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Current Opinion in Structural Biology

Small-angle neutron scattering contrast variation studies of biological complexes: Challenges and triumphs

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Abstract

Small-angle neutron scattering (SANS) has been a beneficial tool for studying the structure of biological macromolecules in solution for several decades. Continued improvements in sample preparation techniques, including deuterium labeling, neutron instrumentation and complementary techniques such as small-angle x-ray scattering (SAXS), cryo-EM, NMR and x-ray crystallography, along with the availability of more powerful structure prediction algorithms and computational resources has made SANS more important than ever as a means to obtain unique information on the structure of biological complexes in solution. In particular, the contrast variation (CV) technique, which requires a large commitment in both sample preparation and measurement time, has become more practical with the advent of these improved resources. Here, challenges and recent triumphs as well as future prospects are discussed.

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Current Opinion in Structural Biology 2022, **74:**102375

This review comes from a themed issue on **Biophysical Methods**

Edited by **David Fushman** and **Dagmar Ringe**

For complete overview of the section, please refer the article collection - Biophysical Methods

Available online 28 April 2022

https://doi.org/10.1016/j.sbi.2022.102375

0959-440X/Published by Elsevier Ltd.

Introduction

Contrast variation (CV) refers to the method by which the neutron scattering intensity from a multi-component complex can be separated into that from the components through the use of hydrogen-deuterium (H-D) substitution in the complex and/or the solvent. The coherent scattering intensity from a dilute solution of homogeneous biological macromolecules in solution can be written as:

$$I(q) = n(\Delta\rho)^2 \langle \left| \int \exp(i\boldsymbol{q}\cdot\boldsymbol{r}) d^3r \right|^2 \rangle, \quad (1)$$

where <> denotes the average over all orientations, n is the number density of molecules, $q = 4\pi \sin(\theta)/\lambda$, where λ is the neutron wavelength and 2 θ is the scattering angle, and $\Delta\rho$ is the contrast. The contrast is written in terms of the neutron scattering length densities (SLDs) of the molecule and solvent, ρ and ρ_s, respectively, as:

$$\Delta\rho = \rho - \rho_s. \quad (2)$$

Thus, when the SLD of the molecule and solvent are equal, $\Delta\rho = 0$ and $I(q) = 0$ at that contrast. This is called the contrast match point of the molecule. If the molecule consists of components with different SLDs, then each component, as well as the entire complex has a contrast match point. This is illustrated in cartoon form for a two-component complex in Figure 1. Therefore, the scattering from one component can essentially be eliminated while that of the other is enhanced and vise versa. By measuring the complex at a number of different contrasts, the scattering from the individual components can be obtained if the data are of sufficient quality. In any case, information on the size of each component and their spatial relationship can usually be obtained. This unique information can be extremely useful as constraints when building model structures of the complex [1••].

While a formal experimental protocol and data treatment has been described for SANS CV experiments of two-component complexes [2–4], the technique can also be applied at some level to complexes with more than two components. The application of SANS with CV has been described in recent reviews and tutorials [5–9], which highlight some of the challenges in performing successful experiments. A critical step is the preparation of the sample. Figure 2 shows the neutron SLDs of several biological molecules as a function of the percentage of D_2O in the solvent. The match point of the protein is at 40% D_2O, where the SLD of the protein and water are the same. The match point of DNA is at 65%

