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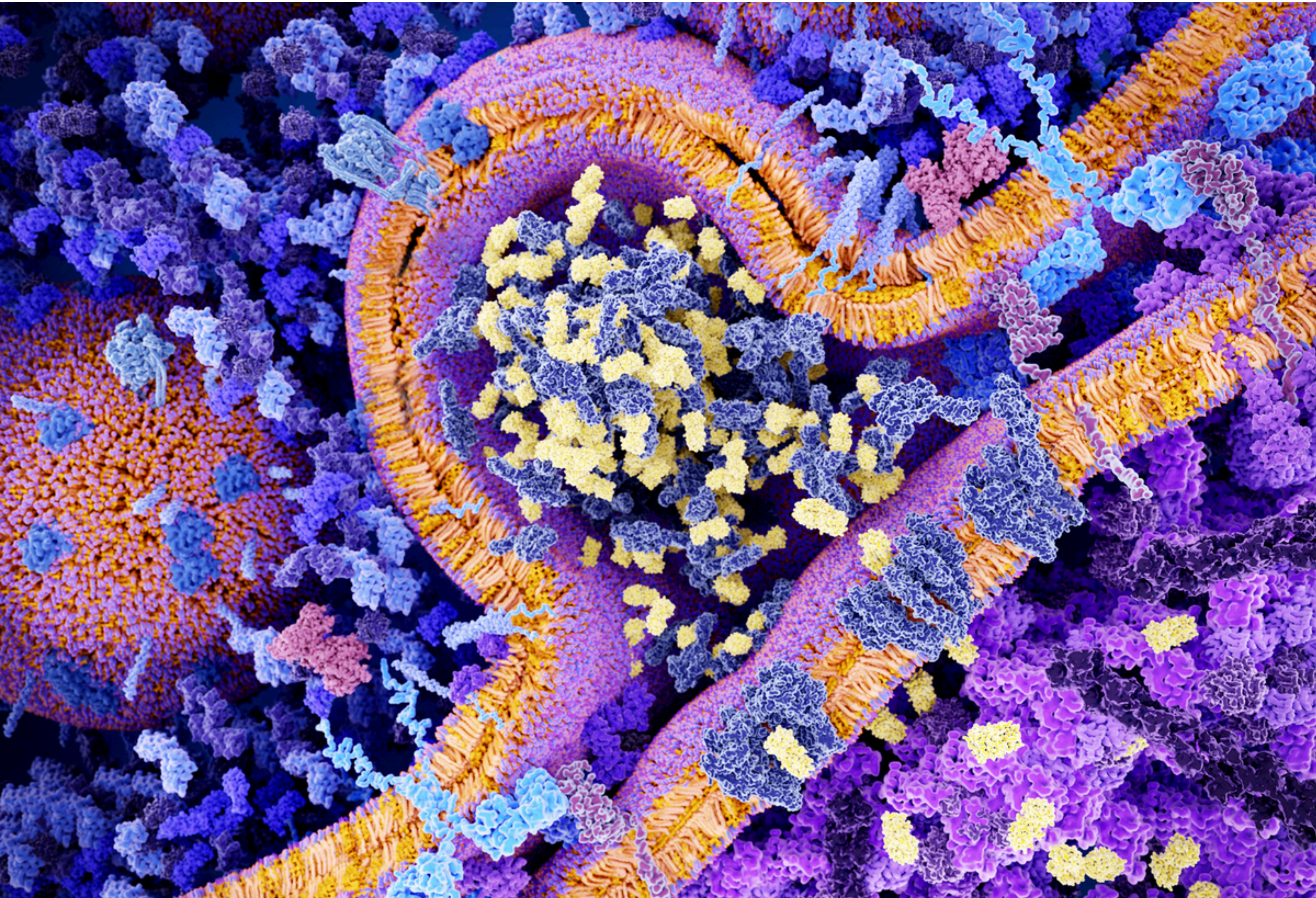
Mass Photometry & Macro Mass Photometry



A new way of characterising biomolecules

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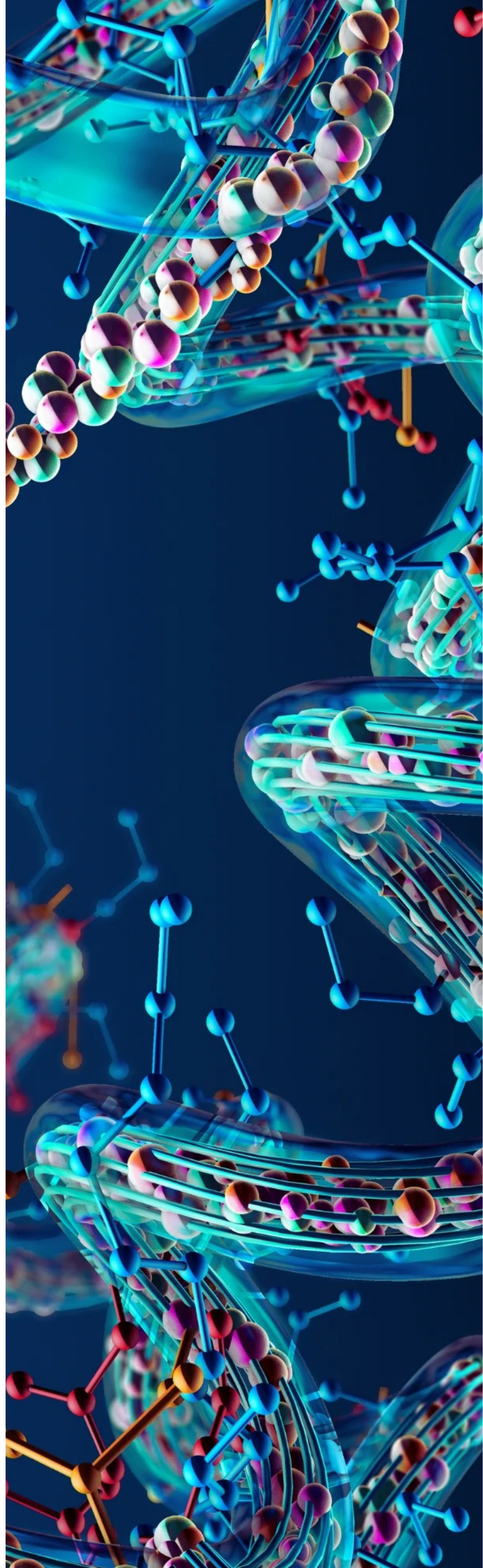

Revolutionary science born in Oxford

Refeyn pioneers analytical instruments that put molecular mass measurement capabilities within easy reach for scientists. Refeyn's unique products measure the mass of individual proteins, nucleic acids, complexes and viruses directly in solution – providing vital insights for scientific discovery, R&D and therapeutics production.

Our instruments feature **mass photometry technology**, which uses light to quantify the mass of single particles in solution without labels, and **macro mass photometry** technology, which uses light to characterize large viral vectors. Providing intuitive data in minutes, mass photometry technologies help scientists solve their research questions, optimize R&D processes and focus on innovation.

> *Our mission is to transform analytical workflows in the life sciences, biopharma and beyond, using the power of light.*

Refeyn was originally spun out of Oxford University in 2018 with the belief that broadening the availability of mass photometry would accelerate science. Today, Refeyn has scaled to be an international player with offices in in the US, China and Japan in addition to its headquarters in Oxford, UK. More than 350 of our instruments have been installed in labs around the world, resulting in more than **300 scientific papers being published so far that use mass photometry.**

Find out more

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Micromolar concentration analysis of samples using mass photometry

The analysis of biomolecular interactions is simple and straightforward with mass photometry. This popular and widely used technology allows for the quantitative analysis of binding, oligomerization, and macromolecular assembly, enabling this both in solution and without any requirement for labels.

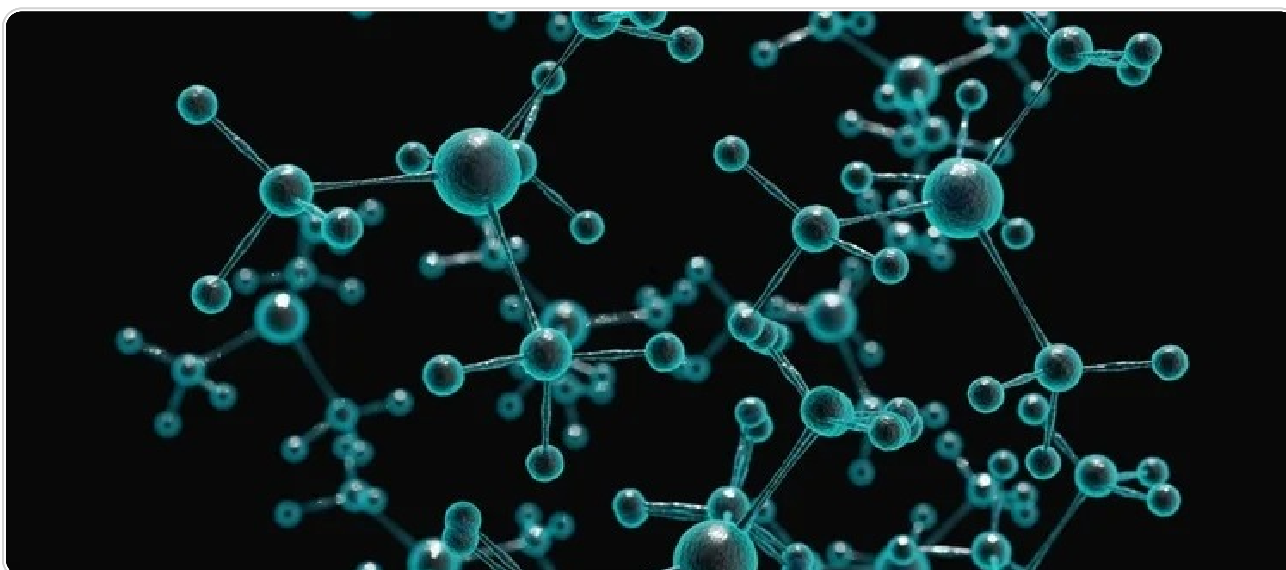

Image Credit: Shutterstock/Siwakorn TH

The MassFluidix® HC microfluidics system from Refeyn offers users a greatly expanded range of sample concentrations agreeable to investigation by mass photometry, enabling applications such as the characterization of low-affinity interactions.

Expanding the scope of mass photometry analysis

This bioanalytic technology is able to rapidly measure the mass distribution of biomolecules in a sample, achieving this in just one minute, in solution, and with no need to use labels.

This technology offers significant value when used in applications such as the

assessment of bindings, sample purity, and oligomerization.

The concentration of the measured sample should ideally be less than 100 nM to ensure optimal mass photometry measurements. This concentration is perfectly viable for a large number of measurement contexts, but it may be necessary to analyze sample behavior at a higher concentration in some instances.

Complexes formed via low-affinity interactions dissociate at low concentrations, meaning they can generally be detected only at higher concentrations.

Refeyn’s microfluidics system MassFluidix® HC successfully addresses this issue, greatly expanding the scope of samples suitable for measurement via mass photometry.

MassFluidix® HC increases the upper sample concentration limit from the nanomolar to the micromolar range. This article explores the system’s operation and outlines an example application whereby it is used to measure interactions between an immunoglobulin G (IgG) antibody and the IgG neonatal Fc receptor (FcRn).[1]

Characterization of low-affinity interactions

Using the MassFluidix® HC system, it is possible to perform measurements at an optimal concentration for mass photometry while simultaneously capturing the state of the biomolecular interactions occurring at micromolar concentrations.

The system achieves this by rapidly diluting the sample and flowing this across the measurement surface - prior to the biomolecular interaction equilibrium being disrupted by the dilution.

This microfluidic system is able to dilute a sample up to 10,000 times, passing this directly onto the measurement surface. Within <50 milliseconds of beginning dilution, the sample has reached the observation window and is ready to be analyzed.

As much as 98 % of the complex will remain intact when the sample is measured under these conditions, even when considering low-affinity (μM KD) interactions with dissociation rates as fast as one second^{-1}.[2,3]

Samples rapidly diluted via the MassFluidix® HC maintain their pre-dilution properties, meaning that users can employ mass photometry to characterize biomolecular activity that takes place only at higher concentrations, for example, low-affinity interactions.

The MassFluidix® HC system

The computer-controlled MassFluidix® HC system includes both fluid controls and a rapid dilution chip. Dilution occurs on the MassFluidix® HC chip, with the sample and buffer combined in a reverse Tesla valve mixer before the diluted sample flows through the observation area. The sample then leaves the chip via an outlet. (Figure 1A).

The wider system sees a central unit, computer, pump, flow rate monitors and pressure regulators working together to ensure that both the sample and buffer enter the chip at the correct flow rates (Figure 1B). This streamlined setup ensures rapid sample dilution that is appropriate for measurement via mass photometry.

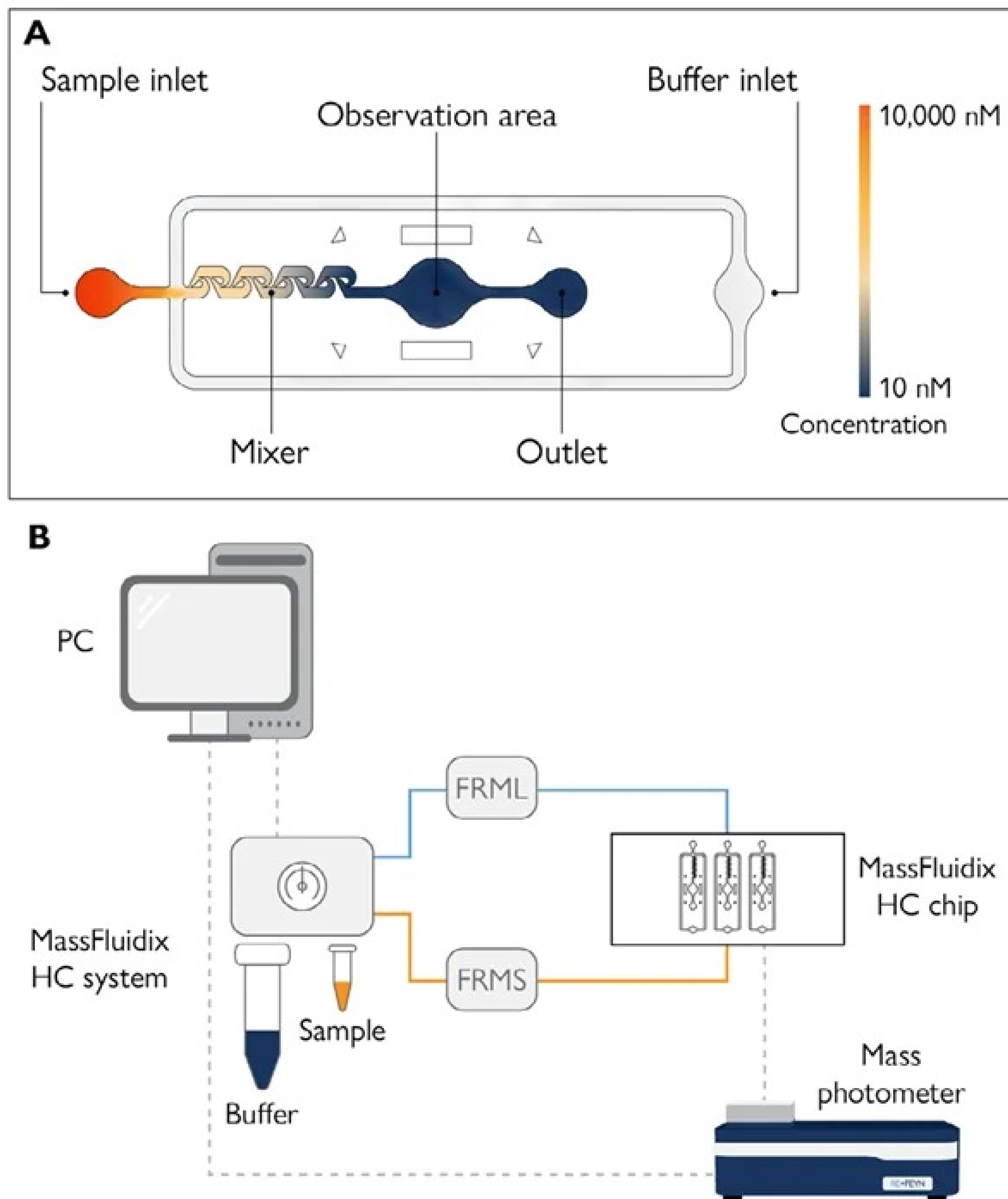

Figure 1. The MassFluidix HC system is designed for rapid dilution*. A: Illustration of the MassFluidix HC chip. The undiluted sample (orange) and buffer (grey) enter through inlets on opposite sides of the chip. The sample is diluted where the channels meet and in the mixer, reaching the target concentration (dark blue) near the observation area. Fluid color indicates concentration (see colorbar). B: The computer (top left) is connected to MassFluidix HC central unit (shown adjacent to the buffer and sample tubes) and the mass photometer (bottom right). Flow through the sample line and buffer line is monitored by the small flow rate monitor (FRMS) and the large*

flow rate monitor (FRML), respectively. The sample and buffer line tubing connects to a channel on the MassFluidix HC chip, which is placed inside the mass photometer. Image Credit: Refeyn Ltd.

A key difference between the MassFluidix® HC and traditional mass photometry is the fact that the molecules being investigated are under flow. Refeyn has verified - both theoretically and empirically - that the flow itself has a negligible effect on the measurement.

From a theoretical perspective, the presence of no-slip boundary conditions means that the velocity of fluid at the measurement surface is zero. It, therefore, remains negligible across the space where biomolecules are commonly measured: up to ~20 nm from the surface.

It is important to note that there is also no variation in concentration across the channel because the sample is homogeneously mixed as it reaches the measurement surface.

From an empirical perspective, measurements of a range of proteins in the system (undertaken by Refeyn) confirmed that counts accumulate as consistently as they do when using conventional measurements.

MassFluidix® HC reveals low-affinity complexes

In the example presented here, the MassFluidix® HC system was employed in an investigation of the binding of the IgG monoclonal antibody trastuzumab to the soluble domain of the FcRn.[1]

A regular mass photometry measurement was undertaken on a sample containing both proteins in a 1:10 ratio (IgG at 2 µM and FcRn at 20 µM). This sample had been manually diluted to 10 nM.

Two main peaks were observed in this instance, consistent with previously published data,[1] one of which corresponded to FcRn monomers and another to unbound IgG (Figure 2). There was no indication that IgG-FcRn complexes were

present.

It was evident, however, that FcRn-IgG complexes were present when the MassFluidix® HC system was used to measure the same proteins. The sample possessed an initial concentration of 22 μM in this measurement and was rapidly diluted 2000-fold to 11 nM in the system.

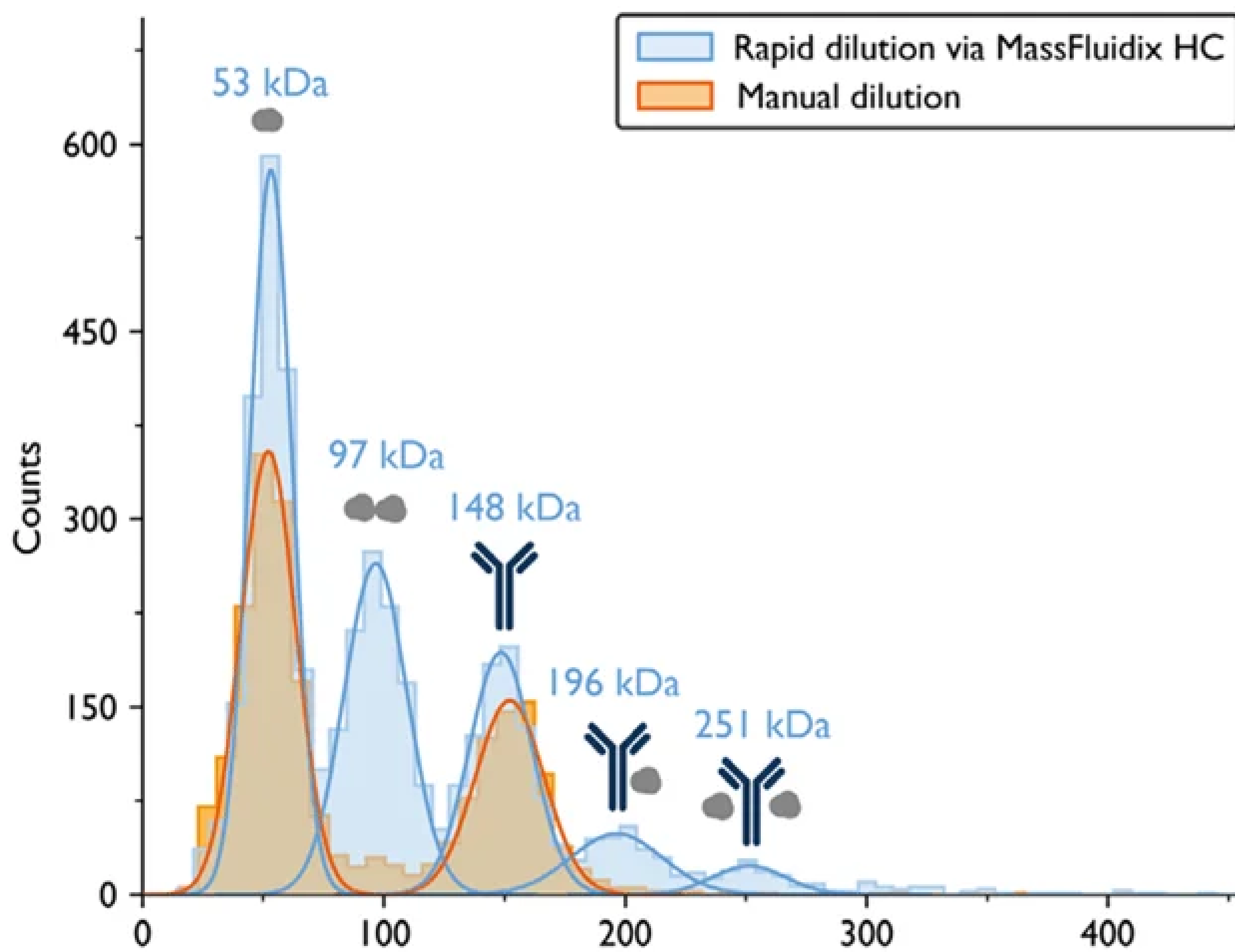

Figure 2. Mass histograms reveal low-affinity complexes only after rapid sample dilution. *Mass histograms and corresponding best-fit Gaussian distributions are shown for measurements of samples containing IgG and FcRn (mixed at 1:10 ratio) following manual dilution (orange) or rapid dilution via the Microfluidix HC system (blue). After manual dilution, peaks corresponding to FcRn monomers and IgG monomers were observed. After rapid dilution, in addition to the FcRn and IgG monomers, peaks corresponding to FcRn dimers and IgG-FcRn complexes with 1:1 and 1:2 stoichiometry were also clearly observed. Image Credit: Refeyn Ltd.*

Peaks corresponding to 1:1 FcRn-IgG complexes and 2:1 FcRn-IgG complexes were also clearly visible in this measurement, as well as the aforementioned peaks corresponding to FcRn monomers and unbound IgG (Figure 2).

This example highlights how mass photometry measurements undertaken using the MassFluidix® HC were able to detect low-affinity complexes that had not previously been observed.

Lower-affinity complexes cannot form unless the interacting biomolecules are present at higher concentrations. In this instance, therefore, results suggested that FcRn-IgG complexes possess relatively low binding affinities.

Differences in complex formation at 10 nM versus 22 µM clearly highlight the value of studying biomolecular interactions at higher concentrations in order to achieve a more comprehensive understanding of biomolecular system dynamics.

The MassFluidix® HC system empowers users to run these measurements using mass photometry.

Conclusion

The MassFluidix® HC system employs rapid dilution in order to expand the range of sample concentrations that can be effectively analyzed using mass photometers, for example, Refeyn's One^{MP} and Two^{MP} mass photometers.

Using mass photometry, measuring the mass distribution of biomolecular samples in solution is quick and easy, without labels and using minimal sample quantities.

This technology and combination of equipment also enable the study of biomolecular assembly, oligomerization and interactions, as well as assessments of sample purity. The MassFluidix® HC also makes it possible to effectively characterize low-affinity interactions, broadening the applicability of mass photometry even further.

Experimental details

- The coverslip was coated with cationic coating
- Samples were at pH 5.0 and diluted in PBS, pH 5.0
- Flow rates were 0.5 µL/minute (sample) and 1 mL/minute (buffer)
- The measurement time was 60 seconds
- A Two^{MP} mass photometer was used for all data collection

References and further reading

1. Soltermann et al., Angew. Chem. Int. Ed., 2020
2. Pollard T.D., MBoC, 2017
3. Jarmoskaite et al., eLife, 2020

Acknowledgments

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Biomolecular characterization using mass photometry

Mass photometry is a powerful and widely regarded analytical technology that is able to accurately measure the mass of single particles in a solution without the need for labels. This technology is used in a diverse array of applications, including biomolecular function research, structural biology, and bioanalytics.

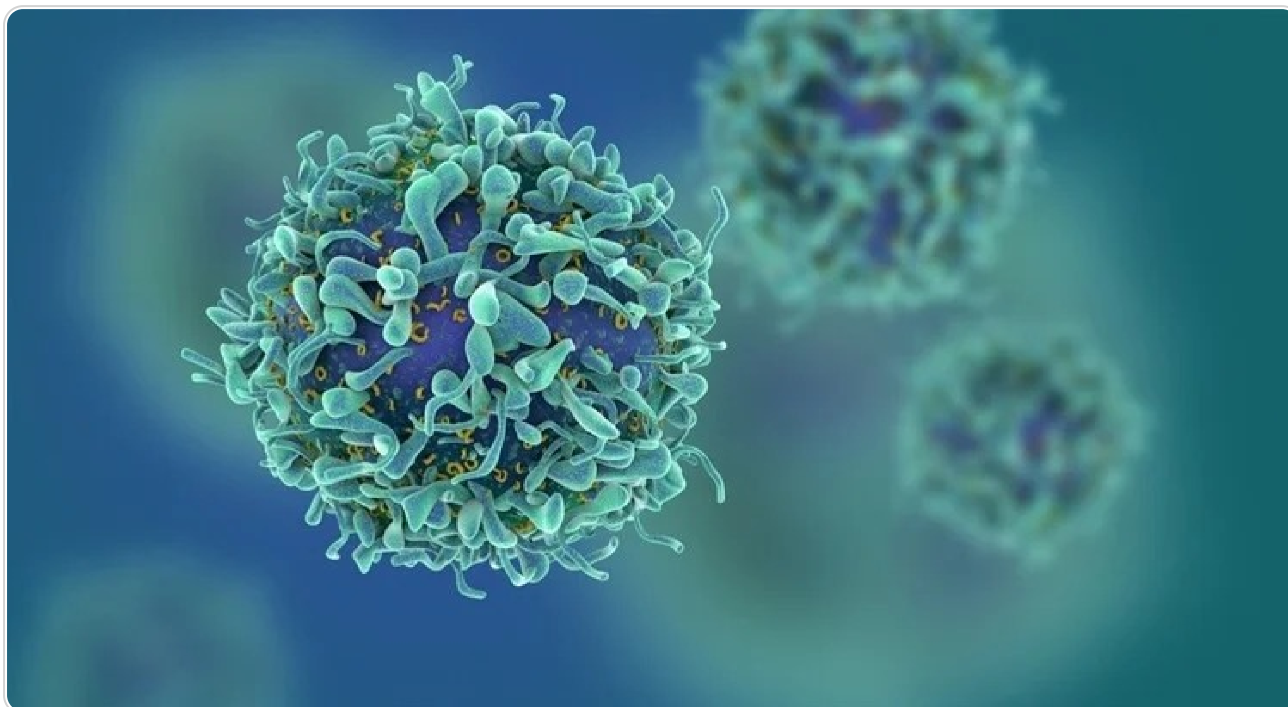

Image Credit: Shutterstock/fusebulb

What is mass photometry?

Mass photometry is an analytical technique used to investigate biomolecules at the single-particle level. It is able to analyze these particles in their native state and without the need for labels.

Mass photometry works by measuring the light scattered by individual particles, using this signal to quantify these particles and measure their mass (Figure 1).

What is mass photometry used for?

Using mass photometry, it is possible to determine the mass distribution and

relative concentrations of all species in a particular sample. This technique can be used to characterize nucleic acids, proteins, and a range of other bioparticles (Figure 2).

Mass photometry can be applied to biomolecules on membranes or in solution membranes,[1,2] and is ideal for streamlining and accelerating an array of experimental workflows. These workflows include:

- Sample characterization, including direct assessment of sample homogeneity and purity during expression and purification.[3]
- Protein oligomerization, including the straightforward validation of protein mono or polydispersity as part of formulation or structural biology studies.[3,4]
- Biomolecular interactions, including the rapid and streamlined quantification of high-affinity interactions among biomolecules.[5,6]
- Macromolecular complex assembly, including monitoring the assembly and disassembly of complexes over a period of time.[7]

Mass photometry provides users with quantitative and easy-to-interpret results, meaning it is an ideal solution to complement existing analytical technologies or provide unique insights or confirmation where required.

