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 D6.3: Report on Various allergen tests

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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

The aim of the activity was to develop an immunoassay for allergen specific Immunoglobulins E on substrates functionalized by copoly(DMA-NAS-MAPS) [1]. In order to assess the quality of the polymeric coating, a set of allergens were spotted on a silicon, flat, substrate with a 100 nm thick thermally grown oxide layer, functionalized by the copolymer. The surface was first tested in model immunoassay experiments to optimize immobilization and assay conditions. Finally the allergen array was challenged with serum samples. It was demonstrated that the polymeric surface preserves the native structure of bound allergens, resists non-specific binding of proteins and provides the analytical sensitivity required in clinics for component resolved diagnosis of allergy.

1.2 Structure of this deliverable

The report is organized according to the following sections:

2. Description of work performed
   2.1. Introduction
   2.2. Model Tests
   2.3. Tests with pools of serum samples
   2.4. Tests with "low unit" serum samples
3. Conclusions
4. Bibliography
5. Abbreviations

2 Description of work performed

2.1 Introduction

The principle of solid phase multiplexed immunoassays for allergy diagnosis consists in surface immobilization of several recombinants or purified allergens as the capture agents. When the serum sample is screened, a multitude of single allergens can be simultaneously probed for Immunoglobulin E (IgE) binding by the use of an anti-IgE secondary antibody. The assay allows the identification of the molecules the patients are sensitized to (Figure 1). The use of a secondary antibody is mandatory in this type of assay to discriminate between allergen specific IgEs, that are diagnostic for allergies, and allergen specific immunoglobulins G (IgGs) that are not.

Measuring IgE levels in serum is a challenging task for several reasons. The first issue is related to sensitivity since IgE is typically the least abundant antibody isotype in blood serum (for example, total IgE levels in a normal individual are ~75 ng/ml, compared to 10 mg/ml for IgG). As a demonstration of the sensitivity required by this kind of test, the classification of allergen specific IgE levels used by ImmunoCAP ® (Phadia, now Thermo Fisher), which is the benchmark for in vitro allergy diagnosis, defines a detection range of specific IgE of clinical relevance from 0.84 to 240 ng/mL [2]. Moreover, most of the IgE-binding sites are conformational epitopes, therefore the allergens must preserve their native structure during the assay so as to be properly recognized by the specific IgE. A surface for allergen specific IgE detection must therefore provide high allergen binding capacity, low non-specific background and maintain native conformation of proteins upon surface immobilization to provide adequate detection sensitivity.

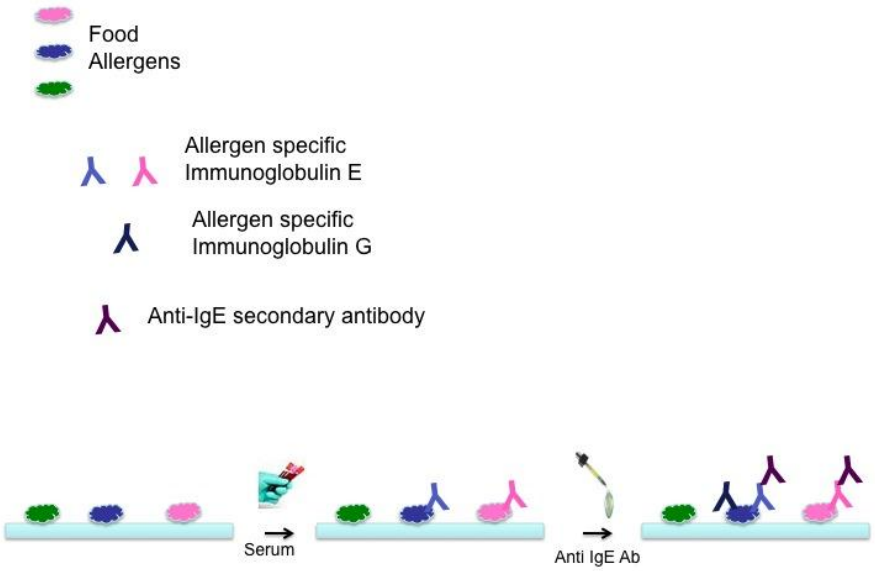

Figure 1: scheme of the immunoassay to detect allergen specific Immunoglobulins E

The binding efficiency of the food allergen panel selected in the POSITIVE project (see D2.3) on planar silicon surfaces coated by copoly(DMA-NAS-MAPS) was characterized by LED-based Interferometric Reflectance Imaging Sensor (IRIS) [3]. Biochemical characteristics of the allergens used in the project and their immobilization yields, expressed as bound molecules per cm^2, are summarized in Table 1 (see D6.1).

