SANS and USANS Investigation of the Structure of Coarse Fibrin Clots

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Abstract

Fibrin is the major protein component of blood clots, forming a cross-linked network of fibers and is important in the blood coagulation process. The relationship of the structure of fibrin networks to their function is crucial to understanding the processes of haemostasis (the halting of bleeding) and fibrinolysis (the breakdown of clots once damage has been repaired). However, studying the structure of these materials has been difficult due to the small size and wide range of size of the structures and to the high turbidity of the materials. Small- and Ultra-small angle neutron scattering (SANS and USANS) will be used to examine the structure of fibrin clots as a function of concentration over a size range of nanometers to micrometers.

Contents

1 Introduction

Biopolymer networks have mechanical properties that are remarkably different from those of most synthetic materials[1]. These properties are directly related to the internal structure of the polymer fibers and of the network. Fibrin, the major protein component of blood clots, has drawn particular interest due to its important role in blood coagulation.[2] It has also been recognized that the relationship between the structure and function of fibrin networks is critical to our understanding of important biological processes including haemostasis and fibrinolysis. Despite its paramount importance, many structural parameters of fibrin have been difficult to measure in unperturbed samples. This is due to the high turbidity of the materials and the small size of these structural features. Thus, previous research has been unable to probe the structure of these materials over a wide range of length scales and protein concentrations. In this experiment, small angle neutron scattering (SANS) will be used to characterize the structure of coarse fibrin clots formed in saline solutions over length scales that extend over five orders of magnitude (1 nm – 20 μm). Furthermore, SANS and Ultra SANS experiments allow us to analyze bulk solvated samples spanning a wide range of concentrations (1 to 15 mg/mL).

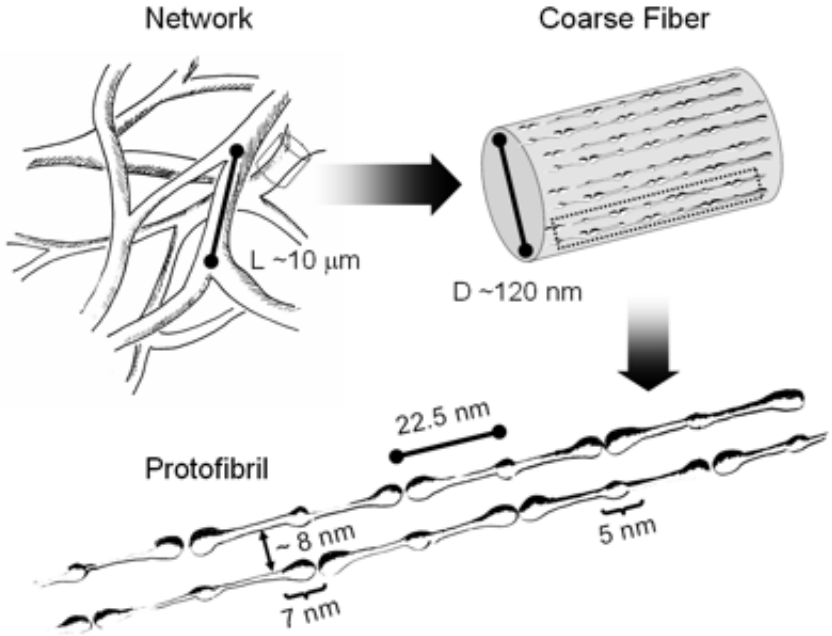

Figure 1: Schematic representation of the structural features found for coarse fibrin clots in length scales ranging from 1 nm to 10 μm.

Figure 1 represents the three-dimensional structure of a coarse fibrin clot over length scales of 1 nm to 10 μm. Fibrin clots are formed when the polymerization of fibrinogen is activated by the enzyme thrombin[2]. After activation, the proteins assemble into highly organized linear arrays

called protofibrils. These protofibrils have a half-staggered linear structure with a repeat distance of half the length of the fully extended fibrinogen molecule (45 nm). Under certain solvent conditions, protofibrils can also aggregate laterally to form a larger coarse fiber.[2, 3, 4] It is also known that the inside of these coarse fibers is mostly composed of water that fills the space between the fibrinogen monomers. The internal volume that is occupied by the proteins has been measured using light scattering and refractive index measurements on dilute clots (¡ 0.25 mg/mL).[5, 6] The estimates of the total volume fraction of protein within the fibers vary between 20-30%. However the composition of the fibers has not been determined in a concentrated system.

