

Measurement of Structural Change in Antitrypsin on Small Molecule Binding

Data first presented as a poster at The 30th Lorne Conference on Protein Structure and Function 6th-10th February 2005, Phillip Island, Australia

Introduction

Dual Polarisation Interferometry (DPI) is an important enabling technology for the rapid and sensitive monitoring of interactions between proteins and small molecules and the differentiation between specific and non-specific binding ⁽¹⁾.

DPI provides density and dimensional measurements, showing mass capture events and revealing structural changes in proteins that are indicative of a response to specific binding. The sub-atomic resolution of DPI allows the detection of small molecules binding to large, immobilised proteins.

This application note demonstrates the use of DPI to monitor the binding of citrate (210 Da) to the protein α -1 antitrypsin (AT, 48,000 Da). AT is responsible for protecting the lungs from protease damage through capture of the target protease by the reactive centre loop (RCL, see **Figure 1**). As well as binding controlling proteases in the lungs, AT is also prone to aggregation ⁽²⁾ as the RCL is also capable of binding into the A β -sheet region of other AT molecules. Tissue deposition of the resulting aggregate leads to both emphysema and liver cirrhosis. One key strategy to counter this is to identify small molecules that can bind to, and stabilise, AT and prevent its aggregation.

Citrate is known to dramatically stabilise AT against aggregation ⁽³⁾. In this study, DPI was used to investigate the nature of citrate binding to AT and to look for resulting structural changes in the protein. The experiments were also designed to demonstrate the feasibility of using DPI to screen for AT binding candidates based on determining whether they are specific binders inducing a structural change in the protein.

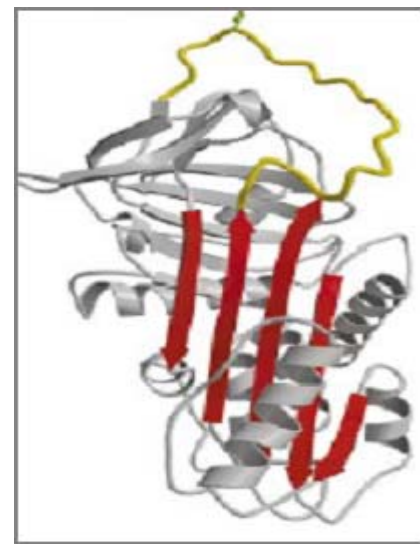


Figure 1: α -1 antitrypsin, showing the RCL (yellow) and the A β -sheet region (red)
(after Huntington et. al., *Nature* 407 923-926)

Experimental

The DPI experiments were performed on a Farfield **AnaLight**[®] instrument. The surface used in all studies was an amine-functionalised silicon oxynitride **AnaChip**[™] with no further pre-treatment. The temperature of the samples was controlled throughout to 20°C. Water used in buffer preparation was deionised and free from organic impurities. All buffers and reagents were analytical grade or higher, and solutions were degassed prior to use.

Protein Immobilisation: The meta-stable nature of AT necessitates careful handling of the protein to maintain activity, as AT becomes inactive at pH4-5. PBS running buffer (10mM, 150mM NaCl, pH7.4) was flowed over the **AnaChip**[™] surface at 100 μ l/min. AT (100 μ g/ml in PBS) was injected into the flow for 3 minutes, followed by a return to running buffer flow at 100 μ l/min until steady state was achieved in the measurements.

Protein-Small Molecule Interactions: The immobilised AT was challenged by three sequential injections of 300 μ l of citrate solution (0.71mM in PBS) under running buffer flow of 100 μ l/min. The measurements from the instrument were allowed to re-stabilise fully between citrate injections. The density, mass and thickness of the AT surface were monitored throughout.

Results and Discussion

Protein Immobilisation: AT immobilisation was repeated ten times onto amine surfaces, giving an average immobilised protein mass of 1.31 \pm 0.25 ng/mm². AT is physisorbed to the positively charged amine surface at physiological pH, and produced good coverage without pH adjustments that might affect AT activity. A stable baseline in running buffer was achieved within 10 minutes post-immobilisation, after which the protein was suitable for challenge by citrate.

Protein-Small Molecule Interactions: Figure 2 shows the change in immobilised AT mass on challenge with the sequence of three citrate injections. There is a net mass gain (after the non-bound citrate is washed away) after the first citrate injection but no subsequent mass gain after the second and third injections of citrate, demonstrating that all available citrate binding sites are saturated by the first injection.

