# Development of a high sensitivity fluorescence immunoassay for Amyloid Beta 42 by a silicon microarray platform

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## ABSTRACT

In this work, we present a highly sensitive immunoassay for the detection of the Alzheimer's disease (AD) biomarker amyloid-beta 1-42 (Aβ42) based on a label/label-free microarray platform that utilises silicon/silicon oxide ($Si/SiO_2$) substrates. Due to constructive interference, $Si/SiO_2$ layered slides allow enhancement of the fluorescence intensity on the surface with significant improvements in sensitivity of detection. The same substrate allows the label-free multiplexed detection of targets using the Interferometric Reflectance Imaging Sensor (IRIS), a platform amenable to high-throughput detection of mass changes on microarray substrates. Silicon chips are coated with copoly(DMA-NAS-MAPS), a ter-copolymer made from dimethylacrylamide (DMA), 3-(trimethoxysilyl)propyl methacrylate (MAPS) and N-Acryloyloxy succinimide (NAS). An optimal antibody pair was selected based on specificity of recognition, binding yield and spot morphology of the capture antibody on the coated silicon surface as analysed by IRIS. Finally, incubation conditions were optimised, and an unprecedented Aβ42 detection sensitivity of 73 pg/mL was achieved using an artificial cerebrospinal fluid (CSF) sample.

***Keywords***: silicon, microarrays, polymer coating, label/label-free detection, Alzheimer's disease

## 1 INTRODUCTION

Alzheimer's disease (AD) affects millions of people around the world. There is an increasing number of cases as the population ages, and currently, there are no treatments available that prevent or slow the disease. AD is characterised by protein misfolding in the brain, and this process, along with the associated brain damage, begins years before the neurodegeneration that accompanies dementia. Three cerebrospinal fluid (CSF) biomarkers (the 42-amino acid fragment of the amyloid-beta protein, the phosphorylated tau protein and total tau protein) have been extensively studied in an attempt to delineate the sequence of events that occurs during Alzheimer's disease (AD) and to diagnose AD in subjects in whom dementia has not yet presented. More specifically, CSF levels of amyloid beta 42 (Aβ42) are lower in AD patients than in normal controls, reflecting amyloid pathology. However, the field still lacks a non-invasive, inexpensive diagnostic tool for population-wide screening to identify individuals with preclinical Alzheimer's disease who are still cognitively healthy. The importance of population-wide screening is supported by recent findings that suggest signs of Alzheimer's disease can be detected in the brains of approximately 20% of cognitively healthy elderly people. In this context, because of the multiplexing capability, low volume sample consumption and sample-to-result time, microarrays are ideal tools, provided they have high sensitivity and specificity.

To the best of our knowledge, no microarray-based methods with high-sensitivity Aβ42 detection have been developed as yet.

Here we present the use of a label/label-free silicon/silicon oxide ($Si/SiO_2$) based microarray platform [1] to develop a highly sensitive immunoassay for Aβ42.

Silicon is a widely available and inexpensive material that is compatible with established glass surface chemistries and suitable as a microarray substrate. By varying the thickness of the $SiO_2$ layer, it is possible to enhance the emission of any fluorophore of choice by constructive interference with significant improvements in detection sensitivity [2] and to employ the Interferometric Reflectance Imaging Sensor (IRIS), a platform amenable to high-throughput screening for label-free multiplexed detection [3]. Silicon technology and tuned modulation of the oxide layer facilitate dual detection of label-based and label-free detection schemes on a single chip. The possibility to detect mass and fluorescence changes in a spot on a single chip is of immense utility during the assay development process to characterise the quality and the quantity of spotted reagents. This information, which is generally not available in glass microarray technology, is crucial for determining the consistency and quality of the spotted array, quantifying the amount of immobilised probes and detecting the fluorescence of bioassays.

In this work, silicon chips were coated with copoly(DMA-NAS-MAPS), a ter-copolymer made with an optimised composition of dimethylacrylamide (DMA), 3-(trimethoxysilyl)propyl methacrylate (MAPS) and N-Acryloyloxy succinimide ester (NAS) [4].

