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# A straightforward method for measuring binding affinities of ligands to proteins of unknown concentration in biological tissues†

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The equilibrium dissociation constant ( $K_d$ ) is a quantitative measure of the strength with which a drug binds to its receptor. Methods for determining  $K_d$  typically require *a priori* knowledge of protein concentration or mass. We report a simple dilution method for estimation of  $K_d$  using native mass spectrometry which can be applied to protein–ligand complexes involving proteins of unknown concentration, from complex mixtures, including direct tissue sampling.

## Introduction

Protein–ligand interactions play a pivotal role in many cellular functions, including enzymatic reactions,<sup>1,2</sup> immune protection,<sup>3,4</sup> and signal transduction.<sup>5,6</sup> Understanding mechanisms of protein–ligand recognition and binding has important implications for the study of protein signalling and function and facilitates the development of novel therapeutics for challenging diseases.<sup>7,8</sup> The efficacy of any drug depends largely on its binding affinity to its target, *e.g.*, receptor, and as such, it is crucial to robustly measure the equilibrium dissociation constant. To date, a variety of techniques including the commonly used isothermal titration calorimetry (ITC),<sup>9,10</sup> surface plasmon resonance (SPR),<sup>11,12</sup> and fluorescence spectroscopy,<sup>13,14</sup> as well as emerging methods such as biolayer interferometry<sup>15,16</sup> have provided thermodynamic and kinetic information of binding events. However, due to specific requirements in terms of sample volume and pre-treatment tasks required (*e.g.*, immobilization, labelling, and purification) these robust methods are often limited to modified simple sample systems. Protein purification is a notoriously laborious task, and efforts have been made to circumvent it for  $K_d$  determinations.<sup>17</sup> However, none of these approaches can be used to provide information regarding target engagement under real physiological conditions.<sup>18</sup> There remains a need for complementary methods that study protein–ligand interactions from a broad range of untreated complex biological samples in a high throughput, label-free and sensitive manner.

Mass spectrometry (MS) has become a powerful and versatile tool for analysing interactions between macromolecules and small ligands, owing to its striking advantages of simplicity, minimum amount of sample consumption, high sensitivity and accuracy.<sup>19–22</sup> Within drug discovery, MS is a well-established method for early-stage high-throughput screening,<sup>23–25</sup> and is also commonly used for lead optimization studies. Mass spectrometry can be used for in-depth characterization of compound binding including determination of the binding site and binding induced structural changes, under native conditions.<sup>26–28</sup> Native MS uses gentle ionization methods to transfer folded proteins and intact protein complexes with non-covalent interactions from solution to the gas phase. It has been widely used in the measurement of protein–ligand binding affinity by either a single-point approach or *via* titration methods.<sup>29–32</sup> Recently, a novel method based on slow-mixing dilution (a variation of the titration approach) has been developed to determine protein–glycan binding affinities independent of ligand concentration through the fitting of ligand titration data.<sup>33</sup> Dilution analysis is a well-established calibration method for accurate quantification.<sup>34–36</sup> Isotope dilution MS has been extensively used to determine the concentrations of various analyte classes, including proteins.<sup>37–39</sup> Nevertheless, compared to the conventional titration fitting model, incorporating a second unknown parameter related to ligand concentration in the new method may lead to greater deviations and increased uncertainty in the determined affinity value. Native MS has also been applied to estimate protein–drug ligand binding affinity from cell lysates without prior knowledge of protein concentration, albeit with poor reproducibility (approximately 100% standard deviation).<sup>40</sup> Accurate affinity measurements can be challenging for certain binding systems due to inherent limitations of the technique. These include in-source dissociation of labile protein complexes, particularly those stabilized by hydrophobic interactions,<sup>41,42</sup> interference from nonspecific binding,<sup>43,44</sup> and non-uniform response