Thus we propose in this experiment to determine the structure of fibrin networks formed from concentrated solutions of fibrinogen.

1.1 Why use SANS?

Generally, static light scattering and small angle X-ray scattering (SAXS) provide the same information about the sample: measurement of macroscopic scattering cross-section $d\Sigma/d\Omega(q)$, as neutron scattering. The contrast in light scattering arises from the difference in the light's refractive index between the particle and water. The wavelength of light limits q < 0.002 Å^{-1} and thus the size range probe to $>\sim$3000. Furthermore, in order to measure the light scattering the sample needs to be dilute and here we wish to study a concentrated network structure. The contrast in X-ray scattering arises from the variation in electron density within the sample. However USAXS does not generally reach as low q as USANS and with protein samples x-rays (particularly at synchrotron sources) can cause damage to the sample as a result of the large amount of energy imparted.

SANS/USANS is therefore an ideal probe for the structure of these biological network structures since it allows measurement at biologically relevant concentrations and conditions over the whole relevant size range with no risk of damage to the sample.

2 The Objectives of the Experiment

To determine the volume fraction of protein within the fibers Making use of the fact that the absolute scattering cross section is obtained from the SANS experiment, the composition of the fibrin fibers will be determined given the known scattering length density of the fibrinogen and heavy water.

To determine the structure of the fibrin clot A suitable model for the scattering at the various length scales will be chosen and fitted to the SANS and USANS data. The dimensions of the coarse fibers will be determined and the structure of the network analyzed.

To determine the concentration dependence of the structure The models developed will be fitted to data taken at varying concentrations and the dependence of the various key parameters on concentration determined.

To determine the pH dependence of the structure The models developed will be fitted to data taken at varying pH and the dependence of the various key parameters on pH determined.

3 The USANS Instrument

Fundamentally, the SANS experiment consists of measuring the number of neutrons scattered per incoming neutron as a function of scattering angle. Since the size probed is inversely proportional to angle, to examine larger objects we need to measure scattering at smaller angles. In the case of a "pinhole" SANS instrument this is achieved by moving a 2 dimensional detector relative to the sample such that a detector element close to the beam center subtends a smaller angle the further the detector is from the sample.

The SANS instruments at the NCNR can measure down to $8{\times}10^{-}4\text{Å}^{-1}$ at their maximum sample to detector distance and using lenses to focus the neutron beam. This implies a maximum size of measurable object of approximately 500nm. One can imagine simply making longer and longer instruments to study larger and larger objects, however there a limitations to that approach. Firstly neutrons have mass and so are affected by gravity. Hence they fall through a parabolic path as they travel from source to detector. Secondly, the collimation required as the instrument gets longer reduces the flux of neutrons on the sample and counting times increase.

There is an alternative to the pinhole instrument and that is to use crystal diffraction to produce a monochromatic beam of neutrons with very good angular collimation and to then use an identical crystal to analyze the scattered beam. This instrument design is known as a Bonse-Hart type or Double-Crystal diffractometer.

Figure 2 show the schematic layout of the NCNR USANS instrument which is located on beam tube 5 (BT-5). A channel cut silicon crystal (monochromator) provides the neutron beam onto the sample, where the neutrons are scattered. A second identical channel cut crystal (analyzer) is then placed in the scattered beam path and rotated to select the scattering angle to be analyzed and diffract the neutrons scattered at that angle into the detector. An experiment consists of rotating the analyzer to a series of angles and counting the number of neutrons that reach the detector.