Key benefits of mass photometry

Mass photometry offers a number of benefits to its users, including:

- The ability to accurately measure the true native behavior of molecules
- Suitability for use in solution and in a range of buffers
- Compatibility with membrane proteins
- A label-free technique with no need for sample modification
- The ability to provide details of all sub-populations in samples
- The capacity for single-molecule counting
- Suitability for working with a wide mass range and high dynamic range
- The ability to use a single assay format while delivering multiple results
- The option to assess homogeneity, structural integrity and activity
- A rapid, straightforward, and user-friendly approach
- A technique that uses a minimal amount of sample

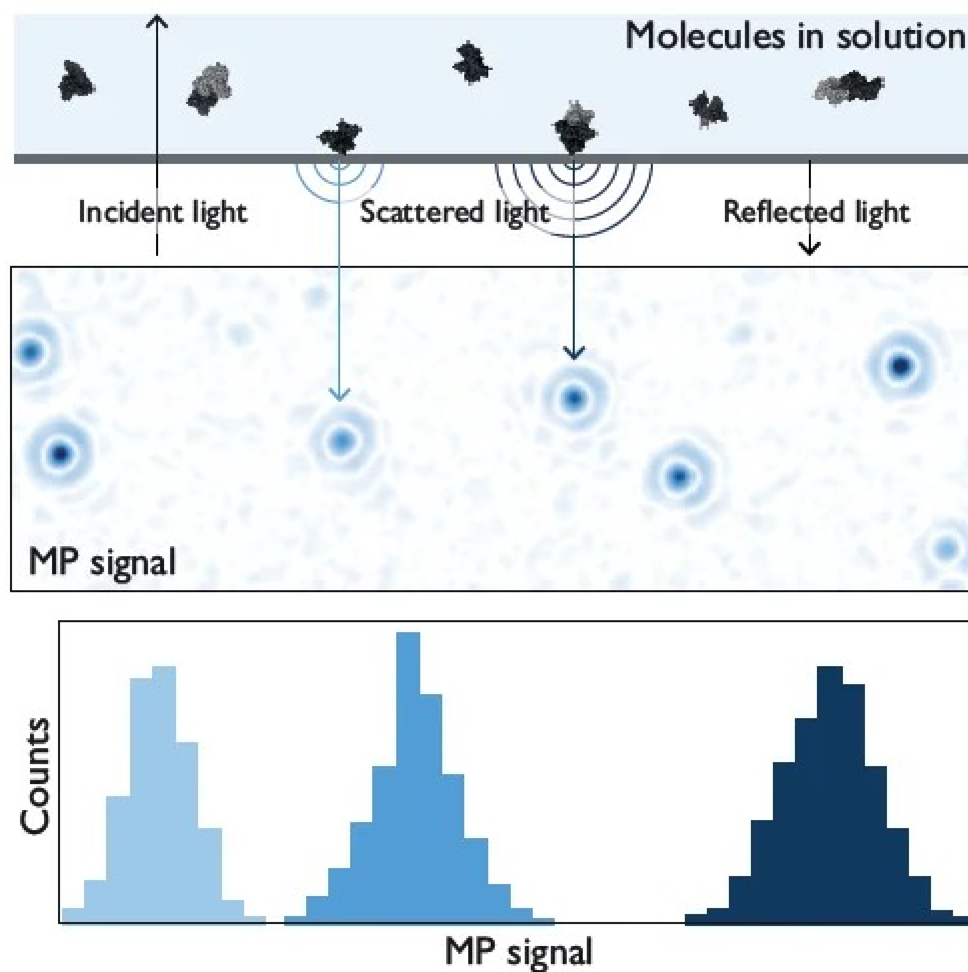

Figure 1. The principle of mass photometry. *The light scattered by a molecule that has landed on a measurement interface interferes with light reflected by that surface. The interference signal (MP signal) scales linearly with the molecule's mass. Image Credit: Refeyn Ltd.*

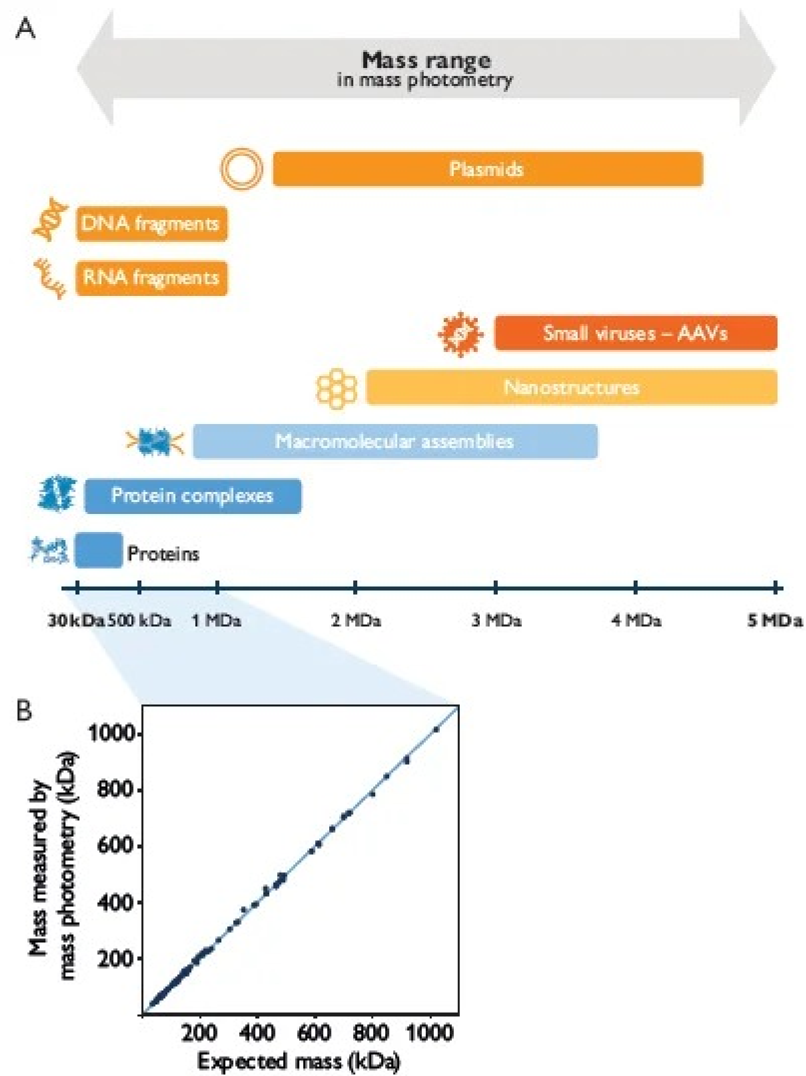

Figure 2. Mass photometry accurately measures diverse biomolecules.
A. Mass photometry works across a broad mass range and for different types of molecules. B. The mass measured by mass photometry agrees closely with the expected mass for a range of proteins, illustrating its accuracy. Image Credit: Refeyn Ltd.

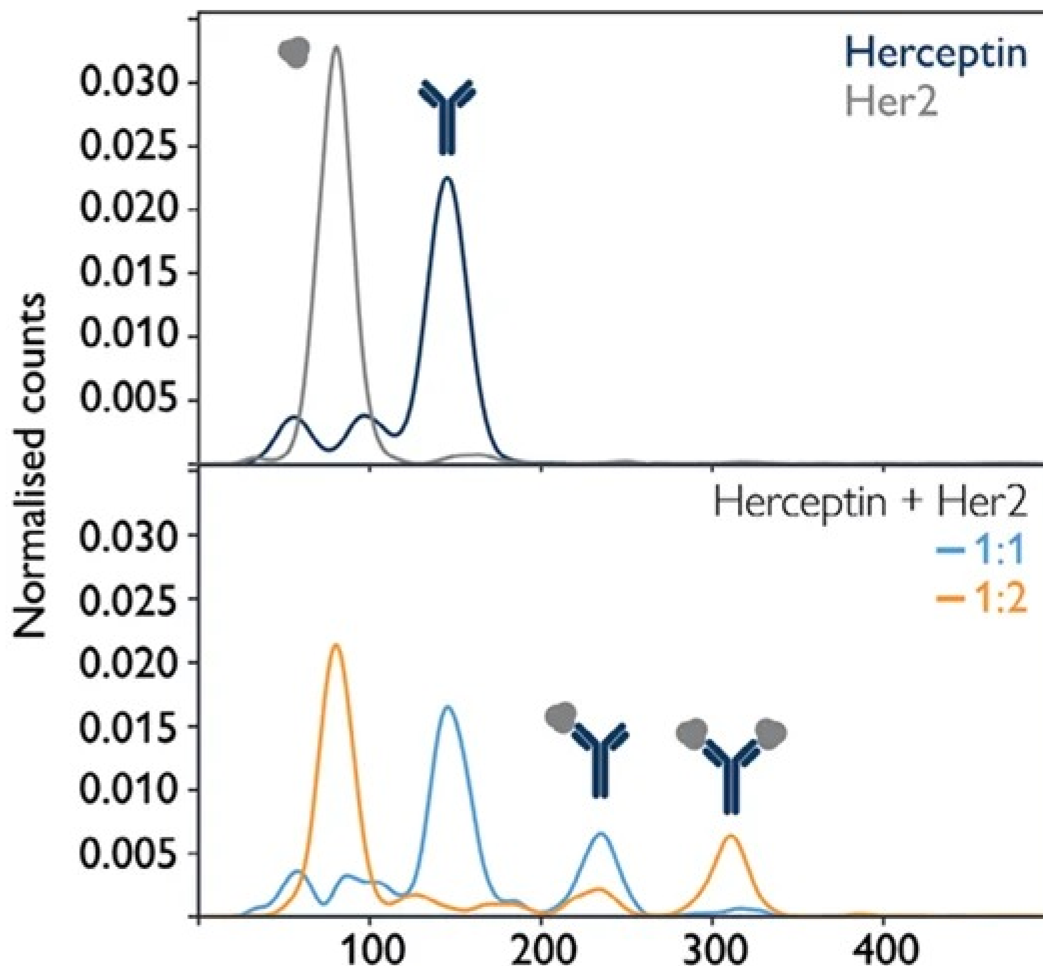

Figure 3. The Two^{MP} can be used to quantify molecular interactions. *The monoclonal antibody Herceptin (trastuzumab) and its target, Her2, were measured individually and in mixtures, demonstrating the instrument's ability to quantify the interactions of individual antibody molecules with target molecules. mage Credit: Refeyn Ltd.*

Mass photometry quantifies binding and assembly

Mass photometry allows users to undertake quantitative studies of biomolecular interactions in solution, such as protein-protein or DNA-protein. As it is able to determine the relative concentrations of both bound and unbound species (Figure 3), mass photometry is ideally suited to the monitoring of interactions and the determination of binding constants for high-affinity interactions.[5]

Mass photometry's ability to readily detect sub-species means it is able to analyze complex, multistep interactions with ease. As users are able to determine intermediate binding constants,[8] they can optimize experimental

conditions as appropriate.

Its broad mass range (Figure 2) also makes it ideally suited to measuring the assembly or disassembly of large, multicomponent complexes (Figure 4) and when looking to follow assembly kinetics.[7,8]

Mass photometry is useful for quality control

Sample homogeneity is informed by molecular mass, and this universal readout also provides information on the structural integrity and activity of biomolecules and biomolecular complexes.

Using mass photometry, it is possible to ascertain the complete mass distribution of a sample in mere minutes while simultaneously maintaining a near-native aqueous environment for the sample.

Mass photometry is a rapid technique that requires very little sample, making it well suited for applications requiring frequent sample characterization, the highest quality results, and straightforward, streamlined analytical workflows.[3]

These characteristics make mass photometry an ideal choice for quality control applications such as protein purification optimization and the preparation of complex samples for analysis by cryoEM.[3] It is also well suited to the assessment of biomolecular oligomerization behavior, allowing users to rapidly optimize buffer conditions, for example.[4]

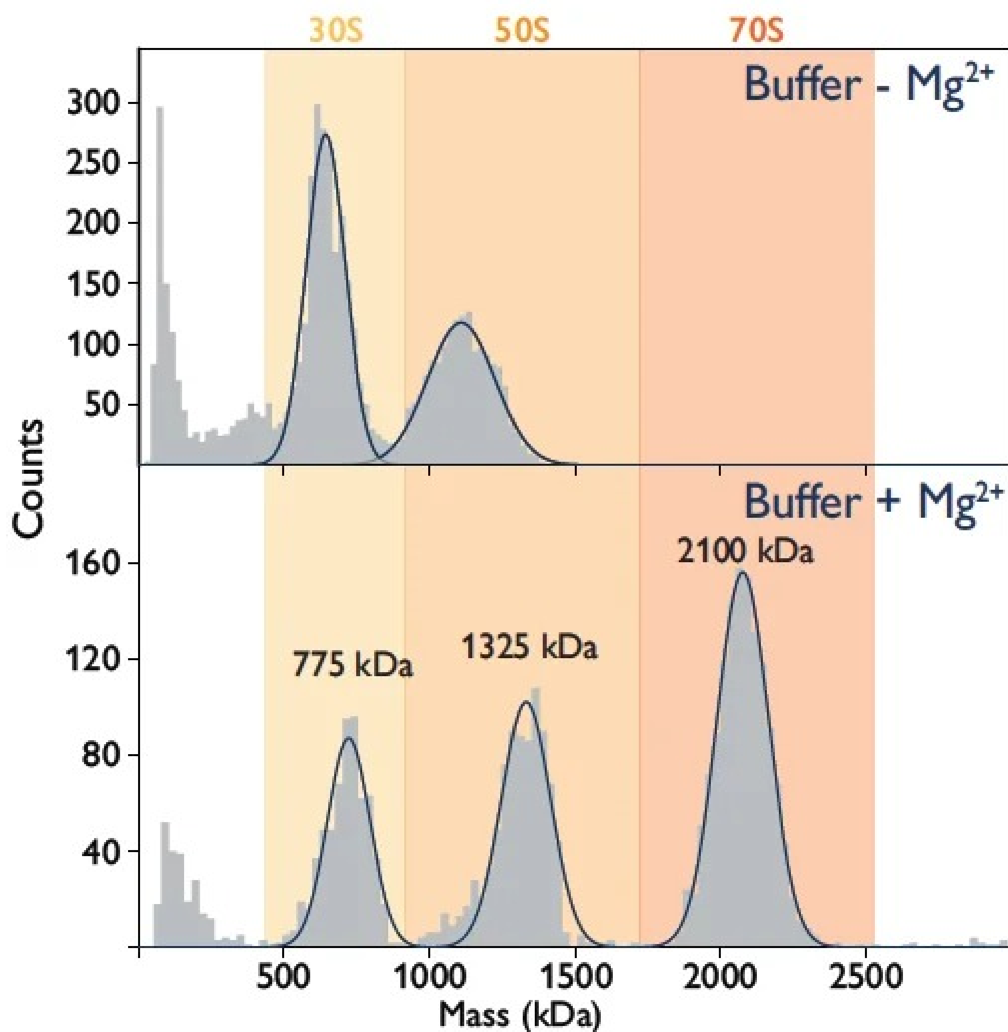

Figure 4. The assembly of bacterial ribosomal complexes monitored. *Mass distributions show that ribosome complexes disassemble completely into two subunits in the absence of* Mg^{2+} *(top). Complexes assemble in the presence of* Mg^{2+} *(bottom). Image Credit: Refeyn Ltd.*

How does mass photometry work?

Mass photometry uses light to weigh single molecules. This technique works because the amount of light scattered by molecules correlates with their mass and this scattering of light scales linearly with the particle's volume and refractive index.

Biomolecules' optical properties and density generally vary by just a few percent, meaning that the scattering signal will be directly proportional to their mass.

In order to accurately detect the tiny amount of light scattered by individual

molecules, mass photometry leverages a combination of carefully controlled illumination, novel spatial filtering in the path of the detection beam,[9] and advanced image analysis.[8]

Mass photometry was developed at Oxford University, building on the underlying principles of interference reflection microscopy[10] and interferometric scattering microscopy.[11]

References and further reading

1. Foley et al., Nat Methods 2021
2. Heermann et al., Nat Methods 2021
3. Sonn-Segev et al., Nat Comm 2020
4. Haeussermann et al., Angew Chem Int 2019
5. Wu et al., Anal Biochem 2020
6. Soltermann et al., Angew Chem Int Ed 2020
7. Malay et al., Nature 2019
8. Young et al., Science 2018
9. Cole et al., ACS Photonics 2017
10. 1Verschueren, J Cell Sci 1985
11. Ortega-Arroyo et al., Phys Chem Chem Phys 2012

Acknowledgments

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Protein sample assessment using mass photometry and size exclusion chromatography

Mass photometry is a popular and robust analytical tool that facilitates the accurate mass measurement of single molecules in solution, in their native state and without the use of labels.

Image Credit: Dark Gel

The studies presented in this article compare the use of mass photometry to size exclusion chromatography (SEC) - the industry's gold standard - for the analysis of protein abundance and antibody aggregation.

As complementary analytical tools, mass photometry and size exclusion chromatography (SEC) can be used to gain detailed and abundant information on the biophysical and biochemical properties of biomolecules within a given sample.

There are a number of important considerations to be aware of when comparing the two techniques' resulting datasets, however, and these are also explored in this article.

Mass photometry and SEC data will be compared via two case studies: one

which showcases the two techniques' ability to quantify the relative abundance of each protein in a sample mixture across a wide mass range and another that looks at how the techniques can be employed in the characterization of antibody aggregation.

Case study 1: Assessing protein abundance within a sample mixture

Figure 1 highlights the inherent differences between SEC and mass photometry via the analysis of a sample mixture comprising four different proteins. These analyses were performed by the laboratory of Professor Justin Benesch (University of Oxford), and all make use of SEC-UV and mass photometry,

An identical sample mix was used for both the SEC- UV and mass photometry measurements in this instance, but there is an obvious disagreement between the techniques in terms of which proteins are the most abundant within the mixture.

SEC-UV suggests that thyroglobulin and ferritin are the most abundant in this example, while mass photometry suggests that conalbumin and aldolase are the most abundant. To properly understand this discrepancy, it is important to consider the fundamental principles of each technique and its use.

Fundamental principles of mass photometry versus SEC

As a single molecule technique, mass photometry provides a particle count versus mass. This means that it detects and counts the number of particles of a given mass.

Essentially, the intensity of a given mass photometry peak (the area underneath the peak) corresponds to the absolute number of molecules with the given mass that were detected, and this measurement is also proportional to the molecular concentration.

SEC-UV analysis works by measuring UV absorbance versus column elution time. Assuming they are known, it is possible to convert data to absorbance versus mass using the species' molecular weights. This approach is also based on the fact that the species of the greatest hydrodynamic volume typically elutes first.

However, it is important to note that absorbance data is not just a function of the molecule’s concentration; this will also depend on the molecule’s UV-absorbing properties. Data in this instance will also be impacted by the specifications of the UV detector itself, such as its sensitivity at a specific UV wavelength.

In cases where the molar extinction coefficient of each molecule is known, it is possible to determine each molecule’s concentration within a mixed sample by evaluating the absorbance data using the Beer-Lambert law.

Mass photometry agrees with normalized SEC-UV analysis

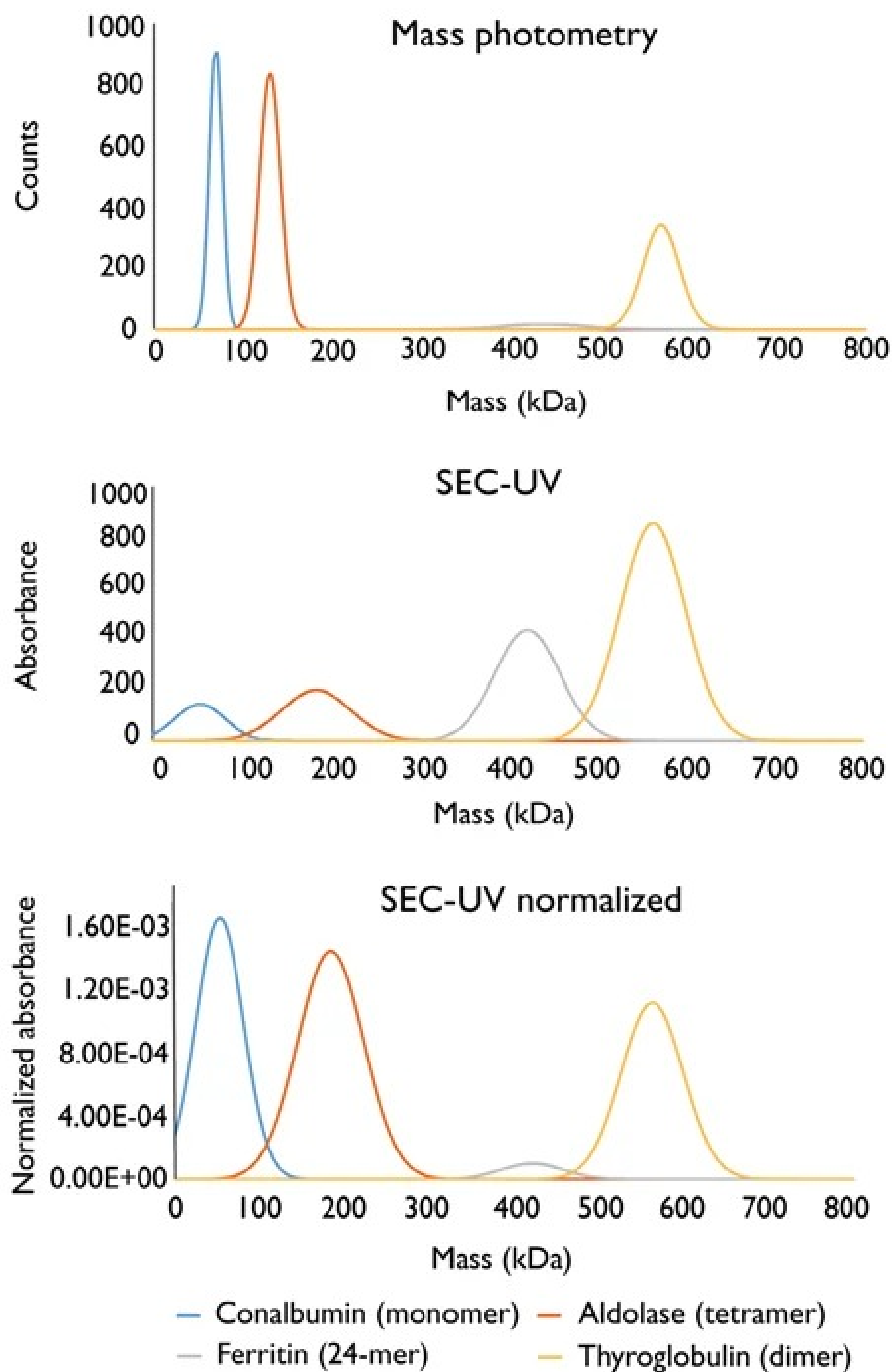

Figure 1. Analysis of the same sample by mass photometry and SEC-UV illustrates fundamental differences between the techniques. *The sample analyzed contained a mixture of four proteins: conalbumin, aldolase, ferritin and thyroglobulin. The molecular weights of each protein within the mixture were known and used to convert the SEC-UV profile from absorbance vs. elution time to absorbance vs. mass. The molar extinction coefficients of each protein were used to normalize the absorbance data. Image Credit: Refeyn Ltd.*

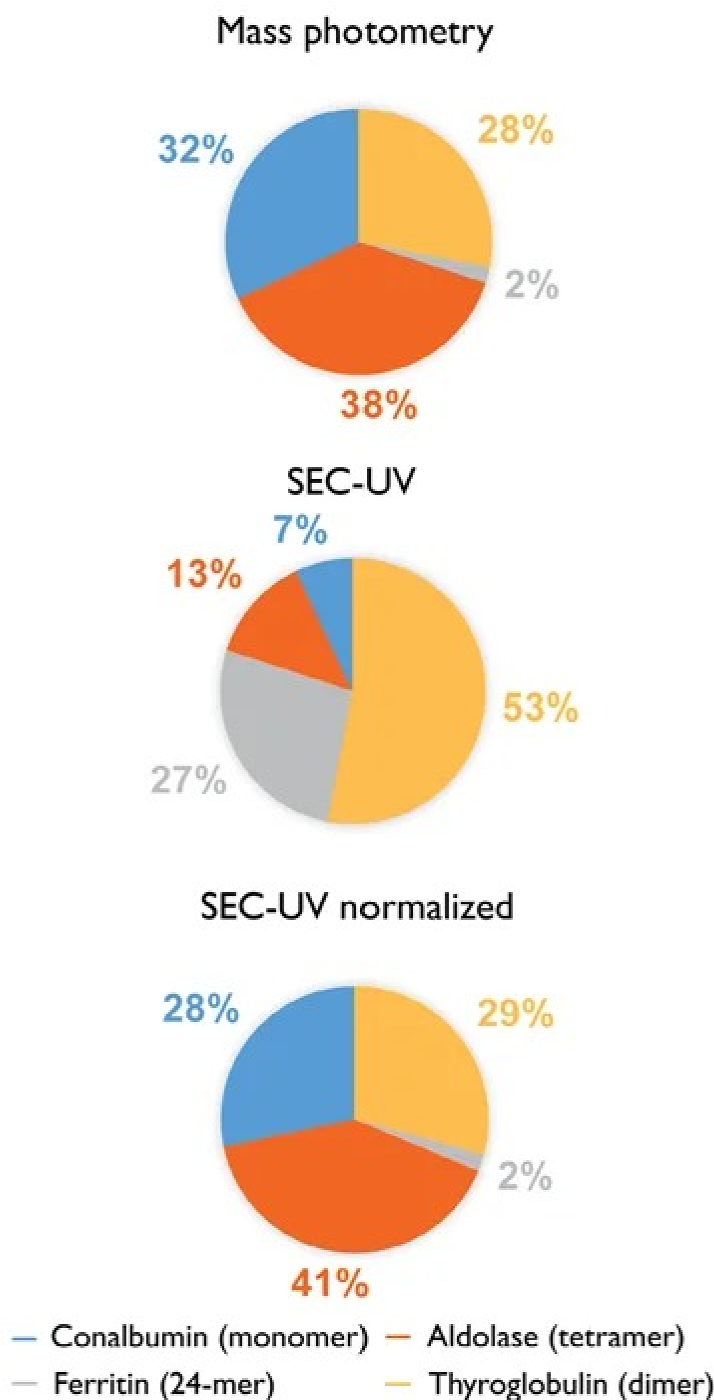

Figure 2. Abundance (%) of each protein within a sample mixture as determined by mass photometry and SEC-UV. *For mass photometry, the number of counts for each molecule is expressed as a percentage of the total number of counts. For SEC-UV, the relative abundances of each molecule are shown before (SEC-UV) and after (SEC-UV normalized), normalizing for the molar extinction coefficient. Image Credit: Refeyn Ltd.*

Using this approach with each individual protein in the sample will result in a normalized SEC-UV data profile that will visually match the corresponding mass photometry data (Figure 1). Quantification of the relative abundance of each

protein will also confirm that the results of SEC-UV and mass photometry are in close agreement (Figure 2).

Case study 2: Monitoring aggregation levels of monoclonal antibodies

Many protein-based biopharmaceuticals rely on aggregation to function in terms of their product efficacy and safety, including monoclonal antibodies (mAbs) and multi-specific antibodies. It is essential that proper monitoring of aggregation levels is performed when developing and evaluating these biopharmaceuticals.

SEC is commonly regarded as the gold-standard analytical tool for the assessment of nanometer-sized aggregates. SEC is regularly used in conjunction with multi-angle light scattering (MALS) in cases where it is necessary to determine molecular weight and size. However, this technique can be complicated by a number of factors, including column and mobile phase optimization.

Mass photometry works by detecting the light scattered by single molecules, allowing the efficient measurement of biomolecules' molecular mass in solution. This technique is capable of analyzing exceptionally small (µL) sample volumes at low concentrations (100 pM up to 100 nM) in just a few minutes while maintaining native conditions.

The example study presented here was conducted by RIC Biologics (Kortrijk, Belgium). SEC and mass photometry were employed in the measurement of aggregation levels of samples of trastuzumab (a monoclonal antibody) and a number of trastuzumab biosimilars.

Trastuzumab is a humanized IgG1 monoclonal antibody with the potential to inhibit HER2 signaling pathways and activate antibody-dependent cell-mediated cytotoxicity. This monoclonal antibody is employed in cancer treatment where there is an overexpression of the HER2 cell surface receptor.

Several trastuzumab biosimilars have been developed in recent years, with chromatography techniques at the forefront of assessing crucial quality attributes during biosimilar development, most notably aggregation.

This case study confidently demonstrates that SEC and mass photometry are

complementary techniques while further highlighting mass photometry's usefulness as an orthogonal technique in monitoring aggregation in biopharmaceuticals such as mAbs.

Experimental methods

An Agilent Technologies 1260 Bio-inert HPLC Infinity II system was used to perform both SEC and SEC-MALS measurements. This system was equipped with a diode-array detector (DAD) and was coupled to both a refractive index (RI) detector and a Wyatt miniDAWN multi-angle light scattering detector.

Sample compounds were initially separated in line with their hydrodynamic radius under native conditions using an SEC column. Detection was then completed consecutively using a DAD, MALS detector, and RI detector.

