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Investigation of surface plasmon resonance biosensor for skin sensitizers studies

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ABSTRACT

Non-animal testing methods are a current challenge in terms of the assessment of skin sensitization potential for new chemicals. Our objective was to investigate a surface plasmon resonance (SPR) biosensor to screen allergens against nucleophilic amino acids (cysteine, lysine and histidine) in a direct binding assay. Amino acids were immobilized on the sensor surface and exposed to different skin allergens (chemicals and fragrances) with varying sensitizing potential.

Cysteine was found to be more reactive than lysine while histidine showed the lowest reactivity. The interactions observed were different depending on the allergen/amino acids involved. It appeared that weak allergens could quickly dissociate from the ligand, whereas strong and extreme allergens remained bound to the amino acids. The SPR report points allowed a good discrimination of the tested allergens.

With this technology, we can observe low energy bindings and get information on the stability of the hapten/amino acid complex which seem relevant for the determination of skin sensitization potential. This prospective experiment showed the potential of real-time SPR to generate specific report points to refine the skin sensitization allergen assessment.

1. Introduction

Skin exposure to allergens can result in allergic contact dermatitis which is an important health issue. Currently, the accepted approaches for sensitization hazard assessment are *in vivo* animal tests such as the local lymph node assay (LLNA) described by Kimber et al. (1994). It is the method of choice for the determination of skin sensitization potential for the purposes of categorization (Basketter et al., 2001; Kimber et al., 2003). However, animal testing of cosmetic ingredients will be banned within the EU in 2009 for several tests, and in 2013 for repeated-dose toxicity including skin sensitization studies in agreement with the seventh amendment of the European Council Directive 76/768/EEC.

As a result of this directive, it has become necessary to develop alternative strategies that are based solely on *in vitro* methods to generate useful data for prediction of skin sensitization potential. Some of the published *in vitro* methods are based on keratinocytes and skin dendritic cell lines (Ashikaga et al., 2006; Hirota and Moro, 2006; Kimber et al., 2001; Ryan et al., 2007; Vandebriel et al., 2005; Van Och et al., 2005). Schoeters et al. (2007) investigated whether the differential gene expression patterns in dendritic cells are relevant for the prediction of the sensitizing potential of chemicals using microarray technology. Alternative approaches to predict skin sensitization are reviewed elsewhere (Casati et al., 2005).

Other strategies are based on electrophilic assays. The electrophilic properties of allergens may enable reaction with skin nucleophiles to form macromolecular immunogens (Aptula et al., 2005). The reactivity with nucleophiles might then be used as a skin sensitizers screening tool. The strongest potential nucleophiles in proteins are the sulfhydryl group of cysteine, methionine, the ε-amino group of lysine and the imidazole group of histidine (Divkovic et al., 2003). Recently published methods are based on human serum albumin (HSA) reactivity to identify the types of amino acids modified and the nature of haptenation by accurate mass shift determinations and sequencing (Aleksic et al., 2007), non-enzymatic thiol reactivity using glutathione in combination with *in vitro* toxicity measurement (Aptula et al., 2006), and synthetic peptides reactivity using HPLC (Gerberick et al., 2004).

Surface plasmon resonance (SPR) biosensor technology has been applied to the study of numerous protein–protein interactions. It involves label-free binding interactions between an analyte in solution and an immobilized ligand being monitored directly by changes in refractive index at the biosensor surface. The response is directly proportional to the mass of the bound analyte. Recently published studies demonstrate the applicability of SPR in characterizing small molecule interactions: drug–protein interactions (Touil et al., 2005; Wear et al., 2005), antigen–antibody interactions (Aizawa et al., 2007; Kawazumi et al., 2005;

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Shankaran et al., 2005), inhibitor–enzyme interactions (Nordin et al., 2005; Stenlund et al., 2006) and small molecule–nucleic acid interactions (Cao et al., 2007; Nguyen et al., 2007). Recent improvements in biosensor instrument hardware, experimental design and data processing have made it possible to detect routinely the binding of low-molecular-mass analytes even though some challenges still exist (Papalia et al., 2006).

In this paper, we describe a method to screen contact allergens against three nucleophilic amino acids (cysteine, lysine, histidine) in a real-time direct binding assay using SPR biosensor technology. A variety of 21 chemicals and 17 fragrances were tested and the interaction was measured. The choice for relevant chemicals and fragrances to test in the present study was based on LLNA, this method being considered as the reference one for assessing the sensitizing potency (OECD Test Guidelines No. 429 adopted on the 24th of April 2002). Samples were chosen on the basis of their EC3 values that covered a wide spectrum corresponding to quite different levels of reactivity and thus covering the different classes of sensitizing potency as defined in the OECD Test Guidelines. LLNA is the only non-clinical validated method widely used as a screening test before performing clinical studies that are, for ethical reasons, only made on non-sensitizing fragrances to be launched on the market. LLNA, when coupled with good exposure data, can be used as the starting point for a quantitative risk assessment (Basketter et al., 2008).

We observed that SPR biosensors can be used to discriminate allergens depending on their specificity for the thiol group from cysteine, the amine group from lysine and the imidazole side chain from histidine. We also report the results of the screening of 17 fragrances supported with LLNA *in vivo* data.

2. Materials and methods

2.1. General principle of SPR

Optical biosensors convert the accumulation of mass at a surface into an optical signal to monitor a wide range of interactions. Biacore's technology relies on surface plasmon resonance which arises when incident light is reflected from a thin gold film between two layers of different refractive indexes (Fig. 1). Measurements are carried out under continuous flow of molecules over the sensor surface mounted in a microfluidic flow cell. Binding and dissociation of molecules in solution to surface-immobilized receptors alter the refractive index of the medium near the surface. These changes are monitored in real-time to measure the amount of bound molecules, their affinity for the receptor and the association and dissociation kinetics of the interaction. Levels of interaction are expressed in arbitrary units named resonance units (RU).

In the direct binding assay, the target (amino acid) is immobilized to the sensor surface and allergens are passed over the surface. In the association phase, the amino acids interact with the allergens, forming a complex. In the dissociation phase, the complex resulting from the interaction starts to dissociate.

2.2. Instrumentation

SPR analyses were performed at 25 °C using a Biacore 3000 optical biosensor equipped with research-grade CM5 sensor chip (GE Healthcare). The chip is covered with carboxymethyl dextran which minimizes non-specific binding of biomolecules to the surface and increases the binding capacity of the surface.

2.3. Allergens

A variety of chemicals were used, selected to represent different sensitizing potentials (non-sensitizer, weak, moderate, strong and extreme) according to Gerberick et al. (2005) as well as a variety of fragrances with sensitizing potentials from strong, moderate to non-sensitizers (according to LLNA *in vivo* data). Allergens are listed in Table 1.

Phosphate buffered saline (PBS) soluble chemicals were stored as 20 mM stock solutions in PBS. Other compounds were stored as 1 M stock solutions in 100% DMSO at −20 °C. Fragrances were stored at 6 °C.

2.4. Buffers

PBS (1.05×) was used as running buffer to immobilize the amino acids and the polypeptides and to screen PBS soluble allergens. PBS (1.05×) 5% DMSO was used as running buffer for 5% DMSO soluble allergens and fragrances and 1.05× PBS 10% DMSO for 10% DMSO soluble allergens. The running buffer contained the same DMSO concentration as the injected samples since DMSO has a significantly different refractive index from the PBS and small differences in DMSO concentration may significantly affect the bulk response.

