New 3D black substrate
for protein microarrays with improved dynamic range

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Introduction

Surge in proteomics research has accelerated the development of new protein arrays as an indispensable tool to study high density protein interaction . The most important factor that determines the performance of protein microarrays is the substrate on which the proteins are immobilised. The microarray substrate, used either in an interaction or capture mode has to provide several key functions. High binding capacity for any proteins without changing their biologically active conformation as well as a low auto fluorescence in order to generate a high signal to noise ratio are essential .This enables the detection of the interaction or capture event within a wide dynamic range. A Microarray slide combining the benefits of increased surface area of 3D cellulose nitrate with the excellent low auto fluorescence of a black substrate has been developed and evaluated. The 3D structure was designed to offer a high binding affinity for proteins and to better stabilize their active conformation even in presence of denaturing temperature conditions. A competitive study analysing the background fluorescence and the detectable concentration range of a chosen antigen is presented. An Affymetrix Spotting roboter 417 arrayer was used for protein dotting. The analysis is based on fluorescence scanning experiments with an Affymetrix 428 array scanner at a gain of 60. Data analysis was made with ImaGene Premium 5.5.3 by Biodiscovery Inc..

Background

The conventional format for arrays is the standard microscopic glass slide (2.5 cm on 7.5 cm). Most of the instruments have been developed in order to work with these standard slides. There are many proposals for 2D and 3D surfaces with better performance than the standard silicate glass surface.

In the 2D family, many different chemistries , some allowing covalent binding, have appeared for glass or plastic slides. In the 3D family , hydrogels and microporous membrane have also been launched.

The cellulose nitrate membrane is a reference substrate because of its high affinity and high capacity for protein binding. Cellulose nitrate membranes are white with a very high reflectance. This is an advantage if the detection system consists of coloured beads but a clear disadvantage when a fluorescent detection system is used. To overcome these limitations, a new black cellulose nitrate membrane has been developed.

Membrane background

For the new black membrane specific non fluorescent black chemicals are added according to a new process in order to have a thorough black internal membrane structure (Figure 1a,b) .
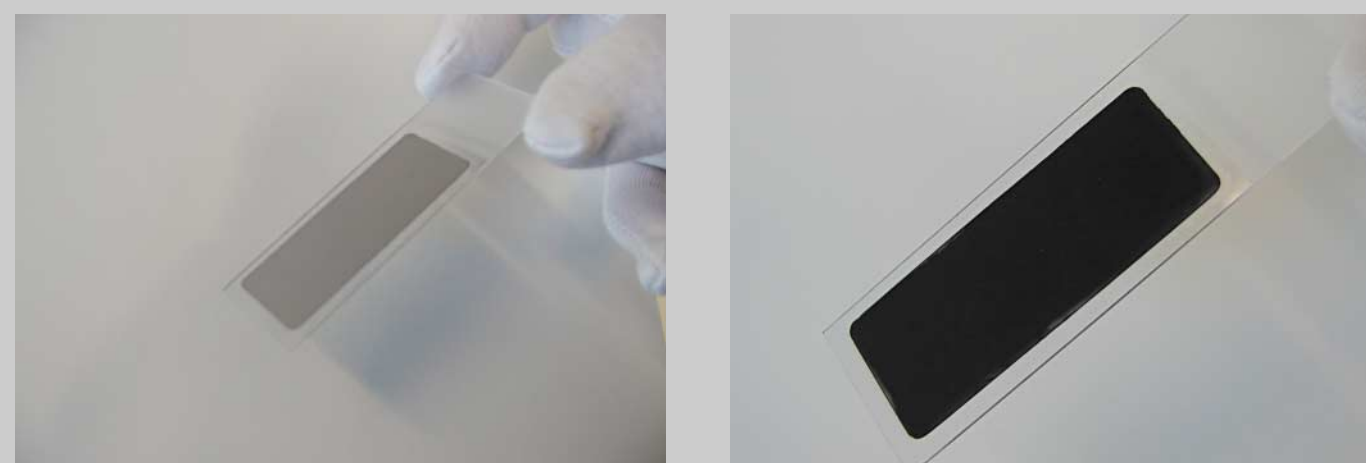
Fig.1a; b: Microarray with a black membrane substrate in dry (a)) and wet (b)) state