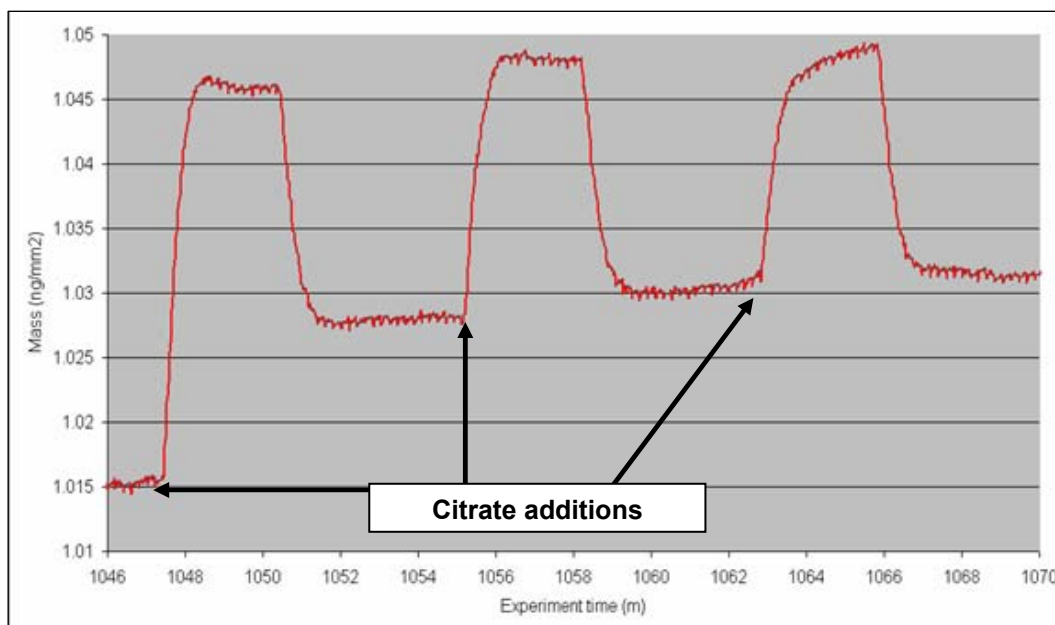


Figure 2: Mass changes to AT on three sequential citrate additions

Figure 3 shows the thickness and density changes to the immobilised AT during the same sequence of three citrate additions. These data demonstrate how DPI goes to a level beyond mass change data, by revealing that the bound citrate causes a **structural change** in the immobilised AT with the protein layer thicker and more diffuse after the first citrate addition. It is this structural change (or response) to binding that is indicative of specific binding to the protein. The lack of structural change after the second and third citrate injections confirms that all available binding sites are occupied after the first citrate addition.

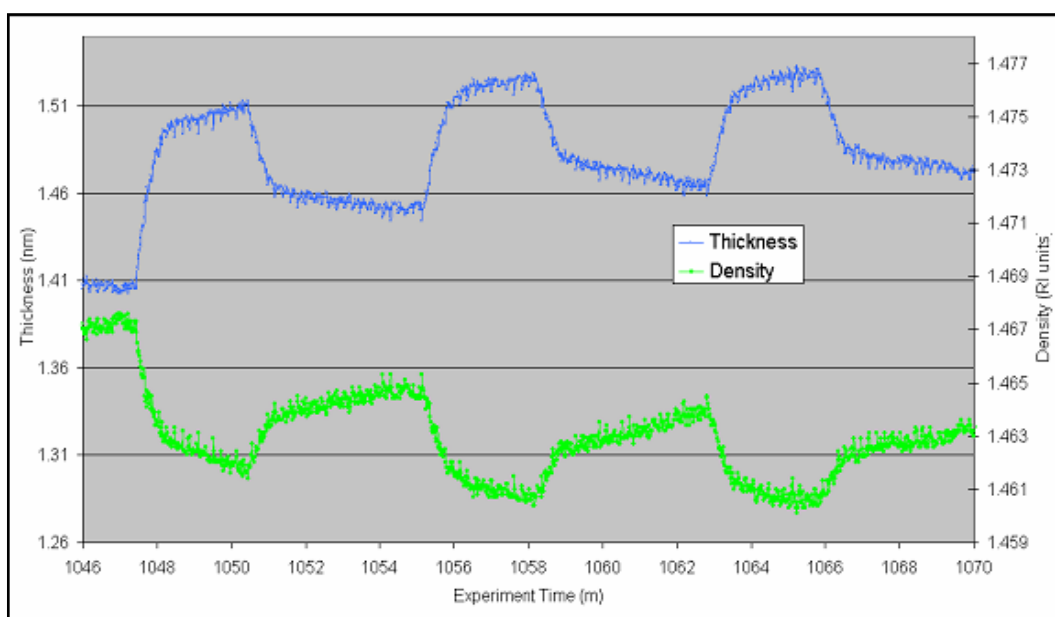


Figure 3: Structural changes to AT (thickness and density changes) on three sequential citrate additions

The increase in thickness and decrease in density observed in AT indicates that specific binding of citrate is 'opening up' the tertiary structure of AT, and allows the postulation that this structural change in AT is the mechanism behind the demonstrated aggregation protective effects of citrate. The DPI data obtained in these studies extends previous work^(2,3) by directly demonstrating, for the first time, the specific nature of binding of the citrate moiety to AT.

Conclusions and Benefits

DPI enables the detailed study of the intimate link between molecular structure and function for a diverse range of molecular systems. These experiments show DPI can bring a new level of understanding to the assessment of small molecules as binding candidates for proteins by giving information on both the **functional** binding events and the **structural** response to binding in the protein. In turn, this allows differentiation between specific and non-specific binding.

The **AnaLight**[®] instruments and their experimental protocols give the researcher a unique combination of high-resolution data in real time on thickness, density (refractive index) and mass from a bench top technique. The **AnaLight**[®] is an important enabling tool for drug discovery researchers, giving them the ability to:

- Rapidly and sensitively detect small molecules binding to large proteins
- Connect functional and structural events in a single set of high-content measurements, in real time
- Differentiate between specific and non-specific binding events
- Measure structural changes in proteins as a result of small molecule binding
- Understand the mechanism of action of known 'binders', and screen for similar modes of action amongst small molecule candidate libraries
- Avoid the ambiguities and long timescales that are inherent in other techniques for structural binding studies

Farfield gratefully acknowledges that these experiments were carried out in collaboration with Dr S.P. Bottomley and his team from the Department of Biochemistry and Molecular Biology, Monash University, Melbourne, Australia.

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

⁽¹⁾ M. Swann, L. Peel, S. Carrington & N. Freeman. *Anal. Biochem.* **329** (2004) 190-198

⁽²⁾ E. James & S. Bottomley. *Arch. Biochem. Biophys.* **356(2)** (1998) 296-300

⁽³⁾ S. Bottomley & D. Tew. *Biochim. Biophys. Acta* **1481** (2000) 11-17