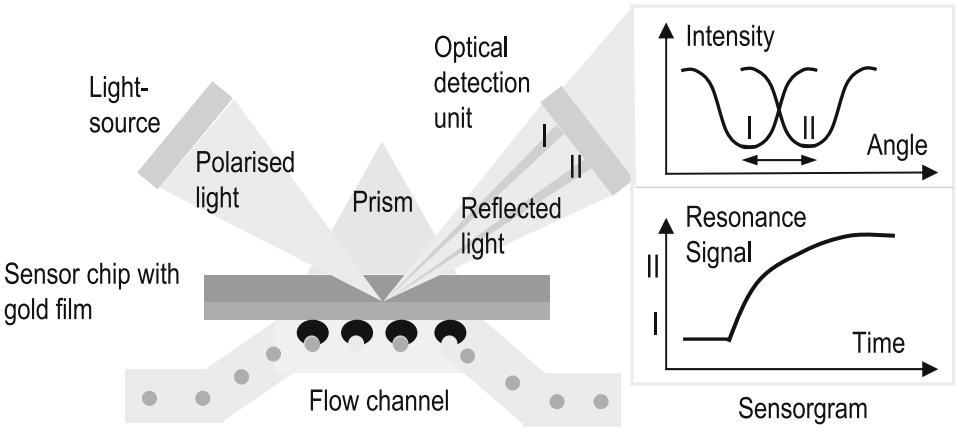

Fig. 1. Scheme of optics and signal generation of the Biacore SPR biosensor. This optical biosensor exploits total internal reflection of light at a surface–solution interface to produce an electromagnetic field, or evanescent wave, from a thin layer of gold into a solution. SPR is seen as a drop in the intensity of the reflected light. One molecule is immobilized onto the surface and the other is flowed past the surface in solution. When the molecules interact to form a complex, the refractive index is changed near the gold surface which alters the angle of incidence required to create the SPR phenomenon. This change in refractive index which is closely correlated to the change in mass near the gold surface is measured in real-time and the result is plotted as response or resonance unit (RU) versus time.

Table 1
Chemical allergens and fragrances tested against nucleophiles.

Chemical	MW	LLNA EC3[a]	Potency category	Dilution buffer	Supplier
Diethylphthalate	222.24	NC	Non-sensitizer	5% DMSO	Sigma
1-Bromobutane	137.03	NC	Non-sensitizer	5% DMSO	Sigma
Hydroxycitronellal	172.77	33	Weak	5% DMSO	Sigma
Cyclamen aldehyde	190.31	22	Weak	5% DMSO	Givaudan
Lilial	204.31	18.7	Weak	5% DMSO	Givaudan
Lyral	210.00	17	Weak	5% DMSO	IFF
Benzyl benzoate	212.25	17	Weak	5% DMSO	Fluka
Citral	152.33	13	Weak	5% DMSO	Fluka
Eugenol	164.20	13	Weak	10% DMSO	Sigma
Hexylcinnamaldehyde	216.33	11	Weak	5% DMSO	Sigma
α-Amyl cinnamaldehyde	202.31	11	Weak	5% DMSO	Sigma
Dihydrocoumarin	148.16	5.6	Moderate	5% DMSO	Sigma
3-Aminophenol	109.10	3.2	Moderate	5% DMSO	Sigma
Phenylacetaldehyde	120.15	3	Moderate	5% DMSO	Sigma
1,2-Benzisothiazolin-3-one	151.20	2.3	Moderate	5% DMSO	Sigma
Dimethylaminopropylamine	102.20	2.2	Moderate	5% DMSO	Sigma
Isoeugenol	164.20	1.2	Moderate	10% DMSO	Fluka
Glutaraldehyde	100.12	0.1	Strong	PBS	Sigma
2,4,6-Trinitrobenzenesulfonic acid (TNBS)	293.17	NC	Strong	PBS	Sigma
p-Benzoquinone	108.09	0.0099	Extreme	5% DMSO	Fluka
2,4-Dinitrobenzenesulfonic acid (DNBS)	270.15	NC	Extreme	PBS	Fluka

Fragrance	LLNA EC3	Potency category	Dilution buffer
Fragrance 1	151.30	Non-sensitizer	5% DMSO
Fragrance 2	119.30	Non-sensitizer	5% DMSO
Fragrance 3	116.50	Non-sensitizer	5% DMSO
Fragrance 4	73.59	Non-sensitizer	5% DMSO
Fragrance 5	66.02	Non-sensitizer	5% DMSO
Fragrance 6	64.30	Non-sensitizer	5% DMSO
Fragrance 7	61.04	Non-sensitizer	5% DMSO
Fragrance 8	60.59	Non-sensitizer	5% DMSO
Fragrance 9	56.41	Non-sensitizer	5% DMSO
Fragrance 10	54.95	Non-sensitizer	5% DMSO
Fragrance 11	38.86	Moderate	5% DMSO
Fragrance 12	34.90	Moderate	5% DMSO
Fragrance 13	32.78	Moderate	5% DMSO
Fragrance 14	18.98	Moderate	5% DMSO
Fragrance 15	16.83	Moderate	5% DMSO
Fragrance 16	13.00	Strong	5% DMSO
Fragrance 17	6.57	Strong	5% DMSO

NC = not calculated.
[a] Gerberick et al. (2005).

2.5. Immobilization of ligands

The immobilized ligands were cysteine (Fluka, Germany), poly-L-lysine (28,200 Da), poly-L-histidine (6700 Da) and poly-glycine (1000 Da) (Sigma, St. Quentin-Fallavier, France). Polypeptides were used as immobilized ligands in order to increase the number of amino acids immobilized onto the sensor surface providing more available binding sites for interaction with allergens and subsequently increasing the sensitivity of the assay. The reagents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS) and 1 M ethanolamine HCl pH 8.5 were obtained from Biacore.

Prior immobilization, CM5 sensor chip was preconditioned in water at 100 μl/min by applying two consecutive 20 μl-pulses of 50 mM NaOH, 0.1% HCl (v/v), 0.1% SDS (w/v) and 0.085% H_3PO_4 (v/v).

Ligands were immobilized by direct amine coupling using their reactive primary amine group leaving other reactive groups free for interaction with the allergens (thiol group for cysteine, amine group for lysine and imidazole side chain for histidine). The surface was activated by injecting a 1:1 mixture of EDC/NHS for 15 min at 5 μl/min. Buffers used for ligand covalent immobilization was optimized prior to the experiment using the pH scouting wizard (GE Healthcare). Cysteine 1 M was diluted 1:2 (v/v) with 10 mM sodium acetate buffer pH 4.0. Polypeptides 1 mg/ml were diluted 1:10 (v/v) with 0.1 M carbonate buffer pH 9.6 for poly-lysine; 1:10 (v/v) with 0.06 M phosphate buffer pH 6.6 for poly-histidine and 1:5 (v/v) with 0.5 M phosphate–citrate buffer pH 4.5 for poly-glycine. Then, 60 μl of the diluted ligand were injected over the surface for 12 min. Injections were repeated until saturation. Finally, the remaining unreacted succinimide esters were deactivated with ethanolamine for 7 min. The surface was then washed with 5 μl 50 mM NaOH and 50 mM HCl to remove any non-covalently bound poly-lysine and poly-histidine, respectively.

An unmodified dextran surface was used as reference surface.

