Multiple epitope presentation and surface density control enabled by chemoselective immobilization leads to enhanced performance in IgE-binding fingerprinting on peptide microarrays

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Abstract

Multiple ligand presentation is a powerful strategy to enhance the affinity of a probe for its corresponding target. A promising application of this concept lies in the analytical field, where surface immobilized probes interact with their corresponding targets in the context of complex biological samples. Here we investigate the effect of multiple epitope presentation (MEP) in the challenging context of IgE-detection in serum samples using peptide microarrays, and evaluate the influence of probes surface density on the assay results. Using the milk allergen alpha-lactalbumin as a model, we have synthesized three immunoreactive epitope sequences in a linear, branched and tandem form and exploited a chemoselective click strategy (CuAAC) for their immobilization on the surface of two biosensors, a microarray and an SPR chip both modified with the same clickable polymeric coating. We first demonstrated that a fine tuning of the surface peptide density plays a crucial role to fully exploit the potential of oriented and multiple peptide display. We then compared the three multiple epitope presentations in a microarray assay using sera samples from milk allergic patients, confirming that a multiple presentation, in particular that of the tandem construct, allows for a more efficient characterization of IgE-binding fingerprints at a statistically significant level. To gain insights on the binding parameters that characterize antibody/epitopes affinity, we selected the most reactive epitope of the series (LAC1) and performed a Surface Plasmon Resonance Imaging (SPRi) analysis comparing different epitope architectures (linear *versus* branched *versus* tandem). We demonstrated that the tandem peptide provides an approximately twofold increased binding capacity with respect to the linear and branched peptides, that could be attributed to a lower rate of dissociation (K_d).

Keywords: peptide microarrays; multiple epitope ligands; click chemistry; polymer coating; IgE detection; surface plasmon resonance imaging; multivalency

Introduction

Peptide microarrays are powerful and versatile tools in many areas of medicine and diagnostics. In particular, their serodiagnostic applications well address the need for profiling individual immune-response in the field of infection, allergy, autoimmunity and provide hints on new biomarkers for cancer or neurodegenerative diseases [1], [2], [3], [4]. Due to the relative small size of molecules immobilized on array surfaces, any application of peptide microarrays demand an attentive strategy to display peptidic epitopes in order to gain the antibody capturing efficiency and specificity required by diagnostic applications.

The use of multi-presenting peptide platforms, i.e. molecular architectures where multiple copies of a peptide sequence are simultaneously displayed, is a well consolidated approach in many fields entailing peptide science [5], [6], [7]. The underlining concept of multiple presentation is that it allows for an increased number of favorable interactions between the peptide and its corresponding biological target, which can reflect on those kinetic, thermodynamic and entropic binding parameters that determine peptide-target affinity. These concepts have found interest in a number of applications, spanning from nanomedicine to bioimaging and biomaterials [8], [9]. In the analytical field, the use of branched peptides in ELISA tests is a widely used strategy to increase sensitivity [10], [11], [12]. Noteworthy, multivalent β-peptide foldamers were recently used as a way to create specific recognition interfaces rather than repeating features for multiple binding in an amyloid-β oligomer ELISA sandwich immunoassay, setting a new paradigm for probe-target recognition [13]. However, multiple epitope presentation (MEP) is still an unexplored path in microarray analysis. Moreover, widespread immobilization strategies for branched peptides on analytical surfaces often rely on non regiospecific binding, so that part of the polyvalent epitope probe is "sacrificed" upon attachment to the surface and consequently it is not fully exposed for favourable target interaction. The positive effects observed in these cases might be ascribed, to a significant extent, to the integrity of residual free epitopes within the polyvalent peptide construct. In addition, possible negative effects on recognition arising from aspecific interactions due to probe overcrowding are not usually considered. Hence, the design and use of multiple architectures as surface capturing agents should not only take into account ligand valency and ability to complement the binding sites, but also entails a fine modulation of probe three-dimensional space arrangement and density onto the analytical surface. Overall, the potential offered by multiple probe display in the design of diagnostic microarrays is likely far from being fully exploited.

Recently, we reported on the use of a polymeric clickable platform to control peptide orientation on microarray surface and demonstrated the highly favorable impact of chemoselective probe binding in a serological application [14].

In the present work we investigate the role of multiple epitope presentation (MEP) in combination with oriented probe display in a peptide microarray, taking into account also the effect of probes density on the analytical surface and the impact of distinct forms of multipresentation. We set this investigation in the challenging context of allergen specific Immunoglobulin E (IgE) detection and, specifically, in the detailed

study of the IgE-binding fingerprints in individuals allergic to the milk protein alpha-lactalbumin (allergen Bos d 4).

To this aim, we selected three known peptidic epitopes (LAC1, LAC2 and LAC8) [15] from alpha-lactalbumin and synthetically realized them in three distinct presenting forms: linear, branched and tandem (Scheme 1). Each probe was synthesized in order to allow following chemoselective immobilization *via* copper-catalyzed-azide-alkyne-cycloaddition (CuAAC)[16] on chip surfaces coated by copoly Azide [17]. A short-chain PEG spacer (O_2Oc) was also included due to its favorable role in maintaining the probe fully accessible [14]. The most reactive epitope of the series (LAC 1) spotted at different concentrations, was used to carefully optimize the probe surface density by measuring the capturing efficiency of an antibody directed against the full protein alpha-lactalbumin. We then investigated the role of MEP in alpha-lactalbumin specific IgE binding, using sera from milk allergic patients and healthy controls, highlighting a favorable trend in diagnostic performance from linear to tandem epitope presentation. To gain insights on the binding parameters that characterize antibody/epitopes affinity, we performed Surface Plasmon Resonance Imaging (SPRi) measurements on the alpha-lactalbumin antibody comparing the peptidic epitope architectures (linear *versus* branched *versus* tandem) for LAC1. Consistently with microarray results, we demonstrated that tandem presentation provided an approximately twofold antibody binding with respect to the linear and branched peptides, that could be attributed to the lower k_d value. In the following sections, results will be described and discussed in depth.

