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Online size-exclusion and ion-exchange chromatography on a SAXS beamline

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TITLE:
Online size-exclusion and ion-exchange chromatography on a SAXS beamline

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SHORT ABSTRACT:
The determination of the solution structure of a protein by small angle x-ray scattering (SAXS) requires monodisperse samples. Here, we present two possibilities to ensure minimal delays between sample preparation and data acquisition: online size-exclusion chromatography (SEC) and online ion-exchange chromatography (IEC).

LONG ABSTRACT:
Biological small angle X-ray scattering (BioSAXS) is a powerful technique in molecular and structural biology used to determine solution structure, particle size and shape, and surface-to-volume ratio of macromolecules. The technique is applicable to a very wide variety of solution conditions spanning a broad range of concentrations, pH values, ionic strengths, temperatures, additives, etc., but the sample is required to be monodisperse. This caveat led to the implementation of liquid chromatography systems on SAXS beamlines. Here, we describe the upstream integration of size-exclusion (SEC) and ion-exchange chromatography (IEC) on a beamline, different methods for optimal background subtraction, and data reduction. As an example, we describe how we use SEC- and IEC-SAXS on a fragment of the essential vaccinia

virus protein D5, consisting of a D5N helicase domain. We determine its overall shape and molecular weight, showing the hexameric structure of the protein.

INTRODUCTION:

BioSAXS is a powerful tool to determine the shape of nano-sized objects[1-4]. The scattering of X-rays by a solution containing macromolecules, sized in the nm range, is recorded at very low angles. This angular range contains information about global parameters: the radius of gyration; the largest intraparticle distance; the particle shape; and the degree of folding, denaturation, or disorder. The technique does not require crystals, and the macromolecule stays in solution and thus can be kept in conditions mimicking certain important parameters of the cell, such as ionic strength, pH, etc. The knowledge of these factors might help to determine, for example, the physiologically-relevant oligomeric state of a protein of interest or to validate a proposed model for a complex. The characterization of protein-protein interactions in different buffer conditions, the creation of models of missing domains, the refinement of homology models, and the determination of discrete folded and unfolded states can be performed quickly and easily[5].

As with any technique, BioSAXS has intrinsic weaknesses: aggregated or denatured samples, mixtures of particles, heterogeneous samples, radiation damage, and buffer mismatches may result in un-interpretable data. For many analysis methods, it is implicitly assumed that the sample is monodisperse, a requirement that is often difficult to obtain in practice. In many cases, the degradation of the sample is subtle and cannot be detected in the data on its own, and any attempt to interpret the data gives inaccurate or even misleading results. To overcome these obstacles, the combination of size-exclusion chromatography (SEC) and SAXS was implemented on many beamlines to ensure data quality and to make this technique more accessible for increasingly difficult samples[6-11]. Recently, we added a new method to the repertoire by developing online ion-exchange chromatography (IEC)-coupled SAXS[12]. Both techniques are opening SAXS to a wide range of biological particles formerly impossible to analyze. The choice of which method to use depends on the biophysical properties of the particles of interest.

SEC separates macromolecules by their size, whereby at least a 10% difference in apparent molecular mass is needed for the separation. Physical limitations of the column and physiological properties of the samples, like hydrophobic surfaces, flexibility, and lack of stability, also complicate data collection, analysis, and interpretation.

Ion-exchange chromatography, which separates molecules based on their charge and, hence, their binding affinity to the IEC column, can be used instead of or in addition to SEC. The total charge can be readily manipulated by changing the pH, and varying the salt concentration of the buffer provides a relatively simple method for the controlled elution of the molecule from the IEC column. By using the charge, the separation of similar types and sizes of molecules, which would otherwise be difficult to separate, can be performed routinely with IEC. Additionally, IEC has the advantage of being able to deal with diluted samples, allowing one to avoid the concentration steps, which carry the potential risk of denaturing the protein.

Unfortunately, as the charge distribution is highly sample-dependent, IEC requires optimization regarding the pH and the salt concentrations[13,14].

For many proteins that are difficult to express, purify, or both, only low quantities of sample are available to study. It is important to be efficient and to minimize the number of purification steps and, therefore, the losses. For this reason, the last purification step is online directly prior to SAXS data acquisition, in order to increase the likelihood of collecting a good data set.

Here, we present and compare online SEC-SAXS and IEC-SAXS. Both techniques were implemented on the BioSAXS beamline BM29 at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France[15]. As a test case, we used the D5N and helicase domain of the vaccinia virus protein D5, which was rather difficult to analyze structurally using other methods. The vaccinia virus is a member of the Poxviridae family and is 98% identical to the variola virus, the cause of small pox. Using the vaccinia system, we study the replication machinery, focusing here on the essential helicase-primase D5.

D5 is a 95-kDa protein with an N-terminal archeo-eukaryotic primase (AEP) domain[16] followed by a cysteine cluster region (res. 240-345)[16]. Further towards the C-terminus comes a D5N domain (res. 340-460), which is always associated with D5-type helicases, and finally a superfamily 3 (SF3) helicase domain (res. 460-785) [17] (Figure 1A). The helicase domain of D5 builds a hexameric ring structure that is needed for tight binding to DNA. Thanks to recent SAXS and EM studies, the low-resolution structures of the primase and the helicase domains are now known[18].

Here, we show how to use the implemented online chromatography techniques on the BioSAXS beamline BM29 at ESRF to gain insights into the structure of the C-terminal fragment (residue 323-785) of D5.

PROTOCOL:

1. Description of the offline preparation and sample generation of a D5 deletion protein

Note: The $D5_{323\text{-}785}$ construct (Figure 1A) was cloned, expressed, and purified as described[18].

1.1 **Clone the construct into the pProEx HTb vector**

1.1.1 Perform a polymerase chain reaction (PCR) on D5R using the 5’-GCGCCATGGGTAATAAACTGTTTAATATTGCAC-3’ and 5’-ATGCAAGCTTTTACGGAGATGAAATATCCTCTATGA-3’ primers and a PCR reaction mix with polymerase, following the manufacturer’s advice.

1.1.2 Purify the PCR fragments via spin columns, following the manufacturer’s advice.

1.1.3 Digest the PCR fragments and the plasmid with the NcoI and HindIII restriction enzymes, following the manufacturer’s recommendations for buffer composition and incubation time.

1.1.4 Run the fragments on a 1% agarose gel in 0.5x TAE buffer (20 mM Tris, 10 mM acetic acid, and 0.5 mM EDTA) and stain the gel with a DNA dye.

1.1.5 Expose the gel to UV light. Caution: Wear personal safety equipment! Cut out the bands of the digested PCR fragments and the digested plasmid and purify the DNA using a gel purification spin column kit, following the manufacturer's recommendations.

1.1.6 Ligate the purified PCR fragments into the plasmid using a fast ligation kit, following the manufacturer's recommendations.

1.1.7 Transform via heat shock the product from the ligation reaction into competent bacteria optimized for plasmid amplification.

1.1.7.1 Add half of the ligation product to a vial of bacteria.

1.1.7.2 Incubate the reaction tube on ice for 20 min.

1.1.7.3 Incubate the tube at 42 °C for 30 s.

1.1.7.4 Place the tube on ice for 2 min.

1.1.7.5 Add 1 mL of Luria Bertani (LB) Broth (Miller) without antibiotics and let the cells recover at 37 °C for 1 h.

1.1.7.6 Spin down the cells at 16,100 x g for 3 s in a tabletop microtube centrifuge and remove most of the medium.

1.1.7.7 Spread the cells onto an LB agar plate with ampicillin (50 µg/mL final) and let the colonies grow at 37 °C overnight.