2.6. Sample preparation

Forty millimolar chemicals in 1.05× PBS 5% DMSO were prepared with 20 μl 1 M stock solution in 100% DMSO, 5 μl 100% DMSO and 475 μl 1.05× PBS. They were then diluted to the required 20 mM concentration using 1.05× PBS 5% DMSO. 10% DMSO soluble chemicals were directly diluted to 20 mM mixing 10 μl 1 M stock solution in 100% DMSO, 40 μl 100% DMSO and 450 μl 1.05× PBS.

Fragrances were diluted 1:500 (v/v) in 1.05× PBS 5% DMSO before analysis.

2.7. Screening procedure

Before injecting the first allergen, 10 consecutive injections of running buffer were performed to equilibrate the Biacore and stabilize the baseline. Allergens were injected for 1 min, followed by a

dissociation period of 7 min. Subsequently, the injection unit and the needle were washed with 30 μl 50% DMSO. Finally, running buffer was injected for 1 min before injection of the next allergen to check for sample carryover. Insufficient wash routines may lead to memory effects, so that an allergen that interacts with the target ligand injected in one cycle may remain attached to parts of the flow system and may reappear in the next injection leading to a false-positive.

At first, all injections passed over the unmodified dextran surface and then passed over the immobilized surface. The reference surface was used for two purposes: firstly as a control for non-specific binding and secondly for subtraction of refractive index effects that occur as a consequence of a non-perfect match of refractive indices of sample and running buffer.

In direct SPR-based binding studies, it is important to consider the possibility of mass transport-limited binding between the allergen in solution and the amino acid on the sensor surface. This may occur when the rate of transfer of an allergen to or from the surface is significantly slower than the kinetic binding rate. Considering the low levels of ligands immobilized, mass transfer limitation can be minimized by using high flow rates to increase the rate of transfer of allergen to or from the biosensor surface. High flow rates were maintained during the analyses depending on the ligand: 50 μl/min for cysteine, 70 μl/min for poly-lysine and 100 μl/min for poly-histidine and poly-glycine.

2.8. Binding analysis

In order to generate binding information that could be analyzed and used for a simple presentation of the results, report points were taken during the association and dissociation phase: 15 s (early binding) and 50 s (late binding) after initiation of sample injection (association phase); 75 s (early stability) and 115 s (late stability) after the end of sample injection (dissociation phase).

To normalize the binding responses from the different allergens, the report points were divided by the molecular mass of the corresponding chemical and multiplied by 100 (R_{cor}) so that chemicals could be compared by their binding and binding stability.

The binding was expressed as $\%R_{max}$, theoretical maximum binding capacity of the immobilized ligand, as defined by the following equations:

$$R_{max} = (\text{MW allergen}/\text{MW ligand}) \times \text{RU immobilized} \times S$$

where MW stands for molecular weight and S for the stoichiometry of the interaction. $S = 1$ for cysteine: one thiol group available for the interaction with the allergens. For the polypeptides, there was more than one binding sites because each polypeptide immobilized on the surface can provide several binding sites even though some might be hidden. S was estimated at 193 for poly-lysine, 43 for poly-histidine and 13 for poly-glycine.

$$\%R_{max} = (R_{cor}/R_{max}) \times 100$$

2.9. Statistical analysis

Data transformation and overlay plots were prepared with BIAevaluation 3.2 software (GE Healthcare) and the resulting data were submitted to multivariate statistical analyses using Splus 6.0. Each report point from binding interaction with the three amino acids was considered to be a statistical variable (maximum of 12 variables) and each analyzed allergen was considered to be an observation.

Principal component analysis (PCA) was used to study the specificity of the tested allergens for the three amino acids. PCA reduces the original dataset into a number of principal components (PCs) that describe the greatest variance over all the data. PCs, which are expressed as linear combinations of the original variables are orthogonal to each other and can be used for an effective representation of the system under investigation with a lower number of variables than in the original case. The combination of variables that explains the greatest amount of variability is the first principal component. The subsequent ones (second and third principal component) describe the maximum amount of remaining variability; they must be independent of the first principal component. The graphical representation allows the identification of groups of samples showing a similar behavior (sample close to one another in the graph) or different characteristics (samples far from each other).

3. Results

3.1. Quality control (data not shown)

The baseline stability was checked throughout the experiment and was shown to remain constant. Using 1.05× PBS 10% DMSO as running buffer resulted in a slight increase of the baseline that was taken into account and did not interfere with the data obtained.

Non-specific binding of haptens on the dextran surface was rare even though some chemicals (*p*-benzoquinone) did sometimes bind to dextran. The data obtained from report point late stability on the unmodified dextran surface (reference flow cell) after injection of chemical gave binding levels lower than 5 RU within the detection limits of the machine and baseline noise (5–10 RU).

No carryover was observed in buffer blanks injected throughout the analysis. The extra wash routines included in the screening procedure were efficient at removing any remaining allergens.

Thus, the observed signals were considered to result from the interaction of the allergen for the immobilized ligand.

3.2. Binding interactions between amino acids and chemicals

Typical immobilizations yielded 500–600 RU of cysteine bound to the biosensor surface. The final amount of poly-lysine, poly-histidine and poly-glycine covalently immobilized on the surface was typically around 3000, 1500 and 200 RU, respectively.

Binding interactions observed for the different chemicals at the end of the association and at the end of the dissociation are resumed in Fig. 2, all the individual report points being presented in Table 2. Report points from association (late binding) gives information about the affinity of the chemical for the immobilized amino acid. The late stability report point (dissociation phase) corresponds to the stability of the interaction and may be used to represent the dissociation rate (in association with the early stability report point). Chemicals binding with high affinity and with slow dissociation rate had high values for report points while chemicals binding with high affinity and fast dissociation rate had a high value for late binding and a low value for late stability report points.

All tested chemicals were shown to bind to cysteine except non-sensitizing allergens (1-bromobutane, diethylphthalate) which did not bind to any residue of amino acids (Fig. 2). The observed variability of the affinity intensity of the tested chemicals for the cysteine is very important and shows specific reactivity depending on chemical. Some chemicals seemed to be specific to one amino acid. Dimethylaminopropylamine, 3-aminophenol, lilial, cyclamenaldehyde, hydroxycitronellal and benzyl benzoate preferentially bound to cysteine. Fig. 3 shows some representative data observed for chemicals binding to immobilized cysteine. Each peak represents the signal change caused by each chemical injection and the baseline represents the signal in the presence of running buffer. It appears that interactions were different during both association

