



Research paper

Surface Plasmon Resonance imaging: A method to measure the affinity of the antibodies in allergy diagnosis

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ABSTRACT

Aim: The biological diagnosis of type I hypersensitivity reactions is based on the quantification of specific IgEs. However, the IgE titer is not always strongly related to the clinical symptoms or predictive of the evolution of the disease. The specificity and affinity of antibodies of other isotypes may contribute to the allergic status of the patients. The aim of the present work was to develop a method that simultaneously detects the complex antibody response to various allergens and measures the avidity of the antibodies directed to each allergen.

Methods: A chip based on a covalent binding of 3 major milk allergens on a gold-activated surface was developed. The binding of specific antibodies to α -lactalbumin, β -lactoglobulin or caseins was monitored using Surface Plasmon Resonance imaging (SPRi). The sensitivity and specificity of the method were compared to those obtained by ELISA, the reference method.

Results: The specificity of the antibodies characterized by SPRi was identical to the one obtained by ELISA. The intensity of the signal was proportional to the quantity of antibodies bound to each allergen. The sensitivity of the SPRi detection was about 8–10 times lower than for ELISA but the SPRi is faster and the analysis of association/dissociation kinetics allowed the determination of the avidity of the antibody response.

Conclusion: The present study shows that SPRi allows a multiplex monitoring of the complex antibody response to the major allergens of an allergenic source. This label-free method constitutes a new tool that may be added to IgE detection to improve allergy diagnosis.

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1. Introduction

The biological diagnosis of various pathologies, including infectious diseases and allergic reactions, is based on the quantification of specific antibodies. Type I hypersensitivity reactions are characterized by a specific IgE response that contains various IgEs recognizing a complex mixture of proteins from an allergenic source (pollen, food, animals...). To detect and quantify

the specific IgE response, the biological diagnosis of allergy usually uses complex protein extracts (e.g. from birch pollen, cow's milk, cat...) that contain allergens and non-allergenic proteins. The use of isolated purified or recombinant allergens is still unusual in routine practice. However, if the titer of specific IgEs is recognized as the most significant biological marker in type I hypersensitivity, it is not always strongly related to the clinical symptoms or predictive of the evolution of the disease. The potential role of IgE affinity on allergic symptoms was evaluated by the use of recombinant IgE of various affinities recognizing various epitopes of the allergen Der p2 (Christensen et al., 2008). This study showed that low affinity IgEs may contribute to the degranulation of basophils harboring allergens captured by high affinity IgEs, supporting the previous hypothesis of Aalberse et al.

Abbreviations: SPRi, Surface Plasmon Resonance imaging; α Lac, alpha Lactalbumin; β LG, beta Lactoglobulin; Cas, caseins

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(Aalberse et al., 2001; Christensen et al., 2008). Added to the IgE response, various other parameters (e.g. specific antibodies of other isotypes, basophil activating molecules...) may contribute to the immunological profile of the patients. Among those, the characterization of the complex antibody response to allergens was focused on its isotypic diversity (Hammarström and Smith, 1987; Chardin et al., 1999; Hidvegi et al., 2002; Niederberger et al., 2002), without measuring the affinity (or the avidity) of the allergen/antibody interactions.

Surface Plasmon Resonance (SPR) was used to detect inter-molecular interactions and measure their affinity in various application areas (Karlsson, 2004). Many SPR systems used antigen/antibody interactions to detect protein biomarkers (Lee et al., 2006; Kim et al., 2009; Oh et al., 2009) or to monitor antibody production, including the detection of specific antibodies and the measure of the affinity of their binding to the antigen (Gibbs and Oger, 2008; Mytych et al., 2009; Oh et al., 2009; Nogues et al., 2010). In the field of allergy, SPR was used to characterize anti-IgE monoclonal (Laffer et al., 2008) or polyclonal antibodies (Pol et al., 2007), to detect free serum IgE in allergic patients (Hamilton et al., 2012) or basophil activation (Yanase et al., 2012). Besides, one previous report followed the affinity of specific antibodies to the major birch pollen allergen Bet v1 during immunotherapy (Jakobsen et al., 2005). In this study, the authors used the Biacore system that allows the measurement of the affinity to only one ligand and requires a purification of the antibodies.

