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# Determination of dissociation constants *via* quantitative mass spectrometry

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The interplay of biomolecules governs all cellular processes. Qualitative analysis of such interactions between biomolecules as well as the quantitative assessment of their binding affinities are essential for the understanding of biochemical mechanisms. As scientific interest therefore moves beyond pure structural investigation, methods that allow for the investigation of such interactions become increasingly relevant. In this perspective we outline classical methods that are applicable for the determination of binding constants and highlight specifically mass spectrometry based methods. The use of mass spectrometry to gain quantitative information about binding affinities however is a still developing field. Here, we discuss different approaches, which emerged over the last years to determine dissociation constants ( $K_D$ ) with mass spectrometry based methods. Specifically, we highlight the recent development of quantitative Laser Induced Liquid Bead Ion Desorption (qLILBID) mass spectrometry for the example of double stranded deoxyribonucleic acids as well as for different RNA–RNA binding protein systems. We show that quantitative laser induced liquid bead ion desorption can successfully be used for the top down investigation of complexes and their dissociation constants values ranging from low nM to low  $\mu$ M affinities.

## KEYWORDS

dissociation constants, native mass spectrometry, method development, RNA protein complexes, biomolecular interactions

## Introduction

Association and dissociation of non-covalently interacting species are fundamental to numerous biological processes, ranging from the formation of deoxyribonucleic acid (DNA) double helices to the self-assembly and functional interplay of molecular machines. To understand the pathways of such reactions it is important to determine the interacting partners as well as their stoichiometry and to further characterize their interactions, e.g. in terms of binding affinities or kinetics. For such interactions, a broad range of methods has been developed which offer different advantages and disadvantages.

Especially the field of affinity assessment has drawn particular interest due to its importance in not only the investigation of biological mechanisms but also its significance for determining the effectiveness of drugs and other bioactive compounds (Kairys et al., 2019). The “gold standard” and most commonly used method to study affinities is isothermal titration calorimetry (ITC). It operates based on the thermal energy that is released or absorbed upon binding. By stepwise titration of one binding partner to the other, the energy needed to keep the temperature constant is monitored and the thermodynamic equilibrium can be determined. Another common group of affinity measurements are fluorescence based

approaches. Dissociation constants can be determined by titration experiments and readout of changes in fluorescence e.g. in Förster resonance energy transfer experiments (Guo et al., 2016; Liao et al., 2021) or by quenching of the fluorophore. (Budhadev et al., 2020). Other well-established methods are surface plasmon resonance (SPR) and electrophoretic mobility shift assay (EMSA). During a SPR experiment, a binding partner is added onto a surface on which the target molecule is immobilized. Binding of the mobile ligand to the immobilized target molecule changes the refractive index of the surface, which can be tracked and analyzed for their affinity and kinetics (Karlsson et al., 1991; Stenberg et al., 1991). EMSAs provide a robust method for quantitative analysis of mostly protein-oligonucleotide interactions (Hellman and Fried, 2007), albeit other combinations of biomolecules can be tested as well. A protein concentration series is combined with a usually  $^{32}\text{P}$ -labeled oligonucleotide, e.g. an RNA, and subjected to electrophoresis. In non-denaturing agarose or polyacrylamide gels, complexes migrate slower than free RNA. The separated RNA and complex bands can then be quantified by autoradiography. The dissociation constant can be assessed from the protein concentration in which the amount of bound and unbound oligonucleotide is equal, i.e. half-stoichiometric saturation (Hellman and Fried, 2007).

The determination of binding affinities with these methods usually requires knowledge of all binding partners as well as their stoichiometry. Therefore, generally an additional step is needed prior to these measurements. Information about the composition of complexes can be gained by several methods, based on the size and mass of these complexes and their subunits (Walport et al., 2021). Examples of commonly used methods are size exclusion chromatography coupled with multiangle light scattering (SEC-MALS) or native mass spectrometry (native-MS). SEC-MALS chromatographically separates molecules and complexes by their hydrodynamic volume and determines the radius by illumination and analysis of the scattering (Wyatt, 1993; Wen et al., 1996). Prior calibration enables a good estimate of the molecular mass and therefore the stoichiometry and composition of the studied complex. (Folta-Stogniew and Williams, 1999). However the methods come with challenges in case of missing prior knowledge of complex stoichiometries, most of all between proteins and RNAs.

