REVIEWS

Emerging applications of small angle solution scattering in structural biology

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Abstract: Small angle solution X-ray and neutron scattering recently resurfaced as powerful tools to address an array of biological problems including folding, intrinsic disorder, conformational transitions, macromolecular crowding, and self or hetero-assembling of biomacromolecules. In addition, small angle solution scattering complements crystallography, nuclear magnetic resonance spectroscopy, and other structural methods to aid in the structure determinations of multidomain or multicomponent proteins or nucleoprotein assemblies. Neutron scattering with hydrogen/deuterium contrast variation, or X-ray scattering with sucrose contrast variation to a certain extent, is a convenient tool for characterizing the organizations of two-component systems such as a nucleoprotein or a lipid-protein assembly. Time-resolved small and wide-angle solution scattering to study biological processes in real time, and the use of localized heavy-atom labeling and anomalous solution scattering for applications as FRET-like molecular rulers, are amongst promising newer developments. Despite the challenges in data analysis and interpretation, these X-ray/neutron solution scattering based approaches hold great promise for understanding a wide variety of complex processes prevalent in the biological milieu.

Keywords: small angle X-ray scattering; anomalous solution scattering; solution neutron scattering; solution structure determination

Introduction

Modern biological applications of small angle X-ray and neutron solution scattering (SAXS/SANS) include low-resolution shape modeling, and the characterizations of protein/RNA folding, intrinsic disorder, conformational transitions, and protein-protein or protein-nucleic acid assembling processes (Fig. 1).[1–5] Even though small angle solution scattering (SAS) typically requires very pure and monodisperse sample, no specialized sample treatments like chemical fixation or crystallization are necessary, making the use of a range of physiologically relevant buffer conditions possible. Furthermore, small sample volume at 1 to 10 mg/mL concentration, rapid data collection at the synchrotron sites (0.5–5 s), practically no size-limitation (depending upon the experimental setup), several dedicated beam-lines at the synchrotron facilities including those with options for automated high-throughput data collection and the availability of user-friendly, automated software for data analysis makes SAXS a

Abbreviations: ASAX, anomalous solution X-ray scattering; FRET, Förster resonance energy transfer; IDP, Intrinsically disordered protein; NMR, Nuclear magnetic resonance; SANS, small angle neutron scattering; SAS, small angle scattering; SAXS, small angle X-ray scattering; SAXS-SEC, small angle X-ray scattering–size exclusion chromatography; WAXS, wide angle X-ray scattering.

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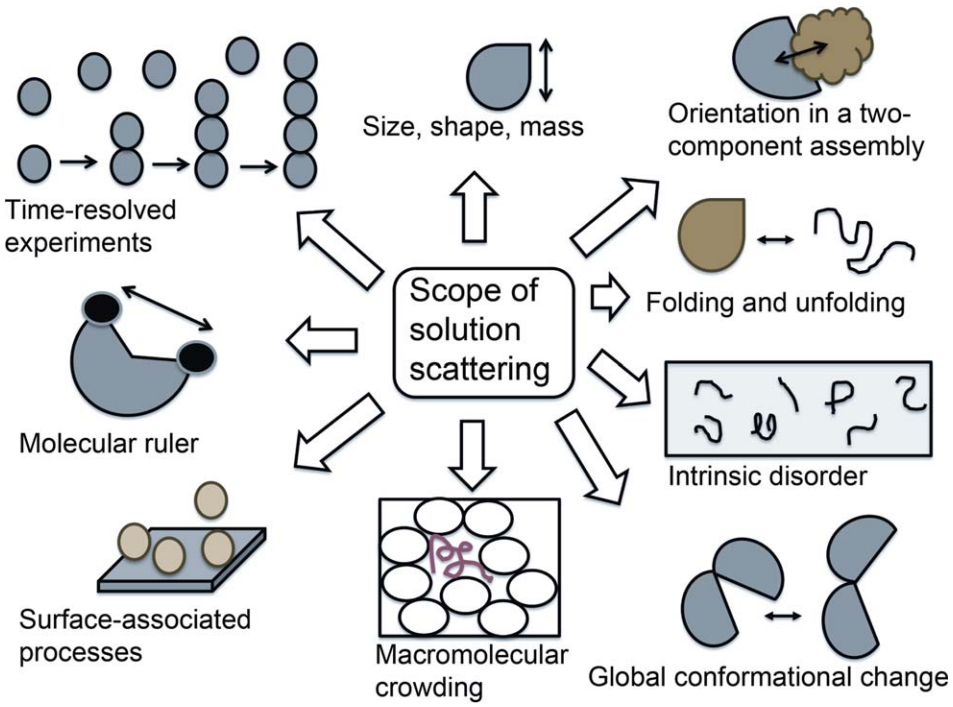

