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The role of small-angle scattering in structure-based screening applications

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Abstract In many biomolecular interactions, changes in the assembly states and structural conformations of participants can act as a complementary reporter of binding to functional and thermodynamic assays. This structural information is captured by a number of structural biology and biophysical techniques that are viable either as primary screens in small-scale applications or as secondary screens to complement higher throughput methods. In particular, small-angle X-ray scattering (SAXS) reports the average distance distribution between all atoms after orientational averaging. Such information is important when *e.g.* investigating conformational changes involved in inhibitory and regulatory mechanisms where binding events do not necessarily cause functional changes.

Thus, we summarise here the current and prospective capabilities of SAXS-based screening in the context of other methods that yield structural information. Broad guidelines are also provided to assist readers in preparing screening protocols that are tailored to available X-ray sources.

1 Introduction

The identification of an optimal ligand-receptor combination by *screening* can be performed by a broad array of methods, in which one searches the chemical space of biomolecular interactions to discover candidates for further investigation. Screening occurs most frequently in pharmacological lead discovery (Ciulli, 2013; Renaud et al, 2016) where a macromolecule relevant to disease is assayed with an existing lead or fragment library containing between $10^2 \sim 10^6$ molecules, seeking candidates that may best be modified to interfere with the receptor's pathogenic function while maintaining clinical viability (Arkin et al, 2014; Erlanson et al, 2016; Payne et al, 2007). Applications in fundamental research also exist, such as in proteomics to screen for potential binding partners of a newly isolated protein, cellular targeting, or in nucleic acid interactions. Since the total chemical space is astronomical relative to existing capability, it is necessary to optimise both throughput and library content. Here, literature applies the term *high-throughput screening* to methodological advances that significantly improve performance via parallelisation, detection speed, and other modifications.

The drawbacks of high-throughput variants often includes a concomitant sacrifice in measurement precision or total information obtained. Thus, secondary screening techniques are included in screening strategies to provide verification of initial leads and further elucidate their observed interactions. For example, in drug discovery contexts a highly parallelisable thermal-shift assay may be paired with dual polarization interferometry to probe both binding kinetics as well as potential structural changes (Grøftehauge et al, 2015). Whereas when investigating protein–nucleic-acid interactions, the initial sequence patterns from an RNA-binding assay may be further filtered by small-angle X-ray scattering (SAXS) to optimise for stoichiometries ideal for structural characterisation (Chen et al, 2018). The judicious use of complementary approaches during screening in-

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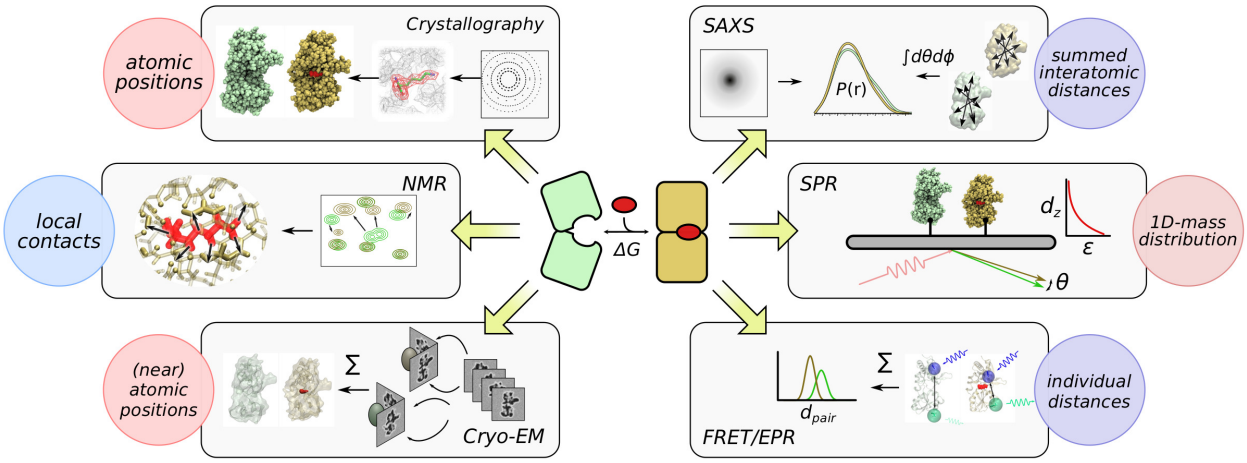

Fig. 1 A simplified schematic depicting structural biology and biophysical techniques that can be deployed in screening applications. Figure prepared with Inkscape-0.92, VMD-1.9.3, and Gimp-2.8.

creases the quality of preliminary information available for decision making.

Since a majority of high-throughput assays work via the detection of thermodynamics or functional effects exerted by bound ligands, secondary screening methods should provide a source of independent information. Here, various structural biology and biophysical methods can yield the requisite data on the nature of conformational or configurational changes triggered by binding, if any (Fig. 1). Notably, their current role as secondary screens is usually dictated by increased sample requirements or protocol complexity relative to other alternatives. The type and amount of structural information depends on the experiment: For example, X-ray crystallography and nuclear magnetic resonance (NMR) methods provide rich, atomistic details on ligand placement and contacts necessary to conduct rational drug design. In contrast, fluorescence labelling and surface plasmon resonance (SPR) provide rapid, coarse structural characterisation ideal for larger-scale applications. Thus a high-level understanding of respective techniques will assisting in choosing an appropriate method for the biological system under investigation (Table 1).