1.1.8 Put a colony into 3 mL of LB with ampicillin (50 µg/mL final) and grow the culture at 37 °C, shaking at 160 rpm overnight.

1.1.9 Follow the instruction of the miniprep kit to extract the plasmid.

1.1.10 Send a sample of the plasmid for sequencing and analyze the sequence for the absence of mutations and for the correct insertion.

1.1.11 Transform the pProEx HTb $D5_{323-785}$ construct into bacteria optimized for protein expression (use 1 µL and follow the steps in step 1.1.7). Spread the bacteria onto an LB agar plate with ampicillin (50 µg/mL final).

1.1.12 To create a starting culture, put one colony in 300 mL of LB with ampicillin (50 µg/mL final) and grow the bacteria at 37 °C, shaking at 160 rpm overnight.

1.2 Inoculate 12 x 1 L of LB in the presence of ampicillin (50 µg/mL final), each with 20 mL of the pProEx HTb $D5_{323-785}$ bacteria starter culture at 37 °C until an OD_{600} = 0.3 is reached. Reduce the temperature to 18 °C and induce expression at an OD_{600} = 0.5 with 1 mM IPTG. Incubate the cultures, shaking them at 160 rpm and 18 ˚C overnight.

1.3 Harvest the bacteria by centrifugation at 50,000 x g and at 4 °C for 30 min. Resuspend the bacterial pellets in approximately 150 mL of lysis buffer (50 mM Tris-HCl [pH 7], 150 mM NaCl, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 10% glycerol) with 1x protease inhibitor and 25 units (U)/10 mL DNase. Lyse the bacteria via sonication on ice (1-inch probe, 50% power, 0.5-s pulse, 0.5-s pause, 3x 5 min).

1.4 Load the supernatant onto a nickel affinity column (Ni-column, 1.5-mL bed volume), and wash it with 10 column volumes (CV) of binding buffer (50 mM Tris-HCl [pH 7], 150 mM NaCl, 10 mM β-mercaptoethanol, and 10% glycerol), 10 CV of washing buffer (50 mM Tris-HCl [pH 7], 1 M NaCl, 10 mM β-mercaptoethanol, and 10% glycerol), and 10 CV of imidazole wash (50 mM Tris-HCl [pH 7], 150 mM NaCl, 10 mM β-mercaptoethanol, 10% glycerol, and 30 mM imidazole). Elute the $D5_{323-785}$ (20 mM Tris-HCl [pH 7], 150 mM NaCl, 10 mM β -mercaptoethanol, 10% glycerol, and 200 mM imidazole).

1.5 Inspect the different purification steps via sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

1.5.1 Load 2-20 µL of each purification step flow-through mixed with 1x loading dye (2x: 4% SDS, 20% glycerol, 120 mM Tris-HCl [pH 6.8], and 0.02% weight/volume bromophenol blue) on a 10% SDS gel and run them at 200 V in 1x Laemmli running buffer (25 mM Tris, 0.192 M glycine, and 0.1% SDS).

1.5.2 Stain the gel with a ready-to-use Coomassie blue solution.

1.6 Exchange the buffer of the eluted protein with binding buffer using a desalting column according to the manufacturer's manual.

1.7 Cleave the His-tag by adding *Tobacco Etch Virus nuclear-inclusion-a endopeptidase* (TEV, 1 mg/100 mg of protein) to the protein solution. Incubate at room temperature overnight.

1.8 Check the digestion by performing SDS-PAGE (see step 1.5)

1.9 Equilibrate the Ni-column in binding buffer. Let the protein solution pass through and wash the beads with 2 CV of imidazole washing buffer while collecting the flow-through.

1.10 Concentrate the protein using a centrifugal concentrator to a final volume of 0.5 mL.

1.11 Inject the concentrated protein onto a size-exclusion column equilibrated with gel filtration buffer (20 mM Tris-HCl [pH 7], 150 mM NaCl, 10% glycerol, and 1 mM dithiothreitol [DTT]).

1.12 Collect the flow-through in 0.5-mL fractions.

1.13 Check the presence and purity of the protein in the peak samples by performing SDS-PAGE (see step 1.5).

1.14 Combine the fractions of the eluted peak of $D5_{323-785}$. Dilute the sample to 25 mM NaCl and 5% glycerol while keeping the Tris and DTT concentrations constant (for 5 mL of protein solution in 20 mM Tris-HCl [pH 7], 150 mM NaCl, 10% glycerol, and 1 mM DTT, first add 5 mL of 20 mM Tris-HCl [pH 7] and 1 mM DTT, and then add 20 mL of 20 mM Tris-HCl [pH 7], 5% glycerol, and 1 mM DTT).

1.15 Load the sample onto an ion-exchange column. Use buffer A (20 mM Tris [pH 7], 25 mM NaCl, 5% glycerol, and 1 mM DTT) and buffer B (20 mM Tris [pH 7], 1 M NaCl, 5% glycerol, and 1 mM DTT) to form a salt gradient from 25 mM to 1 M.

1.16 Collect the eluted protein in 1.5-mL fractions.

1.17 Check the presence and purity of the protein in the peak samples by performing SDS-PAGE (see step 1.5 and Figure 1B)

1.18 Reconcentrate the protein solution in a centrifugal concentrator to a final volume of 0.5 mL.

1.19 Rerun the concentrated protein on a size-exclusion column in gel filtration buffer (20 mM Tris-HCl [pH 7], 150 mM NaCl, 5% glycerol, and 1 mM DTT; see step 1.11).

2. Data collection

Note: Request beam time as early as possible. For ESRF, guidelines on available access types and on how to submit an application can be found at: http://www.esrf.eu/UsersAndScience/UserGuide/Applying. After the acceptance of the proposal and an invitation for the experiment, all participants have to complete a safety training. After validation of the training, fill in the “A-form” (via the ESRF user portal) to declare the researchers visiting the beamline for the experiment, along with the required safety information on the samples. Contact the local contact person to discuss the experiment.

2.1 SEC-SAXS data collection

Note: For online purification, use the high-performance liquid chromatography (HPLC) system installed at BM29. The system consists of an in-line degasser, a binary pump, a valve for buffer selection and gradients, an autosampler, a UV-VIS array photospectrometer, and a conductimeter. It is directly attached to the flow-through capillary of the SAXS exposure unit[19].

2.1.1 Put 1 mL of the gel filtration buffer aside. Connect the buffer bottle to the SEC system (the default is Port A).

2.1.2 Choose the flow rate depending on the column in use. Note: For the size-exclusion column used here, the flow rate is 0.1 mL/min. Define the maximum pressure for the column, take note of the back-pressure, and set the acquisition time to at least 1.2 CV.

2.1.3 Flush the pumps and the upstream tubes via the “auto-purge” function.

2.1.4 Wait until the system is filled with the buffer and connect the column. Equilibrate the column by passing at least 1.5 CV.

2.1.5 Interlock the hutch and measure the buffer set aside in step 2.1.1, using the sample changer to control for variation of the signal due to radiation damage. Only continue if there is no radiation damage to the buffer.

2.1.6 Switch the beamline control system to HPLC mode. Make a test run of buffer, collecting 200 frames with 1 s per frame. Write down the number of counts given by the detector in the summed intensity plot.

Note: The signal should match the previously-measured buffer, and the summed intensity over time should remain constant. If the signal does not match or is not constant, a longer equilibration of the system is required.