Mass spectrometry (MS) is standardly used to analyze molecular masses, if greater accuracy in mass is required. Specifically, native MS allows to analyze the mass of entire complexes as well. This requires a soft ionization or desorption process, during which ions from the sample of interest are transferred into the gas phase and can then be analyzed by their behavior in an electric field. During the ionization or desorption of the sample molecules non-covalent bonds can be preserved which allows the analysis of not only the identity of constituting molecules but also their stoichiometry and can thus provide insight into structural characteristics (Leney and Heck, 2017).

The conventional approach to determine binding affinities, depending on identification of binding partners and the complex stoichiometry prior to the quantitative investigation of the interactions is often time and sample consuming as well as reliant on access to these different analytical methods. To simplify the procedure and ideally minimize the amount of sample and time needed in the process, methods that provide qualitative as well as quantitative information with one experiment are of interest. In our opinion (native) mass spectrometry methods are specifically well suitable to be pushed further to answer questions regarding the interplay of increasingly

complicated and often heterogeneous systems, as it allows precise compositional studies and a mass spectrum's signal intensities relate to the abundance of the correlating species. In the following we will give a brief overview over MS based methods for affinity determination. For a more in-depth discussion of the capabilities of native MS and the challenges posed by different applications the reader is referred to a review by Hopper and Robinson (Hopper and Robinson, 2014).

There are by now several approaches to determine the thermodynamic equilibrium of non-covalent interactions by native mass spectrometry. Most of these are based on Electrospray Ionization (ESI). ESI requires application of a voltage to a capillary containing the sample of interest, which generates an aerosol of charged droplets, which shrink due to evaporation. Repulsion of the accumulating charges in these droplets lead to a break down into smaller, lower charged droplets. During that process the analyte is ionized and transferred into the gas phase. For the analysis of complexes, the non-covalent interactions between binding partners may be preserved. Standardly investigation of biomolecular complexes aims at qualitative assessment of the complex composition. Assessment of binding affinities additionally requires quantitative determination of the present complexes and subunits to allow a statement about their ratio. Challenges for the quantitative assessment by native MS, which should be taken into account, can include differing response factors of different substances (Kitova et al., 2012; Root et al., 2017). The response factor is defined by the correlation factor between signal intensity in the mass spectrum and concentration of a substance, which depends on the substances' ionization efficiency, its ion transportation efficiency, and the detector sensitivity for the respective mass/charge ratio ( $m/z$ ). Additionally dissociation and association during the ionization/desorption process as well as such processes in the gas phase must be considered (Kitova et al., 2012). Generally challenging for MS based methods are low affinity interactions, such as the mM  $K_{\text{D}}$ s which are often found with glycan binding (Dülfer et al., 2021). These however place challenges for other analytical methods as well (Peters et al., 2021).

The most common methods for affinity assessment with native MS are based on titrations. After multiple publications showed that it is possible to qualitatively study non-covalent interactions by ionspray mass spectrometry, Lim et al., (1995) presented a quantitative analysis of the solution equilibrium between a peptide ligand and vancomycin/ristocetin. The fundamental procedure used is still the basic principle of native MS titrations. During these experiments, the two binding partners are incubated with a varying concentration of one of them (Jecklin et al., 2009; Báez Bolívar et al., 2021) (Figures 1A–D). This can be done either by preparation of a titration series or with a continuous ESI source, which allows the gradual combination of the two components (Fryčák and Schug, 2007; Bui et al., 2022). The latter is for example used in the “gap sampler” by the Zenobi group for bioaffinity screening with high-throughput and low sample consumption (Neu et al., 2013; Kaeslin et al., 2021). Additionally, the comparison of peak intensities of the complexes vs. the unbound molecule as a function of the varied concentration allows a determination of the thermodynamic equilibrium as well as the ratio of the response factors of the bound and the unbound species (Gabelica et al., 2003). This has further been improved by the recent establishment of the Slow Mixing Mode (SLOMO) in the Klassen group. It enables the gradual combination of the components while reliably determining their relative response factors and affinity (Bui et al., 2022) (Figure 1E). The hyphenation of capillary electrophoresis

