

Measurement of Conformational Change in P38a/SAPK2a Kinase on Specific Binding of Small Molecules

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 conformational changes in proteins that are indicative of a response to **specific** binding. The high resolution of DPI allows the detection of small molecules binding to large, immobilized proteins. This means that DPI can be used to determine whether small molecule drug candidates change the conformation of a protein upon binding, opening new insights into **structurally informed drug discovery**, giving an enhanced level of information beyond that provided by kinetic parameters alone.

Protein kinases are critically responsible for almost all signal transduction pathways that control the cell life cycle. The enzymatic effects of this super-family of human proteins are marked by the function they play in facilitating the reversible phosphorylation of target proteins, using ATP or GTP as phosphate donor agonists.

The ubiquitous nature of protein kinases in life maintenance associates these enzymes with a variety of indications including inflammatory disorders, diabetes, and cancer. Consequently, kinases have long been considered targets in drug discovery, with 30% of all research spending in therapeutic development focussed on generating and screening libraries of specific small-drug inhibitor binders against myriad kinase proteins.

This application note demonstrates the advantages of DPI for interrogating immobilised P38a protein kinase, providing direct insight into protein function associated with specific and non-specific interactions. This is achieved via interpretation of conformational changes induced upon exposure to a known agonist, antagonist and non-binding inhibitor.

Experimental

DPI experiments were performed on a Farfield **AnaLight**[®] instrument. The surface used in all studies was an unmodified 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.

Immobilisation of P38a Kinase: Calibration of the **AnaChip**[™] was initially performed with an injection of 80% (w/w) ethanol/H₂O into a stream of running buffer (40mM HEPES, 150mM NaCl, 1mM DTT, pH7.2) at a flow rate of 30µl/min to each of the flow channels (**Farfield Technical Note 001**). P38a-kinase (33µg/ml), prepared in running buffer, was then injected into the buffer flow and allowed to physisorb to the working channel of the surface for 20 minutes at 10µl/min. (**Farfield Technical Note 008**)

Protein-Small Molecule Interactions: After stabilising the immobilised P38a-kinase with sufficient rinsing, the running buffer was modified to include 1mM MnCl₂ and 1% (v/v) DMSO. Subsequently, both the working channel and the unmodified reference channel were simultaneously subjected to repeat injections of ATP, SB203580 (a commercially available specific P38a-kinase inhibitor), and rottlerin (a protein kinase inhibitor with no specificity toward P38a), with their associated blank sample injections. All samples were prepared at concentrations greater than their respective K_D values, in HEPES running buffer containing 1mM MnCl₂ and 1% (v/v) DMSO.

Results and Discussion

Immobilisation of P38a-Kinase: **Table 1** shows the physical properties of P38a-kinase protein immobilised by physisorption to the unmodified silicon oxynitride **AnaChip**[™] surface. Protein size and molecular 'footprint' values are in close correlation with crystallographic dimensions of 69 Å and 2244 Å² respectively. This indicates that a non-constrained, and therefore active, conformation of P38a-kinase was maintained during the immobilisation.

	Density (RI Units)	Size (Å)	Mass (ngmm ⁻²)	Area per Molecule (Å ²)
P38a-kinase	1.426	55.1	2.72	2320

Table 1: Structural measurements of P38a-kinase after immobilisation by physisorption

Protein-Small Molecule Interactions: A mass signature alone cannot be used as a reliable indicator to quantify protein-small molecule interactions when considering the binding of low molecular weight drug entities, as the mass change signal becomes diminishingly small as they interact with much larger proteins. The capabilities of the **AnaLight**[®] instrument series allow for unique levels of sensitivity of small molecule detection, by providing direct insight into protein function associated with specific binding through conformational change coupled with density variation.

Whilst looking for the diminutive signals associated with small molecules binding to large proteins, the effects of changes in the buffer or bulk refractive index need to be accounted for if the measurement is to be quantified. Generally speaking, because the **AnaLight**[®] measures in a thin film format, it is adequate to simply subtract the reference channel signals from their DMSO-containing counterparts on the working channel. **Figure 1** shows experimental data from which the DMSO-containing blank data has been subtracted.