Results

A comparison of background fluorescence of five commercial 2D- (Glas, Aldehyd, Cellulose Nitrate) and 3D- slides (Hydrogel, white Cellulose Nitrate Membrane) with the developed black 3D cellulose nitrate membrane measured with an affymetrix scanner at gain 60 is presented in figure 2. The intensity of auto fluorescence is visualised with help of a colour scale. The new black 3D substrate exhibits a very low and homogenous autofluorescence even at this high gain which is typically used for analytes at the lower detection limit. All competitive substrates exhibit significant higher background signals. The white surface of the standard cellulose nitrate membrane is generating too much background under these conditions. As expected this is clear indication that the black substrate will give better detection limits.

3D substrates have been developed essentially for 2 reasons; first to increase the active binding surface area and the total amount of fixed proteins respectively, second to allow proteins to better keep their functional structure in a 3D environment

The risk with a 3D surface compared to a 2D is the loss of fluorescence intensity in the depth of the structure. To verify this phenomenon, we did a confocal signal intensity analysis through the black membrane substrate after spotting 4nL of 10µg/mL Cy-3 labelled anti mouse IgG . The results show that fluorescence photons come from a depth of up to 10 µm (fig. 3) which is conforming with the standard depth of focus of commercial fluorescence scanners. Fluorophors within this depth of focus contribute to the overall signal with a reduced quantum yield because of light absorption inside the membrane. As a consequence spotting of fluorescent protein onto 2D substrates should indeed show higher signal intensity as long as the monolayer concentration of the surface is not exceeded.

Competitive fluorescence intensity analysis of printed mixtures of Cy-3 labelled and unlabelled anti mouse IgG below and above the calculated monolayer concentration is shown in fig. 4.
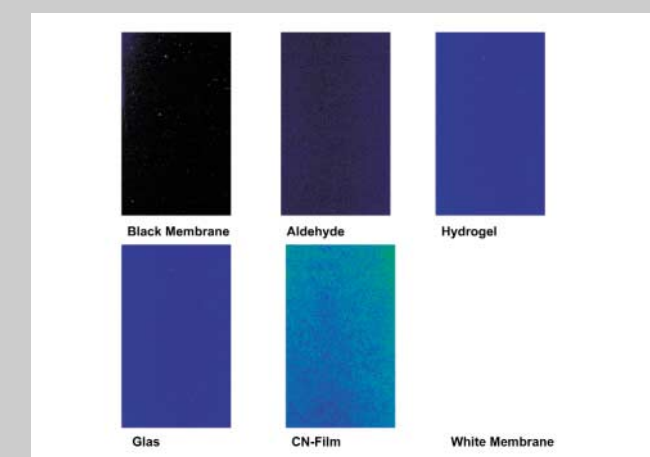

Fig. 2: Background-signal of different commercial substrate materials in comparison to the black membrane measured with an Affymetrix scanner at gain 60; left to right : Black Membrane, Aldehyde glas, Hydrogel, Glas, Cellulose nitrate (CN) film, white CN membrane.
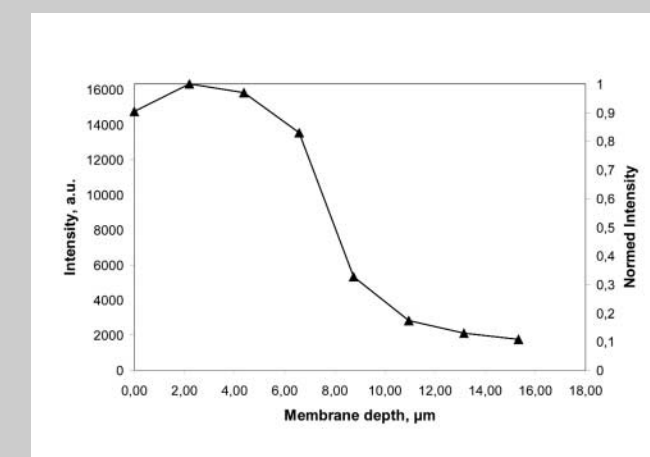

