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# The Characterisation of a Contaminant-free Support Film for MicroPIXE Analysis of Biological Samples

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**Abstract.** A 4 μm thick polypropylene film ($CH_2$) commonly used for sample mounting in XRF experiments appears to be free of the phosphorus and calcium contaminants that are present in the 2 μm Mylar ($C_{10}H_8O_4$) backing film currently used for mounting liquid and crystalline protein samples for microPIXE. After analysing the polypropylene using microPIXE it was found that it contained no contaminants above the minimum detectable limits. The additional thickness of the polypropylene does not significantly affect the PIXE spectra, while the fact that polypropylene contains no oxygen is a great advantage in determining the thickness and composition of the sample matrix from the RBS spectrum. Use of this backing is now allowing us to make accurate trace element measurements of phosphorus and calcium in biological samples of proteins, DNA, and phospho-lipids.

**Keywords:** MicroPIXE, Mylar, Polypropylene, XRF.

## INTRODUCTION

Unique identification of non-organic elements bound to macromolecules is a challenge in structural biology, and although mass spectrometry methods have progressed significantly in the last few years, an unambiguous assignment of endogenous elements is often problematic. These extra elements play critical roles in the structure and function of many biomolecules. MicroPIXE (particle induced X-ray emission) with 2-3 MeV protons on liquid and crystalline proteins has been used successfully in both identifying atoms and in measuring their stoichiometric ratios to an accuracy of better than ±20% on over 50 protein samples [1, 2]. Convenient calibration is achieved using the sulphur X-ray peak and the internal normalisation available from the number of sulphur atoms in the amino acids cysteine and methionione, found naturally in proteins. The number of atoms of element X per protein molecule, $N_{(X)}$ can then be determined in a straightforward manner using the relationship:

$$N_{(X)} = [C_X / C_s] \times [M_{(s)} / M_{(X)}] \times N_{(s)} \qquad (1)$$

$N_{(X)}$ = Number of atoms of X per protein molecule.
$C_X$ = PIXE measured concentration of element X.
$C_s$ = PIXE measured concentration of sulphur.
$[M_{(s)} / M_{(X)}]$ = Mass ratio of element X and sulphur.
$N_{(s)}$ = Number of sulphur atoms per protein molecule, already known from the primary amino acid sequence.

Samples containing DNA offer an alternative internal standard, as the phosphorus content is proportional to the length of the DNA strand. In DNA-protein complexes, this can be used to determine the number of DNA molecules per protein molecule [3], or in place of the sulphur standard as an internal normalisation.

A key advantage of the microPIXE method is the simultaneous detection of Rutherford backscattered protons (RBS) which allows a good determination of the sample thickness and matrix composition [4]. This is essential to correct accurately for X-ray self-absorption and hence in obtaining accurate elemental ratios. Fitting the RBS spectra is often complicated by problems in distinguishing the signal given by the protein from that of the organic support foil.

Liquid (0.2 μL) or crystal (minimum protein size 15 μm) samples are currently mounted on 2 μm thick polyester (commercially available as Mylar) backing film during analysis. The Mylar is known to be

contaminated with calcium and phosphorous: thus problems in determining elemental concentration arise if the biological sample contains either of these elements [5]. Calcium is often functionally important in biochemical processes and many proteins bind it, *e.g.* influenza virus neuraminidase [6]. A 4 μm thick polypropylene film ($CH_2$) (commercially available as Prolene) [7] commonly used for sample mounting in WDXRF (Wavelength Dispersive X-Ray Fluorescence) appears to be free of the phosphorus and calcium contaminants that are present in the 2 μm Mylar ($C_{10}H_8O_4$) backing film historically used in our microPIXE measurements. Experiments on a commercial WDXRF Bruker S4 Pioneer spectrometer revealed the absence of calcium and phosphorus in the Prolene film, and following these initial results, microPIXE analysis was used to check the Prolene film for these and any other contaminants, and to determine its suitability as a replacement for the Mylar film.

## METHODS

Our microPIXE analysis is normally carried out with 2.5 MeV protons focused to a diameter of 2 - 3 μm using the University of Surrey microbeam facility [8]. Characteristic X-rays are detected using an 80 $mm^2$ solid state Si(Li) detector (giving high energy resolution), and the detector is fitted with a 130μm Be filter.

During exposure to the proton beam, samples are held under vacuum on a thin organic film. The film is mounted on an aluminium holder (Figure 1) which is held in a 'ladder' capable of holding up to four samples, within the vacuum chamber. Both liquid and crystal samples can be mounted in this way.

First a coarse scan (150 μm x 150 μm up to 1.0 mm x 1.0 mm depending on the sample size) is collected over the protein sample by scanning the proton beam spatially in X and Y. A software window is placed round the X-ray peak in the spectrum associated with a particular element, and the counts are sorted into an X-Y grid to give individual elemental maps. Simultaneous detection of Rutherford backscattered

(RBS) protons allows the thickness and matrix composition of the sample to be accurately determined. Quantitative information is obtained by collecting spectra at three or four points on the sample and also on the backing film alone for 3-10 minutes each. These spectra are analysed using the program Dan32 [4] to fit the RBS spectra and provide an interface to GUPIX [9, 10] for processing the PIXE spectra. The number of atoms of each element of interest per protein molecule can then be computed from equation (1).

