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Experimental Approaches for Solution X-Ray Scattering and Fiber Diffraction

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Abstract

X-ray scattering and diffraction from non-crystalline systems have gained renewed interest in recent years, as focus shifts from the structural chemistry information gained by high-resolution studies to the context of structural physiology at larger length scales. Such techniques permit the study of isolated macromolecules as well as highly organized macromolecular assemblies as a whole under near-physiological conditions. Time-resolved approaches, made possible by advanced synchrotron instrumentation, add a critical dimension to many of these investigations. This article reviews experimental approaches in non-crystalline x-ray scattering and diffraction that may be used to illuminate important scientific questions such as protein/nucleic acid folding and structure-function relationships in large macromolecular assemblies.

Introduction

Here we review recent research trends and advances in experimental and computational techniques in structural studies of biological macromolecules in non-crystalline forms. We focus on macromolecular solution x-ray scattering (small angle x-ray scattering or SAXS) and fiber diffraction studies (non-crystalline diffraction or NCD) that are playing increasingly important roles in interpreting high-resolution structural information deduced by crystallography, NMR and electron microscopy in the context of molecular physiology. The advent of synchrotron radiation has contributed immensely to technological advances in this field in much the same way as in macromolecular crystallography. Due to space limitations we omit studies of other important biological systems including lipid membranes and refer those interested to a recent review. [1]

Instrumental requirements and experimental considerations

Randomly oriented or partially ordered systems provide only weak and generally more diffuse X-ray scattering primarily at small scattering angles, requiring synchrotron-based instruments for these studies in contrast to strong single crystal x-ray diffraction where diffraction spots can be measured at high scattering angles up to atomic resolution. Undulator radiation at third

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generation storage rings such as the Advanced Photon Source (APS), European Synchrotron Radiation Facility (ESRF), Super Photon Ring-8 (SPRING-8) and other newer sources provide near ideal sources for NCD studies because of their high flux and low beam divergence necessary for high time-resolution and superior accessibility to long characteristic lengths. There are about two dozen experimental facilities for NCD studies at synchrotrons worldwide some of which are devoted to structural biological studies (<http://biosync.rcsb.org>). Such studies require the highest level of overall instrumental stability from x-ray source to detector since generally weak signals can be extracted only after subtracting the contribution of background scattering, especially in solution scattering. Detectors with high photon sensitivity, electronic stability and low noise are essential. High-performance CCD detectors are currently in widespread use in these experiments while silicon pixel array detectors, converting x-ray photons directly to electrons, have recently emerged as the next generation detector. Micro-fluidic chips[2] as well as other sample handling devices have been developed to minimize sample consumption in solution studies. A number of methods have been developed to mitigate radiation damage, particularly acute at high flux third generation facilities, including the use of capillary flow cells in solution scattering and flash-cooling or sample oscillation in fiber diffraction.

X-ray scattering from macromolecules in solution

Small angle x-ray scattering samples contain many copies of a macromolecule in random orientations as a solute. The resulting circularly symmetric scattering profile is usually azimuthally averaged into a one-dimensional profile to improve data statistics. The contribution of solvent x-ray scattering is subtracted to obtain the x-ray scattering from the solute, often called as the form factor, reflecting the three dimensional shape of the macromolecule of interest, including proteins, nucleic acids and biological assemblies. Synchrotron instruments allow solution scattering studies on a variety of macromolecules ranging from long peptides or oligo-nucleotides of a few thousand Da to multi-megadalton assemblies. Most of these instruments can obtain useful data from dilute solutions in the sub mg/ml range and above. Intermolecular interactions, which can complicate interpretation, may be minimized by adjusting solution conditions, *e.g.*, using a moderately high ionic strength. The information content of the form factor and advanced computational methods used to obtain three-dimensional biological structures, at near nanometer resolutions in favorable cases, have recently been reviewed.[3] An increasing number of structural biologists adopt these methods to build 3D models of macromolecular complexes that are unsuitable for crystallographic studies.[4] Here, we emphasize novel experimental approaches that complement this class of studies.