Materials and Methods

Reagents

Reagents for peptide synthesis were from Iris Biotech (Marktredwitz, Germany). Other chemicals were from Sigma-Aldrich (St. Louis, MO, USA) if not stated otherwise. Goat anti-human IgE, ε-chain specific in serum, was purchased from Calbiochem (Merck, Darmstadt, Germany) and labeled with Cy3 (GE Amersham, Chalfont St. Giles, UK) *in-house* according to the manufacturer's instructions. Rabbit anti-goat IgG labeled with Cy3 was from Jackson Immunoresearch (West Grove, PA, USA). Goat polyclonal anti-Alpha-lactalbumin antibody was from GeneTex (Irvine, CA). Silicon slides were from SVM (Sunnyvale, CA).

Polymer synthesis

Copoly Azide was obtained by post-polymerization modification reaction of copoly(DMA-NAS-MAPS) [18] with 3-azido-1-propanamine as reported in [17].

Peptide Synthesis

All peptides were synthesized by stepwise microwave-assisted Fmoc-SPPS on a Biotage ALSTRA Initiator+ peptide synthesizer according to well-established protocols. Briefly, peptides were assembled on a 2-CTC resin. Chain elongation was performed by iterative cycles of amino acids coupling (using Oxyma/DIC as activators) and Fmoc-deprotection using a 20% piperidine solution in DMF. Upon complete chain assembly, peptides were cleaved from the resin using a 2.5% TIS, 2.5% thioanisole, 2.5% water, 92.5% TFA mixture.

Crude peptides were then purified by preparative RP-HPLC. MS analysis was performed separately on purified material. Further details are provided in the Supporting Information

Peptide microarrays

Silicon slides with 100 nm SiO_2 layer were coated according to an established protocol [19]. Briefly, slides were immersed in a polymer solution (1% w/v in 0.9 M $(NH_4)_2SO_4$) for 30 min, then rinsed with water, dried under nitrogen and cured for 15 min under vacuum at 80° C.

Peptides were first dissolved with 50% acetonitrile solution (v/v, in Milli-Q water) to 10 mg/mL stock solution and then diluted into the printing buffer: for CuACC conjugation, 25 mM Na/Acetate pH 4.8, 15 mM trehalose, 100 μM $CuSO_4$, 400 μM THPTA and 6.25 mM Ascorbic Acid. Microarrays were prepared using a non-contact sciFLEXARRAYER S3 (Scienion Co., Berlin, Germany) spotter. Printed slides were placed in a humid chamber, incubated overnight at room temperature and then washed with 2 mM EDTA for 60 minutes.

Label-free quantification of the mass of bound peptides was performed on 500 nm SiO_2 layer chips by an IRIS platform [20]. Images of printed probes were acquired with Zoiray Acquire software and quantified providing a value of spot quality and bound mass density according to previous protocols [21].

Bioassays

Serum samples from allergic patients and healthy controls were collected and characterized as reported in [22] and in the Supplementary Information. Sera were incubated, undiluted, over the peptide array for 120 min. Negative controls included blank arrays incubated only with the secondary antibodies and with incubation buffer (Tris/HCl 0.05 M pH 7.6, NaCl 0.15 M, Tween 20 0.02%) with 1% w/v BSA. The microarray slides were then rinsed for 3 times with washing buffer (0.05 M Tris/HCl pH 9, 0.25 M NaCl, 0.05% v/v Tween 20) and PBS and incubated with 100 μL of 1 ug/mL anti-human IgE secondary antibody in incubation buffer with 1% w/v BSA followed by the same washing steps as described above. For the assay with anti-alpha lactalbumin antibody, chips were incubated for 60 minutes with various antibody concentration in incubation buffer and then with anti-goat IgG labeled by Cy3. Washing steps as described above.

Fluorescence was detected by a TECAN Power Scanner at 100% laser power and PMT adjusted after autogain procedure. Fluorescence intensities were analyzed using the QuantArray software from PerkinElmer. The fluorescence intensities were corrected for spot-specific background, values for replicate spots were averaged. t Student tests over the groups of samples were performed using Prism 7 software from GraphPad.

SPRi measurements

The kinetic-binding analysis was performed by using XelPleX SPRi (HORIBA Jobin Yvon SAS, France). Bare gold SPRi-biochips (HORIBA Jobin Yvon SAS, France) were pre-treated and used to spot the peptides (4 spots each at 100 µM concentration) as described for the peptide microarray (full details in Supplementary Information). Eight serial dilutions of antibody anti alpha-lactalbumin (from 0.95 to 500 nM) were injected (300 µl) at 100 µl/min resulting in 3 minutes of association time. The dissociation was monitored for 7 minutes for all the injected concentrations with exception of the 500nM for which the dissociation was monitored for 4-h. After each injection the chip surface was regenerated with glycine 50mM at pH ranging from 1,5 to 2. The results were preprocessed using EzSuite (HORIBA Jobin Yvon SAS, France) and the kinetic-binding constants values were calculated using Scrubbergen2 (licensed by HORIBA Jobin Yvon SAS).

Results and Discussion

Peptide microarray design

The linear IgE epitopes of the milk protein alpha-lactalbumin (allergen Bos d 4) LAC1, LAC2 and LAC8 were derived from the epitope mapping study reported in [15]. Each peptidic probe was assembled via fully automated MW-assisted solid phase peptide synthesis (see Methods) and modified at the C-terminal region with a short-chain PEG spacer (O_2Oc) bearing a terminal propargylglycine (Prg) to allow subsequent chemoselective conjugation to copoly Azide [17]. Branching was introduced by the use of Fmoc-Lys(Mtt)-OH as previously reported in the literature for branched peptides (see Supplementary Information). Cysteine residues at positions 14 (LAC 1), 7 (LAC 2) and 4, 15 (LAC 8) were mutated into Alanine residues to avoid spontaneous reaction of free thiols with propargyl groups potentially affecting both synthetic yields and immobilization reproducibility [23]. To ensure that full functionality of alanine-modified epitopes was unaltered, preliminary antibody competition assays were run confirming preserved binding efficiency (see Supplementary Information Figure 1-S).