2.1.7 Spin down the sample at 13,000 x g in a tabletop microtube centrifuge for at least 10 min.

2.1.8 Fill the sample into an SEC-compatible glass vial (provided) and put it into the auto-injector. Note the well position.

2.1.9 Interlock the experimental hutch using standard procedure, as explained in the User Safety Training.

2.1.10 Log into and create a folder for data storage through the beamline control software.

2.1.11 Program the HPLC system using the “quick batch” feature. Set the storage location for the UV data to the folder created in step 2.1.10. Make sure to activate the automatic ASCII conversion of the UV data, storing the ASCII file in the same folder.

2.1.11.1. Choose the injection volume and well position. Add the measurement to queue by pressing the “Start” button. The software will ask to save the batch. Prepare for saving, but do not press the “Save” button, as this automatically starts the measurement.

2.1.12 Set up data collection parameters in the beamline control software. Choose the number of frames so that the total data collection time is in slight excess of the acquisition time defined in step 2.1.2.

2.1.13 Open the safety shutter and start SAXS data collection using the beamline control software. Verify that the data are correctly acquired. Compare the newly-collected data to the data collected in step 2.1.6 and check that the number of counts in the summed intensity remains constant for at least 100 frames and matches the value from step 2.1.6.

2.1.13.1. Start the SEC run by pressing the "Save" button, as prepared in step 2.1.11, and note the frame number displayed in the camserver software when the injection is completed and UV data acquisition starts.

2.1.14 After the data collection, open the ISPyB database[20] at http://ispyb.esrf.fr . Log in with the same credentials used when collecting the data. Open the "data acquisition" tab and press the "Go" button to access the data set and the results of the automatic analysis[21].

2.2 IEC-SAXS data collection

Note: Instead of the continuous linear gradient commonly applied in IEC experiments, use a step-wise gradient in which the amount of buffer B is increased in pre-set discrete steps.

2.2.1 Create the HPLC program for a sample-free buffer run. Program a step-wise gradient starting from 0% buffer B and increasing by 5 percentage points after two CV until 100% of buffer B is reached.

2.2.2 Put some of each buffer aside. Connect the bottle with the low-salt buffer A to port A and the one with the high-salt buffer B to port B.

2.2.3 Choose the flow rate and stay below the pressure limit of the column (in this example, 1 mL/min).

2.2.4 As in step 2.1.3, flush the pumps and upstream tubing by using the "auto-purge" function.

2.2.5 Equilibrate the system in low-salt buffer A (see step 1.15) and connect the ion-exchange column. Wait until the column is completely equilibrated (at least 1.5 CV).

2.2.6 Use the buffer set aside in step 2.2.2 to examine buffers A and B for radiation damage using the sample changer, as in step 2.1.5.

2.2.7 Check the buffer coming out of the column, as in step 2.1.6. Compare the signal to the signal of buffer A recorded in step 2.2.6. Note again the number of counts in the summed intensity plot.

2.2.8 Create a folder for data storage for the buffer run.

2.2.9 Set up data collection in HPLC system mode. Choose the number of frames so that the total data collection time is in excess of the total time of the IEC run (see step 2.2.1).

2.2.10 Open the safety shutter and start the SAXS data collection. Verify that it proceeds correctly and double-check that the summed intensity remains constant at the value noted in step 2.2.7 for at least 100 frames.

2.2.11 Use the "Single Run" feature of the HPLC software and start the step-wise gradient programmed in step 2.2.1. For the injection, set the vial number to -1 in order to inject no sample in this step. Write down the number of frames acquired as the program starts.

2.2.12 Create the HPLC program for the sample run. Program a step-wise gradient starting from 0% of buffer B. Estimate the percentage of buffer B at each peak measured offline in step 1.1.5 (Figure 1B). Set at least 3 steps exactly 1 percentage point below and 1.5 points above the peaks of interest, each for 2.5 CV (Figure 1C). Add a final step at 100% buffer B for 2.5 CV.

2.2.13 Spin down the sample at 13,000 x g in a tabletop microtube centrifuge for at least 10 min.

2.2.14 Dilute $D5_{323-785}$ into the salt concentration of buffer A (25 mM) by mixing it with 20 mM Tris-HCl [pH 7], 10% glycerol, and 1 mM DTT.

2.2.15 Load the sample manually onto the column. Put the buffer A input tube into the diluted sample after pausing the pumps to avoid the formation of air bubbles. Disconnect the column from the detectors to directly collect the flow-through from the column.

2.2.15.1 When the sample container is almost empty, return the input tube into buffer A (again pausing the pumps while moving the tube) and restart the pumping with buffer A to transfer the sample from the tubing to the column. Once the entire sample has been loaded, pass 2 CV of buffer A, and then reconnect the column to the detectors.

2.2.16 For the sample run, create a folder for data storage.

2.2.17 Open the safety shutter and start the SAXS data collection. Verify that the data collection proceeds correctly and double-check that the summed intensity remains constant at the value noted in step 2.2.7 for at least 100 frames.

2.2.18 Use the "Single Run" feature of the HPLC software to start the step-wise gradient programmed in step 2.2.12. For the injection, set the vial number to -1 in order to inject no sample in this step. Write down the number of frames acquired as the program starts.

2.2.19 Open “data acquisition” in ISPyB[20] and press “Go” to look at the data set and the automatic analysis[21].

2.2.20 Export the UV data from the HPLC system to ASCII format through the “Postrun Analysis” software.

3. SEC SAXS data reduction & analysis

3.1 Open the data in data acquisition in ISPyB by pressing the “Go” button. Determine in the overview which frames of the SAXS data collection correspond to the elution peak. Verify that the radius of gyration is stable throughout the peak.

3.2 Confirm that the automated buffer correction was performed correctly. Load frames from the central part of the peak into a program that performs manipulations with experimental SAXS data files[22] and average them. Then, load the automatically-generated buffer file and check whether the high *q* regions of the averaged frames and the buffer frames match.

3.3 Compare the frames acquired throughout the peak quantitatively using the CORMAP test[23].

3.3.1 Load the automatically-created subtractions (*_sub.dat files) of frames covering the region of interest.

3.3.2 Scale them to each other by pressing the “Scale” button.

3.3.3 Compare them using the “Data Comparison” feature. There should be no systematic changes between the frames throughout the peak.

3.4 Open all *-_sub.dat frames of interest, merge them using the “Merge” button, and save the merged file in .dat format.

3.5 Determine the radius of gyration with the “Radius of Gyration” tool in the program, noting the first data point in the Guinier range for later cropping of the data at low resolution.

3.6 Determine the pair-distance distribution function with the “Distance Distribution” tool in the program and save the resulting .out file as gnom.out.

4. *Ab initio* modeling

4.1 On a Unix machine, run a 40 x *ab initio* model reconstruction program without symmetry restrictions. Create a new folder and copy the gnom.out file to it. Run the program as follows:

for ((i = 1; i <= 40; i++)); do dammif gnom.out -m s -p dammifp1_$i; done

4.2 Analogously, run an *ab initio* model reconstruction program with symmetry restrictions for six-fold symmetry in a second folder:
for ((i = 1; i <= 40; i++)); do dammif gnom.out -m s –s P6 -p dammifp6_$i; done

4.3 Align all *ab initio* model reconstruction program output files (*-1.pdb). In the two folders from steps 4.1 and 4.2, run damaver –a *-1.pdb.

4.4 Open the union of all models (damaver.pdb) as well as the model filtered to the correct volume (damfilt.pdb) in a suitable molecular visualization software[26] and compare them.

4.5 Open the damsel.log file in a text editor. The model listed as the "reference" at the bottom of the file is the most representative model.

