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Surface plasmon resonance imaging (SPRi)-based sensing: A new approach in signal sampling and management

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ABSTRACT

Surface plasmon resonance imaging (SPRi) is at the forefront of optical sensing, allowing real-time and label free simultaneous multi-analyte measurements. It represents an interesting technology for studying a broad variety of affinity interactions with impact in chemistry, both in fundamental and applied research. Signal sampling and management is a key step in SPRi measurements to achieve successful performances. This work aims to develop a strategy for selecting the sensing areas, called Regions of Interest (ROIs), to be sampled for recording SPRi signals that could results in improved sensor performances. The approach has been evaluated using antigen-antibody interaction: anti-human IgGs are immobilized on the chip surface in an array format, while the specific ligand (hIgG antigen) is in solution. This approach has general applicability and demonstrates that rational selection of sensitive areas and standard management of SPRi data has dramatic impact on sensor behaviour. The criteria of the method are: (a) creation of high density maps of ROIs, (b) evaluation of the SPRi binding signals on all the ROIs during a pre-analysis step, (c) 3D elaboration of the results, and (d) ranking of the ROIs for their final selection in further biosensor analysis. Using standard solution of antigen, three different ROIs selection approaches have been compared for their analytical performances. The proposed innovative method results to be the best one for SPRi-based sensing applications.

1. Introduction

Surface Plasmon Resonance imaging (SPRi) presently represents one of the best sensing platforms for studying a wide variety of affinity interactions (Paul et al., 2009; Scarano et al., 2010) involving nucleic acid sequences (Chen et al., 2009; Fuchs et al., 2010; Lee et al., 2005), peptides (Villiers et al., 2009), proteins (Bellon et al., 2009; Lee et al., 2008), and carbohydrates (Grant et al., 2007). In SPRi the entire biochip surface is displayed during analysis and it is possible to simultaneously monitor up to hundreds of molecular interactions. Here, the conventional SPR is coupled to micro-array format through the introduction of a Charge-Coupled Device (CCD) as detector system instead of a diode array. Open problems in this new sensing transduction approach are mainly related to the lack of standardised strategies for signal sampling and data management, these having dramatic influence on the analytical performances (i.e. sensitivity, selectivity, and reproducibility) of the biosensor, which are a key point for further application of SPRi-based sensing to real analytical problems (i.e. complex matrices). In SPRi bioreceptors are tethered on a chip gold surface in array format, as circular or square spots or even as micro-channels (D'Agata et al., 2008; Ly et al., 2007), and any portion of the biochip could be suitable for SPR signal sampling, since the only limit is represented by the lateral resolution of the CCD detector. As a consequence, any area having a diameter equal to or greater than the CCD resolution may be associated with a SPRi signal, leaving the operator completely free to select number, location and type of sensing areas undergoing signal sampling. Although this selection is a key step for the subsequent analysis, there is a lack of common criteria for doing it despite the high number of papers recently appeared in literature on SPRi. We believe that the valuable information provided by imaging data analysis would be more efficiently exploited, if coupled with well defined criteria for signal sampling and affinity binding data management. Moreover, standardisation of both procedures would eventually build up a common platform for homogenous comparison of SPRi results obtained in different contexts. With this aim in mind, we developed a method the rational and reproducible selection of sensing areas on the biochip (named Regions Of Interest, ROIs) for SPRi signal sampling carried out by a two-step approach performing a fine mapping of the array, followed by identification and selection of the best ROIs to be used for measurements. We report here how improved analytical performances can be thus achieved by using this rational design vs. conventional ROIs selection, in SPRi experiments. As proof of principle we applied