This review aims to report the increasing and potential usage of solution X-ray scattering to conduct small-scale screening experiments, and provide arguments as to its particular role in the gamut of structural-based techniques. As a dedicated solution technique (Grant et al, 2011; Hura et al, 2009; Putnam et al, 2007; Svergun et al, 2013), SAXS is capable of characterising solution stoichiometries (Ando et al, 2012; Chen et al, 2018; Cordeiro et al, 2016; Tuukkanen and Svergun, 2014), complex configuration (Vestergaard and Sayers, 2014; Yang et al, 2010) and even transient structural changes (Cammarata et al, 2008; Cardia et al, 2008; Kantrowitz, 2012; Neutze and Moffat, 2012). Current synchrotron setups offer source intensities that permit precise measurements sufficient to conduct investigation into disordered protein conformations (Cordeiro et al, 2017; Kikhney and Svergun, 2015; Tompa, 2012) as well as weak interactions (Tuukkanen and Svergun, 2014) relevant to current drug targets. These capabilities provide a unique niche for SAXS-based screening. Below, we provide executive summaries of several current and potential alternatives to further contextualise the application of scattering techniques. An overview of scattering theory and practice follows, leading into general advice for tailoring screening protocols to the broad range of X-ray sources.

2 Summaries of four structural biology and biophysical methods

2.1 Nuclear magnetic resonance

NMR spectroscopy is an established approach within drug discovery (Dias and Ciulli, 2014; van Dongen et al, 2002; Erlanson et al, 2016; Harner et al, 2013; Meyer and Peters, 2003) and molecular interactions (Collins et al, 2015; Liu et al, 2016; Williamson, 2013) due to its provision of detailed, spatial information on binding interfaces and in some cases ligand orientations (Skjærven et al, 2013). Here, the seminal publication by Shuker et al (1996) demonstrated the characterisation of structure-activity relationships based on observed chemical-shift perturbations (Williamson, 2013) on the receptor as caused by bound ligand. A range of variant procedures have since been developed based on ligand signals to circumvent size-limitations on NMR signal quality, while losing the ability of directly map the bind-

Table 1 Overview of structure-based screening techniques with two thermodynamics assays for comparison.

Name	Information attained	Advantages	Technique-specific requirements
X-ray crystallography	3D spatial electron density	Extensive information useful for further refinement	Reliable crystallisation conditions with atomic resolution
Cryo-EM	3D spatial electron density	As crystallography without requiring crystallisation conditions	Requires extensive, target-specific protocol optimisation
SAXS	1D pair-distance distribution of electron density	Solution measurement in native environment, applicable to protein disorder	Purifiable to monodisperse or oligodisperse conditions, sufficient conformational changes
receptor-based NMR	local contacts	Extensive information useful for further refinement	Sufficiently small size and μM solution concentrations of labelled species
ligand-based NMR	limited local contacts	parallelisable measurements for fragment libraries	Fast binding and unbinding kinetics, library optimisation to avoid artefacts from non-specific interactions and aggregation
SPR	limited 1D mass distribution	fast measurement and sample reuse	Functionally-neutral tethering site or reliable adsorption mechanism
Fluorescence labelling	pair-distance distribution of two sites	parallelisable measurement and potential for *in-vivo* assays	Functionally-neutral labelling site
Isothermal calorimetry	thermodynamics	High accuracy thermodynamics measurement with relative stoichiometry data	μM solution concentrations of receptor and ligand
Thermal-shift assay	thermodynamics	Simple experiment with massive parallelisability	none

ing site. These include saturation transfer difference (STD, Mayer and Meyer, 1999; Wagstaff et al, 2013), WaterLOGSY (Dalvit et al, 2000, 2001; Huang et al, 2017), and INPHARMA (Sánchez-Pedregal et al, 2005; Skjærven et al, 2013), all of which function by the transfer of magnetization from an arbitrary-sized receptor to the smaller ligand. Thus evidence of binding is demonstrated by presence of magnetization on ligands in the mixture. Alternative formulations based on enhanced relaxation also exist in the form of target-immobilised NMR screening (Vanwetswinkel et al, 2005) and paramagnetic labelling schemes (Jahnke, 2002).

Although a range of NMR methods are available, applicability can be restricted due to inherit size limitations of this method (Felli and Brutscher, 2009; Lee et al, 2014). Receptor-based direct mapping of binding interfaces using chemical shift perturbations are only feasible with signals-to-noise ratios, which still allow for detection of signals after addition of ligands. This method is routinely applicable for systems up to ∼40 kDa of size provided they have globular shape and sufficient solubility and stability. In contrast, ligand based experiments can be measured irrespective of receptor size (Cala et al, 2014). However, binding and unbinding kinetics must be faster than experiment timescales for saturation transfer techniques to work, making this method amenable for detection of ligands binding in a range between 10^{-3} - 10^{-8} M. Both categories see use in fragment-based drug discovery (Friberg et al, 2013; Murray et al, 2010; Prati et al, 2015; Tsao et al, 2006), and analogous techniques can also be applied to investigate protein-protein interactions (Barile and Pellecchia, 2014).

2.2 X-ray crystallography

X-ray crystallography offers the possibility of directly yielding the atomic coordinates of bound ligands within the receptor active site, ideal for downstream rational and *in-silico* drug design. This makes the technique very attractive for lead discovery as long as the target can be reliably crystallised at sufficient resolution both with and without ligand, which may be challenging due to potential conformational changes (Hassell et al, 2007). Where that condition is satisfied, parallised protocols with ligand-cocktail mixtures permit the techniques to be deployed as a primary screen (Davies and Tickle, 2011; Patel et al, 2014). The excess saturation conditions and additional stabilisation of bound complex by crystal contacts also make possible the detection of leads with very weak affinities, which may be difficult to discover using other methods (Schiebel et al, 2016).

