Project Number: 257401

A highly integrated and sensitive PORous SIlicon based lab on a chip for multiple quantitaTIVE monitoring of Food allergies at point of care.

Specific Targeted Research Project

Information Society Technologies

Deliverable D6.1: Report on the receptor immobilization methods on porSi structure

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Dissemination Level		
PU	Public	X
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	

1 About this deliverable

1.1 Introduction

This deliverable reports on the set up of a fuctionalization protocol for porSi structure and on the optimization of binding conditions for food allergens.

1.2 Scope of the deliverable

The scope of the activity has been to devise a strategy to immobilize allergens within the pores of the sensor. The advantage of the probe attachment mechanism proposed in this project is that the polymeric coating, after covalent binding of the specific probes and blocking with ethanolamine, is highly resistant to non-specific binding of proteins.

1.3 Structure of this deliverable

The report is organized according to the following sections

2 Description of work performed

2.1 Introduction

The activity of Workpackage 6 is aimed at modifying the porSi structures in order to obtain a surface for the immobilization of receptors (allergens) with the following characteristics:

1) a high protein binding capacity

2) to allow the binding of allergens whilst preserving their native conformation and allowing them to selectively interact with their specific antibodies

3) a low background noise, which prevents false negative results and a low sensitivity.

The general scheme of the assay is shown in Figure 1: food allergens are immobilized on the modified porSi structures; antibodies from the patient's serum recognise the immobilized proteins on the surface. A secondary antibody allows the detection and quantification of the allergen specific immunoglobulins E which are the target molecules.

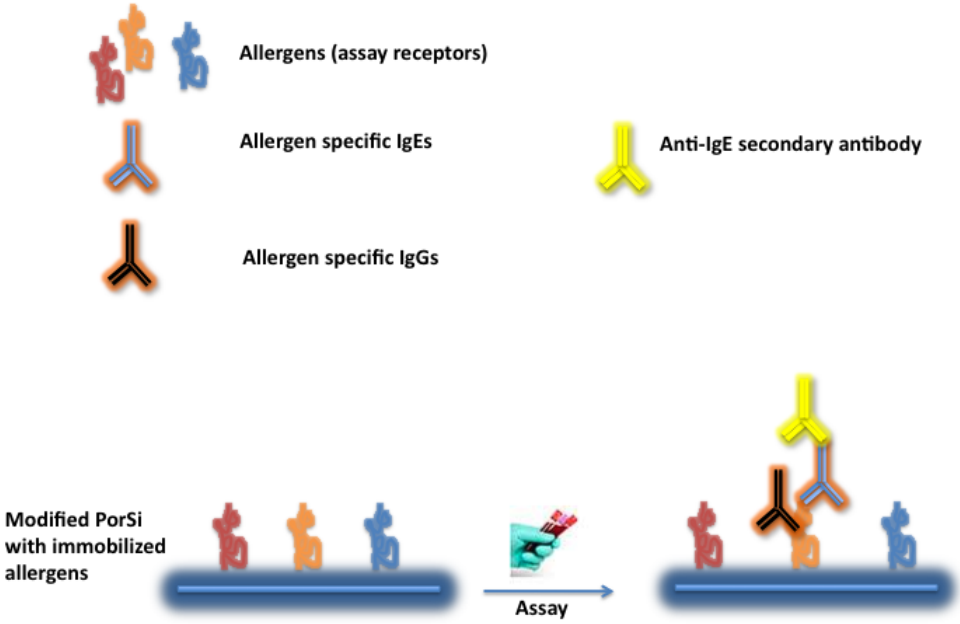

Figure 1: assay scheme

In the first phase, flat silicon oxide substrates, in place of the porSi structures were used to evaluate surface features. The choice of using flat SiO/SiO_2 slides was justified by the availability at the ICRM-CNR laboratory of a LIF (laser induced fluorescence) scanner and a label-free detector compatible with this format. Several model tests were therefore run using flat silicon oxide slides before beginning with the first porSi samples provided by partner UNITN.