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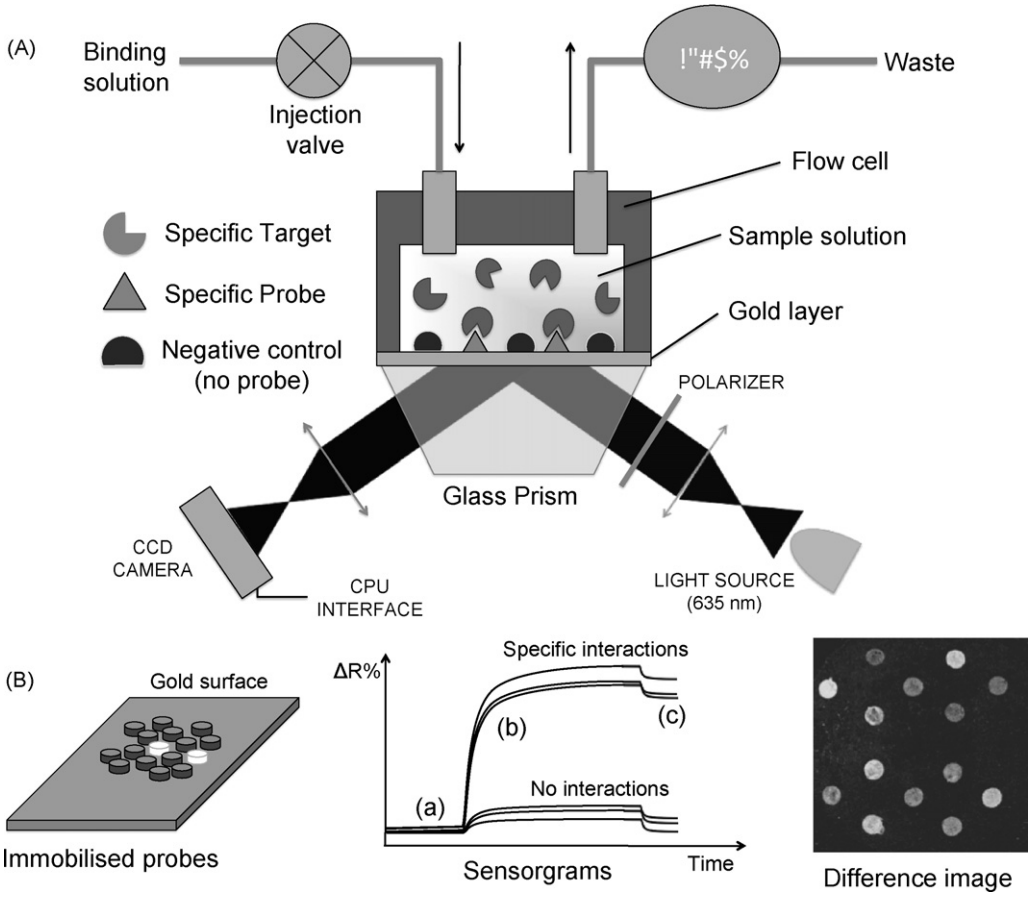

Fig. 1. (A) Scheme of the SPRi apparatus. (B) On the left, an array of probe deposited onto the biochip gold surface is represented; in the centre, a typical shape of a measuring SPRi cycle in which (a) is the signal at the baseline (carrier), (b) is the binding curve, and (c) is the washing step before the regeneration; on the right the difference image as displayed after the binding.

this approach in a paradigmatic immunosensing example involving anti-human IgG antibodies (anti-hIgG) covalently immobilized on gold chip surfaces by a dedicated chemistry, tested with the relative human IgG specific target. However, this approach can be applied to any bio-interaction and demonstrates that rational selection of sensitive areas and standardised management of SPRi data has dramatic impact on sensor behaviour.

2. Material and methods

2.1. Materials

11-Mercaptoundecanoic acid (MUA), 11-mercaptoundecanol, 1-mercaptohexanol, streptavidin, N-hydroxysuccinimide (NHS), sodium acetate trihydrate, ethanolamine hydrochloride (EA), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), Tween 20, biotin, as well as purified human IgG, and the relative biotinylated anti-human IgG were purchased from Sigma–Aldrich (Italy). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) was from Calbiochem (USA). Glycine and hydrogen peroxide 35% were from Merck KGaA (Germany). Ammonia solution was purchased from VWR International (France). Absolute ethanol was form Carlo Erba Reagenti (Italy). 184 Silicone Elastomer kit was from Dow Corning Corporation (USA). Probes for immobilisation were in an immobilisation solution containing 10 mM sodium acetate, pH 5.0 in water. Binding buffer contained HEPES 10 mM, Tween20 (Sigma–Aldrich) 0.1%, pH 7.4. To regenerate the biochip surface after the binding with the analyte a 10 mM glycine solution, pH 1.9, was used. Absolute ethanol, deionised water (18.2 MΩ cm) and all solutions were filtered (Millipore, 0.45 μm) before use. SPR imaging measurements were conducted on SF-10 glass prisms with a 5 nm-layer of chromium coated under a 50 nm gold layer (Genoptics-Horiba Scientific, Orsay, France).

2.2. SPR imaging measurements

SPRi experiments were performed on SPRi-Lab+ instrument (Genoptics-Horiba Scientific, Orsay, France) based on intensity modulation, measuring the reflectivity of monochromatic incident p-polarized light (635 nm) at fixed angle. The optical asset is based on Kretschmann configuration; a high refractive index glass prism is used as resonance coupler. A peristaltic pump (Minipuls 3 M312, Gilson) governs the 6-μl flow cell and the working flow for all measurements was 20 μl/min. Fig. 1A represents the scheme of the SPRi instrumentation.