On the other hand, the lengthy process involved in establishing crystallization conditions for novel proteins imposes a significant limitation on applicability of crystallographic methods, if these have not been found in previous available studies. This biases their application towards known and well-studied systems (Zheng et al, 2015), particularly in membrane-bound scenarios such as G-protein coupled receptors (Ranganathan et al, 2017). Given that transporters and membrane-bound receptors are critical to major cellular processes and thus highly attractive targets, crystallographic screening remains an important source of structural information. While solubilisation in membrane-mimics such as nanodiscs (Bayburt and Sligar, 2010; Ritchie et al, 2009) can bring other techniques into play (*e.g.* NMR and SAXS, Berthaud et al, 2012; Puthenveetil et al, 2017;

Skar-Gislinge et al, 2015), the intensive process involved has so far preferenced its utilisation in ligand screening towards receptor-immobilised platforms (Congreve et al, 2011; Das et al, 2009; Wilcox et al, 2015).

2.3 Cryo-electron microscopy

The recent advent and increasing feasibility of cryo-EM maps at near-atomic and atomic resolutions have inspired discussions on their potential drug discovery applications (Merk et al, 2016; Renaud et al, 2018). The breakthroughs in both resolution required for direct atomic placement (< 3Å) and minimum resolvable particle sizes are regarded as key advances for the technique to become viable as a means to directly visualise bound complexes, particularly for systems that are not known to crystallise. Similar to crystallography, the discovery of such 3D-structure will create starting points for rational drug design not hitherto possible. Although the current throughput is not yet viable for experimental screening, significant efforts are being carried out on automation and sample optimisation similar to that once required to establish crystallographic screening protocols.

2.4 Surface plasmon resonance

Another common biophysical approach to fragment-based screening is SPR spectroscopy (Chavanieu and Pugnière, 2016; Navratilova and Hopkins, 2010; Zeng et al, 2017), where structural and mass changes of biomolecules tethered closely to a metallic substrate alters the local refractive index and thus the total internal reflection behaviour of light beams on the opposing side. Light microscopy for detection brings advantages of millisecond time resolution and thus an ability to directly observe binding kinetics (Daghestani and Day, 2010; Salamon et al, 1997b). The fixation process enables sample reuse across multiple titrations, which is a key advantage in large-scale applications (Maynard et al, 2009; Piliarik et al, 2005). On the other hand, a suitable anchoring site on the receptor must be found that does not perturb its native function or stability significantly. Notably, membrane-embedded targets can either be anchored by tethering the entire membrane above the substrate (Patching, 2014; Salamon et al, 1997a) or attaching nanodisc-solubilised proteins to functionalised surfaces (Congreve et al, 2011; Das et al, 2009). In all, SPR seems to be a persuasive option for intensive screening scenarios where the detection of structural change itself is sufficient for the purposes of candidate selection.

3 Small-angle X-ray scattering

3.1 Basic primer

With the major structure-based and biophysical screening methods introduced above, we devote the remaining space to critical niches that small-angle scattering fulfils. For a thorough coverage of basic principles of scattering, we recommend other excellent reviews both on the theory (Kikhney and Svergun, 2015; Putnam et al, 2007; Rambo and Tainer, 2013) and practical aspects (Bernadó et al, 2017; Gabel, 2015; Skou et al, 2014; Trewhella, 2016) of X-ray (SAXS) and neutron (SANS) scattering. A minimal introduction of scattering is given here to establish an elementary understanding of the information obtained via scattering experiments.

The exposure of a biomolecular solution to an incoming, collimated source results in the elastic scattering of photons by an angle 2θ (Fig. 2A) from all solute and solvent atoms (among other phenomena), with an intensity that decays rapidly as a function of increasing angle. The momentum transfer $q = 4\pi \sin\theta/\lambda$ is generally adopted to remove dependence of this scattering on wavelength λ in the final analysis. To obtain the contribution of solvated macromolecules, scattering intensities of the sample solution and an equivalent buffer solution containing all components except the molecule of interest are measured then subtracted. The difference pattern $I(q)$ due to the macromolecule alone represents the intra- and intermolecular interference patterns summed over all solutions orientations and conformations. Under dilute concentrations where intermolecular effects are insignificant, an $I(q)$ obtained by SAXS/SANS is directly proportional to sample concentration and contains information on the conformational average distances between scattering atoms.

The key distance information provided by small-angle scattering methods in dilute conditions can be summarised in form of a weighted pair-distance distributions $P(r)$ between all scattering centers (§2.3.3 Putnam et al, 2007). $P(r)$ is related to $I(q)$ by indirect Fourier transform, with the weights determined by the product of the net scattering contrast at each scattering site, evaluated as the difference between solute and buffer scattering densities. For X-ray sources, scattering is determined by the electron densities and thus contributions to $P(r)$ is dominated by non-hydrogen nuclei. Whereas, neutron scattering yields an analogous $P(r)$ that is instead dominated by the location and ratio of protons versus almost all other nuclei, due to the proton's negative scattering cross-section. Both SAXS and SANS $I(q)$ patterns can in principle be precisely measured, which yield sufficient accuracy to detect even small pertur-

bations to the conformational ensemble (Kikhney and Svergun, 2015; Rambo and Tainer, 2013).