5. **IEC-SAXS data reduction, analysis, and modeling**

5.1 In the program that performs the manipulation with experimental SAXS data files[22], load about 50 SAXS frames from each mixing step of the buffer run, press the "AVERAGE" button, and save the results.

5.2 Based on the auto-processing results available via ISPyB, identify which frames of the experiment correspond to the elution of the protein and verify that the (preliminary) radius of gyration is stable throughout the peak.

5.3 Load the frames into the experimental SAXS data files manipulation program[22] and average them, as done for the buffer frames (see step 5.1). Then, load the buffer files generated in step 5.1 and compare the high q regions (above 4.2 nm^{-1}) to find a buffer that matches the average from the peak.

Note: There should be no systematic offset between the average from the peak and the buffer. If the sample curve seems to fall between two buffer curves, interpolate their curves by calculating the average.

5.4 Subtract the buffer identified as the best match from the average scattering curve of the peak fraction and save the resulting subtracted file. In addition, create subtracted curves using the average buffer curves from the preceding and following mixing steps to estimate the error margin of the subtraction.

5.5 Repeat steps 5.3 and 5.4 individually for the left and right halves of the peak and compare the results to those from step 5.4 to check for stability of the signal throughout the peak.

5.6 Follow steps 3.5 and 3.6 for all three subtracted curves from step 5.4.

5.7 Compare the results for all three subtracted curves. Note: Only results that remain robust within the error margin of subtraction can be trusted.

5.8 Perform modeling as in steps 4.1 and 4.2 for the best subtraction identified in step 5.3.

5.9 Follow step 4.3 to 4.5 to align the models and to identify the most representative one.

6. Comparison of the results

6.1 Run a program to superimpose 3D structures[12] on the representative models of the SEC (step 4.5) and IEC-SAXS data (step 5.9) with P6 symmetry: supcomb model_sec.pdb model_iec.pdb

6.2 Import the model_sec.pdb and the model_iec-r.pdb into a molecular visualization software.

6.3 Open the .fir files of the modelsec.pdb and the modeliec-r.pdb in a spreadsheet and create a 2D graph of experimental and calculated scattering curves.

REPRESENTATIVE RESULTS:

The results of the model-free invariant analysis are listed in Table 1. The analysis of the $D5_{323-785}$ SEC-SAXS data showed a molecular mass estimate (from a Porod analysis) of 345 kDa versus 338 kDa, observed using IEC-SAXS. Both are in agreement with the expected mass of 6 times 53.5 kDa (321 kDa) for a hexamer. The *ab initio* modeling of both data sets was undertaken with no imposed symmetry (SEC-SAXS: χ^2= 0.88, IEC-SAXS: χ^2=3.1) and using C6 symmetry (SEC-SAXS: χ^2=1.0, IEC-SAXS: χ^2= 4). As the overall fits to the scattering data for both reconstructions are comparable in both cases (Figure 1D and E), C6 symmetry can be assumed. Thus, the model corresponds to a hexagonal cone-like structure with a central channel, which appears partially obstructed (SEC: Figure 1F; IEC: Figure 1G, overlay Figure 1H). An examination of individual models before averaging shows that this obstruction is likely to be an artifact of the averaging process.

Figure 1: SAXS data analysis and comparison of $D5_{323-785}$ (Figure adapted from References 12 and 18).

A) Schematic domain structure of D5 protein and $D5_{323-785}$. B) Offline ion-exchange chromatogram with the indication of the three peaks. Orange curve: Absorbance at 280 nm (au), blue curve: percentage of buffer B. Percentages of buffer B at the peaks are indicated. C) Online ion-exchange chromatogram. Color key as in B. Additionally, the measured intensity is indicated in green. The experimental scattering curve and calculated curve of the model obtained by SEC-SAXS D) and IEC E) data analysis of $D5_{323-785}$. F) Bead model of $D5_{323-785}$ based on SEC data and G) IEC data. H) Overlay of the SEC and IEC models. The panels in E) to G) were created using a molecular visualization software[24].

Table 1: SAXS data parameters.

The table summarizes the parameters of SAXS data acquisition and analysis.

DISCUSSION:

For many macromolecules, a final purification step using chromatography is required prior to SAXS data collection to obtain a good quality data set. However, not all samples remain stable; they may be prone to aggregation or re-equilibration to a mixture of oligomerization states. Therefore, a final online purification step on the beamline is required to minimize the time between purification and data collection in order to obtain the best-quality SAXS data. Depending on the biophysical properties of the protein of interest, SEC-SAXS or IEC-SAXS might be chosen to obtain optimal sample quality. Here, on a protein construct derived from the helicase/primase D5, both techniques are explained and discussed.

Acquisition of SEC-SAXS data is becoming more and more standardized and is available on many BioSAXS beamlines. Data analysis, especially background subtraction, is relatively straightforward and easy. However, a stable buffer signal and the sufficient separation of the macromolecular species remains essential. Therefore, it is critical to reserve enough time to equilibrate the column thoroughly. Failure of this method can be due to persistent contaminants of similar size to the protein of interest, low concentrations, and radiation-sensitive buffers.

In practice, initially, SEC-SAXS is likely to be used as the method of choice for most macromolecular samples. Still, many purification protocols require a prior IEC step due to the presence of contaminants or aggregation. Given that each concentration and chromatography step is associated with losses of sample (estimated at 30-50%) and time, direct IEC-SAXS is advantageous. For samples that cannot be purified by SEC, be it due to the presence of similarly-sized “contaminants” or because they severely aggregate at the necessary concentrations, IEC-SAXS would always be the better-suited approach. Also, the higher flow rates supported by many IEC columns can help to reduce the transit time between purification and measurement. In the example presented here, IEC was used with a step elution, which allows for the separation of the close peaks of $D5_{323\text{-}785}$ from contaminants by carefully choosing the salt concentration steps. In principle, the number of steps is unlimited, but practically, at least 1 step per peak is required, and not too many should be chosen. For the background subtraction method described above, it is crucial to measure a relatively high number of different buffer compositions in order to find the matching one.

A shared downside of both techniques is the lack of precise protein concentration information. Due to this, precise mass determination based on forward scattering is not possible. For globular proteins such as $D5_{323\text{-}785}$, the Porod volume provides an alternative, albeit less precise, mass estimate, but for highly flexible or disordered proteins, this approach would not be valid.

A variation of the step-wise gradient IEC-SAXS method presented here is the use of a linear gradient instead. While it is possible to work with as many steps as desired to isolate sub-peaks using a step-wise elution, in the linear gradient approach, it is required to optimize the gradient conditions carefully in order to separate the peaks entirely before starting the SAXS experiment. Background subtraction in this approach could be done frame-wise and could be verified by the comparison of the individual frames, but it requires more advanced data handling, and a dedicated software does not exist yet.

The choice of a suitable column is critical for both techniques, as it determines the separation of the macromolecular species. Size-exclusion columns differ in loading capacity, the size range of separable macromolecules, and resolution, while ion-exchange columns vary in the kind and the density of their immobilized charges.

While the protocol presented here is specific to the ESRF beamline BM29, adaptation to any other SAXS beamline is, in principle, straightforward. The main requirements are a sufficiently high X-ray flux and a suitable detector (ideally single-photon-counting), to acquire reasonable signal-to-noise data in the range of seconds or less, and an online liquid chromatography system capable of creating gradients. The exact implementation would, of course, depend on the local beamline environment.