Analyte solutions were all prepared in a binding solution (used also as carrier) and injected (175 μl) into by using a Rheodyne 7125 valve (IDEX Health & Science Group, Wertheim-Mondfeld, Germany). The measurement procedure is described all follows (Fig. 1B, centre): The solution flowing on the surface (carrier solution) is taken as reference for the baseline signal (a). Then the chip with immobilised probes was exposed to target solutions for 10 min (b). All SPRi signals were taken after washing the surface with the carrier solution to remove the unbound analyte (c). After each measuring cycle, the biochip surface was regenerated by injecting for 30 s a 10 mM glycine solution, pH 1.9. SPRi signals are recorded in real-time for each area on the biochip surface previously selected as Region Of Interest (ROI), and visualised both as

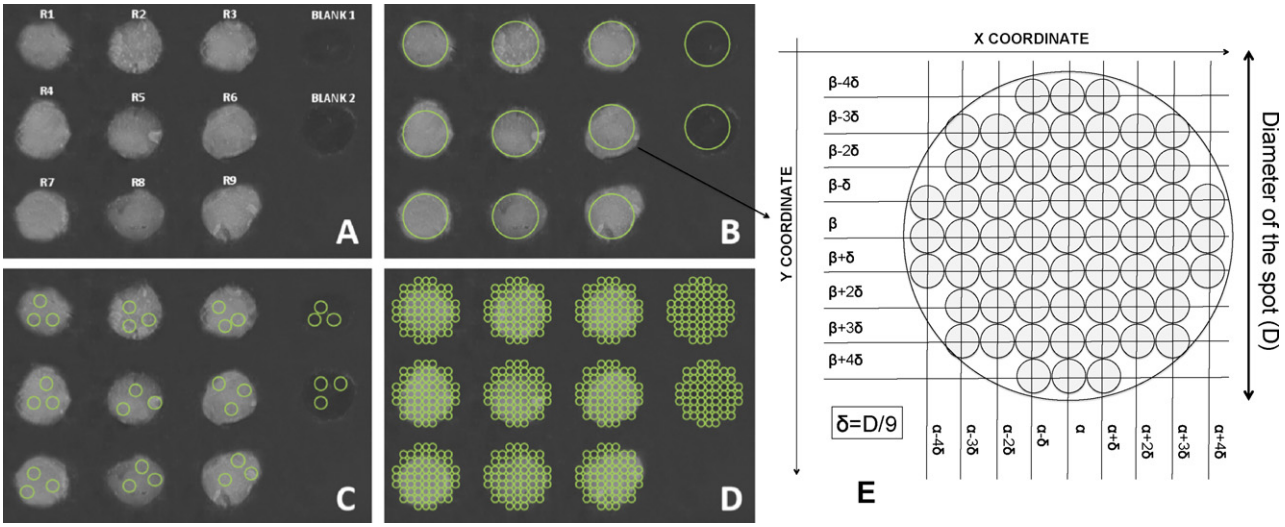

Fig. 2. (A) Image of the array of receptors on biochip surface by the CCD camera. At this stage no ROIs are defined and thus no signal can be recorded. (B) White circles define the initial grid of ROIs, whose coordinates are used by "Grid generator" macro. Each ROI is coincident with one single spot; (C) the grid of the second sampling method applied "C", consists of three ROIs for each spot, arbitrarily selected by the operator; (D) grid obtained using the developed method "D" based on "Grid generator"; (E) zoom of a single spot displaying the ROIs generation using method "D": 61 ROIs/spot are generated by selecting them using radial coordinates reported in the text. δ is the diameter of each small ROI generated an D is spot diameter. In this experiment $\delta = 140\ \mu m$ and $D = 1260\ \mu m$.

sensorgrams and difference image (Fig. 1B). The measuring conditions can vary among different experiments (different type of the receptor/analyte interaction, analyte concentration range etc.) and must be optimized for each single experiment.