Utilizing the DAD or RI signals as a concentration source, it was possible to use the light scattering data from multiple detector angles to ascertain the molecular weight (MW) of analyte peaks. Sample load was increased to 315 µg in this instance in order to enable accurate MW determination of the protein aggregates.

A Two^{MP} system was used to acquire mass photometry data, with measurements performed of both the interference between the scattered light from individual sample molecules and the reflected light of the glass slide measurement surface.

The resulting signal (the interferometric contrast) is directly correlated with molecular mass, meaning that it is simple to convert this using protein standards of known MW.

A comprehensive overview of the experimental conditions and instrumentation used is provided in Tables 1 and 2.

Table 1. Experimental conditions for SEC-MALS. Source: Refeyn Ltd.

SEC-MALS	
System	Agilent Technologies 1260 Bio-inert HPLC Infinity II with RI detector and Wyatt miniDAWN MALS detector
Column	Waters XBridge Protein BEH SEC Column 200Å (7.8 x 300 mm x 3.5 µm)

Temperature	22°C
Mobile phase	0.2 M sodium phosphate, pH 7.0
Flow rate	0.8 mL/min
Run time (Elution time)	24 min
Injection	10 µg (SEC), 315 µg (SEC-MALS)
DAD Detection	
Wavelength	280 nm (band width 4 nm, no reference wavelength)
Peak Width	> 0.2 min (1.25 Hz)
R1 Detection	
Temperature	35°C
Peak Width	> 0.025 min (18.5 Hz)
Data processing	
Software	OpenLAB CDS ChemStation and ASTRA V8

Table 2. Experimental conditions for mass photometry. Source: Refeyn Ltd.

Mass photometry	
System	TwoMP
Temperature	Room temperature (21°C)
Dilution solvent	PBS
Sample concentration (and mass of antibody per sample measurement)	20 nM (30 ng)
Sample carrier slides	Cleaned using water and 2-propanol
Run time (Detection time)	1 min
Data processing	
Software	DiscoverMP

Interpretation of SEC data

Trastuzumab-producing Chinese hamster ovary cell (CHO) clone supernatant samples were purified using Protein A affinity chromatography, and these were then analyzed via SEC.

Figure 3 displays SEC chromatograms of the trastuzumab originator (Herceptin®, essentially the reference product or 'parent molecule') and a total of four selected CHO clones.

There are clear differences in both the high MW and low MW areas. These can

be distinguished by examining the left and right of the main monomer peak, respectively. In this case, the primary high MW peak is associated with a dimer of trastuzumab, which is especially pronounced in clones 3 and 10.

Table 3 shows the relative peak areas for both monomer and dimer species obtained via the SEC-UV chromatogram). These are the key molecules of interest in this example.

An SEC-MALS experiment was run for Herceptin® and CHO clone 10 (Figure 4) to obtain an MW estimation. The latter displayed a MW of 146.5 kDa for the monomer and 295.5 kDa for the dimer, values which show good agreement with the MW of the originator's concurring peaks.

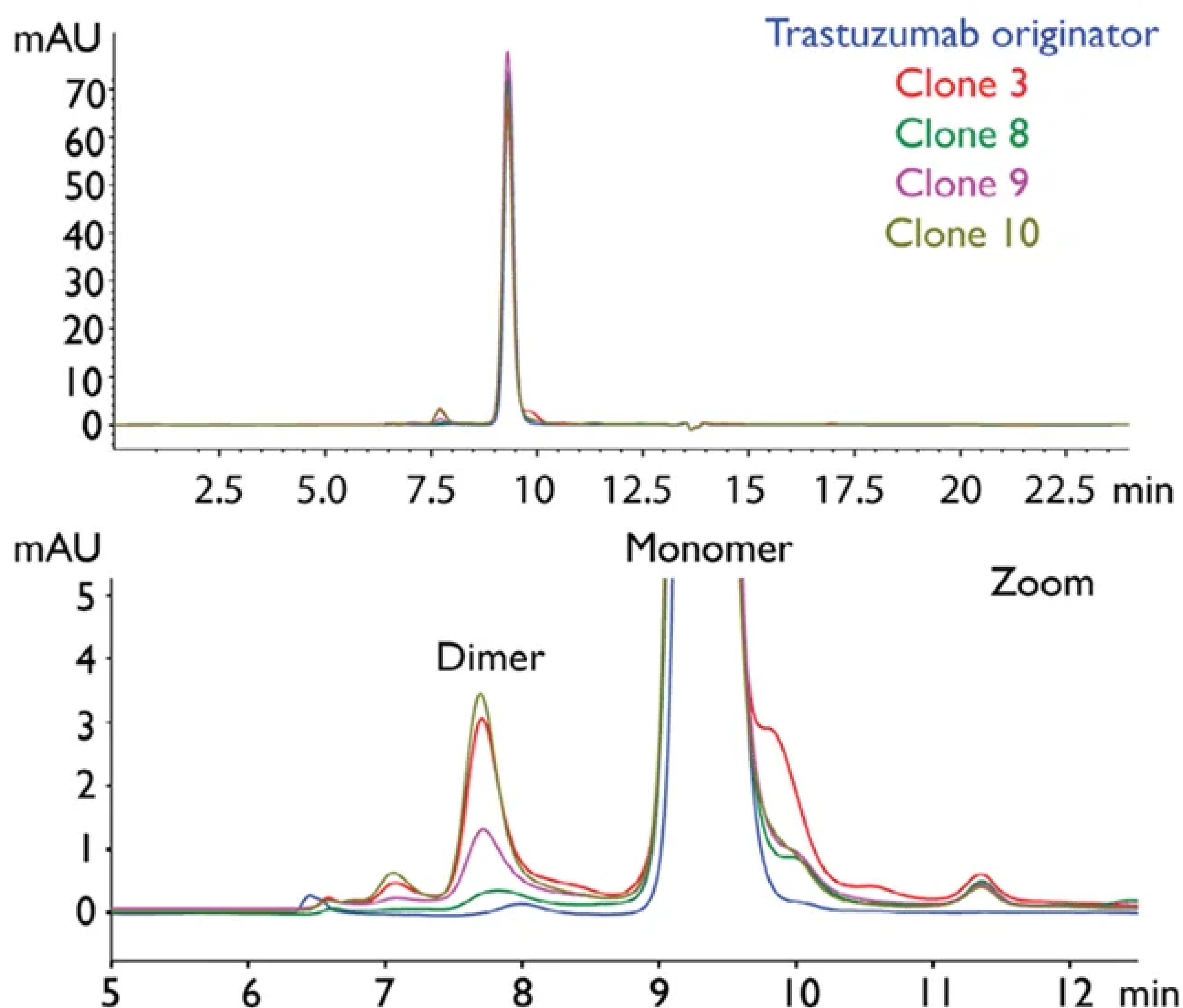

Figure 3. *SEC-UV chromatograms of trastuzumab originator (Herceptin®) and trastuzumab-producing CHO clones (UV 280 nm). Image Credit: Refeyn Ltd.*

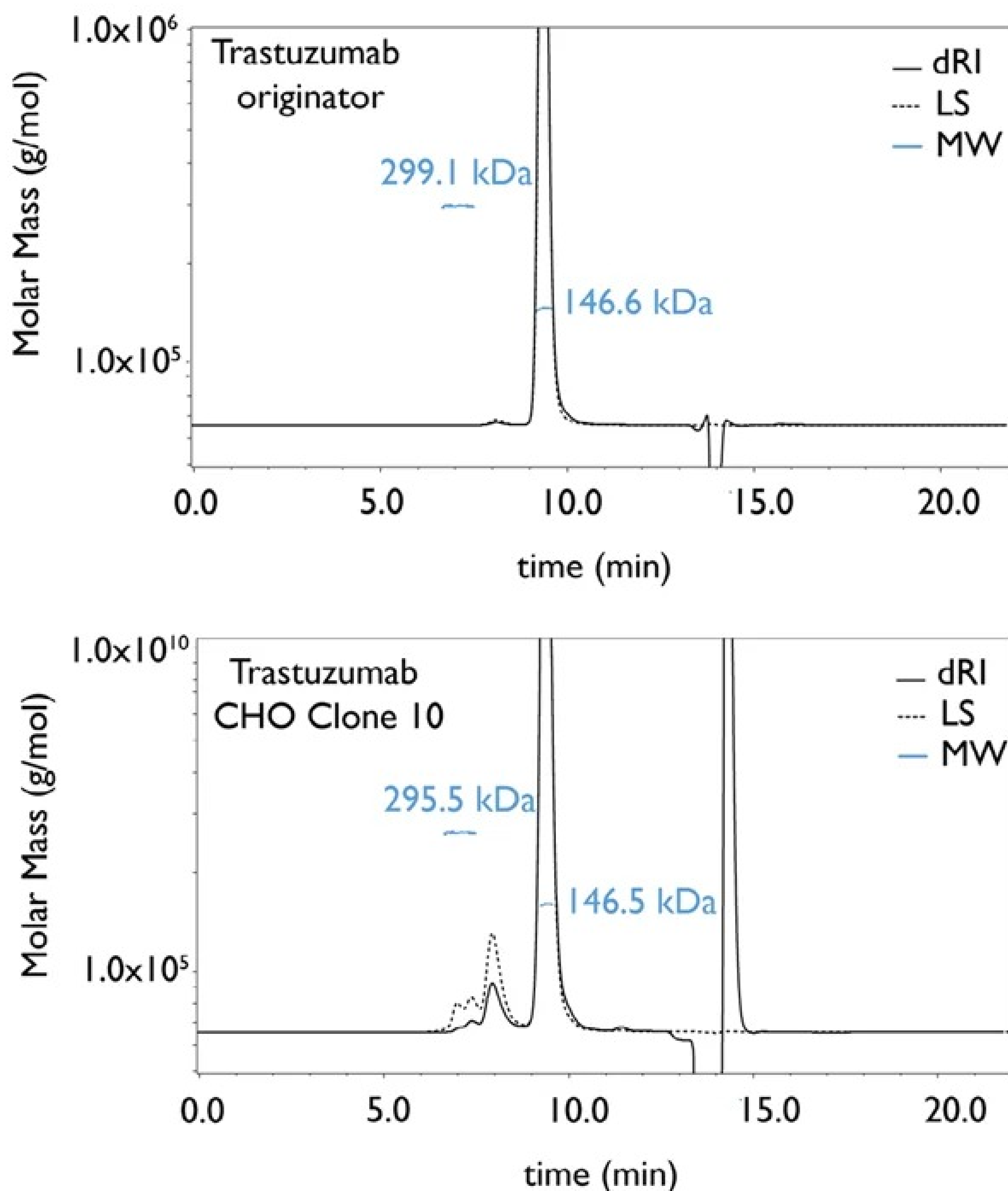

Figure 4. Molecular weight determination by SEC-MALS of monomer and dimer peak for trastuzumab originator (Herceptin®) and trastuzumab-producing CHO clone 10 *(dRI: differential refractive index, LS: light scattering, MW: molecular weight). Image Credit: Refeyn Ltd.*

Denaturing SEC-mass spectrometry analysis showed that noncovalent dimers are present in CHO clone 10, whereas covalently bound dimers are found in the originator product.[1] This also explains the retention time difference between dimer peaks observed in the clones versus the originator.

Trastuzumab sample analysis by mass photometry

Each sample was also analyzed using mass photometry and measured at a final concentration of 10 nM (Figure 5).

Calibrants were used in the form of albumin, α-mannosidase and thyroglobulin, meaning that it was possible to directly interpolate the MW from the data. The MW of the trastuzumab monomer was measured as 155 kDa, and the MW of the dimer was measured as 300 kDa, values of which are both consistent with those determined using SEC-MALS.

Monomer and dimer peaks are defined as the 120–190 kDa and 280–350 kDa mass intervals, respectively. This was done to enable accurate analysis of the mass photometry datasets and to ensure consistency.

Calculations of the percentage abundance of each species were performed by determining the number of counts (within the pertinent defined mass interval) as a proportion of the total count number.

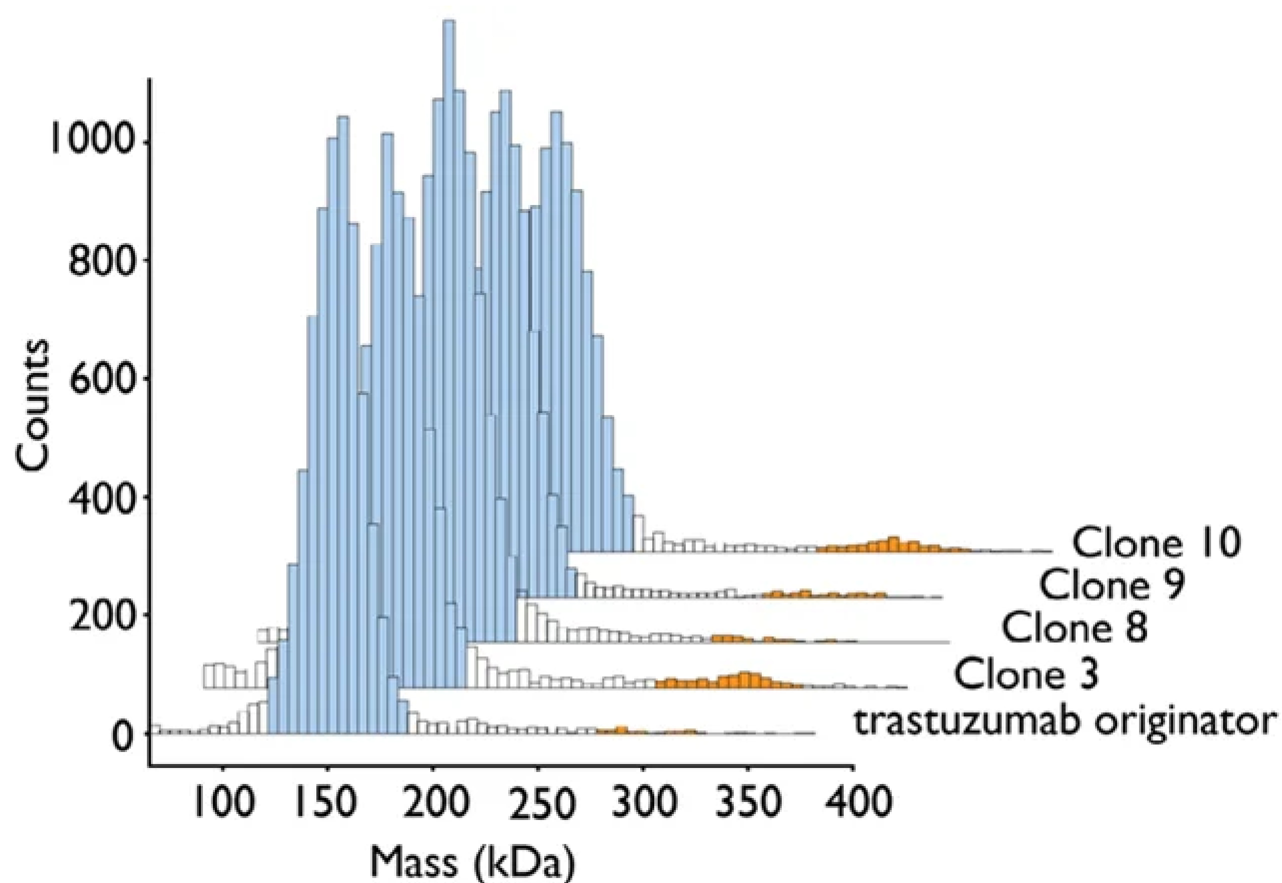

Figure 5. Mass photometry mass histogram of trastuzumab originator

(Herceptin®) and trastuzumab-producing CHO clones. *Monomer peaks are highlighted in blue, dimer peaks in orange. Image Credit: Refeyn Ltd.*

Normalizing SEC-UV data

A comparison of percentage abundance for the monomer and dimer from SEC-UV (Table 3) and mass photometry (Table 5) illustrates that the data is broadly in agreement. Clones 3 and 10 were found to contain the greatest abundance of dimers in relation to the predominant monomeric species.

However, it is necessary to include a correction step to ensure a true quantitative comparison.

When investigating biotherapeutic proteins like trastuzumab, the DAD detector response at 280 nm is principally related to the number of tyrosine and tryptophan residues contained within.

When dimers are formed, a higher number of absorbing residues will be present per molecule with respect to the monomer. This impacts the peak height (detector response) and, in turn, the peak area.

In the example presented here, the Beer-Lambert law was employed in the conversion of measured peak heights in the SEC chromatogram to analyte concentration [g/liter]. A correction factor for the UV flow cell was also applied.

The concentration was then converted into molar concentration [mol/liter] using the MW of the monomer and dimer species, respectively. Table 4 provides a useful overview of both the monomer and dimer abundance for every sample, following the normalization of absorbance for the molar extinction coefficient.

Table 3. Monomer and dimer abundance from SEC-UV chromatograms. To calculate the percentage abundance of both monomer and dimer, the individual peak area for each was calculated and expressed as a percentage of the total/combined peak area. Source: Refeyn Ltd.

	Sample	trastuzumab originator	Clone 3	Clone 8	Clone 9	Clone 10
Abundance (%)	Monomer	99.6	94.9	99.0	97.7	94.7
	Dimer	0.4	5.1	1.0	2.3	5.3

Table 4. Monomer and dimer abundance after normalizing SEC-UV absorbance data. Source: Refeyn Ltd.

	Sample	trastuzumab originator	Clone 3	Clone 8	Clone 9	Clone 10
Abundance (%)	Monomer	99.9	97.7	99.8	99.2	97.6
	Dimer	0.1	2.3	0.2	0.8	2.4

Table 5. Monomer and dimer abundance determined by mass photometry. The 120 –190 kDa mass interval defines the monomer peak and the 280 – 350 kDa mass interval, the dimer peak. Percentage abundance of each species is expressed as a proportion of the total number of counts. Source: Refeyn Ltd.

	Sample	trastuzumab originator	Clone 3	Clone 8	Clone 9	Clone 10
Abundance (%)	Monomer	99.3	96.8	98.8	98.7	97.1
	Dimer	0.7	3.2	1.2	1.3	2.9

Conclusion

Biopharmaceuticals’ product safety and efficacy rely on the proper analysis of the protein aggregates contained therein. Widely regarded as a gold standard technique in this sector, SEC enables the robust determination of protein aggregation, whereby molecular separation is dependent on the difference in hydrodynamic radius.

When coupled to a MALS detector, SEC can be used to identify both the size and molecular weight of analytes, with an analysis run-time of just 30 minutes.

Mass photometry can be used to analyze samples under native conditions within one minute. This analytical technique works by interpreting light-scattering data from individual analyte molecules approaching the glass slide interface.

As the underlying detection principle of each of these methods is fundamentally different, a number of factors must be taken into account when comparing data from both. This is especially important when quantitatively assessing the relative abundance of different molecules in a sample mixture.

In order to properly account for these differences, measured peak area absorbance values must be normalized by converting to molar concentration. By doing so, it is possible to gain an unbiased comparison of samples analyzed by SEC and mass photometry.

Case Study 1 illustrated this with a simple protein mix example, while Case Study 2 explored this further using an example of several trastuzumab biosimilars.

In both cases, SEC and mass photometry were found to provide comparable results. Where there were differences in the data captured by both techniques, it is most likely that these relate to the parameters and/or criteria used for peak integration.

For example, if the monomer-dimer ratio is concentration-dependent when working with trastuzumab biosimilars, differences in the concentration of antibody sample used for SEC versus mass photometry would also affect the relative abundance of monomer versus dimer.

Due to the complexity of biopharmaceuticals such as monoclonal antibodies, it is often necessary to use several analytical tools to provide detailed insight into their efficacy and safety.

As shown in this article, mass photometry and SEC data – for a simple protein mixture, as well as several trastuzumab biosimilars – are in agreement. This confirms and highlights the usefulness and legitimacy of mass photometry as an orthogonal analytical technique.

References

1. Liesa Verscheure, Gerd Vanhoenacker, Sonja Schneider, Tom Merchiers, Julie Storms, Pat Sandra, Frederic Lynen, and Koen Sandra, Analytical Chemistry 2022 94 (17), 6502-6511

Acknowledgments

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Automated mass photometry for protein oligomerization

Oligomerization has the potential to be a key factor in protein function, so properly understanding the function of a protein necessitates the quantification of its oligomerization state.

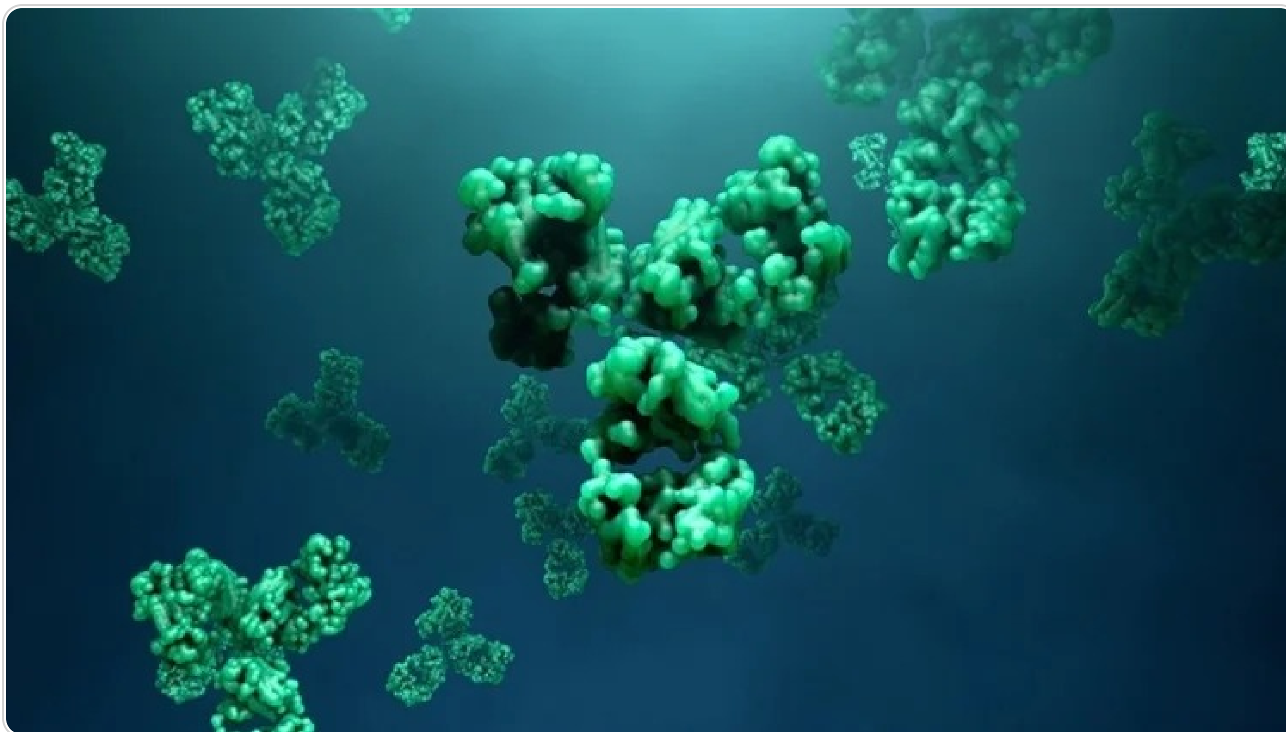

Image Credit: Design_Cells

Mass photometry offers a means of measuring a sample's mass distribution in native conditions and at a single-molecule level, with this technique even being sensitive enough to detect rare species.

Automated mass photometry builds on these capabilities with easy and consistent sample dilution and manipulation, enabling thorough assays of oligomerization behavior.

Oligomerization - best understood as self-assembly into specific quaternary structures - is one of the core processes for many proteins' functions. Proteins that function as monomers are actually in a minority, according to current databases.[1,2]

Oligomer formation has a number of relevant physiological roles. For example,

gene expression regulation typically relies on homo-oligomeric DNA-binding proteins. It is also possible for enzyme activity to be regulated via allosteric interactions between subunits or through the formation of active sites at the subunit interface.

Membrane-associated proteins (such as receptors and channels) often require oligomerization to provide transport across membranes or to enable cell-to-cell adhesion.[2,3] Altered oligomeric states are also a factor in disease development in some instances, making these especially relevant in the context of translational research.[4]

By evaluating and identifying conditions that determine a given protein’s oligomeric state, it is possible to then develop mechanisms for interfering with or stabilizing this process. By doing so, it is possible to explore new and potentially valuable avenues of therapeutic intervention.

Mass photometry and protein oligomerization

It can be especially challenging to properly capture and characterize protein oligomerization if the concentrations of species in the sample are very low.

Mass photometry is one of the most suitable techniques available for the assessment of oligomerization states and dynamics. This powerful technique works by measuring the amount of interference between light reflected by a glass surface and light scattered by a molecule in contact with the glass.

The magnitude of the interference scales linearly with molecular mass. Notably, this interference can be measured without labels, using a wide range of buffers, and with minimal sample amounts.

Mass photometry affords users high-resolution distributions of a sample’s molecular mass directly in solution while maintaining single-molecule sensitivity. The information about the mass distribution of a sample can then be used to infer oligomerization states.

Mass photometry even offers sufficient sensitivity to detect rare species comprising less than 1 % of the main sample population.

Automation of this analytical process via Refeyn’s Auto platform offers a key

advantage that complements mass photometry's inherent strengths. The Auto robotics unit is fully compatible with Refeyn's One^{MP} and Two^{MP} mass photometry instruments, allowing users to automate their mass photometry processes easily.

This combination of sensitivity and efficiency makes this system ideally suited to the rapid screening and analysis of oligomeric states under a range of conditions.

Mass photometry can characterize oligomerization behavior

The experiment series discussed here was performed by GSK using the Two^{MP} Auto. The company's team undertook a preliminary study in order to characterize the oligomerization dynamics of a proprietary protein of interest.

To achieve this, the team assessed the protein's oligomeric status at a number of different concentrations. In the buffer alone, it was noted that the target protein primarily formed monomers at lower concentrations.

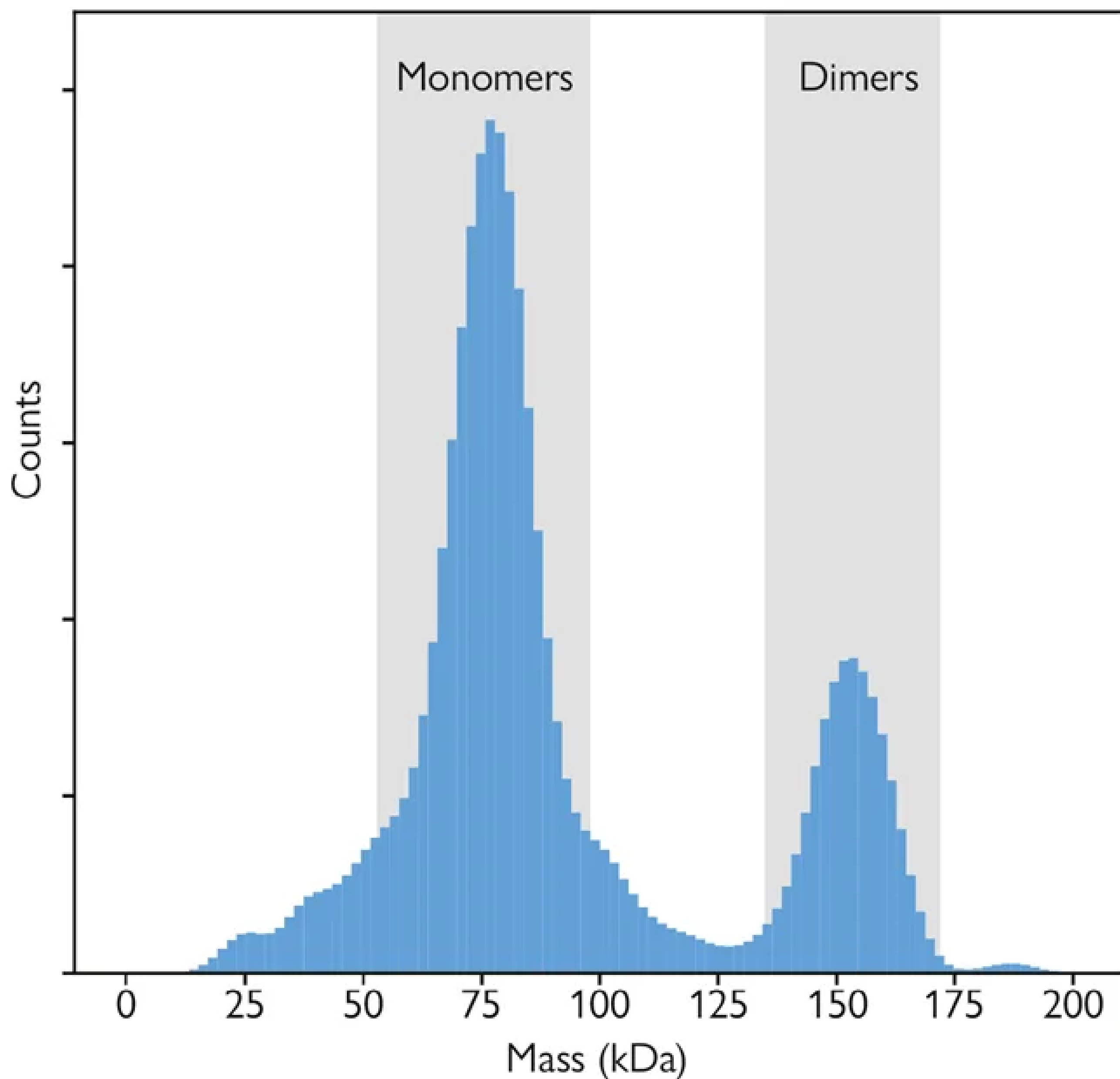

Figure 1. Measuring oligomerization with mass photometry. *Mass distribution of a sample of the target protein measured at a concentration of 6.3 nM. The peak containing the main population (around 80 kDa) corresponds to counted monomers, while the secondary peak (around 160 kDa) corresponds to dimers. Image Credit: Refeyn Ltd.*

It was also observed that as the protein's concentration increased, homodimers became apparent in the mass distribution histogram. These can be seen as a secondary peak at twice the molecular mass of the monomer peak. It was also noted that both peaks were visible at a protein concentration of 6.3 nM (Figure 1).

