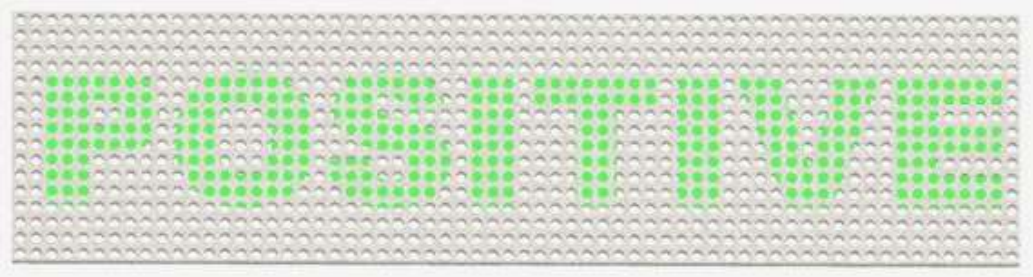

Project Number: 257401

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

D6.1AA - A Second ADDENDUM to Deliverable D6.1: Report on the receptor immobilization methods on porSi structures and conditioning requirements for immobilised receptors.

Due date of deliverable: **January 31 2011**
Actual submission date: **November 6 2012**

Start date of project: 2010-09-01 Duration: 3 Years

Organisation name of lead contractor for this deliverable: **CNR**

Revision **[x]**

Project co-funded by the European Commission within the Seventh Framework Programme		
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 addendum

1.1 Introduction

In D6.1 and in the ADDENDUM to D6.1 previously submitted, we reported on a protocol for the functionalization of i) flat silicon substrates coated by an oxide layer and ii) porSi membranes. A functional coating was formed through chemi/physisorption of a *ter* copolymer, copoly(DMA-NAS-MAPS), obtained by radical polymerization of dimethylacrylamide (DMA), [3-(methacryloyl-oxy)propyl]trimethoxysilane (MAPS) and N,N acryloyloxysuccinimide (NAS). The entire set of POSITIVE food allergens were immobilized in microarray format on the surface of flat silicon slides coated with the polymer. Excellent results were obtained in terms of probe density, reduction of unspecific adsorption and allergen stability (see deliverable D6.1).

During the first year of activity, problems of poor mechanical stability of porous silicon (porSi) membranes emerged which initially delayed the development of flow-through coating procedures. A second generation of porSi membranes became available at the end of the first year from partner UNITN. They were described and characterized in D3.4R. The mechanical strength and permeability of the second generation membranes allowed us to perform the coating in a controlled way (as described in D3.4R). The binding of proteins along the entire pore sections was demonstrated in the ADDENDUM to D6.1 using a home-made micro-photoluminescence (uPL) setup implemented by partner UNITN.

After the first review meeting, the consortium decided to substitute porSi membranes, with an alternative type of porous membranes made of alumina (porAl). The reasons for this choice are extensively discussed in the first intermediate report and the second year report. In this deliverable (D6.1AA) we report on the immobilization of biomolecules within the pores of this alternative type of porous membrane. It is important to note that while the change of the material to porAl has required modifications to the functionalization chemistry initially foreseen for porSi, the use of well characterized, reproducible and permeable membranes has allowed to address problems deriving from the chemical modification of nanoporous materials and to accelerate the development of a flow through biosensor.

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 was aimed at modifying the porSi structures in order to obtain a surface for receptors (allergens) immobilization with high binding capacity, preservation of native conformation of proteins and low background noise. However due to discontinuous supply of permeable membranes with proper mechanical strength the consortium decided to initially carry out bioassay experiments on commercially available porous alumina membranes (Whatman Anodisc, 13 mm diameter, 200 nm pores). Preliminary experiments that were conducted in collaboration with partner Farfield indicated that the alumina layer deposited on top of the surface of silicon nitride chip was removed during the process of coating that is typically used for porSi. Therefore, a different strategy, which will be described in the next sections, was devised for polymeric functionalization of this material, porAl. In addition, a strategy for the introduction of a

chemically reactive group based on the formation of a self-assembled monolayer was pursued to assess the influence of the coating thickness on the sensor performance. This second approach can be seen as a backup strategy in case the thickness of the polymer might contribute to an unacceptable level in reducing the flow rate inside the pores.

2.2 Coating of porous alumina membranes

Coating of the alumina membranes either by a functional polymer or by an epoxysilane monolayer requires organosilanization. In fact the polymer is covalently bound to the surface by a grafting approach which implies the modification of the surface with an allyl moiety followed by radical polymerization of monomers.