The intensity of scattering on the detector after background correction in a USANS experiment is given by

$$I_{cor}(q)_s = \varepsilon I_{beam} \Delta\Omega_A d_s T(\frac{d\Sigma_s(q)}{d\Omega}) \tag{1}$$

where

ε is the detector efficiency

I_{beam} is the number of neutrons per second incident on the sample

d_s is the sample thickness

T is the sample transmission

$\Delta\Omega$ is the solid angle over which scattered neutrons are accepted by the analyzer

$\frac{d\Sigma_s(q)}{d\Omega}$ is the measured scattering cross section, which is the true cross section modified by the instrumental resolution function.

The aim of the experiment is to obtain the differential macroscopic scattering cross section $\frac{d\Sigma}{d\Omega}$ from I_{meas}. How we can go about that process is described later, but first we need to decide how to prepare our sample for the measurement.

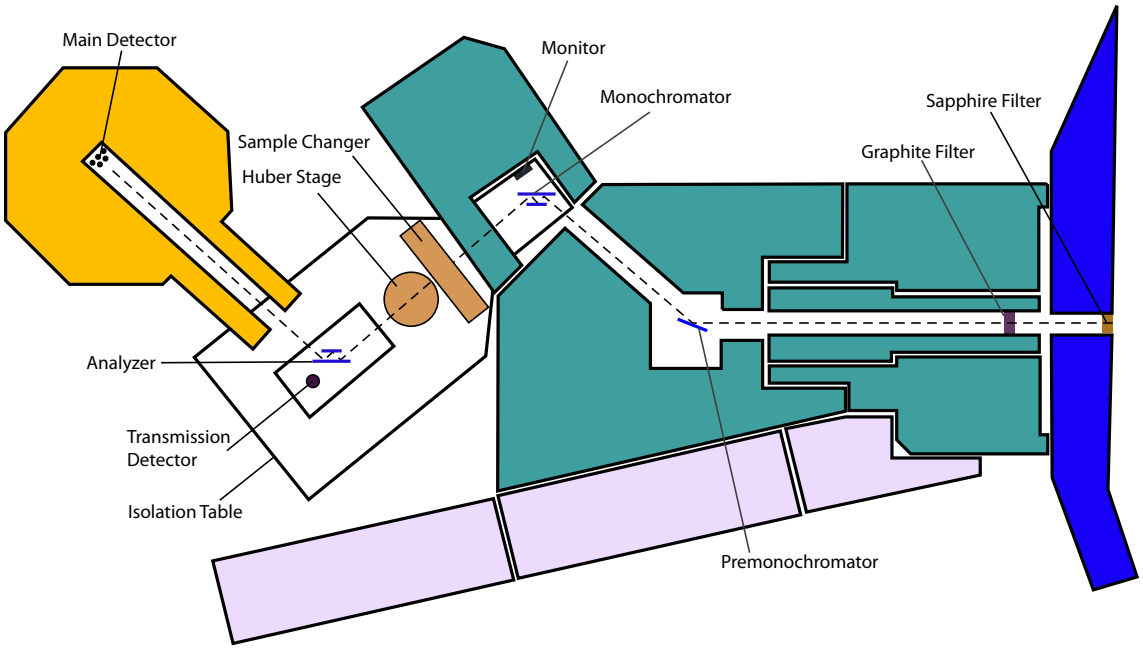

Figure 2: Schematic layout of the BT-5 USANS instrument. The dashed line indicates the beam path. The measured scattering angle, or momentum transfer q, is determined by rotation of the analyzer crystal.

4 Planning the Experiment

Given the stated objectives of the experiment and knowledge of the instrument, how do we go about preparing for the experiment to maximize our chances of success? Here we discuss some of the issues that bear on this question.

4.1 Scattering Contrast

In order for there to be small-angle scattering, there must be scattering contrast between, in this case, the fibrin and the surrounding solvent. The scattering is proportional to the scattering contrast, $\Delta\rho$, *squared* where

$$\Delta\rho = \rho_f - \rho_w \tag{2}$$

and ρ_f and ρ_w are the *scattering length densities* (SLD) of the fibrinogen and the water solvent, respectively. Recall that SLD is defined as

$$\rho = \frac{1}{V}\sum_{i}^{N} b_i \tag{3}$$

where V is the volume containing n atoms, and b_i is the (bound coherent) *scattering length* of the ith atom in the volume V. V is usually the molecular or molar volume for a homogenous phase in the system of interest.