Macromolecular assemblies

biological assemblies of various sizes are formed in response to external factors. The forward scattered intensity provides a direct measure of molecular weight, thus state of assembly. When multiple molecular species exist, the overall scattering signal is weighed by the square of molecular weight. The elimination of aggregated species is thus crucial in obtaining the form factor of a single molecular species. Complementary techniques such as light scattering and gel filtration are useful to identify and purify distinct molecular species. Sokolova *et al.* combined analytical ultracentrifugation and negative stain electron microscopy to identify solution conditions in which intermediate filament vimentin exists as a distinctively monodispersed assembly species.[5] They built 3D structure models of vimentin tetramer, octamer, and unit-length filaments (32-mer) by rigid body modeling of the dimeric crystal structure. A similar approach was used to study discrete 25mer and 100mer assemblies of human prion protein caused by metal-induced oxidation.[6] Inline size exclusion chromatography is another approach to obtain accurate form factor of a single molecular

species.[7] A new method based on singular value decomposition and an assumption of simple association pathways has been developed to obtain both component scattering curves and the equilibrium constants for individual oligomerization steps.[8]

Flexible structures

A number of biological systems are in a dynamical conformational equilibrium. Since all molecules within the volume irradiated by an x-ray beam contribute to x-ray scattering, solution x-ray scattering is both ensemble- and time-averaged. Unlike proteins in a crystal, flexible and mobile portions of a biological structure also contribute to the observed signal. This enables the study of ensembles of flexible protein structures that may take multiple conformations under given conditions. Bernado *et al.* have developed the ensemble optimization method, which fits the experimental scattering curve by an ensemble of 50 to 100 different conformers made up of rigidly folded domains connected by a flexible self-avoiding linker.[9] This approach revealed the dynamical equilibrium between the open active state (15% population) and the closed active conformation (85%) of Src kinase, only the latter of which had been previously identified as active conformation by crystallography.[10] Protein/RNA folding studies present another important example of the effective use of solution scattering [11] on flexible structures which are not suitable for crystallographic studies. Solution x-ray scattering complements local structural probes such as circular dichroism and NMR residual dipole coupling.[12]

Time-resolved studies

Kinetic studies of conformational changes, due to ligand binding or thermodynamic perturbation, at the tertiary and quaternary structural levels are essential for interpreting atomic resolution structures in the physiological context. Such studies require the use of a high-flux synchrotron beam, as well as a fast-readout detector and a method to trigger a conformational change. A number of different experimental procedures are available depending on triggering method and the rate of conformational change. Slow molecular processes of the order of minutes to hours can be studied by manual mixing followed by a series of successive scattering measurements. Large-scale conformational changes in virus and phage particles, which could not be contained in crystallographic lattice structures, have been studied this way. (Figure 1) HK97 bacteriophage maturation kinetics have been found to be remarkably cooperative from the initial to late stage maturation processes despite the involvement of 420 unique protein subunits in the icosahedral shell. [13] Another recent example highlights the direct observation of a 24h period oscillation between two different Kai protein binary complexes in circadian clocks of a cyanobacterium.[14] Remote-controlled rapid mixing is almost universally used for time-resolved solution x-ray scattering studies at faster time scales.[15] Stopped-flow mixers, requiring only as little as a few milligrams of macromolecule, have been successfully used for studying kinetics whose time constants are in the range of milliseconds to minutes and have been particularly valuable in the studies of the folding of proteins and nucleic acids. [11] In favorable cases, only a single mixing event is required to observe structural kinetics at a few ms time resolution.[16] Micro-machined continuous-flow rapid mixers have been recently adopted for solution x-ray scattering to reach sub-millisecond time resolution primarily in fast folding studies. [17,18] Recently, Arai *et al.* monitored a rapid compaction of *E. coli* dihydrofolate reductase within first 0.3 ms after refolding by a urea dilution jump.[19] The use of high brilliance third generation sources is mandatory for such experiments.

High angle scattering studies

Protein/nucleic acid studies in solution at high scattering angles, corresponding to Bragg spacings of 1 nm or smaller, have been thought to bring structural information in this resolution range, but have been hampered by the extremely weak signals. Third generation synchrotron

sources have made this class of studies feasible in recent years. The use of high angle data shows potential for drug screening [20] and most recently as a means of monitoring altered protein quaternary structure due to molecular crowding.[21] High angle data have been shown to be very useful in evaluating various nucleic acid sugar and phosphate backbone structures deduced by NMR and crystallography, in particular, as regards the base pair stacking of DNA. This approach allowed selection of the most realistic solution structure of DNA oligomers among conflicting crystallographic and NMR structures.[22] A similar approach was taken to experimentally assess the accuracy of DNA oligomer structures obtained by molecular dynamics simulation.[23]

Intermolecular interactions

The intermolecular spatial correlation or structure factor can also be observed in solution x-ray scattering even at relatively low concentrations. For instance, a solution of mildly charged molecules exhibits a broad distribution of weakly correlated intermolecular distances in the range of hundreds of Ångströms. The structure factor offers a unique opportunity to study intermolecular interactions, which are central to many biological functions. The physiological importance of intermolecular interactions is exemplified by a series of studies performed on the eye lens protein crystalline.[24] A direct measurement of like-charge attractive interaction of oligonucleotides, essential in nucleic acid packaging in virus particles, has recently been performed.[25] Sato *et al.* demonstrated that the heme binding can induce strong long-range attractive potential of human serum albumin, suggesting the importance of the attractive potential in molecular recognition in protein-protein interaction.[26]