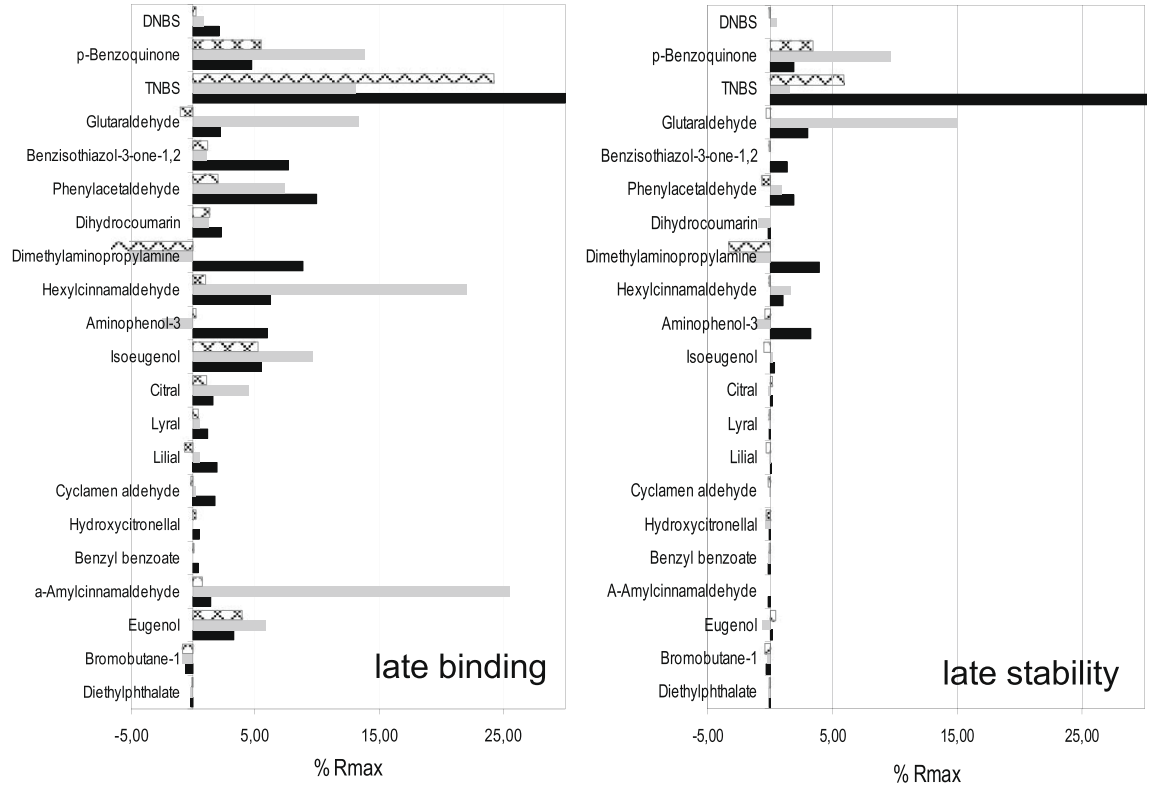

Fig. 2. Binding interactions between 20 mM chemicals and Cys (■), Lys (▒) and His (▒) at the end of the association (late binding) and dissociation phases (late stability). Data are corrected for molecular weight and R_{max}. Report points from association (late binding) gives information about the affinity of the chemical for the immobilized amino acid. The late stability report point (dissociation phase) corresponds to the stability of the interaction.

and dissociation phases depending on the chemical. Some chemicals bound weakly to the immobilized amino acid, as indicated by a lower response at equilibrium and a much faster return to baseline during the dissociation phase. Equilibrium could be reached more or less rapidly and the bound chemical dissociated more or less slowly from the surface. Non-sensitizers and weak sensitizers dissociated completely without the need of any regeneration (Fig. 2, late stability). However, most complexes formed with the extreme, strong and moderate sensitizers were stable and did not dissociate by themselves indicating a tight interaction.

The number of chemicals showing binding to lysine was less important (Fig. 2). Among the weak sensitizers, binding was observed for eugenol, α-amylcinnamaldehyde, and citral. Five of seven moderate sensitizers gave a positive response with lysine: 1,2-benzisothiazol-3-one, phenylacetaldehyde, dihydrocoumarin, hexylcinnamaldehyde and isoeugenol. All strong and extreme sensitizers showed binding to lysine, except DNBS. Glutaraldehyde preferentially bound to lysine. This chemical was chosen as a positive control because it is known to interact with the amino groups. Thus, the observed interaction, which mainly occurred with lysine, contributes to validate the sensor surface and the immobilization procedure (ligand activity and orientation on the sensor surface). As observed with cysteine, the binding of strong sensitizers with the amino groups of lysine were stable.

Fewer interactions were observed with histidine (Fig. 2). Two of eight weak sensitizers showed binding with histidine: eugenol and citral. Five of seven moderate sensitizers gave a positive response with histidine: 1,2-benzisothiazol-3-one, phenylacetaldehyde, dihydrocoumarin, hexylcinnamaldehyde and isoeugenol. TNBS and *p*-benzoquinone bound with histidine and formed a stable complex as with cysteine and lysine.

None of the chemicals reacted with poly-glycine. Glycine is a neutral amino acid and was used as a negative control. The fact that no chemical bound to this amino acid confirms the specificity of the binding interactions observed between chemicals and the sulfhydryl group of cysteine, amino group of lysine and imidazole side chain of histidine.

Principal component analysis showed that 87% of the total variance of the data could be explained using only three principal components. The representation of the first three principal component scores obtained for the 20 chemicals is presented in Fig. 4. Only six variables were used for this multifactorial analysis because of the very high correlations observed between early binding and late binding, early stability and late stability. The first component, which explained 41.3% of the variation in the data, is accounted for by the variables late stability on histidine (lshis), late binding on histidine (lbhis) and late binding on lysine (lblys). The second component (30.6% of variance) is accounted for by the following variables: late stability on cysteine (lscys), late binding on cysteine (lbcys) and late stability on lysine (lslys). The third component explained 15.1% of the remaining 28% variability. It was accounted for by variables late stability on lysine (lslys), late binding on cysteine (lbcys) and late binding on histidine (lbhis). In these dimensions, PCA discriminated the chemicals depending on their potency category (Gerberick et al., 2005). Fig. 4 shows that chemicals of the same potency category were found nearby. Non-sensitizers and weak chemicals were very close to one another. Strong and extreme chemicals were rather far from the others except DNBS. TNBS was not included in the analysis since its high interactions with each amino acid induced a too high scale on the three components and thus did not allow observing the other allergens in those three-dimensions.

SPR report points measured on the three different surfaces are resumed in Fig. 5 depending on the potency category of the tested chemicals. Extreme and strong chemicals could be characterized by their high affinity for the three different amino acids. Those

Table 2
SPR report points of chemicals and fragrances binding to cysteine, lysine and histidine. Results are normalized and expressed as percent R_{max}, theoretical maximum binding capacity of the immobilized ligand. During the association phase: early binding (15 s), late binding (50 s) after initiation of sample injection. During the dissociation phase: early stability (75 s), late stability (115 s) after the end of sample injection.