Figure 1

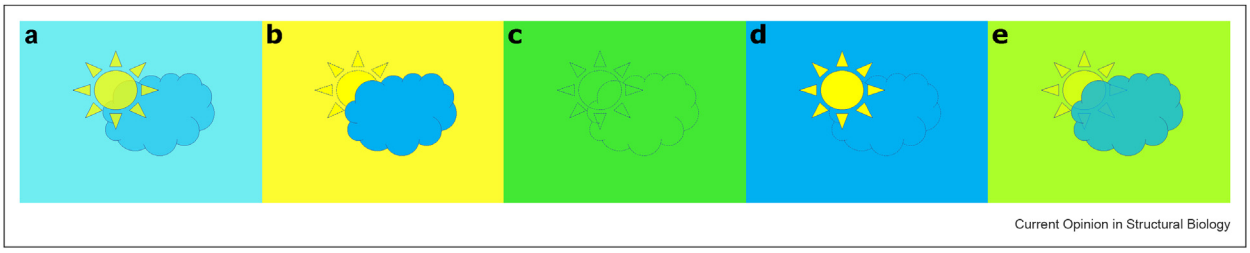
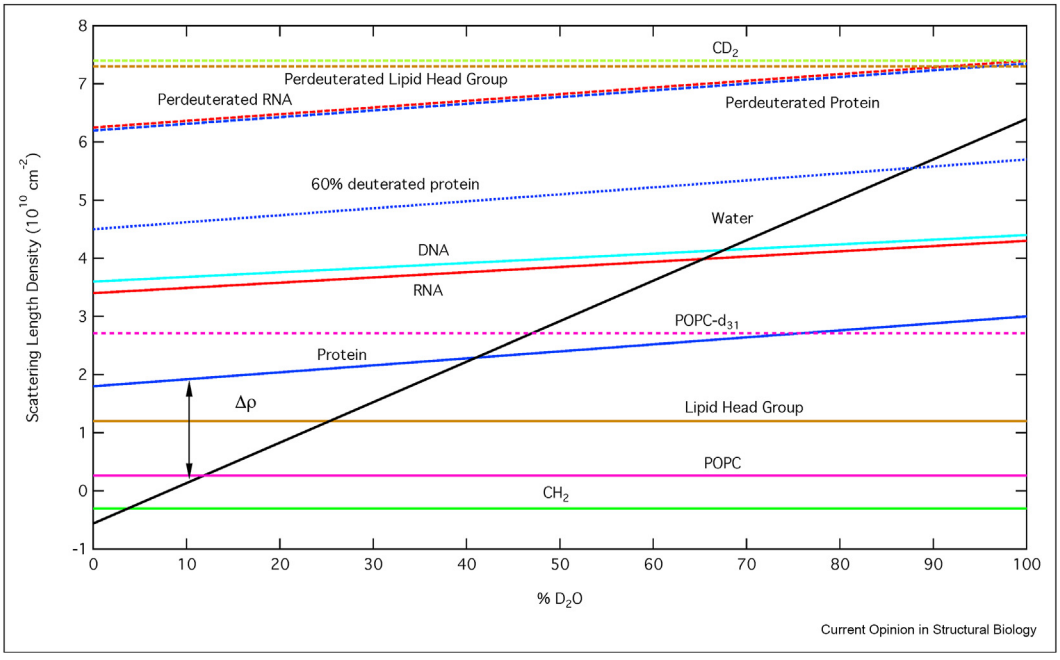


Illustration of contrast variation for a two-component complex represented by the sun and a cloud. (**a**) Both components contribute to the scattering, but the sun contributes more than the cloud. (**b**) The match point of the sun; only the cloud contributes to the scattering. (**c**) The match point of the entire complex; $I(q) = 0$. (**d**) The match point of the cloud; only the sun contributes to the scattering. (**e**) Both components contribute to the scattering, but the cloud contributes more than the sun.

Figure 2



Plot of neutron scattering length densities (SLDs) of several biological molecules as a function of the percentage of D_2O in the solvent, assumed to be water in this case. The SLD of water is also shown for comparison. The contrast, $\Delta\rho$, between a protein and water in 10% D_2O solvent is illustrated by the double-sided arrow as an example. Note that the SLDs for protein, DNA and RNA are representative values, as they depend on the protein or nucleic acid sequence and H-D exchange of the labile hydrogen atoms.