To enable peptides chemoselective immobilization onto microarray surface, we exploited the recently developed copoly Azide polymer (Scheme 2). This is a polymeric coating for microarray silicon slides bearing azido groups for click-type immobilization of *yne*-modified probes; it is obtained by post-polymerization modification of its precursor copoly(DMA-NAS-MAPS) [18] a polymer made of *N,N*-dimethylacrylamide (DMA), *N*-acryloyloxysuccinimide (NAS), and 3(trimethoxysilyl)-propylmethacrylate (MAPS) and characterized by excellent properties for functional coating in microarrays and other biosensing techniques [24]. Chemoselective linkage to the copoly Azide coating allows indeed for accessible probe exposure on the chip surface to favor optimal ligand-target interaction, resulting in a better signal/background ratio [14].

Probe chemoselective immobilization and surface density optimization

Linear (-L), branched (-B) and tandem (-T) forms for the LAC1 peptide were dissolved at concentrations ranging from 4 μM to 80 μM in 100 μM $CuSO_4$, 400 μM THPTA and 6.25 mM ascorbic acid, the optimized spotting buffer for the conjugation on copoly Azide via the azide-alkyne-cycloaddition (CuACC); the IRIS (Interferometric Reflectance Imaging Sensor) technique [20] was used for quantification of peptide mass bound onto the chip surface. This chemoselective immobilization protocol provided a high binding efficiency resulting in an average bound mass of 2 ng/mm^2 for the three peptides, when spotting in optimal conditions (16 - 40 μM). As expected, a dilution of the peptide spotting concentration from 80 μM to 4 μM provided a significant decrease in the binding yields (Figure 1). The IRIS label free imaging of the spotted arrays are reported in Figure 2-S in the Supplementary Information.

Next, we incubated the peptide arrays with an immuno-purified, goat, anti-alpha-lactalbumin polyclonal antibody at a non-limiting concentration (30 ng/mL) and we evaluated the fluorescence responses with a secondary antibody (anti-goat IgG labelled by Cy3). We found that thinning out the peptidic probes resulted in an increased fluorescence signal thus being beneficial for antibody capturing. Accordingly, we correlated the spacing between peptide probes immobilized on the array surface (calculated by the immobilized mass at each spot) with the corresponding antibody capturing efficiency, derived by the detected fluorescence intensity. To estimate the peptide spacing we used mass information detected by IRIS [21] (see Supplementary Information for details).

Figure 2 reports the relative intensity of the fluorescence signal due to antibody capturing as a function of probe spotting concentration (i.e. surface density) for each LAC 1 presentation form (L, B, T).

For the linear and branched forms, a clear trend between antibody capturing efficiency (Figure 2, blu line) and peptide spacing (Figure 2, red line) is observable: at low spotting concentration, peptide spacing is higher and antibody capturing more efficient. At higher spotting concentrations, a presumably probe crowding regime, antibody capturing is diminished. We can ascribe this phenomenon to steric hindrance, as previously reported for anti-phosphotyrosine antibody in their ability to bind phosphorylated peptides [25], and/or to dense peptide packing that could favour hydrophobic interactions leading to aggregation likely altering the accessibility of the epitope sequences to the antibody. In this sense, we hypothesize that this phenomenon, already described by White S.J and coworkers [26], could be more relevant in close proximity of the sensor surface. This could explain why in the tandem presentation form, where the probe likely protrudes out of the surface to an higher extent, the correlation of spotting density on antibody recognition is still present but less evident.

Overall, our data strongly suggest that peptide surface density has to be carefully evaluated to maximize the degree of antibody capturing in microarrays: increasing peptide density is not the obvious choice when capturing large proteins such as antibodies. In this context, the use of a chemoselective strategy to immobilize peptides at one end allows a fine tuning of probe density resulting in optimized performance of microarrays. Indeed, when a random-type immobilization was used for peptides *via* active esters on parent

polymer copoly(DMA-NAS-MAPS), the effect derived from fine-tuned density was undetectable (data not shown).

IgE Epitope fingerprinting in allergic patient's serum

The principle of allergy diagnosis by IgE detection on microarrays consists in an immunoassay run on a panel of allergens to provide the identification of the molecules to which the patients are sensitized (component-resolved diagnostics) [27], [28]. Breaking up the full allergens in their immunoreactive peptides can further detail the knowledge of IgE epitopes [29], [30], and finely dissect the patient's IgE reactivity to correlate with clinical features [31], [32]. The three IgE-reactive epitopes here analyzed for Bos d 4 represent the most frequently recognized peptides of alpha-lactalbumin, they are in close vicinity on the surface of the native protein and seem to define an IgE-reactive patch on the protein [15].

Peptide microarrays displaying three alpha-lactalbumin epitopes (LAC1, LAC2 and LAC8) in the three molecular architectures (L, B and T) were tested in their ability to detect epitope specific serum IgEs. Peptides were spotted at the concentration of 16μM, since we wanted to operate far from saturation concentration (80μM for linear and branched peptides) and given the fact that lower concentrations didn't provide reliable fluorescence signals in this test. The peptide arrays were probed with serum samples (n = 9) from allergic patients previously characterized in their content of allergen Bos d 4 (alpha-lactalbumin) specific IgEs either by ImmunoCAP or ImmunoCAP - ISAC from Phadia (www.phadia.com). The same test was run on controls (n = 9) consisting of sera from patients negative for IgEs specific to Bos d 4. Details on the samples used in this work are reported in the Supplementary Information. We evaluated the peptide specific IgE content by fluorescence detection using a Cy3 labelled anti-human IgE monoclonal secondary antibody as detailed in the experimental section. Figure 3 reports a representative fluorescence image of the peptide array probed with a serum from an allergic patient (panel a) and the same array incubated with the anti-alpha-lactalbumin antibody at 30 ng/mL.