3.2 Applications in structural biology

It is this sensitivity to global distances that makes SAXS and SANS valuable contributors to integrative structural biology. The missing high-resolution information from scattering can be readily furnished via other sources including crystallography (Grant et al, 2011; Putnam et al, 2007) and NMR (Carlomagno, 2014; Grishaev et al, 2005; Hennig and Sattler, 2014; Madl et al, 2011; Rossi et al, 2015). Such combination enables the isolation of complex structures where SAXS can be used as a guide in rigid-body docking (Jiménez-García et al, 2015; Petoukhov and Svergun, 2005; Schindler et al, 2016; Schneidman-Duhovny et al, 2016; Xia et al, 2015), elastic perturbations (Gorba et al, 2008; Zheng and Tekpinar, 2011), atomistic simulations (Björling et al, 2015; Chen and Hub, 2015a; Kimanius et al, 2015), or trivially as *post-hoc* removal of incompatible structural models (Hennig et al, 2013; Karaca and Bonvin, 2013). Moreover, mixtures of monodisperse species can be decomposed (Bernadó et al, 2007; Schneidman-Duhovny et al, 2016) when either the relative populations or the component scattering are already known. Here the decomposition of a five-state ensemble between the trimeric Proliferating Cell Nuclear Antigen plus its disordered binding partner $p15^{PAF}$ (Cordeiro et al, 2016), is a prime example where structural information, population states, and binding affinity can be extracted from a complex oligodisperse system. Additionally, SAXS is often applied to characterise the extent of molecular flexibility in systems ranging from folded proteins (Makowski et al, 2011), linker-connected domains (Receveur-Bréchot and Durand, 2012; Różycki et al, 2011; Yang et al, 2010), to complete disorder (Bernadó and Svergun, 2011; Kikhney and Svergun, 2015; Rambo and Tainer, 2011). Amongst the vast existing structural biology applications of SAXS, we believe that many such cases could potentially be adapted in screening studies. A selection is presented in Table 2 to illustrate the types of structural information that scattering can provide in the context of SAXS-based titrations.

In contrast to SAXS, SANS sees particular uses in situations involving more than two binding partners where the scattering difference between hydrogen and deuterium can be leveraged in contrast variation (Gabel, 2015; Heller, 2010; Jacrot, 1976) studies, initially used to estimate the composition and spatial distribution of large multi-component proteins, nucleoproteins, and viruses by varying H_2O/D_2O ratios (Cusack et al, 1985; Mangel et al, 1990; Svergun et al, 1994). Further distinct measurements can be conducted by selective deuteration, achievable at the sub-component level and more recently the level of individual domains in a multi-domain protein via segmental labelling (Freiburger et al, 2015; Sonntag et al, 2017). The acquisition of such extensive scattering datasets together with NMR restraints (Carlomagno, 2014; Hennig and Sattler, 2014; Madl et al, 2011) provided a critical breakthrough in efforts to characterise flexible protein–nucleic acid assemblies (Hennig et al, 2013, 2014; Lapinaite et al, 2013). While the potential to provide extensive characterisation makes neutron scattering an attractive cousin to X-rays, the lower intensity of neutron sources greatly limit the throughput of SANS measurements. Until significant developments can be made on the level of detection and workflow optimisation such as on microfluidic lab-chip devices (Adamo et al, 2017; Lopez et al, 2015; Pham et al, 2017)), we expect SAXS to remain the primary avenue for high-throughput scattering.

3.3 SAXS-based screening

To summarise the advantages of SAXS-based screening, we briefly describe several examples in the literature starting with our recent work on the binding profile of Sex-lethal protein (Sxl) and followed by other applications and methodologies.

Classification of protein-ligand interactions As a proof-of-principle for SAXS-based screening of protein-ligand interactions, the selectivity profile of Sxl was investigated in a screen of thirty-five oligonucleotides assuming existing biochemical knowledge of its preference versus polyuridine with guanidine substitutions (Chen et al, 2018). The overall procedure is illustrated in Figure 2. Initial selection of candidates are based on sparse sequence space coverage, scanning bipartite and tripartite uridine/cytidine compositions, oligonucleotide length, and guanidine-substitution position (not shown). Twelve eight-point titrations per 96-well plate are conducted to maintain compatibility with existing beamline automation robots, with additional measurements of nucleotide scattering to enable subtraction of unbound ligand contributions.

At concentrations where interparticle scattering are insignificant, the measured SAXS profile of a mixture between unbound proteins, ligands, and their complexes can be expressed by a linear sum of components. Fig. 2B represents the general case where ligand scattering significantly deviates from buffer scattering and the respective receptor and ligand buffers differ. We note that this is reducible to standard buffer subtraction procedures when ligand scattering is not differentiable