D_2O. These two match points are sufficiently different to negate the need for deuterium labeling when preparing a protein-DNA or protein-RNA complex. The same is true for a protein-phospholipid complex such as protein-POPC. Protein-detergent complexes typically don't require deuterium labeling since the main component of detergents is CH_2. However, labeling is often employed to increase the possible contrast matching conditions. To aid in sample preparation, SLDs can be calculated for a wide range of biological complexes using available software [2,4].

On the other hand, protein–protein complexes require deuterium labeling in order to achieve contrast between the components. Figure 2 shows that a protein with random deuteration of about 60% of the non-exchangeable hydrogen atoms has a match point at about 88% D_2O, which would provide sufficient contrast between the two components. While many advances have been made in this area [8], there are still challenges in the cases where post-translational modifications are present or where non-random or segmental deuterium labeling is desired.

Contrast variation implies that the complexes are measured in solvents with a range of H_2O:D_2O content. The more D_2O in the solvent, the higher the chance for unwanted deuterium-induced aggregation. Complexes with deuterated components can also be affected, even in solvents with little or no D_2O. If the aggregation can't be mitigated, the sample can only be measured in a limited range of contrasts, decreasing the amount of information that can be obtained. It is extremely important that the integrity of the sample be checked prior to performing the CV data analysis [5–7] so that only the data from non-affected samples are used.

In spite of the challenges in performing SANS CV experiments, there have been many triumphs using the technique to study the structure of protein–protein and protein-nucleic acid complexes, as well as membrane proteins in biomimetic environments, model biological membranes, nanoparticles for drug delivery and biotherapeutics, and proteins in complex environments. Recent achievements in these areas are highlighted here.

Protein-nucleic acid complexes

Protein-nucleic acid complexes such as the nucleosome were some of the first complexes measured with SANS CV [10]. These were groundbreaking experiments that revealed that the DNA was on the outside of the complex and the protein was on the inside. Studies of the nucleosome have continued [11], with better SANS and SAXS data available to allow detection of differences between different types of nucleosomes. Recently, SANS CV along with SAXS has been used to study an overlapping dinucleosome that forms as a result of nucleosome collisions [12•]. Another study combined atomic force microscopy and SANS CV of isolated chicken erythrocytes nuclei to confirm the bifractal structure of chromatin, with the DNA serving as its framework [13]. Protein-RNA complexes lend themselves particularly well to SANS CV studies combined with NMR, as described in a recent practical guide [14••] that also discusses current modeling approaches.

Protein–protein complexes

Deuterium labeling is required in order to achieve contrast between the components in a protein–protein complex. While the earlier work on the KinA–Sda complex [3] is an excellent example of how to apply SANS CV to a protein–protein complex, it is not always possible to obtain data at high protein concentrations over such a wide range of contrasts. Thus, it isn't always possible to obtain the scattering intensities of the individual components in the complex by the decomposition method [2,3]. However, it is often still possible to obtain the radius of gyration for each component and their spatial arrangement with respect to each other using the Stuhrmann [15] and/or parallel axis theorem [16,17] analysis as shown in a recent study of the structure of vitronectin bound to deuterated plasminogen activator inhibitor-1 [18]. In addition, data can often be obtained at the match point of at least one of the components to obtain the scattering intensity of the unmatched component to aid in the structure modeling of the complex, as illustrated in a study of the chaperone, SurA, bound to perdeuterated unfolded outer membrane protein [19]. This approach was also taken in a systematic study of some of the components that make up the replication transcription complex of SARS-CoV-2 [20•], where deuterated Nsp7 (dNsp7) in complex with protiated Nsp8 was measured at the match point of dNsp7 to compare the structure of Nsp8 in the complex and by itself in solution. A similar tactic was used in a SANS CV study of the cadherin-catenin adhesion complex using deuterated α-catenin bound to protiated β-catenin-cadherin [21•]. These studies [20•,21•] also used small-angle x-ray scattering (SAXS) data as another contrast point and additional constraint for structure modeling.