2.3. Biochip preparation

Polydimethylsiloxane (PDMS) polymer is used for the array fabrication on biochips. A thin film of PDMS is prepared as recommended by supplier and micro-wells (ca. 1 mm diameter) are obtained by piercing the PDMS layer, in agreement with the design of the array. After biochip washing, with absolute ethanol and distilled water and then drying under nitrogen stream, the mask is let adhere to the gold surface of the biochip. Probe immobilisation is carried out on the uncovered gold areas, defined by micro-wells on PDMS mask. Each well is first filled with 1 mM MUA aqueous solution (0.8 μl/well) and let react for 48 hrs in a dark moist chamber. The carboxylic groups of MUA are activated, for covalent coupling of amino group, by aqueous solution of 50 mM NHS and 200 mM EDAC for 20 min. The activating solution is then replaced by streptavidin 200 μg/ml in immobilisation solution for 20 min. After washing, biotinylated anti-human IgGs (150 ppm) in immobilisation solution were added in each well, and the binding reaction lasted for 2 h avoiding drying. Two wells of the array were filled only with immobilisation solution (no biotinylated probe) and were used as control surfaces. Residual reacting sites are blocked with a solution of EA (1 mM water solution, pH 8.0) and then with a biotin solution (2 g/l in water). After removing of biotin solution from wells, the surface was washed with water and dried under nitrogen flow. The PDMS film was finally removed and prism is immersed in an aqueous solution containing 11-mercaptoundecanol and 1-mercaptohexanol at 1 mM concentration each for 8 h to assure gold blocking. Before housing the prism into the instrument, the chip was washed with distilled water and dried under nitrogen.

3. Results and discussion

Using conventional software, it is possible to generate and define a set of ROIs on the biochip. This set can be named as "grid". Information on coordinates, diameters, and identities of all the ROIs of the grid are associated to text files, automatically generated by the software provided with the instrument. However, the selection chosen can vary from a research group to another thus comparison of results is difficult; moreover a selection like this is highly dependent from subjective sensibility. We considered instead standardising the selection process for signal sampling, using eventually an experimental test based on a simple pre-analysis step. The idea is to obtain a set of ROIs, selected after ranking from a high number of starting ROIs, after performing a binding assay using the relative ligands. The criteria for ranking can be identified eventually in the main analytical parameters (sensitivity, signal reproducibility, specificity etc.). In our work here thus we developed a method to create punctual spot mapping, to better define the surface area for signal sampling, for final ROIs sorting out, after ranking. The selected ROIs will be further used in the true analysis. For comparison, the developed method was applied together with two different and non-standardised methods for signal sampling and the analytical performance of the system were investigated. In Fig. 2 are reported the three different spot grids compared in this study, which will be thoroughly discussed in Section 3.3. Here we refer to the methods as "B", "C", and "D" respectively.

3.1. Mapping of the array of probes

The starting point of the procedure is the definition of conventional grid, as displayed in Fig. 2B. Then this selection was further used for achieving the punctual spot mapping of the array, i.e. the generation of the highly defined grid "D". To achieve this goal we used the coordinates of the grid "B", stored in a text file, to create a new grid, characterised by a high density of ROIs (in this study 61 ROIs for each spot on the surface, Fig. 2E, zoom on the right side). To this aim, a dedicated macro (Excel, Microsoft Corporation) named "Grid Generator" was developed. The macro was built considering that the new grid should be adapted to any starting diameter and number of the spots, and to any geometry of the array. For this, the macro uses the centres and the diameters of the starting ROIs as provided by the conventional grid "B" (white circles). The resulting grid "D" is reported in Fig. 2, in which black circles are the new ROIs' group generated by the macro for each spot. The equation applied