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factors between free and ligand-bound proteins.<sup>45,46</sup> While progress has been made to address these challenges—such as using chemical additives,<sup>47,48</sup> maintaining relatively low sampling temperatures, minimizing collisional activation to stabilize hydrophobically bound systems,<sup>49,50</sup> employing reference proteins to correct for nonspecific binding,<sup>51,52</sup> and adopting titration methods with parameters that account for dissociation or response factor discrepancies<sup>33,52</sup>—some bottlenecks remain unresolved. Despite the potential drawbacks, native MS measurements are independent of fast binding kinetics and limited ligand solubility, which hinder SPR and ITC, respectively. Surface sampling and imaging methods using liquid extraction surface analysis (LESA), desorption electrospray ionization (DESI), or nano-DESI and native MS<sup>53–57</sup> are gaining widespread attention for *in situ* detection and imaging of proteins and protein complexes in tissue. Proof-of-concept studies illustrating determination of binding affinity from pre-mixed, surface deposited protein–ligand mixtures have been reported.<sup>57,58</sup> The adjustable sampling time of ~ tens of minutes within LESA-MS experiments allows protein and ligand binding to reach equilibrium, which is the prerequisite for reliable measurement of binding affinity. However, even with controlled protein and ligand concentrations, accurate determination of  $K_d$  during surface analysis remains challenging.<sup>58</sup> To date, accurate determination of  $K_d$  from complex biological systems, with no sample treatment, or protein purification, in which protein concentrations are unknown, has not been reported.

Here we introduce a direct method, based upon a single dilution and several fast infusion ESI measurements, which extends the capability of native MS to the direct determination of the binding affinity of ligands to proteins from a tissue surface. In this work, we demonstrate that without the need for prior knowledge of protein concentration or time-consuming titration measurements, the dilution method for determining binding can be applied to biological tissue samples, and can also be used alongside native surface sampling routines. Furthermore, we propose a model of determining  $K_d$  without protein concentration can also be applied to titration MS and other biophysical methods, such as fluorescence intensity titration, microscale thermophoresis.

## Results and discussion

The pre-programmed, customized workflow (Fig. 1a) consists of surface sampling, protein–ligand mixing, protein dilution, and infusion ESI-MS measurement (details provided in Methods, ESI†). Briefly, with the commercially available surface analysis setup TriVersa NanoMate (Advion Interchim Scientific, Ithaca, USA), a conductive pipette tip containing ligand-doped solvent is positioned by a robotic arm approximately 0.5 mm above the sample surface, where 2  $\mu$ L of solvent is dispensed to form a liquid microjunction between the pipette tip and the surface. After a brief delay, the ligand-doped microjunction liquid, which has extracted the target protein from the sample surface, is re-aspirated into the pipette tip, transferred to a 384-well plate, and serially diluted. Following a 30 minute incubation, the

solutions are infused through conductive pipette tips and analysed using nozzle array chip-based ESI MS. When the protein-bound fraction remains constant upon dilution, our calculation method—which does not require knowledge of protein concentration—enables the accurate determination of binding affinities for surface-deposited standard proteins and the rapid quantification of drug ligand binding to protein receptors in biological tissues. We demonstrate the capability of this new method for ligand screening using the same tissue sample.