In this deliverable, model tests as well as functional tests using characterized patient sera provided by partner C-UB are reported.

Table 1: Biochemical characteristics and immobilization yields on copoly(DMA-NAS-MAPS) coated silicon surface for the POSITIVE allergen panel

Protein	Procured from	Food Source	MW (KDa)	Isoelectric point	Immobilized amount (ng/mm^2)	Immobilized molecules/cm^2
Ovoalbumin	Sigma Aldrich	Hen's egg	42	4,8	6,82 ± 0,65	9,74E+12
Ovomucoid	Sigma Aldrich	Hen's egg	22	4,4/4,6	3,18 ± 0,36	8,69E+12
β-casein	Sigma Aldrich	Cow's milk	23	5,0	1,14 ± 0,27	2,96E+12
α-lactalbumin	Sigma Aldrich	Cow's milk	42	4,2	5,04 ± 0,43	2,16E+13
β-lactoglobulin	Sigma Aldrich	Cow's milk	35	4,6	3,99 ± 0,45	6,83E+12
Ara h 2	Indoor Biotechnologies	Peanut	18	5,2	3,26 ± 0,17	1,09E+13

2.2 Model Tests

A model test to mimic the immunoassay for IgE detection using copoly(DMA-NAS-MAPS) coated silicon surfaces (See D6.1) was developed based on the immobilization of three food allergens (Ovoalbumin, β-lactoglobulin B and α-lactalbumin) followed by the incubation with different concentrations of polyclonal specific antibodies diluted in buffer or rabbit serum. Using fluorescence as the detection method, this panel of tests was devised to assess the sensitivity provided by copoly(DMA-NAS-MAPS) coated silicon surfaces and to set up a protocol for the final bioassay.

Ovoalbumin, β-lactoglobulin B and α-lactalbumin were spotted using a SciFlexArrayer S5 spotter from Scienion (Berlin, Germany) in three different pH conditions (Acetate/NaOH pH 4.4, PBS pH 7.2, Borate/NaOH pH 9) on copoly(DMA-NAS-MAPS) coated silicon slides (SVM, Santa Clara, CA). 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. Arrayed slides were then incubated with the solutions of antibody (anti- Ovoalbumin, anti-β-lactoglobulin B and anti-α-lactalbumin) in incubation buffer (Tris/HCl 0.05 M pH 7.6, NaCl 0.15 M, Tween 20 0.02%) with 1% w/v BSA, for 2 hours or in rabbit serum diluted 1:1 in incubation buffer. Slides were then washed with washing buffer (Tris/HCl 0.05 M pH 9, NaCl 0.25 M, Tween 20 0.05%) for 10 minutes, rinsed with water and then incubated with a Cy3 labelled secondary antibody at the concentration of 0.001 mg/mL in incubation buffer for 1 hour. Slides were then washed with PBS (10 min), rinsed with water and dried with a Nitrogen stream. Scanning for fluorescence evaluation was performed by a ProScanArray scanner from Perkin Elmer (Boston, MA). Fluorescence intensities of 5 replicated spots were averaged. A calibration curve reporting the fluorescent intensities obtained upon incubation with the specific antibodies in the nanogram/mL range was built for each protein specific antibody. Each specific antibody limit of detection (LOD) was extrapolated from the fluorescent value of blank samples plus three standard deviations (3σ). Representative images for these immunoassays are reported in figure 2 for anti-β-lactoglobulin B detection, in figure 3 for anti-α-lactalbumin and in figure 4 for anti-Ovoalbumin.