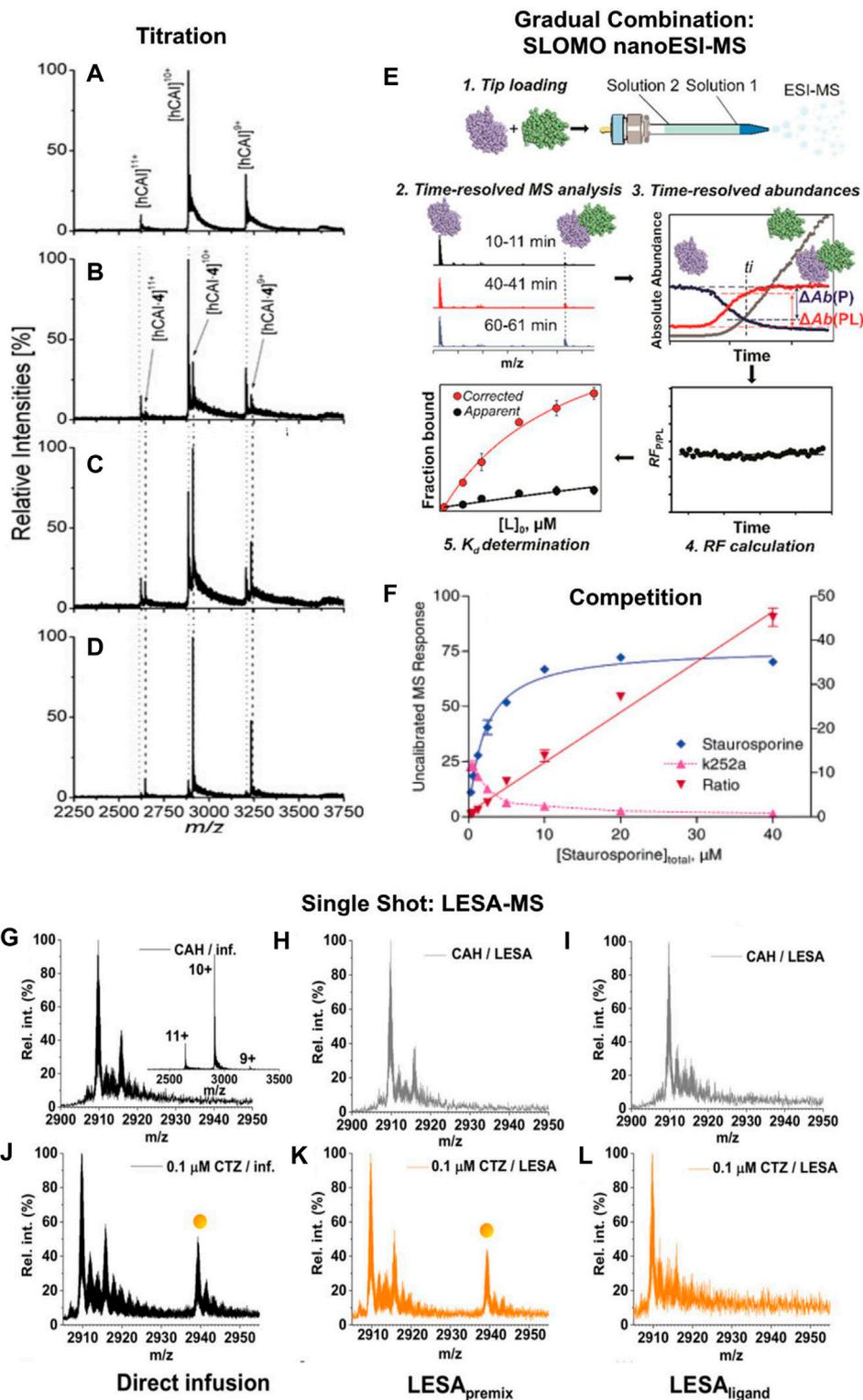


FIGURE 1

(A–D) Mass spectra of 5  $\mu M$  human carbonic anhydrase I with different concentrations of ethoxzolamide: (A) 0  $\mu M$ , (B) 1  $\mu M$ , (C) 2  $\mu M$ , and (D) 5  $\mu M$  for affinity assessment. Unbound protein is marked with a dotted, the complex with a dashed line. A significant shift from free protein to nearly solely complex is observable with increasing ethoxzolamide concentration. Reproduced with permission from (Jecklin et al., 2009). Copyright 2009 John Wiley & Sons. (E) Example of an in-source gradual combination of interacting species. The workflow of a SLOMO experiment for measuring the  $K_D$ s of biomolecular interactions is displayed. Step 1. Tip loading (with solution 1 and solution 2). Step 2. Time-resolved ESI-MS analysis. Step 3. Extraction of time-resolved abundances. Step 4. Calculation of the time-dependent relative RF. Step 5. Calculation of  $K_D$  from RF and  $R_{app}$ . Reproduced under CC-BY-4.0, (Bui et al., 2022). (F) Example graph of competition measurements with affinity ranking and determination of the mode of binding competition for a Zap-70–ligand complex with 20  $\mu M$  K252a against staurosporine. The linear titrant to ligand response ratio plot indicates direct competition between staurosporine and K252a, the decrease of MS response belonging to the K252a complex below 50% of its starting concentration at 20  $\mu M$  staurosporine shows the higher (Continued)

**FIGURE 1 (Continued)**

binding affinity of the latter. Adapted with permission from (Annis et al., 2004). Copyright 2004 American Chemical Society. (G–L) Comparison of spectra resulting from direct ESI-MS and LESA-MS measurements. For LESApremix measurements the substances are mixed prior to being dried and LESAligand measurements are performed by adding the ligand to the dried protein. Spectra shown regard to bovine carbonic anhydrase (CAH) alone (G–I) and in the presence of 0.1  $\mu\text{M}$  chlorothiazide (J–L) following direct infusion, LESApremix, and LESAligand sampling respectively. (Inset) charge state distribution of CAH in its  $\text{Zn}^{2+}$ -bound form with the  $10^+$  ion being the most abundant. Reproduced under CC-BY-4.0, (Illes-Toth et al., 2022).