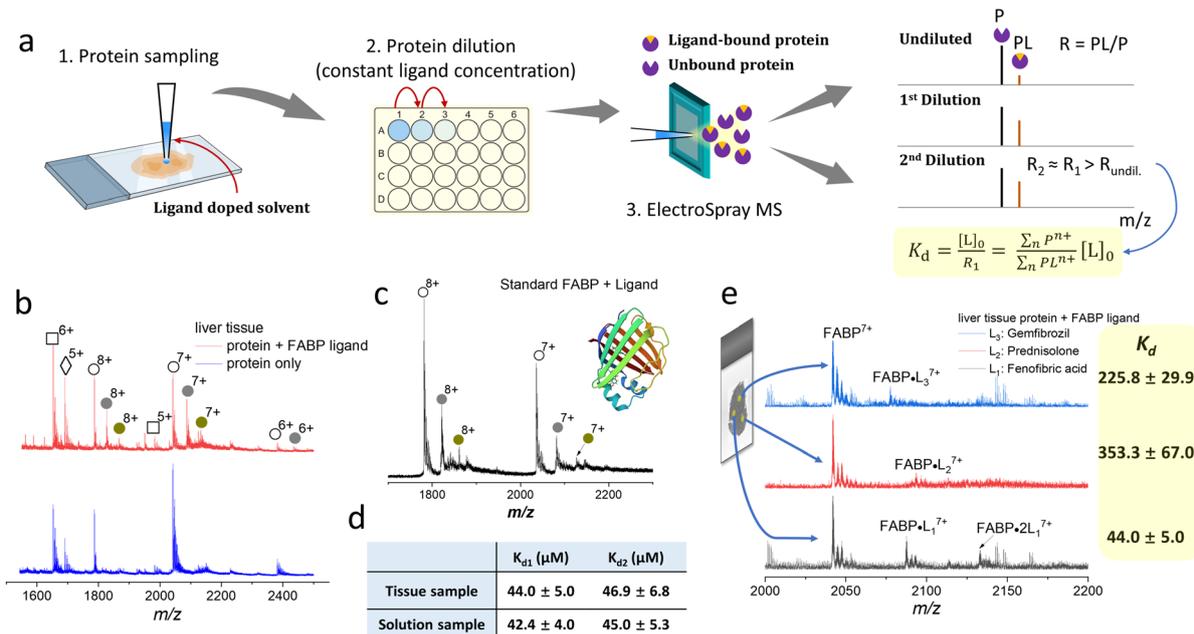
### Determination of protein–ligand binding affinity directly from tissue

To highlight the potential of this novel approach, we selected mouse liver tissue—one of the most complex biological systems—to investigate the binding affinity of therapeutic target fatty acid binding protein (FABP) to several approved drugs for metabolic and immune diseases. The native mass spectrum presented in Fig. 1b from LESA sampled tissues is dominated by peaks related to ions from three proteins, namely Acyl-CoA binding protein, truncated ubiquitin, and FABP between  $m/z$  1600 and 2400. The surface sampling solvent was doped with the drug ligand fenofibric acid. Peaks corresponding to ions of ligand bound FABP were also detected but no significant evidence was observed of the ligand binding to other proteins (Fig. 1b, top panel). FABP was found to form complexes with fenofibric acid in the ratio of both 1 : 1 and 1 : 2, supported by previous reports that liver-FABP has two ligand binding pockets.<sup>59,60</sup>

By comparing the protein bound fractions of the surface extracted and serially diluted protein–ligand mixture samples, slightly larger values were measured from the latter and no significant difference was observed between 2-fold and 4-fold dilutions. Using the simplified approach (eqn (S3), ESI†), the binding affinity of fenofibric acid to FABP, directly from tissue was measured to be  $44.0 \pm 5.0 \mu\text{M}$  and  $46.9 \pm 6.8 \mu\text{M}$  for dissociation constant  $K_{d1}$  ( $\text{PL} \rightleftharpoons \text{P} + \text{L}$ ) and  $K_{d2}$  ( $\text{PL}_2 \rightleftharpoons \text{PL} + \text{L}$ ), respectively (Table S1, ESI†). We also calculated the dissociation constant ( $K_d$ ) values for each charge state separately (Table S1, ESI†). No significant differences were observed between  $K_d$  values obtained from different charge states, indicating that the native MS conditions and parameters used in this study were mild enough to retain most protein complex ions during ion formation and transmission. In addition, the determined  $K_d$  values align well with results obtained by conventional native MS (Fig. 1c) relying on prior knowledge of protein concentration (Table in Fig. 1d). Furthermore, reducing the methanol proportion from 5% to 2% (due to the poor water solubility of the ligand fenofibric acid) did not result in a significant difference in  $K_d$  values (Table S1, ESI†).