		Cysteine				Lysine				Histidine			
		Binding		Stability		Binding		Stability		Binding		Stability	
		Early	Late	Early	Late	Early	Late	Early	Late	Early	Late	Early	Late
Diethylphthalate	Mean	−0.13	−0.20	−0.05	−0.05	−0.05	−0.19	−0.24	−0.10	0.11	−0.07	−0.13	−0.07
	SD	0.64	0.47	0.51	0.35	0.14	0.13	0.07	0.03	0.13	0.02	0.10	0.07
1-Bromobutane	Mean	0.03	−0.58	−0.80	−0.29	−0.51	−0.90	−0.51	−0.22	−0.63	−0.82	−0.57	−0.43
	SD	0.45	0.76	064	0.60	0.09	0.08	0.02	0.07	–	–	–	–
Hydroxycitronellal	Mean	0.34	0.46	−0.03	−0.05	0.37	0.02	−0.65	−0.41	0.50	0.21	−0.46	−0.32
	SD	0.24	0.30	0.33	0.16	0.25	0.04	0.32	0.18	–	–	–	–
Cyclamen aldehyde	Mean	1.76	1.79	0.11	0.04	0.32	0.22	−0.20	−0.07	−0.09	−0.18	−0.17	−0.15
	SD	0.11	0.19	0.04	0.17	0.09	0.03	0.08	0.07	0.67	0.61	0.04	0.12
Lilial	Mean	2.60	1.92	0.17	0.08	0.57	0.51	−0.15	−0.03	−0.60	−0.66	−0.50	−0.34
	SD	0.97	0.87	0.43	0.26	0.14	0.13	0.00	0.04	0.12	0.37	0.89	1.03
Lyral	Mean	0.65	1.16	0.07	−0.09	0.35	0.45	−0.14	−0.09	0.48	0.40	−0.04	−0.03
	SD	0.15	0.17	0.09	0.01	0.48	0.30	0.38	0.26	0.23	0.19	0.12	0.04
Benzyl benzoate	Mean	0.65	0.43	−0.20	−0.12	0.33	0.01	−0.29	−0.15	0.15	0.03	−0.10	−0.04
	SD	0.63	0.57	0.20	0.13	0.09	0.01	0.06	0.05	0.15	0.08	0.11	0.08
Citral	Mean	1.39	1.58	0.20	0.22	4.18	4.44	−0.21	−0.17	1.10	1.07	0.07	0.24
	SD	0.04	0.09	0.04	0.13	3.68	3.05	1.13	0.89	0.10	0.90	0.68	0.54
Eugenol	Mean	1.81	3.25	0.51	0.19	4.89	5.83	−0.01	−0.63	2.11	3.98	1.53	0.48
	SD	1.19	0.84	1.20	0.71	3.23	3.15	0.84	0.65	–	–	–	–
Hexylcinnamaldehyde	Mean	4.96	6.21	1.86	1.06	11.22	22.03	11.56	1.67	1.30	1.03	−0.14	−0.01
	SD	3.09	2.07	2.30	1.28	10.30	25.01	15.20	0.23	–	–	–	–
α-Amyl cinnamaldehyde	Mean	2.30	1.39	−0.16	−0.14	19.61	25.51	0.50	0.02	1.12	0.74	−0.08	0.02
	SD	1.49	0.93	0.38	0.20	25.60	33.79	0.43	0.09	–	–	–	–
Dihydrocoumarin	Mean	1.82	2.30	−0.03	−0.13	1.64	1.30	−1.18	−0.91	1.23	1.33	0.01	0.05
	SD	0.47	0.41	0.32	0.03	0.73	0.64	0.42	0.39	0.19	1.24	0.59	0.16
3-Aminophenol	Mean	4.57	6.02	2.96	3.28	−1.33	−2.45	−1.71	−1.18	1.37	0.24	−1.11	−0.42
	SD	2.46	4.92	4.84	5.54	0.57	1.38	1.34	1.13	–	–	–	–
Phenylacetaldehyde	Mean	8.11	9.94	2.58	1.92	5.88	7.33	1.50	0.94	2.24	2.02	−0.94	−0.65
	SD	1.85	4.22	3.42	2.77	3.22	1.79	1.11	0.82	0.05	0.05	0.11	0.09
1,2-Benzisothiazolin-3-one	Mean	6.85	7.67	1.72	1.43	1.08	1.05	−0.04	0.01	1.21	1.21	−0.30	−0.05
	SD	3.41	3.34	0.63	0.86	0.23	0.58	0.55	0.40	–	–	–	–
Dimethylaminopropylamine	Mean	9.51	8.85	4.71	3.99	−6.68	−8.37	−2.06	−1.77	−5.04	−6.54	−4.80	−3.30
	SD	3.57	2.48	9.92	9.63	2.47	2.40	0.35	0.36	3.46	4.86	3.60	3.35
Isoeugenol	Mean	3.09	5.48	0.85	0.36	7.81	9.60	0.84	0.19	4.19	5.24	0.36	−0.45
	SD	1.20	0.79	0.97	0.52	3.46	2.98	0.02	0.17	–	–	–	–
Glutaraldehyde	Mean	1.20	2.19	2.38	3.06	4.81	13.37	15.00	15.04	−0.78	−1.00	−0.43	−0.31
	SD	1.57	0.79	1.14	2.27	3.78	8.48	9.31	9.44	–	–	–	–
TNBS	Mean	27.81	30.00	39.20	35.40	12.27	13.10	1.54	1.60	20.78	24.24	5.55	5.96
	SD	13.42	60.82	60.86	54.77	1.81	2.44	1.72	1.88	–	–	–	–
p-Benzoquinone	Mean	3.80	4.72	2.04	1.88	7.89	13.83	10.91	9.71	3.91	5.51	3.63	3.48
	SD	2.50	2.65	2.31	1.95	6.38	10.08	7.20	6.07	1.59	3.41	2.55	2.29
DNBS	Mean	1.95	2.09	0.11	0.08	0.55	0.87	0.49	0.53	0.26	0.21	−0.07	−0.03
	SD	1.52	1.53	0.03	0.05	0.09	0.31	0.34	0.42	–	–	–	–
Fragrance 1		1.12	3.18	1.37	0.75	4.66	5.61	1.14	0.41	−0.63	−0.68	−0.21	0.02
Fragrance 2		1.28	2.11	0.90	0.83	1.29	1.50	0.28	0.35	0.04	0.15	0.17	0.13
Fragrance 3		1.52	2.79	1.31	0.92	1.86	1.82	0.29	0.54	−1.19	−0.93	0.05	0.38
Fragrance 4		3.90	5.39	1.77	1.39	1.58	1.73	0.19	0.50	−0.34	−0.27	0.04	0.08
Fragrance 5		6.04	9.41	2.60	1.26	7.93	9.46	2.07	1.17	−1.42	−1.08	0.12	0.11
Fragrance 6		4.19	5.78	1.24	0.79	12.64	12.77	0.48	0.09	−0.61	−0.39	−0.06	0.01
Fragrance 7		1.70	3.57	1.88	1.03	8.62	12.03	4.13	1.49	−0.17	−0.16	0.00	0.07
Fragrance 8		1.62	3.18	2.25	2.50	8.85	9.32	0.56	−0.16	0.07	0.20	0.20	0.12
Fragrance 9		2.82	4.58	1.74	1.05	0.71	0.77	0.31	0.79	−0.22	−0.17	0.09	0.12
Fragrance 10		2.54	4.56	2.02	1.28	4.05	4.35	0.55	0.74	−0.13	−0.16	0.01	−0.04
Fragrance 11		4.40	6.53	2.00	1.22	3.38	4.94	2.23	2.27	−0.17	0.22	0.53	0.81
Fragrance 12		4.62	7.64	2.64	1.27	6.18	6.25	1.10	1.27	−0.31	−0.13	0.08	−0.03
Fragrance 13		2.52	4.62	2.09	1.24	0.76	0.73	0.08	0.45	−0.18	−0.04	0.07	−0.01
Fragrance 14		1.10	1.92	1.02	0.98	3.96	4.80	1.07	0.50	−0.23	−0.13	0.12	0.20
Fragrance 15		2.53	3.44	1.12	0.96	12.76	13.50	0.82	0.56	0.01	0.12	0.18	0.18
Fragrance 16		2.07	4.12	1.97	1.07	7.27	11.05	4.75	1.82	0.50	0.67	−0.04	0.01
Fragrance 17		4.33	8.27	3.61	2.05	12.34	18.24	5.79	2.75	−0.60	−0.51	−0.12	−0.07

chemicals showed high affinities for cysteine, lysine and histidine (high values at late binding) and slow dissociation rates (high values at early and late stability). Moderate chemicals bound with the immobilized cysteine with a high affinity and high stability. Weak chemicals presented a high affinity for lysine together with a high dissociation rate. Non-sensitizers stood in the middle having no binding for the amino acids.