SANS CV studies where data has been obtained at several contrasts are important to constrain model structures in their own right, as the structures need to be consistent with the data at all contrasts measured. One recent study combining SANS CV and modeling [22•] revealed the structure of sulfite reductase heterodimers containing a single copy of flavoprotein reductase and deuterated hemoprotein oxidase subunits. Here, SAXS was used to confirm that there were no deuterium effects by measuring both deuterated and protiated hemoprotein oxidase in solvents with varying amounts of D_2O and observing no change in the scattering curves. SAXS is an ideal method for testing for deuterium effects since x-rays cannot discriminate between hydrogen and deuterium.

SANS CV has also been used recently for kinetic studies by contrast-matching the protiated component in ~40% D_2O solvent while focusing on structural changes in the deuterated component as a function of time. In one study, GFP was perdeuterated to give it high contrast in 42% D_2O, thus obtaining good time-resolved SANS data of its degradation in the presence of a much larger contrast matched complex of PAN-20S proteasome [23••]. In another experiment, the elongation rate and average fibril length of assembling amyloid fibrils of α-synuclein were estimated by adding deuterated monomers to a solution of contrast-matched protiated α-synuclein fibril seeds and monitoring the scattering during the initial stages of seeded growth [24]. The inverse CV conditions can also be used in order to contrast match a partially deuterated component in 100% D_2O to study the protiated component under conditions where there is less incoherent scattering from hydrogen in the solvent. This approach was taken in the study of subunit exchange in αβ-crystallin oligomers [25] and to tease out the mechanism of mosquito-larvicidal binary toxin internalization into the cell membrane [26].

Integral membrane proteins in biomimetic environments

Integral membrane proteins (IMPs) contain hydrophobic transmembrane regions that are embedded in the lipid bilayer. One way to stabilize IMPs for SANS solution studies is in a protein-detergent complex. If the scattering from the detergent is matched out, then the structure of the membrane protein can be studied. However, the match point of many detergents is close to that of H_2O, where the incoherent scattering from hydrogen is high. Nonetheless, cytochrome c6 binding to trimeric photosystem I from the cyanobacterium *Thermosynechococcus elongatus* has been studied in 5% D_2O, the match point of the stabilizing detergent, n-Dodecyl-B-D-Maltoside (DDM) [27]. In another study, both static SANS and SEC-SANS were used to study the *Streptococcus pneumoniae* NADPH oxidase (SpNOX) stabilized by the detergent, lauryl maltose neopentyl glycol (LMNG), using deuterated SpNOX at the match point of LMNG [28••]. A more extensive SANS CV and SAXS experiment measured the ammonium transporter AmtB from *Escherichia coli* in complex with DDM at four different contrasts to differentiate between the individual components (protein, DDM head group, DDM hydrophobic tails) in the complex without the need for deuteration of the protein or the detergent [29].

Another approach to measuring detergent-stabilized IMPs is to use deuterated detergent with a match point near 100% D_2O. Thus, measurements can be made under conditions where the incoherent scattering is much lower. Deuterated DDM was used to stabilize the glutamate receptor, GluA2, so that it could be investigated in its resting state and bound to AMPA and GYKI-53655 [30]. The bacterial holo-translocon (HTL) protein transport machine was studied in the same way, also using deuterated DDM [31•].

Similar approaches can be used for IMPs or peripheral membrane proteins stabilized by vesicles, bicelles or nanodiscs as detailed in a recent perspective article [32]. For example, a fragment of the scaffold protein, dystrophin, known to interact with membrane lipids, was examined when bound to 100% D_2O contrast-matched zwitterionic (DMPC-d_{67}:DHPC-d_{35}) and anionic bicelles (DMPC-d_{67}:DMPS-d_{54}:DHPC-d_{35}), revealing structural changes when bound to the anionic bicelles [33]. Additionally, conformational states of the ABC transporter, MsbA, were obtained from SANS CV measurements of MsbA in 100% D_2O contrast-matched nanodiscs consisting of deuterated MSP1D1 protein and deuterated phosphatidylcholine lipids [34]. Finally, SANS CV structural studies of Gramicidin A and WALPS53 were conducted in the 100% D_2O contrast-matched cubic phase consisting mainly of deuterated monoolein [32].