The SLDs for the two phases in the present case, fibrinogen and heavy water, can be calculated from the above formula, using a table of the scattering lengths (such as Sears,1992) for the elements, or can be calculated using the interactive SLD Calculator available at the NCNR's Web pages (http://www.ncnr.nist.gov/resources/index.html). The SLDs for fibrinogen and light and heavy water are given below in Table 2.

Material	Chemical Formula	Mass Density (g cm^{-3})	SLD ($Å^{-2}$)
Fibrinogen (in D_2O)			3.17×10^{-6}
Light Water	H_2O	1.0	-0.52×10^{-6}
Heavy Water	D_2O	1.1	6.32×10^{-6}

Table 1: The scattering length densities for fibrinogen, light water and heavy water.

You will note that the formula and density for fibrinogen are not quoted. It is difficult to accurately predict what the effective volume and hydration state of a protein in solution will be, so the SLD of fibrinogen was not calculated but measured. This can be done by making solutions of the protein in various mixtures of solvent across the composition range from pure H_2O to pure D_2O. Since

$$I(q) \propto (\Delta\rho)^2 \tag{4}$$

and

$$\rho_{solvent} = \phi_{D_2O}\rho_{D_2O} + (1-\phi_{D_2O})\rho_{H_2O} \tag{5}$$

where ϕ_{D_2O} is the volume fraction of D_2O in the solvent and ρ is the relevant scattering length density, we can plot $\sqrt{I(0)}$ vs ϕ_{D_2O} and there will be a minimum where $\sqrt{I(0)} = 0$ corresponding to the *contrast match point*. This is the point where the SLD of the solvent matches that of the fibrinogen. In the case of a protein account must be taken of the fact that proton exchange can occur (and thus some H atoms on the protein are replaced by D).

If the contrast match point is not required and we want to know the SLD in a given solvent, this can be found from considering a series of concentrations of protein so that the number density of protein molecules at each concentration is known. Thus, assuming the structure does not change, the contrast (and hence the SLD of the protein) can be found from the scattering law at zero angle where

$$I(0) = \frac{N}{V}(\rho_p - \rho_s)^2 V_p^2 \tag{6}$$

where N is the number of protein molecules, V is the total volume, ρ_p is the protein SLD, ρ_s is the solvent SLD and V_p is the volume of a protein molecule. The number of particles is proportional to the concentration so data from multiple concentrations can be used to solve simultaneously to obtain ρ_p.

In this experiment the samples are all prepared in D_2O to obtain good contrast and minimize the incoherent scattering background.

4.2 Sample Thickness

Given the calculated sample contrast, how thick should the sample be? Recall that the scattered intensity is proportional to the product of the sample thickness, d_s and the sample transmission, T. It can be shown that the transmission, which is the ratio of the transmitted beam intensity to the incident beam intensity, is given by

$$T = e^{-\Sigma_t d_s} \tag{7}$$

where $\Sigma_t = \Sigma_c + \Sigma_i + \Sigma_a$, i.e. the sum of the coherent, incoherent and absorption macroscopic cross sections. The absorption cross section, Σ_a, can be accurately calculated from tabulated absorption cross sections of the elements (and isotopes) if the mass density and chemical composition of the sample are known. The incoherent cross section, Σ_i, can be *estimated* from the cross section tables for the elements as well, but not as accurately as it depends on atomic motions and is therefore temperature dependent. The coherent cross section, Σ_c, can also only be estimated since it depends on the details of both the structure and the correlated motions of the atoms in the sample. This should be no surprise as Σ_c as a function of angle is the quantity we are aiming to measure!

The scattered intensity is proportional to $d_s T$ and hence

$$I_{meas} \propto d_s e^{-\Sigma_t d_s} \tag{8}$$

which has a maximum at $d_s = 1/\Sigma_t$ which implies an optimum transmission, $T_{opt} = 1/e = 0.37$. The sample thickness at which this occurs is known as the "1/e length".