While FABP was studied as a proof-of-concept experiment in liver tissue using the surface sampling method, this does not preclude the applicability of the approach to other proteins. The feasibility of extending this method to additional tissue proteins will depend on factors such as ionization efficiency and sampling conditions. Extensive studies have shown that detection sensitivity, specificity, and coverage of proteins in tissue





**Fig. 1** (a) Method developed based on surface liquid sampling, protein-dilution, and native MS for the measurement of protein–ligand binding affinity (b)–(d) from complex samples without prior knowledge of protein concentration and (e) ligand screening of target proteins directly from biological samples. (a) Schematic diagram showing the method workflow consisting of (1) extraction of protein from a surface or tissue sections into a binding–ligand–doped solvent in a conductive pipette through a liquid microjunction formed between the sample surface and the pipette tip, (2) serial dilution of the protein–ligand mixture solution obtained from surface sampling using the same solvent, maintaining a fixed ligand concentration, and (3) nozzle array chip–based nano–infusion ESI MS measurements of protein–ligand mixture solutions obtained from surface sampling and serial dilutions. The binding affinity (dissociation constant  $K_d$ ) can be determined using the simplified calculation method when the protein bound fraction  $R$  (the intensity ratio of ligand–bound to free unbound protein ions) does not change with dilution. (b) Native mass spectra of the mouse liver tissue section analysed by LESA (bottom panel) and with the fatty acid binding protein targeted drug ligand, fenofibric acid, added to the sampling solvent (top panel). Detected proteins and protein complexes were putatively assigned and labelled:  $\square$  acyl–CoA binding protein;  $\diamond$  truncated ubiquitin;  $\circ$  fatty acid binding protein (FABP); ligand bound FABP  $\bullet$  P–L 1 : 1;  $\bullet$  P–L 1 : 2. (c) Native mass spectrum of 5  $\mu\text{M}$  recombinant mouse liver FABP mixed with 15  $\mu\text{M}$  fenofibric acid acquired by nano–infusion ESI analysis. (d) Comparison of determined  $K_d$  values from tissue samples with unknown protein concentration using the developed method and from solution samples using the commonly used single–point MS method. (e) Mass spectra of sufficiently diluted FABP extracted from different locations of the mouse liver tissue section mixed with drug ligands. Binding affinities of FABP to fenofibric acid, prednisolone and gemfibrozil were 44.0, 353.3 and 225.8  $\mu\text{M}$ , respectively, and the binding stoichiometry was 1 : 2, 1 : 1, and 1 : 1.

samples can be significantly enhanced through strategies such as modifications to sample preparation protocols,<sup>61,62</sup> ionization/detection conditions,<sup>53,63</sup> etc. By refining these parameters, the method could be adapted to a wider range of biological targets, extending its utility beyond the current scope.

### Ligand screening from complex sample systems

Gemfibrozil and prednisolone were found to bind to FABP at a ratio of 1 : 1 (Fig. 1e), compared to fenofibric acid which occupied two binding pockets. The  $K_d$  values for FABP and ligands gemfibrozil and prednisolone measured by the concentration–dilution method were  $225.8 \pm 29.9$   $\mu\text{M}$  and  $353.3 \pm 67.0$   $\mu\text{M}$ , respectively. The affinity ranking determined by this approach (fenofibric acid > gemfibrozil > prednisolone) was in agreement with the inhibitor affinity ( $K_i$ ) ranking obtained from the fluorescence assay.<sup>60</sup>

### Affinity measurement independent of protein concentration

We further evaluated our method by analysing several other well characterised protein–ligand complexes, including lysozyme–

NAG<sub>3</sub>, ribonuclease A (RNase A)–CDP, human carbonic anhydrase I (hCA I)–acetazolamide, and hCA I–indapamide. First, we investigated how protein concentration affects the accuracy of binding affinity measurement using solution samples and conventional methods. Our results showed that accurate  $K_d$  measurement was achieved when the initial protein concentration  $P_0$  is lower than the “true (theoretical)” dissociation constant, in agreement with previous reports.<sup>42,64</sup> while deviation occurred and became larger with increasing  $P_0$  (Fig. S1, ESI†).