In a second phase, free standing porSi membranes and porSi supported on silicon wafers were used to set up a coating procedure and to spot fluorescently labelled antibodies

2.2 Polymeric coating

All the methods that deal with the derivatization of SiO_2 surfaces must take into consideration the fact that the number of Si-O-Si and Si-OH groups exposed at the surface and available for derivatization is highly dependent on the past history of the sample. This irreproducibility in surface chemistry makes it difficult to control the coating homogeneity, leading to poor reproducibility and stability of probe attachment. SiO_2 substrates can be modified using methoxysilane or chlorosilane reagents to introduce organic functional groups. However, silane reagents containing only a single surface reactive functionality (e.g. monochloro- or monomethoxysilanes) produce poorly stable films. The stability can be improved by using multiple reactive groups (e.g. trichloro- or trimethoxysilanes) to form more Si-O-Si linkages, but such polyfunctional reagents also lead to poorly controlled surface reactions, compromising surface homogeneity and reproducibility.

Polymeric coatings overcome these problems by providing a more uniform functional coating which increases the resistance to unspecific adsorption. Most importantly, polymeric coatings present a much higher concentration of surface reactive groups leading to more efficient immobilization of probe molecules. In addition, the polymer on the surface acts as a linker that moves the bound probe away from the surface resulting in a much faster reaction with the target.

The coating of SiO/SiO_2 slides as well as the coating of porSi structures was performed by physisorption of copoly(DMA-NAS-MAPS) [1,2], a terpolymer made of *N,N*-dimethylacrylamide (DMA, 97% of moles), *N,N*-acryloyloxysuccinimide (NAS, 2%), and 3-(trimethoxysilyl)propyl methacrylate (MAPS, 1%), synthesized by free radical copolymerization (Figure 2).

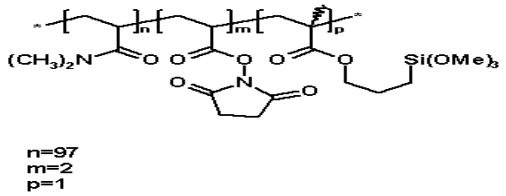

Figure 2: copoly(DMA-NAS-MAPS)

The polymer self-adsorbs onto the surface, the silanating moiety stabilizes the film, whereas the *N,N*-acryloyloxysuccinimide ester covalently binds the bioprobes.
The coating procedure on flat SiO/SiO_2 is usually performed by dip-coating slides into a 1% w/v polymer into water solution of ammonium sulfate at 20% saturation level followed by rinsing with water and drying under vacuum at 80°C. This procedure has been modified for surface modification of porSi.

2.2.1 Coating of porSi structures

Two types of porSi structures were provided by partner UNITN and used for the coating set up: free standing porSi membranes and porSi structures supported on wafers. Free standing membranes, when coupled to microfluidics (see D5.1), allow sealed flow-through protocols of functionalization ensuring infiltration of polymer solution; unfortunately, they still lack the necessary mechanical stability. porSi structures supported on wafers, on the other hand, provide mechanical stability and are easy to handle but do not allow flow-through. It's important to underline that the

surface of porSi is extremely hydrophobic and do not spontaneously soak with the polymer solution.

A coating procedure was therefore specifically devised for the porSi structures in order to assure an efficient infiltration of the polymer solution into the nanopores of the samples and to preserve their physical properties. The procedure is based on the following steps:

1) Oxygen plasma treatment for 10 minutes. (Due to oxidation, this step dramatically enhances the wettability of the samples and reproducibility of functionalization)

2) Immersion of porSi structure into a 1% w/v polymer into water solution of ammonium sulphate at 20% saturation level for 30 minutes whilst applying a controlled (500 mbar) vacuum. Applying the vacuum is essential to let air come out from the nanopores and for the polymer solution to penetrate them.