with mass spectrometry to add the investigated ligand in increasing concentrations has allowed the investigation of stoichiometry and binding characteristics of oligonucleotides to cyclodextrin (Przybylski et al., 2018).

Another common group of methods for the analysis of binding affinities that has first been used by Jørgensen et al. (Jørgensen et al., 1998) and was later described as a general method by Kempen et al. (Kempen and Brodbelt, 2000) bases on the competition between different ligands that bind to the same binding site of a molecule. Here, the molecule is preincubated with a ligand with a known  $K_D$  as reference system. By inserting another binding molecule into the solution, the equilibrium of the reference ligand with the target molecule is shifted (Annis et al., 2004) (Figure 1F). Given the assumption, that the ionization efficiency of the reference system is constant, this shift is solely dependent on the dissociation constant and concentration of the added ligand. Therefore, a measurement of the changing peak intensities of the reference system upon addition of different concentrations of the ligand of interest allows a direct affinity analysis. Additionally, this allows to investigate complexes otherwise not accessible by the used MS method, either due to their ionization efficiency, their mass or due to dissociation during ionization (Wortmann et al., 2008; El-Hawiet et al., 2010). Kopicki et al. were recently able to even make use of a contaminant binding to the Major Histocompatibility Complex (MHC) that is displaced by binding peptides for affinity screening (Kopicki et al., 2022). The use of a well-defined reference system can furthermore be utilized for affinity screening, as shown by Kitov et al. with their competitive universal proxy receptor assay (CUPRA) (Kitov et al., 2019).