A deep investigation of *different surface pre-treatment procedures* to activate surface hydroxyl groups was performed before silanization. In particular, treatments with oxygen plasma and Piranha solution (H_2SO_4:H_2O_2 4:1) were taken into consideration. SEM analysis (performed by partner KTH) demonstrated that both procedures did not affect the physical properties of the alumina membranes. The *oxygen plasma treatment was chosen* as it was faster, easier to control and does not require handling of the fragile supports (Figure 1).

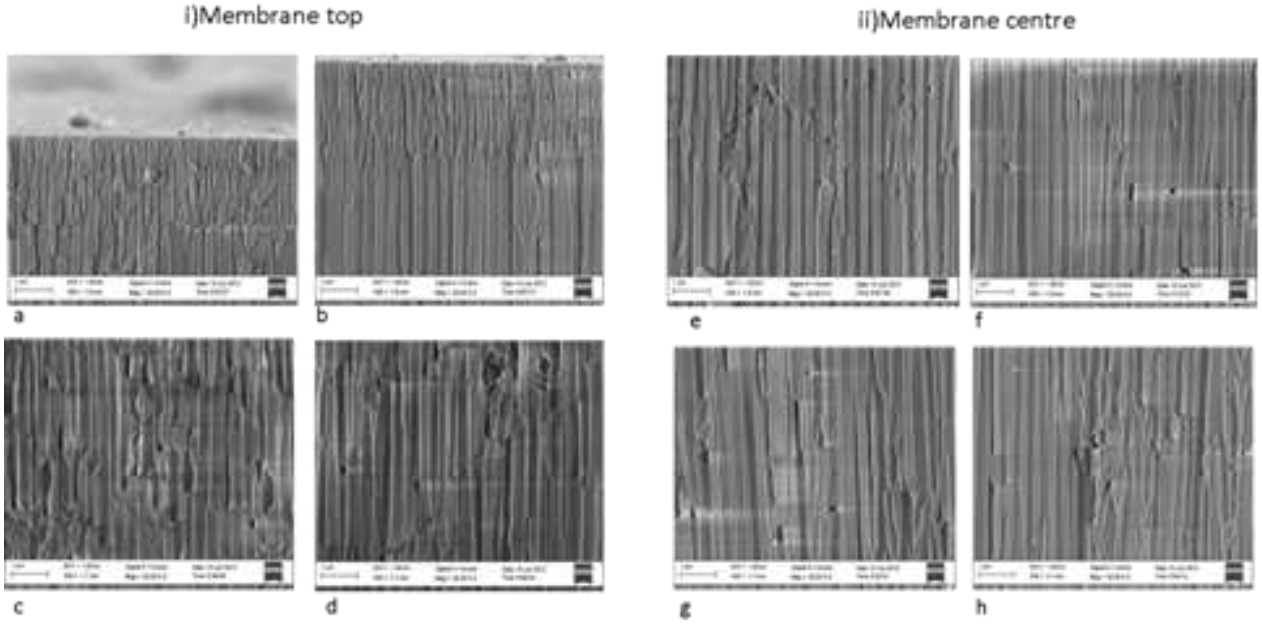

Figure 1 SEM analysis of porous alumina membranes (35K magnification); i) Top view of a) a new membrane b) an untreated membrane c) a membrane treated in piranha solution (H_2SO_4:H_2O_2 4:1) and d) an oxygen plasma treated membrane ii) central view of e) a new membrane f) an untreated membrane g) a membrane treated in piranha solution (H_2SO_4:H_2O_2 4:1) and h) an oxygen plasma treated membrane.

The oxygen plasma treated alumina membranes were coated following two main strategies:

i. **Organosilanization with a silane bearing an epoxy group** i.e. 3-glycidoxypropyltrimethoxysilane, (epoxysilane) or
ii. **Formation of a 3-D polymer coating**. This approach requires an organosilanization with 3 (trimethoxysilyl)propyl methacrylate, -MAPS-) followed by radical polymerization of allyl monomers. Here, the organosilane bears allyl double bonds which allow the covalent attachment of the polymer onto the surface.

In both cases, the silanization step (performed either with epoxysilane, or 3 (trimethoxysilyl)propyl methacrylate, MAPS) was conducted by immersing overnight the membranes in a 1% toluene solution. This step was followed by a curing step at 80°C under vacuum to ensure a high degree of coverage of the surface through a reaction of alumina hydroxyl groups with the organosilane.