3) Rinsing in water under mild vacuum for 15 minutes

4) Drying at room temperature under high vacuum (50 mbar) for 15 minute.

2.2.2 Spotting of coated porSi with a fluorescently labelled antibody

PorSi structures, coated by copoly(DMA-NAS-MAPS) according to the procedure described above were spotted with a Cy3 labelled rabbit antibody at the concentration of 1mg/mL. An array of 35x40 droplets (volume of each droplet is 400pL) of fluorescent protein, equal to a total volume of 560 nanoliters was spotted on each sample and the luminescence of porSi structures was evaluated from the cross section at different sample depth using a Aramis Jobin Yvon Horiba microRaman system, working in microphotoluminescence mode. The achievable spatial resolution is in the order of 1 μ m. Measurements were performed by UNITN.

Figure 3 shows the normalized fluorescence decay detected along the sample depth thereby demonstrating the infiltration of the fluorescent protein into the polymer coated pores. Measurements were taken by looking at sample cross sections after a proper cleavage of the silicon chip. Two different protein concentrations were considered in these measurements. Dataset with filled symbols (samples 33, 41 and 43) were infiltrated with a concentrated protein solution, while sample 35 was spotted with a solution ten times more diluted. On both samples 33 and 35, measurements were performed three times, along different sample sections to check the infiltration homogeneity and reproducibility.

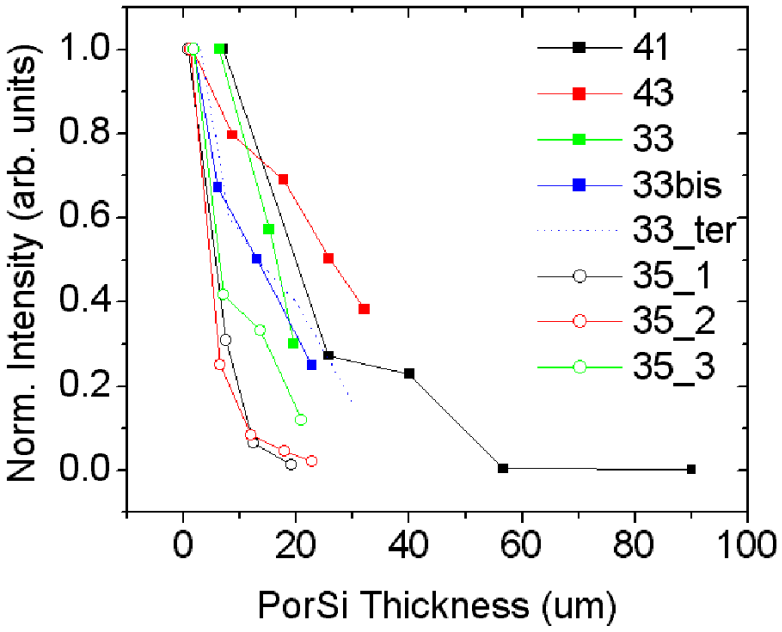

Figure 3 : decay of fluorescence through a porSi sample. Filled dataset represents samples spotted using high concentrated protein solution. Open symbols are for samples spotted with a more diluted solution. All porous silicon samples are fabricated from a (100) oriented silicon wafer.

Even if a quantitative description of the decay length cannot be determined, it is clear that sample 35 shows a systematically shorter decay length compared to the other, more concentrated, samples. This fact gives us a first rough indication about the density of binding sites available inside the porous silicon 'sponge like' material.

Two different silicon crystalline orientation samples were coated and spotted according to the procedure above. Strong fluorescence signal was detected only along the pores in the (100) samples (their pores run perpendicular to sample surface), whereas no fluorescence was detected in the (110) samples (who's pores are smaller and grow at an oblique angle with respect to the surface). Both types of samples show strong and comparable surface fluorescence.