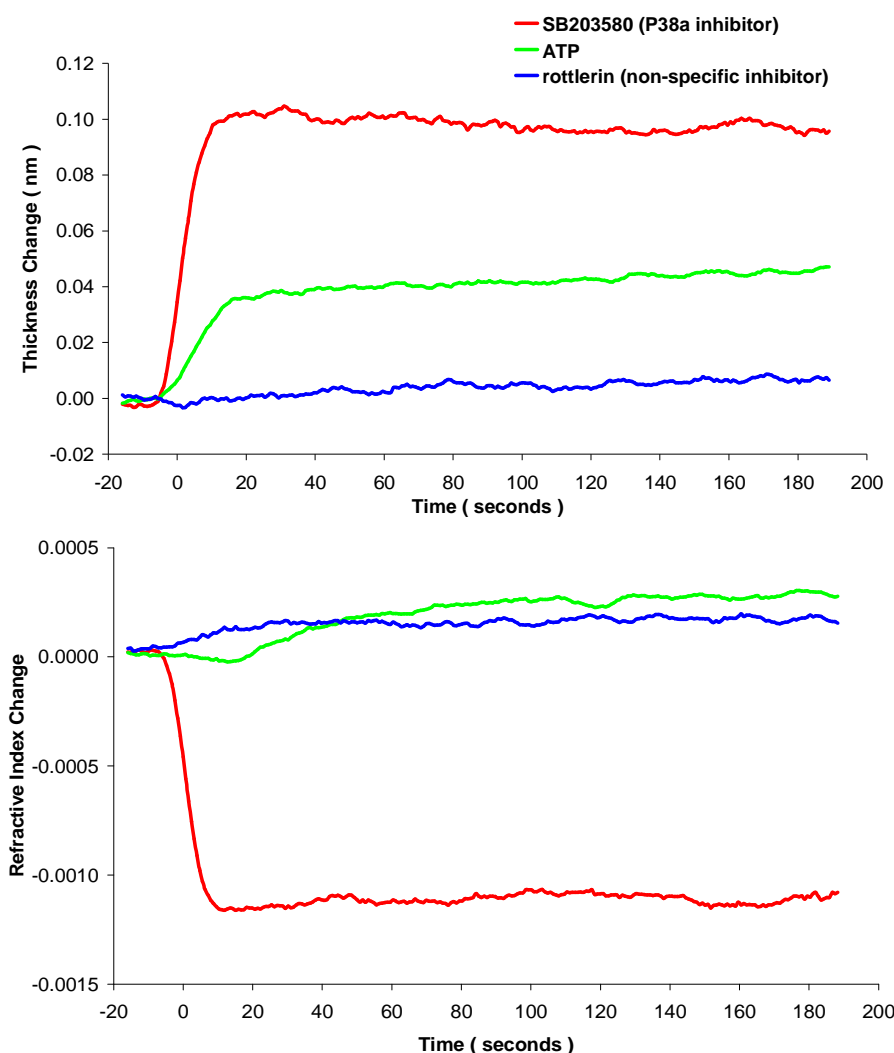


Figure 1: Real time density (RI units) and dimension profiles indicating structural changes of P38a-kinase upon exposure to a range of small molecules. Profiles are the result of subtracting blank (containing DMSO) injections from small molecule injection data

Application Note 033

Inversely proportional changes in size and density of the protein, as measured by DPI, indicate that a conformational change has occurred in the protein resulting from a truly **specific** interaction with a small molecule, in order to maximize binding and cause a functional response. This is clearly demonstrated in the profile for drug SB203580 (red line in **Figure 1**), which is a known specific inhibitor of P38a-kinase. As measured size increases, the density decreases. A schematic representation of this type of specific interaction is shown in **Figure 2**.

Conversely, **non-specific** binding is typically manifested in a DPI profile that is density neutral, but shows an increase in measured size. This is indicated by the profile of the known non-specific inhibitor rottlerin, which causes a near neutral density change and very gradual increase in size (blue line in **Figure 1**). ATP only binds to the active form of P38a-kinase. In the application presented here, an inactive form of the kinase was immobilised. A non-specific interaction therefore occurs upon introduction of ATP to the inactive P38a-kinase. An increased size and near-neutral density change is seen for ATP (green line in **Figure 1**) indicating general adsorption of ATP onto the protein as would be expected from non-specific interactions. This effect is more pronounced for ATP than for rottlerin in this example.

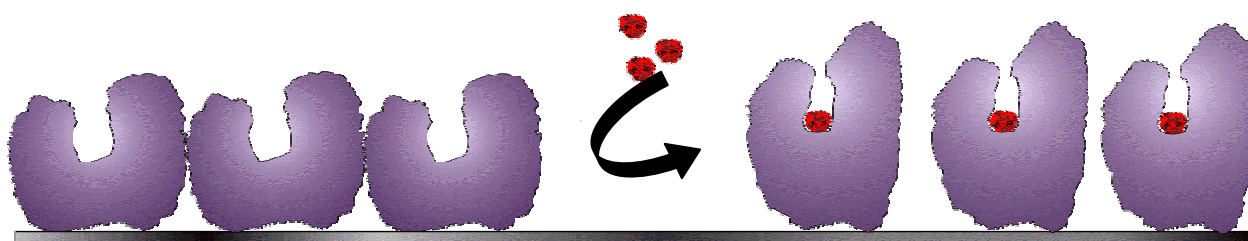


Figure 2: Visual schematic of protein conformational tightening associated with specific small molecule binding, leading to a dimension increase and corresponding density decrease

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 how DPI can bring a new level of understanding to the assessment of small molecules as binding candidates for proteins. DPI gives 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 on measured size, density (refractive index) and mass in real time 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
- Differentiate between specific and non-specific binding events
- Connect functional and structural events in a single set of high-information content measurements, in real time
- Measure structural changes in proteins as a result of small molecule binding, moving the basis for selection of drug candidate molecules beyond simple assessment of binding affinities
- Understand the mechanism of action of known 'binders', and screen for similar modes of action amongst small molecule candidate libraries

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