Fig. 3: Analysis of the depth profile of spotted Cy-3 labelled anti mouse IgG (4 nL; 10 µg/mL) within the black membrane by confocal fluorescence microscopy.
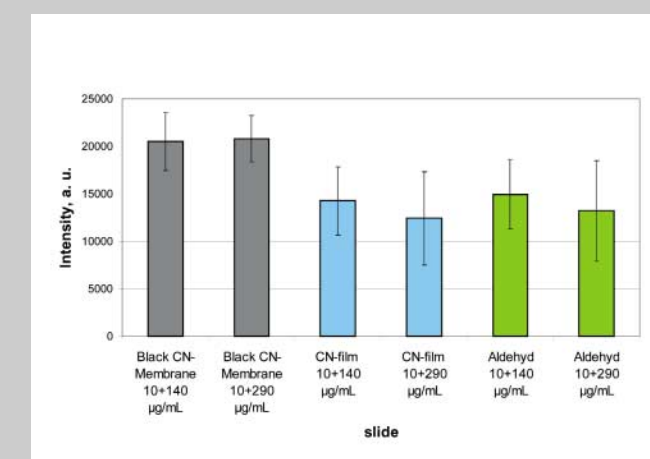

Fig. 4: Competitive Fluorescence intensity comparison of spotted solutions of Cy-3 labelled and unlabelled anti mouse IgG below (10 + 140 µg/mL) and above (10 + 290 µg/mL) the calculated monolayer concentraion of 2D substrates. The Microarrays were blocked and washed before scanning with an Affymetrix 428 array scanner at gain 60.

After spotting the protein solutions, the 2- and 3D-substrates were blocked, washed and finally analysed with an affymetrix scanner at gain 60. A linear intensity increase as a function of fluorescent protein concentration can be noticed for the black membrane substrate. This is the proof that there is no limitation in the protein monolayer formation. The 2D slides exhibit no linear intensity increase but run into an intensity plateau when the monolayer concentration is reached . Any excess protein, piled up in multilayers, is not bound to the substrate and will be washed off during the rinsing step (fig. 5). The higher surface area of 3D structure is able to bind more proteins within the effective depth of 10µm but the total number of bound proteins directly on top of a porous membrane may be lower than on a flat surface film. Furthermore the quantum yield of the fluorophors below the surface decreases to about 20% in 10 µm depth .

Besides the much higher binding capacity, it is also expected that the 3D environment will help to maintain proteins in their biologically active conformation.

This is an essential point for substrate performance besides its structure and binding capacity respectively. A capture experiment was analysed for the interaction between mouse IgG and Cy-3 labelled anti mouse Igg.

At a concentration of 0.0015 µg/µL of Cy-3 the black membrane shows similar signal intensity as a glass slide (Fig. 7).

Conclusion

This new black cellulose nitrate membrane is already showing an improved background at high gain as well as a higher protein binding capacity. Further tests should determine the optimum range where the 3D structure will show increased sensitivity compared to a 2D surface.