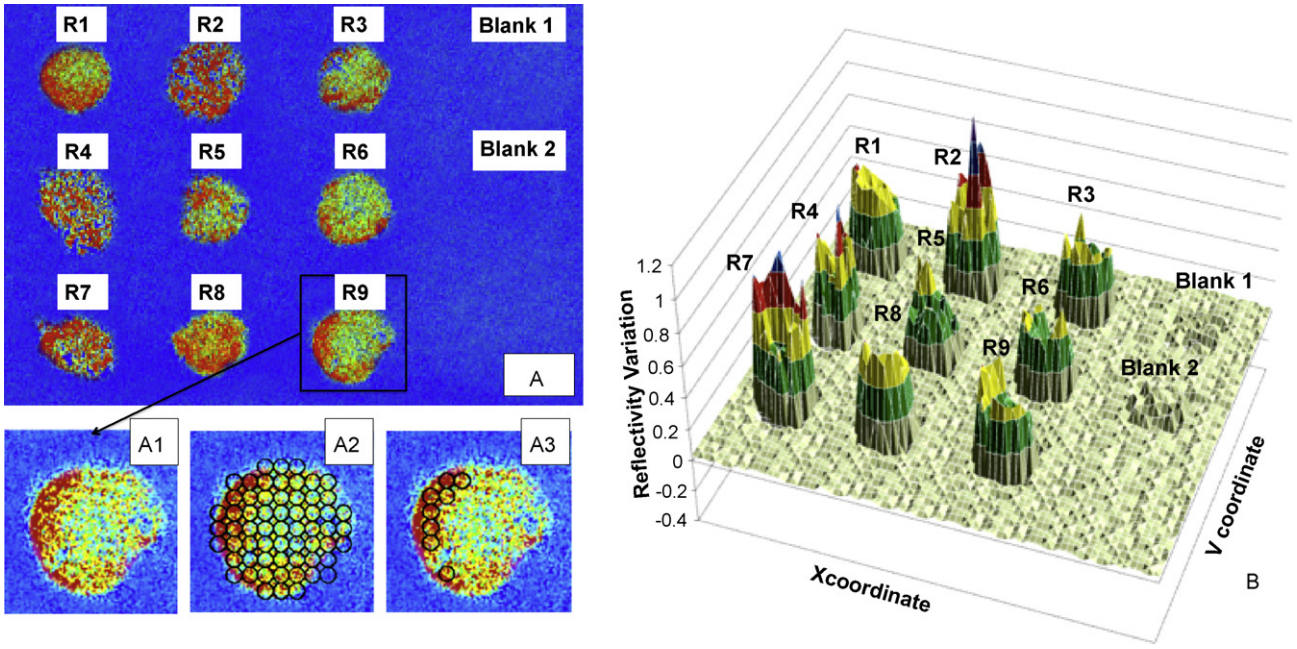

Fig. 3. (A) Experimental difference image displays the spots after hIgG binding; (A1) zoom of R9 spot as example; (A2) mapping of the spots obtained by our method "D" for sampling: 61 ROIs; (A3) highest scored ROIs are selected after the pre-analysis and binding data ranking. (B) 3D view of the same binding data displayed by "Data Analyzer". Reflectivity variations are reported on *Z* axis vs. Cartesian coordinates (*X*, *Y* axes) of each ROI. Each point of the graph corresponds to a SPRi signal and, thus, to a ROI.

to select the ROIs on the base of a radial symmetry was:

$$(X-\alpha)^2+(Y-\beta)^2 \le (D/2)^2 \quad (1)$$

where in the reference system of the instrument, α, β and *X*, *Y* are, respectively, the Cartesian coordinates of the centres of each ROI belonging to the initial grid "B" and the generated grid "D". The resulted geometry obtained for each spot sampled with the grid "D" is represented in Fig. 2E. In this representation *D* is the diameter of the deposited spot, which defines the maximum distance from the centre's coordinates (α, β), for ROIs generation. "Grid generator" originates a text file with the new ROIs coordinates, which replaces the original one. The new grid is then used to carry out the pre-analysis step. This pre-analysis consists in a preliminary surface test, where a standard solution of analytes is injected over the surface for the analysis of the binding with the relative receptors. In other terms it allows to obtain punctual information about affinity binding on the surface in terms of signal intensity, unspecific binding, distribution of the ligands on the spot areas. After the pre-analysis, the affinity-complex is regenerated by using 10 mM glycine solution pH 1.9. To achieve the final ROIs selection, on the base of the experimental pre-test, a ranking of the binding data is performed.

3.2. Ranking and selection of the best ROIs

By using "Data Analyzer", a second in house developed set of Excel macros, the pre-analysis data were ranked. Before performing the ranking, "Data Analyzer" generates a 3D representation of the biochip surface, on the base of the pre-analysis step. As shown in Fig. 3, the experimental difference image of the pre-analysis is compared with the 3D image obtained with "Data Analyzer". The result is the precise correspondence of the coordinates between the two visualisations of the experimental data (respectively Fig. 3A and B) and this resulting correspondence is a confirmation that "Data Analyzer" successfully correlates the ROIs coordinates (*X*, *Y*) with SPRi experimental signals; this is a quite important result. In fact the precise overlapping of difference image and the reconstructed 3D plot is the mandatory pre-requisite for further processing with the final ROIs selection, since it assures that the choice is carried out correctly, on the proper spots.