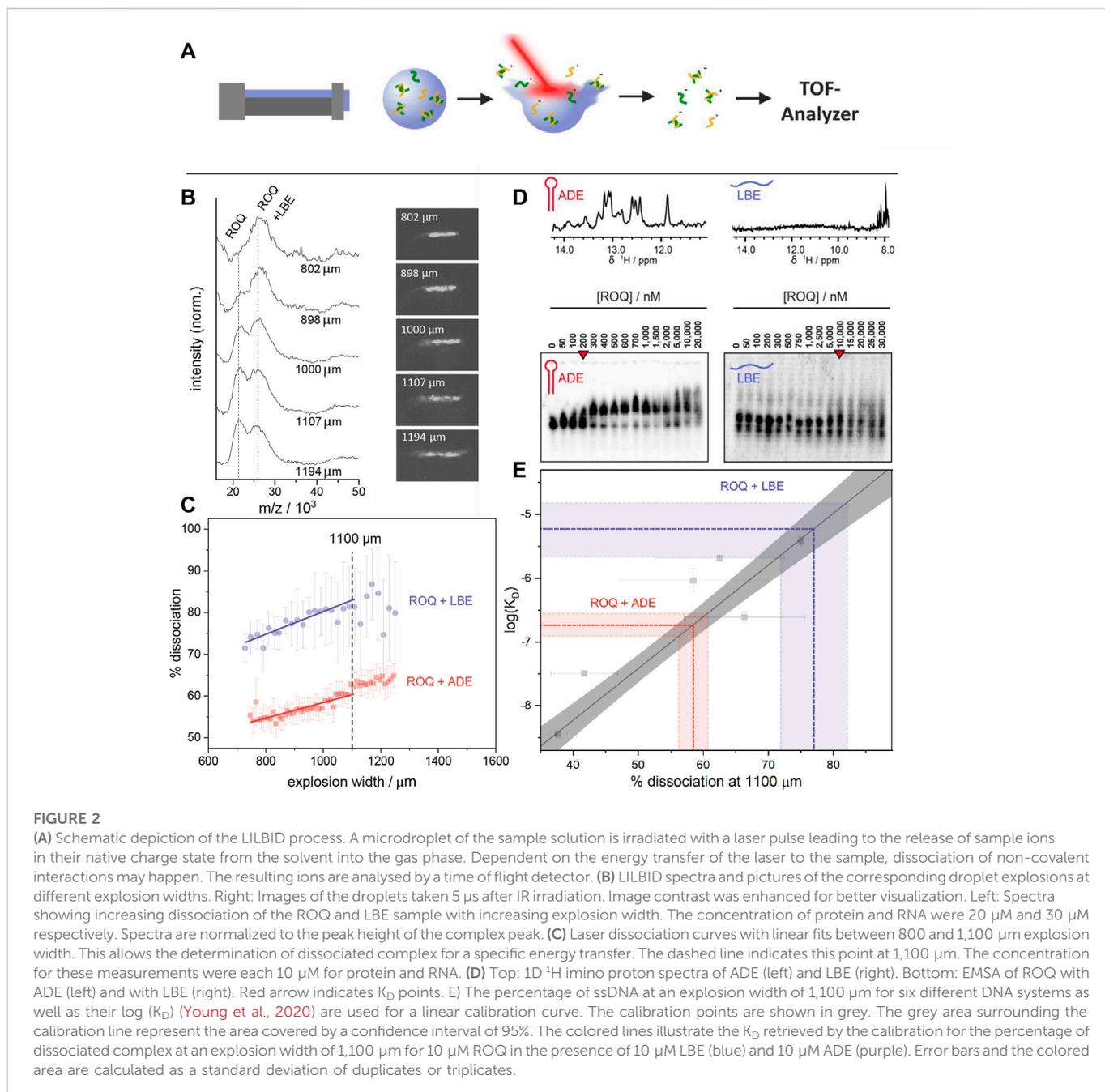
MS based methods less reliant on particularly soft ionization are surface exposure approaches. While not necessarily coupled to native mass spectrometry ion sources, they allow the study of non-covalent binding affinities in native environments. An early example is a method called stability of unpurified proteins from rates of H/D exchange (SUPREX) (Ghaemmaghani et al., 2000; Powell and Fitzgerald, 2003). The method relies on the replacement of surface exposed protons in proteins with deuterium after adding deuterated buffer to the sample. If addition of a ligand promotes the unfolding of the protein, the amount of surface exposed protons will be affected, resulting in a mass shift. This shift can be determined *via* MALDI-MS in a ligand concentration dependent manner, allowing to determine the dissociation constant. A variation of that method is Protein–ligand interactions by mass spectrometry, titration, and H/D exchange (PLIMSTEX) (Zhu et al., 2005). In a similar manner it monitors H/D exchange, but making use of all solvent exposure changes upon ligand binding, either due to conformational changes or due to blocking of the binding site itself. This adds the possibility to monitor binding of large ligands and interactions not interfering with the native structure of the protein. Additionally, conformational information is provided by the amount of deuteration upon binding. A similar approach named LITPOMS (ligand titration, fast photochemical oxidation of proteins and mass