To compare our approach with conventional methods, we determined the binding dissociation constants of all studied systems (Fig. S2, ESI†) at low protein concentrations, with results summarized in Table S2, ESI†. Notably, removing  $P_0$  from the equation did not lead to significant differences in the obtained  $K_d$  values, demonstrating that our method is not only robust but also independent of precise protein concentration measurements. This independence arises because our approach relies on intrinsic ligand binding equilibria rather than absolute protein concentrations, making it particularly



advantageous when protein quantification is challenging in biological samples. Surface sampling coupled with protein dilution (Methods, ESI<sup>†</sup>) enables the rapid and accurate determination of binding affinities from complex sample systems, without the need for prior knowledge of protein concentration. This is based on the fact that the bound fraction no longer changes significantly as the protein concentration is sufficiently diluted.

## Method validation

Using our method, we determined  $K_d$  values of well-studied protein–ligand binding models from surfaces (0.2  $\mu$ L droplets of standard sample mixtures deposited on glass slides and left to air dry) and compared the results with those obtained from solution samples using traditional methods. A narrow distribution of low charge states of free and ligand-bound protein ions was observed (Fig. 2a–d), suggesting that the additional steps of surface deposition and liquid sampling had no significant effect on the preservation of folded protein structures. The ratios of bound to unbound protein are significantly higher after dilutions of surface extractions while ligand concentrations were fixed, suggesting that the protein concentrations obtained from surface sampling were too high to achieve accurate measurement of corresponding binding affinities (Table S3, ESI<sup>†</sup>). Similar bound

fractions are observed in samples diluted 10-fold and 100-fold (Fig. 2a–c) and the binding constant was accurately determined (Table S4, ESI<sup>†</sup>) following the scheme illustrated in Fig. 1a. For the hCA I–acetazolamide complex (Fig. 2d), the sensitivity limitations of the Q-TOF MS prevented measurement at protein concentrations sufficiently below the system's  $K_d$  value to ensure accurate determination of binding affinity. To overcome this, a more sensitive Orbitrap MS was employed to measure up to 1000-fold protein dilution, yielding a  $K_d$  value of  $0.368 \pm 0.046$   $\mu$ M.  $K_d$  values were also determined at different charge states, and similar results were observed across charge distributions, confirming the robustness of our method. In addition, using the conventional titration MS method, the bound fractions of protein as a function of ligand concentrations were measured from solution samples (scatter plots in Fig. 2e). Using eqn (S2) (ESI<sup>†</sup>), the titration curve of each protein–ligand binding system was fitted with the parameter  $K_d$  obtained. Our method demonstrated remarkable reliability and accuracy in measuring binding affinities at unknown protein concentrations (Fig. 2f).

## Negating the need for protein concentration in $K_d$ determinations from other methods

The determination of  $K_d$  without protein concentration can also be achieved using titration MS (see Table S2, ESI<sup>†</sup>). Very close  $K_d$  values are obtained by fitting the titration MS data using routine