The NCNR web based SLD calculator provides estimates of Σ_i and Σ_a and gives an estimate of the 1/e length as well as calculating the SLD.

4.3 Multiple Scattering

The analysis of small angle scattering data assumes that a neutron is scattered only once on passing through the sample and thus that the scattering angle is simply related to structure of the sample. However, if the small angle scattering is strong enough to result in multiple scattering, then the shape of the scattering curve will become distorted [7] and analysis essentially impossible. Thus when Σ_c is significantly larger than $\Sigma_i + \Sigma_a$ the thickness should be chosen such that $T > 0.9$ rather than 0.37 to avoid problems with multiple scattering.

In this experiment, the optimum sample thickness has been determined to be 4mm.

4.4 Required q range

The q range that is routinely accessible using the BT-5 USANS instrument is 5×10^{-5}Å^{-1} to 5×10^{-3}Å^{-1}. Both low q and high q limits are in practice determined by whether there is measurable scattering above background since the analyzer can be set to count at any q. The high q value chosen for an experiment is usually determined by the length scales of relevance to the sample and whether overlap with the SANS measurement regime is required. Figure 3 shows the accessible q ranges of the SANS and USANS instruments.

In this experiment we will be measuring to approximately 3×10^{-3}Å$^{-}1$.

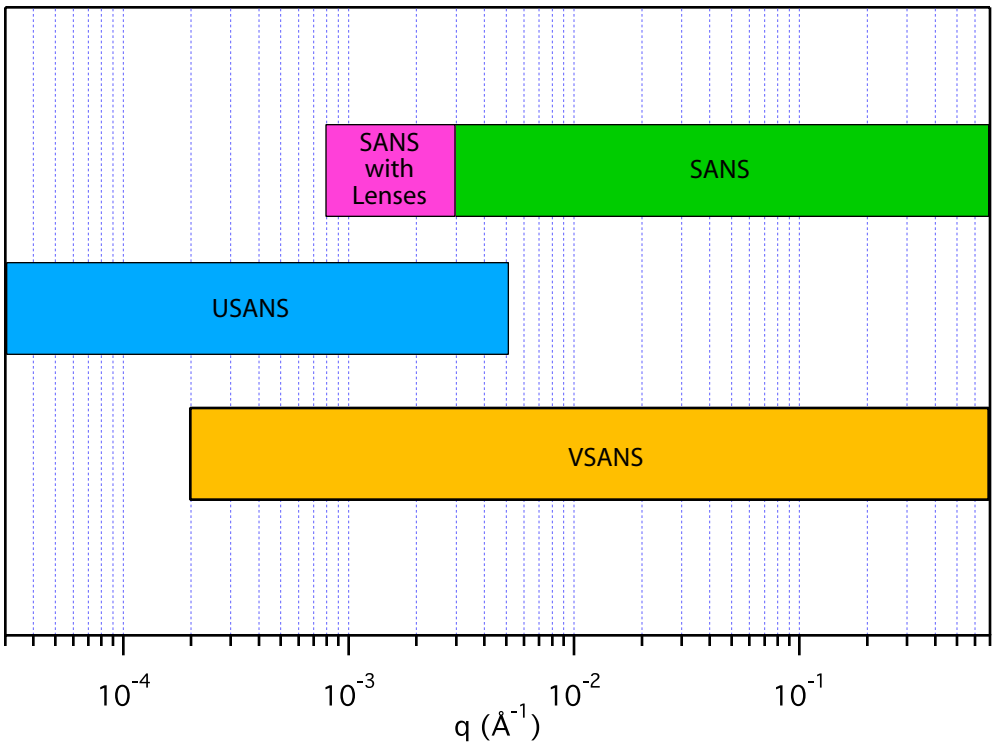

Figure 3: Comparison of the accessible q ranges of the BT-5 USANS instrument, NG-3 and NG-7 SANS instruments and the proposed VSANS instrument

5 Collecting data

As discussed earlier, the experiment consists of scanning the analyzer through a series of angles and counting the scattered intensity on the detector. The first step before collecting the scattering data, therefore, is to decide which angles to measure at and how long to count at each.