Interplay with other techniques

Neutron solution scattering has the advantage of being able to adjust solvent contrast to visualize a selected component of a heterogeneous system made up of different electron densities such as protein-nucleic acid complexes and protein-lipid complexes.[27] Simultaneous fitting of x-ray and neutron scattering data results in more reliable structural models.[28] The long range structural information contained in x-ray scattering has also been used to aid NMR structural solutions of large proteins.[29] The use of a low-resolution macromolecular envelope, derived from solution x-ray scattering, for determining initial diffraction phases in crystallography has been in development.[30] The combined use of molecular dynamics simulation has been beneficial to an increasing number of x-ray scattering studies. Human complement factor fragments have been analyzed on the basis of x-ray solution scattering and thousands of simulated structures by molecular dynamics.[31] The best probable models can be selected among the simulated structures that not only fit x-ray scattering data well, but also fulfill other constraints such as inter-domain interactions. A new computational method to utilize spatial restraints to model protein structures using solution scattering data has been developed as a part of the *Integrative Modeling Platform*. [32] Even though the application of these computational methods is presently limited to relatively small biological systems, we expect them to be more widely applicable as computation costs continue to decline.

X-ray fiber diffraction

Many biopolymers are composed of long helical structures. These macromolecules are typically very difficult to crystallize but have a natural tendency to line up parallel to each other to form fibers. The key difference between fibers and crystals is that in fibers, the fundamental structural motifs are randomly oriented about the fiber axis so that the resulting diffraction pattern is cylindrically averaged resulting in loss of information. Despite this limitation, fiber diffraction has been a powerful technique for the study of a wide variety of biopolymers, ranging from simple polypeptides, polynucleotides, and polysaccharides to filamentous viruses, cytoskeletal filaments, and larger assemblies such as collagen microfibrils and muscle

fibers. The availability of specialized beamlines on third generation synchrotron radiation sources in combination with modern high sensitivity, high spatial resolution CCD-based detectors, have been a boon to fiber diffraction studies since these technologies allow resolution of closely spaced diffraction peaks above the high backgrounds typical of these systems to yield superior, more easily interpreted, diffraction patterns. The ability to focus beams from these sources to micron-scale spots provide new scientific opportunities that are only beginning to be realized.

Wide-angle diffraction of oriented fibers

As in conventional macromolecular crystallography, the ability to produce well-ordered systems is crucial to success. Combinations of centrifugation, controlled drying, and exposure to very high magnetic fields have been shown to produce well-oriented specimens. Even with such approaches, the sizes of well-ordered domains within these samples may be quite small ($\ll 100$ microns) requiring small synchrotron x-ray beams to interrogate them. All these approaches were crucial to success in the determination of the first useful structural parameters from a filamentous virus of the potyvirus family, a previously completely intractable system for structural analysis.[33] While diffraction patterns from large macromolecular systems seldom diffract past *ca.* $3\text{\AA}$, many simpler systems diffract to atomic resolution allowing application of refinement techniques similar to those used in conventional macromolecular crystallography. For instance, Nishiyama *et al.* used a combination of neutron and synchrotron fiber diffraction to determine the crystal and molecular structure, together with the hydrogen-bonding system of cellulose I(α) to atomic resolution ($\sim 1\text{\AA}$).[34]

Small-angle x-ray diffraction of muscle

X-ray diffraction is the only technique that can provide molecular level information in muscle tissue under hydrated, physiological conditions at the physiologically relevant millisecond time scale.[35] Such experiments demand the high intensity and superior collimation of synchrotron radiation and have historically been a driving force in the development of synchrotron-based technologies. The availability of small angle instruments on large third generation synchrotrons have enabled new classes of experiments. One technique exploits the interference between the diffraction from the arrays of crossbridges in the two halves of each thick filament to measure the motions of myosin heads to *ca.* $1\text{\AA}$ resolution.[35] This approach was used to provide a new explanation for the so-called “force velocity curve” of muscle whereby numbers of myosin crossbridges generating similar step sizes and unitary forces are adjusted in the muscle to match the load.[36] The new sources have also permitted other challenging studies including those of the indirect flight muscles in living *Drosophila* during tethered flight.[37] (Figure 2) This system is particularly promising for future studies because of its high degree of structural order and the ease of creating transgenic organisms. A major recent technical development for studies of cardiac muscle has been the development of *in vivo* measurements from beating hearts in living animals. These have allowed relating structural changes to questions of direct clinical relevance.[38] One of the most interesting uses of microbeam diffraction so far was an examination of the filament lattice ordering in cryogenically frozen myofibrils of insect flight muscle from a wide range of species giving insights into how insect flight muscle has evolved.[39]