Mass photometry helps study oligomerization effectors

The impact of varying calcium concentrations was evaluated in this experimental series, and the addition of a candidate inhibitor molecule that had been hypothesized to prevent oligomerization was tested.

A series of measurements were undertaken to increase protein concentrations following the addition of the candidate inhibitor. Both sets of measurements were streamlined via automation, and these were used to calculate the dissociation constants of the homodimers both with and without the presence of the inhibitor (Figure 2).

Results of these experiments implied that the candidate inhibitor impacted protein oligomerization, leading to notably reduced dimer abundance in the presence of the inhibitor.

This change was measured by calculating the dissociation constant (K_D) of protein samples without inhibitor (K_D= 3.55 ± 1.2 nM) and also with inhibitor (K_D= 9.1 ± 1.1 nM). The effect was found to be minimal, suggesting there may be a different inhibition mechanism in this case.

It was also observed that oligomerization was impacted by the presence of calcium. Tetramers were found to be promoted by the addition of $CaCl_2$, appearing as a peak on the mass distribution histogram at four times the monomeric species' molecular mass.

The addition of the calcium chelator EDTA was found to reverse the formation of tetramers, with the histogram reflecting this as the absence of the third peak (Figure 3).

The team further explored the impact of calcium on target protein oligomerization by acquiring repeated mass photometry measurements at increasing protein concentrations while maintaining constant calcium concentrations.

The presence of calcium resulted in a gradual increase in tetramers as protein concentration increased. This also coincided with a reduction in monomers (Figure 4) while the proportion of dimers remained constant. This was in

contrast to the behavior noted in the absence of calcium (Figure 2).

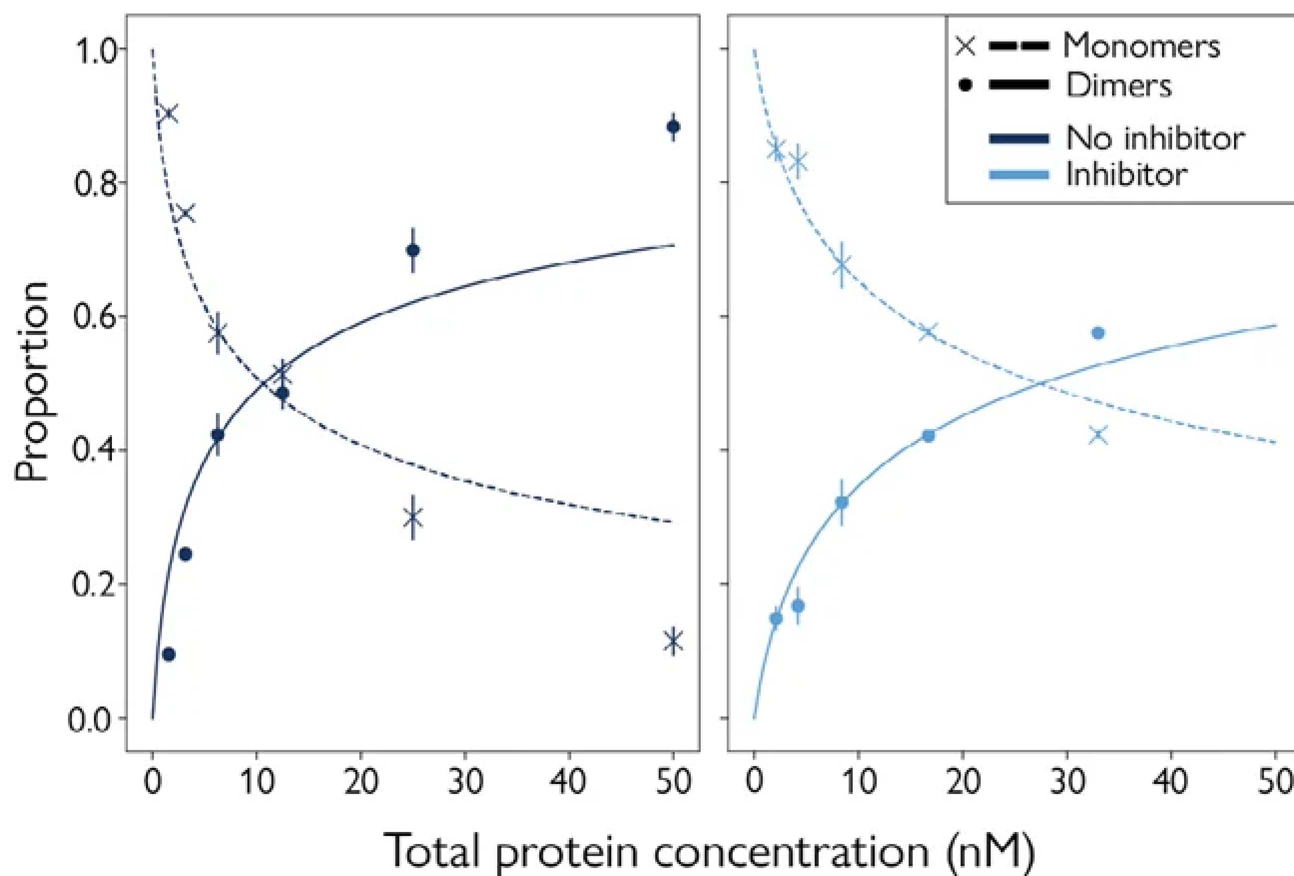

Figure 2. Characterization of protein oligomerization with and without an inhibitor. *Relative proportions of monomers and dimers in the sample, as a function of target protein concentration. Left plot (dark blue) shows measurements in simple buffer, while right plot (mid blue) shows measurements in the presence of an oligomerization inhibitor. Concentrations measured were : 1.55, 3.15, 6.25, 12.5, 25.0 and 50.0 nM for the samples without inhibitor, and 2.1, 4.2, 8.4, 16.7 and 33 nM for the samples with inhibitor. Image Credit: Refeyn Ltd.*

Automated mass photometry streamlines oligomerization research

The Two^{MP} Auto's automated sample manipulations mitigate the variability commonly linked to manual operation between operators and across different experiments.

Reproducibility testing with the Two^{MP} Auto highlighted less than 1 % variabilities in the measured mass and relative proportion of detected species.

This quality was especially beneficial for GSK's preliminary study and its characterization of the investigated candidate inhibitor.

The Two^{MP} Auto's 'in-plate' dilution feature also proved a crucial feature in this series of experiments due to its ability to store the protein sample in each well at a higher concentration before diluting this immediately before measurement takes place.

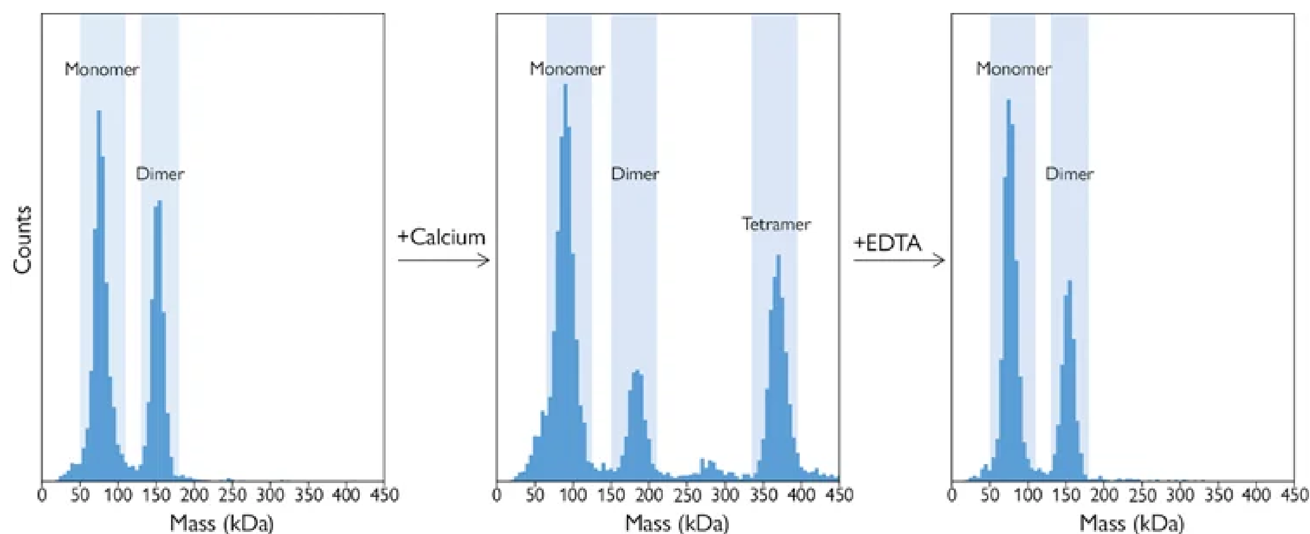

Figure 3. Reversal of tetramer formation. *A: A mass histogram of a protein-only sample, with monomers and dimers present. B: Calcium chloride (present in excess) induced the formation of tetramers and reduced the number of dimers. C: Adding a saturating concentration of EDTA reversed this effect, resulting in the disappearance of the tetrameric species. Image Credit: Refeyn Ltd.*

In-plate dilution also helps to minimize any potential adsorption to the well surface, a significant issue at low protein concentrations.

This product feature minimizes the need to manually dilute samples to the required concentration range for optimal mass photometry measurements. The system also allows users to run several sets of conditions in each run, reducing the required overall screening time.

Using Refeyn's Discover^{MP} software, all files generated from each run can be analyzed as a single batch, further simplifying data reporting and streamlining data analysis.

In summary, mass photometry represents a robust and valuable technique for the accurate characterization of oligomer formation. It is also useful in better explaining factors involved in inhibiting and promoting this crucial biological process.

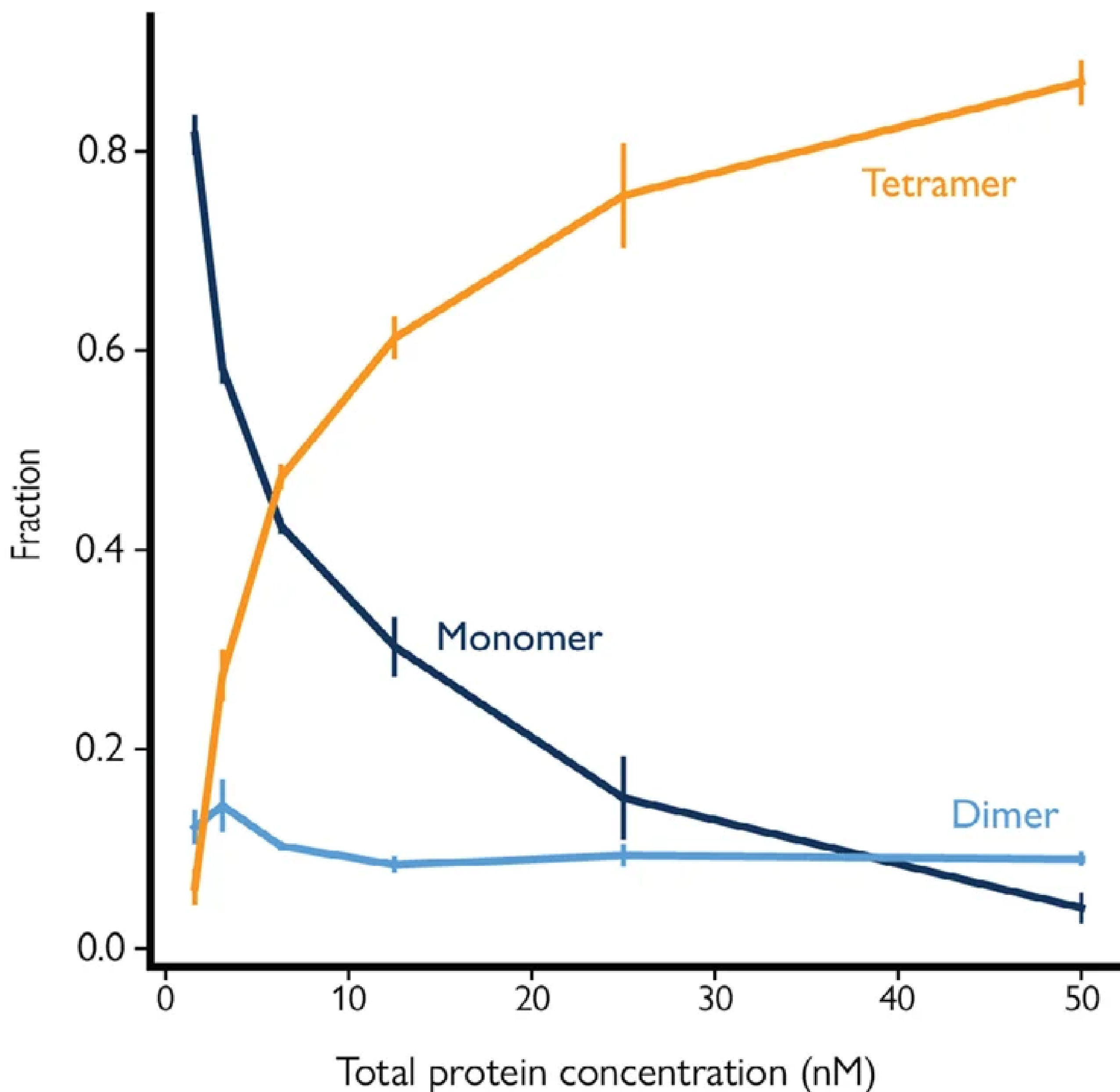

Figure 4. Dilution series showing protein oligomerization under constant $CaCl_2$ concentration. *In the presence of 20 mM $CaCl_2$, increasing the target protein concentration resulted in an increase in tetramer formation, while the proportion of dimers in the sample remained small. Image Credit: Refeyn Ltd.*

This process can be further streamlined via automation, with improved efficiency in steps from sample preparation through to data capture. The use of

automation not only saves the operator time but also promotes the rapid acquisition of reproducible results and a more cost-effective investigation of oligomerization behaviors.

In summary, studying oligomerization using mass photometry offers a range of benefits, including:

- Minimal sample preparation requirements
- The acquisition of accurate results within minutes
- Compatibility with most buffers
- No requirement for the use of labels
- Sensitivity to changes in oligomerization status, even with minimal sample quantities
- Potential to detect even rare oligomeric species
- Rapid, unattended measurement of up to 24 samples via automation
- Suitability for screening and titration, studies of protein interaction and investigation into effector dynamics

References and further reading

1. Danielli *et al*., Sci Rep 2020
2. Hashimoto and Pachenko, PNAS, 2010
3. Marianayagam *et al*., Trends Biochem Sci 2004
4. Sato *et al*. J Biol Chem 2018

Acknowledgments

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Analyzing forced antibody degradation using automated mass photometry

Mass photometry is a powerful, label-free bioanalytical tool that can evaluate antibody fragmentation and aggregation in response to induced stressors such as pH, light, or peroxide.

Image Credit: ustas7777777

The TwoMP Auto from Refeyn allows users to conduct rapid, automated measurements of multiple samples across a wide mass range whilst ensuring consistently high reproducibility.

Aggregation remains a significant issue for antibodies and can have an adverse effect on both product efficacy and safety.[1] A low propensity to form aggregates (from dimers to visible precipitates) is generally desirable throughout manufacturing and product storage.

Forced degradation studies represent a frequently employed approach for the assessment of biopharmaceuticals' physicochemical stability, including antibody-drug conjugates, monoclonal antibodies, and bispecific antibodies.[2]

The use of appropriate analytical techniques is essential in such studies if the right candidates are to be selected in the initial stages of the drug development life cycle.

Mass photometry is an ideal tool for gaining rapid insight into changes in molecular behavior, ranging from protein aggregation and degradation to AAV capsid characterization.[3] Mass photometry only requires small amounts of sample for analysis, and it is also able to accommodate a diverse array of buffer conditions.

Refeyn's Two^{MP} Auto automated mass photometry system is ideal for implementation in wide range of applications, allowing operators to conduct experiments easily thanks to its walkaway automation features.

This article examines the use of the Two^{MP} Auto during a forced degradation study relating to a panel of therapeutic antibodies. These antibodies were provided by an industrial partner.

Mass photometry characterizes antibody stability in response to stress

The data presented here was part of a wider investigation into the effect of three specific stressors - light, pH, and peroxide - on a panel of 14 different antibodies.

This study was undertaken to ascertain whether exposure to these three stress conditions led to fragmentation and/or aggregation, with each condition measured in triplicate for a total of 168 measurements, including specific control measurements.

The user workflow was simplified through the use of Two^{MP} Auto's automation features, with measurements of up to 24 samples per run completed in around 90 minutes. The system was able to do this with only picogram quantities of each antibody.

Data was then analyzed as it was being recorded, with the intuitive software interface allowing the user to begin interpreting results easily and without the need for complicated data analysis routines.

Figures 1 and 2, and Tables 1 and 2, show representative mass photometry data for two of the antibodies from the panel.

When compared to the relevant control sample, it can be observed that each of the antibodies behaved differently in response to the same stress conditions. ‘Antibody 1’ (Figure 1, Table 1) exhibits more pronounced fragmentation, particularly in response to stress condition 2, while ‘Antibody 2’ (Figure 2, Table 2) exhibits a greater degree of aggregation overall.

The results suggest that these antibodies possess very different physiochemical properties while also highlighting the relative abundance of each distinct population of molecules in each antibody sample.

As mass photometry is a single-molecule technique, it has the capacity to detect and count each molecule in a sample. Data acquired, therefore, includes information on a number of rare species, including monomers and dimers.

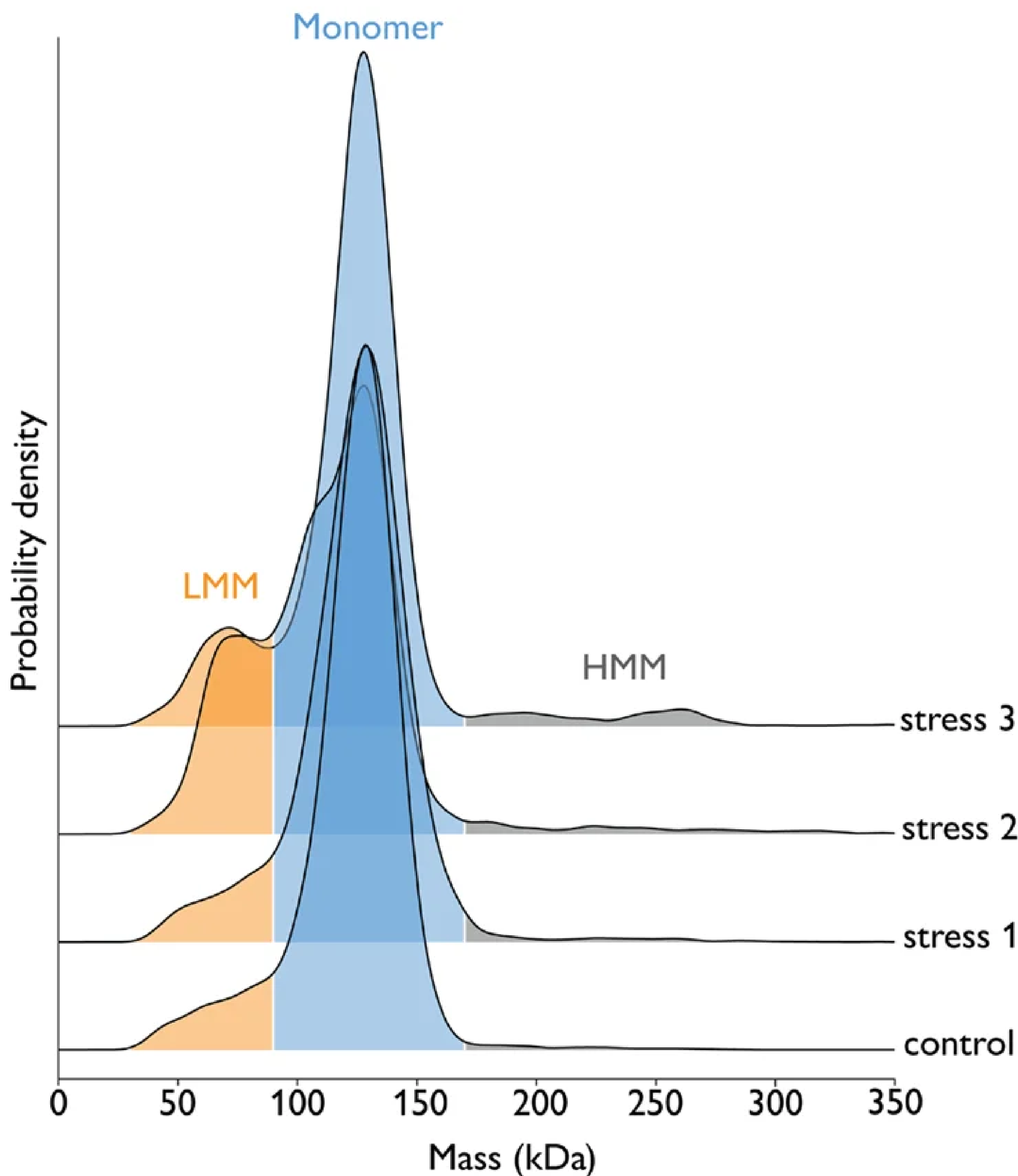

Figure 1. Exposure of 'Antibody 1' samples to three different stress conditions. *The 'Stress 1', '2', and '3' are known to be light, pH change, or peroxide but details of which condition corresponds to which name were not disclosed by the industrial partner. Samples were measured at 10 nM concentration using 5 µl on the Two*^{MP} *Auto. Results show that 'stress 2' promotes the greatest degree of fragmentation of the antibody, as shown by an increase in the % fraction of 'low molecular mass' species (LMM), relative to the control sample. Image Credit: Refeyn Ltd.*

Table 1. Fragmentation and aggregation products in Antibody 1 sample upon exposure to different stressors. Low molecular mass fraction (30 - 90 kD) corresponds to [fragmented antibody], monomer mass fraction (90 - 170 kDa) to [intact antibody] and high molecular mass fraction (> 170 kDa) to [aggregated antibody]. Source: Refeyn Ltd.

Condition	% Fragmented antibody	% Intact antibody	% Aggregated antibody
Control	9% (± 0.7)	90% (± 0.6)	1% (± 0.3)
Stress 1	10% (± 1.1)	88% (± 1.1)	2% (± 0.1)
Stress 2	27% (± 1.8)	70% (± 1.0)	4% (± 0.9)
Stress 3	14% (± 2.2)	81% (± 2.1)	5% (± 0.3)

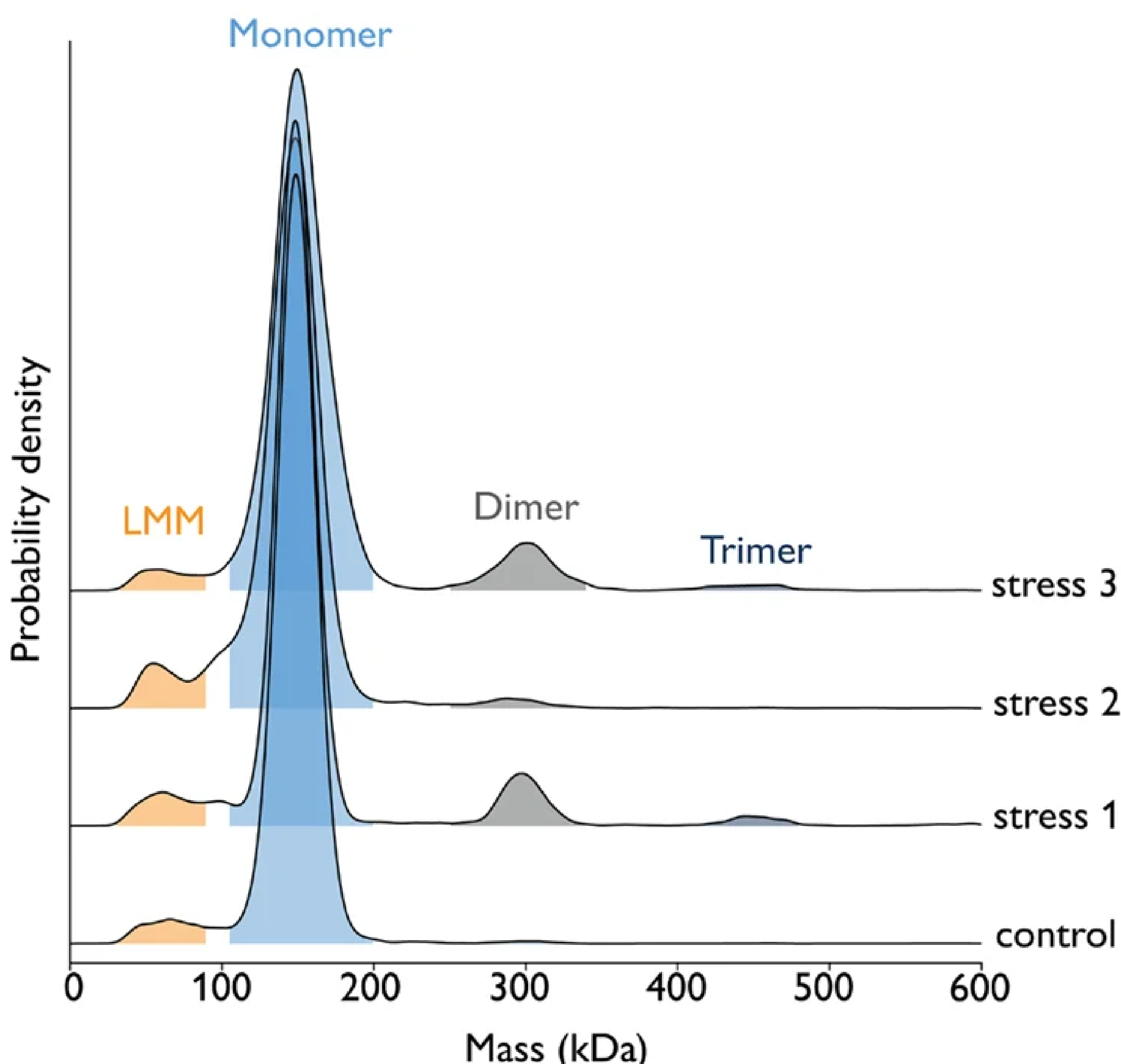

Figure 2. Exposure of 'Antibody 2' samples to three different stress conditions (light, pH change, or peroxide). *Samples were measured at 10 nM concentration using 5 µl on the Two^{MP} Auto. Results show that stress conditions 1 and 3 promote aggregation (specifically, the formation of dimers and trimers), relative to the control sample. Image Credit: Refeyn Ltd.*

Table 2. Fragmentation and aggregation products in Antibody 2 sample upon exposure to different stressors. Low molecular mass fraction (30 - 90 kDa) corresponds to [fragmented antibody], monomer mass fraction (105 -200 kDa) to [intact antibody], dimer (250 - 340 kDa) and trimer (400 - 480 kDa) mass fractions to [aggregated antibody]. Source: Refeyn Ltd.

Condition	% Fragmented antibody	% Intact antibody	% Aggregated antibody (dimer)	% Aggregated antibody (trimer)
Control	4% (± 0.8)	95% (± 0.7)	1% (± 0.1)	0%
Stress 1	5% (± 0.4)	85% (± 0.3)	8% (± 0.4)	2% (± 0.2)
Stress 2	7% (± 1.5)	91% (± 1.4)	2% (± 0.1)	0%
Stress 3	5% (± 0.8)	86% (± 0.5)	8% (± 0.3)	1% (± 0.1)

Summary

A significant number of degradation products or variants may be generated when forced degradation analyses are performed. Due to this, a number of different analytical tools capable of accommodating a wide mass range should be employed to ensure that all appropriate data is captured.

Mass photometry is especially suited for this application area, representing an ideal complementary approach to techniques such as SEC-MALS.

Mass photometry is also a column-free approach suitable for use with a diverse array range of buffers, resulting in very few sample optimization requirements.

As this study highlights, information about a sample may be captured using only picogram quantities of material. These features are augmented by user-

friendly operation and intuitive data analysis, meaning that this technique affords users quick access to valuable new insights.

This data-based decision-making is essential in many areas, such as supporting process development or developability assessments.

Consequently, mass photometry, particularly using the TwoMP Auto, which allows the user to load samples and walk away, can form part of a suite of analytical methods to provide an in-depth characterization of biopharmaceuticals, such as monoclonal and bispecific antibodies.

