

## Exploring Crystallisation of p38 Kinase Using DPI

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### Introduction

The use of X-ray crystallography to derive three-dimensional structures for target proteins in drug design is a common activity in drug discovery today. However, the growth of diffraction-quality protein crystals is a major bottleneck as proteins are not easily amenable to crystallisation, owing primarily to the many parameters that influence this process. Furthermore, to-date, no generic rules that can be applied to protein crystallisation exist.

This application note describes the use of Dual Polarisation Interferometry (DPI) <sup>(1)</sup> to monitor the crystallisation behaviour of the protein MAP kinase p38 in the presence and absence of an inhibitor, and under different conditions which lead to a range of different outcomes.

Kinase activity is of tremendous interest in disease areas including cancer, inflammation, and obesity. Drugs which bind and interact with kinases are therefore of great interest to the drug discovery community. In this respect, the three-dimensional structure of a protein complexed with an inhibitor plays a crucial role in elucidating the binding mode of the inhibitor and the molecular mechanisms underlying the inhibition of protein activity <sup>(2)</sup>.

### Experimental

The DPI experiments were carried out on a Farfield **AnaLight**<sup>®</sup> instrument using an unmodified **AnaChip**<sup>™</sup> (*part no 2007-110c*). Only one instrument channel was used, and three different areas of this channel were monitored simultaneously. The temperature during the experiments was maintained at 20 °C. Reagents were of analytical grade or higher and water was high purity.

Kinase p38 protein in buffer, precipitant and a known p38 inhibitor in buffer were supplied to us in solution form by our pharmaceutical company research partners. The precipitant consisted of 14% (w/v) PEG 1500 in sodium cacodylate (100mM).

Four different experiments were performed using different combinations of the above reagents at pH6 and pH7. The experiments were performed in parallel, with a portion of each sample mixture being left to crystallise in a control sample tube. The different experimental combinations are detailed in **Table 1**.

Experiment	1	2	3	4
Conditions	p38 + precipitant at pH 6	p38 + inhibitor + precipitant at pH 6	p38 + precipitant at pH 7	p38 + inhibitor + precipitant at pH 7

**Table 1: Experimental Matrix for p38 Crystallisation Studies**

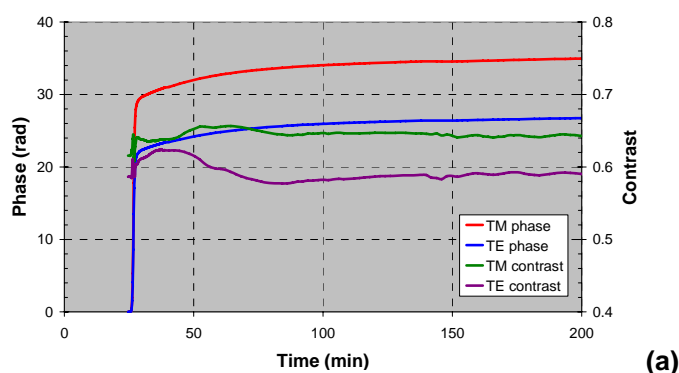
In all experiments, the precipitant was mixed with the protein solution in a 1:1 (v/v) ratio a few minutes before the final mixture was flowed over the **AnaChip**<sup>™</sup> surface. In cases where the inhibitor was used, this was added to the protein solution before the precipitant. The final solutions were gently stirred by rocking the container (to avoid the formation of foam) before being run over the **AnaChip**<sup>™</sup> surface at a flow rate of 50 µl/min. The flow was stopped when more than half of the sample had reached the **AnaChip**<sup>™</sup> surface. This experimental approach was designed to be as similar as feasible to the batch mode crystallisation technique.

Additionally, the surface of the **AnaChip**<sup>™</sup> was monitored with a microscope mounted above the flow cell and operating in polarising reflection mode. Only light caused by birefringence was reflected back to the CCD camera attached to the microscope.