The core of "Data Analyzer" consists then in the ranking of the SPRi signals obtained during the pre-analysis followed by the sorting out of the "best performing" ROIs on the basis of their analytical performances. To perform the ranking of the single ROIs, the recorded signal considered is the SPR of each single ROI, averaged on three replicates ($n = 3$) of the tested concentration of the target during pre-analysis (3 ppm in this case). These ROIs responses are evaluated on the base of the main analytical parameters: SPRi signal vs. specific target, averaged coefficient of variation% (CVav%), and specific/unspecific signal ratio. The following equation was applied to perform the normalization between the different parameters:

$$N_i = \frac{X_i - \min_{1\to n}}{\max_{1\to n} - \min_{1\to n}} \quad (2)$$

N_i indicates the normalized value of the *i*th ROI for each considered parameter, X_i is its corresponding value on the series, and min and max indicate the lowest and the highest value in the series, respectively. All the values correlated to the considered analytical parameters are thus normalized in a 1–100 range and can be now directly compared.

All the parameters were then combined and elaborated by a polynomial function as follows:

$$S_i = a \times \text{sensitivity}_{(i)} + b \times \text{variability}_{(i)} + c \times \text{specificity}_{(i)} \quad (3)$$

S_i represents the weighted combination of the all considered parameters for the *i*th ROI. Sensitivity$_{(i)}$, variability$_{(i)}$, and specificity$_{(i)}$ are the normalized values obtained by Equation 2 for the *i*th ROI corresponding to averaged specific SPRi signal, CVav%, and specific/unspecific signal ratio respectively. Coefficients *a*, *b*, *c* are arbitrary numbers from 0 to 1 and define the weight of each parameter in the combination. Their sum must be = 1.

The best resulted ROIs are listed at the top of the ranking.

To each ROI corresponds the set of relative Cartesian coordinates, diameter, and identity. From these data, "Data Analyzer" automatically generates a new text file containing all this information for the selected ROIs. This text file thus describes the final

grid that will be used for further measurements by the software of the instrument under the optimized selection of ROIs.

The possibility to automatically select a defined number of highly performing surface areas, following rational analytical criteria, represents an important achievement. The spot surface is in fact evaluated taking into account eventually present inhomogeneity, intrinsic with the deposition procedure, even performed by automatic spotting. Furthermore, by combining the different parameters in ROIs ranking, it also possible to weight the priority criteria to be considered in the experiments. It should be stressed that in this approach the signal selection occurs on the base of real fast analysis, moving from *ex ante* approach to *ex post* selection.

3.3. Application of the method and analytical results

To demonstrate the validity of our approach, in terms of improvement of analytical parameters of the sensor, the binding results of this immunosensor were compared with those obtained by applying other two approaches widely used for ROIs selection and displayed in Fig. 2.

Fig. 2A shows the digital image of the biochip carrying the anti-hIgG array (R1-R9) after immobilisation; reference surfaces (blanks 1 and 2) with no receptor are also included. At this stage (before interaction with the relative human IgG target), ROIs are usually defined by direct selection of the spots on this image (white circles, Fig. 2B), as afore mentioned. In this choice, each ROI is coincident with one whole single spot and its area depends on the single spot diameter. SPR signals, i.e. the percentage in reflectivity ($\Delta R\%$) change, will be then recorded on these areas as the average of all the values obtainable within each ROI, expressed as grey scale variation. This example is reported as paradigmatic of a fast, easy but far from optimal ROIs selection. In fact the receptor could not be homogeneously immobilized within the ROI's area, thus resulting in an unpredictable binding efficacy, which affects the distribution of the signal. The second considered sampling method is represented in Fig. 2C (white circles). A sufficient number of ROIs (e.g. $n = 3$) is representatively sampled within each spot to obtain the averaged SPRi signal for each spot and the corresponding standard deviation (SD). Although this approach provides better results than previous one, the arbitrary choice of ROIs can lead to a low reproducibility of the method. Fig. 2D reports ROIs map created using our method in which each spot of the array is sampled with 61 ROIs (70 μm diameter each), following Eq. (1), and the final grid comprises Nx61 ROIs, where N is the number of spots (11 in this experiment), i.e. by using a set of 671 ROIs simultaneously. Pre-analysis was thus applied by injecting the target analyte (hIgG 3 ppm) which was let to interact with the immobilized anti-hIgG for 10 min and the 671 relative affinity bindings were monitored on the corresponding ROIs in real-time both on the sensorgrams and difference image (data not reported).