The aim of the present work was to develop a tool to simultaneously detect and measure the avidity of the various antibodies recognizing the major allergens of an allergenic source. Surface Plasmon Resonance imaging (SPRi) is a sensitive label-free method to detect and visualize the region of interest of the biochip. The high resolution CCD video camera provides real-time difference images across an array of immobilized molecules. This design allows the use of a pattern of various immobilized molecules and simultaneously follows up the kinetic curves of each spotted molecule. In this SPRi configuration, the measured local changes in light reflectivity are proportional to the amount of target molecules caught on each spot, and detailed information on molecular binding and kinetic processes may be obtained. In the present study, cow's milk allergy was chosen as a model. Thus, a chip based on a covalent binding of α -lactalbumin, β -lactoglobulin and caseins on a gold-activated surface was developed, and used for the multiplex monitoring of various sera by SPR imaging.

2. Material and methods

2.1. Characterization of the sera by ELISA

Specific antibodies to α -lactalbumin (a-Lac), β -lactoglobulin (b-LG) and caseins (Cas) were quantified by an in-house ELISA. Briefly, the microplate wells (NuncMaxisorp, Kamstrup, Roskilde, Denmark) were coated overnight at 4 °C with a-Lac

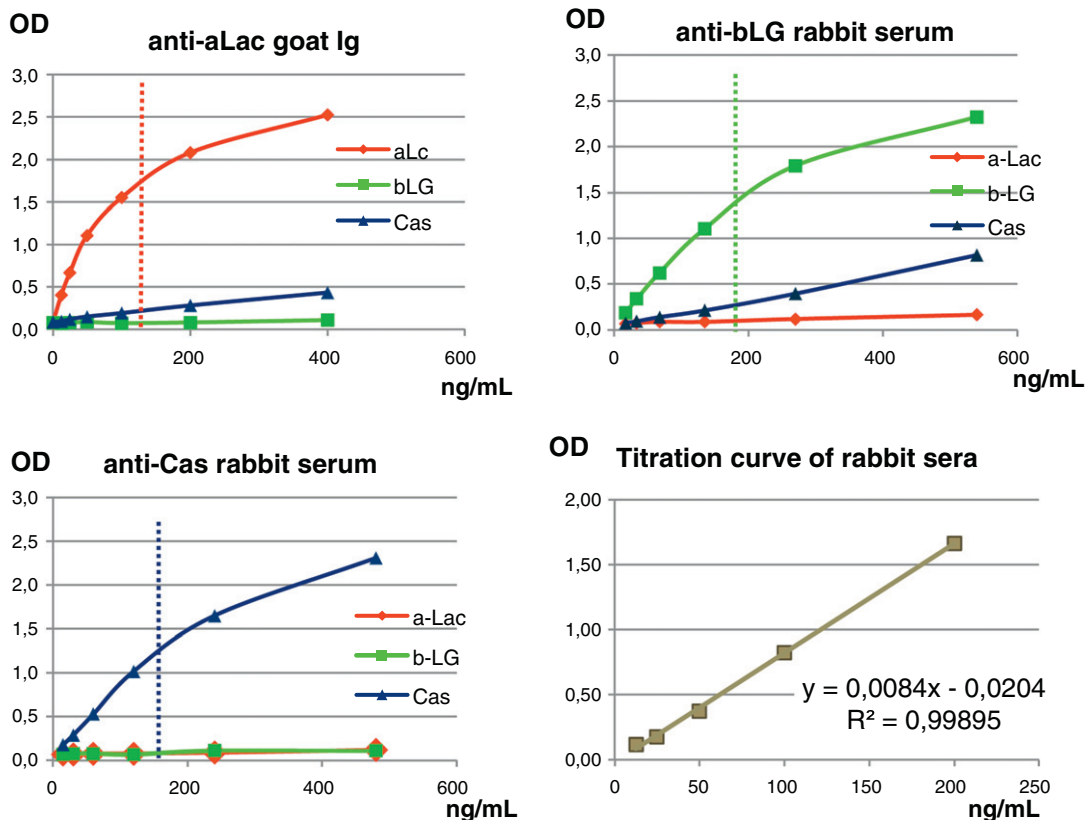


Fig. 1. Detection of the antigen/antibody reaction by ELISA. Specific and cross-reacting antibodies from goat and rabbit sera were detected by ELISA. A vertical dot line for each antibody source indicates the smallest antibody concentration used for SPRi monitoring and affinity measurement. The titration curve allows the quantification of the specific antibodies of anti-bLG and anti-Cas rabbit sera.

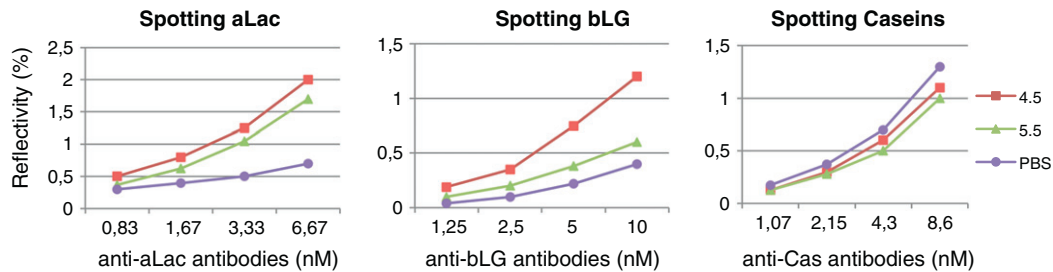


