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Characterising biomolecular interactions and dynamics with mass photometry

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Abstract

We review recent advances in our ability to characterise biomolecular structure, interactions and associated dynamics by mass photometry (MP), the label-free detection and mass measurement of individual biomolecules in solution. Molecular counting and identification provides direct access to relative abundance, and thereby affinities, while associated dynamics yield on- and off-rates. The molecular resolution afforded by MP enables these measurements as a function of stoichiometry and assembly at equilibrium, as opposed to the majority of existing solution-based methods. Together with future improvements in terms of assays and technological performance, MP is likely to provide mechanistic details of complex biomolecular processes.

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Mass photometry, Single molecule, Biomolecular interactions, Label-free detection.

Background and introduction

Biomolecular interactions are fundamental to the majority of cellular processes [1]. The interacting building blocks, such as proteins, nucleic acids and lipids form a variety of functional structures over different length scales and levels of complexity, ranging from simple stoichiometric interactions such as ligand-receptor or antibody–antigen recognition [2], the formation of functional entities such as viruses [3], all the way to micron sized dynamic architectures of the cellular cytoskeleton [4] (Figure 1a–c).

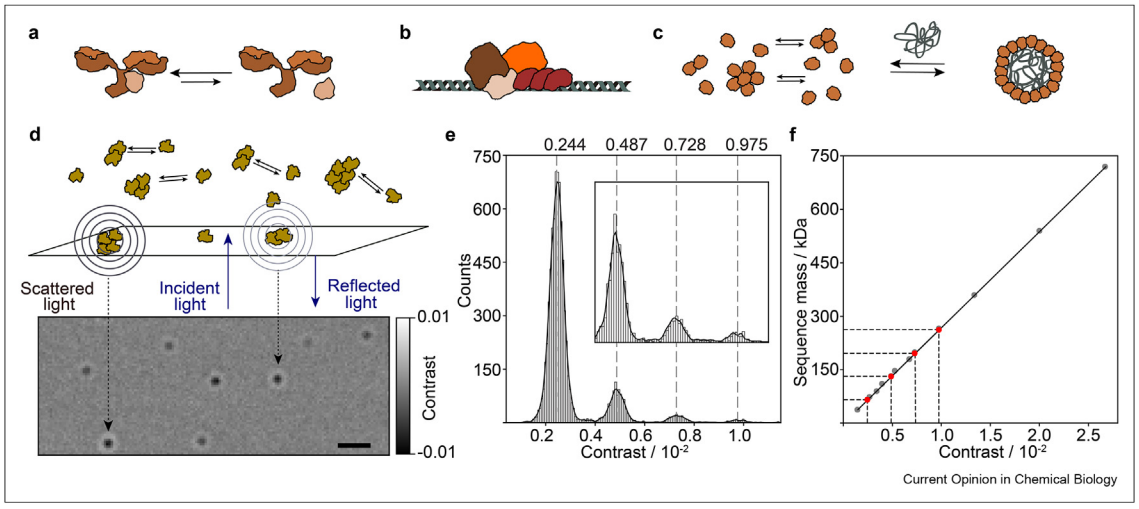
A central objective of molecular biophysics is to resolve and quantify the nature, strength and dynamics of these interactions, and to translate this knowledge into a detailed understanding of the molecular mechanisms governing cellular processes.

Characterisation of inter-molecular interactions requires quantitative *in vitro* techniques that provide information on structure, stoichiometry, energetics and kinetics. Elementary 1:1 interactions are sufficiently simple to be characterised by bulk methods since the ensemble-based average provides the required information for resolving one or few interaction parameters [5–7]. This is not the case, however, when considering macromolecular complexes involving multiple interacting subunits or complex stoichiometries. Here, extracting mechanistic information from ensemble-based measurements relies heavily on modelling and fitting, an approach that becomes challenging as the number of degrees of freedom increases.

X-ray crystallography and cryo-EM provide detailed information on structure and stoichiometry of macromolecular interactions. These methods, however, are static, and inherently biased towards structures that can be crystallised or captured, and thus limited in their ability to tackle highly heterogeneous and dynamic systems [8]. Heterogeneity, however, is ubiquitous in many biological processes and is often related to function, such as in self-assembly where the heterogeneity in the aggregation state is directly linked to the symmetry of the higher-order structure, the energetics associated with inter-subunit interactions, and to the mechanism of assembly [9,10]. Similarly, protein-DNA complexes often involve multiple proteins that interact in an hierarchical manner with affinities regulated by ATP binding and hydrolysis, resulting in both highly dynamic and heterogeneous complexes [11,12].