Table 2 Examples of SAXS-based titrations or screening

System	Source	Type	Description and notes
Protein conformational change			
Leucine/Isoleucine/Valine binding protein	Olah et al (1993)	titration	contraction of bi-lobe domain upon ligand binding. Early example using lab-based source.
Transient photoexcitation of haemoglobin	Cammarata et al (2008)	time-resolved SAXS	laser-triggered detachment of carbon monoxide enables solution characterisation of the relaxed state(s).
Aspartate transcarbamoylase	Cardia et al (2008), Kantrowitz (2012)	time-resolved SAXS	self-regulation of enzyme by substrates and regulators
Protein domain assembly			
B_{12}-dependent methyltransferase	Ando et al (2012)	titration	association of two domains in a mixture of 1:1 and 2:1 stoichiometry
Ribonucleotide reductase	Ando et al (2016)	titration	α subunit in dimer-hexamer equilibrium, and β subunit dimer
Antibody self-association	Fukuda et al (2017); Tian et al (2014)	screening	technical studies on antibody stability as a function of concentration, pH, buffers and viscosity, important in clinical applications
Linker-connected domain configurations			
Src kinase	Jamros et al (2010); Yang et al (2010)	two-point measurement	Assembly state of regulatory domains in the presence of SH3 signal peptide
Sxl-lethal RNA-binding protein	Chen et al (2018)	titration	Equilibrium between apo-protein, 1:1 protein-RNA complex, and 2:2 protein-RNA complex.
Disordered protein interactions			
Proliferating Cell Nuclear Antigen, $p15^{PAF}$	Cordeiro et al (2016)	titration and population modelling	association of trimer-of-dimer PCNA with disordered p15 in 3:0–3:3 stoichiometries
α-synuclein	Herranz-Trillo et al (2017)	time-resolved SAXS and population modelling	decomposition of species during amyloid fibril genesis
Micellular compositions			
various detergents used in membrane protein solubilisation	Lipfert et al (2007), Oliver et al (2013)	titration	Shift in micellular size distributions and associated shape.
Nucleic-acid conformations			
dT_{30} with $MgCl_2$	Meisburger et al (2013)	titration	Alteration of polymer properties due to coordinating ions.

from buffer scattering and buffer conditions are shared between protein and ligand. By fitting the scattering profiles of individual components to the final mixture scattering profile, we derive a residual quantity χ_{lin} that represents departure from the linear sum (Fig. 2C):

$$\chi^2_{lin} = \frac{1}{N_q}\sum_q^{N_q} \frac{\left(I_{mix}(q) - \sum_i^{comp.} f_i I_i(q)\right)^2}{\delta I_{mix}(q)^2 + \sum_i^{comp.} \delta I_i(q)^2} \quad (1)$$

χ_{lin} is linearly-proportional to the amount of formed complex and magnitude of structural perturbations. Under conditions that permit ligand-saturation, the bound complex curve can be unambiguously derived and associated with a saturated χ_{lin}. The effective affinity $K_D^{eff.}$ can be computed from the χ_{lin} titration curve. In the current scenario where RNA-excess conditions trigger sample oligomerisation, $K_D^{eff.}$ was computed instead by assuming a shared binding mechanism across all RNA-titrations. This enables simultaneous fitting across multiple titrations to derive respective affinities and shared saturated χ_{lin}.

The buffer-subtracted curves can also be directly compared using a number of curve similarity metrics (Fig. 2D, *c.f.* Franke et al, 2015; Hura et al, 2013). Hierarchical clustering can be further performed on the metrics that preserving linear distance such as symmetrised pairwise-χ to more clearly summarise binding mechanisms. When aggregated across all titration points, the overall "distance" $\overline{d_{ab}}$ between titrations of two ligand a and b is useful for classifying binding interactions without assuming any structural knowledge:

$$\chi^2_{pair,ab} = \frac{1}{N_q}\sum_i \frac{\left(I_a(q_i) - (f\, I_b(q_i) + c)\right)^2}{\delta I_a(q_i)^2 + \delta I_b(q_i)^2} \quad (2)$$

$$\overline{d_{ab}} = \frac{1}{2}\sum_{titration} \left(\chi_{pair,ab} + \chi_{pair,ba}\right) - 1 \quad (3)$$

When clustering was performed on the Sxl-RNA interactions screen, we uncovered a range of solution stoichiometries consisting of 1:1, 2:2, and mixtures thereof, corresponding to low and high apparent $K_D^{eff.}$ resulting from combined analysis. Results were further confirmed by EOM modelling using the published crystallographic conformation(Handa et al, 1999), Notably, this stoichiometry switching was not evident in accompanying isothermal calorimetry and NMR measurements. Thus SAXScreen provides useful structural information on optimising species purity for downstream structural biology work, and we expect it to be useful in other applications as a complement to thermodynamics-based assays.

