

## Structural and Functional Characterisation of Hydrocortisone - Anti-Hydrocortisone Antibody Interactions

### Introduction

Dual Polarisation Interferometry (DPI) is a major enabling tool for the study of protein-small molecule and antibody-antigen interactions. Farfield's **AnaLight**<sup>®</sup> DPI instrument embodies a truly quantitative analytical technique, rather than a simple 'mass sensor' response, providing absolute mass, dimensional and protein fold density measurements of biomolecules and their complexes at high resolution<sup>(1)</sup>. Changes in these physical parameters can be directly related to both the **structural** and **functional** elements of the interactions of biomolecules and can be used to accurately measure binding events.

This application note describes the immobilisation of anti-hydrocortisone antibody and the measurement of the function and structural changes accompanying hydrocortisone binding to the antibody.

The example shown here demonstrates the unique ability of **AnaLight**<sup>®</sup> to characterise the molecular arrangement and orientation of a biomolecular surface. Whilst this application note shows an oriented IgG3 antibody immobilisation, this rationale can clearly be applied to any other antibody systems. The binding of hydrocortisone, a low molecular weight molecule (362 Da) is then thoroughly characterised. The resultant analysis is applicable to a wide range of protein-small molecule interactions.

### Experimental

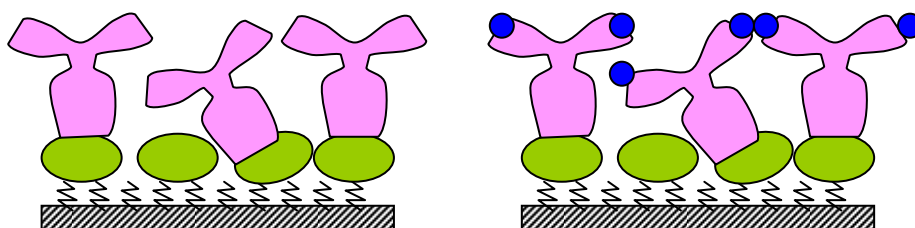
The DPI experiments were performed on a Farfield **AnaLight**<sup>®</sup> instrument. The surface used was a thiol modified **AnaChip**<sup>™</sup>. The temperature of all samples was controlled throughout to 20°C. Water used in buffer preparation was HPLC grade. All reagents were analytical grade or higher, and solutions were degassed prior to use.

**Anti-hydrocortisone Immobilisation:** The **AnaChip**<sup>™</sup> was calibrated with injections of 80% (w/w) ethanol/water. Bulk calibration was then performed on a stream of PBS running buffer (10mM sodium phosphate, 150mM NaCl, pH7.4) (**Farfield Technical Note 001**). Sulpho-GMBS crosslinker (2.5mgml<sup>-1</sup> in PBS) was added to both channels for 3 minutes at 50µlmin<sup>-1</sup>, rinsed with PBS and Protein G (0.4mgml<sup>-1</sup> in PBS) added (50µlmin<sup>-1</sup>) for 6 minutes (**Farfield Technical Note 006**). The anti-hydrocortisone IgG3 antibody (40µgml<sup>-1</sup> in PBS) was bound to the Protein G on the surface during a 14 minute injection at reduced flow rate (20µlmin<sup>-1</sup>) followed by rinsing with PBS running buffer for 30minutes.

**Hydrocortisone Binding and Affinity Calculation:** Hydrocortisone was dissolved in a minimum of DMSO and diluted in PBS. Following establishment of a baseline, hydrocortisone (concentration range 0.1, 0.5, 1.125, 2.25, 7.5 and 22.5µM) was injected into the flow over the immobilised anti-hydrocortisone for 1 minute with 5 minutes rinsing with PBS between samples. Data was corrected for the RI effects of DMSO (**Farfield Technical Note 020**).

### Results and Discussion

**Figure 1** shows the schematic for the **AnaChip**<sup>™</sup> surface on hydrocortisone binding.



**Figure 1: Schematic of the Molecular Structure of the Layer Deposited on the AnaChip<sup>™</sup> Surface**

**Anti-hydrocortisone Immobilisation:** The surface structure of the immobilised antibody can be followed step by step in real time. **Figure 2** shows final layer values for the sequential assembly of the antibody surface. The stepwise formation of the sulpho-GMBS, Protein G and antibody layers during immobilisation can be clearly seen.