When the silanization was conducted with MAPS, allylic double bonds, free to react through radical polymerization with other monomers, in particular N, N-dimethylacrylamide (DMA) and N-acryloyloxysuccinimide (NAS) are exposed on the surface. DMA represents the 90% of the polymer backbone and increases polymer stability onto the surface through hydrogen bonds, while NAS monomer reacts with protein amino groups enabling their attachment onto the surface.

The membranes were analyzed by FT-IR after each step, to demonstrate modification had occurred. The signal of an uncoated membrane (blank sample) was subtracted to the spectra of the modified membrane so to highlight the peaks corresponding to the coating agents.

Regarding the epoxysilanization, only the peaks corresponding to epoxy-silane were clearly visible on the silanized membrane (Figure 2). Peaks at 2850-2980 cm^{-1} were present which corresponds to stretching vibrations of ether v(CH), v (CH2) and v (CH3), respectively. The weaker band at 1450 cm^{-1} is due to asymmetric bending d(CH), d(CH2) or d(CH3). The band at 1258 cm^{-1} corresponds to the epoxy ring breathing while the band at 1150 cm^{-1} is due to the C-O stretching. Signals in the finger print region are not visible because of alumina properties.

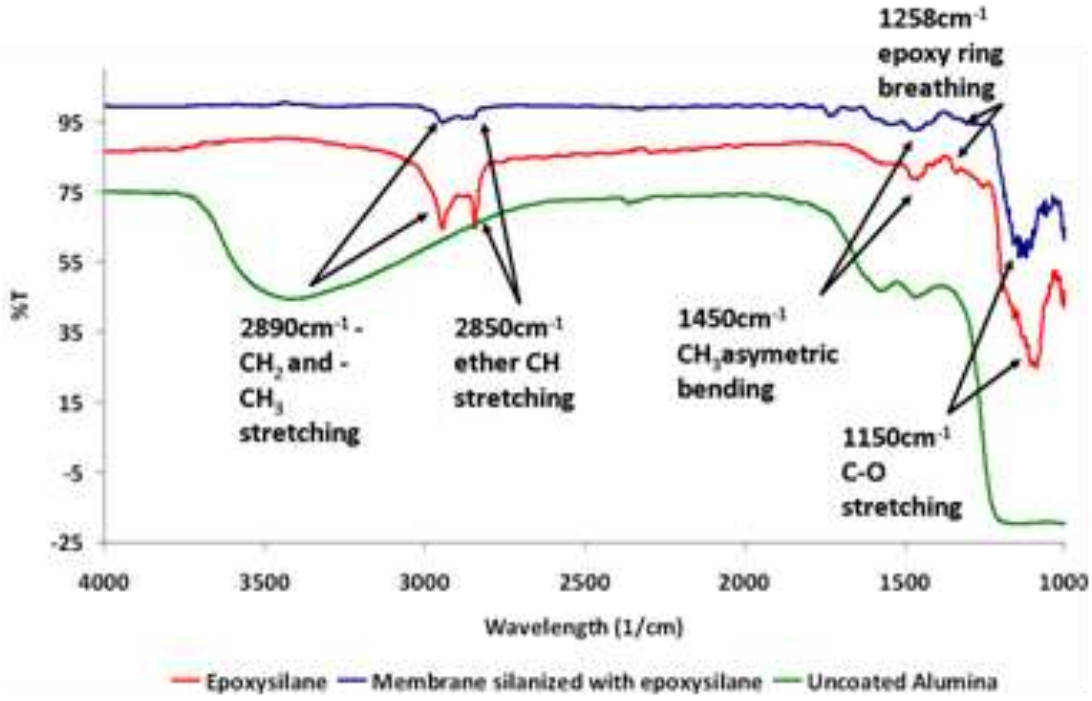

Figure 2 FT-IR analysis of a porous alumina membrane modified with epoxysilane compared to an uncoated alumina membrane. Peaks corresponding to epoxysilane monomer are easy to observe on the functionalized membrane.

The FT-IR spectrum of the *polymer coated membrane* (Figure 3) shows a peak at 1740 cm^{-1} corresponding to the stretching of carbonyl group of MAPS which increases when NAS is added onto the surface. On coated membranes, together with the NAS peak, it is possible to observe a peak at 1640 cm^{-1} which corresponds to the stretching of the DMA carbonyl group. For comparison, an FT-IR of the polymer used to coat the membrane (poly(DMA-co-NAS-co-MAPS)) has been registered as well, so to confirm the presence of the coating onto the membrane.