2.3 Characterization of allergen (receptors) binding

When multiple proteins are immobilized on a surface they share the same surface chemical environment regardless of their broad range of physicochemical properties and their different binding vocations. Protein array techniques, on the other hand, assume that the entire panel of arrayed proteins is reproducibly and quantitatively immobilized on the probing surface. Moreover, for each protein, immobilization conditions must be carefully optimized to keep solubility and structure, favour binding and obtain a good spot morphology. In-depth investigation on the absolute amount of surface bound probes is indeed of utmost importance for protein chips, especially if quantitative data are required as it is in clinical applications, such as antibody profiling for allergy diagnosis.

Allergen binding was characterized on copoly(DMA-NAS-MAPS) coated flat SiO/SiO_2 in place of porSi structures using a label-free detector available at ICRM-CNR, the LED-based Interferometric Reflectance Imaging Sensor (IRIS) [3].

Briefly, the principle of detection for the LED-IRIS is based on quantifying the shifts in the spectral reflectance signature to calculate the added biomass by sampling it at specific wavelengths and measuring the characteristic reflection intensities using a CCD camera. The surface is sequentially illuminated by four independently driven LEDs with peak emission wavelengths of 455nm, 518, 598nm, and 635nm. The position of the LEDs' emission spectra with respect to the reflectance curve is critical for allowing the accurate measurement of the shift in this curve due to a change in the thickness (biomass accumulation) of the top layer. After acquiring images of the substrate for each of the four wavelengths each pixel of the CCD represents an individual measurement of the reflective interference intensity at each wavelength, forming a 3-dimensional array of data (pixel, wavelength, intensity) for the entire sensor. The data points for each pixel are fitted to a curve derived using well-known formulations (Fresnel equations). To determine optical spot heights, the average value from pixels in an annular region outside of the spot (background) is subtracted from the average value of pixels inside the spot. By using previously determined calibration factors, this information can be converted to mass densities at each spot location on the chip to determine binding [4].

The immobilization conditions for the panel of food allergens of the POSITIVE project (see D2.3) were studied. Ovalbumin, β-lactoglobulin B, α-lactalbumin, Casein, Ara h 2 and Ovomucoid were patterned using a piezoelectric spotter. Every protein was spotted in three different buffers: PBS (pH 7), borate (borate/NaOH 50 mM pH 9) and acetate (6-EACA acetate 20 mM pH 4.4) at 1 mg/mL except for caseins that required special solubilization protocols (casein solubility is pH and ionic strength dependent) and Ara h 2 which is commercially available at the concentration of 0.6 mg/mL. Printed slides were placed in a humid chamber and incubated at room temperature overnight. The slides were then blocked by 50 mM ethanolamine in TRIS/HCl 1M pH 9 for 1 hour, washed with water and dried by a stream of nitrogen. Table 1 in the appendix reports the quantification of the amount of allergen bound for each tested immobilization condition onto a copoly(DMA-NAS-MAPS) silicon surface measured by the LED-based Interferometric Reflectance Imaging Sensor (IRIS).

Retention of the allergen structure under the different binding conditions was verified using model sandwich immunoassays for the detection of anti-allergen polyclonal antibodies to be detected by LIF scanning. As an example of these studies, the investigation of solubilization and binding of casein from bovine milk, is detailed in Figure 4. In this experiment, 1 mg/mL casein was added to PBS with 0.1% w/v SDS (1), PBS with 1% w/v SDS (2), PBS with 0.01% v/v Tween (3), borate pH 9 with 0.1% w/v SDS (4), borate pH 9 with 1% w/v SDS (5), borate pH 9 with 0.01% v/v Tween (6), saline-sodium citrate (SSC) buffer (7), phosphate/NaOH 50 mM pH 8.5 buffer (8) and NaOH 10 mM (9) and allowed to dissolve under stirring overnight. The casein solutions were then filtered and spotted (4 replicated spots for each condition) on the silicon chip. After one night of incubation in a humid chamber, the surface was washed and the unreacted sites were blocked as reported in the experimental section. The surface was then imaged and the amount of immobilized protein quantified.

