

Quantifying the Effects of Dimethyl Sulphoxide (DMSO) on Protein Structure using DPI

Introduction

Dual Polarisation Interferometry (DPI) offers a truly quantitative analytical technique, rather than a simple 'mass sensor' response, providing absolute density measurements and structural dimensions of immobilised proteins at high resolution⁽¹⁾.

The use of combinatorial and parallel chemistry has made high-throughput screening the major tool for discovery of pharmaceutically active compounds. Large collections of low molecular weight organic molecules (< 500 Da) represent a primary source of discovery chemistry for bioscreening. Dimethyl sulphoxide (DMSO) is an important solvent for small molecule studies as it provides a nearly universal approach for the solubilisation of small molecules. Because of its physicochemical properties, high solvent power, low chemical reactivity and relatively low toxicity, DMSO has become the solvent of choice for sample storage and handling in the pharmaceutical industry, particularly in the initial stages of high-throughput screening where storage of candidate molecules in DMSO is routine.

The effects of DMSO on protein structure and function are extremely varied. When used as a co-solvent at high concentration DMSO has great potential to give misleading results in bioavailability measurements, by acting as a permeation enhancer, a denaturant, or even an inhibitor⁽²⁾. Additionally, DMSO has a much higher refractive index (RI) than common buffer solutions, which can make it difficult to produce reliable readings from DMSO-containing samples using angular-based optical biosensor technology such as SPR. The combination of density changes and DMSO effects on the protein has made experimental design very arduous and interpretation of results rather complex. This is compounded by the limited dynamic range first-generation biosensors have for high RI solvents such as DMSO, making some experiments simply unworkable.

In the pharmaceutical industry, knowledge of the three-dimensional structure of a specific protein target facilitates the **structurally informed drug-discovery** process. This application note explores the effects that DMSO as a co-solvent has on lysozyme structure through conformational changes induced upon exposure to a concentration gradient of aqueous DMSO solutions. This work also demonstrates the wide dynamic range of DPI and its capability for analysis even when using high RI buffer solutions, without the need for complicated pre-calibration procedures.

Experimental

DPI experiments were performed on a Farfield **AnaLight**[®] instrument. This method assumes that the **AnaLight**[®] fluidic system has been cleaned according to the recommended procedures (**Farfield Technical Note 002**) and that the injection needle and syringes are free of contaminants before use. The surface used in these studies was an amine functionalised silicon oxynitride **AnaChip**[™] activated with bis(sulphosuccinimidyl)suberate (BS³). 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.

Calibration: The amine-functionalised **AnaChip**[™] was calibrated using 80% ethanol and water (**Farfield Technical Note 001**). DMSO was prepared as a co-solvent in PBS (10mM phosphate, 150mM NaCl, pH7.4) at 1%, 2%, 5%, 10% and 20% (v/v) and then flowed over the untreated **AnaChip**[™] at 50µl/min. As a control, similar concentrations of H₂O in PBS were injected over the **AnaChip**[™] surface at the same flow rate.

Immobilisation of Lysozyme: After activating the surface with BS³ linker (3mg/ml in PBS), lysozyme (2mg/ml) prepared in PBS was introduced into the buffer flow and allowed to covalently couple to the **AnaChip**[™], via NHS-ester reactive groups and primary amines on the protein, for 6 minutes at 30µl/min. To block any unreacted linker or surface groups, an injection of ethanolamine (0.1M in PBS) was passed over the **AnaChip**[™] (**Farfield Technical Note 003**).

DMSO–Lysozyme Interactions: After stabilising the immobilised lysozyme with sufficient rinsing, the protein layer was exposed alternately to PBS and increasing concentrations of DMSO (1%, 2%, 5%, 10% and 20% (v/v)) in PBS for 4 minutes per cycle at a flow rate of 50µl/min. A similar experiment was performed in which H₂O was substituted for DMSO in PBS at the same concentrations.