References and further reading

1. Vázquez-Rey & Lang, Biotechnol Bioeng, 2011
2. Blessy, Patel *et al*., JPA, 2014
3. Refeyn, How does mass photometry work? [Blog], 2021

Acknowledgments

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Measuring protein binding affinities using mass photometry

Mass photometry is a label-free bioanalytical tool that evaluates the dynamics and strength of protein-protein interactions via the direct measurement of mass. It also provides valuable insight into the relative abundance of individual proteins and the complexes they form in solution.

Image Credit: Shutterstock/Christoph Burgstedt

Protein interactions are central to a diverse array of biological processes, but these are typically highly complex and involve the binding of multivalent ligands to several binding sites. It can, therefore, be extremely challenging to identify the various complexes formed and evaluate the strength of their interactions.[1]

Mass photometry is a rapid, label-free technology ideally suited to measuring the molecular mass of individual biomolecules in solution. It is also an excellent choice for the study of protein-protein interactions.

Mass photometry provides useful information on the mass distribution of biomolecules in a sample, affording users a comprehensive overview of binding partners present, complexes formed, the relative abundance of each species, and the associated strength of their interactions.[1,2,3]

This article explores the use of mass photometry in the characterization of interactions between Immunoglobulin G (IgG) antibodies of different origin species (human and bovine) and protein A.

The relative abundance of each protein and the complexes they form in solution are quantified. The automated pipetting feature of Refeyn's Two^{MP} Auto mass photometer was used to generate highly reproducible data, with the equilibrium dissociation constant (K_D) calculated for each interaction using this data.

Mass photometry measures sample homogeneity

It is possible to utilize mass photometry in assessing the purity and quality of control samples, though it is important to ensure that aggregation or protein oligomerization is identified and accounted for when interpreting results.

In the example presented here, mass photometry measurements verified the anticipated mass of the two IgG antibodies from human and bovine origin (~150 kDa for each), and protein A (~42 kDa)[4] (Figure 1).

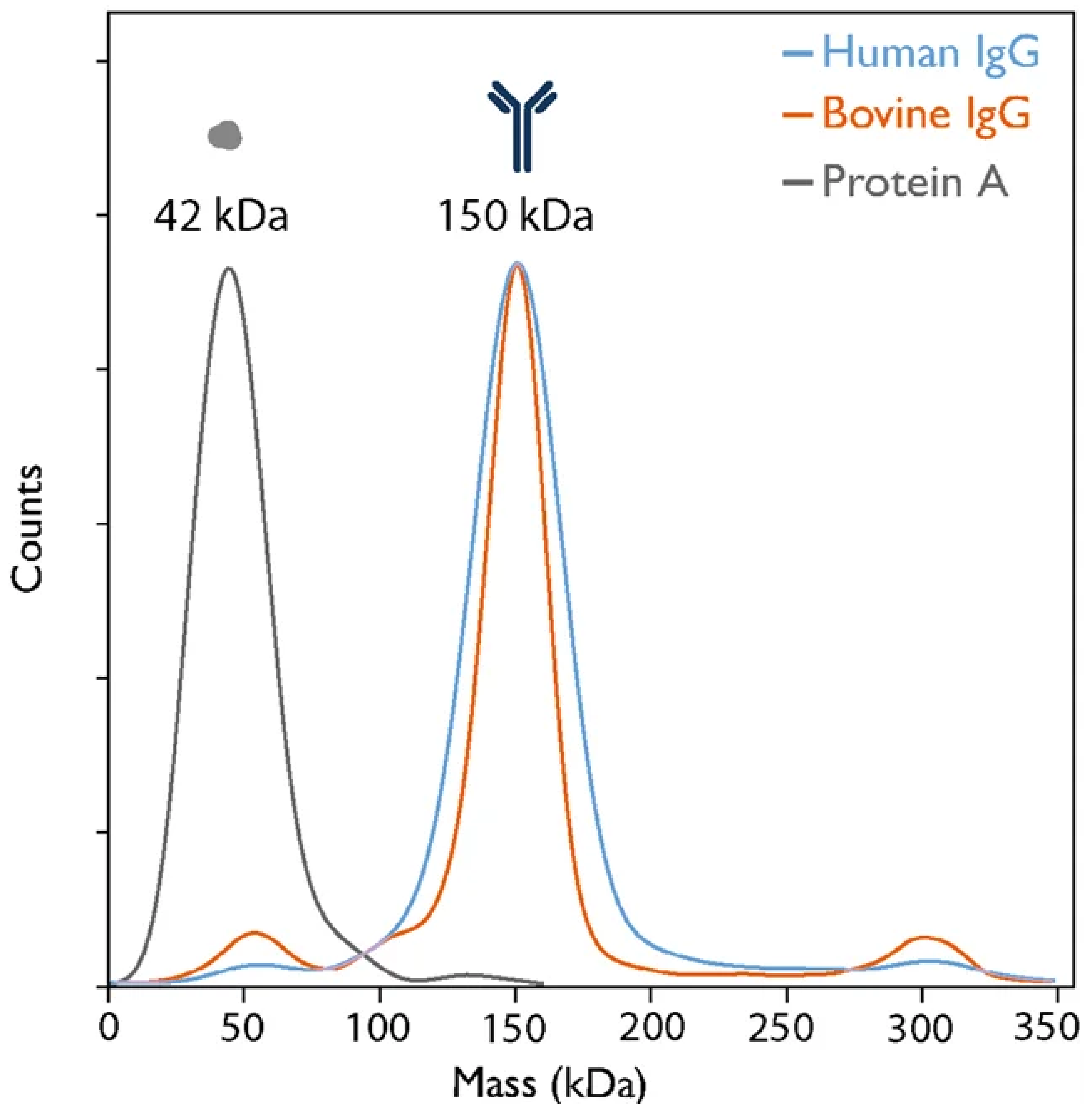

Figure 1. Mass photometry measurements of human and bovine IgG antibodies and protein A. *Both the human (blue) and bovine (orange) IgGs had a molecular mass of 150 kDa, while protein A (grey) had a mass of 42 kDa. Image Credit: Refeyn Ltd.*

Mass photometry resolves complex equilibria

Mass photometry is also well suited to the investigation of binding and the analysis of complex formation. In this instance, IgGs of differing origins were combined with protein A and mass photometry was used to ascertain differences between the two IgGs in terms of complex formation and their

relative abundance (Figure 2).

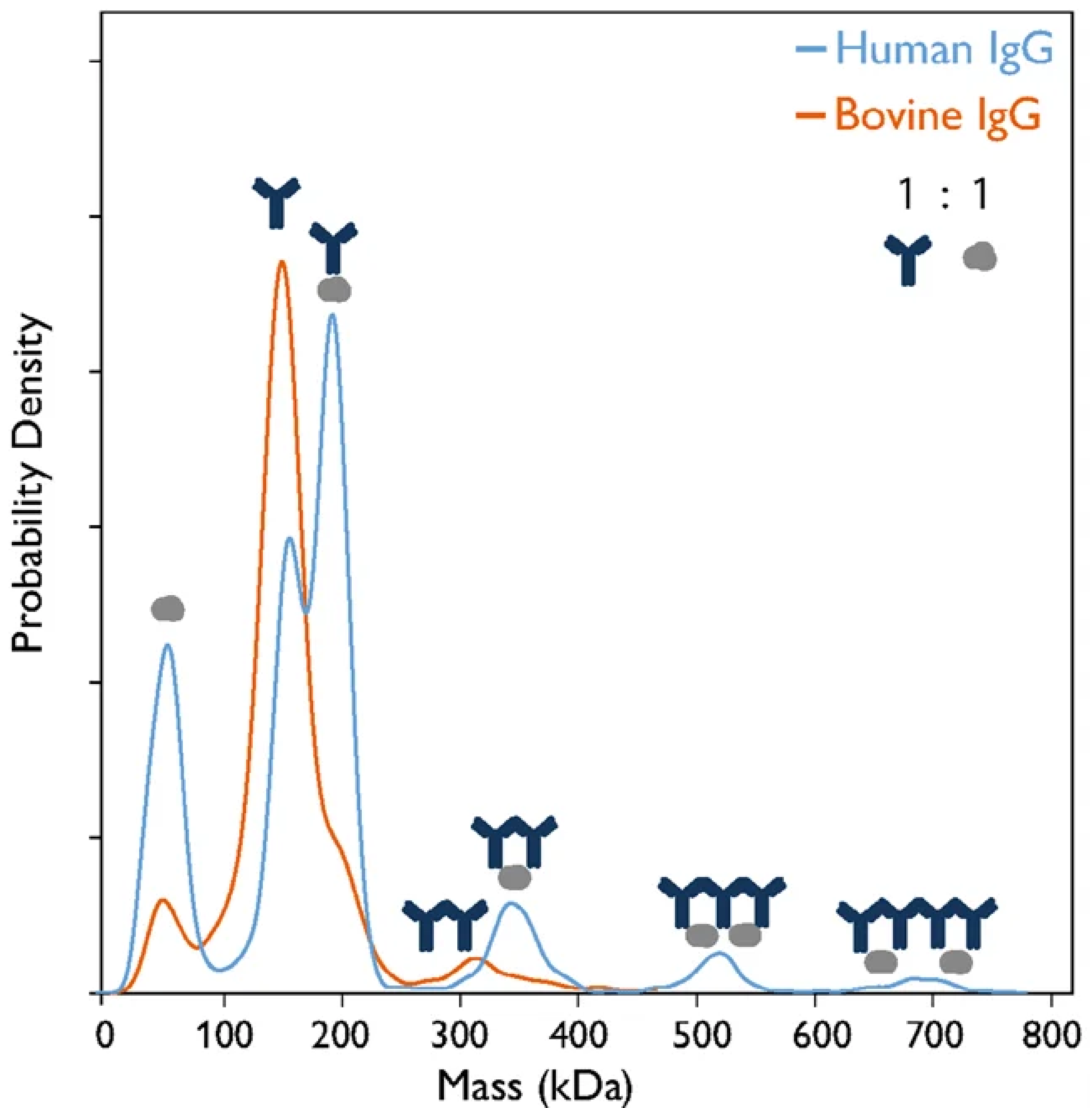

Figure 2. Mass photometry reveals differences in complex formation between protein A and IgG antibodies of differing origin. *IgGs of either human or bovine origin were mixed with protein A in a 1:1 molar ratio at 20 nM concentration. When human IgG was mixed with protein A (blue line), the following species could be resolved: Protein A (42 kDa); IgG (150 kDa); and protein A: IgG complexes of stoichiometry 1:1 (192 kDa), 1:2 (342 kDa), 2:3 (534 kDa) and 2:4 (684 kDa). When bovine IgG was mixed with protein A (orange line), the resolvable species were: Protein A (42 kDa), IgG (150 kDa), 1:1 protein A: IgG complex (192 kDa) and IgG dimers (300 kDa). Image*

Credit: Refeyn Ltd.

The IgGs were mixed with protein A in a 1:1 molar ratio before being measured at concentrations of 5, 10, and 20 nM. Both IgGs formed 1:1 complexes with protein A at each measured concentration, but differences emerged for higher-order complexes.

It was observed that protein A exhibited a strong interaction with human IgG, as shown by the formation of higher-order protein A:IgG heterocomplexes and a relatively low percentage of free human IgG (33 %).

It was also noted that protein A bound to bovine IgG to a lesser degree. Higher-order protein A:IgG complexes were not observed in the case of bovine IgG, where most bovine IgG remained unbound (82 %).

Results from this study confirmed that mass photometry successfully resolved and quantified the relative abundance of multiple species coexisting in complex equilibrium reactions.

Mass photometry measures interaction strength

Protein A was observed as forming more complexes and higher-order complexes with the human IgG antibody versus the bovine IgG, highlighting a notable difference in affinity between the two interactions.

The K_D for each interaction (Figure 3) was calculated to quantify this difference, with mass photometry measurements performed at 20 nM concentration (Figure 2, and two repeats not shown).

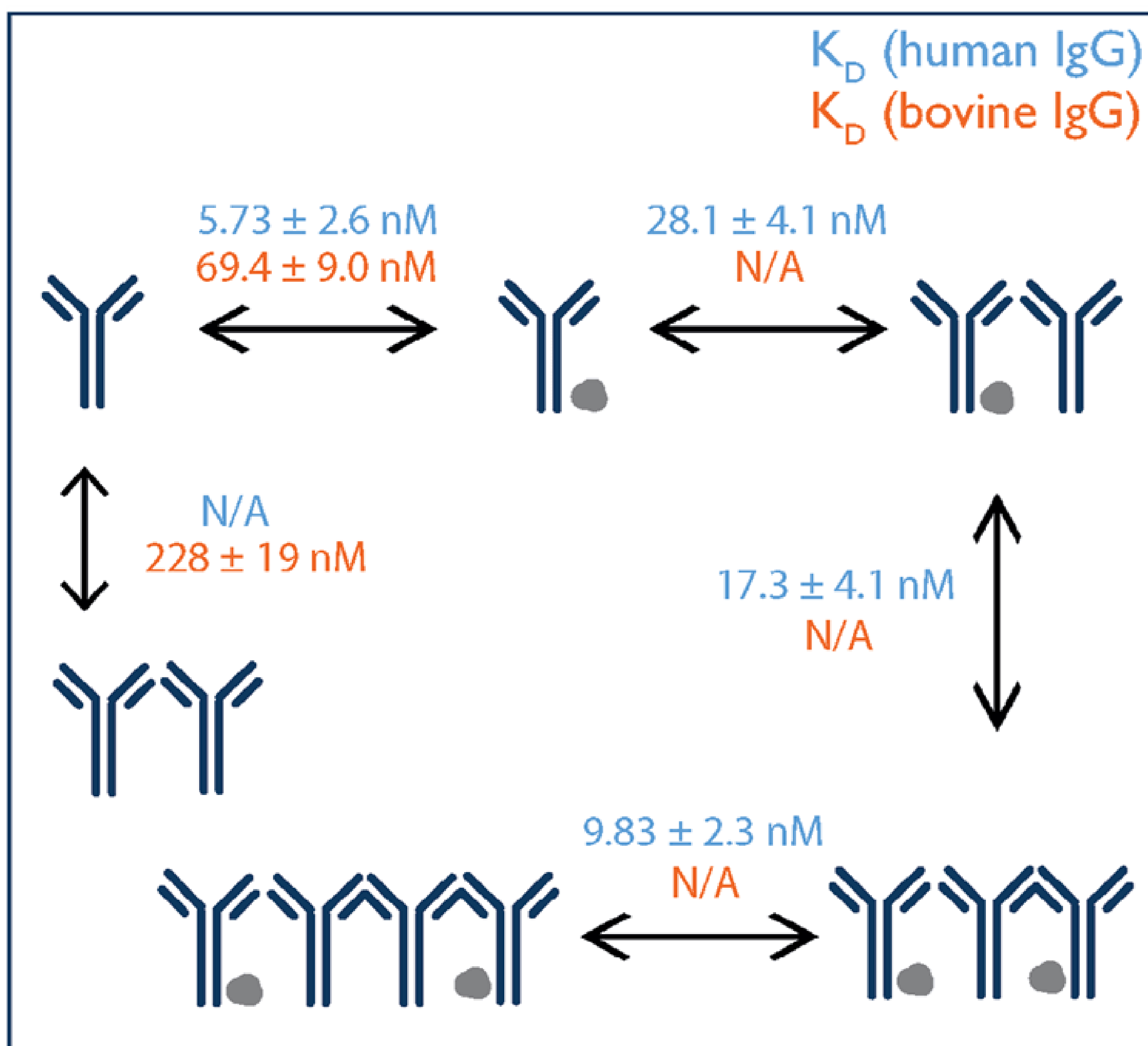

Figure 3. *Multiple K_d values can be calculated from a single mass photometry measurement. Schematics show the complex formation depicted in Fig. 2. K_D values for interactions involving the IgG of human origin (blue) and of bovine origin (orange) are both shown. Since IgGs of bovine vs. human origin did not form all the same complexes, the K_D calculation was not applicable for certain interactions (depicted as N/A). The K_D values were calculated for each of three repeated mass photometry measurements, and the mean ± standard deviation is given. Image Credit: Refeyn Ltd.*

These calculations verified the binding of protein A to the bovine IgG with lower affinity (K_D = 69.4 ± 9.0 nM) than the binding of protein to the human IgG (K_D = 5.73 ± 2.6 nM).

It was also noted that for the human IgG-protein A interaction, somewhat lower

K_D values were seen for larger complexes. This difference implied the presence of cooperativity effects involving IgG and/or protein A.

The experiment was repeated to improve measurement accuracy in this instance, but in most cases, a single mass photometry measurement is enough to provide the data required to calculate K_D, even when working with complex equilibria.

Mass photometry is also able to measure the K_D values of interactions where both bound and unbound species are observable at the concentrations employed in mass photometry measurements.

Conclusion

Mass photometry is a flexible, label-free bioanalytical tool ideally suited to the investigation of complex equilibria at a wide range of physiologically relevant concentrations.

It affords users the ability to rapidly measure the mass of individual biomolecules and the complexes they form in solution while requiring only minimal sample amounts.

The data needed to calculate K_D values can be acquired with a single mass photometry measurement, even in the case of complex interactions that generate multiple species.

Refeyn's Two^{MP} and Two^{MP} Auto mass photometers are both well suited to the study of protein-protein interactions. Data in this experiment was generated using the Two^{MP} Auto, which offers automated pipetting features for improved reproducibility.

Methodological details

- Protein A, bovine & human IgG antibodies were acquired from Sigma-Aldrich.
- Mixtures of IgG and Protein A (1:1 molar ratio) were incubated for 1 hour at room temperature before measurement. These were transferred to a 96-well plate which was loaded onto the Two^{MP} Auto mass photometer.

- Measurements were conducted within 40 minutes using PBS as the buffer for droplet dilution to find focus.
- K_D values were calculated as described.[3]

References and further reading

1. Wu and Piszczek, Anal Biochem 2020
2. Wu and Piszczek, JoVE 2020
3. Soltermann et al., Angew Chem Int Ed 2020
4. Young, Biswas and Chen, Biophys J 2003

Acknowledgments

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Mass photometry techniques with detergents

Detergents are commonly used throughout the biochemistry industry, but these tend to display complex behavior in aqueous solutions. Mass photometry is tool well suited to the study of biomolecules in solutions that contain detergent, as well as the evaluation of micelle formation and molecular aggregation.

Image Credit: Shutterstock/Mongkolchon Akesin

This article explores how detergents impact mass photometry measurements, and summarizes several key recommendations around the optimization of conditions for mass photometry experiments with detergents.

Detergents have a wide range of uses throughout the industry, including the extraction and solubilization of membrane proteins, the prevention of nonspecific binding, and the control of conditions affecting protein crystallization.

Detergents exhibit remarkable chemical properties and complex behavior in aqueous solutions; however, the presence of detergents imposes considerable downstream limitations on many widely utilized analytical technologies.

Mass photometry measures the mass of individual biomolecules in solution, making it an ideal solution to this particular challenge. It is also compatible with a diverse array of buffers, effectively eliminating the need for complete detergent removal.

Properly used, mass photometry provides a rapid, streamlined means of determining the impact of detergents on sample solubility and the ways in which detergent behavior varies in different buffers and at different concentrations.

The effect of detergents on mass photometry

Mass photometry is unable to detect individual detergent molecules as the amount of light they scatter is typically below the technique's detection threshold. Detergents may also produce noise (signal fluctuations) across ratiometric mass photometry images.

This can be the result of water molecules forming large solvation shells around detergent molecules, detergent molecules generating dynamic structures on the glass surface, or a range of other factors that may impact the refractive index at the glass-water interface.

Detergents can also impact mass photometry measurements via the formation of micelles when the detergent concentration in an aqueous solution is greater than the critical micelle concentration (CMC).

Smaller micelles (those below the detection limit) behave in a similar fashion to individual detergent molecules in that these generate noise in a mass photometry measurement.

It is possible to visualize larger micelles directly[1] in mass photometry in the same way as biomolecules,[2] but many standard protocols necessitate the use of high concentrations of detergent, leading to micelle concentrations that are too high to permit individual micelle masses to be quantified by mass photometry.

Many overlapping events will be observed in this scenario as the micelles meet the glass-water interface. These overlapping events produce a pattern of noise that is similar to the pattern generated by individual molecules and smaller

micelles but with a stronger signal.

The overall result is a random noise pattern in the ratiometric mass photometry image (Figure 1, upper panel), which prevents the detection of macromolecules with a signal in the same range or lower, effectively raising the lower limit for mass detection (Table 1).

It is also important to note that these noise patterns can result in incorrect or misleading mass photometry signals, much like the signals generated by macromolecules.

When this occurs, conventional mass photometry image analysis will incorrectly read these patterns as a macromolecule landing on the surface. Should the patterns occur more than once, they will result in a peak in a histogram with a certain apparent mass but no biological significance.

The lower panel of Figure 1 highlights the presence of a mirror-image peak with an equivalent, ‘negative’ apparent mass and the same height. It is possible to use this signature mirror-imaging to differentiate peaks arising from noise to those representing biomolecules that land on the measurement surface because negative mass results from particles moving away from the glass surface, not particles landing on it.

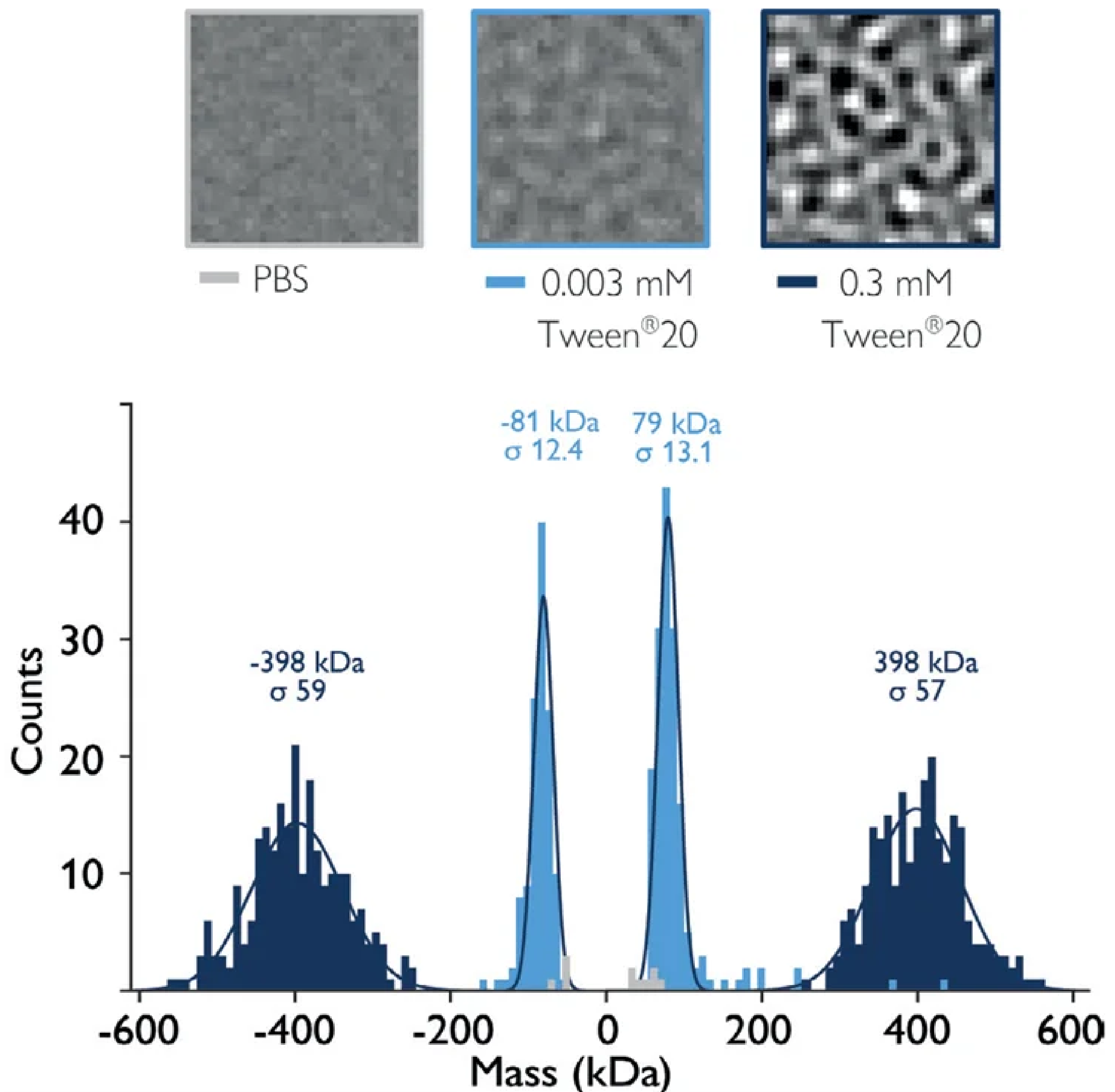

Figure 1. Typical detergent noise signature. *Top: PBS buffer alone and with detergent Tween®20 at two concentrations. Bottom: Superposition of histograms of PBS (grey) with PBS supplemented with Tween®20 at concentrations below (0.003 mM, mid blue) and above (0.3 mM, dark blue) the CMC. Apparent mass and sigma values of Gaussian fits are indicated. Values measured on the One^{MP}. Image Credit: Refeyn Ltd.*

It is important to note that mass photometry measurements of proteins and similar biomolecules generally yield no, or very small, negative peaks as the biomolecules will usually interact with the glass surface rather than moving away from this.

Measuring samples containing detergents

Mass photometry will only image biomolecules with mass significantly greater than the apparent mass corresponding to the detergent noise peak. Therefore, a lower detergent concentration will usually result in improved resolution, superior accuracy and a lower mass detection limit (Figure 2).

Mass photometry measurements should be conducted at the lowest possible detergent concentration. In certain cases, however, this may still correspond to a mass detection limit that is too large for meaningful measurement via mass photometry.

If the mass detection limit is too large for meaningful measurement but the detergent and protein are sufficiently bonded, it is possible to employ an in-drop, fast dilution procedure to facilitate mass photometry measurements of proteins at detergent concentrations below that which is otherwise considered the minimum for protein stability.[5]

This straightforward procedure can only be used if the detergent-protein interaction remains stable for the entire 1-minute duration of the measurement and the protein does not aggregate. The procedure is completed using the following steps:

1. Load buffer with no detergent onto the coverslip
2. Find the focus
3. Add protein with detergent to the original buffer. This should then be mixed by using the pipette to gently aspirate in and out.
4. Conduct the mass photometry measurement

To assess the apparent mass of the detergent noise peak, this procedure should be completed following an appropriate control measurement of the buffer at an identical detergent concentration.

Table 1 provides approximations of the effective detection limits for different detergents after dilution in PBS. This should be regarded as a general guide, since actual detection limits may vary based on the buffer’s characteristics; for example, ionic strength or pH.

It is, therefore, prudent to assess the mass detection limit of detergent-

containing solutions on a case-by-case basis. This is best achieved by conducting control measurements of the solution without the biomolecules of interest present.

Certain proteins are only soluble above a specific detergent concentration, and these proteins will form large aggregates when below this concentration. Optimal conditions for each individual protein/detergent combination are difficult to anticipate due to their dependence on a number of different factors.

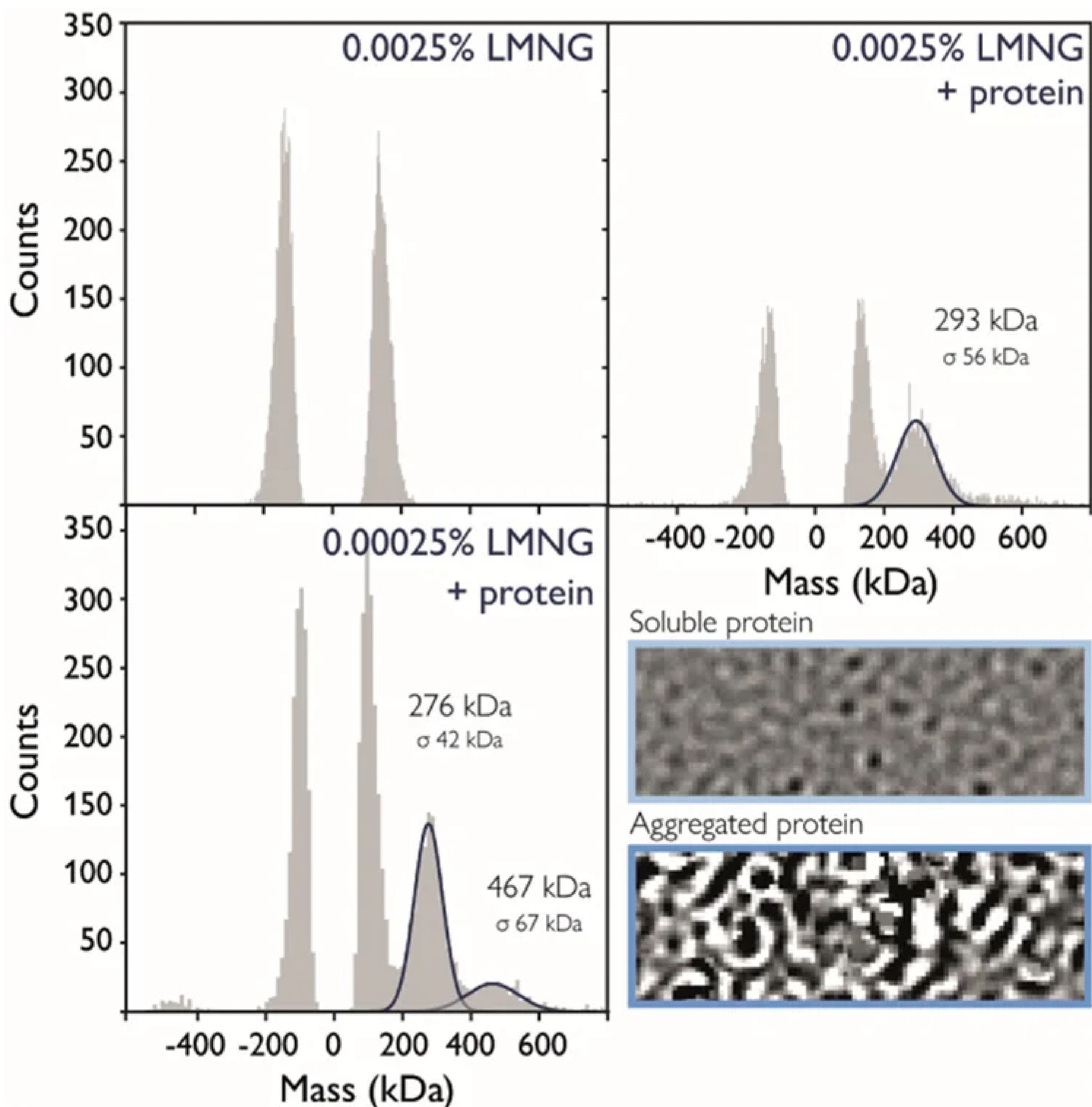

Figure 2. Mass photometry measurement of a protein in detergent. *Histograms represent measurements of buffer with 0.0025% LMNG alone, and 10 nM protein in buffer with 0.0025% and 0.00025% LMNG. Excessive dilution*

of detergent may result in protein aggregation, as illustrated in ratiometric frames showing soluble protein (light blue) and aggregated protein (mid blue). Data courtesy of Blanca López Méndez and Vadym Tkach, University of Copenhagen. Values measured on the One^{MP}.