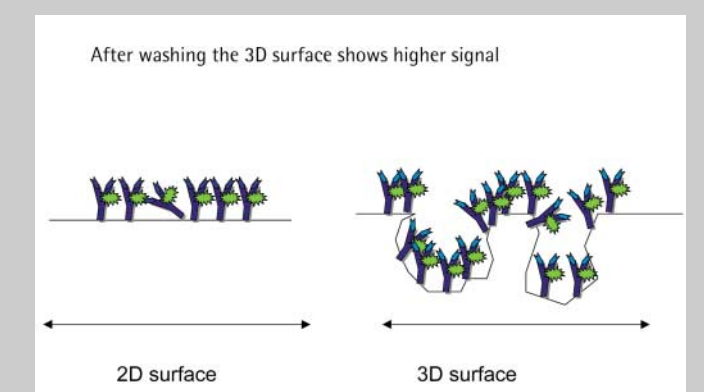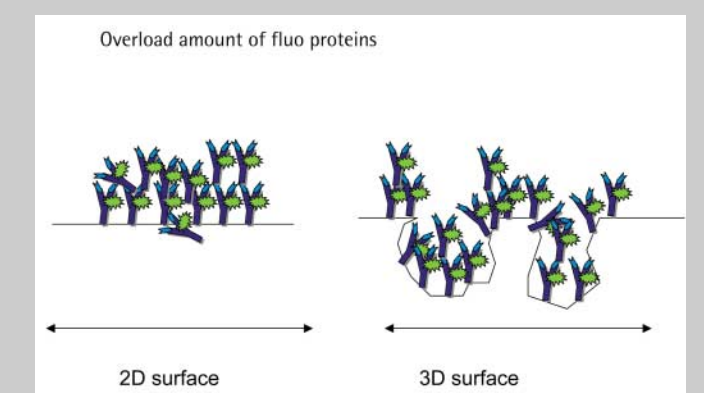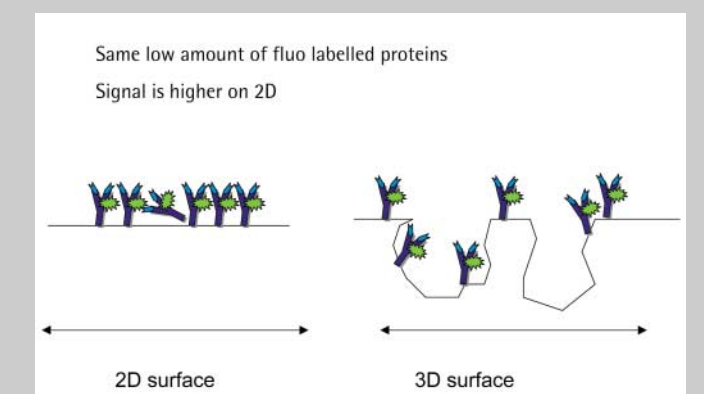...Come to the dark side of arrays!

Fig. 5: Schematic illustration of protein immobilisation on 2D and 3D substrates below and above the monolayer concentration of 2D substrates.
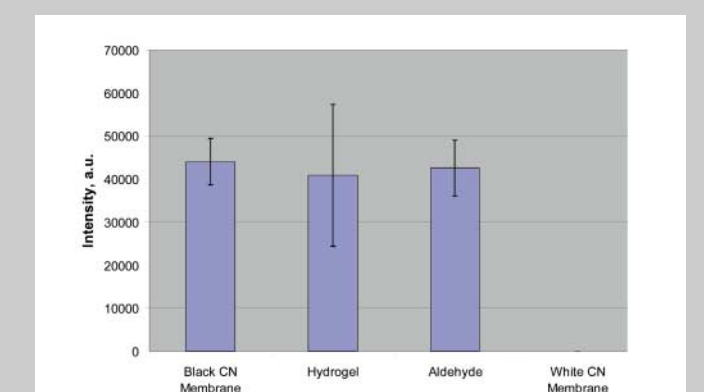

Fig.6: Fluorescence intensity analysis of a capture experiment of mouse IgG (1,0mg/mL) immobilised on the substrate and Cy-3 labelled anti mouse IgG (0,0015µg/µL).