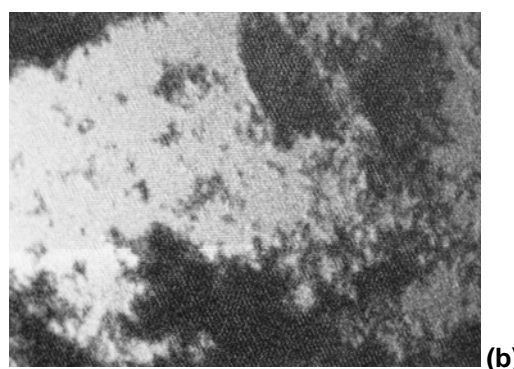
## Results and Discussion

### Experiment 1: p38 + precipitant at pH6

The evolution of the phase and contrast signals (for both TE and TM polarisations) is depicted in **Figure 1a**. Initially, the phase increased rapidly as a result of the refractive index (RI) of the bulk solution and the adsorption of a protein monolayer to the surface of the **AnaChip™**. The phase data then reached steady state and remained unchanged for the remainder of the experiment (more than 20 hours). The contrast also remained unchanged. Examination of the surface of the chip, (**Figure 1b**), showed the formation of white precipitates and no crystals.



**Figure 1a: Phase and contrast changes during first 200 minutes of Experiment 1**

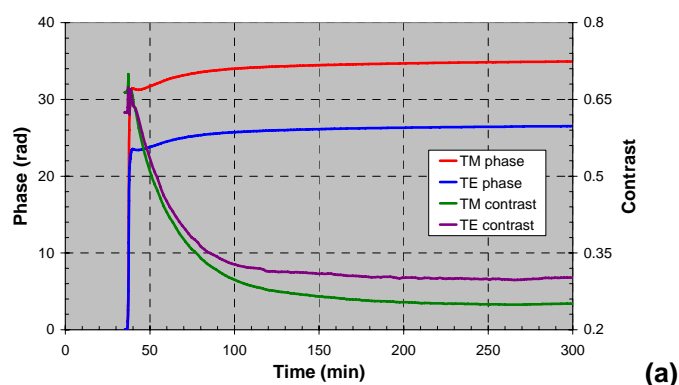


**Figure 1b: High-resolution image of *AnaChip™* surface showing p38 precipitation at end of Experiment 1**

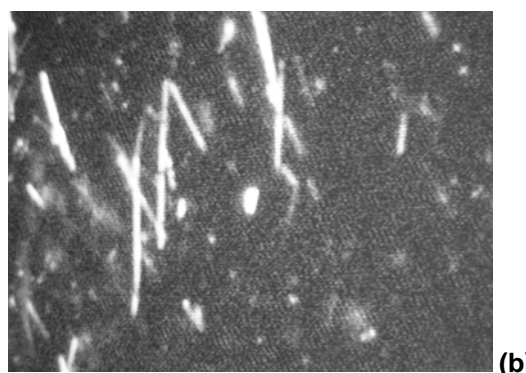
### Experiment 2: p38 + inhibitor + precipitant at pH6

As shown in **Figure 2a**, the phase is characterised by three distinct stages: an initial rise caused by the bulk RI and the adsorption of a thin protein monolayer, followed by a small drop for a short period of time (most likely caused by removal of material from the surface), then a slow increase to reach a plateau. However, the contrast started dropping rapidly after the flow was stopped. Contrast reached a minimum (<40% of the starting value) after approximately three hours. Subsequently, it remained almost unchanged for more than 68 hours.

High resolution microscopy analysis showed the formation of needle-like crystals mixed with white precipitates (**Figure 2b**).