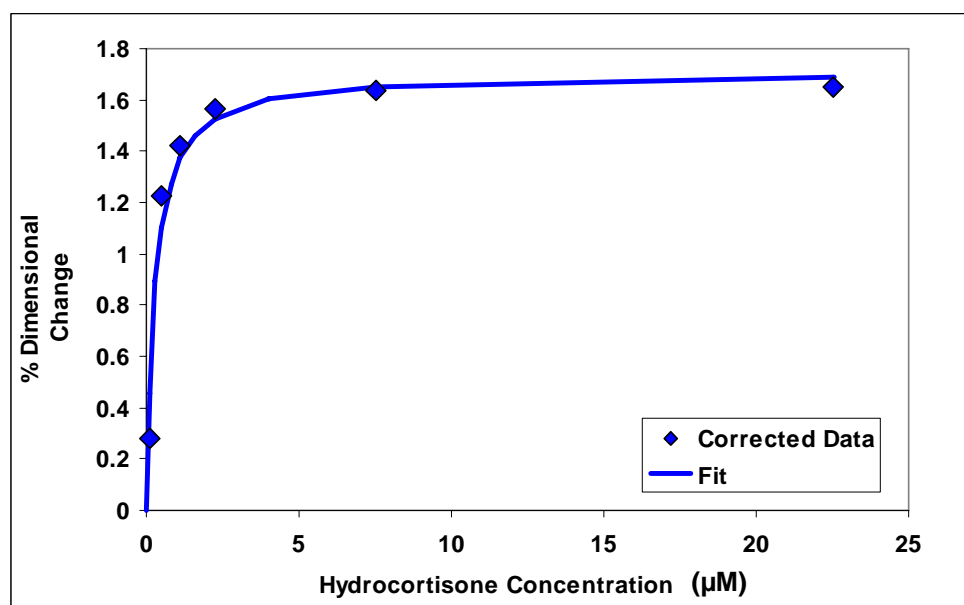
	RI	Thickness (nm)	Density (gcm <sup>-3</sup> )	Mass (ngmm <sup>-2</sup> )
<b>Sulpho-GMBS</b>	1.44142	0.509	0.5773	0.2940
<b>Protein G</b>	1.48843	0.789	0.8316	0.6559
<b>IgG3 anti-Hydrocortisone</b>	1.36889	15.173	0.1849	2.8054

**Figure 2: Layer Values for the Assembled Antibody Surface**

The sulpho-GMBS linker dimensions are as expected and indicate good layer coverage. Immobilisation of the Protein G produces a thin, dense layer, with the 19kDa protein immobilised prone on the **AnaChip™** surface. The antibody is resolved as a separate layer on top of the Protein G, and the completed immobilisation produces a diffuse layer 15nm thick. This is consistent with the longest axial dimension of an IgG3 antibody, and shows that the intact antibody is predominantly oriented vertically onto the Protein G sub-layer. This is confirmation of the expected orientation, since Protein G is specific for the F<sub>c</sub> domain in antibodies

**Hydrocortisone Binding and Affinity Calculation:** **Figure 3** illustrates the affinity binding curve for hydrocortisone using the change in protein dimension. The dimension of the anti-hydrocortisone layer increases by 0.26nm on addition of hydrocortisone, which indicates a conformational change in the antibody in response to hydrocortisone binding. The K<sub>D</sub> calculated from this conformational response is 0.23µM, which was confirmed by the supplier to be within the expected range for the interaction, confirming that the structural change observed can be traced to the hydrocortisone binding event.

Using the mass data, it was calculated that R<sub>max</sub> is reached at 0.006ngmm<sup>-2</sup> and the stoichiometry of the interaction was 2 hydrocortisone to 1 antibody (**Figure 1**).



**Figure 3: Affinity Plot Based on Conformational Response to Addition of Hydrocortisone to Oriented, Immobilised Anti-Hydrocortisone**

## Conclusions and Benefits

These experiments show how DPI can be applied to the study of affinity measurement in antibody-small molecule antigen interactions. The **AnaLight**<sup>®</sup> 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 benchtop instrument.

The binding response of hydrocortisone with anti-hydrocortisone has been quantified, providing conventional binding parameters. In addition to this, the unique measurements provided by the **AnaLight**<sup>®</sup> instrument allow a characterisation and understanding of the molecular orientation of the surface, such that the assumed assembly can be verified. The dimensional data provided for hydrocortisone binding define the conformational signature of this binding event. This system can be viewed as a model for protein-small molecule interactions, where different types of interactions can be defined in terms of binding affinity, stoichiometry, and also structural response.

The **AnaLight**<sup>®</sup> is an important enabling tool for protein biochemists and biophysicists, giving them the unique ability to calculate accurate affinity data and also:

- Understand the structural nature of interactions which measuring mass changes alone cannot elucidate
- Link structural and functional events directly in real time through a single set of high-content measurements
- Monitor the deposition of a complex multilayer for certainty of experimental starting point
- Confidently orientate immobilised molecules for optimal binding
- Use quantitative mass measurements to calculate the stoichiometry of an interaction

**For further applications information contact: [applications@farfield-group.com](mailto:applications@farfield-group.com) or  
Telephone the applications team on +44 (0) 870 950 9717**

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