Table 1. Effective lower detection limits corresponding to relative concentrations of detergents. Estimates of lowest detectable protein mass in kDa for One^{MP} (light blue) and Two^{MP} (mid blue) are based on the noise peak detected at the respective detergent concentration (mM, grey gradient). Detergents were diluted in PBS. CMC is indicated in grey. N/A: Detection limit of the instrument applies. Source: Refeyn Ltd.

%CMC	1%	5%	20%	100%	500%	2000%	OneMP TwoMP
	82E-3	0.41	1.6	8.2	41	160	[mM]
SDS	N/A	70	70	170	180	180	kDa
	N/A	110	120	120	230	230	kDa
	1.2E-3	6E-3	24E-3	0.12	0.6	2.4	[mM]
DDM	N/A	N/A	N/A	560	560	560	kDa
	N/A	N/A	N/A	120	480	480	kDa
	0.23	1.2	4.6	23	120	460	[mM]
OG	N/A	N/A	N/A	220	460	760	kDa
	N/A	N/A	40	250	250	330	kDa
	0.8E-3	4E-3	16E-3	0.08	0.4	1.6	[mM]
NP-40	N/A	50	90	260	430	430	kDa
	N/A	N/A	N/A	60	500	500	kDa
	0.6E-3	3E-3	12E-3	0.06	0.3	1.2	[mM]
Tween 20	90	120	240	430	430	430	kDa
	100	110	210	270	270	270	kDa
	3.5E-3	18E-3	0.07	0.35	1.8	7	[mM]
Triton X-100	90	110	190	620	620	620	kDa
	30	50	210	480	480	480	kDa
	0.08	0.4	1.6	8.0	40	160	[mM]
CHAPS	N/A	N/A	90	210	210	300	kDa
	70	80	100	230	230	320	kDa
	0.1E-3	0.5E-3	2E-3	0.01	0.05	0.2	[mM]
LMNG	N/A	N/A	60	210	410	500	kDa
	N/A	N/A	280	280	400	550	kDa

These should also be assessed experimentally on a case-by-case basis, ideally using mass photometry. This technique represents an ideal choice for the screening of solubility conditions because it requires very little sample and can be performed quickly. Aggregates are also easily identifiable when viewed in a ratiometric mass photometry movie (Figure 2).

Using mass photometry to assess the CMC

Detergents commonly generate a low mass photometry background below the CMC, with the mass photometry background increasing abruptly above the CMC.

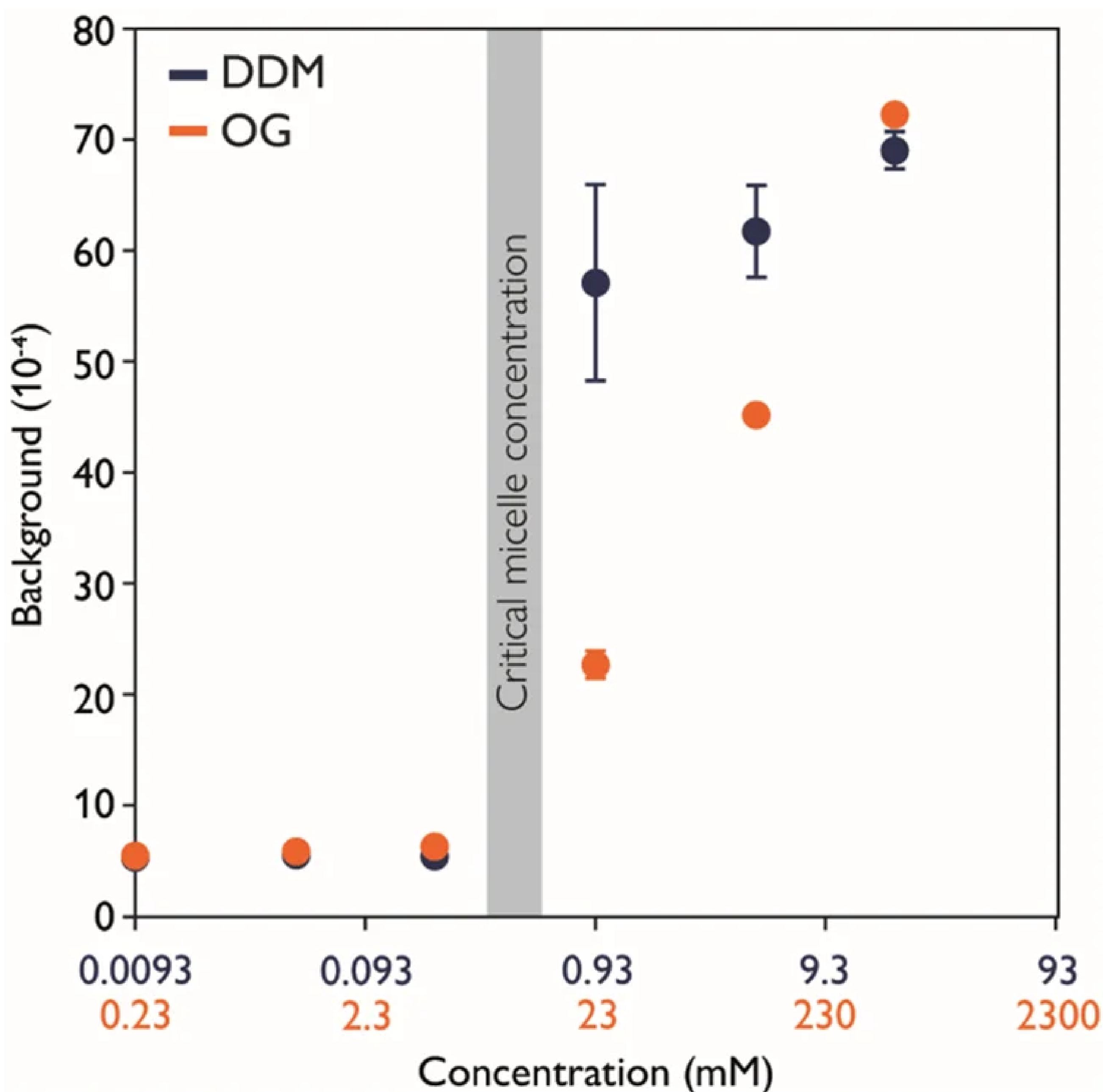

Figure 3. Detergent behavior above the CMC varies by detergent. *Mass photometry measurements of two different detergents, DDM (dark blue) and OG (orange), show sigmoidal (DDM) vs linear (OG) increases in background as detergent concentration is increased above the CMC. The approximate CMC (in PBS) is indicated in grey. The background was quantified as the standard deviation of contrast for each ratiometric image, averaged over 3000 frames. Values measured on the One^{MP}. Image Credit: Refeyn Ltd.*

Background intensity can be seen to plateau for detergents that form micelles of a single size, for example, DDM (n-dodecyl-ß-D-maltoside). This background may also continue to increase with some detergents, should the micelle size increase with concentration; for example, in the case of OG (octyl glucoside) (Figure 3).

The CMC in these cases in largely dependent on factors such as the nature of the biomolecules or the ionic strength and pH of the buffer. These factors can lead to a detergent’s observed CMC to vary significantly from the CMC reported for that detergent in water, even when investigated under experimental conditions.

This variability is especially apparent in ionic detergents like SDS (sodium dodecyl sulfate)[3] (Figure 4, top).

Detergents are also prone to displaying complex micelle formation behavior. For example, CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulf onate) has been found to possess two distinct CMCs (around 7 and 32 mM) and has been seen to form micelles 1.8 times larger above the second CMC[4] (Figure 4, bottom).

The capacity to monitor micelle formation under a range of experimental conditions is valuable because this allows users to optimize detergent concentration, using only the necessary amount of detergent in each instance.

There are a number of challenges associated with measuring the CMC in practice; however, experiments are generally performed with detergent concentrations far above the required concentrations.

Mass photometry offers a potential solution to this issue. It offers a rapid, convenient means of evaluating detergent behavior under a range of

experimental conditions, making it easy to establish the optimal parameters for any given experiment.

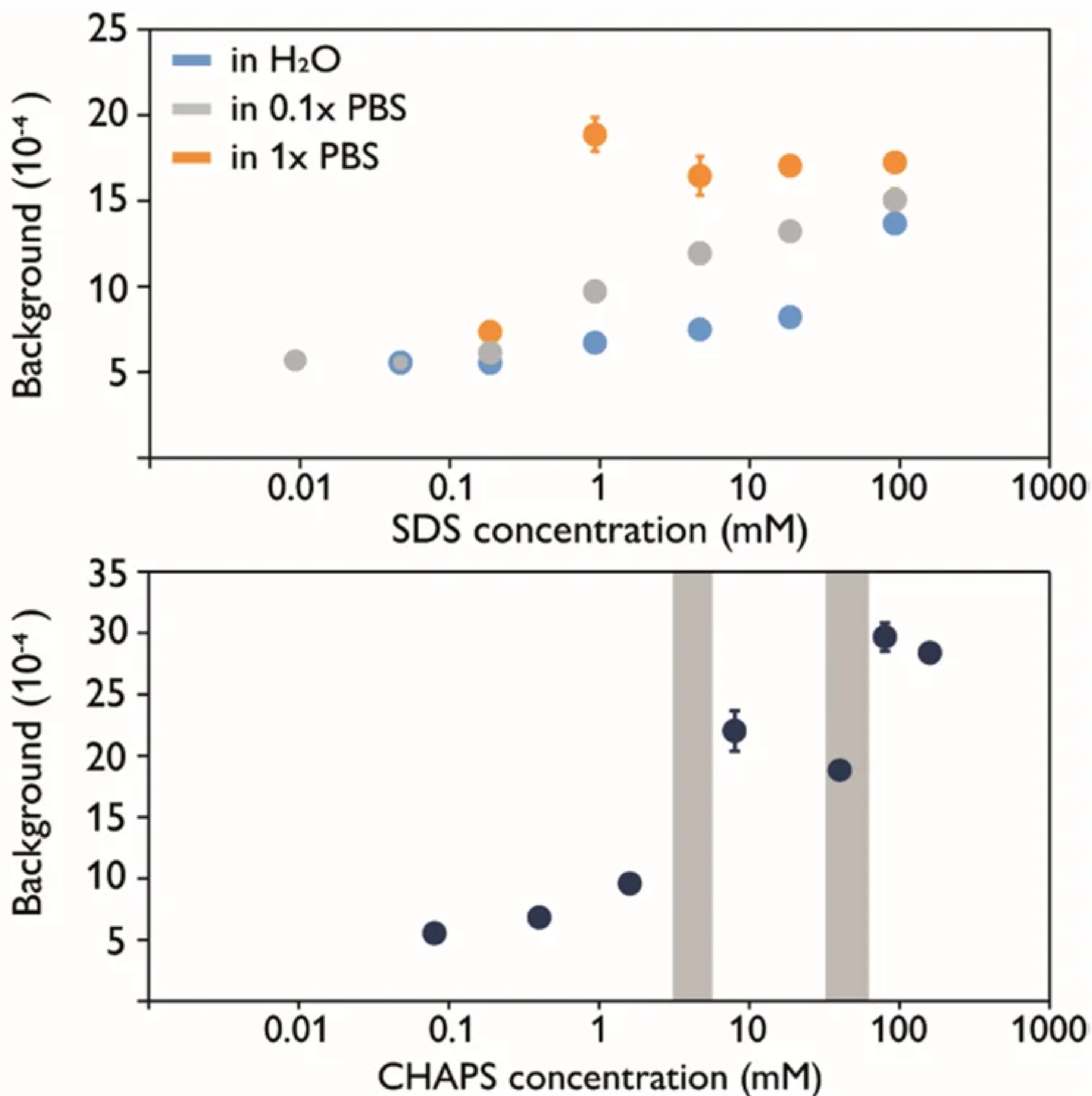

Figure 4. Detergent micelle formation may be sensitive to buffer composition or display complex behavior. *Top: The background measured using mass photometry for increasing concentrations of SDS in water (blue), 0.1x PBS (grey) and 1x PBS (orange). Bottom: Similar measurements for increasing concentrations of CHAPS in PBS. CMCs reported in the literature[3] are indicated as grey areas. The background was quantified as in Fig. 3. Values measured on the One^{MP}. Image Credit: Refeyn Ltd.*

References and further reading

1. Lebedeva *et al*., ACS Nano 2020
2. Young *et al*., Science 2018
3. Danov *et al*., Adv Colloid Interface Sci 2014
4. Qin *et al*. J Phys Chem B 2010
5. Olerinyova *et al.*, Chem 2021

Acknowledgments

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Characterizing membrane proteins in nanodiscs with mass photometry

Mass photometry is able to offer users efficient and accurate characterization of complex samples, including those that feature membrane mimetics. This technique is ideal for streamlining membrane protein purification processes, a feature highlighted by the example application of mass photometry measurements presented here.

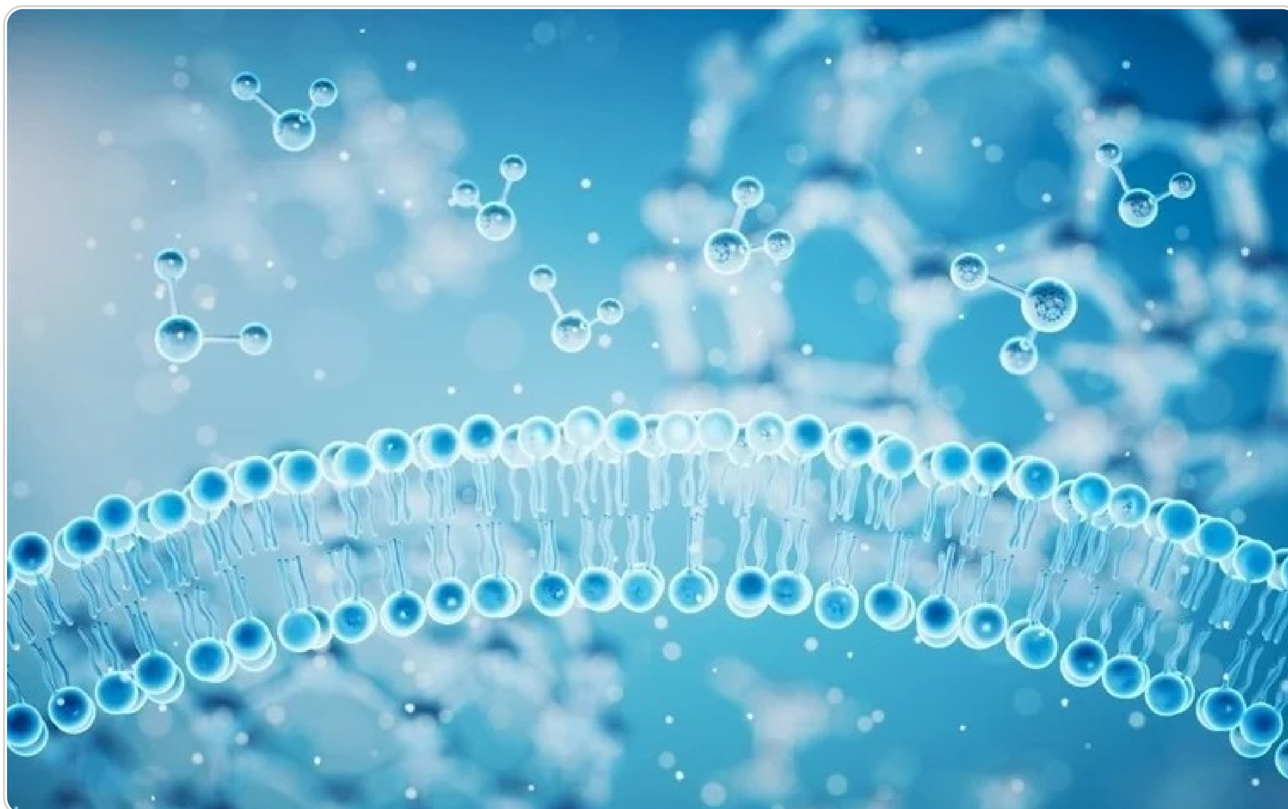


Image Credit: stock_wichel

This case study sees measurements undertaken at various points throughout the process of purifying SMALP-embedded AQP4 samples.

Membrane proteins fulfill a range of essential biological roles, including signal transduction, immune system recognition, and molecule/ion transport across membranes.[1]

It is often necessary to extract, solubilize, and stabilize membrane proteins embedded in a lipid bilayer, which often involves using membrane mimetics.[2]

Nanodiscs are garnering increased interest in this area due to their capacity to provide improved stability and their potential for dilution to low concentrations

without losing shape or integrity.

Styrene maleic acid polymer lipid particles (SMALPs) represent especially attractive options, as these function by encapsulating pieces of the lipid bilayer. This particular extraction process affords the studied proteins a native-like, detergent-free environment.[3]

Mass photometry and the AQP4 membrane protein

Mass photometry is a robust analytical approach that measures the mass of molecules in a solution. This technique functions rapidly and with very little required sample while being largely compatible with membrane mimetics such as nanodiscs. These factors make mass photometry an ideal addition to membrane protein purification processes.

Aquaporin-4 (AQP4) is an integral membrane protein that functions as a bidirectional water channel. AQP4 is typically present in the central nervous system (CNS), making this a promising drug target for CNS edema.[4]

This article looks at the purification of a sample of M1-isoform AQP4 embedded in SMALP nanodiscs as part of a functional and structural characterization process.

Mass characterization of AQP4 purification fractions

Mass photometry was utilized to investigate the composition and quality of protein samples at various points during the purification process. This experiment aimed to identify sample components and characterize isolated nanodisc-embedded AQP4 singlets before engaging in further study.

Once the embedding process had been completed, size exclusion chromatography (SEC) was employed in the separation of nanodisc-embedded AQP4 proteins from any impurities, such as empty discs or aggregates.

Mass photometry was used to characterize any fractions expected to contain the nanodisc-protein complex to identify the fraction with the greatest abundance of nanodisc-embedded AQP4 singlets (Figure 1).

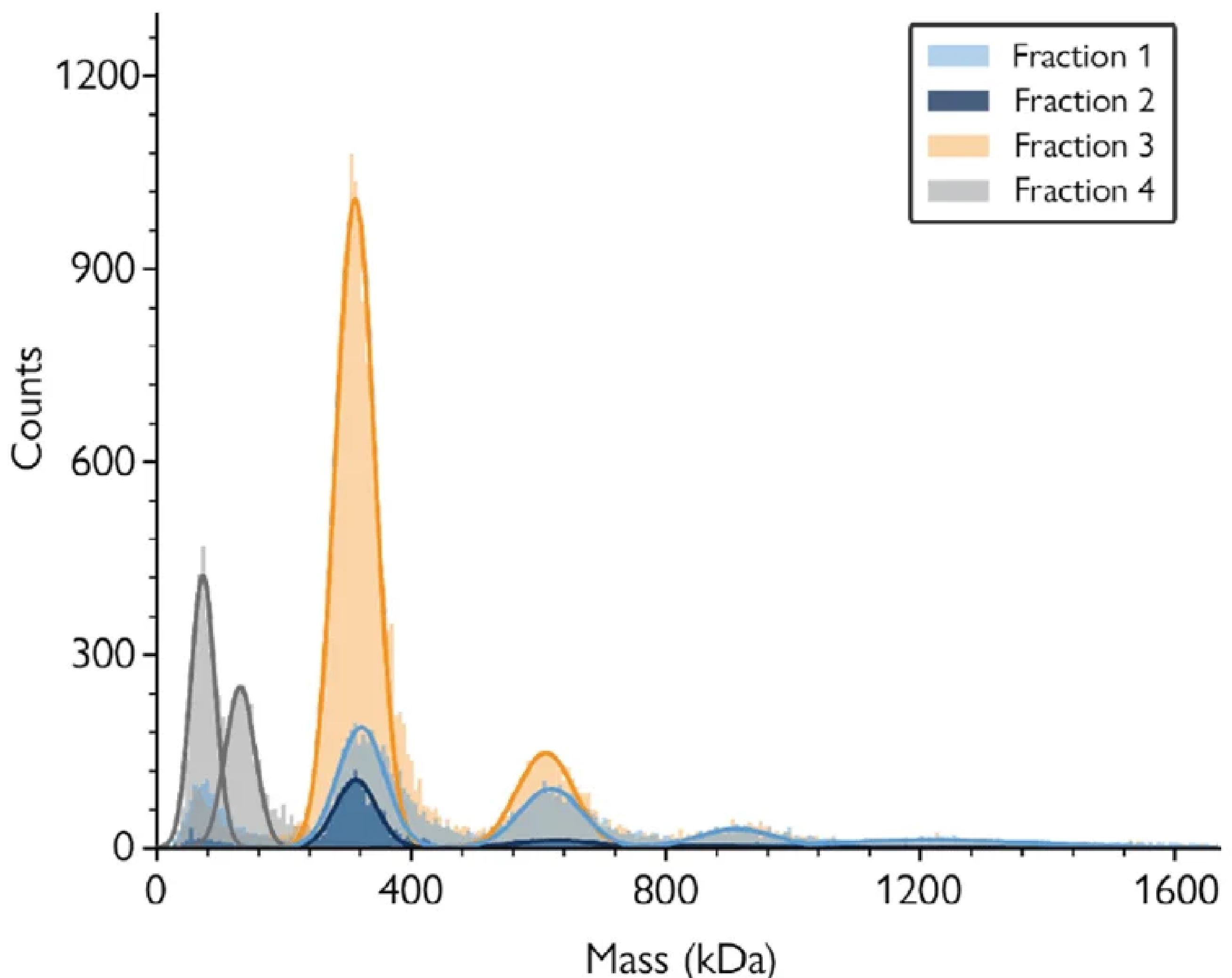


Figure 1. Mass photometry characterization of SEC fractions collected in the purification process of SMALP-embedded AQP4. *Mass histograms show four selected fractions with varying proportions of the different complexes (detailed in Box 1). Fraction 3 (orange) had the highest proportion of AQP singlets (mass of ≈310 kDa). Image Credit: Refeyn Ltd.*

Peak interpretation

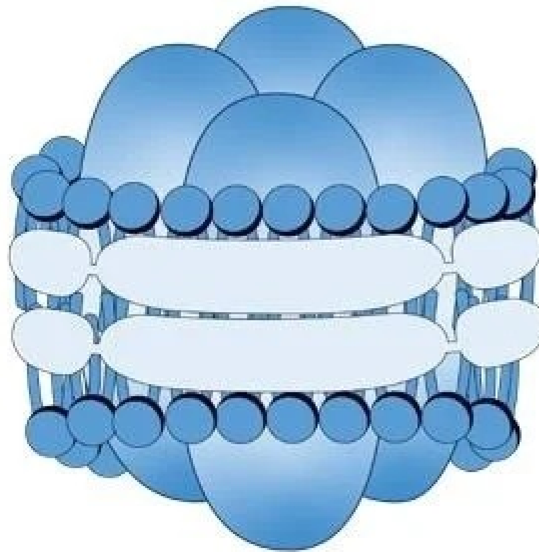
The mass histogram peaks in Figure 1 correspond to:

≈70 and 140 kDa: Purification contaminants

≈310 kDa: One tetramer of AQP4 (singlet)

≈585 kDa: Two tetramers of AQP4 (doublet)

≈862 kDa: Three tetramers of AQP4 (triplet)



This image shows an example of a single AQP4 tetramer embedded in a SMALP (singlet). Groups of several tetramers (doublets, triplets etc.) can be found embeded in SMALPs of variable sizes. Image Credit: Refeyn Ltd.

Identification of desired species

Within this study, it was possible to confirm the presence of natively folded AQP4 singlets in the affinity-purified sample through the use of an anti-AQP4 antibody.

Analyzing the sample incubated with anti-AQP4 antibody using mass photometry illustrated the presence of a new population at ≈449 kDa, corresponding to anti-AQP4 antibodies that had bound to nanodisc-embedded AQP4 singlets (Figure 3, blue histogram).

Scattered events present in higher mass regions imply the presence of antibodies bound to doublets and triplets. Variable SMALP nanodisc masses resulted in the absence of clearly resolved peaks, however, preventing proper quantitative analysis in this instance.

A similar mass distribution to the untreated sample (Figure 1) was observed when employing negative control using a non-specific antibody (anti-GFP), though an additional peak at 145 kDa was noted, corresponding to an unbound antibody. Figure 3 shows the combination of both experiments, verifying the presence of natively-folded AQP4 in the sample.

Discussion

Mass photometry addresses many of the challenges encountered during membrane protein purification, as this analytical technique requires very small amounts of sample and is compatible with the majority of membrane mimetics.

Measurements take minutes using mass photometry, making this an ideal tool for the characterization of different fractions during purification.

SMALPs are known to have variable sizes, and this variability is reflected in the data presented here, which shows the presence of additional broad peaks in the higher mass ranges.

These peaks may represent larger complexes where relative protein and SMALP masses are unknown (Figure 2), meaning it can be especially difficult to draw conclusions about the SMALP:AQP4 proportions of higher mass components in these instances.

Masses of the complexes identified here do remain consistent with theoretical predictions, however, and the SMALP and protein masses of AQP4 singlets are in line with results acquired via other analytical methods like SEC and SMA-PAGE.[5]

This article showcased the ways in which mass photometry provides valuable information about complex samples, affording users greater insight into their nature and empowering them to make more informed decisions during membrane protein purification.

Experimental details

- All measurements were conducted using the Two^{MP}.
- Clean sample carrier slides and Refeyn 6-well sample cassettes were used.
- Samples and supplementary data were kindly provided by Philip Kitchen and Caolan Browne from Aston University.