Figure 1. A schematic diagram showing various biological applications of small angle solution scattering technique.

very popular technique.[5–9] Due to the immense popularity of SAXS, guidelines for publications are actively discussed in the research community.[10,11] For the theoretical basis and technical details of small angle scattering, we refer the readers to an excellent, recently published text-book.[12] In this review, we will focus our attention on the modern applications of SAXS and SANS in a variety of problems in structural biology.

Size and shape of a macromolecule

Analysis of SAS data allows ready estimations of mass, radius of gyration, and maximum diameter of a monodisperse macromolecule in solution.[1,2,13] In addition, cross-sectional size and linear mass density (mass per unit length) of a rod-shaped filament and thickness of a disk-shaped particle can be estimated from SAS data.[13–15] These quantities and the pair-wise distance distribution profile computed from SAS data are useful for testing various structural hypotheses and can be used as restraints for structural modeling. While small angle X-ray/neutron scattering[1–4] provides information about the global size and shape of the macromolecule in solution, wide-angle X-ray scattering (WAXS) data obtained at higher scattering angles contains information about its finer structural features.[16] On the other hand, SAXS at ultra-low scattering angles can aid in the elucidation of large-scale, higher-order structures, such as the nucleosome fiber.[17]

When a crystal structure is not available, SAXS provides a quick way to compute a low-resolution, average molecular shape of a mono-disperse, globular macromolecule from its scattering profile.[1,2,18–21] Impositions of anticipated point-group symmetry, connectivity and compactness conditions, and multiple *ab initio* shape computations with consistent results, aid in obtaining an average, low-resolution shape model.[22,23] Due to the inherent ambiguity of SAS-based shape modeling, validations of the model using additional experimental data that are "not seen by the model" are endorsed.[12] Comparisons of the SAS-derived shape models with corresponding known structures indicated excellent agreements for a number of cases.[24] Recently, methods were developed to model RNA structures using experimental SAXS data in conjunction with the structure prediction algorithms.[25,26]

SAS as a complementary technique in structural biology

While X-ray crystallography is a routine tool for the atomic resolution structure determinations of biomolecules held in crystal lattices, SAS can readily reveal their oligomeric states and domain organizations in solution.[1,2] Solution scattering profiles are computed from the atomic co-ordinates of available crystal structures for comparisons with the experimental SAXS/WAXS data.[27–32] In addition to *ab initio* shape modeling, SAS can be used in conjunction with the existing substructures to model their overall tertiary or quaternary organizations.[33–39] Furthermore, SAS-based shape computations with known partial structures can help to predict the location of a missing component within the structure.[40]

Despite the tremendous methodological advances in structure determination techniques, many

multicomponent or multidomain biological entities continue to pose considerable challenge to structure determination. An effective way to tackle this challenge is to combine information obtained from complementary methods, such as crystallography, nuclear magnetic resonance spectroscopy (NMR), transmission electron microscopy, mass spectrometry and SAS, to learn about the structural organizations of proteins and their assemblies at data-dependent levels of details.[41–45] For example, Wang *et al.,* used information about overall shape, relative orientations of the components and the footprints of buried, interface residues obtained from SAXS, NMR and mass spectrometric data to learn about the oligomeric states of a chemokine CCL5.[44] Hybrid methods are developed to integrate SAS data with other experimental data for structural modeling.[46–49] In particular, NMR with SAS has emerged as a powerful approach to elucidate the structural organizations of various biological entities.[44,49–52] Thus, SAS in combination with other complementary techniques can be an effective tool to address structural problems that are not easily amenable to a single technique.