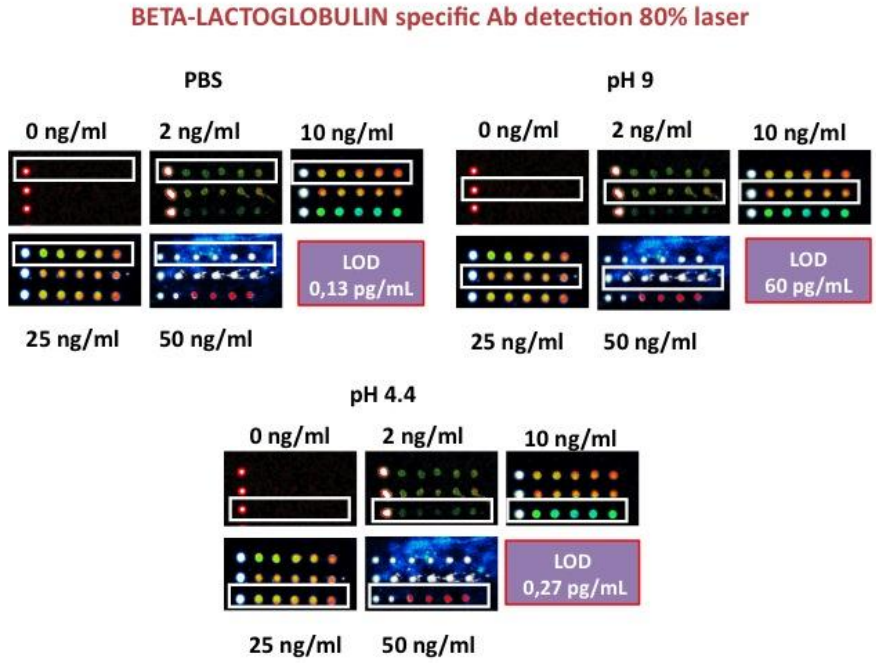

Figure 2: representative fluorescence images (obtained using 80% laser power and PMT gain) and LOD for anti-β-lactoglobulin B specific antibody detection in three immobilization conditions used for the the allergen β-lactoglobulin B.

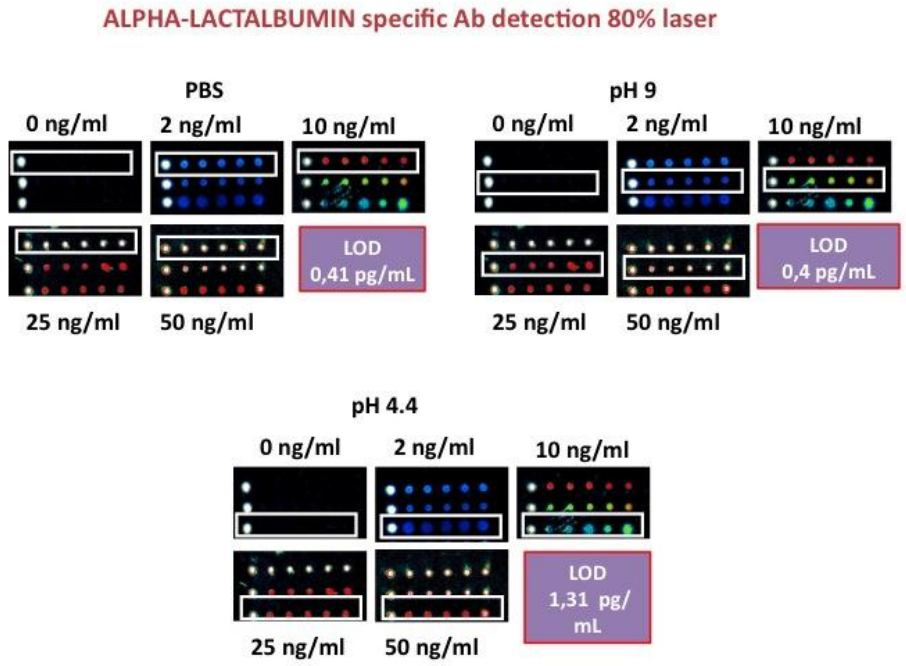

Figure 3: representative fluorescence images (obtained using 80% laser power and PMT gain) and LOD for anti-α-lactalbumin specific antibody detection in three immobilization conditions used for the allergen α-lactalbumine.