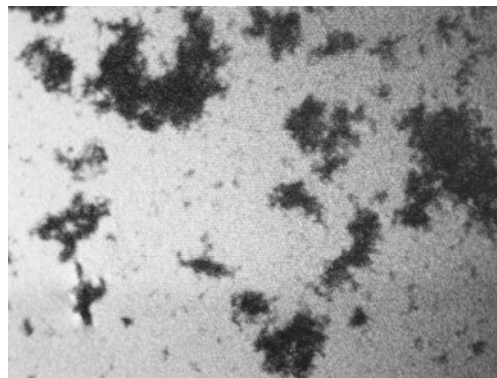
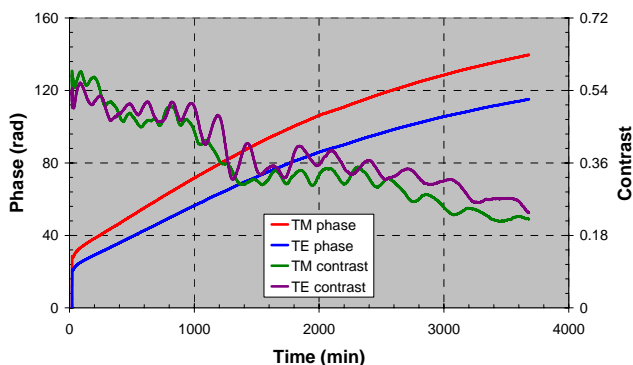
**Figure 2a: Phase and contrast changes during first 300 minutes of Experiment 2**



**Figure 2b: High-resolution image of *AnaChip™* surface showing needle-like p38 crystals at end of Experiment 2**

**Experiment 3: p38 + precipitant at pH7**

**Figure 3a** illustrates the phase and contrast signals for this experiment. The phase shows a continuous and monotonic increase for an incubation period of more than 60 hours. This is indicative of a steady increase of the build up of protein on the **AnaChip™** surface. This was accompanied by a slow decrease of the contrast. The inspection of the surface reveals the formation of islands of birefringent material (**Figure 3b**) which is interpreted as protein precipitates of semi-crystalline form.



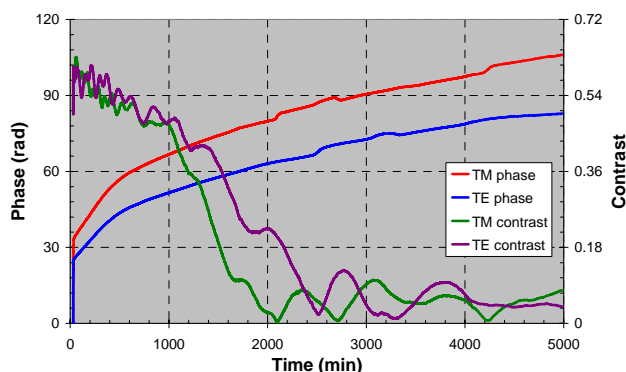
**Figure 3a: Phase and contrast changes during Experiment 3**

**Figure 3b: High-resolution image of AnaChip™ surface showing birefringent material, probably a semi-crystalline precipitate of p38, at end of Experiment 3**

**Experiment 4: p38 + inhibitor + precipitant at pH7**

**Figure 4** shows the changes in phase and contrast as the experiment progressed over a period of more than 86 hours. The evolution of these parameters for the first 1000 minutes is similar to Experiment 3 (no inhibitor present, **Figure 3a**). Subsequently, the contrast dropped off sharply and ultimately the fringes completely disappeared. However, the phase continued increasing, albeit very slowly.

The investigation of the surface with a microscope showed the growth of a single, large, needle-like crystal with a few small ones branching out in addition to birefringent precipitates (image not available).



**Figure 4: Phase and contrast changes during Experiment 4**

These experiments give an excellent indication of the effects of changing the pH and the presence or absence of an inhibitor on p38 kinase crystallisation. To summarise:

1. At pH 6, the presence of the inhibitor (Experiment 2) resulted in the formation of a shower of crystals compared with a precipitate in the absence of the inhibitor (Experiment 1). One explanation for this is that the bound inhibitor causes a conformational change in the protein, which subsequently facilitates crystal formation.
2. In the absence of the inhibitor, the increase from pH6 (Experiment 1) to pH7 (Experiment 3) appears to bring the protein closer to crystallisation.