The results obtained on $D5_{323-785}$ using the two methods differ slightly. The radius of gyration is slightly smaller for the IEC data than for the SEC data, and the local minima of the scattering curve are shifted to slightly larger scattering vectors. This means that the $D5_{323-785}$ measured with IEC-SAXS is slightly more compact than the $D5_{323-785}$ measured with SEC-SAXS. This might be due to differences in the sample preparation, in the time between purification and measurement (IEC is faster), or, less likely, to a contaminant in the SEC-purified sample. The bead models obtained completely independently with both methods are comparable (Figure 1H). $D5_{323-785}$ shows the expected hollow, hexameric structure[18].

In conclusion, online ion-exchange and online size-exclusion chromatography are important biochemical purification methods that can be coupled directly to SAXS[6,7,9-12,25,26]. The background subtraction of IEC-SAXS data is slightly more difficult and ambiguous than for SEC-SAXS, but it is nevertheless possible. Depending on the biophysical properties of the protein of interest, both SEC and IEC-SAXS allow for the optimization of species separation with inherent advantages. Providing that the validation steps (as described) are correctly observed, the resulting data can be analyzed with confidence, and models can be determined using the standard tools available within the community. Together, both techniques allow online separation for a broad range of biological macromolecules, yielding data not accessible via standard static measurements.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure

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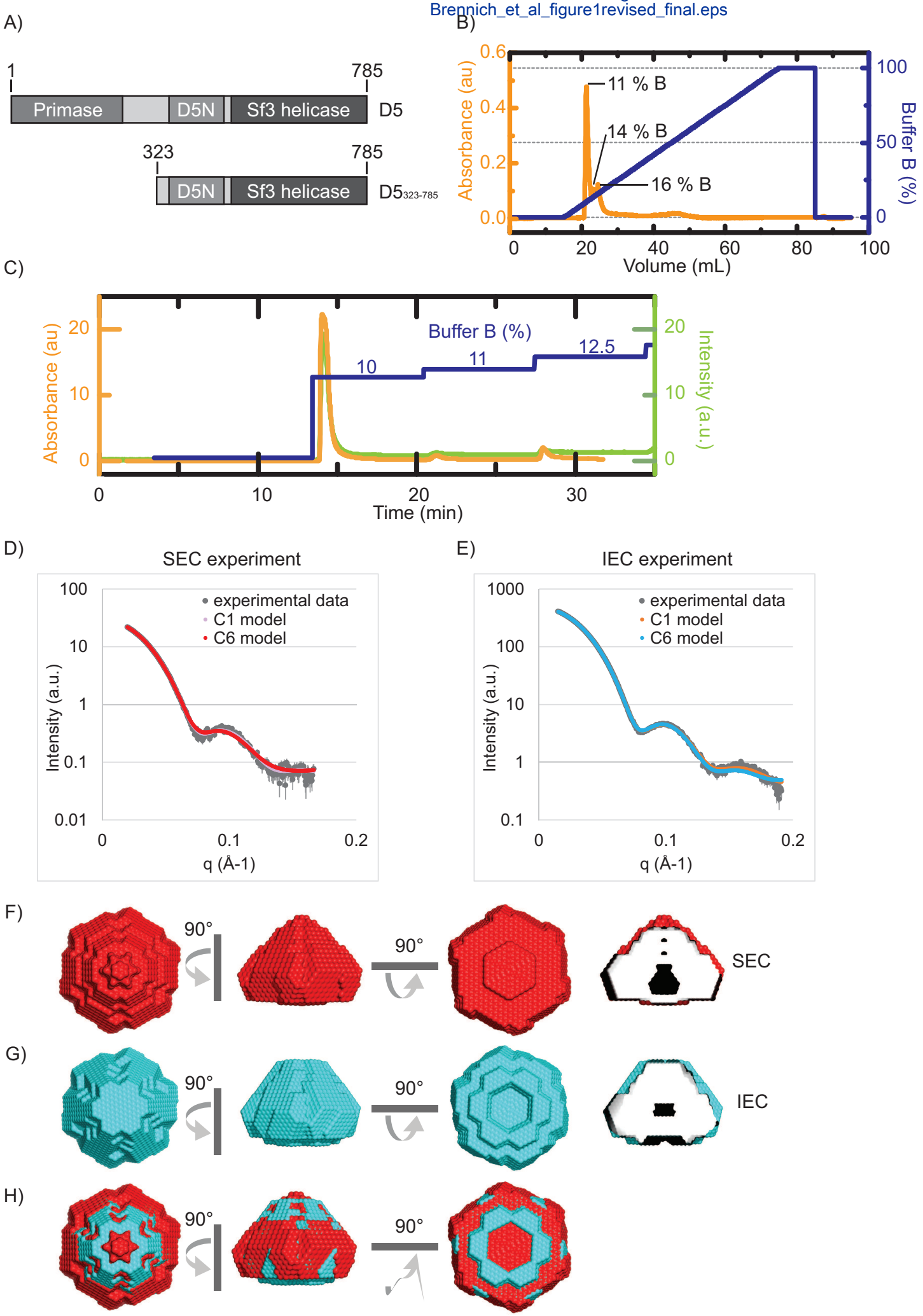

Table

Data-collection parameters	
Instrument:	ESRF BM29
Wavelength (Å)	0.99
q-range ($Å^{-1}$)	0.0032 – 0.49
Sample-to-detector distance	2.864 m
Exposure time (sec)	1 per frame
Concentration range	n.a.
Temperature (K)	293
Detector	Pilatus 1M (Dectris)
Flux (photons/s)	$1 \cdot 10^{12}$
Beam size (μm^2)	700 × 700
Structural parameters for $D5_{323-785}$, SEC	
I_0 (cm^{-1}) [from Guinier]	0.0237
Rg (Å) [from Guinier]	48
$q_{min}R_g$ – $q_{max}R_g$ used for Guinier	0.77 - 1.24
D_{max} (Å) [from p(r)]	145
q-range used for p(r) ($Å^{-1}$)	0.02 - 0.17
Porod volume V_p ($Å^3$) [from Scåtter]	$(570 \pm 5) \cdot 10^3$
Molecular mass M_r (kDa) [from V_p]	345 kDa
Calculated monomeric Mr from sequence (kDa)	321 kDa
Structural parameters for $D5_{323-785}$, IEC	
I_0 (cm^{-1}) [from Guinier]	0.386
Rg (Å) [from Guinier]	46.5 ± 0.1
$q_{min}R_g$ – $q_{max}R_g$ used for Guinier	0.44 - 1.29
D_{max} (Å) [from p(r)]	120
q-range used for p(r) ($Å^{-1}$)	0.03 - 0.18
Porod volume V_p ($Å^3$) [from Scåtter]	$(577 \pm 5) \cdot 10^3$
Molecular mass M_r (kDa) [from V_p]	339 kDa
Calculated hexameric Mr from sequence (kDa)	321 kDa