The ability of each epitope presentation form to capture specific antibodies (IgEs), thus discriminating between controls and allergic patients, was evaluated based on the quantification of the peptide-specific fluorescence signals detected in the two sample groups, allergic and negative (Figure 4 and Figure 3-S). We observed that the use of MEP generally provides an increase of fluorescence intensities in the group of allergic patients and, especially for the tandem peptides, a compression of the distribution of fluorescence values among the negative patients as compared to the linear peptides (see Figure 4). In our conditions, these two distinct effects resulted in the outcome of an increasing capability to distinguish the two sample groups from the linear to the branched and tandem peptides. This feature can be quantitatively evaluated by the *p*-values (statistical significance) of the unpaired t-Test for the peptide specific IgE detection in the two sample groups which is reported in Figure 5 for each peptide.

As compared to the linear peptides, only the branched and tandem forms of LAC1 and LAC8 gained statistical significance ($p<0.05$) in distinguishing among allergic and negative patients; LAC2 increases its diagnostic capability as well. Overall, our results demonstrate that MEP is potentially beneficial to the

diagnostic capabilities of the peptide array, with tandem peptides performing at the highest statistical significance. The evidence that branched peptides do not perform the same level of diagnostic discrimination of tandem repeats further supports the need for a full probe accessibility that in the case of branched peptides could be hampered by close spatial proximity of the two epitope sequences, possibly aspecifically interacting with each other. It is worth remarking that the diagnostic context in which we set our investigation is highly challenging. The IgE titer in blood is about 100,000 times lower than IgG and IgM level, making IgE detection much more demanding, with required sensitivity down to the ng/mL level and below [33]. A further difficulty originates from the presence in serum of both allergen specific IgEs and IgGs competing for the binding to the same immobilized allergen, thus hindering the detection of IgEs [34], [35]. The use of silicon chips with an optimized SiO_2 layer to enhance fluorescence [22], [19] and of a polymeric coating which efficiently suppress matrix non-specific interactions [36] were crucial to achieve in this assay a low background for sera analysis and the necessary detection sensitivity.

The enhanced performance in IgE fingerprinting provided by MEP peptides could be ascribed to a combination of favorable features including, but not restricted to higher apparent affinity (possibly due to multivalent binding of IgE and/or increased probe accessibility) and by amplified probe selectivity. In order to gain deeper insights on the different behaviour of the three epitope presentations, we performed a complementary analysis by SPR imaging aiming to obtain kinetics information on antibody-probes interaction that could support our hypotheses.

SPRi experiments

Surface Plasmon Resonance Imaging (SPRi) was used to characterize the binding between the anti-alpha-lactalbumin antibody and the different peptidic epitope architectures (linear *versus* branched *versus* tandem) of epitope LAC1. Kinetic-binding analyses were performed by flowing different concentrations of anti alpha-lactalbumin onto a SPRi-chip coated with copoly Azide and arrayed with LAC1_L, LAC1_B and LAC1_T. In fact, a simple coating procedure described in the experimental section allowed us to apply a common chemoselective immobilization method on both silicon microarray and gold SPRi-chips thus minimizing experimental differences.

Considering the intrinsic very high affinity of the antibody tested here, the experiments design was consequently optimized in order to perform kinetic measurements according to similar studies reported in literature [37]: first, the dissociation curves were monitored for a long time (4 hours) to determine very low dissociation rates; second, the antibody solutions were injected at high flow rate (100 µl/min), in order to control and limit mass transport effects. The concentration of peptides spotted on SPRi chips is 100 µM, higher than those used on microarray chips (4 - 80 µM) because lower concentrations did not give significant ligand immobilization and detectable SPRi signal (data not shown). By observing the kinetic data in Figure 6, the first clear feature observable is that the R_{max} (i.e. maximum binding capacity) related to the tandem (LAC1_T) architecture is almost doubled (251%) if compared with the R_{max} of the linear peptide (LAC1_L).

The same result was not observed for the branched configuration (LAC1_B), which is rather associated with a slightly lower binding capacity if compared with LAC1_L. This evidence may be justified by the close proximity of peptide chains in the branched form which may lead to unfavorable interactions that could slow down antibody-probe recognition and is in accordance with data on peptide microarrays incubated with the anti-alpha-lactalbumin antibody. Indeed, after normalization by the number of bound molecules determined by the IRIS technique, arrays showed the highest antibody binding on LAC1_T followed by LAC1_L and LAC1_B (Supplementary Information, Figure 4-S).

Considering the binding kinetics (Table 1), the association constants (K_a) related to all the peptides are similar, however maximum association rates were measured for the linear architecture (LAC1_L). The probe-antibody association rate seems therefore not depend on the peptide geometry, but might rather be determined by probes accessibility to the antibody which in the case of linear peptides is higher.

The study of dissociation curves confirmed the hypothesis of very slow rates ($K_d < 2 \times 10^{-5}\ s^{-1}$) and very long residence time of the antibody-peptide complex (>15 h) due to high antibody affinity, for all the peptides. Intriguingly, LAC1_T dissociation rate is around 3-fold slower than those of LAC1_L and LAC1_B with a consequent residence time of 47.5 h, to be compared with 16.0 h and 17.3 h for LAC1_L and LAC1_B, respectively. All these data are further summarized by the affinity constants related to the three different architectures, in particular showing higher affinity (242%) for LAC1_T epitope ($K_A = 3.75 \times 10^9\ M^{-1}$) if compared with that related to LAC1_L ($K_A = 1.55 \times 10^9\ M^{-1}$) and LAC1_B ($K_A = 1.46 \times 10^9\ M^{-1}$)(94%) as reported in Table 1.

Since the dissociation of species interacting polyvalently require breaking multiple interactions, the kinetics for polyvalent interactions often show a decrease in the rate of dissociation (K_d) of the two polyvalent entities, rather than an increase in the rate of association [38]. Therefore, the evidence that antibody-LAC1_T dissociation occurs more slowly than the others, could suggest a bivalent interaction between the antibody and the tandem construct.