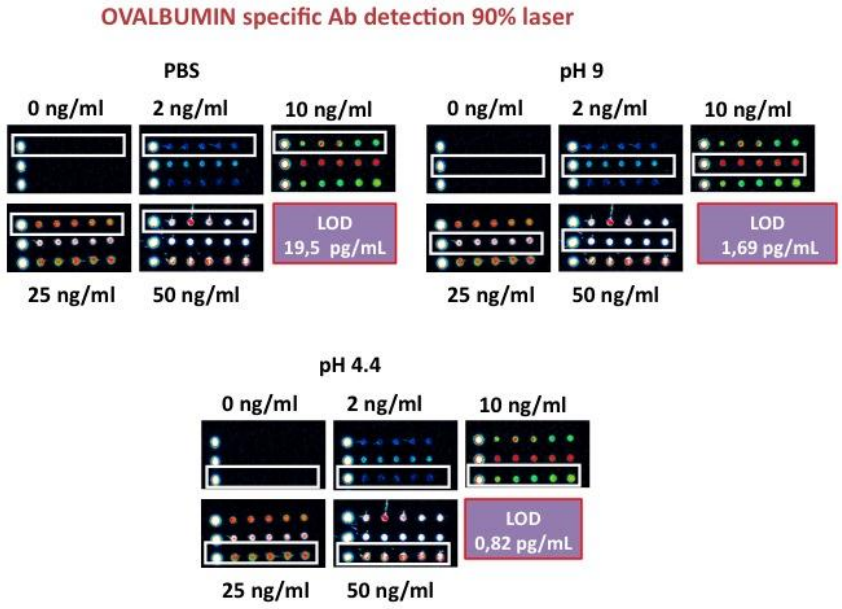

Figure 4: representative fluorescence images (obtained using 90% laser power and PMT gain) and LOD for anti-ovoalbumin specific antibody detection in three immobilization conditions used for the the allergen ovoalbumin.

For each allergen spotting condition, the LOD of the corresponding specific antibody was determined leading to the values reported in Molar concentrations in Table 2.

Table 2: Limits of Detection (LODs) reported in Molar concentration for β-lactoglobulin B, α-lactalbumin and Ovoalbumin specific antibodies when the corresponding food allergens were immobilized in three different pH conditions.

Immobilization conditions	**pH 4.4**	**pH 7.2**	**pH 9**
LOD (M) β-lactoglobulin B sAb	1,80E-15	8,67E-16	9,20E-15
LOD (M) α-lactalbumin sAb	8,73E-15	2,73E-15	2,67E-15
LOD (M) Ovalbumin sAb	5,47E-15	1,30E-13	1,13E-14

These experiments demonstrated that, for the three food allergens tested, the copoly(DMA-NAS-MAPS) coated surface provided an efficient method of immobilization able to detect, when using fluorescence detection, femto-Molar concentrated antibodies.

The same experiments described above were repeated diluting the specific antibodies into a rabbit serum in order to verify how the presence of a biological matrix affects detection sensitivity.

Table 3 reports the LODs determined for each specific antibody (anti-Ovoalbumin, anti-β-lactoglobulin B and anti-α-lactalbumin) in rabbit serum diluted 1:1. As expected the LODs determined in physiological conditions are influenced by the presence of interfering biomolecules; however sensitivity still resides below the picoMolar range due to the low unspecific background provided by the polymeric coating.

Table 3: Limits of Detection (LODs) reported in Molar concentration for β-lactoglobulin B, α-lactalbumin and Ovoalbumin specific antibodies diluted in rabbit serum.

Immobilization conditions	**pH 4.4**	**pH 7.2**	**pH 9**
LOD (M) β-lactoglobulin B sAb	8,00E-14	2,07E-13	2,00E-13
LOD (M) α-lactalbumin sAb	5,13E-12	9,89E-12	4,40E-12
LOD (M) Ovalbumin sAb	8,07E-13	1,23E-12	1,01E-12

In summary, the model immunoassays here reported demonstrated that a silicon surface functionalized by copoly(DMA-NAS-MAPS) presents all the necessary characteristics to act as an efficient substrate for allergen specific IgE immunoassays:

1) it provides a high immobilization yeald for allergens,

2) it keeps the functionality of the proteins during the assay

3) it resists non-specific interactions and

4) when the devised protocol was used, can provide a sensitivity in the femtoMolar range

2.3 Tests with pools of serum samples

The silicon surfaces coated by copoly(DMA-NAS-MAPS) and the protocol for antibody detection described above were tested in a real assay for IgE detection using characterized patient's sera.
Partner C-UB pooled several human serum samples in order to provide six sero-analytical tools to asses specificity and sensitivity of IgE detection on silicon coated by copoly(DMA-NAS-MAPS). The sera were characterized for IgE content specific for hen's egg and cow's milk using ImmunoCAP ® System from Phadia (Thermo Fisher) and accordingly classified from "Low" to "Medium" to "High". The amount of specific IgEs in the six pooled sera is reported in Table 4 and expressed in KU/l (1 KU equals to 2.4 μg of specific IgE)

Table 4: pool of sera characterized by ImmunoCAP ® System. 1 KU equals to 2.4 μg of specific IgE.