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
1,4 dithiothreitol [DTT]	Euromedex	EU0006-B	
10% Mini-PROTEAN TGX Precast Protein Gel	Biorad	4561033	
2x Phusion Flash PCR Master Mix	ThermoFisher scientific	F 548	
acetic acid glacial	VWR Chemicals (BDH Prolabo)	20104.298	
Agarose D-5	Euromedex	LF45130653	
Amicon Ultra -15 Centrifugal filters Ultracel -30K	Millipore Ltd	UFC903024	
Ampicillin sodium salt	Euromedex	EU0400-D	
Benzonase	Novagene	70750-3	
bromophenol blue	MERCK	8122	
Complete protease inhibitor cocktail	Roche	11231400	
Econo-Pac 10 DG Desalting column	Bio-rad	732-2010	
EDTA	Sigma	E-5134	
Glycerol	VWR Chemicals (BDH Prolabo)	24388.295	
Glycine	Euromedex	26-128-6405-C	
HindIII	Roche	656313	
HisSelect HF Nickel Affinity Gel	Sigma	H0537	
Hydrochloric acid (HCl)	VWR Chemicals (BDH Prolabo)	20252.295	
Imidazole	AppliChem Panreac	A1073,0500	
InstantBlue	Expedeon	ISB1L	
LB broth Miller	Fluka Analytical	L3152	
MgCl2	ICN Biomedicals Inc.	191421	
NaCl	VWR Chemicals (BDH Prolabo)	27808.297	
NcoI	Fermentas	ER0571	
One Shot BL21 Star (DE3)	ThermoFisher scientific	C6010-03	
pProEx HTb vector	Addgene	10711018	
Primer	Eurofins mwg operon		

QIAprep Spin Miniprep kit	QIAGEN	27106	
QIAquick Gel extraction kit	QIAGEN	28706	
QIAquick PCR purification kid	QIAGEN	28106	
SDS	Sigma	75746	
Superose 6 10/300 GL	GE Healthcare	17-5172-01	
SYBR Safe DNA gel stain	Invitrogen life technology	S33102	
T4 ligase	ThermoFisher scientific	EL0011	
TEV			home made
TOP 10	Invitrogen life technology	C404003	
Tris base	Euromedex	26-128-3094-B	
Uno Q 1R column	Bio-rad	720 0011	
β-mercaptoethanol	MPBiomedicals, LLC	194834	

Softwares

Beamline control software BsXCuBE	ESRF	Pernot et al. (2013), J. Synchrotron Rad. 20, 660-664	local development
Camserver software	Dectris	n.a.	detector control software
DAMAVER	ATSAS 2.6.0	http://www.embl-hamburg.de/biosaxs/download.html	program to align *ab initio* models
DAMMIF	ATSAS 2.6.0	http://www.embl-hamburg.de/biosaxs/download.html	ab initio model reconstruction programm
HPLC program Biologic Duo Flow	Bio-rad		
HPLC program LabSolutions	Shimadzu	n.a.	

ISPyB	ESRF	De Maria Antolinos et al. (2015). Acta Cryst. D71, 76-85.	local development
PRIMUS	ATSAS 2.6.0	http://www.embl-hamburg.de/biosaxs/download.html	program, which performs the manipulation with experimental SAXS
PyMOL	DeLano Scientific LLC	https://www.pymol.org/	software for visualization.
SUPCOMB	ATSAS 2.6.0	http://www.embl-hamburg.de/biosaxs/download.html	program to superimpose 3D structures

Equipment

BioLogic Duo Flow	Biorad	
BioLogic Biofrac Fraction collector	Biorad	
HPLC system	Shimadzu	
Labsonic P	Sartorius Stedim biotech	BBI8535108

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Dear Dr Nguyen and dear reviewer,

Thank you very much for your kind and thorough revisions. We appreciate your input and tried to address all your comments. Please find our changes in the manuscript and our remarks in blue in this document:

Editorial comments:

Please maintain the current formatting throughout the manuscript. The updated manuscript (54861_R1_050916.docx) is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. Please download the .docx file and use this updated version for any future revisions.

1. Formatting:
-Section 1 heading – Please use a single statement/phrase as a heading.

We changed the header to: **Description of the offline preparation and sample generation of a D5 deletion protein.**

-Please define all abbreviations at first occurrence (ie LB, etc.).

Thank you for the remark, we defined them in the text now.

-It is only necessary to define the abbreviation DTT once. Please use the abbreviation after it is first defined (1.11).

We removed the unnecessary full terms.

-Please include all software used in the materials table.

Please find them in the table.

2. Grammar:
-Please copyedit the manuscript for numerous grammatical errors, which is required prior to acceptance. Some errors are described below.
-Please use American English throughout the manuscript. For instance, “analyse” should be “analyze”.

It is changed.

-1.2 – “Express D5323-785 in 12 L LB with Ampicillin bacterial culture”

It is changed.

-Please do not capitalize “ampicillin”. It is not a proper noun.

It is changed.

-1.5, 1.8, 1.13, 1.17, 2.2.6, 2.2.7, 3.2 – Please use “Check” or similar rather than “Control” here and look for similar uses throughout the manuscript. “Control” is not used correctly.

It is changed.

-2.2.11 – “software start the”

There was an and missing.

-Line 509 – “is a required”

The a is removed.

-Line 526 – “as method of choice and work”

The “and work” part is removed.

3. Additional detail is required:
-1.1.7.7 – Are antibiotics used? If so, at what concentration?

Information is included in the text.

-1.1.8 – What concentration of ampicillin?

Information is included in the text.

-1.2 – Are bacteria grown at 18 degrees the entire time?

No, first they were grown at 37°C. We clarified that in the text.

-1.3 – What are the sonication settings?

The settings depend largely on the machine and probe used in each laboratory. Still, we gave our settings and included the machine in the material list.

-2.1.1 – What buffer is used?

The gel filtration buffer.

-3.1 – How is ISPyB used to do this?

We hope the changed text elucidates it.

-3.3.2 – How is scaling performed?

By pressing the Scale button in the program

-3.4 – How are the files merged?

By pressing the Merge button in the program

-3.5, 3.6 – How are these determined? Detail is required to include these in the video.

We have added which parts of the software need to be used. Details on how exactly to determine a "good" distribution function are outside the scope of this paper (typical at least a few paragraphs in SAXS textbooks).

4. Unnecessary branding should be removed:

-1.3 – benzonase

We removed the name and used the generic: DNase.

-Please remove all trademark symbols from the materials table.

-3.2, 3.3, 5.1, 5.3 – PRIMUS

We replaced it.

Reviewers' comments:

We would like to thank all the referee once again for their insightful comments that helped us to improve the paper. Please find changes, explanations and comments to each of your raised points.

Reviewer #1:
Manuscript Summary:
The topic covered in the current manuscript is very significant to the future of small angle X-ray scattering (SAXS) of macromolecules and macromolecule complexes. Having chromatography systems linked to the beam line set up will surely aid in data collection for transient protein complexes and partially aggregated samples. Here are some suggestions to improve the manuscript for the video.

The protocol presented by the authors although quite detailed is very specific to the BioSAXS BM29 beam line at ESRF at Grenoble, France. The title suggests the protocol will be more general. A more general protocol will better serve the scientific community at large.

We added a paragraph to the discussion

For example: Explain about the reason and probable challenges one may face while adopting such systems at other beamlines.

We added a paragraph to the discussion.

Why choose step-wise gradient over continuous gradient? PS: Figure B show a continuous gradient while text insists in use of step-wise gradient.

The figure is as described in the text the offline profile of the protein, where we used a linear gradient, on with we based later our choice of gradient steps.

Detailed protocol for protein purification is not relevant to the current work.

We agree that it is not absolutely necessary but we were specifically asked to put it in by our first editor.

Maybe worthwhile to mention an overall range of dilution factors and minimum volumes required when performing size exclusion chromatography linked SAXS.

As both the dilution factor and the minimum volumes required depend on the type of column and the sample (the authors have seen injections volumes as low as 10 µL and as high as 1 mL), we decided not to add this kind of information.

What is the rationale behind the automated buffer profile generation?

We are not sure what the referee is referring to with automated buffer profile generation. If he refers to the automated buffer subtraction in the SEC experiment, we added a relevant citation in 2.1.14 and 2.2.19.