In order to avoid the generation of spurious signals from the material of the target chamber, the beam is stopped in a Faraday cup fabricated from spectroscopically pure graphite. Beam currents are in the range 100 – 300 pA.

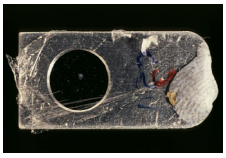

**Figure 1**: Aluminium sample holder with backing film stretched across face. A drop of dried liquid protein (0.2-0.3 μl) can be seen on the surface of the backing film.

The Prolene and Mylar were each mounted on the holders as described above. The RBS and X-ray spectra were collected and analysed for trace elements. The measurements were carried out at 2.5 MeV and the charge was 20 nC for the Mylar and 8 nC for the Prolene.

## RESULTS

In our tests, we were looking for any contaminants specifically phosphorus and calcium. The Prolene backing gave a remarkably clean spectrum, and there are no peaks seen above the LOD (limit of detection). Peaks were detected for impurities of both phosphorus and calcium in the Mylar film. Contamination layers were assumed to be thin and results were obtained as areal densities. The system was calibrated using the lead glass standard BCR-126A in the procedure described in [11]. Fitted RBS and X-ray spectra from points on both the Prolene and Mylar films are shown in Figures 2 and 3 below. The results of the quantitative analysis are displayed in Tables 1 and 2.

### An example of a protein - DNA complex: microPIXE analysis of the third KH domain of hnRNP K and single-stranded DNA.

hnRNP K is one of the major proteins found in hnRNP particles which are ribonucleoprotein complexes containing protein and pre-messenger RNA. hnRNP K contains hnRNP K homology (KH) domains which bind both Cytosine-Thymine rich single-stranded DNA (ssDNA) and Cytosine-Uracil rich ssRNA. Co-crystallization of the third KH domain of human hnRNP K with a 15-mer ssDNA gave both rod shaped and square plate crystals in the same crystallization drop. The square plates were similar in morphology to native crystals obtained previously.

Using X-ray diffraction data from the rod shaped crystals, initial molecular-replacement trials using the structure of the native protein alone as a search model failed to allow the structure of the putative protein-DNA complex to be solved. No heavy-atom derivatives could be obtained to enable phase

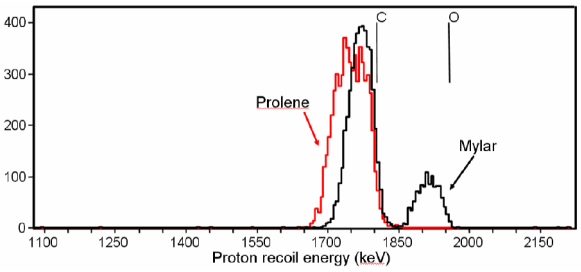


**Figure 2:** RBS Spectrum-4 µm Prolene (red) and 2 µm Mylar (black).

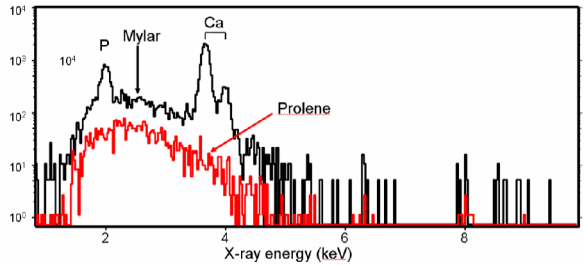


**Figure 3:** X Ray Spectrum-4 µm Prolene (red) and 2 µm Mylar (black).

**Table 1**: Areal density of calcium and phosphorus on 4 µm Prolene film (ng cm$^{-2}$).

| Prolene film 4 µm | | |
|---|---|---|
| **Element** | **Conc** | **LOD** |
| | | |
| P | nd | 117.2 |
| Ca | nd | 21.0 |

nd = not detected

**Table 2**: Areal density of calcium and phosphorus on 2 µm Mylar film (ng cm$^{-2}$).

| Mylar film 2 µm | | |
|---|---|---|
| **Element** | **Conc** | **LOD** |
| | | |
| P | 755.9 | 51.8 |
| Ca | 1424.2 | 19.0 |

P/Ca Mylar = 0.53 (std dev 0.04) w/w (0.66 P/Ca atoms/atoms) measured on a typical run.

determination. MicroPIXE experiments showed that these crystals contained large amounts of phosphorus (Figure 4).The phosphorus to sulphur atomic ratio was measured to be 4.4 and 4.7 in two different rod shaped crystals. This agreed well with the theoretical ratio of 4.67 corresponding to a stochiometry of three KH3 domains (protein molecules) per 15-mer ssDNA, taking into account the fact that the synthetic DNA has only 14 phosphates [3]. For the square plate crystal form (Figure 5), the microPIXE measurements gave a phosphorus to sulphur atomic ratio of 1.1 x $10^{-2}$ , showing that DNA was not bound.