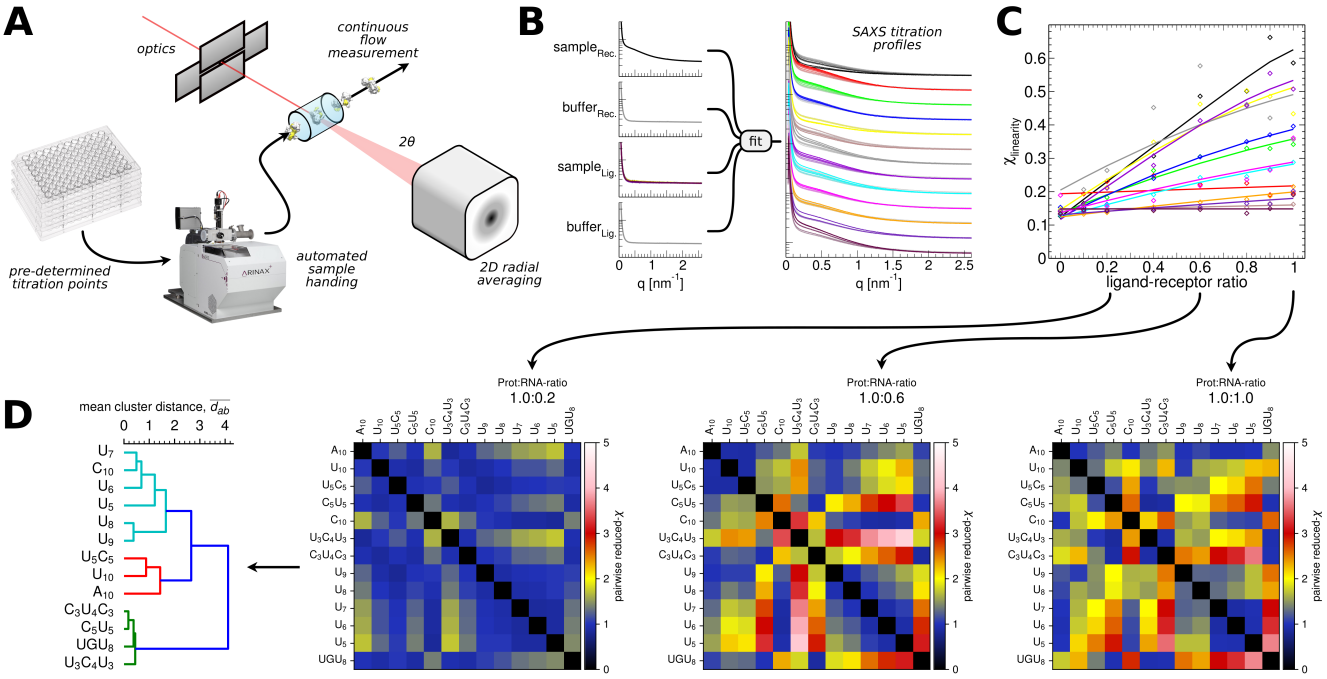

Fig. 2 A proposed method to conduct SAXS-based screening (see main text for details). Prepared mixtures between receptors and ligands are submitted to SAXS measurements at beamlines with automated liquid handling capabilities (**A**). Scattering profiles of individual components and respective buffers are used to fit the mixture scattering profiles (**B**), where the goodness-of-fit χ_{lin} is taken as a proxy for complex formation (**C**) and subsequent affinity calculations. Direct comparison metrics between scattering profiles at equal ratios can be used to visualise ligand-dependence (**D**), as well as compute a titration similarity metric for classification of binding responses (**E**). Figure prepared with Inkscape-0.92, Xmgrace-5.1.23, and matplotlib-2.2.2. Permission to use image of the Arinax sample-handling robot kindly provided by Ralf Siebrecht.

Screening for concentration and environmental effects One direct application of screening is to investigate in detail the dependence of the structural ensemble upon environmental conditions such as temperature, buffer composition and pH tolerance. Several studies included in Table 2 have conducted small-scale screens in this manner such as Tian et al (2014) and Fukuda et al (2017), who examine optimal conditions for the formulation and delivery of antibody therapeutics. The particular sensitivity of SAXS towards inter-particle scattering is leveraged to identify conditions that avoid unwanted oligomerisation and associated viscosity. Here, the sample requirements for scattering measurements is significantly smaller than that required for then-current rheological methods. Similarly, labs such as Lipfert et al (2007) and Oliver et al (2013) leverage SAXS to probe the solution morphology of detergent micelles commonly used in membrane protein solubilisation. Although individual micelles might be highly diverse in shape, essential parameters can be captured in ellipsoidal models that describe the average size, shape, and approximate packing as a function of total concentration. Numerous other applications such as protein disorder, protein-detergent complexation, and solvation layer densities showcase the ability of scattering methods to detect small magnitude perturbations as long as they are sufficient to influence the entire $P(r)$ distance distribution (Chen and Hub, 2015b; Cordeiro et al, 2016; Henriques et al, 2018; Kim and Gabel, 2015; Koutsioubas et al, 2013; Møller et al, 2013). These perturbations can in principle also be screened to study environmental influences on molecular structure.

Microfluidic chip screening platform Although our own work utilises broadly-available 96-well plates setups for screening, it is well worth mentioning the ongoing improvements in microfluidic chip platforms as a means to conduct screening. For purposes of illustration, we restrict ourselves here to two sister reports by Teychené lab in the understanding that multiple labs have also contributed towards this sub-field (Lafleur et al, 2011; Møller et al, 2013; Pham et al, 2017; Schwemmer et al, 2016; Watkin et al, 2017), with alternative on-chip mixing schemes (Lee et al, 2011; You et al, 2015).

Pham et al (2017) reports the fabrication and initial testing of a four-channel mixing device intended for screening crystallisation conditions, whereas Rodríguez-Ruiz et al (2017) reports its immediate implications for on-chip structural characterisation. The authors' implementation of chip-based SAXS screening relies upon the

creation of stable water-in-oil droplets by joining three aqueous channels into a primary channel containing immiscible fluorous oil. Precise regulation of relative rates between the aqueous channels determine final concentrations in the droplet, and thus enabling coverage of protein concentration and crystallisation conditions, or equivalently titration points in a protein-ligand-buffer setup. Droplet size is itself regulated by relative oil-water velocities and synchronised with the X-ray beam to minimise damage and interface scattering artefacts. This results in the measurement of one to three SAXS measurements per droplet, each equivalent to individual exposure frames of a capillary-based setup. The authors use lysozyme to demonstrate the dependence of inter-particle interactions on salt concentration, as well as a standard concentration series.