Results and Discussion

Immobilisation of Lysozyme: Table 1 shows the physical properties of lysozyme covalently immobilised to an amine functionalised **AnaChip™** surface prior to DMSO treatment. Protein size and molecular ‘footprint’ values are in close correlation with crystallographic dimensions of 45Å (long axis) and 30Å (short axis) and 900Å² respectively, suggesting a non-constrained conformation of lysozyme post-immobilisation.

	Density (RI Units)	Size (Å)	Mass (ngmm ⁻²)	Area per Molecule (Å ²)
Lysozyme	1.457	40.2	2.692	882

Table 1: Structural properties of covalently coupled lysozyme protein, immobilised to an amine functionalised surface activated with BS³ NHS-ester reactive groups

DMSO-Lysozyme Interactions: Whilst looking for the diminutive signals associated with molecular reorientation, effects of differences in the buffer, particularly density, need to be accounted for if the measurement is to be quantified. This is simply and unambiguously achieved with the **AnaLight®** instrument by subjecting the untreated **AnaChip™** surface to a series of trial solutions before immobilisation of protein. Bulk density differences are corrected by subtracting values for initial injections of DMSO over the untreated **AnaChip™** from the corresponding immobilised lysozyme data. Conformational changes within the protein structure induced by exposure to the different solvent regimes may then be accurately measured. **Figure 1** shows concentration dependent effects of DMSO on lysozyme protein, and molecular reorientation upon returning to PBS.

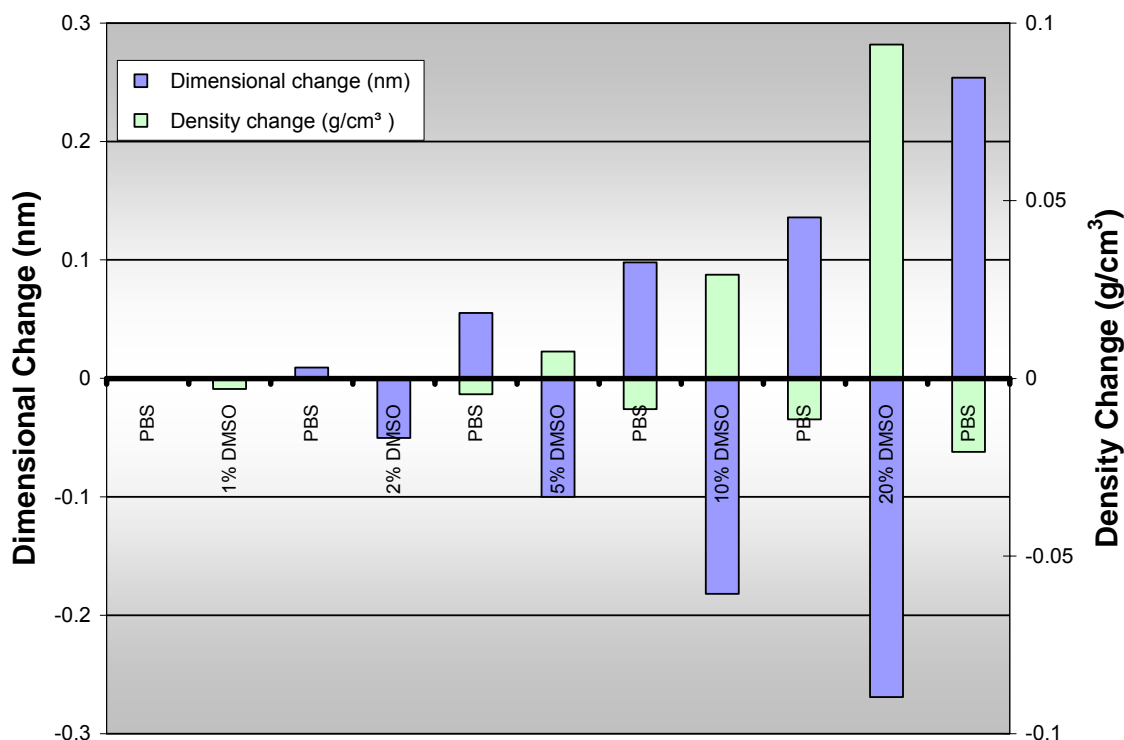


Figure 1: Dimensional (blue) and density (green) changes indicating conformational rearrangement of lysozyme during exposure to a range of DMSO concentrations and upon returning to PBS running buffer