## 2 MATERIALS AND METHODS

Poly(DMA-co-NAS-co-MAPS)-coated silicon microarrays were fabricated as previously described [3]. For the label-free IRIS imaging, antibodies against Aβ1–42 were patterned on IRIS chips using a SciFlexArrayer S5 spotter from Scienion (Berlin, Germany). Each protein was spotted in PBS in one array corresponding to one area on the chip.

Printed chips were placed in a humid chamber and incubated at room temperature overnight. The chips were then blocked with 50 mM ethanolamine solution in 1 M TRIS/HCl pH 9 for 1 hr, washed with water and dried under a stream of nitrogen. IRIS images were acquired and fitted with Zoiray Acquire software. For each protein, signals from 35 replicate spots were averaged.

For the fluorescence detection of Aβ1–42 and Aβ1–39, capture antibodies against Amyloid-β and Cyanine 3-labelled with Streptavidin (reference) were patterned Printed chips were placed in a humid chamber and incubated at room temperature overnight. The chips were then blocked with 50 mM ethanolamine solution in 1 M TRIS/HCl pH 9 for 1 hr, washed with water and dried under a stream of Nitrogen.

The chips were then incubated with Amyloid-β 1-42 or Aβ1–39 diluted in artificial cerebrospinal fluid (ACSF) Chips were then washed with washing buffer (0.05 M Tris/HCl pH 9, 0.25 M NaCl, 0.05% v/v Tween 20) for 10 minutes with stirring, rinsed with water, and incubated with the Biotin- labelled secondary antibody at 1 µg/ml in PBS for 1 hr. Chips were then washed with PBS and water for 10 min each and then incubated for 1 hr with Cyanine 3 labelled with Streptavidin at 2 µg/mL in PBS. Chips were washed again with PBS and water for 10 min each. Fluorescence was determined by a ProScanArray scanner (Perkin Elmer, Boston, MA), and silicon chips were analysed using 80% or 90% Photomultiplier (PMT) gain and laser power.

For detection limit experiments, 9 chips were incubated for 2 hrs in either dynamic or static conditions with Amyloid-β at 100, 50, 20, 5, 2, 1, 0.5, 0.1 and 0 ng/mL in ACSF. Calibration curves (dose–response curves) were generated using a three-parameter equation in the OriginLab software. The detection limit was defined as the analyte concentration corresponding to a signal 3 SD above the background signal as calculated from the linear range of the calibration curves.

## 3 RESULTS AND DISCUSSION

Several commercial antibodies directed against Aβ42 were evaluated by IRIS for their binding yield on copoly(DMA-NAS-MAPS) coated silicon and spot morphology. The antibodies evaluated in this study, their entry and the amount bound (ng/mm$^2$) when the antibody was spotted on the functionalised silicon are presented in Table 1.

| Antibody | Short code | Bound protein (ng/mm$^2$) |
|---|---|---|
| Santa Cruz D17 | SC-D17 | 2.895±0.22 |
| Millipore W0-2 | W0-2 | 0.562±0.12 |
| Millipore G2-13 | G2-13 | 0.681±0.32 |
| Invitrogen A11 | A11 | 3.644±0.25 |
| Nanotools 1E8 | NT-1E8 | 1.930±0.24 |
| Nanotools 11H3 | NT-11H3 | 1.043±0.12 |
| Nanotools 8G7 | NT-8G7 | 0.776±0.09 |
| Covance 4G8 | Cov-4G8 | 5.062±0.42 |
| Covance 12F4 | Cov-12F4 | 1.515±0.16 |
| Covance 6E10 | Cov-6E10 | 2.81±0. |

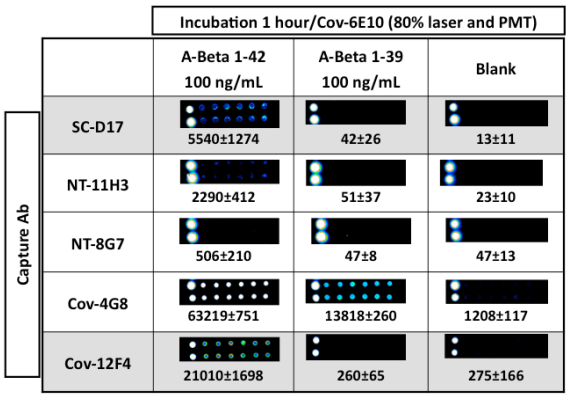
Table 1: list of the antibodies used in this work

Of the 10 antibodies analysed, 6 were selected for subsequent functional tests: SC-D17, NT-11H3, NT-8G7, Cov-4G8, Cov-12F4 and Cov-6E10. The antibodies against W0-2 and G2-13 were omitted due to their low binding (0.56 and 0.68 ng/mm$^2$, respectively), NT-1E8 for the poor spot morphology, and A11 for being specific for soluble oligomers.