The chief advantages of microfluidic platforms include sample-efficiency, consistency and immediate measurement. For a theoretical application to ligand screening, the estimated consumption of $2\,\mu$l per titration point [1] is an order of magnitude smaller than minimum volumes in the well-plate setup. On-chip generation of droplets followed by UV-characterisation prevents stochastic sample handling errors that can occur while preparing plates. The main challenges in adapting microfluidics for screening consists of a higher sample concentration required to produce an equivalent angular q-resolution and throughput, and rapid exchange of ligand identity between titrations.

10-100 nm ultrastructural organisation It should also be noted that the applicable length-scales of SAXS reaches above the single molecule interactions covered here: a simple adjustment of sample-detector distances enables investigations of cellular structures, meso-scale phenomena, and beyond. We highlight here the application of SAXS-based screening by von Gundlach et al (2016) towards tracking the effect of antibiotics on ultrastructural changes in *E. coli* cells, by measuring scattering between $0.05 < \mathrm{q} < 3.5\,\mathrm{nm}^{-1}$, corresponding to length scales of $3 \sim 120\,\mathrm{nm}$.

The authors demonstrate that alterations in physical characteristics such as membrane integrity and nucleoid distribution can be indirectly detected and used to classify mechanisms of action by novel agents, showing by example the unique scattering changes of polymerase inhibitors and membrane disruptors versus protein synthesis inhibitors. The principle-component analysis used here is another valid *ab-initio* method that does not invoke structural assumptions, and can be utilised alongside hierarchical clustering to distinguish between ligand responses. The observed differences in SAXS was also shown to correlate with visual changes of TEM images. Independently, the authors also make efforts at mapping the time-dependence of structural alterations with decreasing cell viability. This work exemplifies the potential for SAXS to offer real-time study of cellular and sub-cellular ultrastructure.

[1] Based on observed droplet volume of order 20 nl and ~ 100 droplets required to produce the reported average using 100 ms exposure time at BM29, ESRF Grenoble.

4 General considerations for screening

Due to the relative uniqueness of synchrotron platforms in terms of beamline characteristics, sample automation and other aspects, it is not feasible to include in this review a comprehensive formulation of a SAXS-based screening protocol. These hardware and software characteristics influence the optimal sample volumes, concentrations and expected throughput. As such, we recommend initial contact with beamline staff and research labs for assistance in tailoring experimental proposals to the specific advantages of respective beamlines. A number of notable items will be discussed below to cover broadly applicable aspects.

4.1 Available screening platforms

In terms of screening capabilities, most synchrotron beamlines around the world possess automated sample measurement and analysis capabilities. The authors have recently tested the BioSAXS sample changer (Round et al, 2015) that is currently implemented at ESRF and PETRA-III, which is also available at Diamond Light Source. These utilise 96 well-plates as the means of sample preparation followed by robotic loading and measurements. We include a representative list of equivalent automation setups below for other beamlines: SIBYLS (Classen et al, 2013), SOLEIL (David and Pérez, 2009), Stanford SRL (Martel et al, 2012), Australian Synchrotron (Kirby et al, 2013), CHESS (Acerbo et al, 2015), and the Shanghai SRF (Li et al, 2016). At the time of writing, the biological SAXS beamlines at MAX-IV Sweden, SESAME, and Taiwan Photon Source remain in various stages of preparation, for which we also expect feasibility of screening applications.

Although the above list suggests that screening can be carried out at numerous beamlines, there currently exists no detailed performance comparisons specific to biomolecular titrations. We note here that lab-based sources may serve as a readily available and more reproducible platform to conduct screening. While the lower intensities extend required measurement times by $10^2 \sim 10^3$-fold and thus impose relatively strict limits on

protein stability, measurement protocols are in principle identical to synchrotron sources.

4.2 Sample consumption

For screens conducted using small-angle X-ray scattering, a balance must be struck between information gained versus total throughput. The precision and total information gained from a single titration is proportional to sample concentration and volume, number of titration points, and measurement time. Since synchrotron sites currently recommend protein sample parameters of volumes $20 \sim 60\,\mu$l and concentrations of 0.2∼10.0 mg mL^{-1}, a single ten-point titration theoretically consumes $100\,\mu$g of materials. The destructive nature of measurement implies that sample consumption scales directly with the size of the screen, – thus for large-scale efforts and low-yield targets it may be more cost-effective to adopt alternatives that re-use valuable binding partners.

Further improvements in these consumption rates are largely contingent upon hardware upgrades. Minimum sample volumes for standard capillary-flow measurements are formulated based on sample cell dimensions as dictated by the incoming beam geometry. For example, the beam geometry provided by G1 at MacCHESS (Acerbo et al, 2015) consists of a $250 \times 250\,\mu$m cross section with an optimised path length of 2 mm, resulting in an instantaneous exposure volume of $0.125\,\mu$l. The sample is flowed through this volume to minimise radiation damage during measurement, resulting in a final recommendation of 15 or 30μl. Similarly, the geometry of P12 at PETRA ($120 \times 200 \times 1700\,\mu$m) results in a recommendation of $20\,\mu$l. Sample-handling robotics may also implement dead volumes to prevent collisions, which further increase the practical minimum volume. In these contexts, a broader adoption of microfluidic platforms as discussed above is likely to significantly improve future sample utilisation by an order of magnitude.