Model biological membranes

Important insight into the function of membrane-binding proteins can be obtained using model biological membranes. A good example is peptide-mediated vesicle fusion. The use of SANS CV with deuterated lipids allows for even more flexibility in the contrast-matching conditions. The transformation of large unilamellar vesicles (LUVs) into multilamellar vesicles with a collapsed interbilayer spacing due to membrane adhesion induced by antimicrobial peptides was studied using a strategic mixture of POPE-d_{31}, POPG and POPG-d_{31} with a match point the same as that of the peptides (43.7% D_2O) [35]. Thus, a scattering signal was detected only when there was peptide fusion-induced lateral separation of the lipids.

In another SANS CV study where data were obtained from LUVs at three different contrasts, it was shown that a variant of the gp41 fusion peptide that doesn't strongly promote fusion associates with the headgroup region of a POPC-d_{31}:POPS:cholesterol lipid bilayer and distorts it in a manner that still promotes fusion to a limited extent [36]. Other recent studies of protein interactions with model lipid bilayers include measurements of DPPC-d_{75} LUVs in 100% D_2O, which showed that amyloid beta monomers interacted with LUVs in the fluid phase and not in the gel phase, penetrating mainly the outer leaflet [37], and measurements of melatonin interacting with contrast-matched, phase-separated DSPC:DSPC-d_{70}:POPC:DOPC:cholesterol LUVs, which revealed that melatonin stabilized the liquid-ordered/liquid-disordered phase coexistence over a wide temperature range [38•].

SANS CV can also be used to study transport phenomena in model membranes, as detailed in a recent study of inter-membrane transport between contrast-matched LUVs consisting of POPC-d_{31} or POPS-d_{31}, which discovered that the energetic barrier of cholesterol exchange is enthalpic in POPC membranes, but has a significant entropic component in POPS membranes [39]. Additionally, peptide-induced lipid flip-flop between asymmetric bilayers with a DMPC-d_{54} tail-deuterated outer leaflet and a POPC-d_{13} head-deuterated inner leaflet was studied for LUVs interacting with several different antimicrobial peptides by tracking the loss of scattering signal as the lipids spread more uniformly in the bilayer over time [40•].

It also can be useful to use SANS CV to study model biological membranes themselves. In one such study, small unilamellar vesicles (SUVs) with asymmetric bilayers produced using DPPC-coated silica nanoparticles that exchanged lipids with DPPC-d_{62} symmetric SUVs were studied in 100% D_2O using SANS CV and the distribution of DPPC in the resultant DPPC:DPPC-d_{62}

vesicles was obtained [41]. In another study, the structural parameters of palmitoyl sphingomyelin (PSM) and stearoyl sphingomyelin (SSM) LUVs were determined using a combination of SAXS, SANS CV at four contrasts, including PSM-d_{31} in 100% D_2O, and MD simulations [42•].

Therapeutic nanoparticles

Therapeutic nanoparticles include carriers for drug delivery and gene therapy as well as nanoparticle vaccines. They are well-suited for SANS CV studies since they are multi-component complexes consisting of protein, nucleic acid, detergent and/or lipid, perhaps in addition to other molecules used to functionalize their surfaces. Earlier work on MS2 phage particles showed how to use SANS CV to separate the scattering from the protein outer shell and the RNA in the core of the particle [43] and to obtain the molecular weight of each component. When nanoparticles can be approximated as spheres, the scattering can also be modeled as a core-shell particle. A global fit to the model using the data at all contrasts enables the determination of the radius of the core and thickness of the shell(s), as well as their SLDs, which can help determine the amount of water or other molecules in a given layer. This approach was used recently to determine the location of a partially-deuterated anti-inflammatory compound (AIC) in mRNA-containing cationic lipid nanoparticles (LNPs) consisting of dilinoleylmethyl-4-dimethylaminobutyrate (DLin-MC3-DMA), abbreviated as MC3 [44••]. By fitting the data at five different contrast to a core-shell model, it was determined that a high percentage of the AIC is in the shell of the MC3 LNP. Another study used SANS CV to determine the structure and composition of four mRNA-containing MC3 LNPs containing components with different amounts of deuteration, including two LNPs that were contrast-matched near that of protein (~43% D_2O) [45••]. Measurements of all of the samples at four or five different contrasts enabled the determination of the distribution of the lipid components in the absence and presence of Apolipoprotein E.