- In the presence of the inhibitor, the increase of the pH6 (Experiment 2) to pH7 (Experiments 4) led to the formation of a large crystal compared to a shower of crystals. This was accompanied by a slowing of the drop in the contrast measurement.
- A complete or partial decrease in the contrast measurement is observed only when crystals, or a mixture of crystals and precipitates) are deposited on the **AnaChip™** surface. This effect is the result of the loss (or decrease) of the intensity of the light propagating along the measurement waveguide within the **AnaChip™**. These losses are caused by a sharp (and well-defined) change in the RI at the locations where the crystalline matter is in close contact with the surface of the waveguide, as illustrated in **Figure 5**.
- Both TE and TM phase signals changed differently for the four experiments, especially during the initial stages of the experiments. Taking into account the fact that the phase is very sensitive to the way proteins behave at the surface, this suggests that the phase measurements could be used as a very early-stage indicator as to the possible outcome of the experiment in advance of the contrast loss measurements.

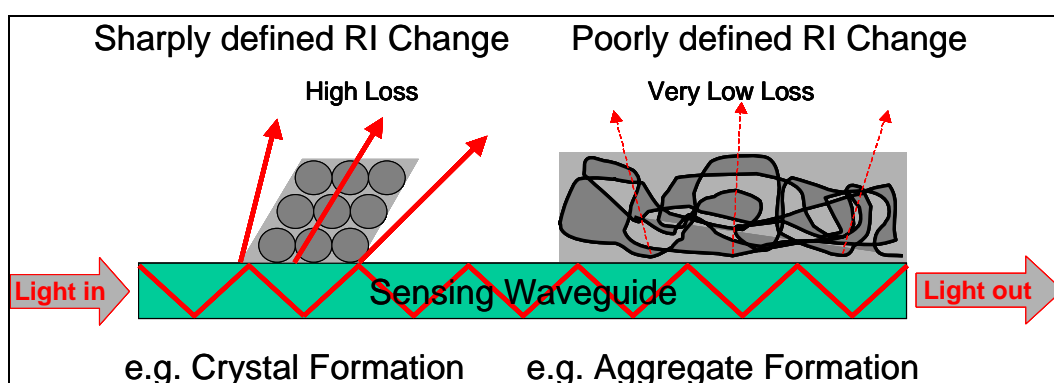


Figure 5: Effects of crystalline and non-crystalline material on light propagation within the measurement waveguide of an **AnaChip™**

## Conclusions and Benefits

The **AnaLight®** instrument and experimental protocols give the researcher a unique combination of high-resolution data in real time on the phase and contrast of the interference fringes generated by the DPI technique. These experiments demonstrate the capability of the DPI technique in the field of protein crystallisation. In particular, it was shown that DPI can play an important role in:

- Detecting the formation of protein crystals 10-100 times faster than using polarised microscopy (see also [Farfield Application Note 037 Detection of Early-Stage Protein Crystallisation using DPI](#))
- Distinguishing easily between protein crystallisation and other macromolecular physical processes, such as aggregation and precipitation
- Rapidly exploring the combinatorial conditions that favour and promote protein crystal formation
- Optimising the conditions that lead to the growth of high-quality crystals for X-Ray diffraction analysis
- Investigating of the effects of specific small molecule binders and inhibitors on the crystallisation of proteins

## References

- (1) Cross et. al. *Biosens. Bioelectron.* **19** 383-390 (2003)
- (2) Pargellis et. al. *Nature Struct. Biol.* **9** 268-272 (2002)

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**Note: The use of Dual Polarisation Interferometry for the detection of early-stage protein crystallisation is the subject of Patent Application PCT/GB02/02185**