While in sera epitope mapping on microarrays both branched and tandem architectures provided an increase of diagnostic performance, the SPRi results pointed only to the increased affinity of the tandem construct with branched peptides performing at a lower level than the linear form. This non uniformity of results for the branched peptides can be reconciled considering the intrinsic different nature of the two biosensing experiments and that the discrimination potential of a probe is not necessarily correlated with affinity. Measuring the probe affinity for a purified and high-affinity antibody is indeed not the same as screening the probe towards a multitude of different antibodies in the context of a complex biological sample with high cross-reactivity potential. In fact, probes of low affinity can in some cases discriminate better than do probes with high affinity if weaker cross-reactions will tend to be below the detection sensitivity [39].

Conclusions

The aim of this study was to evaluate the role of epitope multipresentation on the performances of serological assays in microarray analysis. To this aim we investigated a case study using a set of three peptides derived from the milk allergen alpha-lactalbumin presented in three distinct forms (linear vs branched vs tandem) and analyzing a panel of sera representative of different levels of specific IgEs, demonstrating an increased discriminating potential for the MEP peptides. Improvements were particularly relevant for the tandem presentation form. Our results point also to the importance of accurate peptide density tuning in order to reduce probes overcrowding effects and maximize microarrays analytical performance. The role of probe orientation and surface crowding when using low-molecular weight capturing elements was indeed reaffirmed for multivalent peptide foldamers in relation to analyte-binding accessibility in ELISA [13]. To gain insights into the mechanisms determining antibody/MEP affinities we ran SPRi experiments with a purified antibody. Even if the use of an antibody with such an intrinsic high affinity for the tested probes is probably not the optimal model to highlight the differences between the various peptidic constructs, we found that the tandem presentation results in the highest affinity due to lower K_d value. This could be indicative of a multivalent interaction.

More in general, the better performances that we observed for the MEP forms in this serological assay is not necessarily related only to increased affinity. Weak affinity interactions are less tight but not automatically less specific compared to stronger binding pairs. Branched peptides indeed showed a slightly lower affinity than linear ones but still are more capable of diagnostic discrimination.

A common immobilization chemistry (copoly Azide) for silicon and gold chips enabled the use of a multi-sensing platform (SPRi, label-free and high-sensitivity fluorescence microarrays) to deliver effective diagnostic assays while providing insights into the molecular recognition mechanisms involved.

The approach here reported, applied in the frame of allergy, may certainly be extended to other fields. For example, to better investigate the high autoantibody heterogeneity in Multiple Sclerosis [40], Rheumatoid Arthritis [41] and other autoimmune diseases [42].

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Figure captions

Scheme 1: alpha-lactalbumin IgE epitopes were synthesized as linear aminoacidic sequences terminated by PEG spacer bearing a propargylglycine for the CuACC click-type immobilization on copoly Azide. The linear peptide was compared to peptides bearing the same sequence repeated twice in a branched and tandem form

Scheme 2: A) Amino acid sequences for LAC epitopes. Cysteine residues at positions 14 (**LAC 1**), 7 (**LAC 2**) and 4, 15 (**LAC 8**) that were mutated into Alanines are underlined in bold. B) Representative structures for C-terminal modification of peptides (-$(O2Oc)_2$-Prg) and for copoly-Azide. C) Representative structure for epitope branching.

Figure 1: binding efficiency (ng/mm^2) for peptides LAC1_L, LAC1_B, LAC1_T spotted at concentrations ranging from 4 μM to 80 μM on Copoly Azide assessed by IRIS. The CuACC click strategy provided high binding efficiency for the whole set of probes at any spotted concentration.

Figure 2: correspondence between probe spacing expressed in nanometers (red line) and relative fluorescence intensity (RFI, blue line) detected after antibody capturing on peptides LAC1_L, LAC1_B, LAC1_T immobilized on Copoly Azide. A common trend between antibody capturing efficiency and peptide spacing is observable.

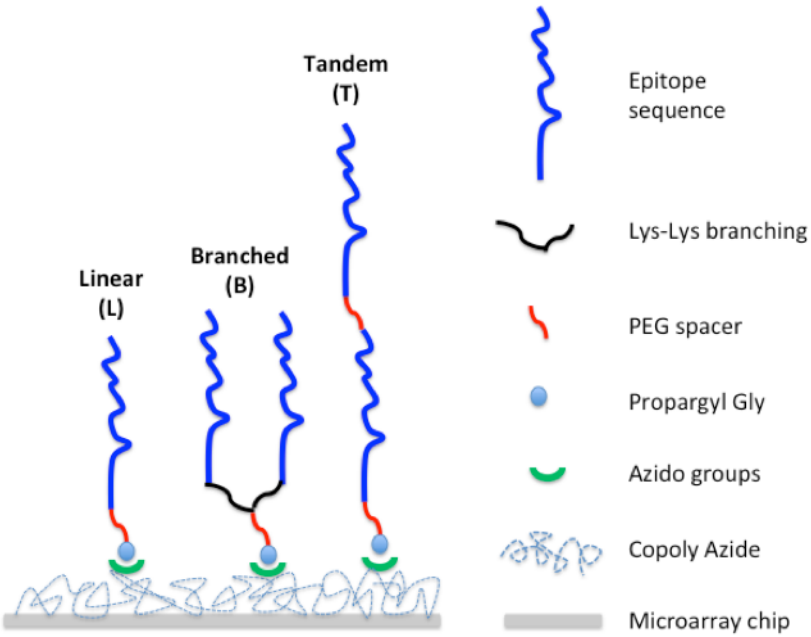
Figure 3: Panel a): representative image of the peptide array incubated with an allergic paptient's serum samples followed by incubation with anti-IgE labelled with Cy3, analyzed at 100% laser and 900% gain. Panel b) representative image of the peptide array incubated with the anti-alpha-lactalbumin antibody (30 ng/mL) followed by incubation with anti-Goat IgG labelled with Cy3, analyzed at 50% laser and 50% gain.

Differences in spot size (less than 20%) is due to slight changes in the surface tension of the spotted droplets for different solutes.