Resolving the solution distribution of interacting species and the associated time dependence is therefore key to revealing molecular mechanisms, as it is closely linked to the underlying free energy landscape. Achieving this aim remains challenging considering the need to detect and quantify different species in dynamic mixtures. In particular, tight sub-μM affinities that often drive the formation of molecular machines,

Figure 1



The importance of biomolecular interactions and their characterisation by mass photometry. Range of biomolecular interactions from simple 1:1 interactions (a), to macromolecular machines (b), and assemblies (c). (d) Working principle of mass photometry. Binding events appear as individual diffraction-limited events (scale bar 1 μm) in the rolling averaged ratiometric images, emphasising that intensity changes caused by reflectivity changes originate from binding of biomolecules to the glass surface. The contrast of each event is proportional to the mass of the bound macromolecule. (e) Recording the contrast for many molecules results in a histogram, which is representative of the distribution of species in solution. (f) Contrast measurement for a series of biomolecules allows for the accurate conversion of optical contrast to mass.

require measurements at very low analyte concentration, which is difficult for bulk methods.

These challenges are in principle addressable by single molecule techniques that are able to detect individual species, quantify their interactions and provide sufficient statistics to accurately estimate solution distributions. At the same time, single molecule biophysics in the context of interactions has been dominated by fluorescence-based approaches, which often struggle with quantification once more than a few species are involved, in addition to the need for the introduction of extrinsic labels.

The advent of mass photometry (MP), the label-free detection and mass measurement of individual biomolecules in solution [13], removes the need for labelling, provides a universal detection and identification approach through elastic light scattering and mass, and is intrinsically quantitative [14]. Here, we review recent efforts using MP for the characterisation of biomolecular structures, interactions, and dynamics.

Principles of mass photometry

In a mass photometry experiment, a drop of solution containing the biomolecules of interest is placed on a glass coverslip illuminated by a laser impinging on the glass–water interface. Individual molecules are detected through the change in light scattering at the interface when biomolecules (un)bind from said interface (Figure 1d). The interference between the light scattered from the biomolecule and the reflected light from the glass–water interface is then imaged on a camera. The resultant interference contrast scales linearly with the polarisability of the molecule, which is proportional to its refractive index and volume (Figure 1d and e).

Optimising the relative amount of detected scattered relative to reflected light, combined with data analysis based on rolling average ratiometric imaging, makes it possible to accurately determine the change in the local reflectivity caused by individual proteins [15,13]. As a result, the molecular mass of individual proteins can be determined with few percent accuracy to its sequence mass. In addition, the single molecule measurement precision is sufficient to resolve different oligomeric states, which is central to any attempts at revealing and quantifying biomolecular complexes and dynamics [13]. The linear relationship between polarisability and mass includes both single- and double-strand DNA [16]. Following calibration with a mass standard (Figure 1f), MP thus provides mass quantification for individual species with up to 20 kDa (FWHM) resolution and 2% mass accuracy [13].

Current status of mass photometry

MP has been applied to a vast variety of biomolecular systems since its inception. These include the investigation of protein oligomerisation [17,18] and heterogeneity [19], the characterisation of interactions between integral-membrane proteins in membrane