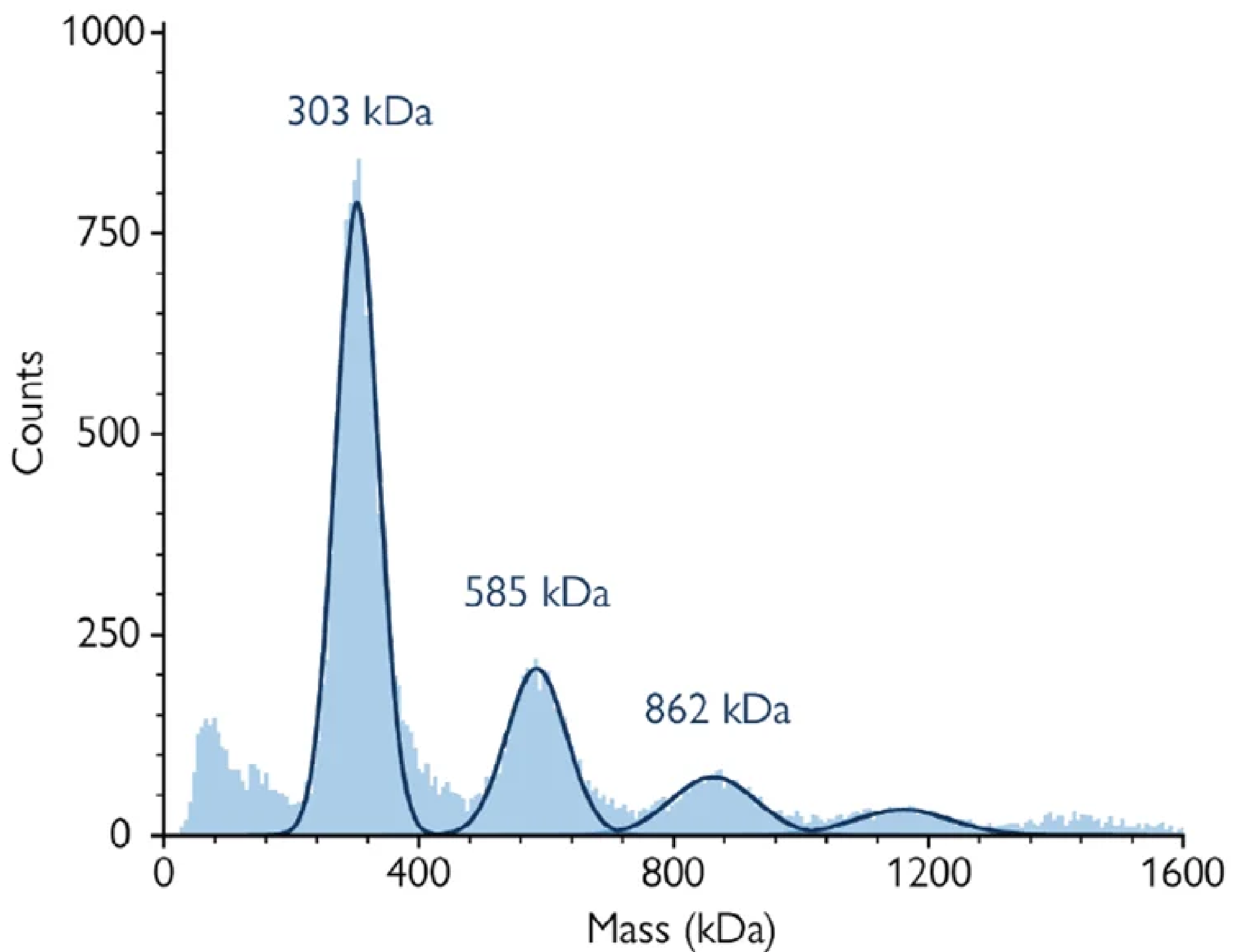


Figure 2. Affinity-purified sample of nanodisc-embedded AQP4. *The mass photometry histogram shows four peaks corresponding to the main components described in Box 1. Image Credit: Refeyn Ltd.*

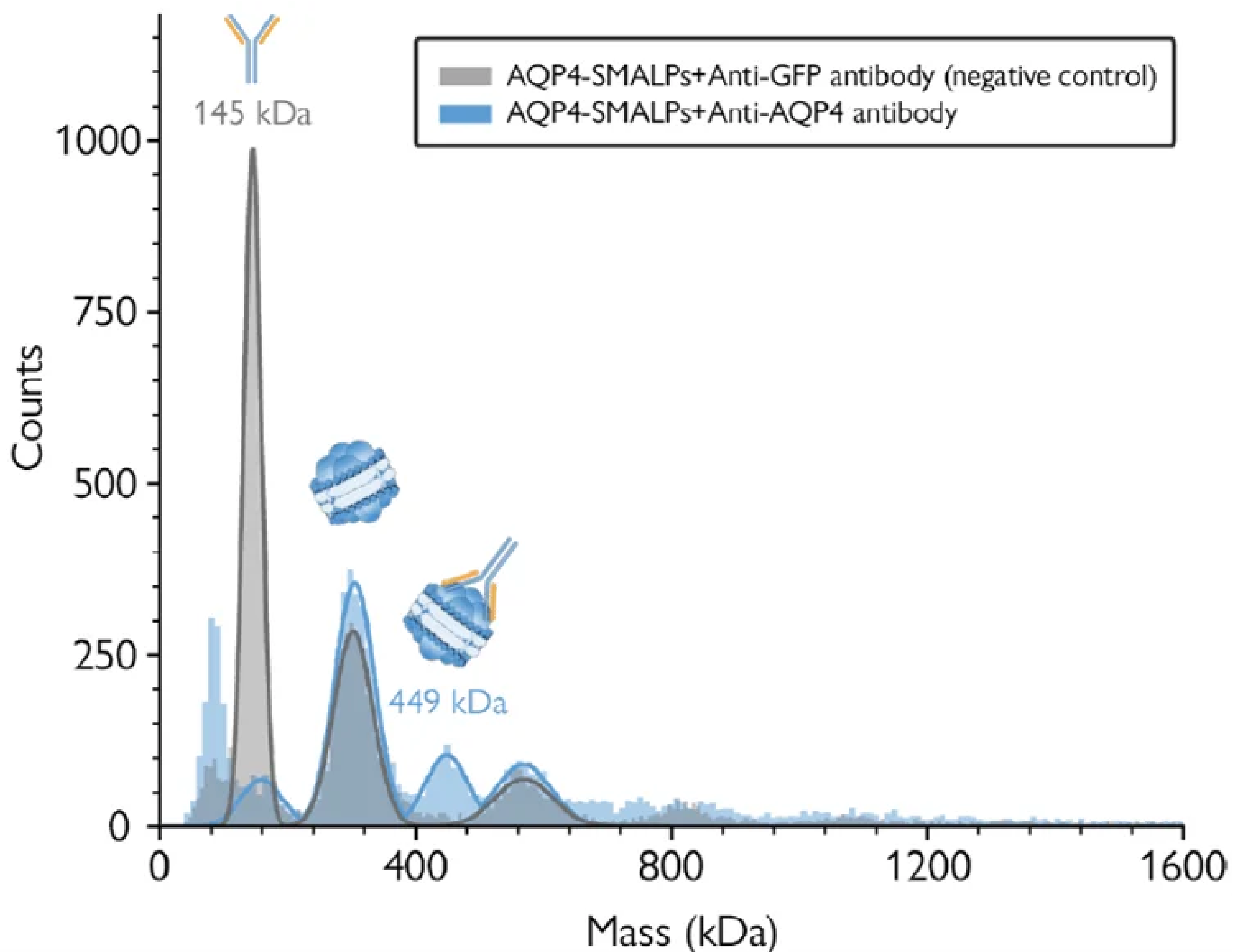


Figure 3. Antibody binding assay confirms the presence of AQP4 in the purified sample. *Blue histogram: Antibody binding assay using an anti-AQP4 antibody. The peak at 449 kDa corresponds to AQP4 singlets bound to anti-AQP4 antibody. Grey histogram: Negative control with an anti-GFP antibody. The peak at 145 kDa corresponds to free anti-GFP antibody. Image Credit: Refeyn Ltd.*

References and further reading

1. Almeida *et al*., Biochim Biophys Acta Biomembr. 2017
2. Majeed *et al*., Membranes. 2021
3. Young, Biochem Soc Trans. 2021
4. Kitchen *et al*., Cell. 2018
5. Supplementary tests by Caolan Browne and Philip Kitchen

Acknowledgments

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Advances in automated mass photometry

Automated mass photometry combines the unparalleled simplicity and sensitivity of Refeyn's molecular mass measurement technology with the efficiency and ease of automation.

This approach offers enhanced precision while freeing up valuable operator time, facilitating the rapid measurement of multiple samples with minimal sample requirements.

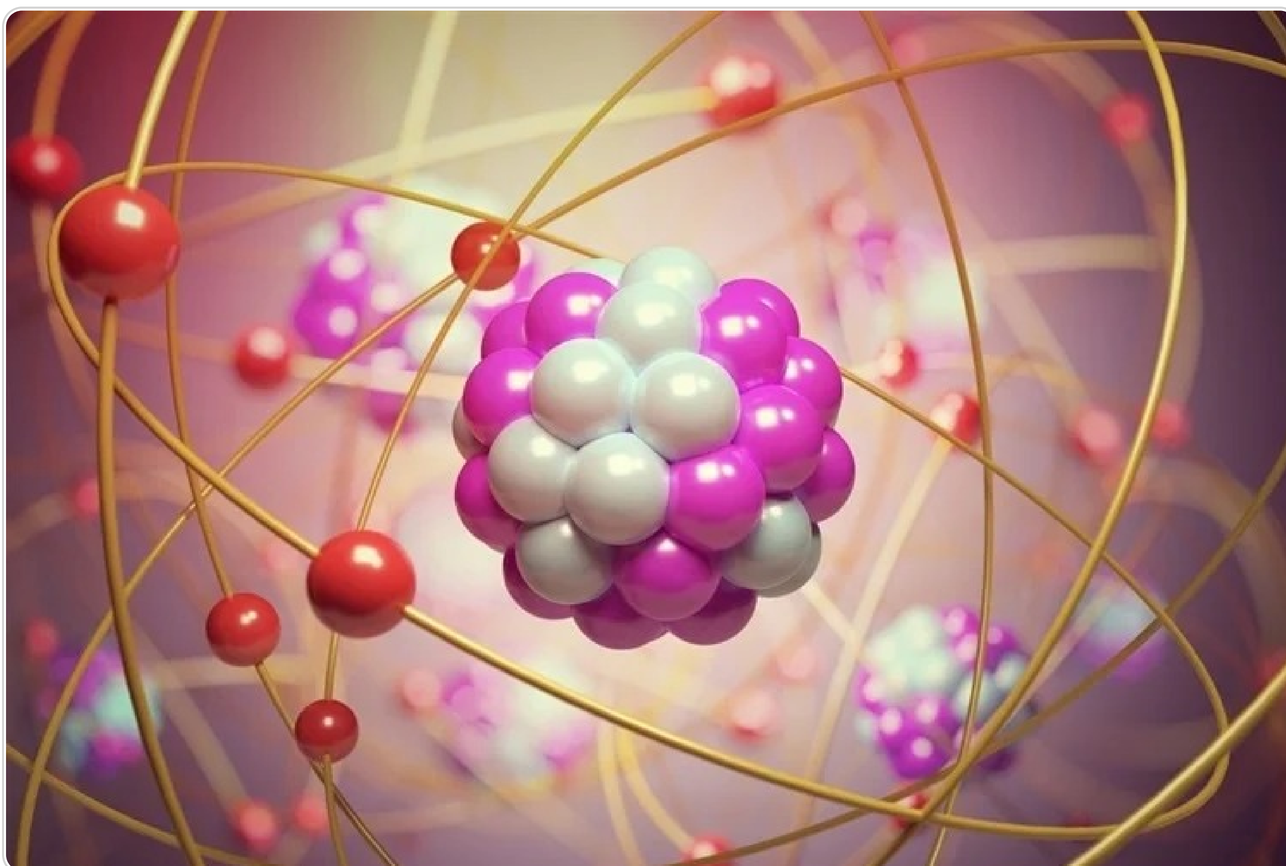

Image Credit: vchal

About mass photometry

Mass photometry is a powerful analytical tool that investigates biomolecules at the single-molecule level, without the need for labels, and in the biomolecules' native state.

This technique is used to measure the interference between light reflected by a glass surface and light scattered by individual molecules adhering to the glass, utilizing this signal to measure the molecules' mass by counting them (Figure 1).

Mass photometry is employed in a wide range of application areas, including the study of oligomerization, molecular interactions, and macromolecular assembly. It also facilitates easy sample quality and stability assessments as a core element of rapid and responsive process optimization cycles.

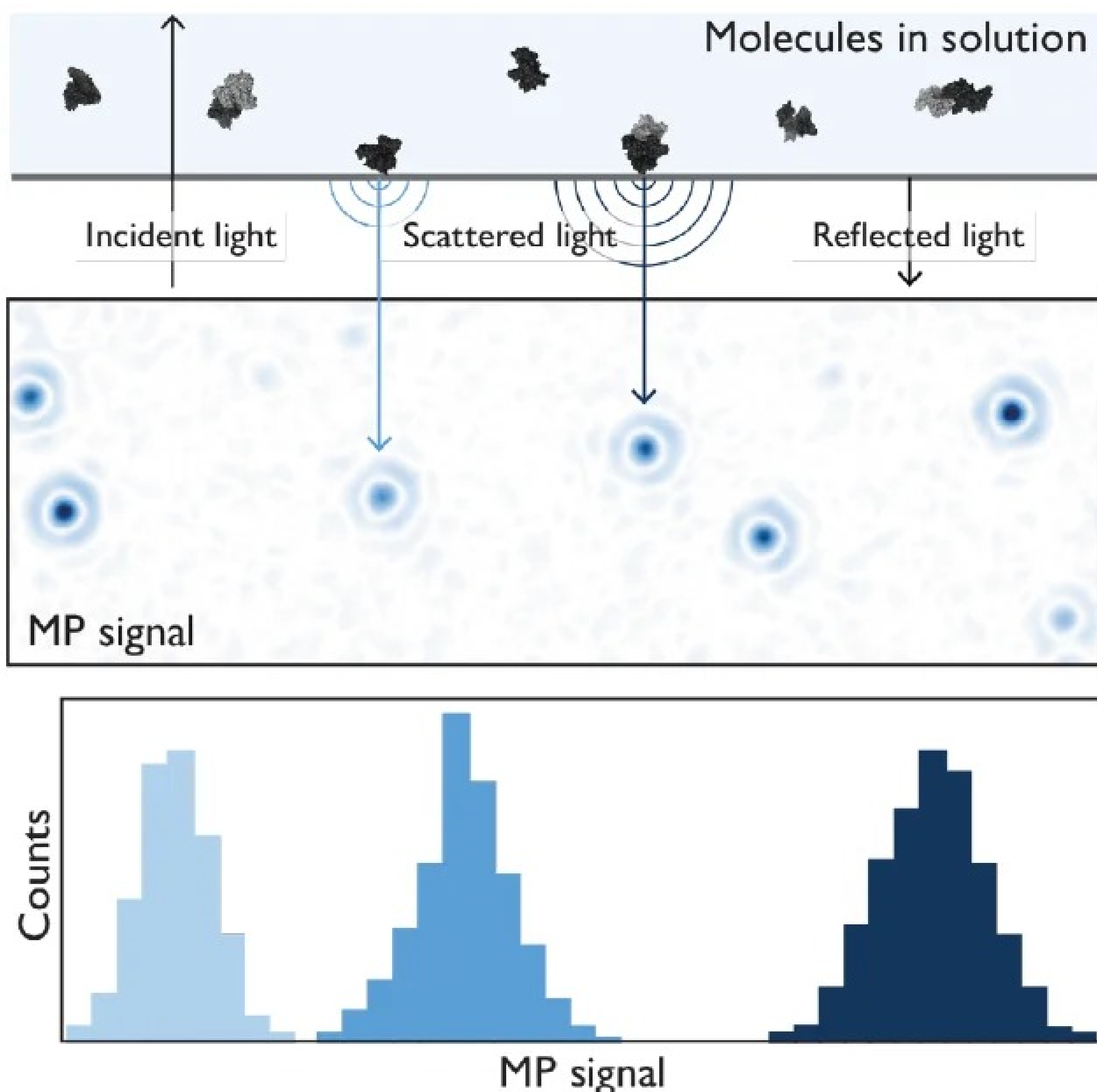

Figure 1. The principle of mass photometry. *The light scattered by a molecule interacting with the measurement interface interferes with light*

reflected at that interface. The signal intensity scales linearly with mass. Image Credit: Refeyn Ltd.

Automated mass photometry

The automated mass photometry solution from Refeyn is comprised of a robotically-controlled mass photometer able to autonomously measure 24 samples, including calibrants, in just 90 minutes.

This technology is ideally suited to a wide range of applications that require precise and efficient molecular mass characterization on a repeated basis, including titration assays and screening.

Automated mass photometry is ideal for any task that requires precise and efficient repeated molecular mass characterization. The robotics unit can be retrofitted to both the One^{MP} and Two^{MP} mass photometers produced by Refeyn. It is also available together with the Two^{MP} mass photometer as the Two^{MP} Auto.

The Two^{MP} Auto from Refeyn is easy to use thanks to an intuitive single software interface that allows the operator to directly control the whole data acquisition process.

The operator defines a sample mixing and measurement protocol before loading the sample and any required buffers, calibrants and solutions onto a 96-well plate. It is also possible to automatically dilute samples immediately before measurement, minimizing the possibility of well adsorption.

Once it has been defined, the robot follows this protocol to transfer components from the 96-well plate to a cassette on the mass photometer. This multi-sample well cassette is able to accommodate 24 samples (Figure 2), with its first and final wells typically used for calibrants, ensuring the presence of an internal validation control.

The robot will autonomously run the mass measurements, returning relevant data to the operator for further analysis.

All the benefits of mass photometry are afforded by automated mass photometry; for example, each experiment takes just minutes, uses a minimal amount of sample, and the results can be intuitively interpreted, all with the more consistent and convenient sample manipulation inherent to automated processes.

Figure 2. A close-up of the robotic pipetting arm used in automated mass photometry. *The liquid-handling robot uses the pipette tips (1) to transfer sample from the 96-well plate (2) to the multi-sample well cassette (3), in preparation for a measurement. Image Credit: Refeyn Ltd.*

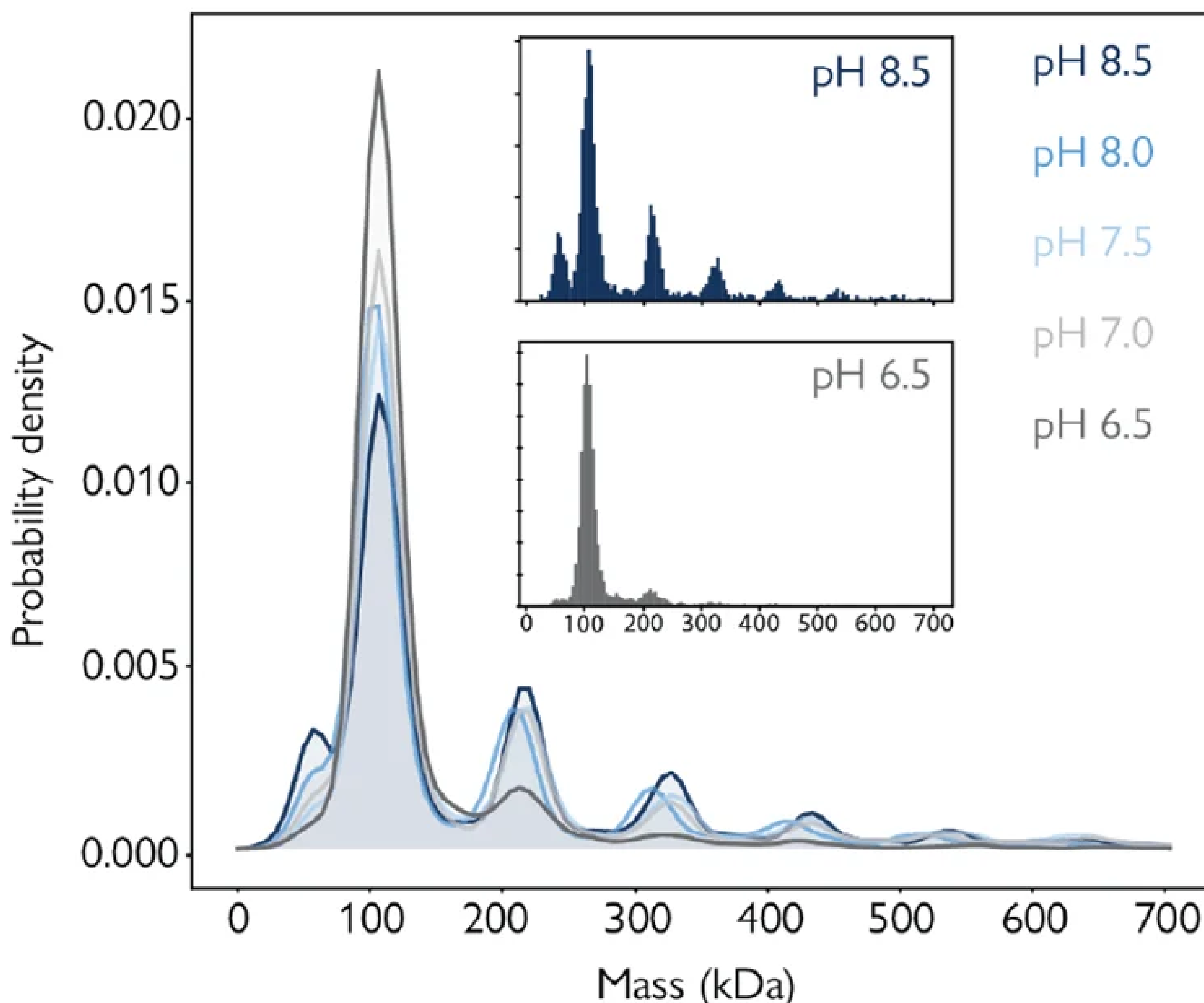

Figure 3. Studying protein oligomerization with automated mass photometry. *Mass distributions of citrate synthase samples in a pH titration series performed using the Two^{MP} Auto. The inset plots show detailed molecular mass distribution histograms for the highest (8.5) and lowest (6.5) pH values measured. Data courtesy of Stefano Lometto and Dr. Georg Hochberg of the Max Planck Institute for Terrestrial Microbiology, Marburg. Image Credit: Refeyn Ltd.*

Oligomerization studies

Oligomerization is a key process in the function of a significant number of proteins, but effectively capturing and characterizing protein oligomerization can be challenging. This is especially the case where oligomeric species are only present at very low concentrations in the sample.

Mass photometry is a powerful tool that provides high-resolution distributions of molecular mass directly in a solution while maintaining single-molecule sensitivity. These qualities make it ideally suited to detecting a protein's different oligomeric states, including rare species representing less than 1 % of a sample population.

The Two^{MP} Auto builds on the strengths of mass photometry, allowing users to rapidly screen a protein's oligomerization states with ease. This is possible under a range of different experimental conditions, including variations in buffer pH (Figure 3).

In the example presented here, the Two^{MP} Auto was employed in the measurement of mass distributions in citrate synthase samples in a pH titration series. At certain pH values, citrate synthase will oligomerize into inactive filaments that register in a mass photometry histogram as peaks at higher molecular masses, therefore representing higher-order oligomers.

Straightforward titration

Running titration assays like the one displayed in Figure 4 is simple and straightforward using automated mass photometry. In the example shown here, mass photometry analysis revealed that the protein inosine-5'-monophosphate dehydrogenase (IMPDH) exists in two different oligomeric states: a tetramer (known to be catalytically active) and an octamer (known to be inactive).[1]

Protein concentration was held constant throughout this experiment, while the ligand diadenosine tetraphosphate (Ap4A) concentration was varied (this ligand is known to promote octamer formation).

It was observed that octamer concentration rose sharply before becoming saturated at higher Ap4A concentrations. Mass photometry measurements exhibited high consistency in this experiment, with close values confirmed between repeated measurements.

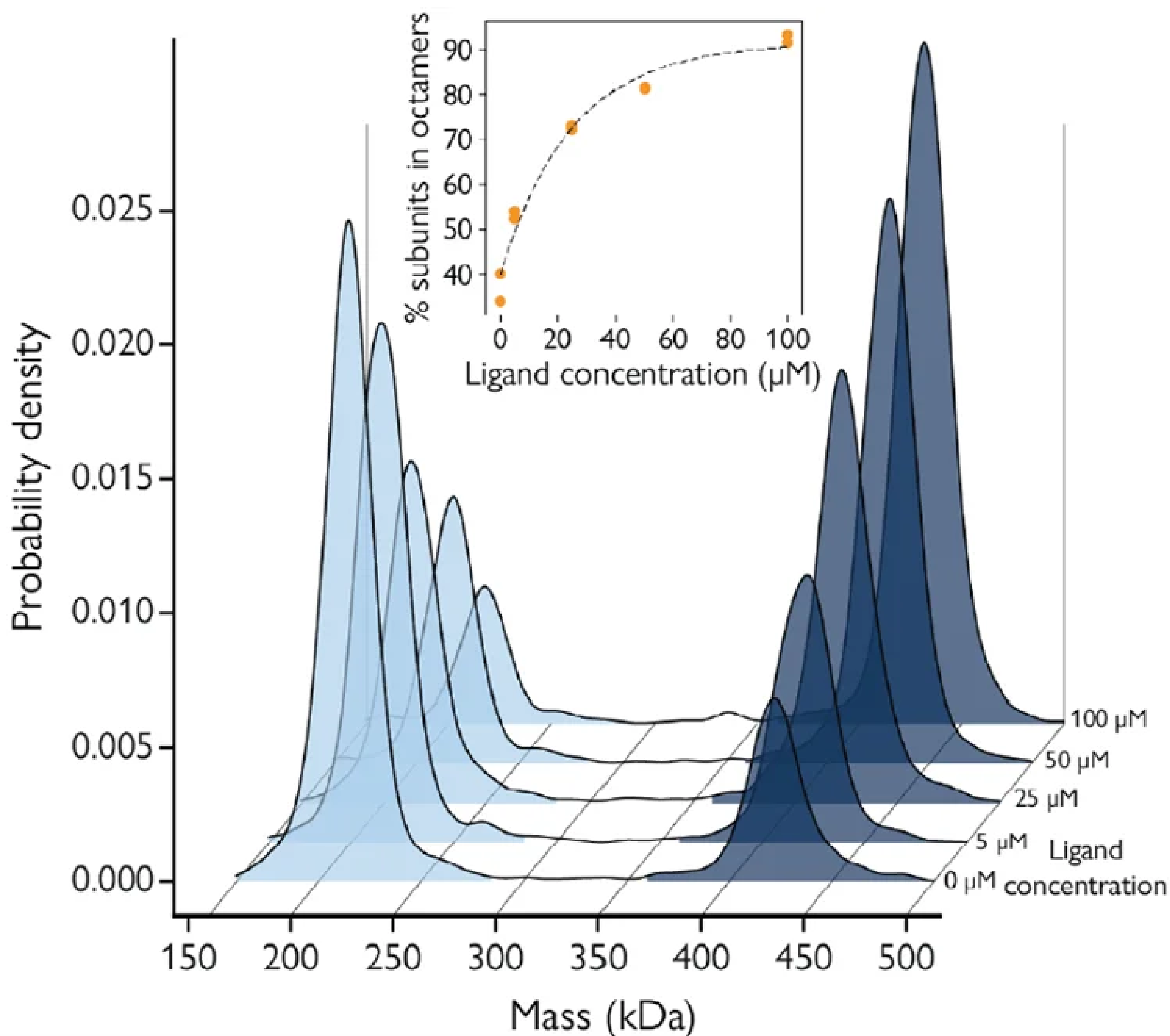

Figure 4. A ligand titration series. *Mass photometry analysis shows two oligomeric states of IMPDH: a tetramer (light blue peaks) and an octamer (dark blue peaks). Inset: The proportion of octamers to tetramers increased as the concentration of the ligand Ap4A was increased. Each measurement was performed twice, as shown in the inset, and one set is shown in the main plot. Data courtesy of Pietro Giammarinaro, Prof. Dr. Gert Bange and Dr. Georg Hochberg, Max Planck Institute for Terrestrial Microbiology, Marburg. Image Credit: Refeyn Ltd.*

Two^{MP} Auto: The automated mass photometer

Combining the efficiency and ease of automation with the simplicity and sensitivity of mass photometry, the Two^{MP} Auto enables rapid measurement of multiple samples while maintaining consistently high reproducibility.

Image Credit: Refeyn Ltd.

Reproducibility

A combination of automated liquid handling and the capacity to create and use standardized protocols is key to the simplification of mass photometry experiments. These characteristics also ensure improved reproducibility of results versus manual operation (Figure 5).

There was clear consistency across measurements between mass measurements (147 ± 1.0 kDa for monomers, 304 ± 2.4 kDa for dimers) and the proportions of monomers and dimers (90 % monomers ± 0.7 %), indicating the reproducibility and reliability of mass photometry. Mass measurements were

found to match the reported mass of both IgG monomers (150 kDa) and dimers (300 kDa).