5.1 Configuring the instrument

We need to measure over a range of angles spanning two orders of magnitude in q and an appropriate q spacing for around q=0 would lead to a huge excess of data points at around q=1×10^{-3}. Thus we divide the data collection into six separate equally spaced scans, with each scan having roughly double the q spacing of the previous one. The first scan spans the main beam and the peak intensity from that scan is used to determine the q=0 angle, to scale the intensity into absolute units and to determine the sample transmission.

5.2 What measurements to make

To correct for instrument "background" measurement of scattering without the sample is needed. Counts recorded on the detector can come from three sources: 1) neutrons scattered by the sample itself; 2) neutrons scattering from something other than the sample, *but which pass through the sample*; and 3) everything else, including neutrons that reach the detector *without passing through the sample* (stray neutrons or so-called room background) and electronic noise in the detector itself.

In order to separate these contributions we need to make three separate measurements:

1. Scattering measured with the sample in place (which contains contributions from all three sources listed above), $\mathbf{I_{sam}}$
2. Scattering measured with the empty sample holder in place (which contains contributions from sources 2 and 3 above), $\mathbf{I_{emp}}$
3. Counts measured with a complete absorber at the sample position (which contains only the contribution from source 3 above), $\mathbf{I_{bgd}}$

The $\mathbf{I_{bgd}}$ on the USANS instrument is predominantly due to fast neutrons. This background is independent of instrument configuration as the fast neutrons are not coming along the beam path. It has been measured and is $0.018 s^{-1}$, which equals 0.62 counts per 10^6 monitor counts. Thus we do not usually measure a blocked beam run on USANS but use a fixed value for $\mathbf{I_{bgd}}$

5.3 How long to count

A SANS experiment is an example of the type of counting experiment where the uncertainty, or more precisely the standard deviation, σ, in the number of counts recorded in time, $I(t)$ is given by $\sigma = \sqrt{I(t)}$. Thus increasing the counting time by a factor of four will reduce the relative error, σ/I by a factor of two. If there are 1000 total counts per data point, the standard deviation is $\sqrt{1000}$ which is approximately 30, giving a relative uncertainty of about 3%, which is good enough for most purposes.

A related question is how long should the empty cell measurements be counted relative to the sample measurement. The same $\sigma = \sqrt{I(t)}$ relationship leads to the following approximate relationship for optimal counting times

$$\frac{t_{bgd}}{t_{sam}} = \sqrt{\frac{\text{Count Rate}_{bgd}}{\text{Count Rate}_{sam}}} \tag{9}$$

Hence if the scattering from the sample is weak, the background should be counted for as long as (but no longer than!) the sample scattering. If, however, the sample scattering count rate is, say, 4 times greater than the background rate, the background should be counted for only half as long as the sample.

Since the scattering usually becomes much weaker at larger q, the time spent per data point increases with angle and the high q scans dominate the overall counting time.

5.4 Sample Transmission

The sample transmission is determined in two ways.

5.4.1 Wide angle transmission

A separate transmission detector (see figure 2), located behind the analyzer, collects all neutrons not meeting the Bragg condition for the analyzer. When the analyzer is rotated to a sufficiently wide angle from the main beam orientation the transmission detector counts both the direct beam intensity and the coherently small angle scattered intensity. Thus the ratio of count rate on the transmission detector with and without the sample is the sample transmission (T_{wide}) due to attenuation from incoherent scattering and absorption.