SANS CV was also used to study how modifications in the manufacturing procedure affect the internal structure of mRNA-containing LNPs consisting of DOTAP and differing amounts of positively-charged protamine [46]. The SANS results were correlated with mRNA release and transfection efficacy measurements. Finally, a nanocarrier-cargo co-assembly was studied by SANS CV using sub-20 nm three-helix micelles (3HMs) consisting of peptide polymer, PEG and alkyl chains, with a cargo molecule, doxorubicin (DOX) [47]. Particles with both deuterated and hydrogenated alkyl chains were measured in 100% D_2O to deduce that DOX intercalates into the 3HM alkyl core, near the core-shell interface.

Non-spherical therapeutic nanoparticles can also be examined with SANS CV. A recent example is the characterization of the Novavax, Inc. nanoparticle vaccine for respiratory syncytial virus (RSV) [48•]. SANS CV and SAXS data showed that the vaccine consists of a cylindrical micellar core of approximately 350 polysorbate 80 (PS80) monomers with a shell consisting of five RSV fusion protein trimers arranged in an antiparallel configuration around the core such that the PS80 stabilizes the transmembrane region of the protein.

Proteins in multi-component environments

A particular challenge for structural characterization of compound systems with SANS is the delineation of the various components. While lipids and proteins in a cell have different SLDs, all of the lipids and proteins will scatter and it is not possible to differentiate between proteins in the membrane and those in other parts of the cell, for example. This issue has been tackled recently to study a cell membrane *in vivo* by judicious labeling of the gram-positive bacterium *Bacillus subtilis* to make only the cell membrane visible to neutrons by re-introducing hydrogenated fatty acids while growing the organism in deuterated media [49]. While this type of manipulation isn't always possible, this work paves the way for future experiments where unlabeled lipids or proteins can be incorporated into similar systems.

A simpler system where deuterium labeling can be incorporated consists of monoclonal antibodies (mAbs) in protein A chromatography resins. A recent study used SANS CV to study IgG1 in a flow cell containing contrast-matched silica-based protein A resin, demonstrating the feasibility of the technique to study mAbs in other resins containing agarose and cellulose [50•].

An area of interest to both the pharmaceutical and food industries is the study of proteins in the frozen state. The presence of ice complicates the interpretation of SANS data since there can be significant scattering from the ice-air interface. However, through the use of SANS CV, the contribution from the ice-air interface can be matched out in 8% D_2O [51]. A recent study demonstrates that, when combined with data in 100% D_2O, a complete picture of the aggregation behavior of a protein can be obtained during an *in situ* freeze-thaw cycle [52].

Concluding remarks

The SANS CV technique is alive and well as evidenced from the recent work highlighted here. With new algorithms for atomic structure prediction such as AlphaFold [53,54] and RoseTTAFold [55], continuing innovations in deuterium labeling [56], including in multi-component environments [49], improvements in instrumentation and sample environments, including

simultaneous or sequential measurements using SANS along with other techniques such as SEC [57], UV-Vis and fluorescence [58], FTIR and DLS [59], electric fields [60], low temperatures [61] and high pressure [62], to mention a few, SANS CV will have even more applicability in the future. Thus, there will be more triumphs to come.

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Conflict of interest statement

Nothing declared.

Acknowledgements

The author acknowledges Dr. Joseph E. Curtis for helpful discussions in organizing this paper.

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- •• of outstanding interest

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