Neutron and X-ray scattering with contrast variation

Small angle neutron scattering or SANS contrast variation experiments reveal the relative dispositions of components within a multicomponent assembly.[4,13,53–56] A difference in the neutron scattering length density of hydrogen (H) and deuterium (D) is exploited in a SANS contrast variation experiment to vary the excess scattering density or contrast of the scattering macromolecule.[53–57] A typical contrast variation dataset is obtained by collecting SANS data at a number of different H/D ratio, from which individual scattering profile for each component in the assembly as well as the cross-term describing their relative orientations in a two-component assembly can be retrieved.[57] In addition, distance between the centers of mass of the two components can be estimated from SANS contrast variation dataset.[57] Thus, SANS with H/D contrast variation is very well suited for analyzing relative positioning of the components within an assembly and an induced-fit or a mutual induced fit mode of molecular recognition.

In an alternative contrast matching experiment, neutron scattering contribution from one of the components can be selectively "matched out" or abolished by adjusting the H/D ratio of the buffer. The "matching out" conditions for lipids, proteins, carbohydrates, and DNA/RNA are at about 10 to 14%, 40 to 45%, 47%, and 65 to 72% D_2O, respectively, which can be changed by deuteration.[1,4,13,57] Neutron scattering with contrast matching is an exceptional tool for observing a selected component in the presence of another "invisible" component in a heteromeric assembly.

Scattering with contrast variation aided in elucidating the organizations of many two-component assemblies, such as nucleoprotein assemblies and protein-protein assemblies with a deuterated protein component.[58–64] Owing to the differences in scattering densities between proteins and lipids as well as proteins and detergents, SANS is useful for studying membrane proteins.[65,66] Recently, a hybrid strategy to model membrane proteins in lipidic environment by combining SAXS and SANS data was described.[67] Thus, the feasibility of deuterium-labeling the macromolecules for changing contrasts and the power of H/D contrast variation allows SANS to characterize a range of two-component assemblies, which is not possible to achieve solely based on SAXS.

Due to a limited number of neutron scattering facilities, a large amount of sample requirement (150 μL or more at 5–10 mg/mL concentration),[12] noisier data due to in-coherent scattering and long data collection times (a few hours), resurgence of neutron scattering is gradual**.** In comparison, SAXS typically requires only 20 to 30 μL of sample and a few seconds of exposure at a powerful synchrotron source. Although less potent than SANS with H/D contrast variation, SAXS with varying amounts of sucrose provides another avenue to protein contrast matching and a limited range of contrast variation.[68–72] Moreover, sucrose can reduce the effect of radiation damage at the powerful synchrotron sources.[73] However, unaddressed issues such as enhanced viscosity at higher sucrose concentration may limit the utility of these SAXS-based contrast variation experiments and care should be taken to account for these effects.[72] High salt conditions are effective for SAXS-based contrast variation experiments, but have limited applicability in biological problems.[1,72] Due to the relative ease of obtaining SAXS data in comparison to SANS, a renewed search for additional contrast variation agents for SAXS that are suitable for biological samples will be rewarding to the SAS research community.