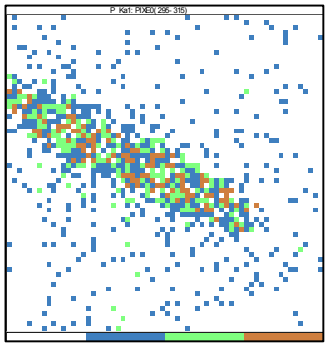

**Figure 4**: Phosphorus elemental map (150 µm x 150 µm). ssDNA binding crystal: DNA bound (100µm crystal). Result: 4.4 phosphorus/sulphur atom (1 methionine per protein molecule). 3 protein molecules per 15mer ssDNA

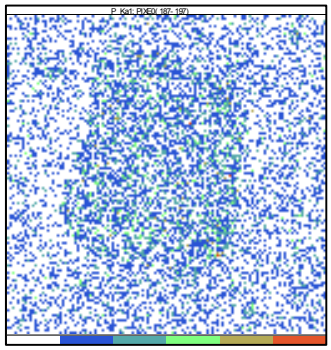

**Figure 5**: Phosphorus elemental map (250 µm x 250 µm). ssDNA crystal: DNA not bound: the faint crystal outline is due to an increase in the background bremsstrahlung when scanning over the crystal. Mounted on Mylar backing so phosphorus contaminant is visible in the elemental map.

Knowledge of the protein to DNA molecular ratio from the microPIXE measurements forced a re-examination of the X-ray data and of the self-rotation calculations. This added further evidence for a crystal asymmetric unit containing three KH3 domains per 15-mer of DNA in the rod shaped crystals. Subsequent molecular-replacement trials using just one copy of the

KH3 domain gave a cross-rotation function plot revealing three peaks, in good agreement with the self-rotation function. An electron density omit map was then calculated and gave clear difference density for the DNA, showing that there were two KH3 domains in contact with each DNA molecule and one KH3 domain positioned away from it (Figure 6). Thus the structure of the KH3-DNA complex was solved with the aid of microPIXE analysis [3].

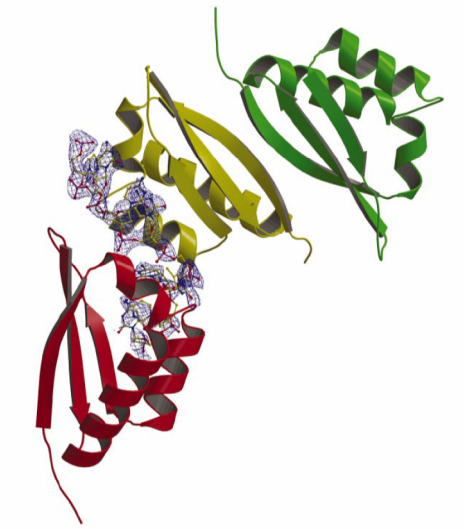

**Figure 6**: Ribbon representation of the three KH3 domains in the asymmetric unit (coloured red, yellow and green) and an omit map showing the electron density around the single-stranded DNA drawn at the 1σ level. Only two of the KH3 domains (red, yellow) interact with the DNA. The third KH3 domain (green) interacts with the second (yellow) *via* the strand $\beta$1 edges of their respective $\beta$-sheets, an interaction also seen in crystals of the uncomplexed protein [3].

## CONCLUSIONS

A 4μm thick Prolene film [7] ($CH_2$) routinely used for sample mounting in WDXRF experiments appears to be free of the phosphorus and calcium contaminants that are present in the 2 μm Mylar ($C_{10}H_8O_4$) backing film we currently use in microPIXE analysis of protein samples. A further compelling advantage of the Prolene is that unlike other easily available polymer films, it contains no oxygen, making RBS spectra interpretation much more straightforward. At present RBS spectra are fitted using a two layer model in which the oxygen in the Mylar film overlaps with the oxygen in the biological sample, and this gives uncertainty in assigning a thickness to the sample. This factor is critical since it affects the accuracy of the X-ray self-absorption correction. A further advantage RBS spectra from Prolene mounted samples are less complex to analyse, which improves the accuracy of the results and also allows the use of thicker and thus more mechanically robust films (4 μm rather than 2μm), greatly facilitating sample handling. We have not experienced any sample loss due to mechanical damage during the beam irradiations we have carried out thus far. MicroPIXE analysis of the Prolene showed that it contains no contaminants above the minimum detectable limit. From Table 1 it can be seen that the phosphorus concentration in the Prolene is at least an order of magnitude less than that present in the Mylar, and is not at a level which affects protein or DNA trace element analysis. This film is now allowing us to perform accurate analysis of the calcium and phosphorus in biological samples of proteins, DNA, and phospho-lipids for the first time.

## ACKNOWLEDGMENTS

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