5.4.2 Rocking curve transmission

Rotating the analyzer through the main beam allows the intensity at q = 0 to be measured. The ratio of this intensity with and without the sample gives the transmission of the sample (T_{rock}) due to attenuation from incoherent scattering, absorption and *coherent small angle scattering.*

5.5 Multiple scattering estimate

The ratio of these separate transmission measurements can be used to estimate the amount of multiple scattering by determining the scattering power ($\tau = \Sigma_{SAS} d_s$) by

$$T_{SAS} = \frac{T_{\text{Rock}}}{T_{\text{Wide}}} = e^{-\tau} \tag{10}$$

where ideally $T_{SAS} > 0.9$

6 Data reduction

Data reduction consists of correcting the measured scattering from the sample for the sources of background discussed in section 5.2 and rescaling the observed, corrected data to an absolute scale of scattering cross section per unit volume. This is done via equation (1) presented previously and reproduced here for reference:

$$I_{cor}(q)_s = \varepsilon I_{beam} \Delta\Omega_A d_s T \left(\frac{d\Sigma_s(q)}{d\Omega}\right) \tag{11}$$

The beam intensity, εI_{beam}, is measured by rotating the analyzer through the direct beam at q = 0 with the empty cell in the beam path. The transmission, T, is measured by taking the ratio of the

count rate observed on the transmission detector with and without the sample in the beam path. The solid angle of scattering accepted by the analyzer, $\Delta\Omega_A$, is given by

$$\Delta\Omega_A = \left(\frac{\lambda}{2\pi}\right)^3 (2\Delta q_v)\Delta q_h \tag{12}$$

where $2\Delta q_v$ is the total vertical divergence of the beam convoluted with the angular divergence accepted by the detector and Δq_h is the horizontal divergence accepted for diffraction by monochromator and analyzer crystals. The instrument accepts scattered neutrons with $\pm\Delta q_v = 0.117\text{Å}^{-1}$. The horizontal resolution Δq_h is measured from the full width at half maximum (fwhm) of the main beam profile obtained by rotating the analyzer through the direct beam. The fwhm when the crystal is properly aligned is 2.00 arcsec, equating to $\Delta q_h = 2.55 \times 10^{-5}\text{Å}^{-1}$. Thus the solid angle over which neutrons are accepted by the analyzer is $\Delta\Omega_A = 8.6 \times 10^{-7}$

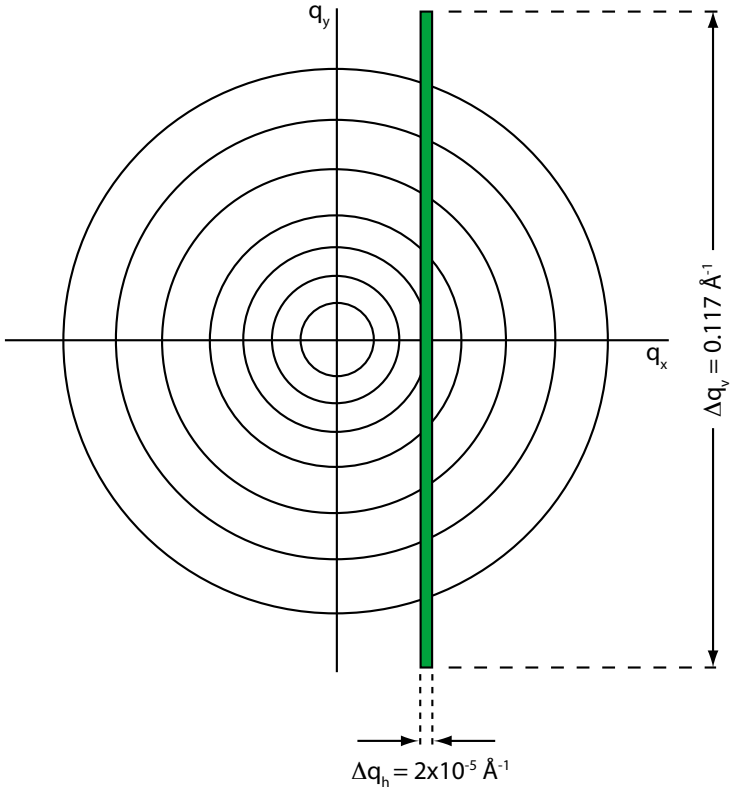

Figure 4: View of scattering with axes q_x and q_y collected by the analyzer on the BT-5 USANS instrument. The circles represent iso-intensity contours from isotropic small angle scattering. The narrow slit represents the scattering region collected by the analyzer.