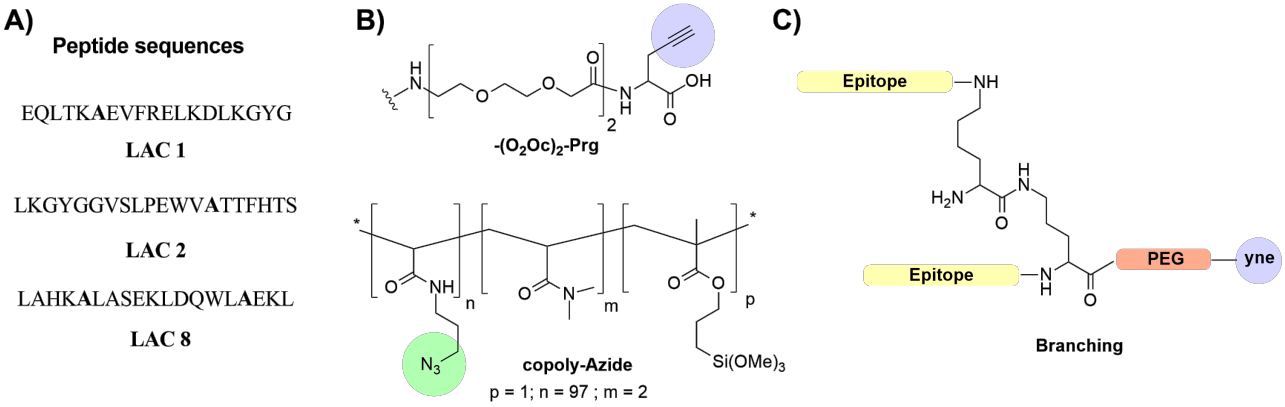
Figure 4: Fluorescence intensity (RFI) for the peptide specific IgE detection on each peptide. Peptide arrays displaying LAC1, LAC2, LAC8 in the linear, branched and tandem form were probed with serum samples and pools of sera from allergic patients (n = 9) and control patients (n = 9).

Figure 5: unpaired t-Test results for the peptide specific IgE. Peptide arrays displaying LAC1, LAC2, LAC8 in the linear, branched and tandem form were probed with sera (n = 9) from allergic patients and control patients (n = 9). ns = not significative. Significative: $p<0.05$; * = $p<0.05$; ** = $p<0.01$.

Figure 6: SPRi kinetic-binding study. Binding of anti-alpha lactalbumin antibody to immobilized alpha lactalbumin IgE epitopes LAC1_L, LAC1_B, LAC1_T spotted in replicate (four spots each) on a copoly-azide coated SPRi chip. The binding responses were simultaneously monitored at 25 °C by injecting the antibody at 8 different concentrations ranging from 0.98 to 500 nM (different colored lines). The association rates were calculated by monitoring 3 minutes of association for all the concentrations injected (A-C;); the dissociation rates were calculated by monitoring 4-h of dissociation of the antibody injected at 500 nM (D-F). The orange traces represent the fit resulted from a global fit assuming a 1:1 Langmuir binding.



Scheme 1



Scheme 2

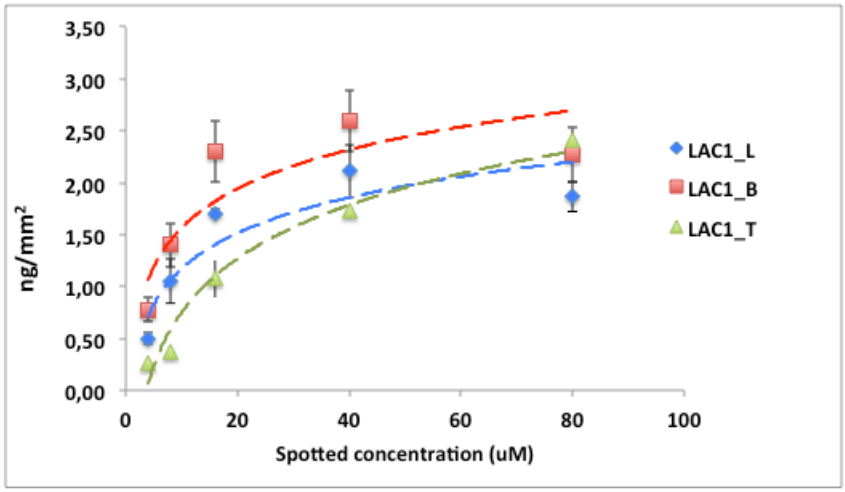

3,50
3,00
2,50
2,00
1,50
1,00
0,50
0,00
ng/mm²
0
20
40
60
80
100
Spotted concentration (uM)
LAC1_L
LAC1_B
LAC1_T

Figure 1

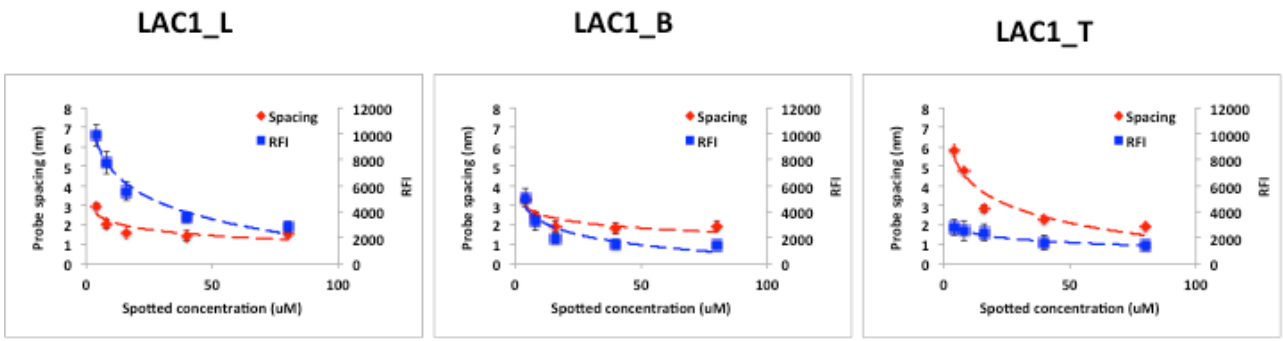

LAC1_L
LAC1_B
LAC1_T
Spacing
RFI
Probe spacing (nm)
RFI
Spotted concentration (uM)
0
50
100
0
1
2
3
4
5
6
7
8
0
2000
4000
6000
8000
10000
12000