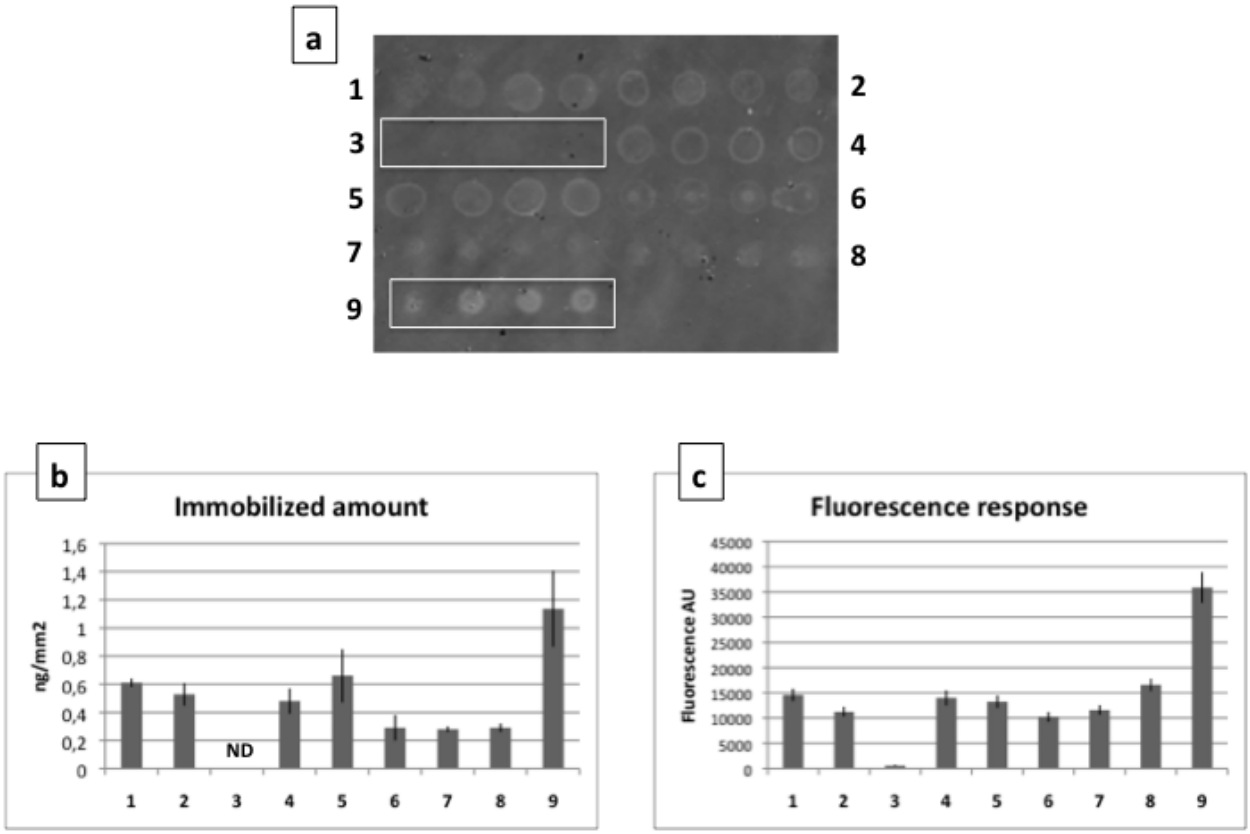

Figure 4-a: label free image of casein from bovine milk dissolved and spotted in PBS with 0.1% w/v SDS (1), PBS with 1% w/v SDS (2), PBS with 0.01% v/v Tween (3), borate pH 9 with 0.1% w/v SDS (4), borate pH 9 with 1% w/v SDS (5), borate pH 9 with 0.01% v/v Tween (6), saline-sodium citrate (SSC) buffer (7), phosphate/NaOH 50 mM pH 8.5 buffer (8) and NaOH 10 mM (9)

Figure 4-b: quantification of the amount of casein immobilized in the different conditions (expressed as ng/mm^2).