Fig. 2. Chip development. For each protein, 3 buffers adjusted at pH 4.5 (■), 5.5 (▲) and 7.4 (●, PBS) were tested for optimal spotting onto the gold surface. The % of reflectivity obtained for increasing concentrations of specific antibodies (nM) was evaluated. The diagrams show the results obtained for a spotting at a protein concentration of 5 μ M and are representative of the 3 tested concentrations.

(Sigma-Aldrich, St Louis, MO, USA), b-LG (Sigma-Aldrich) or Cas (Calbiochem, Merck Chemicals Ltd, Nottingham, UK) diluted in PBS pH 7.4 at 500 ng of protein/well. After saturation with PBS containing 1% PolyVinylPyrolidone (PVP, Sigma-Aldrich) and 0.05% Tween 20, the wells were incubated with one of the 3 following antibody sources: affinity-purified goat anti-aLac antibodies (GTX77275, GenTex, San Antonio, USA), an in-house rabbit serum to bLG or a rabbit serum to caseins (generously given by Dr JM Wal, CEA, Saclay, France). After extensive washing, the binding of antibodies was detected by incubation with alkaline phosphatase-conjugated rabbit anti-goat IgG (A4187, Sigma-Aldrich) or alkaline phosphatase-conjugated mouse anti-rabbit IgG (A2556, Sigma-Aldrich). All antibodies were diluted in PBS containing 0.1% PVP and 0.05% Tween 20 (PBS-PVP-Tw), and incubated 2 h at 37 °C. After extensive washing, the alkaline phosphatase activity was detected by *p*-nitro phenyl phosphate (Sigma-Aldrich), and the OD measured at 405 nm (Multiskan RC, Thermo Electron Corp., USA). Each test was done in triplicate. Controls were done by incubation with PBS-PVP-Tw instead of the diluted primary antibodies.

Standard curves were established to quantify the specific antibodies of the rabbit sera in absolute amounts. Briefly, wells were coated with affinity-purified goat anti-rabbit IgG (R4880, Sigma-Aldrich) and saturated as described above. Various quantities of rabbit IgG were added and then detected by the corresponding conjugated antibodies as described above.