Figure 2

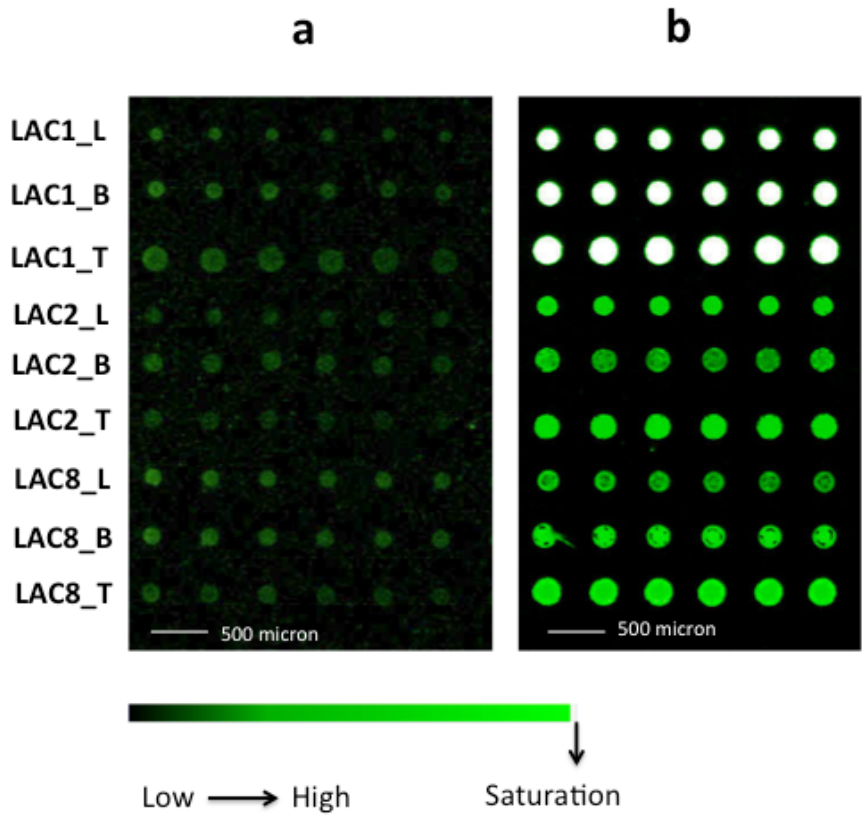

a
b
LAC1_L
LAC1_B
LAC1_T
LAC2_L
LAC2_B
LAC2_T
LAC8_L
LAC8_B
LAC8_T
500 micron
500 micron
Low
High
Saturation

Figure 3

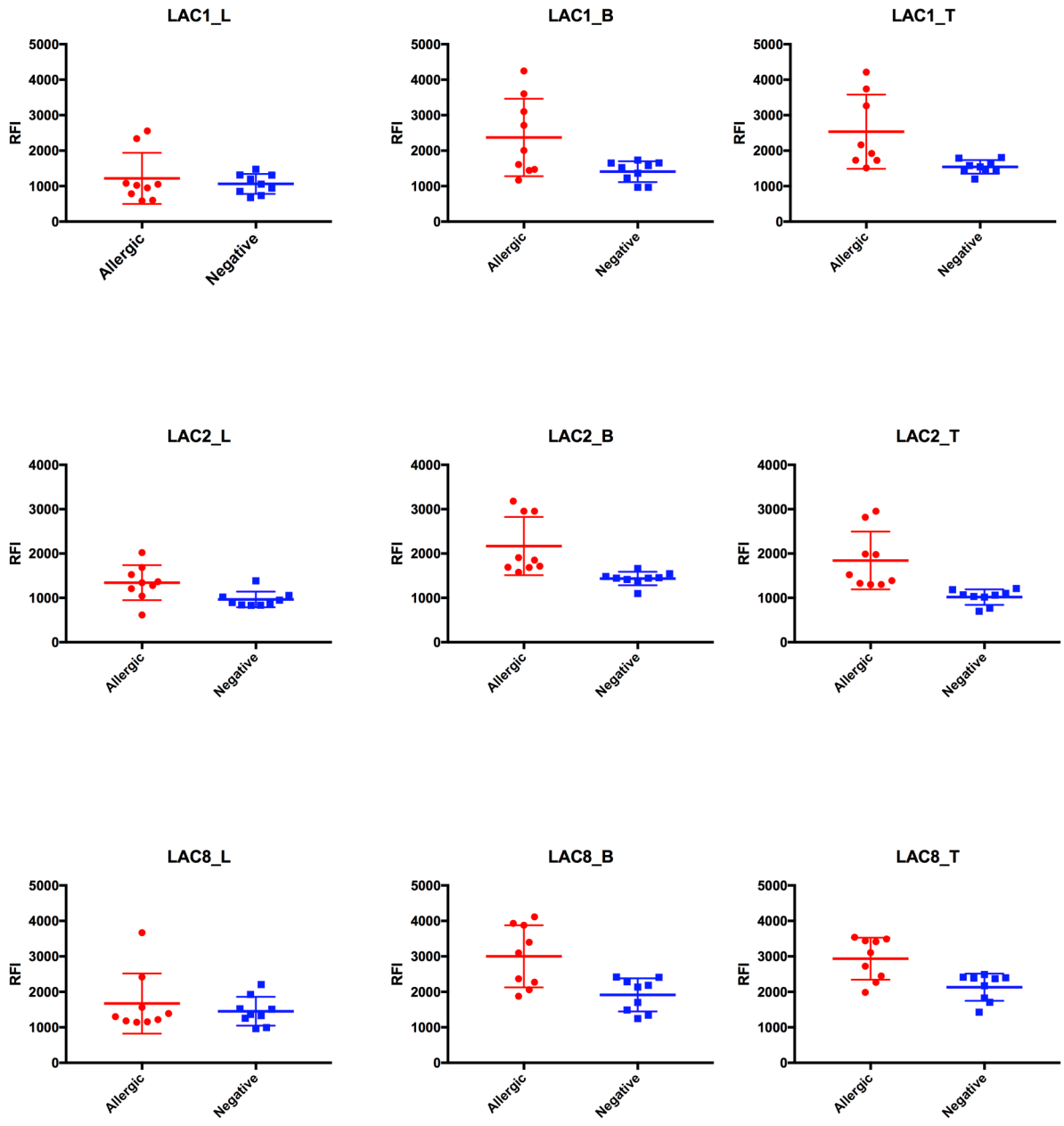

LAC1_L
LAC1_B
LAC1_T
LAC2_L
LAC2_B
LAC2_T
LAC8_L
LAC8_B
LAC8_T
RFI
Allergic
Negative