spectrometry) uses fast photochemical oxidation of proteins by UV-irradiation of a sample containing hydrogen peroxide (Liu et al., 2019). The irradiation leads to a sub-milliseconds oxidation of solvent exposed molecular surfaces, which can be monitored and evaluated by MS.

The fastest and simplest way to use mass spectrometry for quantitative binding analysis are single point measurements. These methods directly link the relative peak intensities of intact and dissociated complexes with their affinity and have first reportedly been used by Loo and co-workers (Loo et al., 1993). A limiting factor for application of such approaches is the usually restricted knowledge of the response factor of a non-covalent complex. Therefore, mainly systems of a large molecule and a smaller ligand are accessible based on the experience, that the response factor of the complex and the larger molecule alone are identical. Another limitation of single point measurements is the unknown amount of dissociation during the ionization. This requires especially soft ionization techniques (Kitova et al., 2012). Therefore it is also a valuable approach to determine the softness of an ionization technique, as recently shown with Liquid extraction surface analysis (LESA). LESA is an ion source that is easy to automate and by deducing apparent  $K_D$ s when using different preparations Illes-Toth et al. were able to determine the most native approach (Illes-Toth et al., 2022) (Figure 1G–L). Garcia-Alai et al. were able to determine macroscopic dissociation constants of proteins containing an epsin N-terminal homology domain with phosphatidylinositol 4,5-bisphosphate by single point measurements. Cytochrome C was added to the samples to detect and correct for unspecific clustering during ionization (Garcia-Alai et al., 2018). An alternative approach uses intentional complex dissociation by collision-induced dissociation to characterize complex stability (Yefremova et al., 2017).

An ion source which has been used for single point measurements in the past is laser-induced liquid bead ion desorption mass spectrometry (LILBID-MS). The basic method, utilizing a liquid sample beam was established by Kleinekofort et al. (Kleinekofort et al., 1996) in 1996 and has been further developed by Morgner, making it suitable for the analysis of a broad range of biological systems, using sample droplets (beads) (Henrich et al., 2017; Lieblein et al., 2020; Vu Huu et al., 2022). The method has shown to be tolerant regarding the sample environment e.g. in terms of salts, detergents or pH and is hence able to investigate samples in a particularly native state. This is underlined by the lowly charged ions that are generated by LILBID-MS and the range of non-covalent interactions detectable (Hellwig et al., 2022).

In the LILBID ion source, a piezo driven droplet generator produces aqueous droplets with a diameter of 50  $\mu\text{m}$ –70  $\mu\text{m}$  at a rate of 10 Hz containing the sample of interest. These droplets are transferred into a vacuum chamber and irradiated by a 6 ns IR-pulse. Its wavelength of 2.8  $\mu\text{m}$  corresponds to the asymmetric OH-vibration of water and leads subsequently to an excitation of the irradiated volume into a supercritical state. During the following explosive



expansion of the droplet, contained ions are desorbed from the solvent and transferred into the gas phase for mass analysis (Figure 2A).