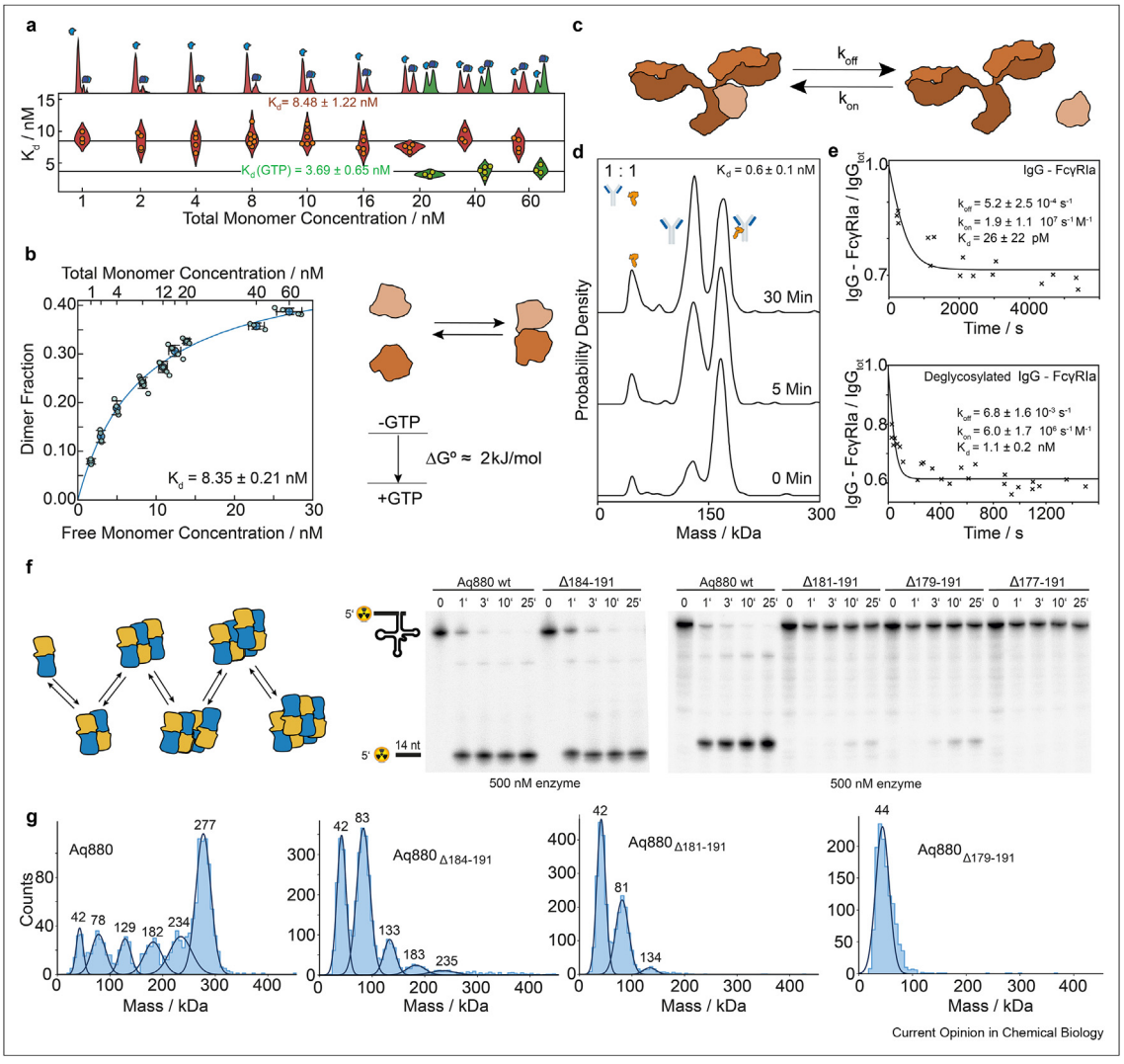
mimetic systems [20], as well as nucleic acids and their complexes [21,16]. MP was also shown to be applicable to study antibody–antigen interactions, many of which have affinities in the nM range [14,22]. The ability of MP to resolve the mass of highly glycosylated and heterogeneous antibody–antigen complexes and quantify their associated affinities and dynamics was shown to provide unique and complementary information to traditional techniques such as size-exclusion chromatography multi-angle light scattering and native mass spectrometry [23]. It was further applied, in combination with charge detection mass spectrometry, to study the interactions between antibodies and the SARS-CoV-2 spike receptor, providing information on affinity, avidity and cooperativity [24].

All of the above depend on the fundamental capabilities of MP to quantify molecular interactions by resolving the stoichiometry and dynamics of the interaction, as well as the abundance of the interacting species, which yields the associated energetics and molecular mechanisms.

Extracting thermodynamic quantities

The arguably simplest example of self-assembly is dimerisation, which often acts as the first step prior to

Figure 2



Examples of MP-based studies of the energetics, kinetics and function of biological macromolecules. (a–b) Quantifying the free energy of the formation of $\alpha\beta$-tubulin and changes associated with GTP presence. (c–e) Following the dissociation kinetics for sub-nM affinity complexes. (f–g) The effect of oligomerisation on pre-tRNA processing enzymatic activity. Adapted with permission from Fineberg et al. [25] Copyrights 2020 Elsevier, reference [14] Copyrights 2020 Wiley-VCH and reference [26] Copyrights 2021 Feyh et al. All under Creative Commons Attribution 4.0 International License https://creativecommons.org/licenses/by/4.0/.