In situ studies of connective tissue

Connective tissues are particularly complex biological materials, being composed of various combinations of collagen fibers, complex glycoproteins and mineralized tissues. A recent advance was the publication of a MIR structure for rat-tail collagen showing the packing of the collagen molecules within the 67 nm long unit cell providing essential structural information for revealing likely mechanisms of proteolysis.[40] For this work, techniques from

macromolecular crystallography were successfully adapted to the study of natural fibers *in situ*. In most studies of connective tissue, however, the emphasis is on the hierarchical arrangement of molecules within the tissues, rather than on the structure of the molecules themselves, with the aim of explaining their physical properties. Diffraction techniques have been used for the study of bone[41], cartilage[42], and cornea[43]. All of these kinds of studies could benefit from using microbeams in order to interrogate the different ordered regions that make up the substructure of these complex materials.

Fiber diffraction and protein misfolding diseases

Amyloid fibers are formed when normally soluble proteins change conformation to form insoluble filaments that may aggregate and deposit in tissues, contributing to pathologies such as Alzheimer's and Huntington's disease among many others. Because of their complexity and insolubility, amyloid fibers have largely resisted characterization by protein crystallography and NMR, so fiber diffraction has been the method of choice for structural studies.[44] Although most amyloid proteins share a cross- β structure it is becoming apparent that there is no reason to assume that all amyloids have the same tertiary or quaternary structure. For instance fibril-forming peptides with a cross- α structure were recently reported.[45] Mammalian prions also cause disorders due to protein misfolding but unlike other amyloid systems, they are infectious. The central event appears to be a change from a predominantly α -helical, non-infectious form of the protein PrP to a predominantly β -sheet form that is then infectious. Illnesses caused by prions include Creutzfeldt-Jakob disease, bovine spongiform encephalopathy ("mad cow disease"), and scrapie. Structural analysis of prions using diffraction and other techniques has recently been reviewed.[46] An example of how microbeams could bring these kinds of studies into a new era is given by Yagi *et al.*[47] who used a 6 μ m beam to interrogate different parts of the structure of individual insulin spherulites. In the future, similar techniques could be envisioned to obtain structural information from amyloid structures *in situ* using sectioned tissue samples.

Conclusions

Third generation sources have finally fulfilled the promise of synchrotron radiation for non-crystalline diffraction. Flux and beam quality, for most studies, are no longer limiting. New sources on the horizon such as the NSLS II (Brookhaven) and Petra III (Hamburg) and proposed energy-recovery linacs and ultimate storage rings such as PEP-X (Stanford) can provide extremely small source sizes and beam divergences, much more suitable for nano-beam studies than current 3rd generation sources. This will enable whole new classes of NCD studies of complex materials. It is also fair to say that the ability to obtain high quality data for SAXS and NCD has outstripped our ability to analyze it fully. The challenge for developing suitable analytical techniques has always been the diversity of the systems studied and the approaches needed. Solution studies have great potential, as yet not realized, for high-throughput structural characterization. The availability of fully coherent 4th generation sources such as XFEL (Hamburg), SPring-8 XFEL (Harima) and the LCLS (Stanford,) in the near future raises the possibility of collecting continuous diffraction patterns from single molecules and using various phase retrieval algorithms to recover their structure. One approach proposes to use laser alignment to orient a stream of macromolecules that passes through the x-ray beam as a means of mitigating radiation damage to allow reconstruction of secondary protein structures to $\sim 7\text{\AA}$ resolution with relatively short exposure times [48].

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Figure 1.

HK97 bacteriophage maturation kinetics studied by time-resolved solution x-ray scattering. A stopped-flow mixer (SF) initiates the maturation by a pH-jump, followed by a series of successive x-ray scattering pattern measurements. A synchrotron storage ring (SR) provides an intense x-ray beam. The direct beam is intercepted by a beam stop (blue disk) and surrounding circularly symmetric x-ray scattering is recorded as a function of time, and azimuthally averaged into one-dimensional pattern as shown in the time course illustration. The peak position shifts toward small angle reflect the larger particle size in the final stage of the maturation. Courtesy of Dr. Kelly Lee, the Scripps Research Institute.

Figure 2.

X-ray diffraction pattern from living *Drosophila* during tethered flight. The high intensity of the 19.3 nm first row line reflections (circles) indicates a high occupancy of actin binding sites by myosin heads poised for active contraction during the downstroke of the wingbeat.