3.3. Binding interactions between amino acids and fragrances

Results obtained with fragrances are resumed in Fig. 6 for late binding and late stability. All tested fragrances bound with cysteine and lysine independently of their sensitizing potential. No interaction was observed with histidine. Dissociation was more or less rapid depending on the amino acid/fragrance complex and

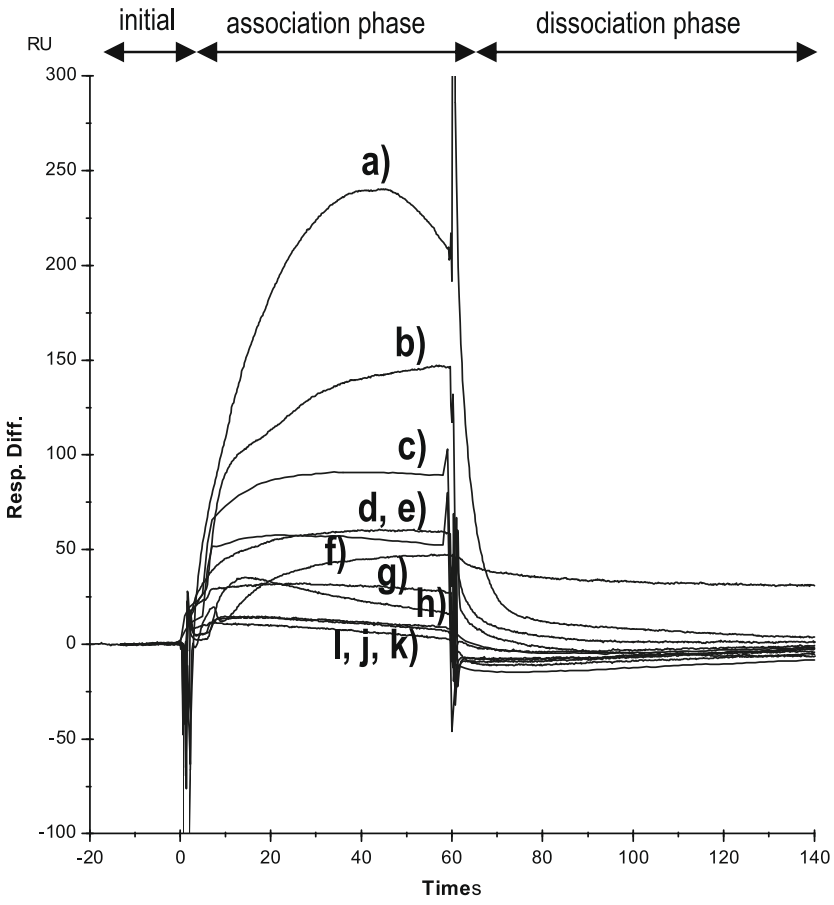

Fig. 3. Sensorgram overlays of skin sensitizing chemicals binding to immobilized cysteine. (a) Hexylcinnamaldehyde, (b) phenylacetaldehyde, (c) isoeugenol, (d) *p*-benzoquinone, (e) eugenol, (f) lyral, (g) dihydrocoumarin, (h) α-amylcinnamaldehyde, (i) citral, (j) diethylphthalate, (k) hydroxycitronellal. 20 mM chemicals were injected in 1.05× PBS 5% DMSO running buffer at 50 μl/min flow rate. Reference data are subtracted but data are not normalized. The initial part of these curves represents a buffer flowing past the sensor surface. The second and rising part corresponds to the response of the sensor surface observed as a chemical volume flows past the immobilized cysteine. The final portion of the curves corresponds to the dissociation of bound chemical after the sample volume has finished and the buffer flows past the sensor surface again.

some interactions did not dissociate within the 7 min of the dissociation phase. Non-sensitizing fragrances which showed a high affinity for lysine bound weakly to the amino acid and totally dissociated from the amino acid while strong sensitizing fragrances with a high affinity seemed to bind more tightly. This can also be observed in Fig. 7 where all SPR report points are resumed depending on the potency category of the tested fragrances (non-sensitizers (EC3 range: 54.95–151.30), moderate (EC3 range: 16.83–38.86) and strong (EC3 range: 6.57–13.00)). Strong sensitizers were distinguished from the two others. They presented a much higher affinity for lysine, and a lower dissociation rate which characterized really stable bindings. They also had a higher affinity for cysteine. Among the fragrances tested here, it was not possible to differentiate between non-sensitizers and moderate.

4. Discussion

A prospective study based on a SPR direct binding assay was developed to discriminate different chemicals depending on their specific interaction with amino acid residues. Different reactivities of the nucleophilic amino acids toward the tested allergens were observed. Cysteine was found to be more reactive than lysine and histidine was the least reactive. These results are in general agreement with what has been described in the literature (Basketter et al., 1995; Divkovic et al., 2005; Gerberick et al., 2004). Protein thiol reactivity appeared like a good indicator of skin sensitization hazard. Some authors have investigated electrophilicity assays based on thiol reactivity as a model nucleophile for identification of electrophilic skin sensitizers (Aptula et al., 2006; Schultz et al., 2005, 2006). Nevertheless, *in vitro* assay development should not be limited to one nucleophile. Results obtained showed that each nucleophile tested was useful for screening allergens. The interest of cysteine and lysine in quantifying hapten reactivity has also been mentioned by Gerberick et al. (2004). When in the 3D environment of the protein, allergens may bind with different nucleophiles depending on the accessibility of the nucleophiles. Aleksic et al. (2007) showed that one sensitizer could modify a broad spectrum of HSA nucleophiles (lysine, cysteine, histidine and tyrosine) and that different sensitizers could modify different HSA residues.

Furthermore, the Biacore biosensor used in this experiment is characterized by four available flow channels. The use of multi-channel sensing thus seems relevant. Since each channel on the sensor surface can be addressed individually, it is possible to have one reference channel and one flow channel for each nucleophile. With a single injection step, the allergen comes through each surface being able to bind with either nucleophile in 7 min. This can be used to compare simultaneously an allergen's selectivity for different target amino acids. Where other technologies can provide single end point data, Biacore offers numerous real-time data assisting in the understanding of how skin sensitizers interact.