the formation of larger structures. In these cases, the associated affinities are high, with dissociation constants in the nM regime. Quantifying such tight interactions is non-trivial due to the associated requirement for measurements at either very low analyte concentrations, or long term measurements. A simple example is the $\alpha\beta$-tubulin dimer, the fundamental building block of microtubules, where a broad range of affinities has been reported using a variety of techniques. Mass photometry is ideally suited to study such interactions because single molecule operation requires nM total protein concentrations. The resulting mass distributions recorded at total tubulin concentrations between 1 and 60 nM (Figure 2a) reveal a clear trend in the molar fractions and can be used to extract the respective dissociation constant (8.48 $\pm$ 1.22 nM). Owing to the large number of detected particles in a typical MP measurement, the extracted dissociation constant based on individual measurements compared well to a full titration procedure (Figure 1b), demonstrating the ability of MP to extract an accurate measure for the affinity from a "single shot' experiment. The high measurement accuracy from individual measurements makes it possible to resolve even small changes in the interaction free energy, such as heterodimer formation in the presence of GTP(K_d = 3.7 $\pm$ 0.65 nM). This change in the equilibrium constant corresponds to a standard free energy change, $\Delta\Delta G^\circ$ = 2 kJ/mol, which is equivalent to 0.8 k_BT. Given the high confidence with which this shift can be observed, and the reproducibility of the measurement, one can envision that even smaller changes in the interaction free energy could be potentially revealed. Considering the relative errors of the reported affinities (between 14% and 18%), we can expect to be able to resolve subtle changes in dimerisation free energies down to about 0.2 k_BT. The demonstrated sensitivity and reliability of MP in not only quantifying affinities, but also resolving small changes in reaction free energy differences will enable the exploration of very subtle changes in solution conditions (such as added salt or osmolytes), post-translational modifications or small molecule binding on protein–protein interactions using similar, simple and quick experimental procedures.

Binding stoichiometry and kinetics

Despite the importance of thermodynamic characterisation to our understanding of molecular mechanisms, it only provides information on the equilibrium state of the process. At conditions far from equilibrium, we expect that kinetic factors dictate the course of an interaction. This is particularly important when considering strong interactions, where local reversibility cannot be assumed owing to very slow dissociation rates. In this case, kinetic studies can validate whether equilibrium can be achieved at relevant timescales and conditions. Mechanistically, the additional dimension provided by a time-resolved experiment allows us to deconstruct the overall reaction into its elementary steps, where the rates are governed by the concentration of molecules and the forward and backward rate constants, $k_{on/off}$.

The concept of counting free and bound species can be applied to kinetic studies as long as mass distributions can be recorded on a time scale shorter or comparable to the process of interest [14,23]. This can be achieved, for example by monitoring the time evolution of the abundance of the bound and unbound species (Figure 2d), during the (dis)assembly processes, induced by either a concentration drop (disassembly) or mixing the interacting species (assembly), as shown here for the interaction between the monoclonal antibody trastuzumab and the soluble domains of IgG Fc receptors [14]. Since all involved species can be reliably detected and resolved, the mole fraction of the complex can be used as the reaction coordinate (Figure 2e). Here, the reported temporal resolution of 30 s is sufficient to resolve the initial exponential decay of the complex, from which the respective rate constants can be extracted. In the example shown in Figure 2d and e, the effect of glycosilation on antibody–antigen interactions could also be resolved. Here, N-glycan removal led to an order of magnitude increase in the off-rate constant (decreased affinity), which confirms the critical role of glycans for binding affinities. In addition to mechanistic information, kinetic measurements performed by a concentration drop method, has the potential to expand the quantitative affinity measurement towards the μM range [23].

Linking oligomeric state with function to understand enzymatic mechanisms

MP can resolve mass distributions in close to native solution conditions with high mass sensitivity and resolution, which has been used, for example, to assess sample heterogeneity for structural analysis [19]. Therefore, MP can in principle extend structure–function analysis by introducing additional information on polydispersity and its dynamics with respect to function, in particular when combined with structural methods and biochemical analysis. In the case of the prokaryotic, Homologs of Aquifex RNase P (HARP) system, responsible for pre-tRNA processing, MP was employed to elucidate the relation between the enzymatic activity of Aq880, a HAPR protein, and its solution polymerisation state (Figure 2f). Aq880 is known to assemble into functional, higher order oligomers from a single polypeptide subunit of $\sim$23 kDa. The structure of the Aq880 oligomer, however, could not be resolved using X-ray crystallography or NMR. In this work, MP measurements taken from a single peak eluted from Ni-ion affinity chromatography and SEC revealed a high degree of

polydispersity, which is most likely the reason for the failure of structural approaches.