Figure 4

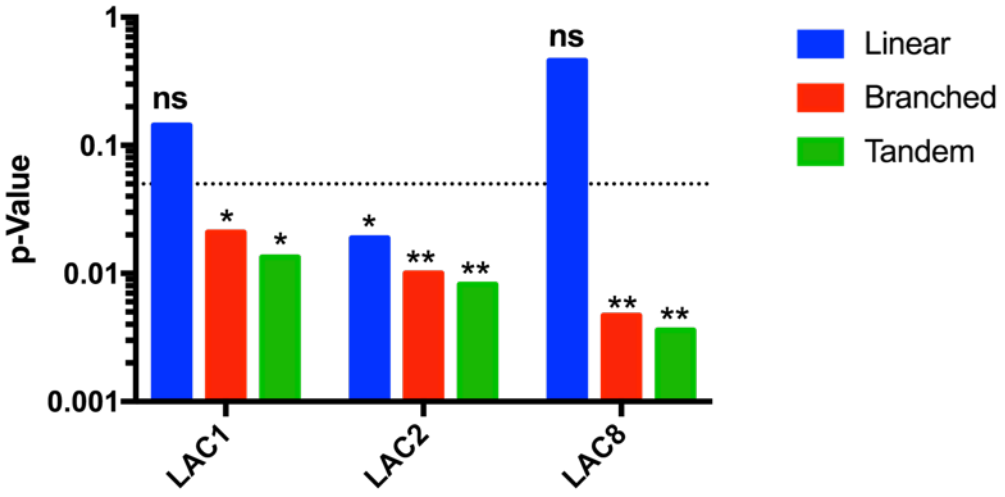


Figure 5

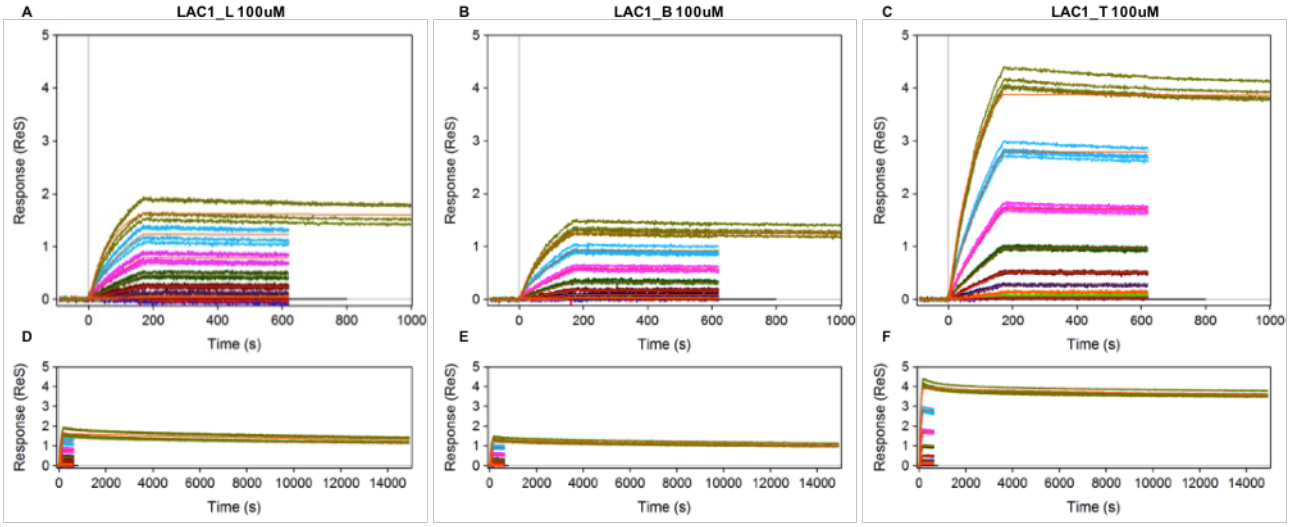


Figure 6

Table 1: Kinetic values related to the SPRi kinetic-binding study reported in Figure 6.
([a]) Percentage values are related to linear epitopes (LAC1-L) kinetic constants used as reference (100%)
([b])"Res. SD" is the average residual standard deviation of the global fitting and is referred to the signal (ReS).
([c]) "relative Res. SD" is calculated as ratio between Res. SD and R_{max}.

Epitope	K_a (M^{-1} s^{-1}) x10^4	K_d (s^{-1}) x10^{-6}	Residence time, 1/K_d (h)	R_{max} (ReS)	K_D (pM)	K_A (M^{-1}) x10^9	Res. SD[b] *(relative Res. SD)[c]*
LAC1_L	2.690±0.010	17.360±0.005	16.0	1.804±0.002	645±3.0	1.55±0.007	0.128 *(7.0 %)*
LAC1_B	2.347±0.007 *(87%)[a]*	16.040±0.005 *(92%)[a]*	17.3 (108%)[a]	1.475±0.002 *(82%)[a]*	683±2.0 *(106%)[a]*	1.46±0.004 *(94%)[a]*	0.066 *(4.5 %)*
LAC1_T	2.194±0.004 *(75%)[a]*	5.85±0.010 *(33%)[a]*	47.5 (274%)[a]	4.528±0.003 *(251%)[a]*	267±0.5 *(41%)[a]*	3.75±0.007 *(242%)[a]*	0.116 *(2.6 %)*