A Prototype System for Dynamically Polarized Neutron Protein Crystallography

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Abstract

The sensitivity of Neutron Macromolecular Crystallography to the presence of hydrogen makes it a powerful tool to complement X-ray crystallographic studies using protein crystals. The power of this technique is currently limited by the relative low neutron flux provided by even the most powerful neu-

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tron sources. The strong polarization dependence of the neutron scattering cross section of hydrogen will allow us to use Dynamic Nuclear Polarization to dramatically improve the signal to noise ratio of neutron diffraction data, delivering order of magnitude gains in performance, and enabling measurements of radically smaller crystals of larger protein systems than are possible today. We present a prototype frozen spin system, built at Oak Ridge National Laboratory to polarize single protein crystals on the IMAGINE beamline at the High Flux Isotope Reactor (HFIR). Details of the design and construction will be described, as will the performance of the system offline and during preliminary tests at HFIR.
Keywords: polarized target, dynamic nuclear polarization, protein crystallography, neutron scattering

1. Introduction and Motivation

Macromolecular crystallography is a powerful tool to measure the structure of proteins. This is usually done by measuring the diffraction from a protein crystal in a high intensity x-ray beam. The incredibly high flux of these beams has resulted in the determination of tens of thousands of protein structures. Unfortunately, since x-rays scatter primarily from the high Z atoms in a sample, these structures often lack direct information about the location of the lighter elements, especially hydrogen. This remains true even when the diffraction data extend to atomic resolution. Neutron Macromolecular Crystallography (NMC) presents a potential solution to this problem. The relatively high cross section for neutron scattering from hydrogen enables neutron diffraction to determine the hydrogen positions in proteins even at

medium resolutions (2.0Å), information that is otherwise unattainable [1][2]. The greatest hurdle to the wide spread utilization of this technique is the relatively low neutron flux available at even the most intense neutron sources in the world [3][4]. This flux limitation requires data sets to be taken over longer time, typically days or weeks, and more critically, using crystals that are several orders of magnitude larger than those required at an x-ray facility. This crystal size limitation ends up the limiting factor for the technique, requiring researchers to spend considerable amounts of effort to grow large enough crystals, often without success. The spin dependence of the neutron-hydrogen cross section presents a possible resolution to this problem [5].

The cross section for neutron scattering can be split into coherent and incoherent parts. For a crystal sample, the coherent scattering contains information about the positions of atoms in the crystal. Incoherent scattering enters as a background. Both the coherent and incoherent cross sections display a large spin dependence, especially in the case of polarized neutrons scattering from polarized hydrogen. The coherent cross section is given by equation 1 and the incoherent is given by equation 2, where b_0 is the spin independent part of the scattering amplitude, b is the spin dependent part of the scattering amplitude, p is the neutron polarization, P is the nuclear polarization, and I is the spin of the nucleus [6]. A plot of the spin dependence of the cross section is shown in Figure 1. The flux (or crystal size) needed for a particular measurement, is reduced by the square of any increase in the signal to noise ratio. This can result in order or magnitude or greater reductions in crystal size without the need for deuteration.

$$\left(\frac{d\sigma}{d\Omega}\right)_{coh} = b_0^2 + 2b_0 bIpP + b^2 I^2 P^2 \quad (1)$$

$$\left(\frac{d\sigma}{d\Omega}\right)_{inc} = \frac{b^2}{4}[I(I+1) - pPI - P^2 I^2] \quad (2)$$

The relatively low magnetic moment of the hydrogen nucleus means that even for a high magnetic field (5 T) and low temperature (1 K), the nuclear polarization for hydrogen is low (about 0.5%). Dynamic Nuclear Polarization (DNP) [7] uses a combination of high field, low temperature and microwave irradiation to produce very high (potentially 90% or higher) nuclear polarization in samples which have been prepared by the addition of unpaired electrons. This technique has been applied previously to Small Angle Neutron Scattering (SANS) measurements [8][9]. Tests with polarized single crystals of small molecule systems have also been conducted [10]. In order to test the effectiveness of DNP in improving NMC measurements of protein crystals, a prototype system was built to polarize these crystals, and measurements were made using a beam of polarized neutrons. This prototype will be described below.