Folding, conformational flexibility, and intrinsic disorder

SAS is a powerful global sensor of the folding states and conformational changes in proteins and nucleic acids. A global indication of "folded-ness" of the biomolecule can be obtained from the Kratky plot ($I.q^2$ vs. q, where I is the scattering intensity, and q is the momentum transfer).[74] Kratky plots, together with the changes in sizes and pair-distribution function profiles, are used to track folding-unfolding behaviors of biomolecules under varying experimental conditions.[74] Furthermore, changes in sizes and pair-distributions functions are model-free indicators of large-scale conformational changes in folded, non-aggregated proteins, which can be combined with shape or structural modeling. Although the

applications of SAXS are limited to detections of large movements,[75–79] WAXS can sense small amplitude structural changes in proteins.[80,81]

A large number of naturally occurring proteins are either disordered or contain long stretches of unstructured regions, and are generally classified as intrinsically disordered proteins (IDP).[82,83] The disordered regions often fold in the presence of a binding partners(s) and play crucial role in molecular recognition.[82,83] Due to a lack of rigid, well-defined structure, characterization of the IDPs pose a challenge to structural biologists. SAS is naturally suitable for studying these IDPs, as the scattering profile represents an ensemble of conformations of the IDP in solution.[82] An IDP or a flexible protein can be identified from the global features in the SAS data, such as the Kratky plot, a left-skewed pair-distribution function or a Porod-Debye plot.[82,84,85]

In addition to the global analysis, a number of different computational methods were developed to generate an ensemble of protein conformers from the SAS data.[86–89] These methods typically involve computational generations of multiple conformers followed by their selections based on their compatibility with the experimental SAS data.[86–89] Ensemble modeling with SAS data yielded structural information on the spatial occupancy of glycans in glycosylated proteins that are notoriously difficult to crystallize.[90] Thus, SAS in combination with suitable computational methods can provide potentially unrestricted access to the conformational spaces of flexible proteins under different conditions, which is a clear advantage over crystallography.

Due to the low information content of SAS and a large number of parameters required for an exhaustive description of an IDP, combining SAS with additional complementary experimental data is a sensible approach, when possible. SAS analysis of IDPs is typically complemented with solution NMR, circular dichroism, dynamic light scattering and other hydrodynamic data analyses.[91] Synergy between NMR and SAS were exploited in numerous studies of IDPs, which is recently reviewed elsewhere.[92] Bertini *et al*. used SAXS with NMR on lanthanide-labeled proteins to determine the most abundant conformers of highly flexible calmodulin, which might be needed for a better explanation of its function in solution.[93] In a recent article, exhaustive SAXS and NMR based ensemble analyses, together with extensive cross-validations, were used to predict conformations of IDPs relevant to neurodegenerative disorder.[94] SAS, particularly in combination with NMR, will continue to play a pivotal role in providing key structural descriptions of IDPs.

Macromolecular crowding

SAS provides an elegant way to examine the important but often neglected effect of macromolecular crowding on proteins.[95] WAXS data suggested that concentrated conditions inhibit breathing motions in proteins.[96] Furthermore, SAS was found to be a suitable tool to study the effects of crowding on RNA folding.[97,98] SAXS-based studies indicated that crowding promotes compactions in the tertiary and quaternary structures of certain modular enzymes and their complexes.[99,100] Quite significantly, this compaction correlates with higher enzymatic activity.[99,100] On the other hand, a recent, remarkable SANS study on the intrinsically disordered, deuterium-labeled protein N in the presence of two different un-labeled proteins as crowding agents under their "contrast matching" conditions indicated minimal effect of crowding on the protein N.[101] Valuable new insights on the effects of crowding on nucleic acids and deuterated proteins, both folded and intrinsically disordered, can be obtained from SANS in the presence of these "invisible" contrast-matched, unlabeled protein crowding agents that realistically mimics the intracellular environment.

