

Measurement of Conformational Change in MAP Kinase on Small Molecule Binding

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 sub-atomic resolution of DPI allows the detection of small molecules binding to large, immobilised proteins. This means that DPI can be used to determine whether small molecule drug candidates change the conformation or structure of a protein upon binding, opening new insights into **structurally informed drug discovery**, giving a 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 is palpable 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. It comes as no surprise therefore, that 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 mitogen-activated protein kinase (MAP kinase), by providing direct insights into protein function associated with specific and non-specific interactions through conformational changes induced upon exposure to known agonists, antagonists, and non-binding inhibitors.

Experimental

The 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 MAP Kinase: The **AnaChip**[™] was calibrated by injecting an 80% (w/w) ethanol/water solution into a stream of HEPES running buffer (40mM, 150mM NaCl, 1mM DTT, pH7.2) at a flow rate of 100µl/min. MAP kinase (690µg/mL) prepared in running buffer was then injected into the buffer flow and allowed to physisorb to the working channel of the **AnaChip**[™] surface for 30 minutes at 10µL/min.

Protein-Small Molecule Interactions: After stabilising the immobilised MAP kinase with sufficient rinsing with HEPES running buffer, both the working channel and second, unmodified reference channel were simultaneously subjected to repeat injections of AMP-PNP (ATP analogue), *Drug X* (a commercially-sensitive specific MAP kinase inhibitor), *Drug Y* (a commercially-sensitive non-specific MAP kinase inhibitor), and nicotinamide (non-binder). All were prepared at concentrations greater than their respective K_D values, in HEPES running buffer.

Results and Discussion

Immobilisation of MAP Kinase: The physical properties of MAP kinase protein immobilised by physisorption to the unmodified silicon oxynitride **AnaChip**[™] surface are presented in **Figure 1**. Thickness and molecular 'footprint' values are in close correlation with crystallographic dimensions of 69Å and 2244Å² respectively, suggesting a non-constrained conformation of MAP kinase post immobilisation.

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 sufficiently low molecular weight drug entities, as the signal becomes diminishingly low with their interactions with much larger proteins. The capabilities of the **AnaLight**[®] instrument series allows for unique levels of sensitivity to small molecule detection, by providing direct

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insight into protein function associated with specific binding through conformational change coupled with density variation.

Layer	Density (RI Units)	Thickness (Å)	Mass (ngmm ⁻²)	Area per Molecule (Å ²)
MAP kinase	1.429	57.9	2.97	2236

Figure 1: Structural properties of MAP kinase upon physisorption to a silicon oxynitride *AnaChip*TM surface

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 counterparts on the working channel. **Figure 2** therefore shows the difference between the two channels, and demonstrates the structural changes that MAP kinase undergoes when subjected to a series of small molecule binding candidates.