The antibodies SC-D17, NT-11H3, NT-8G7, Cov-4G8 and Cov-12F4 were tested as Aβ42 and Aβ39 capturing antibodies using biotinylated Cov-6E10 for the detection, followed by incubation with Cy3 labelled streptavidin. The aim of this experiment was to select the best capture antibody in terms of intensity and specificity for the Aβ42 signal. Fluorescence images for the analysis of 100 ng/mL of Aβ42, Aβ39 and a blank sample with a similar fluorescence value at 80% laser and PMT are shown in Figure 1. Antibodies SC-D17 and Cov-12F4 resulted in higher signal intensity and specificity for Aβ42 as indicated by the absence of fluorescence for Aβ39.

In addition to being most specific for Aβ1–42, the antibody matched pair SC-D17/Cov-6E10 and Cov-

12F4/Cov-6E10 resulted in absence of fluorescence for the blank samples and, therefore, were chosen as the optimal matched reagents for Aβ42 detection in this study.




(1)

Figure 1: Fluorescence images and intensities (80% laser and PMT) for the detection of Aβ42, Aβ39 and a blank using SC-D17, NT-11H3, NT-8G7, Cov-4G8 and Cov-12F4 antibodies as capture reagents and biotinylated Cov-6E10 as the detection antibody.

The antibodies SC-D17 and Cov-12F4 were immobilised on copoly(DMA-NAS-MAPS) coated silicon slides. The chips were incubated with several concentrations of Aβ42 dissolved in an artificial cerebrospinal fluid (ACSF) that mimics the composition of endogenous CSF, in static (using a cover slip) and dynamic (in a petri dish on a horizontal shaker at 50 rpm) conditions, followed by incubation with Cov-6E10 detection antibody and Cy3-streptavidin. Calibration curves (dose–response curves) to determine the fluorescence intensities obtained upon incubation with Aβ42 in the ng/mL range were produced for each tested condition, and the data were fitted to the three-parameter logistic equation. Figure 2 shows the fitted dose response curves obtained using a 2-hr dynamic incubation with matched antibody pairs Cov-12F4/Cov-6E10 at 90% laser power and PMT. LODs were extrapolated from the average fluorescent value of the blank samples plus three standard deviations (3σ) using the linear range of the calibration curves. A limit of detection of 73.07 pg/mL was obtained

Figure 3 shows the fluorescence results of arrays for the detection of 0, 0.1, 0.5, 1, 2, 5, 20, 50 and 100 ng/mL of Aβ42 in ACSF using the 2 tested capture antibodies SC-D17 and Cov-12F4 and 2 hrs of dynamic incubation. Cov-12F4 gave a clear fluorescence response even at the lowest concentration tested, whereas SC-D17 yielded detectable signals only for concentrations higher than 5 ng/mL. These data indicate that a detection limit one order of magnitude lower can be achieved by using Cov-12F4. Notably, intense fluorescence signals coupled to low non-specific background resulted from the fluorescence-enhancing effect of the constructive interference of the 100 nm silicon oxide layer and the non-fouling properties of the polymeric coating, which gives a very low background even at high laser power.