SAXS-based molecular ruler

SAS provides global information on size and shape but no local structural information on a particular site can be generally obtained. However, for certain biological applications, such site-specific details can be very instructive. One way to obtain local information from SAXS is to label the protein with electron-rich elements.[102,103] DNA coupled to gold nanocrystal as a probe was successfully used to measure the length of DNA using scattering interference.[104] In another recent SAXS study, gold cluster-labeled DNA was used to track conformational changes induced by a DNA mismatch repair protein, leading to new insights into the repair mechanism.[105] Under the experimental conditions of low concentrations, scattering from the electron-dense gold-component dominated the total scattering.[105] Grishaev *et al*. determined the structural organization of lead-substituted calmodulin-peptide complex using a combination of sucrose contrast matching, conventional SAXS/WAXS and NMR residual dipolar coupling measurements.[70] Scattering contribution from the protein parts was contrast matched by sucrose, leading to the heavy-atom dominated scattering profile, which was critical for the correct positioning of structural components.[70] Unlike the Förster resonance energy transfer or FRET-based molecular rulers that are restricted around the Förster distance of the available donor-acceptor pairs, SAXS-based molecular rulers can be potentially used to measure a much larger range of distances.[104] Therefore, use of labeled samples for SAXS analyses of localized conformational changes, molecular recognition processes and multimeric organizations, especially coupled with time-resolved SAXS experiments,[104]

electron microscopy,[104] and contrast matching,[70] will almost certainly become more common in future.

Anomalous solution X-ray scattering

Anomalous scattering properties of metal ions, which are routinely exploited in multiwavelength anomalous dispersion phasing methods in macromolecular crystallography, has been used to a limited extent in SAXS.[106–109] Strong anomalous solution X-ray scattering (ASAXS) of terbeium at the L_{III} edge was exploited to measure the mean distance between the terbiums substituting the calcium binding sites within parvalbumin and the center of mass of the protein, which was consistent with the crystallographic results.[107] Makowski *et al*. recently used anomalous contribution from iron in the solution scattering data at the Fe K edge to determine the distance between centers of mass of hemoglobin/myoglobin and the metal ion.[109] Anomalous contribution due to the metal label to the total scattering is typically quite small, requiring very careful measurements at a tunable-wavelength, powerful synchrotron X-ray source. Although small anomalous contribution due to the cross-term between the label and the protein segment is usable, scattering contribution due to the interference within the anomalous scattering metal component itself is negligible.[109] Theoretical calculations predict that the use of metal clusters, such as a gold nanocrystal, may alleviate this problem for ASAXS studies.[110] With further explorations of dissimilar anomalous scattering atomic clusters as labels to measure specific distances, and concomitant development of theories,[110] applications of ASAXS-based molecular rulers to probe intricate conformational changes and molecular recognition processes may become routine.

Mixed systems

Sample heterogeneity pose challenges to routine SAS data analysis that generally assumes stable molecular species of one kind in dilute, noninteracting solution state. However, many interesting biological phenomena take place in a mixture of interacting particles in solution, which might include weak and transient interactions. SAXS is one of the very few structural techniques available to study these mixed systems.

For a separable mixture, such as a monomer with a stable dimer, SAXS at a powerful synchrotron source coupled with in-line size-exclusion column chromatography (SAXS-SEC) allows an elegant way to study individual entities while being eluted.[111,112] In an extension of this coupled approach, scattering from a low-affinity complex of actin with a peptide ligand was measured using SAXS-SEC in a buffer saturated with this peptide ligand, while effectively excluding any scattering from the higher aggregates.[113,114] Sokolova *et al*. modeled intermediate filament assembling pathway from SAXS data by finding out conditions dominated by each of the constituents.[115] However, separation of individual entities in a mixture may not be possible or desirable in all cases.