2.2. Chip development

The biochip surface chemistry was based on a previously defined protocol for covalent protein binding (Bellon et al., 2009) and was slightly modified to improve the activation. Briefly, the gold surface of the SPRI-Biochip™ (Horiba Scientific, Palaiseau, France) was activated by a sulfo-N-Hydroxysuccinimide solution. Proteins were immobilized on the activated surface using the SPRI-Continuous Flow Microspotter™ (SPRI-CFM™, Horiba). The SPRI-CFM™ printer allows molecules to be immobilized using flow deposition, leading to increased spot homogeneity.

Three milk allergens (a-Lac, bLG and Cas) and two negative controls (the birch allergen Bet v1 and Human Serum Albumin) were spotted on a SPRI-Biochip™. To determine the best experimental conditions of spotting for each protein, 3 buffers

(acetate pH 4.5, acetate pH 5.5 and PBS pH 7.4) and 3 protein concentrations (2 μ M, 5 μ M and 10 μ M) were compared.

2.3. Characterization of the sera by SPRI

The 3 sources of antibodies characterized by ELISA were also characterized using a SPRI-Plex apparatus (Horiba). Each allergen spot was selected and identified. The SPRI technique allows the monitoring of the reflectivity variation at a single incident angle. This variation of reflectivity is correlated to the amount of analyte that reacts on each spot per surface unit.

Affinity-purified goat anti-aLac antibodies were injected at concentrations of 1 μ g/mL, 500 ng/mL, 250 ng/mL and 125 ng/mL. Anti-bLG rabbit serum was injected at concentrations of specific antibodies of 1.5 μ g/mL, 750 ng/mL, 375 ng/mL and 187.5 ng/mL. Anti-Cas rabbit serum (generous gift from Dr JM Wal) was injected at concentrations of specific antibodies

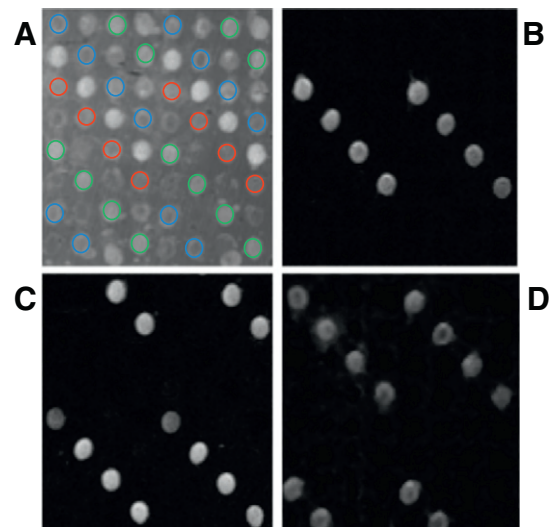


Fig. 3. Flowcell and difference images of the biochip. (A): Flowcell image of the spotting on the biochip. ● a-Lac, ● b-LG and ● Caseins. Other spots represent negative controls spotted in the same conditions of concentration and pH. Left 4 columns: 5 μ M of proteins/spot, right 4 columns: 10 μ M of proteins/spot. (B–D): Difference images of the biochip incubated with antibodies to a-Lac (B) or rabbit serum to b-LG (C) or caseins (D).

of 1.3 $\mu\text{g/mL}$, 650 ng/mL, 325 ng/mL and 162.5 ng/mL. The running buffer used was PBS-PVP-Tw (as defined in the ELISA section) with a flow rate of 50 $\mu\text{L/min}$ and a sample loop volume of 190 μL . The biochip was saturated with PBS containing 1% PolyVinylPyrrolidone (Sigma-Aldrich) and 0.05% Tween 20 before specific antibody injections. The 3 sources of antibodies were analyzed with one biochip by successive biomolecular interaction monitoring. After each injection of antibodies or serum, the SPRI-biochip was washed with the running buffer. After washing, the biochip was regenerated with 0.1 M glycine HCl at pH 2.0, for a further analysis of another antibody solution. Spots of Bet v1 (a birch pollen allergen) and human serum albumin (HSA) were used as negative controls.