When would one chose Ion exchange versus size exclusion SAXS?

In our introduction we describe the different use of the two chromatographic methods ("By using the charge, the separation of similar types and sizes of molecules, which would otherwise be difficult to separate, can be made routinely with IEC. Additionally IEC has the advantage to be able to deal with diluted samples, avoiding concentration steps, which carry the potential risk of denaturating the protein. Unfortunately, as the charge distribution is highly sample dependent, IEC requires optimization regarding the pH and the salt concentrations [13,14].

For many proteins, which are difficult to express, purify or both, only low quantities of sample are available to study. It is important to be efficient and minimize the number of purification steps and therefore the losses. For this reason, the last purification step is online directly prior to SAXS data acquisition in order to increase the likelihood to collect a good data set.")
We think that a more detailed discussion would be too long in the context of this publication and can be found easily in text books or online.

Furthermore the 3rd paragraph of the discussion ("In practice, in first instance, SEC-SAXS is likely to be used as method of choice for most macromolecular samples. Still many purification protocols require a prior IEC step due to the presence of contaminants or aggregation. Given that each concentration and chromatography step comes along with a loss of sample (estimated 30-50%) and time, direct IEC-SAXS is advantageous. ") mentions our reasoning again.

We added "For samples which cannot be purified by SEC, be it due to the presence of similarly sized "contaminants" or because they severely aggregate at the necessary concentrations, IEC-SAXS would always be the better suited approach. Also, the higher flow rates supported by many IEC columns can help to reduce the transit time between purification and measurement."

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

Reviewer #2:

Thank you very much for your comments. They are highly appreciated.

Manuscript Summary:
The authors of the manuscript (JoVE54861) report the practical application of SEC- and IEC-SAXS at ESRF BM29 using the vaccinia virus protein D5. The protocol covers from gene cloning to protein purification of D5323-785 protein, a deletion mutant of D5, as well as SAXS experiments and analyses. A striking feature of this manuscript is that the authors devise and implement a strategy for background adjustment of IEC-SAXS data. An IEC-SAXS data of D5323-785 protein was reasonable and comparable to SEC-SAXS data.
However, the readability of the manuscript is very hard particularly in protocol section. Below are major and minor comments for the author's consideration that absolutely need to be addressed before publication.

Major Concerns:
1. (overall in protocol session) Add product name listed in table, especially if manufacturer's protocol is referred.

We agree that it would be easier but JOVE explicitly asked us to remove all product names. They can be found in the table of material.

It would be a good idea to add a short title in each paragraph as much as possible (particularly secession 2.2 is hard to understand).
For example, 1.9 (line 203) could be:
1.9 [Ni-Affinity Chromatography] Equilibrate the Ni-column (His-Select HF Nickel Affinity Gel) in binding buffer. Let the protein solution….
2. (SEC- & IEC-SAXS parts in protocol session) Model name of HPLC system (also software name and its version) should be described in the text since there are some system-specific terms in protocol.

As mentioned above we had to remove all product names. Regarding titles in 2.2, it is indeed the workflow as it is described here and we rather feel that it would interrupt the flow by adding more titles.

The details of the column used for D5323-785 protein are also missed in text, especially column volume. It would be a good idea to repeat the column and buffer information even if the details are duplicated.

We agree that it would be easier but JOVE explicitly asked us to remove all product names. They can be found in the table of material.

3. (Line 269) What is "the number of counts"? Transmission intensity measured by photo diode? Need to describe the details.

We clarified “Write down the number of counts given by the detector in the summed intensity plot”

4. (2.2.12, 2.2.18 & Fig 1B) If I understood the protocol correctly, the protein was eluted by 2% step gradients whose volume is 2.5 CV/ gradient. The gradient used for Fig 1B doesn't look like 2.5CV step if the column volume is 1.3 ml. Figure 1B should be replaced with the profile at IEC-SAXS experiment (not that of purification). Otherwise add such a profile in addition to Figure 1B. The profile should be magnified around elution peaks (e.g. 10-40mL).
Salt concentration of SAXS data used for modeling should be mentioned as well.

We added a figure showing the actual profile of the IEC SAXS run (1C) and precised the explanation of the steps we used.

5. (Line 550-) Two SAXS curves are obviously different as the authors mentioned. The authors assumed that the difference mainly comes from the different salt concentrations. In my opinion, I think that protein conformation and/or SAXS profile are more susceptible to high concentration of glycerol (e.g. >5%) than salt concentration. I guess that difference of salt concentration between SEC and IEC is probably less than ~30mM. It is hard to imagine that SAXS profiles are dynamically changed like Figure 1C and 1D by small difference of salt concentration. High concentration of glycerol can alter inter- and/or intra-molecular interactions and changes the degree of hydration shell which is ideally taken account during SAXS modeling.

We clarified it in the text in both cases SEC and IEC SAXS we used 5% glycerol in the buffer. Another slight difference is that the proteins were prepared on different days.

After careful evaluation of the data we agree that the salt concentration is less likely to cause the differences, but maybe the differences in the sample preparation, in the time between purification and measurement (IEC is faster) or, less likely, to a contaminant in the SEC purified sample.

A sentence is added to the text.

The authors mentioned that D5323-785 protein measured by IEC-SAXS is more compact than that of SEC-SAXS, based on Rg and Dmax. However, Porod volume is opposite. Together with above, more careful discussion should be required.

The differences between the curves are quite small and do not affect the conclusions on the shape of $D5_{323\text{-}785}$ as mentioned in the text.

6. (Line 555-) The chi square value is the error-dependent value. It is irrelevant to compare experimental SAXS curves based on chi or chi square value by model fitting. Remove the sentence.

Done.

7. (IEC-SAXS) The zero-extrapolation is not performed in this protocol. Effect of interparticle interactions would be remaining in the profile. Also this procedure tends to have over-subtraction

when sample is very elongated or flexible. Accordingly, careful validation, ideally by different methods, should be required. The authors should discuss such potential risks in text.

The zero-extrapolation was not explicitly mentioned in the manuscript. However, just as for SEC-SAXS we verified a stable radius of gyration (based on a preliminary buffer subtraction) before proceeding. We now explicitly mention this in step 5.2

Minor Concerns:
8. (overall) Name of all software (beamline control, etc) should be also indicated to clarify currently working software/program. "beamline control system" and "beamline control software" are same? Also need to indicate the version of ATSAS package.

We unified the term and the version can be found in the material table.

9. The title of the manuscript sounds somewhat generic. The words of both "D5" and "ESRF BM29" would be included in the title.

We did not change the title of our manuscript given that we believe that D5 and BM29 are served as an example to show that the method is working and that this can be implemented on different SAXS beam lines.

10. No word of "SAXS" in short abstract.

We included SAXS in the short abstract.

11. (Line 73) Need reference regarding separation of SEC. In general, it is impossible to separate monomer and dimer by SEC. "10% difference" sounds weird.

There are plenty of examples for the separation of mono and dimers by SEC in the literature. See for example J.A.P.P van Dijk, J.A.M Smit (2000)

12. (Line 141-) It is awkward that individual protocol is only provided for the competent cell (Top 10?). Change to "the manufacture's recommendation" in the same fashion?

JOVE explicitly asked us to remove all product names. They can be found in the table of material. The paragraph 1.1.11 was elaborated.

13. (Line 189) 1x Laemmli running buffer. Convert to concentration.

It is changed.

14. (Line 216-) 1.14. Need rewording.

We hope is now better understandable.

15. (Line 288) 3.10 \ 2.1.10?