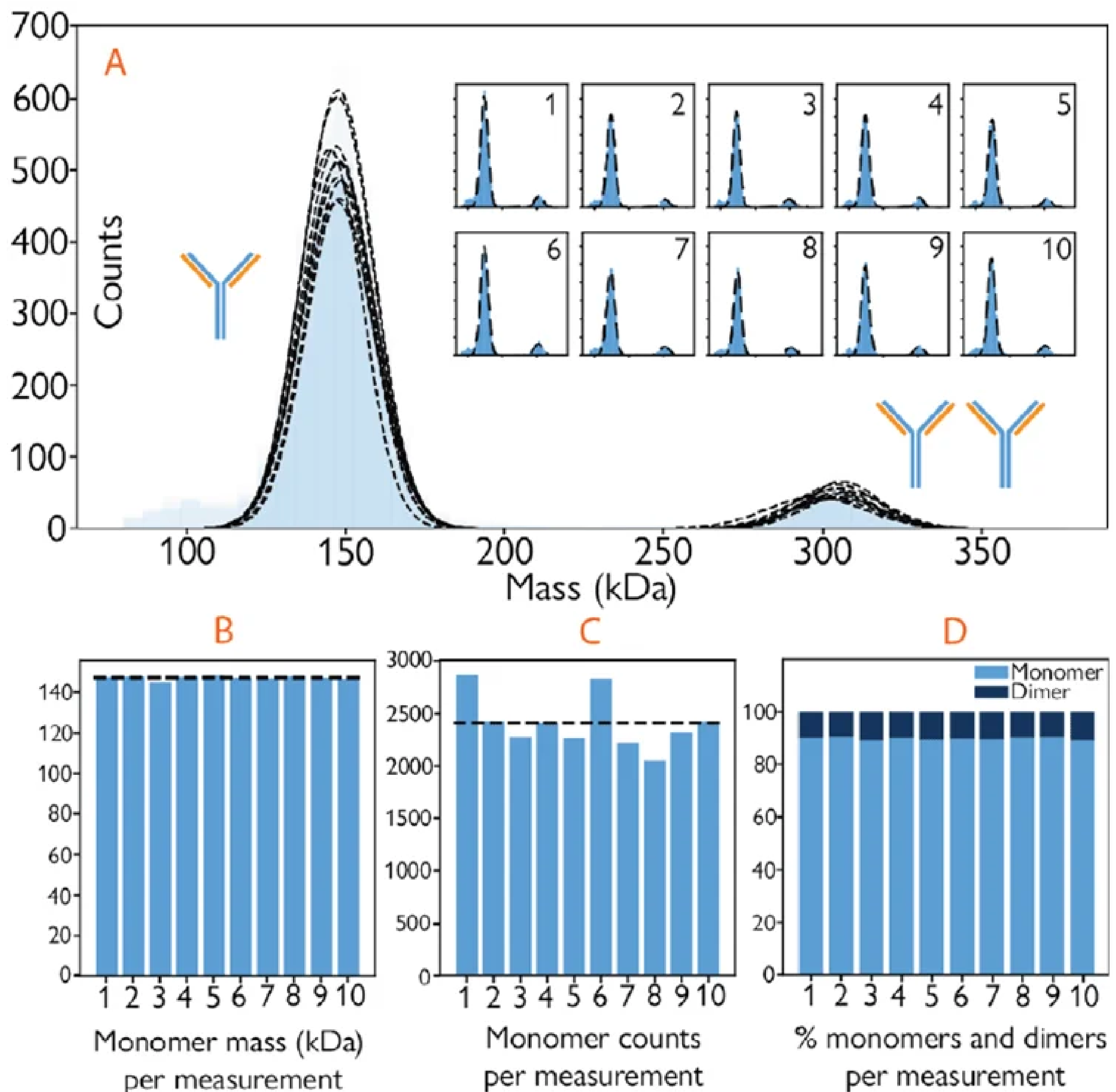

Figure 5. Automated mass photometry measurements are highly consistent. *Autonomously run mass photometry measurements of IgG (10 nM) illustrate the reproducibility of the system. A: The mass distributions (inset: mass histograms for each of 10 wells, shaded light blue) form two peaks – monomers and dimers. The measured mass, calculated from the location of the maximum of the lower-mass peak in each measurement (B), counts associated with the lower-mass peak (C), and relative proportions of monomers and dimers (D) are shown across the 10 wells. Image Credit: Refeyn Ltd.*

Accessories and consumables

Refeyn offers a number of accessories and consumables that are ideal for mass photometry measurements.

These products afford users confidence in their data by helping maintain consistent measurement conditions while simultaneously reducing the number of steps required to obtain each measurement.

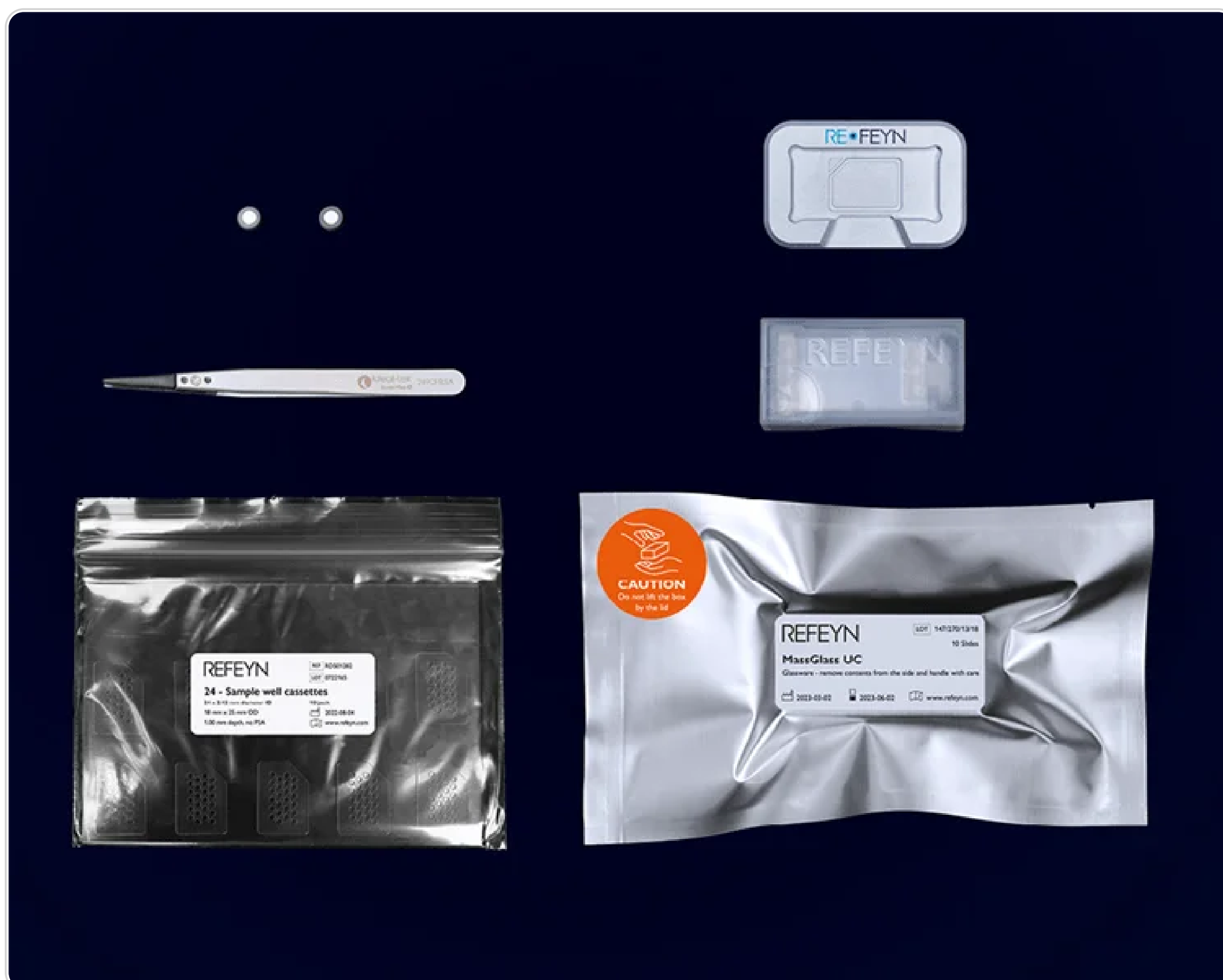

Image Credit: Refeyn Ltd.

Refeyn's range of accessories and consumables includes:

- Ready-to-use sample carrier slides
- Multi-sample well cassettes
- Magnetic slide holders
- An alignment tool and tweezers

References and further reading

1. Giammarinaro, Pietro I., *et al*. Nat Microbiol, 2022

Acknowledgments

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Exploring single molecule detection with mass photometry

This article outlines and explores the use of mass photometry for detection. It also summarizes the most common errors that can adversely affect mass photometry measurements, supporting users in the acquisition of the highest-quality results.

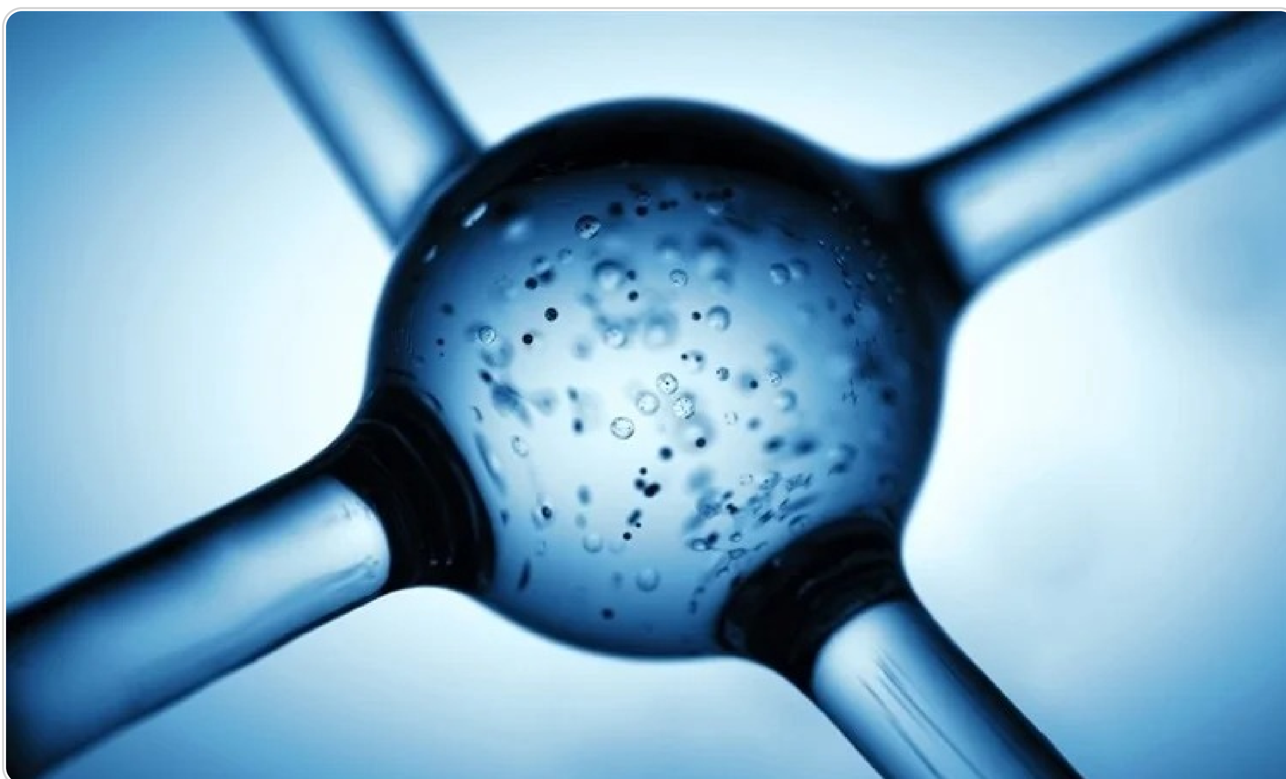

Image Credit: stock_wichel

Mass photometry is a widely used, label-free, single-molecule analysis technique that works by detecting the light scattered by a single particle at a glass-water interface.

A single protein exhibits a weak scattering signal, but this is quantified against an orders-of-magnitude stronger background signal from reflection at the interface.

Mass photometry data is returned as a series of images (Figure 1). For each image, the average of N frames is taken and divided by the average of the N subsequent frames to illustrate the amount of signal change observed when a molecule lands on the glass surface.

The signal generated by a single molecule is known to scale linearly with the molecule's mass, so standard samples with previously recorded molecular masses can be used to calibrate the mass to the signal. Due to this, mass photometry is able to accurately measure mass, even when working with unknown samples.

Noise sources in mass photometry

Mass photometry is limited by shot noise originating from the quantum nature of the light. This limitation results in random fluctuations in the number of photons arriving at the detector, a limitation that is further compounded by the reflection at the glass surface, generating an inhomogeneous image.

These two factors represent the primary source of signal variation (Figure 1), with background fluctuations originating from mechanical vibrations or drift in the lateral stage position also impacting data quality.

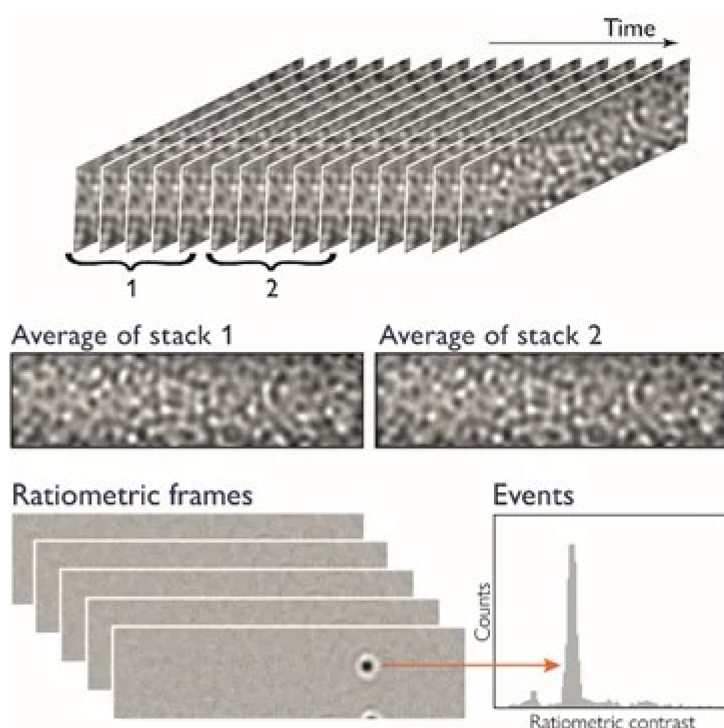

Figure 1. Generation of the mass photometry signal. *Images of the glass surface, taken over time, are divided into two stacks of N consecutive frames (typically N=5). These stacks are averaged to calculate a single ratiometric frame. The process is repeated for stacks of frames shifted by one frame at a time, generating a ratiometric movie. The signals from molecules landing on the glass surface throughout the movie contribute to the MP histogram. Image*

Credit: Refeyn Ltd.

Single-molecule detection

Single molecules will produce features in ratiometric images as they interact with the glass surface. As these molecules are much smaller than the wavelengths typically used for imaging, they will be represented as a point-spread function (PSF) (Figure 2) in most instances.

The PSF defines the imaging system's response to a point source, which will depend on the objective lens's numerical aperture (NA) and the imaging wavelength employed. The higher the NA and the shorter the wavelength, the smaller the PSF of the device.

If the same instrument is used to measure molecules of different masses, the PSFs will have identical shapes but varying intensities (Figure 2). For example, thyroglobulin possesses a mass ten times greater than bovine serum albumin (BSA), so its signal intensity will be ten times stronger.

Several hundreds of molecule-glass landing events can be detected during a 1-minute acquisition, affording users direct information on the molecular mass distribution of a sample containing both molecule types (Figure 2).

Events must be well separated in terms of time and space to avoid fitting errors due to overlapping PSFs that may obscure the desired results. Sample concentration must, therefore, be maintained at a level that allows single molecule detection (generally <100 nM). This is why mass photometry measurements typically require very low sample volumes.

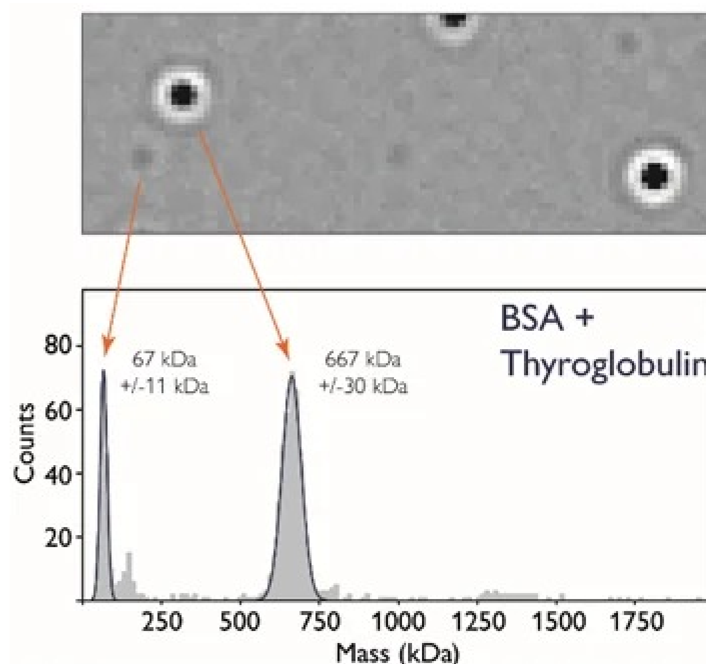

Figure 2. Single molecule detection with mass photometry. *Upper panel: a single ratiometric frame of a mixture of BSA and thyroglobulin, showing PSFs with different intensities. Lower panel: a mass histogram. An MP movie recorded for one minute typically consists of 6000 frames, during which several hundreds of molecules can be detected as they land on the glass surface. From the resulting series of ratiometric frames, a mass histogram can be created. Image Credit: Refeyn Ltd*

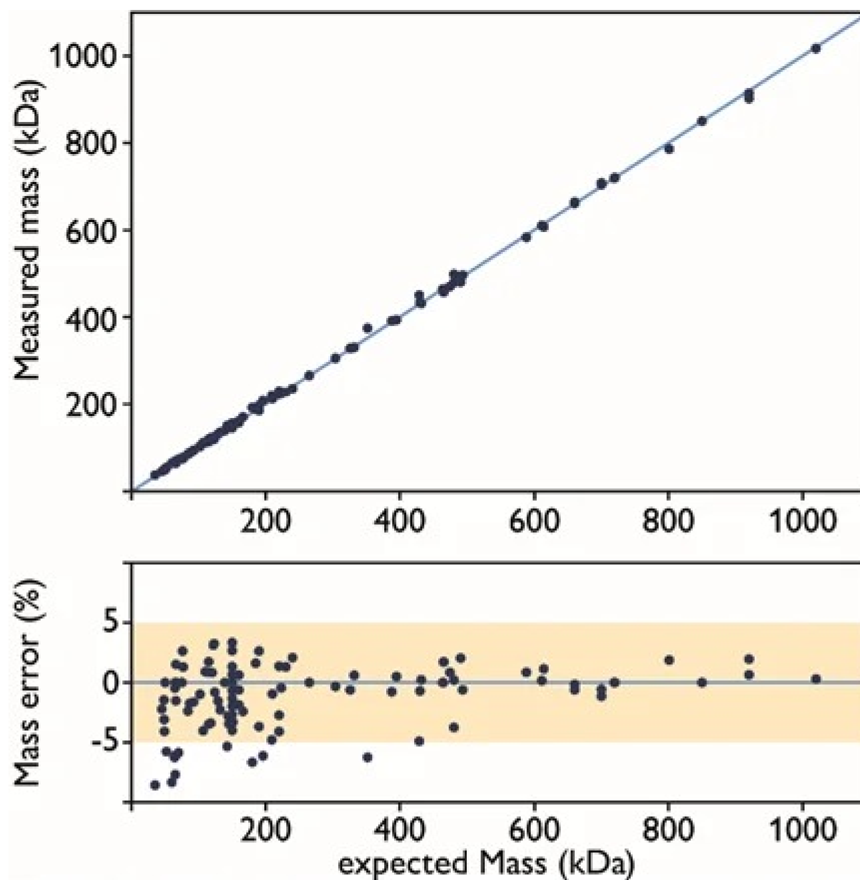

Figure 3. Accuracy of mass photometry measurements. *Upper panel: correlation of expected vs measured molecular mass (in kDa) of molecules. Lower panel: mass error shown as a percentage of the expected mass (N=150). Image Credit: Refeyn Ltd.*

Accuracy

Peaks in a mass photometry histogram can be fitted using Gaussian approximations. The peak center represents the measured mass of the species in these instances.

Mass measured by mass photometry can be found to deviate from the anticipated mass due to a number of factors; for example, errors in measurement, calibration and fitting.

These errors are generally within 5 % of the expected mass, however (Figure 3), meaning that it is possible to improve accuracy by repeating an experiment and averaging errors of individual experiments to gain an improved estimation of the calibration error.

Detection range

Noise levels in the ratiometric image will impact the user's ability to accurately detect and quantify PSFs. Averaging an increased number of frames in order to calculate each image in the ratiometric movie will reduce the shot-noise-induced fluctuations in each ratiometric image, therefore increasing the signal-to-background ratio and allowing for the detection of lower mass molecules.

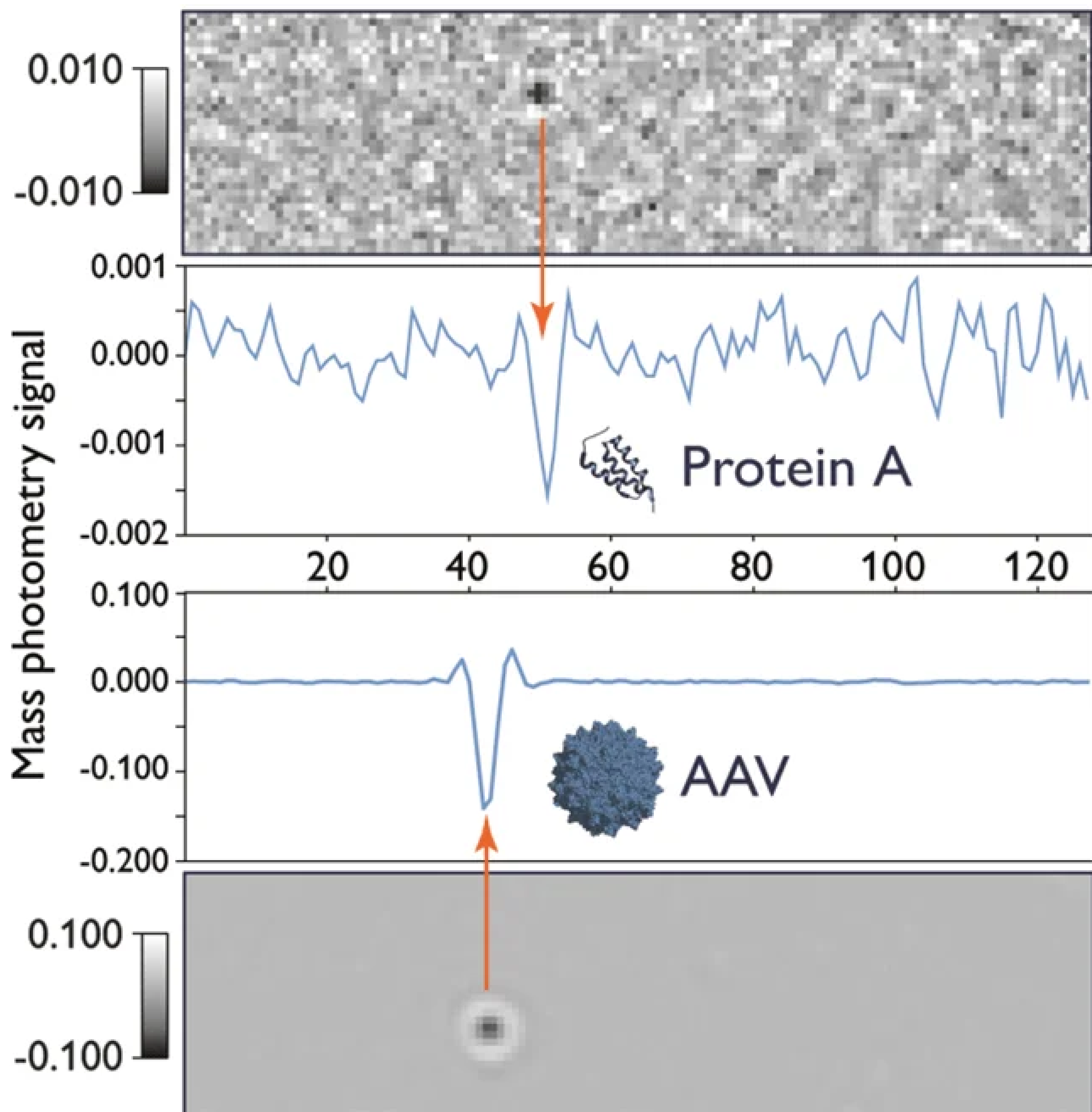

Figure 4. Mass photometry signal scales with particle size. *Signal traces of landing events for protein A (42 kDa, upper panel) and an adeno-associated virus (AAV) particle (5 MDa, lower panel) are depicted. Image Credit: Refeyn*

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There is a notable downside to averaging more frames, however, as this will lead to reduced temporal resolution. In this case, it may be necessary to decrease protein concentrations in order to prevent PSFs from overlapping.

Longer averaging times may also hinder data quality due to lateral drift, meaning that in order to achieve accurate measurement of molecules with a mass close to the detection limit, averaging more frames may be necessary (while still paying attention to drift).

As larger molecules yield a stronger signal, these do not need as much frame averaging. The mass range that can be accommodated by mass photometry is clearly demonstrated by measurements of protein A (42 kDa) and an AAV particle (-3.7 MDa). As these samples possess almost 100 times greater mass, they will generate a signal with almost 100 times greater intensity (Figure 4).

Resolution

Resolution can be understood as the minimum separable distance between peaks in a multi-peak distribution. In this context, resolution is directly related to the width of individual peaks.

Standard deviation is generally -10 % of the mass in these scenarios, as indicated by the center of the peak. Resolution is also highly dependent on sample purity and composition, as well as on the relative concentrations of the species present.

It is possible to resolve two peaks when the separation between their centers is larger than the sum of their full width at half maximum (FWHM), assuming these have equal peak heights.

Where proteins exhibit low mass, mass photometry enables the separation of peaks that differ by only -25 kDa (BSA and protein A) (Figure 5). Where proteins exhibit greater mass (thyroglobulin, 670 kDa), the resolvable distance would increase to -85 kDa due to thyroglobulin’s larger FWHM (which partially stems from the presence of a high level of glycosylation).

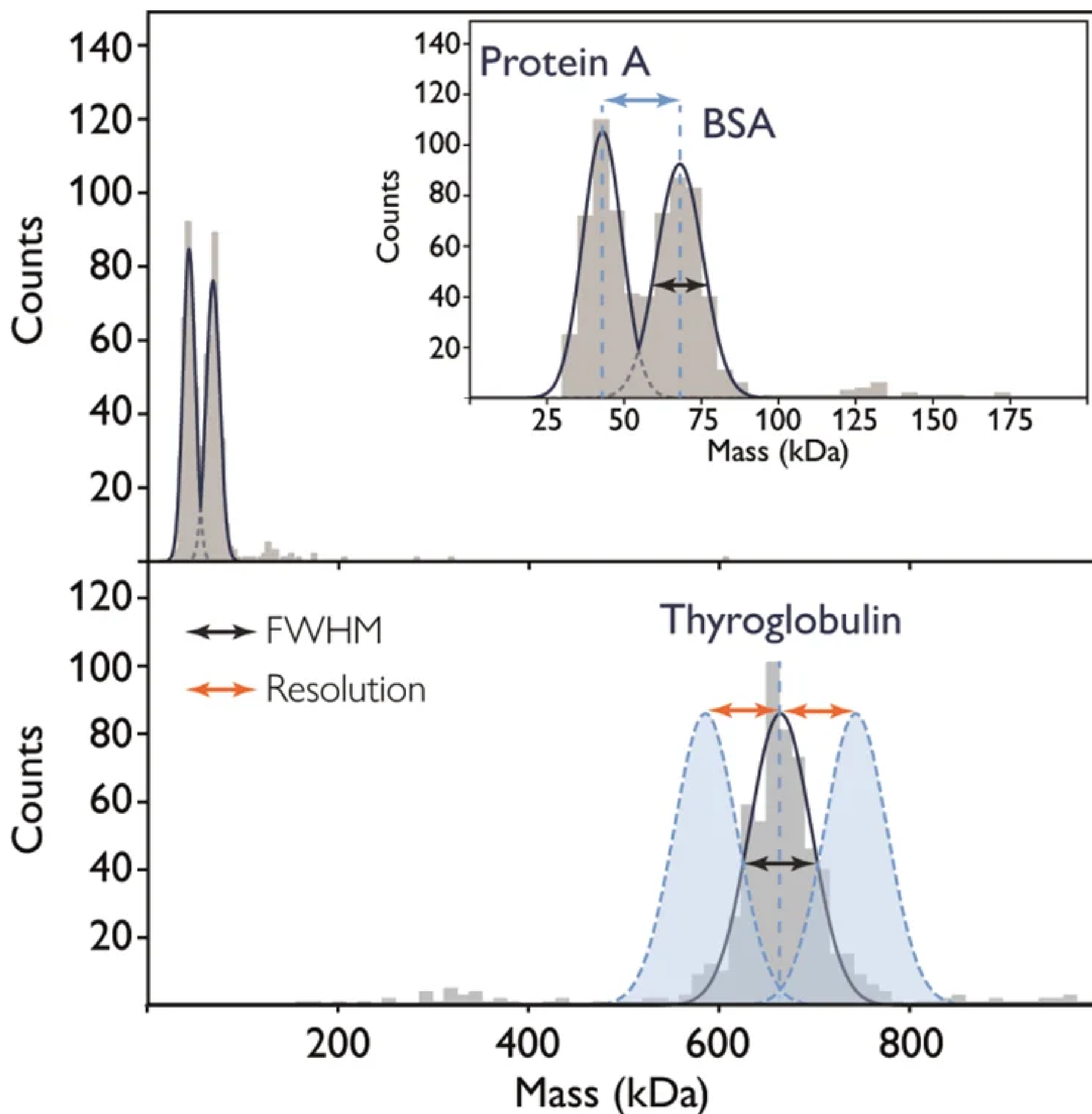

Figure 5. Resolution and full width at half maximum. *Top: the mass histogram for a 1:1 mixture of protein A (42 kDa) and BSA (66 kDa). Inset: the FWHM (black arrow) indicates the resolving power, allowing for clear peak separation (blue arrow). Bottom: thyroglobulin (670 kDa, glycosylated) with FWHM (black arrow) indicated. Theoretical Gaussians (blue) indicate possible resolution (orange arrows), assuming similar peak heights (i.e. numbers of events). Image Credit: Refeyn Ltd.*

Acknowledgments

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