Binding analysis used the SPRI-Analysis software (Horiba) and avidity was determined by using the ScrubberGen software. To assess the repeatability of the measurement, the rabbit sera and the goat antibodies were tested onto 3 different biochips and the determination of the avidity was done in triplicate for each kinetics.

3. Results and discussion

3.1. Characterization of the sera by ELISA

ELISA was used as the reference method to characterize antigen/antibody reactions. Fig. 1 shows the results obtained for the 3 sources of antibodies. The titration curve allows the determination of the concentration of specific antibodies in absolute amounts.

The results show the strong reactivity and the good specificity of each antibody source, with a limited unspecific binding to casein for the highest concentrations of anti-aLac antibodies or anti-bLG rabbit serum.

3.2. Chip development

Various concentrations (2 μM , 5 μM and 10 μM) and buffers (pH 4.5, 5.5 and 7.4) were tested for each protein. The results show that the optimal conditions were an acidic buffer at

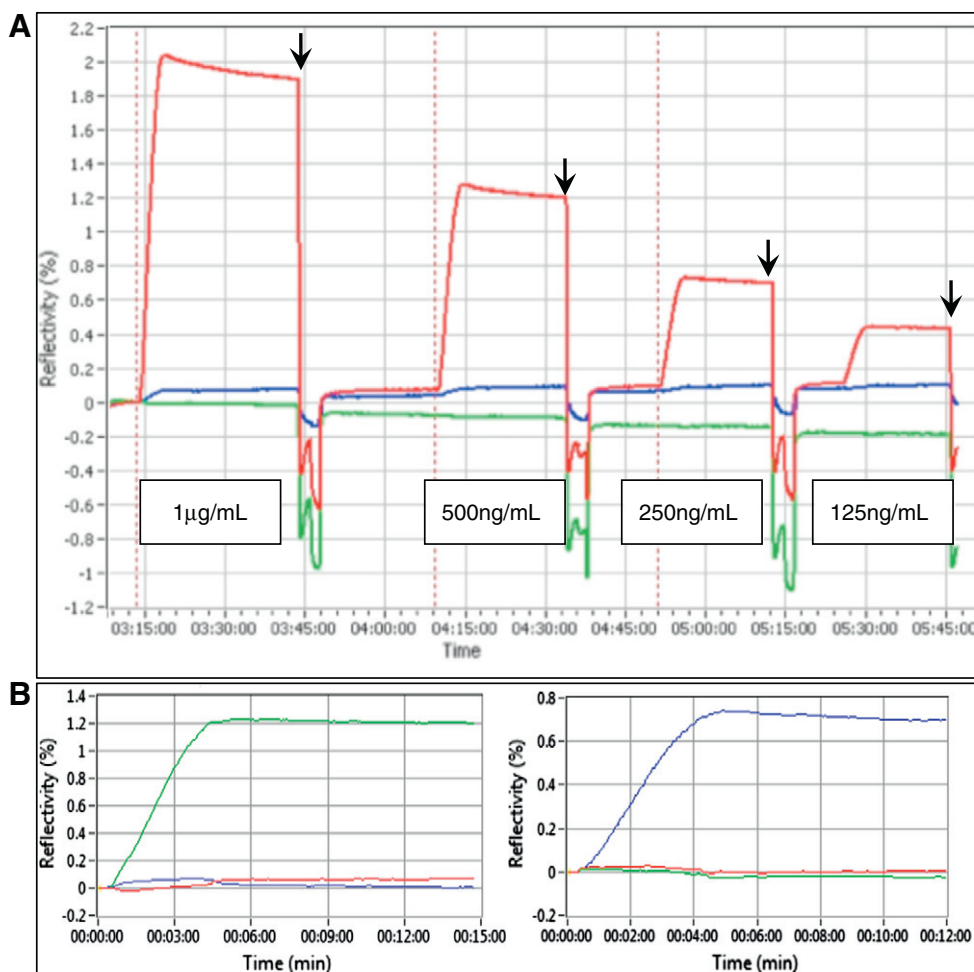


Fig. 4. Detection of antibody binding to milk allergens by SPRiPLEX. (A) Binding capacities of goat anti-aLac antibodies: the intensity of the signal was proportional to the concentration of antibodies indicated in the boxes. Regeneration of the chip by 0.1 M glycine HCl at pH 2.0 was performed between each injection (arrows). (B) Kinetics of association/dissociation obtained with rabbit sera to b-LG (left) and Cas (right) containing 1.5 $\mu\text{g/mL}$ and 1.3 $\mu\text{g/mL}$ of specific antibodies respectively. Red lines = binding to aLac spots, green lines = binding to bLG spots, blue lines = binding to casein spots.

