). During the dissociation phase, H-NS rapidly dissociated from the DNA surfaces containing the 1A fragment.

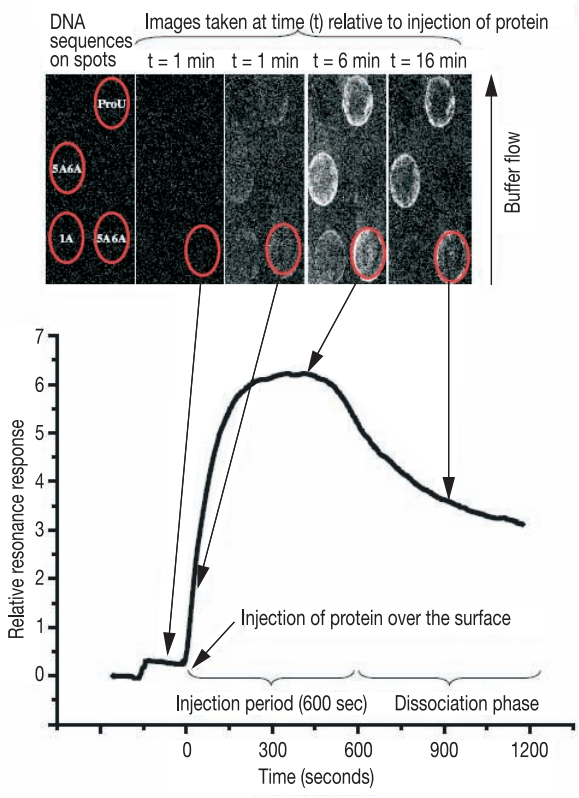

Figure 1:
1) Difference Images of functionalized SPRi-Biochip™ containing immobilized DNA fragments. The spots have been circled in this representation with the name of the respective DNA fragment in each circle.
2) Kinetic curve of the binding of H-NS (500 nM) to the prism surface. The arrows show the image associated with a specific time point on the curve.

A single binding site model for the 5A6A containing fragment allowed calculation of apparent rate constants and of an equilibrium dissociation constants of 3.7 10^{-8}M, that were similar to those calculated on the BIAcore surface (2.7 10^{-8}M). Since H-NS is generally perceived to bind in a sequence non-specific manner with a preference for bent DNA, they decided to look at the binding of IHF, a heterodimeric *E.coli* protein that binds several specific DNA sequences. DNA fragments containing a single IHF binding site have been immobilized to both BIAcore and SPRi–Plex surfaces. After injection of IHF, binding constants were measured (Fig. 2).

Technique	k_d (s^{-1})	k_a ($M^{-1}s^{-1}$)	K_d (M^{-1})
Biacore	4.8 ± 3.0 x 10^{-3}	1.7 ± 0.9 x 10^{5}	2.6 ± 0.3 x 10^{-8}
SPRi-Plex™	3.1 ± 0.5 x 10^{-3}	1.6 ± 0.4 x 10^{5}	2.0 ± 0.7 x 10^{-8}

Figure 2: Apparent kinetic parameters for IHF binding to immobilized DNA fragments on Biacore and SPRi–Plex™ surfaces

The kinetic parameters of binding of IHF to immobilized DNA were the same, independent of the surface or the SPR technology used, in agreement with what we observed with H-NS. These values are in full agreement with those in the literature (22).

2) Recovery of material from SPR and SPRi surfaces for mass spectrometry analysis

A fresh Biacore sensorchip surface was prepared with immobilized DNA containing the 5A6A fragment and H-NS (500 nM) passed across the BIAcore apparatus. The material was collected on a reverse phase column, desalted and analysed directly on line by an electrospray mass spectrometer (Analytica of Branford). Results indicated that it is almost impossible to identify m/z values indicative of H-NS alone due to the presence of Tween in the elution buffer (data not shown).

A different approach using SPR imaging coupled with SELDI has been studied.

A surface was prepared using the SPRi–Plex™ apparatus with the configuration shown in Figure 3 with DNA fragments

containing 5A6A [Fig. 3 (i)], proU [Fi.3 (ii)] and 1A [Fig. 3 (iii)], but this time the flow was stopped after 2 min into the dissociation phase. The surface was removed and air dried. Each spot was then treated with 1ml of a solution of NaCl (1 M),

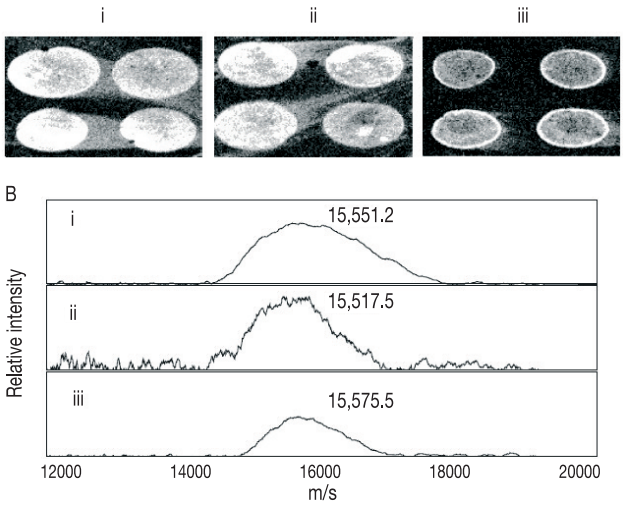

Figure 3: Recovery of material after SPRi for mass spectrometry analysis. Difference images of H-NS retained on SPR-Biochip™ surface by immobilized DNA. (i) 5A6A sequence (ii) proU sequence (iii) 1A sequence; B) Mass spectra of proteins removed from this surface, adsorbed onto a H4 ProteinChip™ array and read on a ProteinChip™ reader (SELDI): (i) 5A6A sequence (ii) proU sequence (iii) 1A sequence.

the material from four similar spots was pooled and the salt solution transferred to and adsorbed onto a H4 hydrophobic ProteinChip™ surface (Ciphergen). The air-dried ProteinChip™ surface was then analysed using a SELDI ProteinChip™ reader (SELDI) (Fig. 3B). The amount of H-NS present at the moment of dissociation from the surface containing the 5A6A fragment estimated as being 2 pmol/mm^2. No peaks were obtained from material eluted from areas of the surface that did not contain DNA (data not shown).

Masses from all the three surfaces studied here (average mass 15547Da) corresponded with that expected for H-NS showing that it was able to recover material from the surface. The transfer protocol thus allowed quantitative recovery of material from the SPRi surfaces to the SELDI ProteinChip™ surfaces and detection by mass spectrometry.

Conclusion

Results demonstrate that monolayer surfaces permit accurate SPR analysis of macromolecular interactions and that the SPRi–Plex™ configuration is well suited to a simple recovery protocol that provides rapid sensitive mass spectrometry analysis. Clearly at these levels of surface saturation more than 100 spots could be rapidly visualized and the material recovered for analysis by SELDI even with the manual approach used here.