By using "Data Analyzer", the 3D visualisation of the SPRi biochip after pre-analysis is generated, in which each point of the graph corresponds to a SPRi signal and, thus, to a ROI (Fig. 3B). It clearly evidences spot coverage, specificity of response and unspecific binding presence on reference surfaces. Finally, the selection of the best performing ROIs is automatically obtained ranking the 671 binding responses resulting from the pre-analysis. Ranking was achieved using the best scored ROIs for each spot in terms of the S_i value (see Eq. (2)). In this way, only a final optimized pool of ROIs will be used for signal sampling (Fig. 3A3), generated by exploiting experimental binding data. The choice of the parameters for ROIs ranking, as mentioned above, can be driven by selecting one parameter at a time (i.e. highest signal, lowest CV%, highest S/N, lowest unspecific adsorption) or by choosing a compromise among all the different parameters. Best results were achieved in this example by their combination in the polynomial function (Eq. (3)) (data not shown). Finally a set of best performing ROIs was extracted (i.e. 8 per spot as in Fig. 3A3), on the base of tested parameters. These parameters (corresponding to Eq. (3) coefficients) were respectively $a = 0.3$ for sensitivity, $b = 0.2$ for variability, and $c = 0.5$ for the specificity (respectively as test weight of 30%, 20% and 50%). These present a compromise in the performances, however they can be eventually varied depending on the need of the assay. Eventually even the number of finally selected ROIs can be flexible.

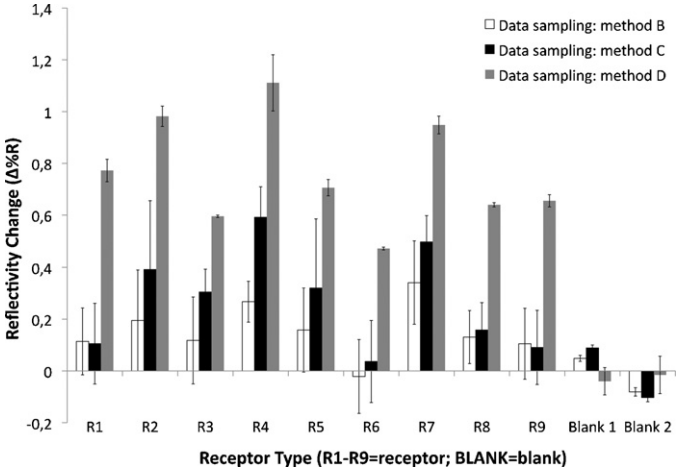

Fig. 4. SPRi results obtained for each spot of the array by comparing the three signal-sampling methods (B–D) displayed in Fig. 2. SDs are the average calculated on the total number of the selected ROIs for each considered method, and for 3 ppm hIgG injections in replicates ($n = 3$).

In Fig. 4 the IgG binding responses using the three different ROIs sampling methods described above (Fig. 2B–D) are compared. The bars represent the behaviour of each spot of the array after an injection of human IgG at 3 ppm concentration, when binding data are analysed with the three different sampling methods. The first significant result achieved with the method D is that all SPRi signals are higher than those obtained when the selection was done using methods B and C. In particular the averaged increase obtained with method D *vs.* B and C was of 4- and 3-fold, respectively. Moreover, even signals that would have been considered not significant, result in positive binding as clearly illustrated in the case of R6 spot. The other remarkable result is that SD generally decreases for all spots and, consequently, also the reproducibility, expressed as the averaged CV% (CVav%), was improved. Furthermore the specific vs. unspecific-binding ratio is much enhanced. This value was 3.4 for method B, 3.1 for C, but 19.2 using our approach, resulting in 6-

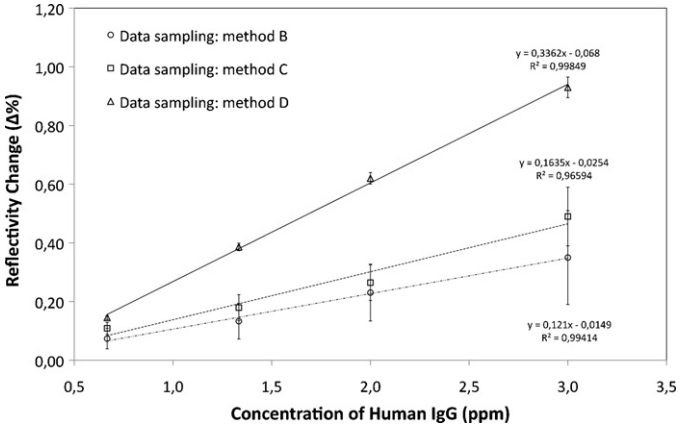

Fig. 5. Calibration curves of hIgG showing the comparison of the system's performance within the concentration range 0.7–3.0 ppm for the three signal-sampling methods considered. Here is reported the data obtained from spot R7.