Figure 4-c: quantification of fluorescence after incubation with a polyclonal anti-casein antibody from sheep (1 ng/mL) followed by incubation with an anti-sheep IgG antibody labelled with Cy3 for fluorescence dection.

In Figure 4-a, the label free imaging of casein dissolved and spotted in different conditions (labelled from 1 to 9) is shown. Poor morphology of spots for conditions (2), (4), (5) and (6) can be observed; moreover one can notice that the dissolution condition (3) led to an absence of interferometric signal on the chip whereas condition (9) provided the highest signal with an acceptable spot morphology. The quantification of the amount of casein immobilized in the different conditions (expressed as ng/mm^2) is reported in Figure 4-b, showing that dissolving and spotting casein in NaOH 10 mM, condition (9), provided more than 1ng/mm^2 immobilized protein on copoly(DMA-NAS-MAPS) coated Si/SiO_2 slides. The array was then incubated with a polyclonal anti-casein antibody from sheep (1 ng/mL) followed by incubation with an anti-sheep IgG antibody labelled with Cy3 for fluorescence detection. The quantification of detected fluorescence signals is shown in Figure 4-c; as expected, condition (9) provided the highest fluorescence intensity confirming that, among the tested conditions, dissolution of casein in NaOH 10 mM yields the highest amount of protein available in spotting solution and an efficient immobilization environment.

Similar studies were performed on the entire panel of food allergens of the POSITIVE project. Figure 5 summarizes the amount of protein bound for each allergen in their best conditions expressed as ng/mm^2. For each of the allergens in this panel, pH 9 (Borate/NaOH 20 mM buffer) provided the highest yield of immobilization which is 4 ng/mm^2 in average. Binding of casein is lower due to its poor solubility.

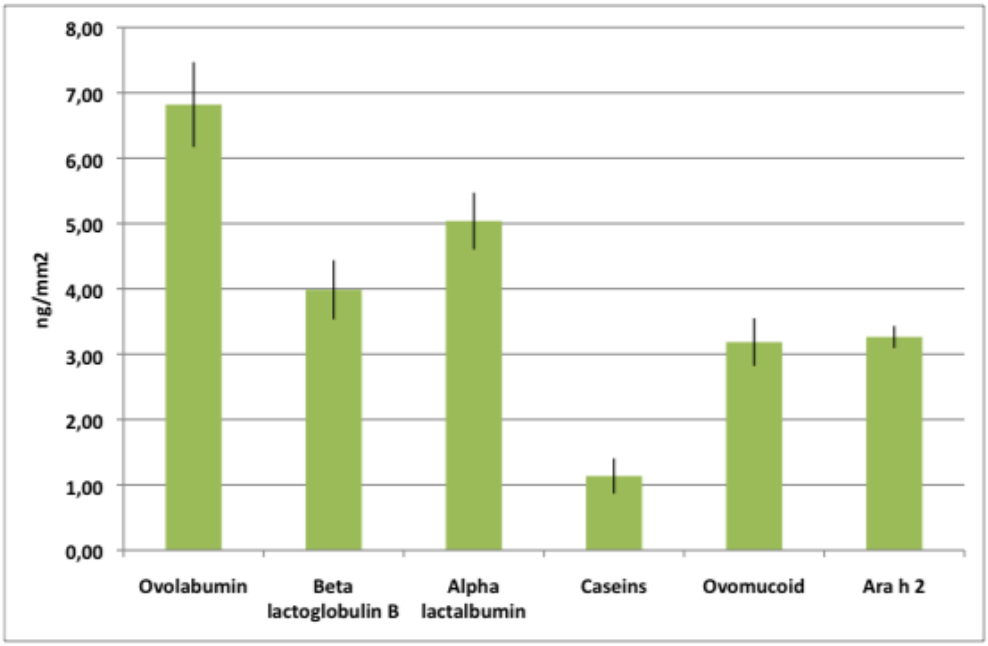

Figure 5: absolute amount of protein bound (in the best conditions) for each of the allergen of the POSITIVE project on copoly(DMA-NAS-MAPS) coated silicon.