Sample ID	**Egg Low**	**Egg Medium**	**Egg High**	**Milk low**	**Milk Medium**	**Milk High**
Phadia CAP f 1 (hen's egg)	0,97 kU/l	18,9 kU/l	44,1 kU/l	0,96 kU/l	12,0 kU/l	27,8 kU/l
Phadia CAP f 2(cow's milk)	1,56 kU/l	12,0 kU/l	63,1 kU/l	0,94 kU/l	21,0 kU/l	>100 kU/l

Food allergens were spotted using a SciFlexArrayer S5 spotter from Scienion (Berlin, Germany) according to the scheme reported in Figure 5.

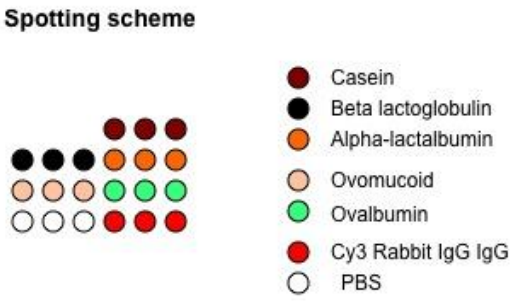

Figure 5: spotting scheme used in the immunoassays for IgE detection in human sera

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 1 M pH 9 for 1 h, washed with water, and dried by a stream of nitrogen. Arrayed slides were incubated for 2 h with serum samples (20 µl, undiluted), washed by the washing buffer (Tris/HCl 0.05 M pH 9, NaCl 0.25 M, Tween 20 0.05% v/v) for 10 min under stirring, rinsed with water, and incubated with the CY3 labeled secondary anti-IgE antibody at 0.01 mg/ml in the incubation buffer for 1 h. Slides were washed with PBS (10 min) and water (10 min). Scanning for fluorescence evaluation was performed by a ProScanArray scanner from Perkin Elmer (Boston, MA) at 70% and 80% of laser power. Fluorescence images are shown in Figure 6 (sera from patients allergic to milk) and Figure 7 (sera from patients allergic to egg). The images showed a low background (even using a high laser power) and several fluorescent spots resulting from the presence of IgEs specific for the tested allergens demonstrating the feasibility of the use of copoly(DMA-NAS-MAPS) as a functional surface for IgE detection assays in real samples and the efficacy of the proposed protocol.

Figure 6: representative fluorescence images of the analysis of pools of sera from patients allergic to cow's milk

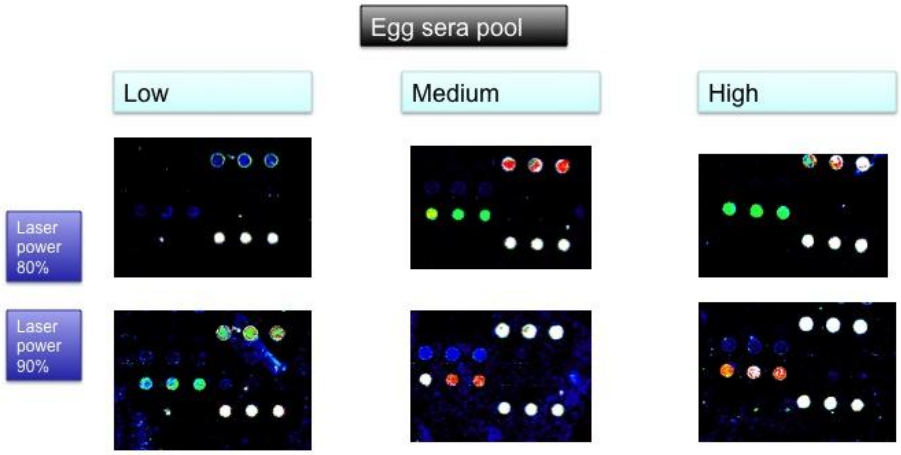

Figure 7. representative fluorescence images of the analysis of pools of sera from patients allergic to hen's egg

Negative controls such as atopic (allergic patients without food allergy neither to against hen's egg nor milk) and non-allergic (no reported food allergy) sera were analysed and didn't provide any fluorescent signal.

Fluorescence responses in arbitrary units (Fluorescence intensities with background subtracted) were calculated at 90% laser power and are reported in Figure 8 for pools of patients allergic to cow's milk and in Figure 9 for patients allergic to hen's egg.