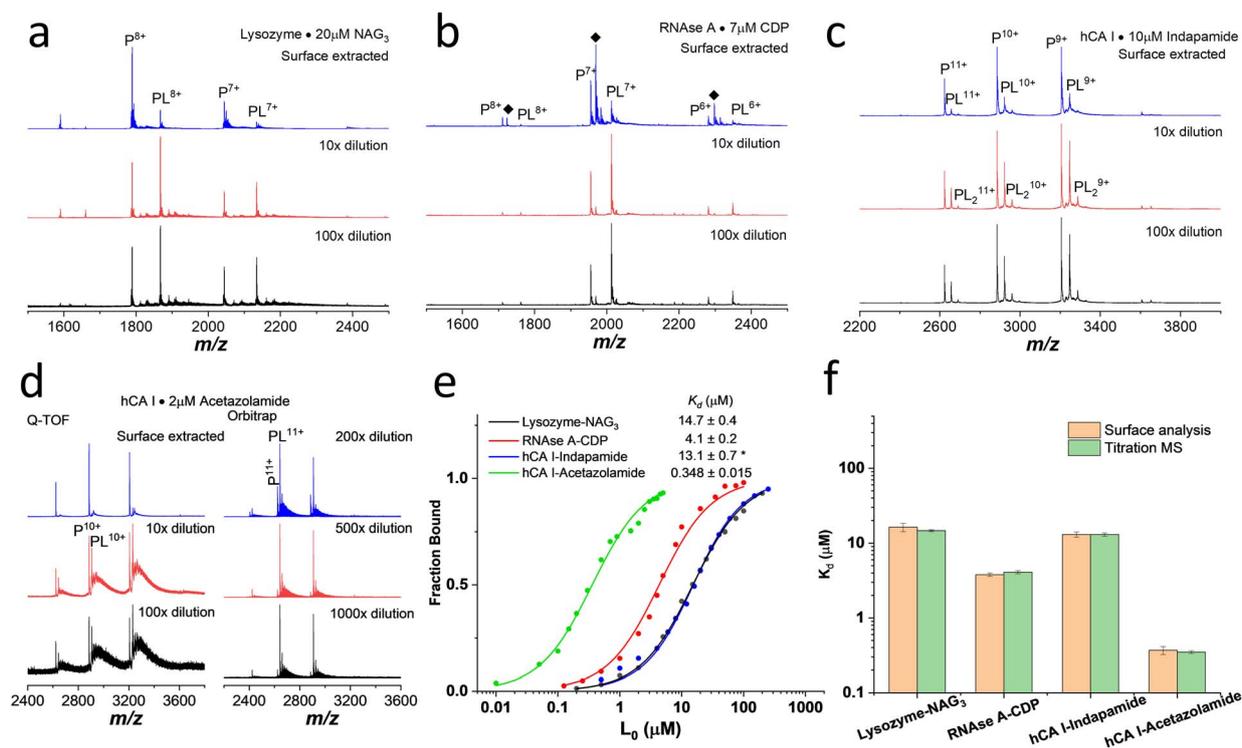


Fig. 2 Measurement of protein–ligand binding affinities from standard samples on surfaces with no prior knowledge of protein concentrations. Native mass spectra of surface-extracted and serially diluted proteins mixed with binding ligands (a) lysozyme–NAG<sub>3</sub>; (b) RNase–A–CDP; (c) hCA I–indapamide; (d) hCA I–acetazolamide collected using the method illustrated in Fig. 1a. (e) Determination of protein–ligand binding dissociation constants through nonlinear curve fitting of titration MS data, *i.e.* bound fraction of protein as a function of ligand concentration, using eqn (S2), ESI<sup>†</sup>. (f) Comparison of  $K_d$  values obtained from surface analysis using the newly developed method and solution measurement using the conventional titration MS method. \*Ion intensities of both 1:1 and 1:2 protein–ligand complexes were taken into account to calculate the summed bound protein fraction  $F$ .



eqn (S2) (ESI)<sup>†</sup> and simplified eqn (S4) (ESI).<sup>†</sup> Using data available from published sources, we further evaluated this method on other biophysical techniques and indeed found that similar  $K_d$  values could be obtained without the need for protein concentration (Table S5, ESI<sup>†</sup>). In studies employing fluorescence spectroscopy titration,<sup>65,66</sup> the measured  $K_d$  varied slightly from 7.7 to 9.6  $\mu\text{M}$  and from 4.3 to 4.9  $\mu\text{M}$  for lysozyme–NAG<sub>3</sub> and HusA–Haem complexes, respectively. Using microscale thermophoresis,<sup>67,68</sup> the  $K_d$  values for SAM-II riboswitch–SMA and AhR–ARNT complex–1-hydroxyphenazine complexes changed from 0.14 to 0.16  $\mu\text{M}$  and from 0.88 to 1.03  $\mu\text{M}$ , respectively.