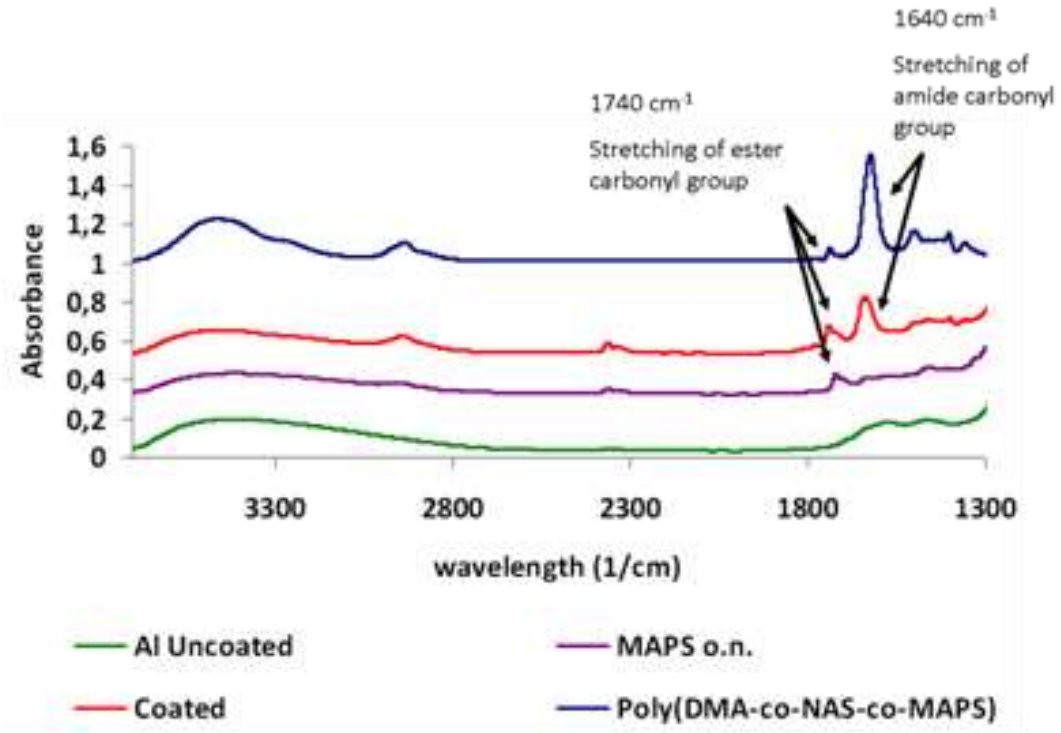

Figure 3 FT-IR analysis of a porous alumina membrane modified with functional polymeric coating. The membrane is silanized overnight with MAPS which bears allyl group which allow a polymerization with DMA and NAS. Peaks corresponding to DMA and NAS carbonyl moities are easily observed if compared to an uncoated membrane.

2.3 Immobilization of protein at specific locations of the membrane by piezoelectric spotting

The permeability of coated membranes was verified to ensure that the functionalization steps do not cause unacceptable constriction within the pores. Membranes were layered onto a nitrocellulose coated slide and spotted with several droplets of a fluorescent protein. By naked eye it was possible to see the infiltration of protein solution into the alumina membrane. After the spotting, the alumina membrane was removed and the nitrocellulose support analysed for the presence of fluorescence using a scanner. In both cases (epoxy-silane and polymer coated membranes) fluorescent spots were detected on nitrocellulose demonstrating the permeability of the functionalized alumina. The experiment described in this section also demonstrates that it is possible to create arrays of proteins on the surface of the membrane and that the amount of protein solution delivered onto the surface by a piezoelectric spotter is sufficient to fill the membrane pores and does not diffuse laterally.

3 Conclusions

WP6 was aimed at functionalizing the pores of the membranes used in biosensing experiments to allow the covalent attachment of bioprobes to the inner surface of the pores. A polymer coating was formed on a flat silicon slide with a layer of thermally grown silicon oxide. The slides were successfully used in microarray experiments and tested in the Analight platform by partner Farfield.

In a separate set of experiments a method was devised to coat a porSi membrane. It was shown that, after coating, the inner surface of the pores binds proteins evenly along their entire length.

In the last part of the work that is reported in this second ADDENDUM to deliverable D6.1, a nanoporous membrane of alumina was functionalized using two different methods. In both cases FTIR analysis showed the presence of functional groups on the membrane. It was also demonstrated that a fluorescent protein, spotted on the surface penetrates through the membrane pores simply by gravity without the need of applying pressure or vacuum. The coated alumina membranes were used by partner UVEG in biosensing experiments whose results are reported in the 2°year report.