4.3 Throughput

Our current experience with the capillary-based measurements at ESRF Grenoble and PETRA-III Hamburg indicate a performance of 4∼5 hours per plate in practice (Chen et al, 2018). The majority of time is spent conducting necessary capillary cleaning and transport using the Arinax sample handler, noting that recommended exposure times (1∼10 seconds) permit a theoretical floor of 3∼15 minutes per plate. This latter value is stated to be achievable according to SIBYLS's

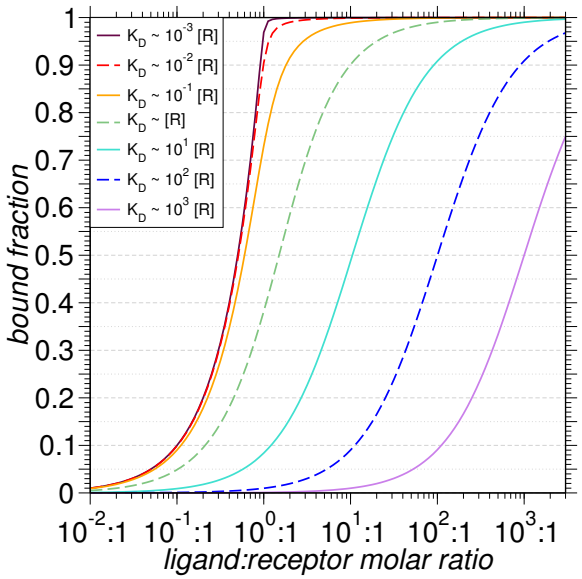

Fig. 3 Family of titration curves at constant receptor concentration $[R]$, for a simple two-state interaction. Colors indicate expected bound-fractions for a range of K_D values relative to $[R]$: 10^{-3} (maroon), 10^{-2} (red), 10^{-1} (orange), 10^{0} (green), 10^{1} (turquoise), 10^{2} (blue), and 10^{3} (violet). Figure prepared with Xmgrace-5.1.23.

setup[2] for solution-based SAXS. In powder-diffraction applications, the Australian Synchrotron[3] proposes a benchmark of 5 minutes per plate. Thus, significant increases can be achieved with more specialised setups that minimise downtime between sample exposures. Given an optimised logistics profile, practical throughput can be increased to 10^4 measurements per day at current beamline intensities.

4.4 Sensitivity and titration coverage

A final limitation in throughput is the choice of titration points that ideally covers conditions spanning between *apo*- and saturation. In the context of a simple two-state, receptor-ligand binding where one partner possesses buffer-like scattering (featureless over the q-range relevant to SAXS,) the receptor concentrations used in the measurement and the precision at which the bound fraction can be evaluated determines the strongest distinguishable K_D (Figure 3). For instance, a $\sim 5\%$ uncertainty on the bound fraction implies that all ligands possessing K_D stronger than $\sim 10^{-2}$ of receptor concentration $[R]$ are indistinguishable. Here, beamline-specific reference data can be utilised to estimate uncertainty values. In contrast, detection of weak K_Ds is a

[2] https://bl1231.als.lbl.gov/2017/03/sibyls_installs_new_tecan_free.php

[3] http://www.synchrotron.org.au/images/AOF2017/10-SAXS-Charlotte-Conn.pdf, slide 47 of 63.

matter of including relevant titration points potentially followed by subtraction of detectable unbound ligand scattering. Thus, we suggest a higher concentration of titration points around 1:1-ratios for improved coverage of strong-affinity ligands.

Although the base method does not assume any knowledge about molecular structure, external knowledge can significantly improve the accuracy of estimated bound fractions. For instance, available atomistic structures can be utilised to generate predicted scattering curves for the *apo-* and *holo-* states (Franke et al, 2017; Knight and Hub, 2015; Schneidman-Duhovny et al, 2016), which can be used to directly model the relevant populations. We also note that Sedlak et al (2017) has proposed a quantitative error model to recapitulate the measurement process. When used together with predicted scattering curves, this can assist with determining optimal concentrations at which to perform titrations.

5 Summary

Small-angle scattering has a unique niche amongst the structural biological screening techniques in that it supplies information on solution conformations at low material costs per measurement, albeit using a destructive measurement. Since the common high-throughput assays detect binding thermodynamics or biological function, the structural information can be used to independently confirm affinity predictions and binding mechanisms of initial hits. These range from distinguishing agonists versus inhibitors to optimising binding partners for downstream structural biology. However, serial measurements limit the current SAXS throughput to 10^3 titrations per day with current improvements focussed upon reducing the downtime due to sample handling and cell cleaning. This restricts SAXS-based approaches currently to smaller-scale investigations and screening with curated libraries.

At the time of writing, there remains numerous avenues to further investigate SAXS-based screening such as feasibility on lab-based sources, parallelisation and protocol reproducibility across synchrotron sites. This review has collated the essential information necessary to conduct small-scale screening, with which we hope to popularise the utilisation of existing beamline automation capabilities and encourage further development in throughput. We note that data curation support remains relatively underdeveloped compared to structural modelling applications, – the current formatting of SAXS repositories SASBDB (Valentini et al, 2014) and BIOISIS do not yet support composite entries for reporting titrations. Nevertheless, we recommend contribution of summary titration data and the adoption of recent publishing standards (Trewhella et al, 2017). These actions will ease the development of comparable protocols between different setups. As the ongoing efforts to improve throughput and efficiency bear fruit, we hope to see an increasing role of small-angle scattering within the arsenal of structural biology screening methods.

Author Contributions

P.C. and J.H. co-wrote the article.

Conflict of Interest

P.C. declares that he has no conflict of interest. J.H. declares that he has no conflict of interest.

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Supplementary Material

None.