2. DNP Apparatus

2.1. Requirements

The DNP system used for NMC measurements shares aspects from the more commonly seen systems used in medium energy nuclear physics [11][12][13] or NMR [14]. In order to not block the acceptance required for diffraction measurements, the system has to run in frozen spin mode. The refrigerator, magnet, microwave, and NMR systems will be described in the following

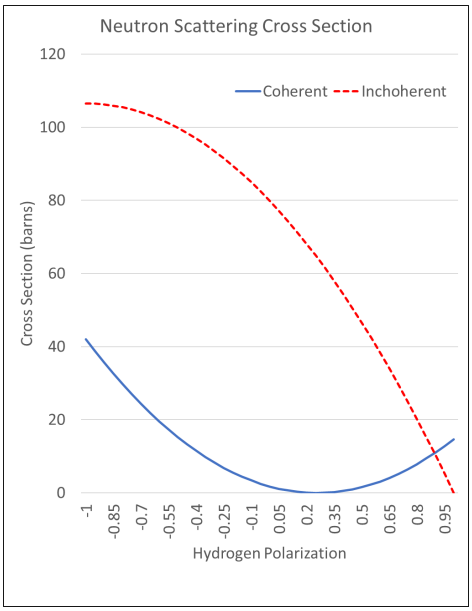

Figure 1: The coherent and incoherent cross sections for polarized neutrons scattering from a hydrogen nucleus, as a function of the nuclear polarization, as calculated in [6].

sections, as will the custom sample interface which was required for compatibility with the protein crystal samples to be polarized.

2.2. Refrigertator

The refrigerator used was a commercially available, cryogen-free, dilution refrigerator[6]. This was fitted with a custom tail and sample interface, including a sample loading system (see section 2.6). Before the addition of the sample interface, the refrigerator was capable of reaching a base temperature of 7 mK. The refrigerator has a listed cooling power of 400 μW at 100 mK. Tests at higher temperatures showed that it was capable of 12 mW of cooling

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at 1 K.

2.3. Magnet

The magnet used was a 5 T, warm bore, cryogen-free, vertical magnet[7]. The magnet has a 100 mm clear bore, and a better than one part in 10^4 uniformity over a 2 cm diameter spherical volume. In order to facilitate the transition from the polarizing configuration to the data taking configuration, the magnet was mounted on a custom designed lift table. This table was capable of manually raising or lowering the magnet while it was at full field. The minimum time to raise or lower the magnet was limited by the eddy current heating in the refrigerator and sample space, but usually was less than ten minutes. A diagram of the system in polarizing mode and data taking mode is shown in Figure 2.

2.4. Microwaves

The small sample size (< 1 mm^3) allowed the use of a relatively inexpensive solid state microwave source[8]. This source was capable of generating 300 mW of microwave power at 140 GHz. The frequency was extremely stable, and set via USB communication with the device. A variable attenuator and a power meter were used to control the amount of microwave power sent into the system. For the cryogenic wave-guide, a 0.188 in. O.D. copper nickel tube was used. A sheet of brass at the end of the wave-guide was used

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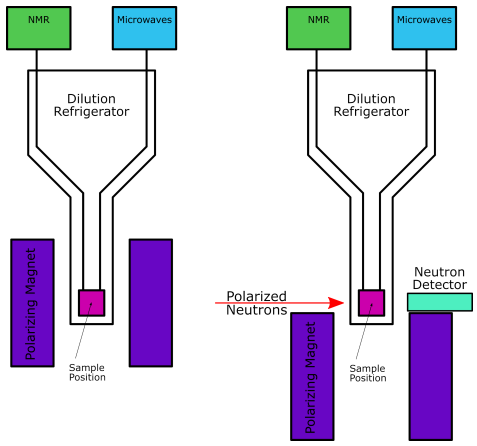

Figure 2: A diagram of the system in polarizing mode (left) and data taking mode (right). In order to move between the two configurations, the magnet was moved down, and a detector moved in close to the sample.

to reflect the microwaves at 90° so that they would intercept the sample position.

2.5. NMR System

The polarization was monitored using a Liverpool [15] q-meter and a pre-amplifier card which provided a gain of 500. This amplification was necessary to see the thermal equilibrium (TE) polarization signal for the extremely small samples used. Even with this amplification, the TE signal area could not be measured reliably enough to calibrate the NMR system through the standard technique of comparing the TE signal to the known TE polarization (although increases in the signal size were clear evidence of polarization). In order to estimate the maximum polarization, larger (a few μL) frozen samples (of a solution which had the same chemical composition as the crystals) were polarized. These samples were large enough to use the

TE signal for calibration. A number of these samples were measured, and with some variation, showed maximum polarizations of about 50%, usually after 8 hours or more of polarizing. The error on these measurements was still quite high (20% or higher relative error), as the low cooling and microwave power of the system limits the maximum sample size significantly. This error, combined with the unknown systematic error that comes from comparing the large sample polarizations to that of the crystal, results in a system that is only capable of qualitative estimates of the polarization. The NMR system was, however, sufficient to monitor the relative decay of the polarization, since the polarized signals are two orders of magnitude larger than the TE signals needed for calibration.