At elevated desorption laser energies an energy transfer from the water to the analyte molecules may take place and lead to a partial dissociation of non-covalent bonds. This effect is mainly correlated to the strength of the interaction and the absorbed energy during the irradiation. Hence, stronger bonds need higher laser energy for dissociation and weaker bonds are already broken at low laser intensities. Controlling the laser energy transferred into the sample is however not trivial due to e.g. fluctuations in the laser pulse power or variations in positions of each droplet relative to the laser focus. Young was able to show, that the transferred energy correlates to the size of the plume created during the explosive expansion of the droplet. (Young et al., 2020). Illuminating and recording a picture of the plume

5  $\mu\text{s}$  after the IR-irradiation allows monitoring the diameter of the visible droplet dispersion (explosion width) as a proxy for the transferred laser energy (Figure 2B). Young was accordingly able to associate larger explosion widths with more dissociation of the bonds of a non-covalent complex (Young et al., 2020).

These findings enable the examination of different non-covalent interactions for the same laser energy transfer. To minimize the error and maximize the number of spectra usable for the analysis, dissociation is monitored in dependence of increased laser energies (laser ramping). This is done by plotting the percentage of dissociated complex against the explosion width of each droplet irradiation. For explosion widths between 600  $\mu\text{m}$  and 1,100  $\mu\text{m}$  a linear relation between dissociation and explosion width can be seen. With a linear fit, these relations can be interpolated for a specific explosion

width (e.g. 1100  $\mu\text{m}$ ) and hence a specific laser energy transfer. By keeping the influence of the laser constant for each system, Young showed that it is possible to determine the thermodynamic equilibrium for the dissociation of double stranded DNAs by single point measurements after prior calibration. The reference affinity values for the calibration as well as for the evaluation have been done by ITC and UV melting curves and show a good correlation to the results gained by Young with quantitative LILBID (qLILBID).

To enable a generally applicable top down method for composition and affinity studies this approach has to be suitable not only for DNA but for other substance classes and combinations as well. For an overview of mass spectrometric analysis of nucleic acid complexes, the reader is referred to this excellent review and references within (Largy et al., 2022).

Here we show as a proof of concept that the above mentioned laser ramping approach can be widened to complexes of RNA and RNA-binding proteins (RBPs). As a model system, we use the immune-regulatory protein Roquin and RNA fragments of previously described targets, e.g. found in the *Ox40* 3'-UTR. Roquin recognizes stem-loop structures (termed CDE and ADE, constitutive and alternative decay elements, respectively) and recruits degradation machineries to initiate mRNA decay, both *via* deadenylation and decapping (Glasmacher et al., 2010; Leppek et al., 2013; Janowski et al., 2016). As many Roquin targets encode for key players in the immune system, Roquin function is crucial for immune responses in form of immune system homeostasis and T cell differentiation (Heissmeyer and Vogel, 2013). As we and others have recently shown, Roquin binds the *Ox40* ADE with nanomolar affinity through its unique ROQ domain (Tants et al., 2022). Roquin also recognizes specific unstructured RNAs, termed linear binding elements (LBE), to which it binds with low micromolar affinities *via* the very same ROQ domain (Essig et al., 2018). We wondered whether this versatile RNA-binding capability can -in its full range-be quantitatively represented in LILBID and thus proves the method's applicability to challenging RNA-protein complex systems.