As you may have noted above, the analyzer has very good resolution in the horizontal direction and very poor resolution in the vertical direction as depicted graphically in figure 4. This is referred to as "slit geometry" as opposed to the "pinhole geometry" of a standard SANS instrument - you may be familiar with this from using a Kratky camera for lab-based small angle x-ray scattering. The large difference between the horizontal and vertical resolutions means that the smearing can be treated as that from an "infinite" slit. The measured cross section, $d\Sigma_s/d\Omega(q)$, obtained from data

reduction as described above is related to the true differential macroscopic cross section, $d\Sigma/d\Omega(q)$ by the relation [8]

$$\frac{d\Sigma_s}{d\Omega}(q) = \frac{1}{\Delta q_v}\int_0^{\Delta q_v} \frac{d\Sigma}{d\Omega}(\sqrt{q^2+u^2})du \tag{13}$$

Figure 5 compares the scattering from a 1 volume % dispersion of 2 μm silica particles with 5% polydispersity in D_2O using pinhole and slit geometries. Note the damping of the oscillations, the change in slope and reduction in intensity. Desmearing the data directly can be done by an iterative convergence method [9] but the desmeared result is very unstable, being sensitive to noise in the data. The preferred method is to make use of equation (13) to smear a model function and fit the smeared data *directly*. The latter is the method we will employ in the analysis of our data.

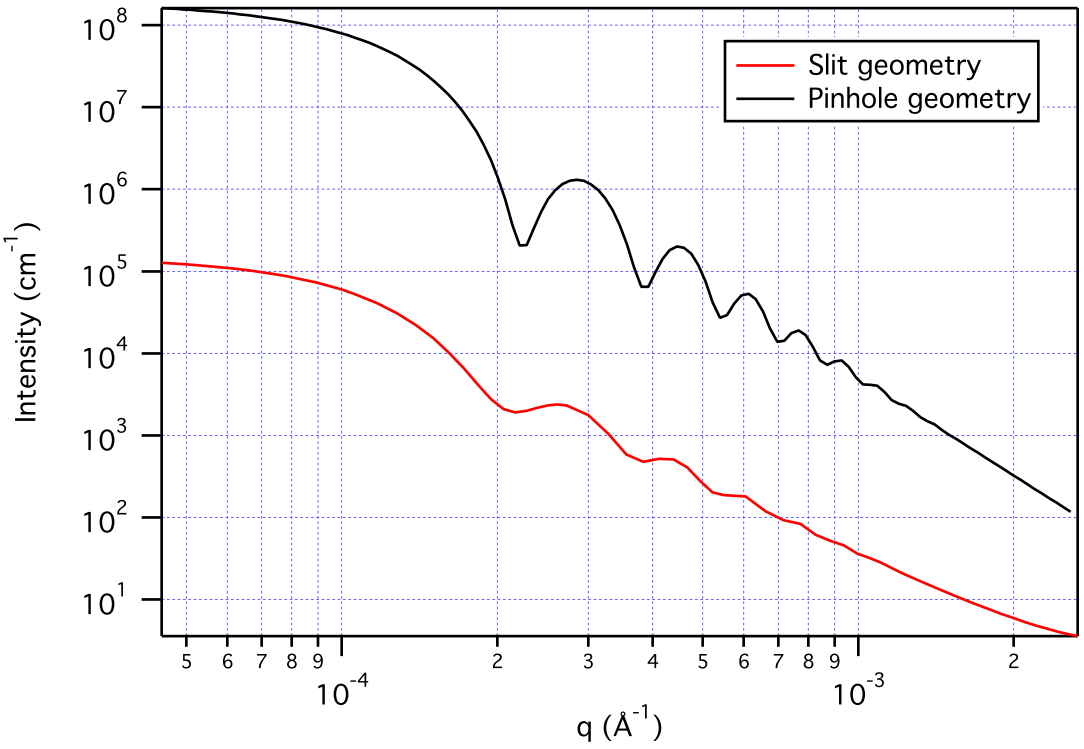

Figure 5: Comparison of the modeled scattering from a 1 volume % dispersion of 2 μm silica particles with 5% polydispersity in D_2O using pinhole and slit geometries

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