Sorry, we do not understand the meaning of the question.

16. (Line 365) What kind of detector? UV detector?

All detectors.

17. (Line 425) paragraph 3.8 and 3.9 should move to paragraph 4. Accordingly, the title of paragraph 3 needs to be changed. Then paragraph 4 should be something like "ab initio modeling".
Title of paragraph 5 would be "IEC-SAXS data reduction and analysis (or modeling)".

We followed the excellent suggestion of the referee.

18. (Line 171 and175) Missing a step of centrifugation? Need to mention speed and time. Also those values should be mentioned in all centrifugal places.

We added the procedure to the step and added the speed and time to 1.1.7.6

19. (Table 1) The number of flux is correct?

No, the formatting was wrong. It should be 10^{12}.

20. (Figure 1B) Why does vertical axis have arbitrary unit?

Because the units in the plot are arbitrary (albeit with a known scaling factor to cm^{-1}).

Additional Comments to Authors:
N/A

Reviewer #3:

Last but not least thank you so much for your help in improving this manuscript!

Manuscript Summary:
This manuscript describes SAXS data acquisition and analysis with on-line, pre-SAXS measurement SEC or IEC purification. An example of the vaccinia virus protein D5 is given to demonstrate the performance of the two different purification options. Overall it can be a valuable contribution and the comparative analysis of the two methods and their results is instructive.

The points below are essential to be address adequately in a revised submission.

1. The first sentence of the abstract overstates the importance of BioSAXS and should be pulled back some to be more in line with the language of first few sentences of the introduction, which acknowledges it as a powerful (as opposed to the arguable "indispensable") technique and provides an accurate description of the information inherent to the scattering data (which falls short of "the solution structure" but does provide solution structural parameters that can be powerful constraints or tests).

We changed it to powerful.

2. Dilute solution conditions are not "close to physiological" - they can mimic certain important parameters such as ionic strength, pH etc. Paragraph 1 needs to be corrected on this point.

It is changed to: The technique does not require crystals and the macromolecule stays in solution, thus can be kept in conditions mimicking certain important parameters of the cell such as ionic strength, pH etc..

3. In paragraph 2 the conditions that can lead to uninterpretable data are noted, but more significantly these conditions can lead to biased data where the bias is undetected without great care in the data reduction and analysis. Hence the data are interpreted but give inaccurate results - e.g. subtle aggregation or inter-particle interference. This should be noted.

We added this additional issue to the paragraph.

4. Reference 12 is "in submission" it seems important to this paper. Its acceptance and hence availability to the reader of this paper I presume would be important.

We are of hope that at the time this manuscript will be published the submitted paper will be available to the public (the paper is accepted).

5. lines 84-85; are the authors saying dilution can cause denaturation? Is this common? Can they clarify?

No, what we say is that IEC has the advantage to be able to deal with diluted samples without a potentially denaturating concentration step. This means that during the concentration step samples might denature and yes, that happens often. For example; some protein precipitate on the membranes of concentrators or if they are too concentrated. We changed the formulation.

6. line 417 point step 3.7 states that one should "crop the data" before the beginning of the Guinier region. This is a potentially dangerous step as it can minimize without actually removing the effects of subtle but significant aggregation or inter-particle interference. While it is a common practice, it is one that should not be approach blithely as routine. There is no objective

way of determining "the beginning of the Guinier region" - rather it takes careful consideration of the scattering profile overall. From this point the paper proceeds with the automated ATSAS data processing and interpretation. This automated pipeline might be helpful for getting some measure of feedback on your data during the experiment, but given the many subtleties in SAXS data, the requirement for careful post experiment analysis and validation cannot be underestimated. This point needs to be made very clear at this stage in the protocol and in the discussion.

We agree with the reviewer that is a common practice that requires some kind of experience and given that we use the p(r) we removed the paragraph from the paper.

7. line 485 "model free" is a bit misleading. The data in Table 1 are derived from the Guinier model, P(r) model, and a model for the high-q scattering form Porod all of which make assumptions about the shape and surface of the scattering particle. It is correct to say that the results are independent of any specific shape or atomistic model and can be derived directly from the scattering data.

The only assumptions for Guinier and P(r) are that the system is biphasic and that the Guinier range is accessible. Hence, the values themselves are not based on any model (although their interpretation may be). For the Porod volume, there is the additional globularity requirement, but even this is not a "model". We did however add the term "invariant" to the text to be more specific.

8. A significant limitation of the combined SEC-SAXS and IEC-SAXS method is that one does not know the protein concentration in the sample at the point of measurement, which precludes using I(0) to determine the molecular mass of the scattering particle, an essential validation parameter given the strict requirement for monodisperse particles of the same size for accurate structural interpretation. To cope with this limitation, the Porod volume and now the somewhat arbitrary half of the volume of the dummy atoms in the bead modelling is used by many. The Porod volume is fraught by its dependence on the scattering invariant which requires integration from 0 to infinity, and hence assumptions about the high-q behavior beyond the measurement range and in the range where background subtraction errors and flexible/unfolded regions in the structure can be a problem. This issue should be acknowledged and appropriately noted.

We added a paragraph addressing this issue to the discussion.

9. The authors talk about "good" background subtraction and need to describe how they assess "goodness." Background subtraction at increasingly powerful synchrotron sources is notoriously becoming more difficult and common practice in batch measurements is to measure solvent backgrounds before and after the sample, subtract one or the other and average of both and by some criteria decide which is best. Given the contributions of internal density fluctuations in the high-q scattering, how is a "good" background assessed. It would be valuable to describe here given the emphasis on improved background subtractions - why are they good with this method and how do they compare to the batch mode.

We agree with the referee that the question of what constitutes a correct background subtraction is indeed complex. For this reason, we actually subtract different backgrounds in step 5 and only trust results robust against background subtraction. We therefore do not claim to have an improved background subtraction.

The answer to the question of what would constitute a "good" background in our case is actually provided in the note of step 5.3: "There should be no systematic offset between the average from the peak and the buffer." The reason for this is that while the contribution of the density fluctuations is often not negligible, it generally does not give a q-independent signal.

As we don't think that generalized statements about which method allows the best background subtraction are possible, we would like to refrain from adding such a discussion.

10. lines 552-555 The authors need to consider if the difference in the results reflect the fact that the SEC sample is contaminated with a small amount of aggregate by the time it reaches the SAXS measurement cell. This seems counter-intuitive as the I(0) data indicate the SEC sample is more than and order of magnitude more concentrated than the IEC sample. The authors should comment on this.

Based on the forward scattering, the SEC sample is 10 times less dilute than the IEC sample. Hence, one would expect the IEC sample to be more prone to aggregation. We did extend the discussion of possible reasons for the observed differences.

11. In regard to 10 above, what is the distance and time taken for transit from the SEC or IEC to the SAXS sample chamber? This would potentially be where samples highly prone to aggregation could start to aggregate, and hence point 8 above is more significant as with accurate concentration determination (possible with careful A280 measurements and accurate extinction coefficient assessment) one can measure molecular masses reliably with an accuracy of 5-10%. Obviously this paper cannot solve this problem, but it should be acknowledged as a limitation and if people are working on solutions, referenced.

We have added the difference in transit time as one reason why one might choose IEC in the discussion. We also address this as a possible source for the observed differences between the two curves.

12. In the table it says "monomeric Mr" when it seems it should be "hexameric Mr"?

It indeed should be and is now corrected.

While there are many detailed points made above, they are made with the view of improving the paper and contributing to strengthening confidence in SAXS as a valuable structural biology technique when used with care.

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A