2.6. Sample Space

The sample space and loading system has unique requirements, owing to the nature of the samples used. Protein crystals contain about 50% solvent by volume, and in order to retain their crystal structure, must be frozen very quickly to vitrify this solvent. This is generally accomplished by immersion in liquid nitrogen. Once frozen, the samples are mechanically very robust, but must remain below around 140 K to prevent the formation of crystalline ice, which would disrupt the protein lattice. Frozen crystals must therefore be loaded with the refrigerator already cooled. Protocols for NMC at cryogenic temperatures have been developed [16] and demonstrated previously on IMAGINE [17][18]. As an additional requirement, while the sample is being polarized, it must have sufficient thermal contact with the mixing chamber to remove the heat deposited by the microwaves. The very high neutron capture cross section of ^{3}He makes it impossible to simply locate the sample

in the mixing chamber, so some other thermal link must be established. The most effective thermal link from the mixing chamber to the sample is a high conductivity tube, filled with super-fluid helium.

The sample was loaded though a thin walled stainless steel tube, which led from the atmosphere side of the the refrigerator top plate, down to the mixing chamber plate. This tube was thermally anchored at the 40 K, 4 K, and still plates of the refrigerator using copper clamps and commercially produced C101 copper braided connections. At the mixing chamber position, the tube switched from stainless steel to copper, with a thick flange attaching the copper to the mixing chamber plate. This thick walled copper tube extended down the tail section of the refrigerator, into the magnet. The last section of the tube was made of PCTFE (polychlorotrifluoroethylene, a material similar to Teflon) and attached via an indium seal to a stainless steel flange at the end of the copper tube. The PCTFE sample space had a thin walled (0.025 $in.$) section to accommodate the beam and the diffracted neutrons. The bottom of the PCTFE sample space was fitted with a second indium seal, where a ceramic, SMA style, RF feed-through was connected in order to get an NMR coil as close to the sample as possible. A picture of the PCTFE sample space can be seen in Figure 3. The vacuum can and radiation shields had thin aluminum windows along the beam path to minimize the background as much as possible. The vacuum windows were 0.01 $in.$ thick, while the three radiation shields were less than 0.001 $in.$ thick along the beam path.

The sample itself was located in a 0.125 $in.$ O.D. PCTFE capillary, mounted via a short aluminum transition piece, to the sample stick. The stick consists of a long, fiberglass reinforced epoxy (G10) shaft, glued into

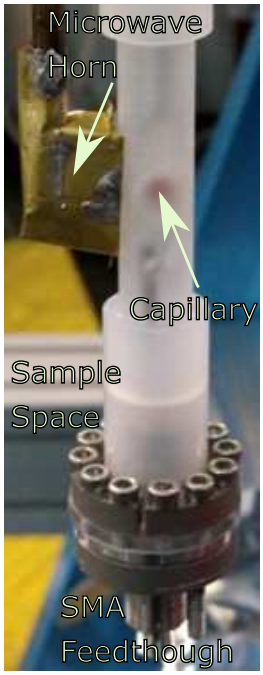

Figure 3: A photograph of the PCTFE section of the sample space. The microwave reflector is visible in the left side of the image. The SMA feed-through and lower indium seal are also visible. The sample has been indicated in this case by painting the capillary red (visible through the PCTFE).

an aluminum top section. The aluminum top section is keyed, allowing it to be inserted or removed using a tool designed specifically for this purpose. The top of the stick is located at the level of the dilution refrigerator mixing chamber. The top of the stick also has a spring energized Teflon seal, which causes it to make tight contact with the inner wall of the sample space at the position of the mixing chamber interface. This seal reduces the amount of gas and superfluid that entered the section of the loading tube which leads to room temperature. A thin walled 0.125 *in*. stainless steel tube is used to

supply helium gas below this seal. This tube was thermally anchored at the 40 K,4 K, and still plates of the refrigerator.