pH 4.5 for a-Lac and b-LG, and a PBS pH 7.4 for caseins (Fig. 2). Among the 3 concentrations tested, similar results were obtained for 5 and 10 μM , with a stronger signal than 2 μM . Thus, the concentration of 5 μM was chosen, as it was the minimal concentration of proteins giving the strongest signal.

3.3. Antibody detection by SPRI

Antibodies from hyper immunized animals were used to assess the specificity and the sensitivity of the SPRI. The results obtained in SPRI were compared to those obtained by ELISA, the reference method. SPRI allowed the detection of a specific antigen/antibody reaction visualized on the chip (Fig. 3A) by the selective binding to a-Lac, b-LG or casein and the absence of visible cross-reaction with the other proteins (Fig. 3B–D). The intensity of the signal was proportional to the quantity of the antibodies bound to each spot, as it is illustrated for anti-aLac antibody source (Fig. 4A). The kinetics of association/dissociation obtained for high concentrations of anti-bLG and anti-caseins rabbit sera (Fig. 4B) shows a strong, stable and specific allergen/antibody binding, and the very limited unspecific binding to the other allergens that dissociate after washing with the running buffer. To be detected, the minimal needed antibody concentration was about 10 ng/mL in ELISA and 80–100 ng/mL in SPRI. Thus, the sensitivity of the SPRI system was about 8–10 times lower than the one of ELISA.

A previous study comparing ELISA and SPR to monitor an antibody response showed that the ELISA was more sensitive than the SPR, but also that the sensitivity of the method depends

on the affinity of the antibodies, the ELISA working better for the antibodies of the highest affinity (Lofgren et al., 2007). Similarly to what we obtained, a detection limit of 70 ng/mL was estimated in a recent study that used SPRI to detect anti-bovine immunoglobulins in human serum and milk (Scarano et al., 2011).

Comparing the respective sensitivities of the ELISA and the SPRI system, and the amount of serum needed for each system, it may be evaluated that a biochip that contains 12 different allergens utilizes the same amount of serum as the one needed for a similar screening in ELISA. As the SPRI-Continuous Flow Microspotter™ used in the present study allows the printing of 144 spots on the sensitive surface of the biochip, more than 12 allergens may be easily screened in one biochip. Thus, comparing the two methods for a screening to multiple allergens, it may be concluded that the SPRI system presented in this study is faster, label-free and consume less biological reagents than the ELISA. Otherwise, it was shown that the combination of a microfluidic system with SPRI detection may provide immunodiagnostic devices that approach the sensitivity of the ELISA, with lower biological reagent consumption (Suzuki et al., 2007; Luo et al., 2008).

3.4. Determination of the avidity of the specific antibody responses

The avidity of each specific antibody response was determined using a molecular mass of 150,000 Da that corresponds to the one of the IgG molecule. The avidities were determined