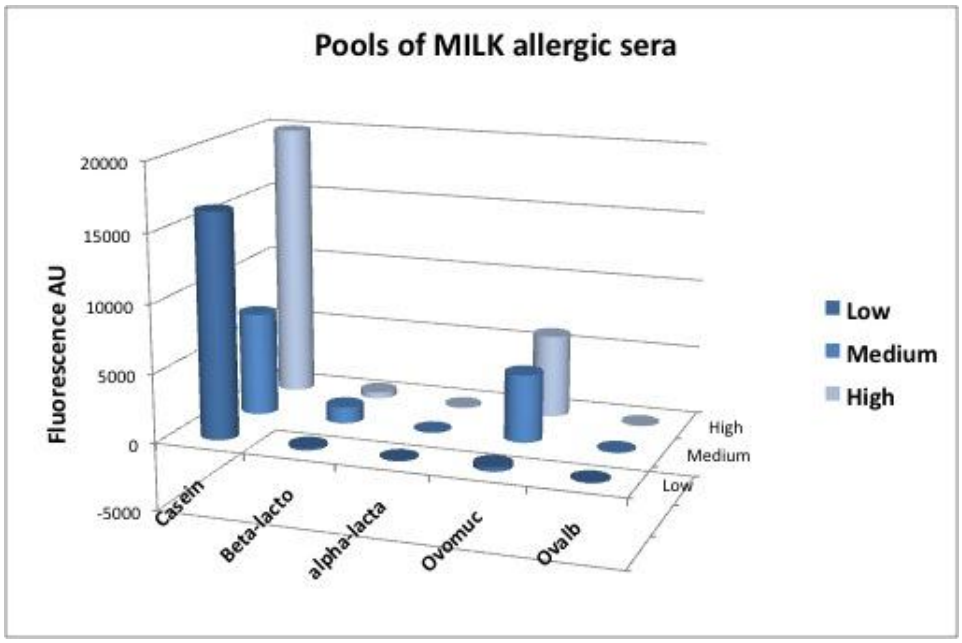

Figure 8: fluorescence detected for each spotted allergen in pools of sera of patient's allergic to cow's milk

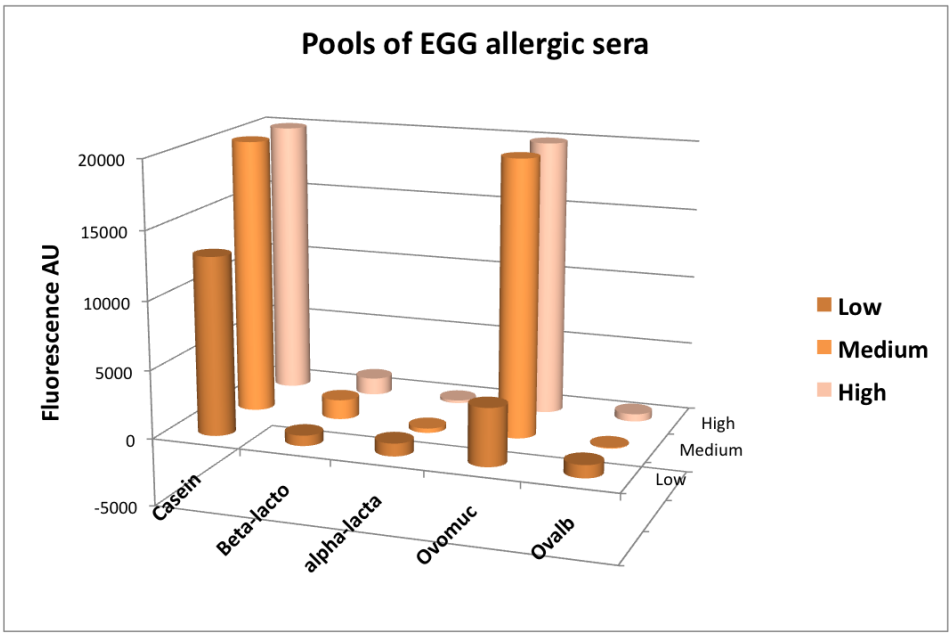

Figure 9: fluorescence detected for each spotted allergen in pools of sera of patient's allergic to hen's egg.

It is worth to say that, unlike ImmunoCAP ® System, allergen arrays provided a component resolved allergy diagnosis in which depending on the spotted proteins, allergy to cow's milk for example can be resolved in the proteins that really elicit an allergic response in patients (caseins, β-lactoglobulin B or α-lactalbumin) and allergy to hen's egg can be resolved in ovalbumin or

ovomucoid.

