

DPI Characterisation of Antibody Immobilisation, Orientation and Activity

Introduction

The surface chemistry of proteins plays a major role in several biophysical application areas within the pharmaceutical, biotechnology and diagnostic industries. Dual Polarisation Interferometry (DPI) is a major enabling tool for the study of protein immobilisation and molecular orientation. Farfield's **AnaLight**[®] DPI instrument range embodies a truly quantitative analytical technique, rather than a simple 'mass sensor' response, providing absolute mass, dimensional and density measurements of biomolecules and their complexes at interfaces ⁽¹⁾.

The example shown here demonstrates the ability of **AnaLight**[®] to monitor the construction and molecular arrangement of a biomolecular interface, in this case an immobilised antibody-antigen surface, and its structural and functional changes throughout the assay. Similar methodologies are applicable to, and have been observed for, other systems across a wide range of industrial and academic disciplines, eg. electrochemical sensors.

An amine modified **AnaChip**[™] was treated with BS3 linker to facilitate antibody binding. Antibody was then allowed to bind to the **AnaChip**[™], after which the surface was blocked with ethanolamine to prevent non-specific binding in later stages of the protocol. The antigen (itself an antibody) was then added followed by glycine at pH2. The latter two steps were repeated to allow investigation of the regenerative properties of the assembled layer.

Results and Discussion

Antibody Immobilisation:

Figure 1 shows the antibody layer being deposited between points **a** and **b**. Blocking with ethanolamine caused a reversible mass increase. When the system was returned to running buffer a stable antibody layer resulted (**c-d**). Good layer coverage of immobilised antibody was achieved as indicated by the density of 3.2gcm^{-3} (**Figure 2**). The thickness of the blocked antibody layer was measured at 10.4nm (**Figure 2**) indicating that a significant proportion of the antibody layers were oriented perpendicular to the surface. Antibody layers 3-5nm thick are commonly observed when the molecules lie prone on the surface.

Antigen Binding and Regeneration:

At point **d** (**Figure 1**) the antigen was added. The antigen bound specifically to the antibody, causing a mass increase accompanied by an increase in layer dimensions and decrease in density (**Figure 2**), and

could not be eluted with PBS buffer (**e-f**). The > 6nm increase in thickness of the layer together with the drop in density showed that the antigen was binding at the top of the immobilised antibody layer. The mass increase was lower than expected showing, that the immobilised antibody is approximately 40% active, assuming a 1:1 stoichiometry for the interaction.

At point **f**, regeneration of the antibody layer with glycine (pH2) caused a loss of mass and thickness to levels below the initial antibody layer, indicating that some of the original layer was stripped away along with the bound antigen. There was also a substantial increase in density, indicating a rearrangement of the antibody layer. Returning the system to running buffer (**g**) caused an apparent slight mass increase but this was an effect of the different refractive index (RI) values of glycine and PBS.

A second antigen challenge (**h**) resulted in a much lower binding response. During this step, the density of the antibody layer increased (rather than the decrease observed previously) and there was only a comparatively small thickness increase (**Figure 2**). This indicated that the antigen penetrated the immobilised antibody layer. Antibody activity fell to 19%. A small mass increase was observed, which was reversed on readdition of glycine (**i**). The amount of antibody lost from the surface on the first regeneration step correlates with the reduction in antibody binding capacity.

Further challenge with antigen (**j**) caused a very slight mass increase. Subsequent antigen challenges (not shown) produce a more consistent response at this lower binding capacity

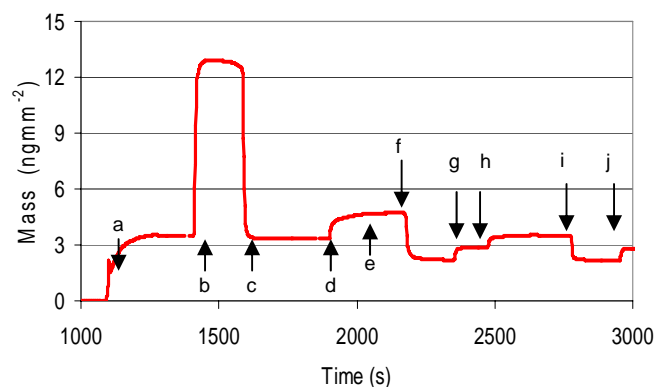


Figure 1: Mass Changes Occurring on Progressive Deposition of the Antibody-Antigen Layer

Figure 2 shows final layer values for the assembled antibody surface and antibody-antigen complex.