Compared with mass spectrometry, the deviation of  $K_d$  obtained by other techniques without protein concentration is slightly larger, *i.e.*, 14–25% in fluorescence and thermophoresis measurements *vs.* less than 10% in MS single-point measurement, titration measurement, as well as the dilution method coupled to surface sampling and applied to tissue analysis. Nevertheless, regardless of the technique, the deviation does not exceed one order of magnitude, indicating that the method of calculating  $K_d$  without protein concentration is a widely applicable complementary tool for measuring binding affinity in drug development and protein biochemical studies.

### Affinity determination under binding competition

Many ligand binding studies are performed using complex biological samples in which the protein of interest exists in a mixture with other proteins. Therefore, the potential interference of off-target binding on  $K_d$  measurement from surfaces was also assessed. Using the simplified approach (eqn (S6), ESI<sup>†</sup>), from surfaces deposited with the mixture of lysozyme, ubiquitin and myoglobin (Fig. S3, ESI<sup>†</sup>), the dissociation constant of lysozyme–NAG<sub>3</sub> was determined to be  $18.1 \pm 0.8 \mu\text{M}$ , which was very similar to that measured without interference binding. This is unsurprising, as neither ubiquitin nor myoglobin forms a specific interaction with the ligand NAG<sub>3</sub>, which targets lysozyme.

Notably, when considering the much weaker, non-specific binding pair myoglobin–NAG<sub>3</sub> ( $K_d \sim 345.6 \mu\text{M}$ , determined in solution using the conventional native MS method) as the system of interest, our method was still able to determine its binding affinity ( $K_d \sim 355.2 \mu\text{M}$ ), even in the presence of a much stronger binding competitor (*i.e.*, lysozyme). This indicates that strong off-target interactions did not substantially impact  $K_d$

measurements for the intended protein–ligand pair. However, it is important to note that sufficient dilution is required to minimize protein concentration-dependent ligand depletion, ensuring accurate  $K_d$  determination.

### Summary and conclusions

It is disappointing that many promising compounds generated in early stages of drug development programmes show limited success in preclinical and clinical trials. One of the important reasons may be the significant difference in the buffer environment, which leads to a large deviation in the binding affinity between the potential drug ligand and the therapeutic target protein from laboratory screening to biological research. Therefore, methods which can be used to determine protein–ligand binding affinity for proteins sampled directly from their native environment would prove highly beneficial, providing more accurate guidance for the selection of effective drug candidates. Our dilution-based method has the advantages of low sample consumption, high measurement throughput, and simple data analysis. More importantly, it eliminates the need for labour-intensive steps such as protein extraction, purification, and quantification, which are often challenging in complex biological matrices. With minimal sample manipulation, this method can be directly applied to *in situ* or surface analysis of complex systems (Table 1), such as tissue sections, extracts, or cell lysates. It is also well-suited for ligand screening from these heterogeneous samples.

To evaluate the method for binding affinity measurement, we compared the dissociation constant ( $K_d$ ) values of FABP with various ligands obtained in our work with inhibitor constant ( $K_i$ ) values reported in previous competitive fluorescence displacement assays. While the rank order of ligand binding affinities determined by our approach (fenofibric acid > gemfibrozil > prednisolone) aligns with that of fluorescence-based  $K_i$  values, we observed notable absolute differences between  $K_d$  and  $K_i$  values. A thorough literature review and database search did not identify previously reported  $K_d$  values for FABP and its related ligands, making a direct comparison unavailable. Instead, we referenced  $K_i$  values from fluorescence displacement assays, which provide an indirect measure of binding strength. However, it is essential to recognize that  $K_d$  and  $K_i$  represent fundamentally different parameters:

- $K_d$  reflects the equilibrium dissociation constant, solely determined by the binding affinity of the paired system.