In the literature, many chemical groups with electrophilic properties are shown to react with various nucleophiles to form stable

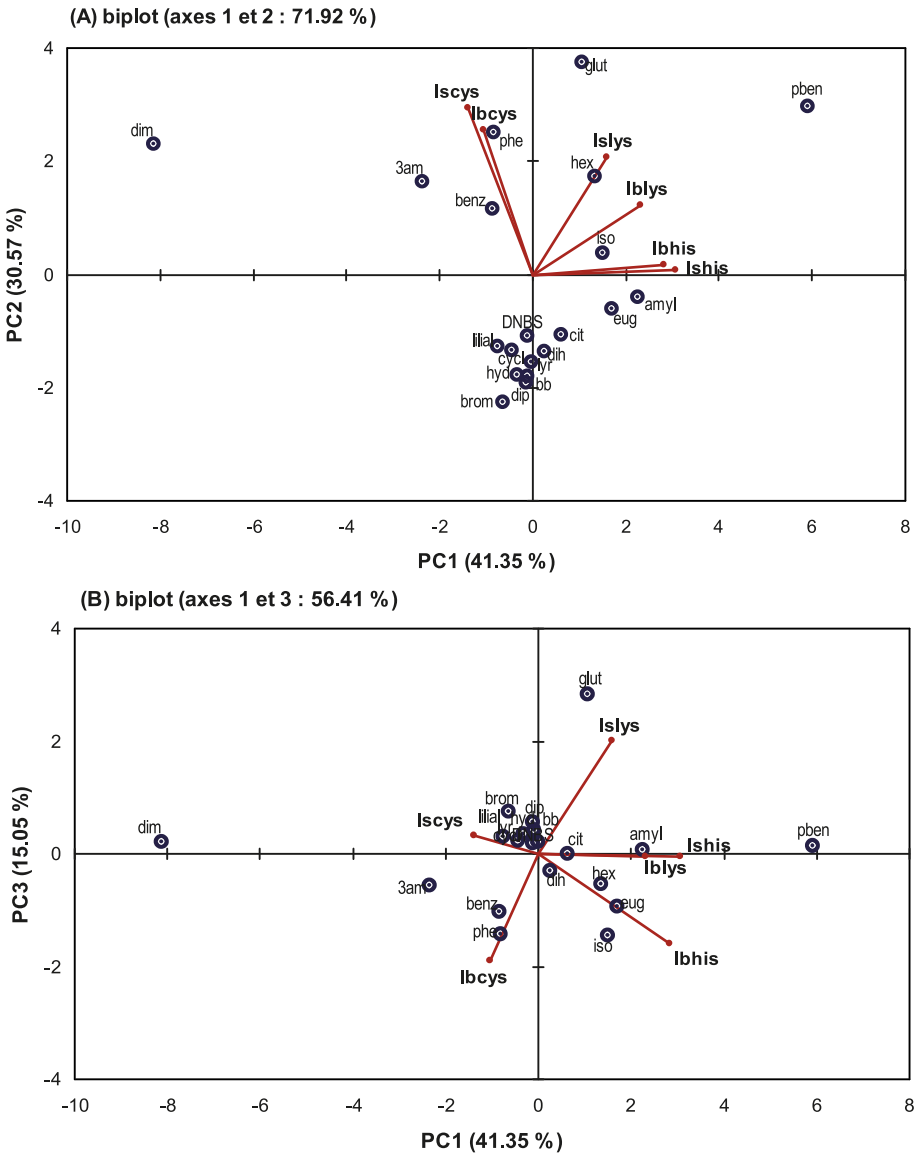

Fig. 4. Principal component analysis similarity map determined by principal components 1 and 2 (A), 1 and 3 (B) for SPR data set for chemicals. Six variables were used: lb = late binding, ls = late stability, cys = cysteine, lys = lysine, his = histidine. Twenty chemicals: dip = diethylphthalate, brom = 1-bromobutane, hyd = hydroxycitronellal, cycl = cyclamen aldehyde, lilial, lyr = lyral, bb = benzyl benzoate, cit = citral, eug = eugenol, hex = hexylcinnamaldehyde, amyl = α-amylcinnamaldehyde, dih = dihydrocoumarin, 3am = 3-aminophenol, phe = phenylacetaldehyde, benz = 1,2-benzisothiazolin-3-one, dim = dimethylaminopropylamine, iso = isoeugenol, glut = glutaraldehyde, pben = *p*-benzoquinone, DNBS.

covalent bonds (Basketter et al., 1995). Depending on their chemical structure, related mechanisms of reaction are well-documented (Aptula et al., 2005; Basketter et al., 1995; Divkovic et al., 2003, 2005; Roberts et al., 2007). Aleksic et al. (2007) demonstrated covalent haptenation of extreme and moderate sensitizers while non-sensitizers and irritants gave no evidence for covalent binding. The authors did not experiment with low sensitizers binding to HSA but their results suggest different nature of haptenation depending on sensitizing potential.

Using SPR-based technology biosensor, we observed different kinds of interactions between allergens and immobilized amino acids. The reactions of chemicals with amino acids lead to the formation of bonds of different strengths. It was shown that weak chemicals could be washed off by the flowing buffer, while strong and extreme chemicals remained bound to the immobilized amino acids. Moderate chemicals showed stable bindings only with cysteine. This is indicative of the "strength" of the binding between chemicals and amino acids. Moreover, moderate chemicals may produce a significant binding response when compared to strong chemicals, which may be further evidence of their differing modes of action. These binding properties were also observed with fragrances. Thus, it seems important to consider the affinity of the hapten for the protein as well as the nature of the binding involved.

It is known that complexes of low stability which characterize weak interactions are of great biological importance (Basketter et al., 1995). Some authors (Basketter et al., 1995; Pichler, 2001) proposed other hypotheses of protein haptenation in favour of non-covalent modes of protein–hapten association. Basketter et al. (1995) reported the possibility of strong non-covalent binding between metal salt and electron-rich residues of proteins. Pichler (2001) concluded that certain drugs are able to bind directly in a non-covalent way and can stimulate T-cells. He assumed that small molecules may bind via Van der Waals or hydrogen bonds. This hypothesis is in agreement with the observed SPR binding interactions. Most of the tested allergens (among weak and moderate) formed complexes which dissociate within seconds, back to

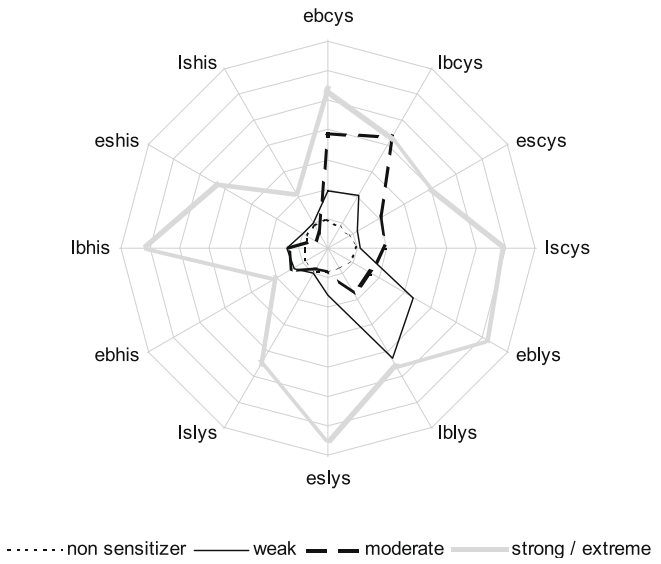

Fig. 5. SPR report points measured on the three different surfaces depending on the potency category of the tested chemicals. Eb = early binding, lb = late binding (represents the affinity for the corresponding amino acid), es = early stability, ls = late stability (represents the stability of the interaction, together with the early stability represents the dissociation rate), cys = cysteine, lys = lysine, his = histidine.

baseline, indicating that the interaction is fully reversible in 7 min observation time.

Our experiment also shows that it was possible to immobilize sufficient amino acids and polypeptides by direct amine coupling while maintaining their binding capacity. These molecules have amine groups available for covalent coupling to the dextran of the biosensor. This amine coupling technique allows orientation of the immobilized molecule and thus gives available nucleophilic sites, lysine amino groups, cysteine sulfhydryl groups and histidine imidazole groups, for binding interactions with electrophilic haptens. The amino acids and polypeptides were immobilized at the highest possible density on the biosensor surface in order to maximize the sites that permit binding of allergens. One advantage is the simpler analytical process, which has the potential for a high throughput assay to be developed.