Layer	Mass (ngmm ⁻²)	Thickness (nm)	Density (gcm ⁻³)
Antibody	3.424	10.504	0.3260
Blocked Antibody	3.282	10.412	0.3152
Antigen	4.644	16.605	0.2797
Glycine Regeneration	2.799	6.832	0.4096
Antigen	3.470	8.098	0.4285

Figure 2: Final Layer Values for the Assembled Antibody-Antigen Surface

The combination of mass, dimensional and density data allows elucidation of the likely mechanism of layer formation, regeneration and subsequent rearrangement.

Figure 3 shows the proposed sequence for the **AnaChip™** surface on immobilisation of antibody. As layer deposition proceeds, only small unoccupied sites remain on the surface. These sites will only accept antibody molecules which are vertically oriented, and therefore present the active site to the bulk solution, resulting in a layer with relatively high activity.

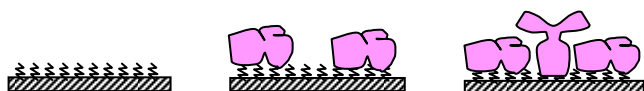


Figure 3: Schematic of the Molecular Structure of the Antibody Layer Deposited on the AnaChip™ Surface

Figure 4 shows the proposed behaviour on antigen binding, regeneration and rebinding. Initially, antigen binds to vertically oriented antibody. Glycine regeneration causes removal of vertically oriented antibodies, along with their bound antigen. Subsequent challenge with antibody results in a different binding pattern, as antigen integrates more intimately into the reduced-activity layer.

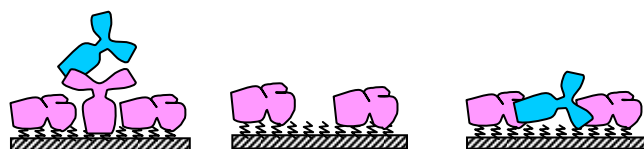


Figure 4: Schematic of the Molecular Structure of Antigen Binding, Regeneration and Repeat Binding

Conclusions and Benefits

These experiments show how DPI can be applied to the study of the construction and function of sensing and assay surfaces. The **AnaLight®** instruments and their experimental protocols give the researcher a unique combination of high-resolution data in real time on dimension, density and mass in a bench-top instrument.

The immobilisation of an antibody layer has been quantified, showing a degree of complexity in its deposition. This results in the favourable orientation of a portion of the antibody. This oriented antibody has higher activity than average, accounting for a significant portion of the total antibody response. The unique measurements provided by the **AnaLight®** instrument provide a characterisation and understanding of the molecular orientation of the constructed surface, allowing the assumed assembly to be verified. Further to this, the activity and subsequent loss of activity of the surface can be related to the structural measurements and the molecular orientation on the surface.

The **AnaLight®** is an important enabling tool for biophysicists, surface scientists and protein biochemists, giving them the unique ability to:

- Understand structural events during interactions which mass change alone cannot reveal
- Connect structural and functional events directly and in real time through a single set of high-content measurements
- Monitor the build up of a complex multilayer for elucidation of experimental starting point
- Confidently measure the orientation of immobilised molecules for the optimisation of surface coating
- Characterise the functioning of an immobilised protein interaction surface

Farfield gratefully acknowledges that these experiments were performed by Dr David Cullen and Dr Ming Xu from the Institute of BioScience & Technology, Cranfield University, Silsoe, Bedfordshire, UK.

For further applications information contact: applications@farfield-group.com

⁽¹⁾ Cross et al., *Biosens. Bioelectron*, **19** (2003) 383-390.