3 Conclusions

The work described here led to a protocol specifically devised for porSi coating using copoly(DMA-NAS-MAPS) and to the optimization of immobilization conditions on polymer coated silicon for the entire set of POSITIVE food allergens. The results are in line with what was expected, taking into consideration the robustness of the film forming process on silicon oxide. Problems of poor mechanical stability of porous silicon membranes have emerged. The fragility of the self-standing membranes is an obstacle to the development of flow-through coating procedures. A strong corrective activity is in place in the WPs devoted to membrane production and microfluidics.

4 Bibliography

[1] Cretich M.; Pirri G.; Damin F.; Solinas I.; Chiari M.; A new polymeric coating for protein microarrays. *Anal Biochem* **2004,** 332, 67-74

[2] Cretich M.; Di Carlo G.; Longhi R.; Gotti C.; Spinella N.; Coffa S.; Galati C.; Renna L.; Chiari M. High sensitivity protein assays on microarray silicon slides. *Anal Chem* **2009**, 81, 5197-5203

[3] Daaboul G.G.; Vedula R.S.; Ahn S.; Lopez C.A.; Reddington A.; Ozkumur E.; Unlu M.S. LED-based Interferometric Reflectance Imaging Sensor for quantitative dynamic monitoring of biomolecular interactions. *Biosens Bioelectron* **2011***,* 26, 2221-2227

[4] Özkumur E.; Yalçın A.; Cretich M.; Lopez C. A.; Bergstein D. A.; Goldberg B. B.; Chiari M.; Ünlü M. S. Quantification of DNA and protein adsorption by optical phase shift. *Biosensors and Bioelectronics* **2009,** 25, 167–172

5 Abbreviations

CCD – charge coupled device

Cy3 – reactive water-soluble fluorescent green dyeof the cyanine dye family

IgE – Immunoglobulin E

IgG - Immunoglobulin G

IRIS - Interferometric Reflectance Imaging Sensor

LED – Light emitting diode

LIF – Laser induced fluorescence

porSi – porous silicon

Appendix

Table 1: quantification of the amount of bound allergens on copoly(DMA-NAS-MAPS) coated silicon

Allergen	Immobilization conditions	ng/mm^2	SD	molecules/cm^2
Ovoalbumin 42KDa	PBS	2,39	0,52	3,41E+12
	pH 9	6,82	0,65	9,74E+12
	pH 4.4	0,21	0,39	3,53E+11
Beta lactoglobulin B 35KDa	pH 4.4	0,206	0,391	3,53E+11
	PBS	1,226	0,151	2,10E+12
	pH 9	3,986	0,452	6,83E+12
Alpha Lactalbumin 42KDa	pH 4.4	0,197	0,137	7,89E+10
	PBS	1,948	0,680	8,35E+12
	pH 9	5,037	0,435	2,16E+13
Caseins 23KDa	PBS + SDS 1%	0,527	0,196	1,37E+12
	NaOH	1,136	0,269	2,96E+12
	pH 9 SDS 1%	0,260	0,088	6,79E+11
Ovomucoid 22KDa	pH 4.4	0,210	0,057	5,73E+11
	PBS	0,443	0,044	1,21E+12
	pH 9	3,185	0,365	8,69E+12
Ara h 2 18 Kda	pH 9 (0,3 mg/mL)	3,264	0,168	1,09E+13
	PBS (0,6mg/mL)	0,463	0,146	1,54E+12