2.3 Tests with "low unit" serum samples

Patient's serum samples that provided low reactivity and were classified as "low unit" by ImmunoCAP ® System were provided by partner U-CB and analysed by allergen microarrays as described above. The aim of these tests was to challenge the devised protocol and surface chemistry with samples in the inferior limit of the detection range. Fluorescence responses in arbitrary units (Fluorescence intensities subtracted by the background) were calculated at 90% laser power and are reported in Figure 10 for 4 serum samples of patients allergic to cow's milk containing 0.18, 1.74, 2.12 and 4.30 KU/L specific IgE and in Figure 11 for patients allergic to hen's egg containing 0.11, 0.37, 1.78 and 3.52 KU/L specific IgE.

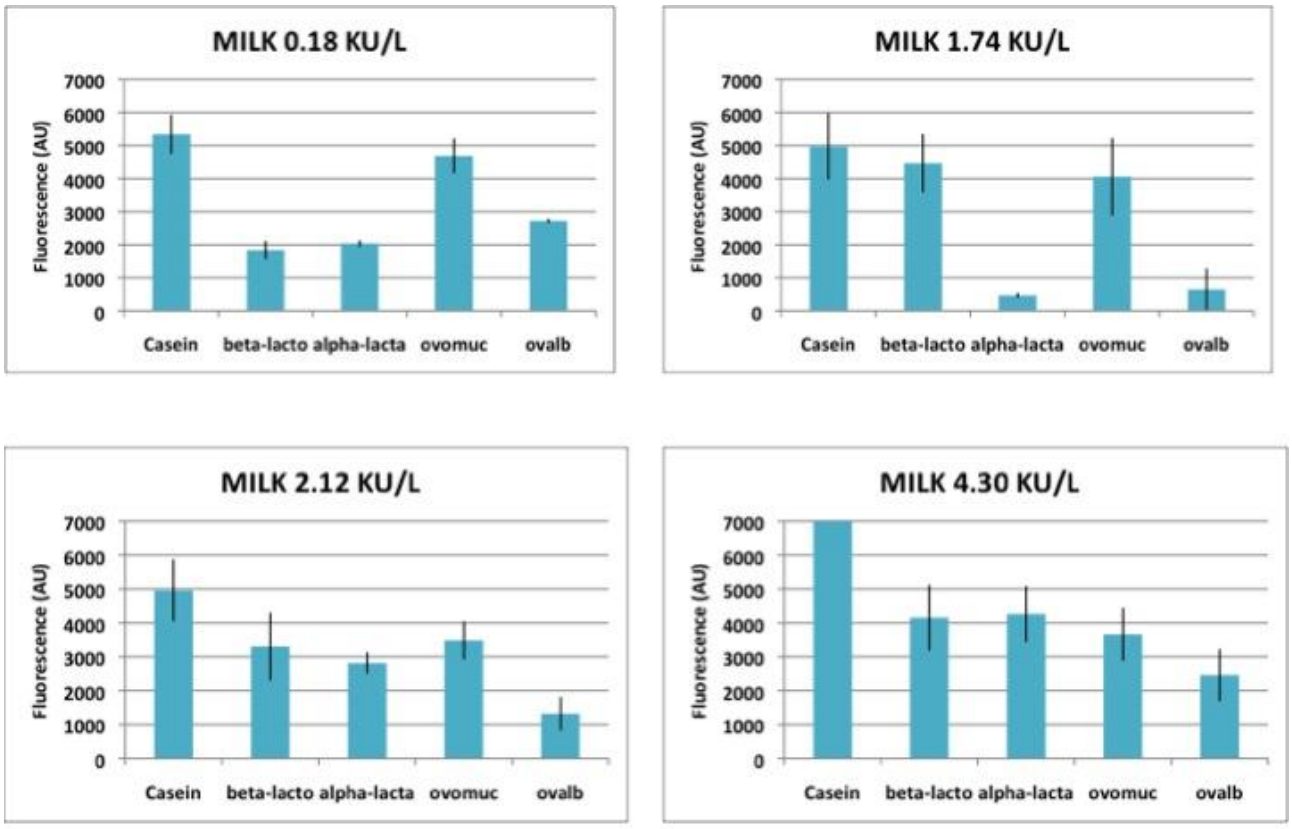

Figure 10: fluorescence intensities detected for each spotted allergen in "low unit" sera form patients allergic to cow's milk