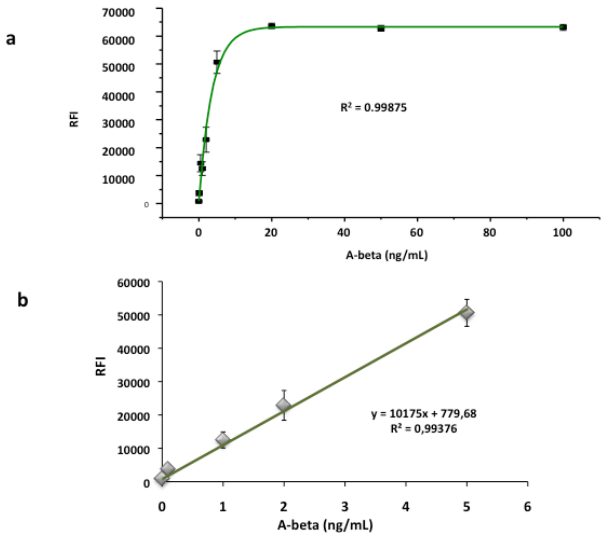



Figure 2: a) Calibration curve for Aβ42 detection in ACSF with Cov-12F4 as the capture antibody and Cov-6E10 as the detection antibody and 90% laser and PMT gain. Aβ42 was incubated under dynamic conditions for 2 hrs. b) Close-up of the liner range of the curve, which yielded an LOD of 73.07 pg/mL.

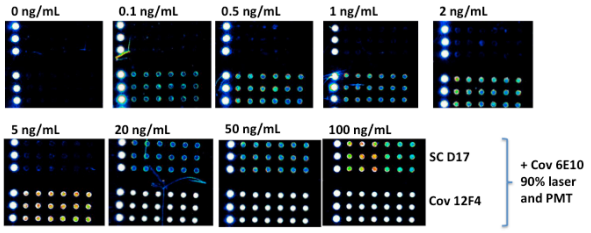



Figure 3. Fluorescence images (at 90% laser power and PMT) of Aβ42 detection using SC-D17 (upper array) or Cov-12F4 (lower array) as the capture antibody and Cov-6E10 as the detection antibody. Samples with concentrations ranging from 0 to 100 ng/mL were analysed.

In summary, the best conditions developed for Aβ42 detection consisted of the use of Cov-12F4/Cov-6E10

matched antibody pair and 2 hrs of dynamic incubation and resulted in the unprecedented microarray LOD of Aβ42 in ACSF of 73.07 pg/mL.

This value is compatible for the use of the proposed assay in diagnostics, where controls show a concentration of Aβ42 in CSF of 794±20 pg/mL and AD patients show a concentration lower than 500 pg/mL

## 4 CONCLUSIONS

A label/label-free silicon/silicon oxide ($Si/SiO_2$)-based microarray platform was used to develop a highly sensitive immunoassay for Aβ42. In summary, the best conditions developed consist of the use of a 100 nm $Si/SiO_2$ chip coated with copoly(DMA-NAS-MAPS) and spotted with Cov-12F4 as the capture antibody. Aβ42 was detected with Cov-6E10 antibody and 2 hrs of dynamic incubation resulting in the unprecedented microarray sensitivity of Aβ42 in ACSF of 73 pg/mL.

Notably, Aβ42 has a high propensity to form oligomers in *in vitro* environments that can lead to a decrease in its capture efficiency and difficulties in evaluating LODs accurately. When using real samples, longer incubation times could be beneficial and further enhance sensitivity of the proposed assay.

Microarrays are ideal tools due to their multiplexing capability, low volume sample consumption and sample-to-result time for population-wide screening to identify individuals with preclinical Alzheimer's disease who are still cognitively healthy. The high sensitivity of this assay format coupled to a pre-concentration step or signal-enhancing protocols could lead to a non-invasive, inexpensive diagnostic tool for population-wide screening of AD biomarkers in biological fluids other than CSF, such as serum or plasma.

### REFERENCES


[1] Cretich M, Reddington A, Monroe M, Bagnati M, Damin F, Sola L, Unlu MS, Chiari M, 2011 Biosensors and Bioelectronics , 26, 3938-3943

[2] Cretich M, Di Carlo G, Longhi R, Gotti C, Spinella N, Coffa S, Galati C, Renna L, Chiari M, 2009, Anal Chem, 81, 5197-5203

[3]Cretich, M, Monroe, MR, Reddington, A, Zhang, XR, Daaboul, GG, Damin, F, Sola, L, Unlu, MS, Chiari, M, 2012, Proteomics, 12, 2963-2977

[4] Cretich M, Pirri G, Damin F, Solinas I, Chiari M, 2004, Anal Biochem, *332, 67-74*



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