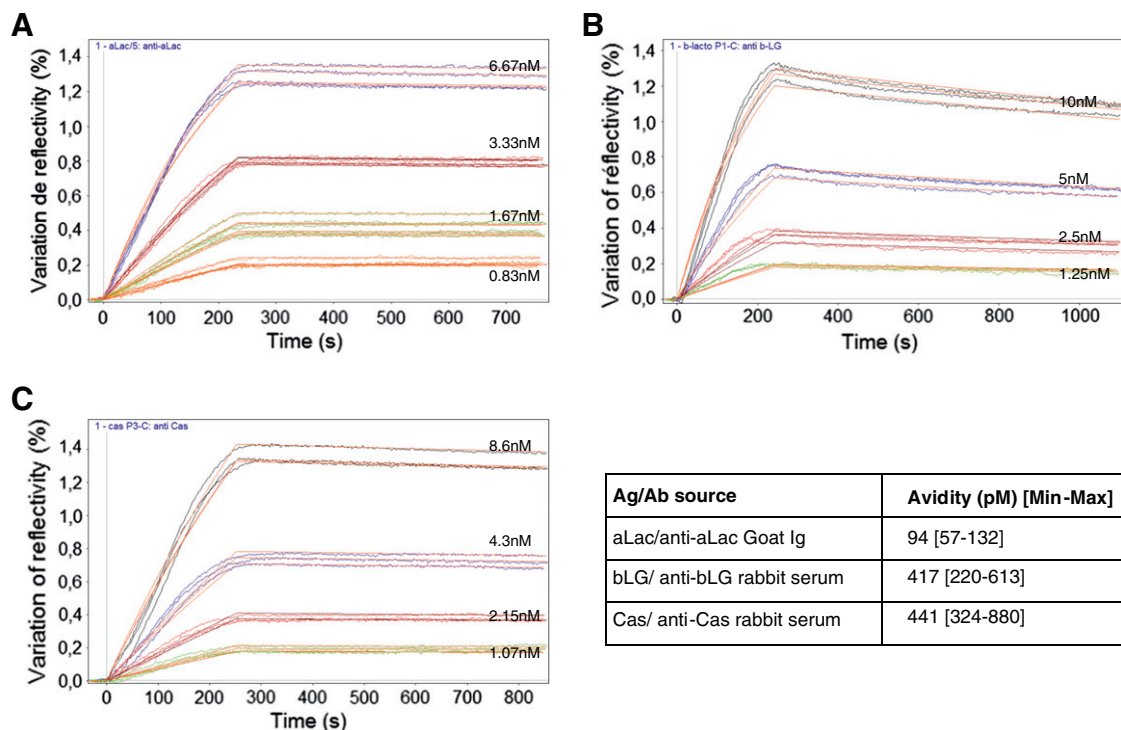


Fig. 5. Evaluation of the avidity of the antibody responses using the ScrubberGen software. The determination of the avidity of anti-aLac goat Ig (A), or the avidity of the rabbit serum to bLG (B) or to caseins (C) was done using the ScrubberGen software. A molecular mass of 150,000 Da, corresponding to the IgG molecule, was chosen. The concentration of antibodies that were injected in each plot is expressed in nM.

as follows (median [min–max]): 94 pM [57–132] for goat anti-aLac antibodies, 417 pM [220–613] for rabbit anti-bLG antibodies and 441 pM [324–880] for rabbit anti-Cas antibodies. Fig. 5 illustrates representative results of avidity measurements by the ScrubberGen software for the 3 sources of antibodies.

The variation in the avidity of the response among the three tested sources of antibodies (anti-aLac > anti-bLG = anti-Cas) is consistent with what was expected. Indeed, anti-aLac antibodies were purified by affinity, thus selecting the antibodies of the highest affinity. Moreover, it is well known that the avidity of an antibody response increases with boosting stimulations suggesting that, in hyper immunized animals, the avidity of the response increases in parallel with the antibody titer. This was observed in the present study as the anti-bLG and anti-Cas rabbit sera displayed similar titer of specific antibodies and similar avidity of the response.

4. Conclusion

The present study shows that SPRI allows a multiplex monitoring of the complex antibody response to the major allergens of an allergenic source. Even less sensitive, the SPRI monitoring is faster, label-free and more informative than the usual methods derived from ELISA. In order to know whether high-affinity specific antibodies might modulate the IgE-mediated symptoms of allergy, the next step of this work will be the use of SPRI to determine the avidity of the specific antibody response of milk-allergic children. Thus, added to the classical quantification of the allergen-specific IgE response, the SPRI monitoring of the antibody response to isolated allergens should constitute a new tool of interest to improve allergy diagnosis.

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