By solving the structure of the closely related HAPR, Hhal2243, using cryo-EM, the authors showed that this minimalistic, protein-only, RNase P system is built from a monomeric subunit that forms stable dimers, which further assemble to form a screw-like homo-dodecameric structure. The resulting structure and assembly model correlates well with the observed 295 kDa and 277 kDa masses of the larger oligomer for Hhal2243 and Aq880, respectively, as well as with the ~40 kDa spacing between different species detected by MP (Figure 2g). Further structural analysis identified the importance of the C-terminal α-helix in stabilising dimer–dimer interactions responsible for oligomer formation. Stepwise truncation of the C-terminus of Aq880, reduced or even completely eliminated oligomerisation (Figure 2g). For the least perturbed protein, Aq880$\Delta_{184-191}$, assemblies identified by MP included octamers (183 kDa), decamers (235 kDa), and a small fraction of dodecamers. The corresponding enzymatic activity was 90% of wtAq880. Aq880$_{\Delta_{181-191}}$ formed tetramers and a low fraction of hexamers with enzymatic activity reduced to ~10%. More significant mutations ($\Delta_{179-191}$ and $\Delta_{177-191}$) resulted in dimer as the only observed species, which exhibited no pre-tRNA processing activity. Thus, these experiments demonstrate that the activity of the enzyme in pre-tRNA processing assays depends on its ability to form higher-order oligomers. Together with the structural analysis of the enzymatic catalytic site, these results led to a molecular mechanism in which oligomerisation is required for HARP activity, enabling the cooperative actions of two adjacent dimers in the complex. As the size of the oligomeric state increases, the stability of the dimer–dimer interaction increases, leading to more productive pre-tRNA processing.

Summary and outlook

In the few years since its introduction, MP has shown to be a promising approach for characterising biomolecular interactions. Owing to the universality of light scattering, it is applicable to a broad range of biomolecules, providing free energies, affinity, stoichiometry, and dynamics, while operating in native solution, thus facilitating integration with structural and biochemical assays. Recent advancements that have the potential to dramatically expand the scope of the technique include dynamic-MP, where mass measurement at the single protein level is applied to laterally diffusing species on lipid bilayers, rather than non-specific binding to microscope cover glass [27,28]. These studies extend the scope of MP beyond the measurements of solution distributions to include means for the characterisation of reaction dynamics through the extended observation of individual complexes. Similarly, these studies pave the way to studies of integral membrane proteins and their interactions in their native environment.

Some of the near future fundamental challenges in advancing the capabilities of MP as well as extend its use for the investigation of much more complex systems will rely on improvements in detection sensitivity, mass resolution, dynamic range, and an expanded concentration range of operation. In its current form, detection is possible down to ~30–40 kDa, which limits detection to a subset of biomolecules, but more importantly makes quantification challenging in the <50 kDa range, which is critical for accurate biophysical characterisation of heterogeneous mixtures. Weak scattering signals from small biomolecules cannot be distinguished from other noise sources contributing to the overall measurement background. These include illumination shot-noise and other scattering sources and instabilities in the imaging path, resulting in background noise fluctuations whose spatial pattern resemble those from biomolecules themselves. Further improvement in the hardware aimed at increasing the signal-to-noise ratio (SNR) by using higher illumination powers or optimised manipulation of the relative amplitudes and phases of the back reflected and scattered light fields, could potentially improve the detection capabilities as well as the resolution. Additionally, new approaches for data analysis of the ratiometric images aimed at background rejection using improved spatio-temporal filtering or more advanced machine-learning classification procedures, could increase the counting accuracy for low mass species.

Dynamic-range is an essential parameter in any bioanalytical assay. Quantification of self assembling systems such as filaments or viruses requires a dynamic range >100 in mass detection and counting statistics. Similarly, multi-protein complexes may involve multiple interactions of different strengths, which results in exponentially decreasing abundances for weakly interacting species. One of the advantages of MP is its lack of bias in detecting mass. As such, the resulting dynamic range in mass detection and counting depends on the solution abundance of each species and on how many molecules can be detected in a single experiment. The current limit on the order of a few thousand particles per measurement could be increased significantly by optical developments enabling larger fields of view, and faster frame rates, as well as incorporating high-throughput sample delivery and loading systems with automated data acquisition procedures. These will increase the precision of mass detection, mass separation and quantitative counting for both low (tens of kDa) and high (few MDa) mass species.

Lastly, current experiments are limited to ~100–200 nM object concentration, where the binding density limits

the ability to detect and extract the contrast of individual species. The ability to quantify interactions is therefore limited to the low nM range. While increasing the frame rate could somewhat alleviate this limitation, surface passivation will likely be required to sufficiently reduce binding rates and push detection capabilities towards the affinities in the μM range. In combination with specific surface functionalisation, these assays could provide means to detect specific species or interactions out of complex mixtures, by rejecting the vast majority of non-specific background.

Declaration of competing interest

The authors declare the following financial interests/personal relationships that may be considered as potential competing interests. Philipp Kukura reports a relationship with Refeyn Ltd. that includes: board membership, consulting or advisory, and equity or stocks.

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