3. Operation

Crystals were soaked for 30 to 60 minutes in a solution containing $100mM$ hydroxy-TEMPO and flash frozen (with some of samples first being passed through a glycerol cryo-protectant solution) in liquid nitrogen before being placed in the capillary and stored under liquid nitrogen. In order to ensure that the crystals didn't warm during loading, samples were loaded into the refrigerator while it was in standby mode (no helium circulating in the dilution unit, pulse tube at base temperature). After the refrigerator recovered from the heat associated with inserting the sample (which was at liquid nitrogen temperature), helium was condensed in the dilution unit. Once the dilution unit was running, helium was flowed through the small stainless steel tube into the sample space, through a mass flow controller. After approximately 16 mL of helium had been condensed in the sample space, the mixing chamber and sample temperature (as measured by a pair of resistors mounted to the SMA feed-through) were within about 35 mK of each other, corresponding to the lowest achievable sample temperature. This temperature varied from sample to sample, but generally the lowest sample temperature attainable was between 200 and 250 mK. This variation is presumed to be primarily related to the effectiveness of the energized Teflon seal, which can change due to the presence of ice or other contaminants, either on the seal itself or on the mating surface in the sample space.

With the magnet at full field, and raised till the sample was in the uniform

region, microwaves would be transmitted to sample. As measured at the top of the cryostat, the optimum microwave power to produce high polarization was 10-20 mW, corresponding to about a mW at the sample position. Polarization was generally quite slow, requiring eight or more hours of microwave irradiation, so the samples were usually polarized overnight in order to optimize operational efficiency. The microwaves were then turned off, and the sample allowed to cool. Re-cooling was relatively quick, requiring about 30 minutes. The magnet was then lowered, and neutron diffraction data was collected for a time equivalent to the spin lattice relaxation time T_1. This means that the effective average in-beam polarization was about half of the starting polarization. The magnet could then be moved back up and the process repeated. Optionally, the sample temperature could be increased while in the data taking position by means of a heater located on the mixing chamber plate. Raising the temperature shortens T_1 greatly, allowing for unpolarized data to be taken for comparison to the polarized results.

4. Performance and Results

Over a dozen protein crystal samples were tested at the IMAGINE beamline over two separate HFIR operating cycles under different conditons (including room temperature tests, cold tests, and polarized tests) in order to first commission the apparatus and the detector, and then measure the effect of the polarization on the diffraction. The samples were hen-egg-white lysozyme and T4-lysozyme, well characterized standard proteins that are typical of the medium unit cell (around 100Å) systems that are amenable to neutron analysis. The crystals were of varying size, with most of them being

Figure 4: Apparatus in the data taking position. Detector shown with a 3-D printed shield to reduce background.

roughly cubic and approximately 600 μm on edge. IMAGINE is a quasi-Laue diffractometer which utilizes a pair of elliptical neutron mirrors to produce a focused neutron beam spot [19]. The standard detector was not utilized for these proof of principle tests, since it was not possible to integrate it with the polarizing magnet. Instead, a single arm detector was built, using a prototype Anger camera which has been developed at ORNL. This detector uses silicon photo diodes instead of the usual photo-multiplier tubes. This allowed the detector to operate in the fringe field of the polarizing magnet. Data was taken with the detector approximately 20 cm from the sample position, over a 116 mm by 116 mm area. The detector was positioned at

different angles, between 0° and 60°. The neutron beam was polarized using a V-cavity supermirror polarizer[9].

About half of the crystals both polarized and produced sufficient diffraction to test the effect of polarization on the diffraction pattern (the crystals that both polarized and diffracted were all T4-lysozyme crystals, which is the sample material that was used for early offline tests). The spin relaxation time for the four best diffracting samples varied from 0.7 to 3.0 hours. All of the T4-lysozyme samples polarized to some extent. Measurement of the diffraction showed a substantial enhancement in the intensity for the case where the sample was polarized anti-parallel with the beam, and a reduction in intensity for polarization parallel with the beam (relative to the unpolarized intensity). Figure 5 shows a diffraction image for both the polarized and unpolarized data and an intensity profile for an individual reflection. Due to the limited acceptance of the detector and the unknown orientation of the crystal, diffraction patterns were not indexed. Instead, the integrated intensity for sub-sets of reflections was compared to average enhancements calculated using estimates of the sample polarization (determined from NMR measurements). For the anti-parallel case, the integrated intensity of the diffraction pattern typically increased by factors of 2 to 3, with enhancements of individual reflections within the diffraction pattern varying, depending on the relative contribution of hydrogen to that particular reflection. Comparing individual diffraction measurements this way has the potential for large systematic errors, but the results of these averaged

[9]SwissNeutronics AG, Bruehlstrasse 28, CH-5313 Klingnau, Switzerland

measurements were consistent with samples starting with about 50% polarization in the highest cases and decaying over the course of the data taking as expected. This qualitative consistency between NMR and diffraction data provides some confidence as to the level of polarization being produced in the samples. A detailed description of the sample preparation and the experimental results will be discussed in a future publication.