Clear changes are measured in the lysozyme structure each time the solvent is switched. Measured size of the lysozyme molecule decreases when it is subjected to DMSO-containing buffer compared to PBS alone. Density increases correspondingly, particularly at higher concentrations of DMSO. In neat DMSO the structure of lysozyme is

known to approach a random coil with complete lack of tertiary structure ⁽³⁾, a process that appears to be initiated in the lysozyme well before 100% DMSO, arguably at concentrations used in this study. However, the exaggerated compression of the protein structure caused by higher DMSO concentrations eventually leads to a progressive and irreversible reordering of the lysozyme structure on returning to PBS, as shown by the increasing protein size from left to right in **Figure 1**. Data in **Figure 1** also demonstrates the ability of the **AnaLight**[®] to resolve structures in high RI solvents such as 20% DMSO in PBS (measured by DPI to have a RI of 1.3667). In fact, the upper limit for the RI of a solvent that may be successfully resolved in DPI experiments is approximately 1.50, a value higher than that achievable using other optical techniques such as SPR.

A parallel experiment was performed where water, rather than DMSO, was added to PBS in order to correct for salt concentration effects. Changes in pH and salt concentration can alter electrostatic interactions between charged amino acids in a protein structure. Increasing salt concentration (by reducing the concentration of H₂O in the sample) reduces the strength of ionic binding by providing competing ions for the charged residues, as pairing of salt ions with charged groups of the protein shields intra-molecular repulsion. Measured size of the lysozyme molecule **increases** when it is subjected to water-containing buffer compared to PBS alone. Initial structural dimensions are spontaneously resumed each time the protein is returned to PBS buffer (data not shown), suggesting that these structural processes are **completely reversible**.

This confirms that DMSO-containing buffer is having a structural effect on the protein that is distinct from salt concentration effects.

Conclusions and Benefits

AnaLight[®] DPI systems offer next-generation technology, providing valuable insights into the relationship between protein structure and function under different buffer regimes, eg. pH, salt concentration and organic buffer constituents. 'Biosensor' techniques such as SPR and QCM cannot reveal this level of quantitative structural information. This application note clearly demonstrates that DPI is a powerful technology with the sensitivity and dynamic range to give reliable data on protein structural changes even in high RI background solutions. DPI has also revealed that DMSO can cause irreversible conformational changes in target proteins. Whilst structural effects are seen in equivalent concentrations of water, changes cause by water, are completely reversible. It is likely that DMSO has an effect on the structural integrity, and therefore function, of a wide range of proteins. The implications of these structural changes in therapeutic target proteins should be carefully considered during high-throughput screening.

These experiments show DPI can bring a new level of understanding to the assessment of protein structure by giving real time information on immobilised protein dimensions, density, surface coverage and orientation. **AnaLight**[®] gives the researcher a unique combination of high-resolution data in real time on thickness, density and surface coverage from a bench top technique. **AnaLight**[®] is an important enabling tool for biophysicists, giving them the ability to:

- Connect functional and structural events in a single set of high-content measurements, in real time.
- Measure the structural changes in proteins that result from solvent effects.
- Qualify false positives in high-throughput screening that are caused by solvent effects
- Design experiments in which the solvent regime is ideal for the target protein rather than being limited by instrument capabilities

For further applications information contact: applications@farfield-group.com or
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⁽¹⁾ G.Cross, A Reeves, S Brand, J Popplewell, L Peel, M Swann & N Freeman. *Biosens. Bioelectron.* **19** (2003) 383-390

⁽²⁾ H Johannesson, V Denisov & B Halle. *Protein Science* **6** (1997) 1756-1763

⁽³⁾ T Knubovets, J Osterhout & A Kilanov. *Biotech. Bioeng.* **63** (1999) 242-248