**Table 1** Comparison of methods using native MS for the determination of protein–ligand binding affinity. \*Assuming protein molecular weight is 20 kDa and ligand molecular weight is 300 Da

	Dilution-based MS	Titration MS	Single-point MS	Slow-mixing MS
Suitable for surface analysis	✓	✗	✗	✗
Protein concentration required	✗	✓	✓	✓
Protein consumption*	1.11 $\mu\text{g}$ (5, 0.5, 0.05 $\mu\text{M}$ , 10 $\mu\text{L}$ )	10 $\mu\text{g}$ (5 $\mu\text{M}$ , 10 titrations, 10 $\mu\text{L}$ )	1 $\mu\text{g}$	1 $\mu\text{g}$
Ligand concentration required	✓	✓	✓	✗
Ligand consumption*	90 ng (10 $\mu\text{M}$ , 30 $\mu\text{L}$ )	700 ng (1–100 $\mu\text{M}$ , 10 $\mu\text{L}$ )	30 ng (10 $\mu\text{M}$ , 10 $\mu\text{L}$ )	30 ng (10 $\mu\text{M}$ , 10 $\mu\text{L}$ )
Sample preparation time (min)	5	20	3–5	3–5
Data acquisition & analysis (min)	8–10	30	5	30–60



•  $K_i$  represents the inhibitory potency of a ligand, influenced by both binding affinity and its ability to disrupt protein function, often measured in the presence of competing molecules.

Additionally, differences in experimental conditions—such as solvent composition, presence of competitors, and assay format—can contribute to discrepancies between  $K_d$  and  $K_i$  values. In our study, the presence of 5% methanol in the native MS system may have influenced the measured  $K_d$  values, though we found no significant variation when reducing methanol to 2%. However, 10 mM ammonium acetate in 5:95 methanol/water was used in our study while 10% (v/v) DMSO in the buffer of 20 mM MES, pH 5.5, 100 mM NaCl, 1 mM DTT, and 0.5 mM EDTA was used in the literature fluorescence displacement assays. These solvent effects, along with differences in assay principles, likely explain the observed deviations from  $K_i$  values reported in the literature. Similar discrepancies have been noted in previous studies comparing  $K_d$  with  $K_i$ -based affinity measurements.<sup>69</sup>

While our method offers a rapid, straightforward, and label-free strategy for determining  $K_d$  values directly from complex biological samples, a number of challenges remain. The detection sensitivity of native MS for folded proteins and intact protein complexes typically ranges from nanomolar to low micromolar, making it challenging to accurately measure ultra-high-affinity interactions ( $K_d$  in the sub-nanomolar range). Nonetheless, estimation based on sensitivity of MS indicates that our method is applicable to more than 80% of protein–small molecule complexes recorded in the BindingDB database,<sup>70,71</sup> making it broadly useful for studying biologically relevant binding affinities.

However, tissue complexity poses additional challenges, including interference from lipids and salts, which may impact the efficiency of protein extraction, ionization, and detection. To enhance the applicability of our approach, future work should focus on refining ionization strategies, optimizing sample treatments, and assessing the method's performance across various tissue proteins and conditions. By integrating established MS-based enhancement techniques to improve sensitivity and specificity, such as advanced ionization/detection strategies and modified solvent systems, our approach could be extended to a broader range of biological targets. This would pave the way for more comprehensive applications in tissue-based drug screening and biomolecular interaction studies.

## Data availability

Data for this article are available at Open Science Framework at <https://doi.org/10.17605/OSF.IO/TBY45>.

## Author contributions

B. Y. – conceptualization, formal analysis, investigation, methodology, validation, visualization, writing – original draft, writing – review & editing; J. B. – conceptualization, methodology, funding acquisition, supervision, writing – review & editing.

## Conflicts of interest

There are no conflicts of interest.

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