5. Conclusion and Outlook

Tests using the system described in this paper have shown that it is possible to increase the intensity of diffraction from a protein crystal by manipulating the nuclear polarization. The test apparatus built is sufficient to polarize these samples and allow neutron scattering data to be taken, although the data collection time is limited by the relatively high sample temperature in frozen spin mode. Since the tests described were completed, the sample interface has been redesigned to lower the sample temperature and correspondingly increase the spin relaxation time, with the goal of increasing the average polarization during data collection. The upgraded apparatus is scheduled to be tested in the IMAGINE beam-line in 2019.

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[1] O'Dell, William B., Annette M. Bodenheimer, and Flora Meilleur, Archives of Biochemistry and Biophysics 602 (2016): 48-60.

[2] Schrder, Gabriela C., et al., Acta Crystallographica Section D: Structural Biology 74.8 (2018).

[3] Blakeley, M. P., Crystallography Reviews 15.3 (2009): 157-218.

[4] Coates, Leighton, et al., Review of Scientific Instruments 89.9 (2018): 092802.

[5] Stuhrmann, Heinrich B. Reports on Progress in Physics 67.7 (2004): 1073.

[6] A. Abragam and M. Goldman, "Nuclear magnetism: Order and Disorder" Clarendon Press (1982).

[7] A. Abragam, "The Principles of Nuclear Magnetism " Oxford University Press. (1961).

[8] Knop, W., et al., Journal of applied crystallography 25.2 (1992) 155-165.

[9] Zhao, J., et al., Nuclear Instruments and Methods in Physics Research Section A 356.1 (1995): 133-137.

[10] Piegsa, F. M., et al., Journal of Applied Crystallography 46.1 (2013) 30-34.

[11] C.D. Keith, et al., Nuclear Instruments and Methods in Physics Research Section A 501 (2003) 327.

[12] C.D. Keith, et al., Nuclear Instruments and Methods in Physics Research Section A 684 (2012) 27.

[13] Pierce, J., et al., Nuclear Instruments and Methods in Physics Research Section A 738 (2014) 54.

[14] Hall, D. A., et al., Science 276(5314) (1997) 930.

[15] G.R. Court, et al., Nuclear Instruments and Methods in Physics Research Section A 324 (1993) 433.

[16] Myles, Dean AA, et al., Journal of Applied Crystallography 45.4 (2012) 686-692.

[17] Blakeley, M. P., et al., Proceedings of the National Academy of Sciences 101.47 (2004) 16405-16410.

[18] Li, Le, et al., Protein Science 26.10 (2017) 2098-2104.

[19] Meilleur, Flora, et al., Acta Crystallographica Section D: Biological Crystallography 69.10 (2013) 2157-2160.

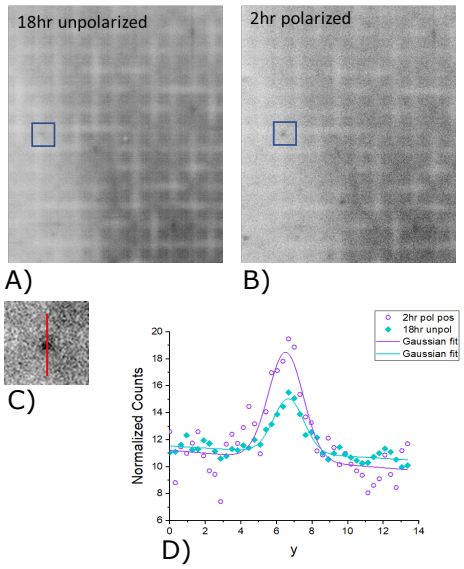

Figure 5: A) Eighteen hours of diffraction data with an unpolarized sample, showing a large fraction of the detector coverage. B) Two hours of diffraction data in the same configuration, but with the sample polarized anti-parallel to the polarization of the neutron beam. C) The time normalized profile of the detector counts, along with a Gaussian fit, in a single reflection from two different images, showing an enhancement in the polarized case. D) A detail showing the profile that was plotted. Both of these detector images were acquired using a T4-lysozyme crystal, a quasi-Laue polarized neutron beam, and the detector positioned at approximately 45 degrees. The contrast has been scaled to the data collection time.