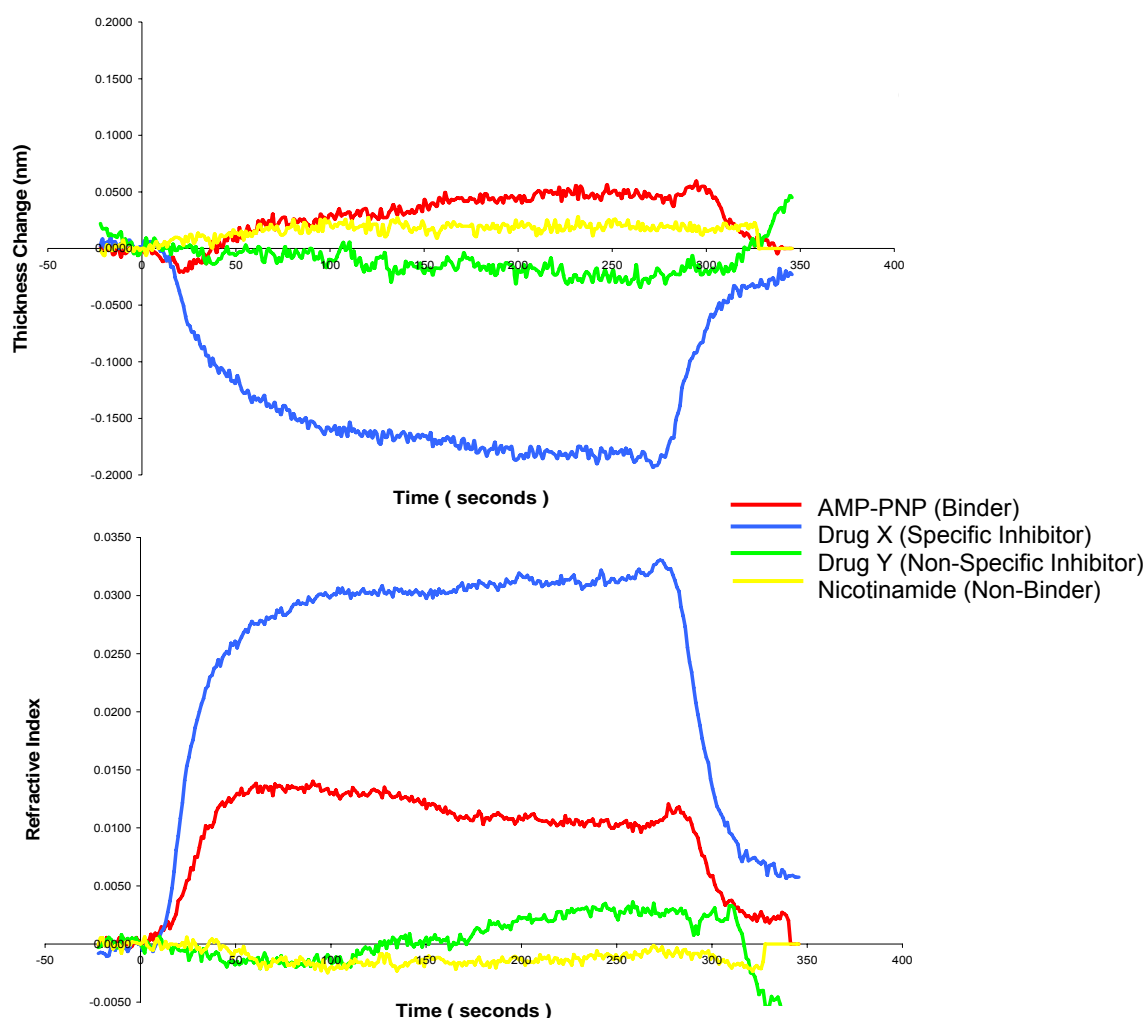


Figure 2: Real time density (RI units) and thickness profiles indicating structural changes of MAP kinase upon exposure to a range of small molecules. Profiles are the result of subtracting reference channel data from working channel data

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The specific interaction between protein and small molecule will generally elicit a conformational tightening to maximise binding potential, resulting in a thickness reduction and density increase of the protein. This is epitomised in the profile for *Drug X* (blue line in **Figure 2**), which has known MAP kinase inhibition specificity. A schematic representation of this type of specific interaction is shown in **Figure 3**. Inhibitor *Drug Y* (green line in **Figure 2**) shows a similar trend although to a much lesser extent, characteristic of its reduced specificity toward MAP kinase at the same concentration.

Conversely, non-specific binding is typically manifested in a DPI profile showing neutral density change and an increase in thickness. This is evident in the profile of the known 'non-binder' nicotinamide (yellow line in **Figure 2**). AMP-PNP, an ATP analogue, only binds to the active form of MAP kinase. In the application presented here, an inactive form of the kinase was immobilised. A non-specific interaction therefore occurs upon introduction of AMP-PNP to the inactive MAP kinase. An increase in both thickness and density is seen for AMP-PNP (red line in **Figure 2**) indicating general adsorption of AMP-PNP on top and around the protein as would be expected from non-specific interactions.

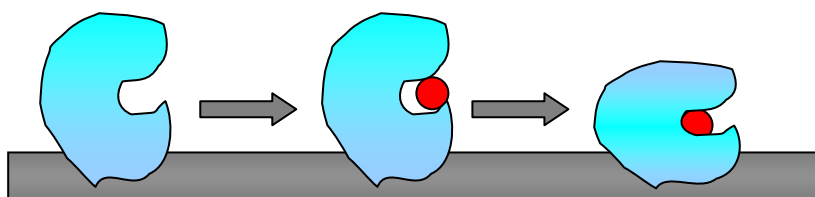


Figure 3: Visual schematic of protein conformational tightening associated with specific small molecule binding, leading to a thickness decrease and corresponding density increase

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 and differentiate between specific and non-specific binding events
- Connect functional and structural events in a single set of high-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 measurement of binding affinities
- Understand the mechanism of action of known 'binders', and screen for similar modes of action amongst small molecule candidate libraries

Farfield gratefully acknowledges that these experiments were carried out using samples provided by AstraZeneca GSI, Alderley Park, Macclesfield, Cheshire, United Kingdom.

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