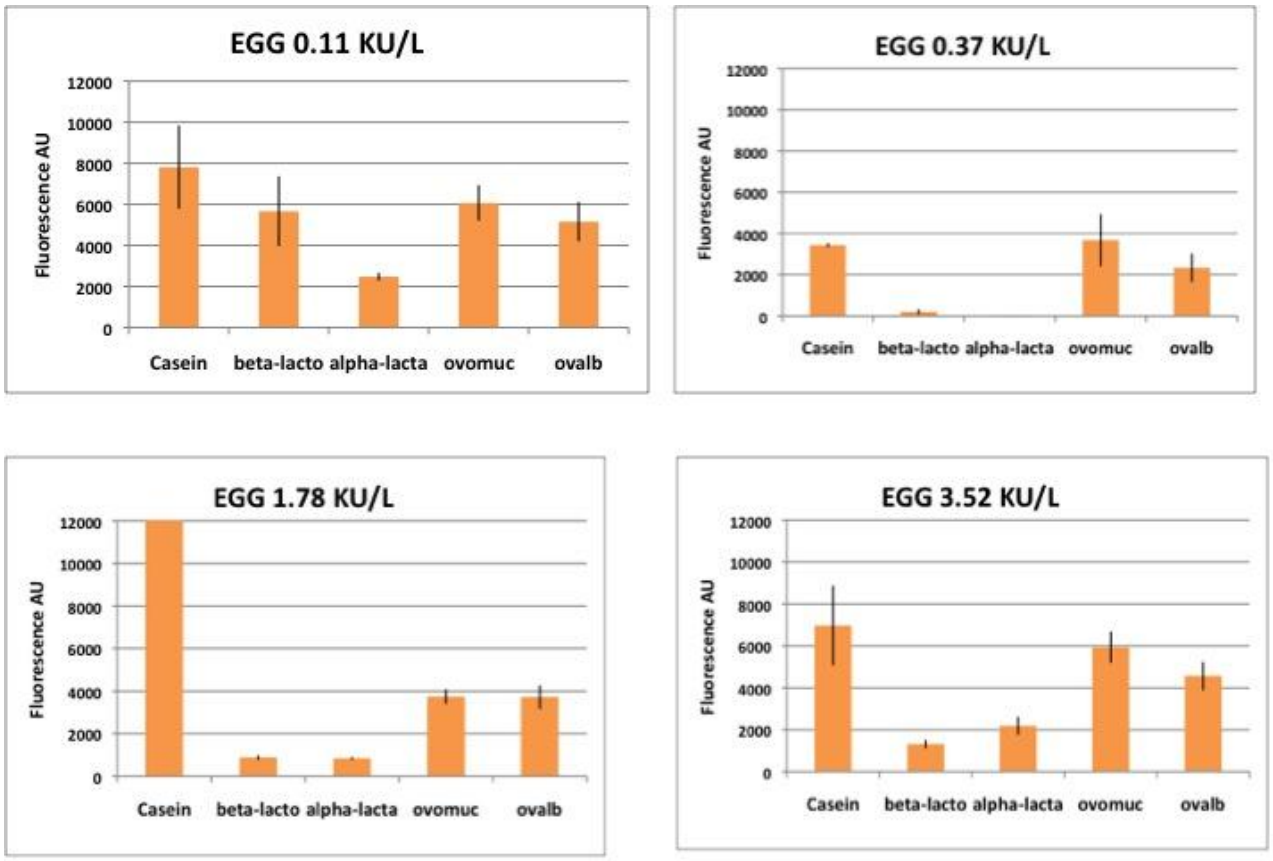

Figure 11: fluorescence intensities detected for each spotted allergen in "low unit" sera form patients allergic to cow's milk

These tests demonstrated the ability of the proposed allergen microarray to detect concentrations of specific IgEs even in the inferior limit of the sensitivity range required by clinicians and to resolve the components of the food allergies to cow's milk and hen's egg.

3 Conclusions

The allergen tests reported in this deliverable demonstrated the ability of the polymer coating based on copoly(DMA-NAS-MAPS) to act as an effective surface chemistry in allergen microarrays. In particular the functional surface and the related protocol described here and in D6.1 provided a substrate able to immobilize food allergens with high yield, keep their active structure and resist non-specific adsorption in biological matrices. The features described were demonstrated by the realization of a component resolved diagnosis for cow's milk and hen's egg allergy even in samples with low content of specific IgE.

3 Bibliography

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4 Abbreviations

IgE: Immunoglobulins E

IgG: Immunoglobulins G

PMT: Photomultiplier

LOD: Limit of Detection

copoly(DMA-NAS-MAPS): copolymer made of *N,N*-dimethylacrylamide (DMA), *N,N*-acryloyloxysuccinimide (NAS), and 3-(trimethoxysilyl)propyl methacrylate (MAPS)