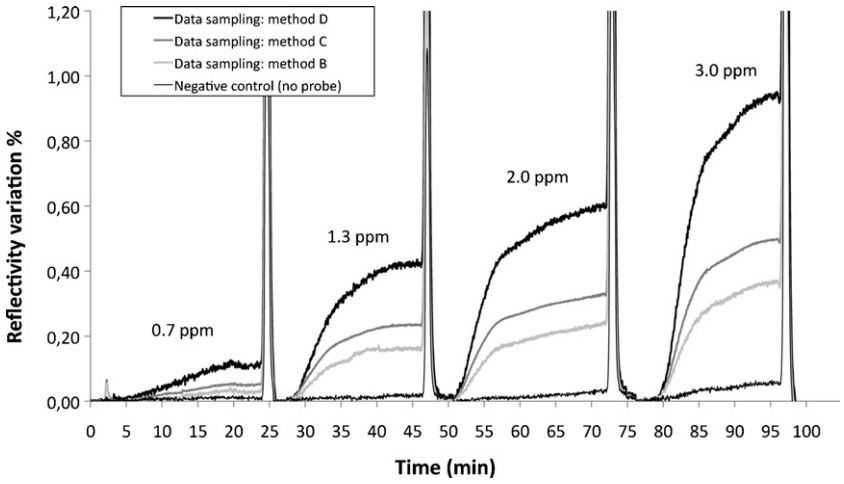

Fig. 6. Sensorgrams relative to the calibration curves (spot R7) together with the signal recorded on control surfaces. After each binding the surface is regenerated (spikes in the sensorgrams) using glycine 10 mM pH 1.9.

fold increase. We believe this impressive improvement obtained in sensor performances can be attributed to the rational procedure developed for ROIs selection and ranking. The biosensor was then calibrated by testing different concentrations within the concentration range 0.7–3.0 ppm of hIgG. In Fig. 5 is reported the plotted curves obtained during calibration by applying the three different signal-sampling methods simultaneously, in which the improvement obtained by applying the method "D" is evident at all the analyte concentrations tested. As paradigmatic example, we report the calibrations obtained for on one spot of the array (spot R7), using different sampling approaches however the same behaviour can be observed for all the spots of the array. The sensor binding profiles obtained during calibration on spot R7 are reported in Fig. 6 as sensorgrams; here the binding curves are visualised together with the signal obtained from the spot used as control surface. A higher slope in the association phase using sampling method "D", could be observed. This behaviour is particularly evident by comparing the methods "B" and "D". Since method "D" allows to select the most performing areas of the array, a possible explanation of the observed differences could be found in the ability to sample areas of the immobilized receptor that are more accessible to the analyte, resulting thus in high binding signals. From this preliminary evaluation, the developed method allows the improvement of binding profiles of the molecular interaction, and thus can be useful also for accurate kinetic evaluation.

4. Conclusions

We developed an innovative approach to signal sampling and affinity data management applied to SPRi technique. It consists in a fine mapping of all the probing areas of the biochip taking into consideration numerous parameters playing a role in the interaction (i.e. receptor homogeneity within the spot, sensitivity, S/N). The method has a general validity and can be applied to any interaction for affinity sensing. In the first part of the proposed approach, a dedicated algorithm was drawn and optimized to obtain a detailed mapping of the array, which consists in the definition of a high number of small ROIs covering the whole area of each spot. In the second part, all ROIs mapped onto the array are tested by a pre-analysis to obtain SPRi experimental signals for each ROI. All signals are then sorted, by using a different customised set of macros, on the base of selected analytical parameters (i.e. desired selectivity, reproducibility, etc.) and, finally, only the best ROIs are selected for further analyses. This final ranking of SPRi signals is the keystone of the method. It is achieved by inserting in the algorithm some crucial parameters for real sensor application, *i.e.* signal intensity ($\Delta R\%$), specificity (specific signal vs. blank), signal precision and reproducibility (in terms of standard deviation (SD) and coefficient of variation (CV%)) within and among different spots, and signal to noise ratio (S/N). By the combination of all these parameters, a rational, automatic and standardised ROIs selection is achieved. On this ROIs' pool, further SPRi-based biosensor analysis is achieved. The peculiarity of this approach is that final ROIs selection is made after a real and fast pre-analysis with the specific target analyte. In contrast, the selection of ROIs is traditionally carried out simply defining the entire (or part of) spot area before recording the binding with the target. Moreover, the 3D representation of the pre-analysis clearly evidences spot coverage, specificity of response and unspecific binding presence on reference surfaces. In other words, by this two-step method it is possible to analyze, select, and finally extract only the best performing ROIs for each spot, on the base of chosen and standardised parameters.

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