As described above, the percentage of dissociated ROQ-RNA is plotted against the explosion width and interpolated to an explosion width of 1,100  $\mu\text{m}$  (Figure 2C). A prior affinity calibration of the qLILBID measurements with oligonucleotides allowed for a good  $K_D$  evaluation of the investigated systems. Figure 2E shows the obtained  $K_D$  values correlate well with reference affinity values, obtained by EMSA measurements as previously described. The resulting percentage of dissociated protein at an explosion width of 1,100  $\mu\text{m}$  were  $(58 \pm 3) \%$  for ROQ and ADE and  $(78 \pm 6) \%$  for ROQ and LBE. With the oligonucleotide calibration these correspond to dissociation constants of  $K_D = (180 \pm 40) \text{ nM}$  in the case of ADE and  $K_D = (6 \pm 3) \mu\text{M}$  for LBE. These are in good agreement with the results obtained by EMSA and literature results of approximated  $K_{D,EMSA} = 200 \text{ nM}$ , and  $K_{D,Lit} = (170 \pm 20) \text{ nM}$  for ADE (Tants et al., 2022) and  $K_{D,EMSA} = 5 \mu\text{M}$  and  $K_{D,Lit} = (3.6 \pm 0.4) \mu\text{M}$  for LBE with a shorter ROQ version (Essig et al., 2018). These findings confirm the utilizability of LILBID-MS in these quantitative affinity investigations, covering a broad range of affinities (low nM to low  $\mu\text{M}$ ) and both structured and unstructured RNAs.

The use of 20 mM NaCl in the sample buffer exemplifies the tolerance of LILBID regarding buffer conditions which can be challenging for other ion sources. With only about 50 pmol of substance required per measurement and only little method dependent preparation needed, it has furthermore proven to be highly time and sample efficient. A principle advantage of this

method is that measurements do not rely on prior separation of the binding partners, as it is generally the case for established  $K_D$  assessment methods, i.e. to mix samples at different concentrations to do titration experiments. This provides the opportunity of investigating volatile complexes.

In particular for RNA-protein complexes, our approach provides advantages over classical methods to determine binding affinities. First, LILBID does not require fluorescence- or radioactive isotope-labeling of RNAs, which is a clear gain in time and financial burden. Especially fluorescence tags may further interfere with the natural interaction and yield a different apparent affinity. The minimum amount of RNA needed is a clear benefit seeing the comparably high costs for commercial RNAs (relative to DNA) as well as for nucleotides used for in-house *in vitro* transcription.

As shown for the example of Roquin interacting with two completely different types of RNAs, LILBID allows in general to address the full spectrum of RNA-protein complexes. Clearly, the Roquin ROQ domain uses an induced-fit/conformational selection processes to shape-specifically engage with folded RNAs (Schlundt et al., 2014), i.e. driven by entropy and with very low complex off-rates. Differently, it uses charge-charge interactions to engage with linear RNAs, supposedly driven by on-rates. Thus, we suggest that our approach is able to cover quantitative analyses on RNPs based on very different types of complex formation, also seeing the constant increase in identification of so-far unknown RNA-binding proteins and their target sequences (Hentze et al., 2018)). LILBID comes with an additional mentionable advantage in future applications. In fact, for many RNA-protein complexes the type of interaction and its affinity is difficult to anticipate and predict (Schlundt et al., 2017) and the process of examining an initial order of magnitude for their binding constant is usually not desired to consume excessive amounts of sample. LILBID might become a valuable tool for the initial screening of affinity ranges for those.

In future the method will be widened to include other biomolecular complexes. This might require specific calibration for the interaction type of interest (soluble or membrane proteins might require a different calibration curve than oligonucleotides) or a correction for the relation between explosion width and dissociated complex. As well the parallel determination of  $K_D$  values for complexes with more than one binding partner will be established.

## Discussion

With growing interest not only in biomolecular structures but in biological reaction pathways and mechanistic understanding of cellular processes, the further development of methods that allow a precise analysis of the partaking interactions is still of high relevance. As presented, there is a broad spectrum of mass spectrometry based methods that allow for qualitative as well as quantitative insights into non-covalent interactions. These combine the ability to identify binding partners and compositions and to determine their affinity for effective top-down investigation of biomolecular interactions. We expect further developments in instrumentation and adaptation of current methods in the following years to broaden the applicability towards larger, heterogeneous systems with more than two interacting species or with higher order stoichiometries.

## Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: <https://zenodo.org/record/4088567#.Y-5RFXbP1dg>

## Author contributions

NM and JS: conception and design; draft manuscript preparation, JS, J-NT and JE data collection, analysis and interpretation of results, J-NT and AS review of the manuscript; all authors approved the final version of the manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/frans.2023.1119489/full#supplementary-material>

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