However, we have to note that the influence of the three-dimensional protein environment is not represented in the same way as the nucleophile position in the 3D protein. The surrounding hydrophobic and non-polar side chains, the amino acids which influence the local pH and thus the degree of nucleophilic side chain ionization (Aleksic et al., 2007; Divkovic et al., 2005) are not present. An attempt has been made to study allergens binding to an intact protein, human serum albumin (HSA) used as a model protein (Aleksic et al., 2007). The authors investigated protein–hapten binding mechanisms and hapten selectivity for nucleophiles that are presumably in an environment more conducive to reactivity than others or perhaps more accessible in the 3D globular folding of the protein. But, cysteine nucleophile is underrepresented in HSA and the choice of a model protein is a critical point. It is interesting to note that interactions between HSA and drugs of different affinities have been investigated with biosensor based-SPR technology (Liu et al., 2006; Rich et al., 2001). This method was proved to be applicable for screening a large number of compounds binding to HSA as well as for characterizing in detail the binding properties of selected compounds. Therefore, investigating binding interactions between allergens and a model immobilized protein using SPR may be a future challenge assuming a suitable

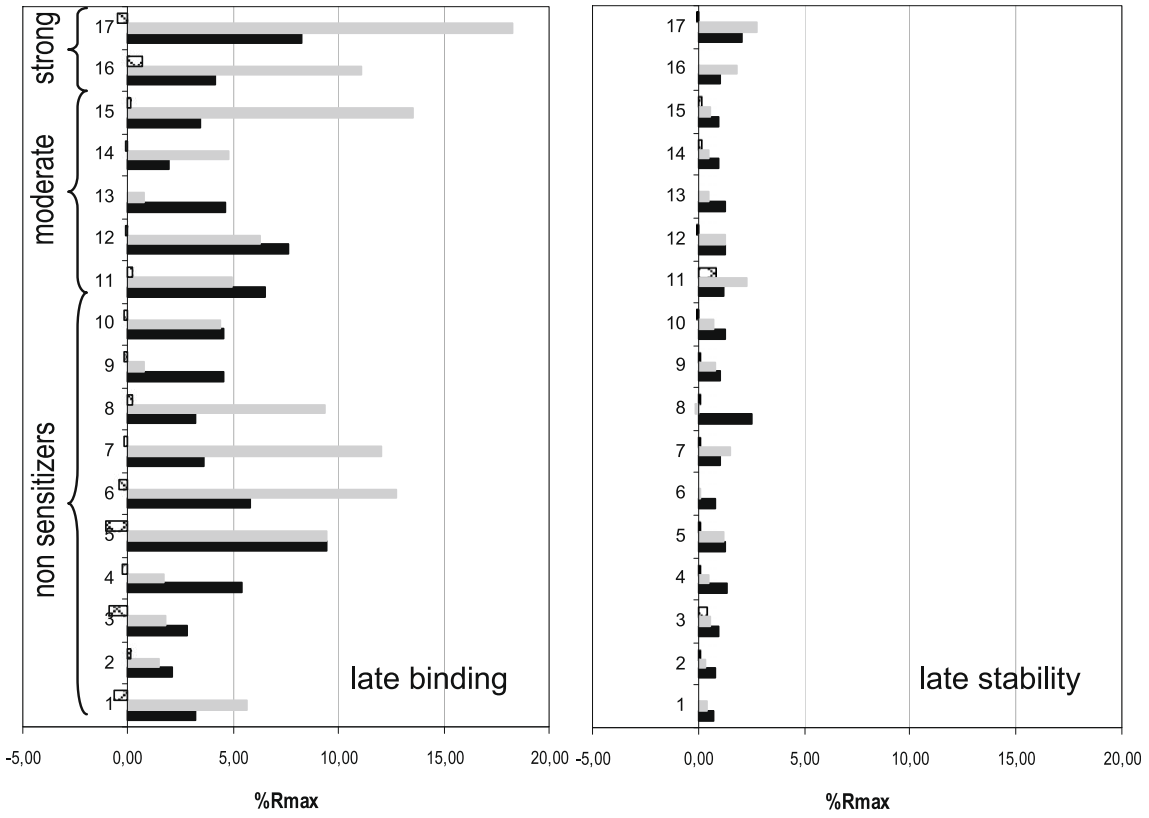

Fig. 6. Binding interactions between fragrances and Cys (■), Lys (▨) and His (▨) at the end of the association (late binding) and dissociation phases (late stability). Data are corrected for molecular weight and R_{max}. Report points from association (late binding) gives information about the affinity of the chemical for the immobilized amino acid. The late stability report point (dissociation phase) corresponds to the stability of the interaction.

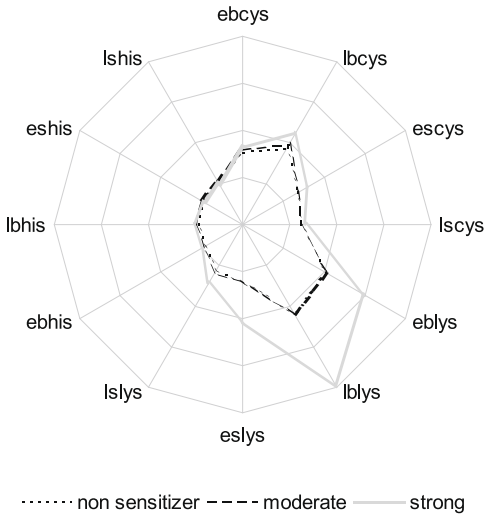

Fig. 7. SPR report points measured on the three different surfaces depending on the potency category of the tested chemicals. eb = early binding, lb = late binding (represents the affinity for the corresponding amino acid), es = early stability, ls = late stability (represents the stability of the interaction, together with the early stability represents the dissociation rate), cys = cysteine, lys = lysine, his = histidine. Tested fragrances were distributed in three groups: non-sensitizers (EC3 range: 54.95–151.30), moderate (EC3 range: 16.83–38.86) and strong (EC3 range: 6.57–13.00).

simple model of skin proteins containing all potential nucleophiles simultaneously can be found.

Another drawback of the present assay relies on its capacity to detect pro-haptens that will be transformed into allergens by skin metabolism. This is also the limitation of most of the *in vitro* techniques proposed for screening the sensitizing potency of chemicals (Gerberick et al., 2004; Aptula et al., 2006). Therefore the present method is not better but not worst than the concurrent ones already published.

On the other side, SPR-based biosensor is a simple and rapid tool without the need for pre-concentration and labeling and requires only small amounts of interacting partners, pure or mixed ingredients. This technology provides information on the stability of the hapten/amino acid complex. However, it remains to be seen whether this approach is sufficiently robust to allow the discrimination of a wide range of allergens. It is difficult to envisage that an accurate single *in vitro* method of prediction could be developed as there are many factors contributing to the manifestation of allergic contact dermatitis including exposure level, chemical structure, protein binding affinity and specificity and immune responsiveness (Divkovic et al., 2003; Kimber et al., 2001). This study shows the interest of SPR technology considering that factors other than electrophilic reactivity are involved in the sensitizing potential of an allergen. The Biacore technology could be a test integrated in a tiered set of tests giving specific complementary endpoint on the affinity and the stability of the interaction between allergen and amino acid in order to refine the assessment of skin sensitization potential of chemicals. The possibility to apply this technology to complex mixtures as cosmetic finished products needs to be further studied.

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