Computational approaches were developed for extracting individual scattering profiles of the constituents from concentration-dependent SAXS dataset obtained from mixed systems involving weak homo- or hetero-oligomers co-existing with monomers.[116–118] These approaches are original in a sense that they allow derivations of low-resolution shape models as well as the association constants and assembling pathway from the same experiment.[116–118] Petoukhov *et al*. recently published a method for composition analysis and shape modeling of oligomeric assemblies from condition-dependent scattering curves for a variety of user-defined mixed system scenarios.[119] However, conformational changes upon binding can make simple interpretations of SAS data obtained from mixed systems difficult. Above computational approaches involving equilibrium mixtures can be potentially used in conjunction with time series of SAXS to provide a rich variety of information on the mixed system.[116]

Time-resolved SAS to track biological processes

Time-resolved SAXS, SANS, and WAXS studies can provide direct mechanistic insights on the course of a biological reaction/process in terms of its constituent components, including the pathway intermediates. Data collection capability up to about 100 ps time resolution at the modern, powerful synchrotron sources equipped with better detectors and better methods to trigger an event make it possible to observe the time progressions of SAXS/WAXS profiles of a variety of evolving systems.[120–124] SANS provides another avenue for time-resolved studies, albeit at a longer time scale, which can be judiciously combined with contrast matching for a two-component system.[125,126] The target biological process can be initiated by a number of ways, such as laser light for a light-initiated process (pump-probe) or by using a mixing device or by quickly removing one component from the mixture, depending upon the nature and time-scale of the process under investigation.[120–124] Thus, time-resolved SAS is a valuable tool for investigating a range of processes including the slow assembling (second or longer time-scale) as well as fast folding kinetics (second to 100 μs) and ultra-fast protein movements in the subnanosecond region.[12] In recent times, 10 to 100 fs X-ray laser pulses are opening up the first-time possibility to observe even faster, light-triggered processes, such as a "protein-quake."[124,127]

Time-resolved SAS is suitable for probing many biological processes, such as capsid maturation and fibrillation pathways that are difficult to study by traditional means. Examples include movements in

light-driven proton pumps,[128] microtubule formation,[129] allostery and structural dynamics in hemoglobin,[120,130] fibrillation pathway in insulin amyloid and alpha-synuclein,[131,132] viral capsid maturation,[133–135] protein folding,[136–138] and RNA folding.[139,140] In a seminal study by Vestergaard *et al*., insulin fibrillation process was modeled using time-resolved SAXS data, leading to an elongation pathway for amyloid fibril formation from a helical nucleus.[131] In another study, global size information obtained from time-resolved SAS was effectively complemented with local solvent accessibility data from time-resolved hydroxyl radical footprinting to provide unique insights into the RNA folding pathway.[141] Recently, Chen *et al*. performed time-resolved SAXS under protein contrast matching conditions to track salt-induced DNA unwrapping in the nucleosome core particle.[72] An ability to probe unrestricted movements of label-free biomolecules in near-physiological conditions at a wide range of temporal resolutions is a huge advantage of time-resolved SAS. Furthermore, time-resolved SAXS/WAXS on proteins with site-specific heavy atom labeling can be used to track local changes, while simultaneously monitoring the global changes in the protein.[142]

Concluding remarks

Advances in SAS methods opened up the exciting opportunities to learn about size, shape, folding, recognition, flexibility and disorder of soluble single/multidomain proteins, membrane proteins, glycoproteins, intrinsically disordered proteins, DNA/RNA, and their assemblies (Fig. 1). Feasibility of time-resolved SAXS/WAXS studies of biological processes, contrast variation for multicomponent assemblies, convenient applications of SAXS or ASAXS on heavy-atom labeled samples as molecular rulers and appropriate combinations of above are some of the key advantages and future promises of SAS technique. Furthermore, grazing incidence solution scattering and related reflectivity techniques are suitable for studying membrane-associated proteins in near-natural environment.[143] Although SAS is essentially a low-information technique, combining SAS with complementary techniques can circumvent this limitation in many cases.[47–50] However, much work remains in method developments for data acquisition, analysis and model validation.[144–146] In the coming years, modern X-ray sources and methodological advances will probably open up unprecedented ways to reconstruct solution structures of biomacromolecules, and will conceivably reveal unforeseen wealth of information on protein motion, which will be a giant step forward.[114,124,147,148]

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