

Denaturing mass photometry for straightforward optimization of protein-protein cross-linking reactions at single-molecule level

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1 Denaturing mass photometry for straightforward optimization of protein-protein

2 cross-linking reactions at single-molecule level

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17 ABSTRACT:

18 Mass photometry (MP) is a versatile, fast and low sample-consuming biophysical technique that gained 19 interest in structural biology to study noncovalent assemblies in native conditions. We report here on a novel 20 method to perform MP analysis in denaturing conditions (dMP) and its application for fast, accurate and 21 straightforward optimization of chemical reactions in cross-linking mass spectrometry (XL-MS) workflows. 22 dMP consists in a robust 2-step protocol that ensures 95% of irreversible denaturation within only 5 min. The 23 proposed single-molecule method clearly overcomes the limitations and outperforms gold standard SDS-24 PAGE, as illustrated on several biological complexes. dMP provides an unprecedented and unmatched insolution quantification of all coexisting XL species, including sub-complexes and non-specific XL aggregates, 25 26 along with identification of significantly higher numbers of XL dipeptides in MS. We anticipate single-molecule 27 dMP to be a high-impact game-changer for the XL-MS community with the potential to leverage the quality 28 and reliability of XL-MS datasets.

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35 MAIN

36 Characterization of protein-protein interactions (PPI) are essential for the understanding of biological 37 processes that drive life in different organisms. Although technological breakthroughs in well-stablished atomic-resolution biophysical techniques such as X-ray crystallography (XRC)¹, nuclear magnetic resonance 38 39 (NMR)², and electron microscopy (EM)³ have boosted structural biology, several limitations (requirement of highly purified systems, extensive sample preparation protocols, production of high-quality crystals or cryo-40 41 EM grids, large amount of sample material, etc.) still remain. To circumvent those limitations and for 42 NMR/XRC/cryo-EM-resistant complexes (highly flexible, disordered, membrane solubilized, etc.), structural mass spectrometry approaches nicely complement the classical structural biology toolbox^{4,5}. Structural MS is 43 44 a generic term that encompasses a series of MS-based strategies adapted for the characterization of non-45 covalent assemblies. Among them, protein cross-linking followed by mass spectrometry (XL-MS)⁶ is a covalent labelling proteomics-based technique that has drastically progressed this last decade from in vitro⁷, to 46 47 proteome-wide⁸ or even *in-situ* and *in-cellulo* applications^{9–11}. For XL-MS, the native (multi)-protein complex is 48 usually incubated with a chemical reagent (cross-linker) in order to stabilize complexes (both stable and labile 49 or transient), which allows to take a snapshot of non-covalent interactions, spatial proximities and provides 50 insights on tertiary/quaternary structure and PPIs. The first step of chemical XL is of utmost importance: before 51 going to MS analysis, optimal XL conditions have to be determined, ranging from the selection of the optimal 52 XL reagent to screening of best adapted XL conditions ensuring proper XL while not generating non-specific XL 53 aggregates⁷. According to latest published community-wide guidelines for XL-MS¹², denaturing sodium dodecyl 54 sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is recommended to monitor and optimize XL 55 conditions, allowing visualization of high molecular weight (MW) bands on the upper part of the gel 56 corresponding to cross-linked species, and concomitant vanishing of the bands corresponding to individual 57 lower MW free protein partners. Even if SDS-PAGE analysis is easy, low cost and available in all laboratories, it 58 also has some limitations, among which being: i) low mass-resolution, ii) time consuming (gel casting, sample 59 denaturation, gel migration, staining); iii) not suited for high-mass complexes visualization as they do not enter 60 the gel.

Mass photometry (MP), that has been recently introduced as a single-molecule biophysical technique¹³ 61 62 operating in native conditions (nMP), can be positioned as alternative or to complement native mass 63 spectrometry (nMS) for "nMS-resistant" protein complexes. MP is based on the principle of the interferometric 64 scattering microscopy (iSCAT) and uses the contrast originated as a result of the destructive interaction between the scattered light and reflected light of biomolecules in solution upon irradiation with a visible laser 65 light^{14–16}. As the contrast intensity linearly scales with the mass, MP can serve to estimate masses of 66 67 biomolecules after proper calibration with reference molecules. MP offers several advantages: i) a fast (minute 68 scale), straightforward analysis of samples in solution, ii) multiplexing and automation capabilities, iii) no extensive sample preparation (e.g. buffer exchange)¹⁷, iv) low sample quantity requirements (pM-nM 69

concentration range, compared to μM range for nMS), v) a broad mass range (30 kDa to 5 MDa)^{17,18} and iv) single molecule detection which enables to relatively quantify the detected populations leading to the possibility to estimate affinity constants in the nM-μM concentration range^{19,20}. Although nMS mass accuracy is unmatched compared to MP, nMP has already emerged as a valuable asset to characterize highly heterogeneous complexes²¹, membrane proteins solubilized with different types of membrane mimics^{18,22,23}, ribosomes²⁴, and viral capsids^{25,26}.

76 Despite its routine use to study noncovalent machineries, MP is scarcely reported for the characterization and 77 quantitation of covalent cross-linked assemblies. MP has been used after glutaraldehyde cross-linking of 78 molecular machineries to verify the enrichment of a targeted oligomer before electron microscopy 79 experiments²¹. As nMP gains popularity in the structural biology community, it looks appealing for 80 straightforward XL reaction optimization. In this context, we report on the development of a groundbreaking 81 and robust single-molecule protocol to perform MP analysis in denaturing conditions (dMP). We first used reference protein complexes of increasing sizes and complexities as proof-of-concept and dMP performance 82 83 assessment. We then evaluated our dMP protocol for XL reaction monitoring and benchmarked it against the reference gold standard denaturing SDS-PAGE gel method. Due to its single molecule detection capabilities, 84 85 we demonstrate here that dMP can be proposed as a straightforward, and more precise alternative to 86 conventional SDS-PAGE analysis to monitor and optimize XL reactions, as illustrated on a real-case study for 87 optimization of R2SP XL reaction.

88

89 **RESULTS**

90 Development of a denaturing mass photometry (dMP) protocol

91 To develop a fast, efficient and non-reversible denaturation protocol while maintaining good quality MP signal 92 intensities, we step-by-step optimized several parameters and first focused on the choice of the denaturing 93 agent. We selected as potential denaturing agents urea and guanidine HCl, two well-known chaotropic agents 94 that disrupt H-bonds, increase solubility of hydrophobic regions, and finally lead to the disruption of tertiary and quaternary structures^{27–29}, along with the H₂0/ACN/FA mix (50/50/1) classically used in intact MS mass 95 96 measurement in denaturing conditions. The H₂0/ACN/FA mix was discarded, as incompatible with stabilization 97 of MP droplets (which might be due to modification of the droplet surface tension related to presence of 98 organic solvent and acid). We next assessed the impact of urea/guanidine HCl solutions on the quality of MP signal using "protein-free" droplets (Fig. 1A) by monitoring three output indicators (signal-Si, sharpness-Sh 99 100 and brightness-Br) reflecting the quality of the MP images/frames. Si translates the level of activity in each 101 frame that can be due to either protein binding, or contaminants/salts/surfactants presence. Si values should be as low as possible and always <0.05 % to avoid extensive peak broadening¹⁷. Sh refers to the level of detail 102 103 visible in each frame, which influences the aptitude to find and maintain the good focusing position, and 104 should be as high as possible. Finally, Br characterizes the amount of light available in images: as low Br will

105 cause peak broadening by increasing the noise, Br value has to be maximized. With the aim to reach similar 106 MP measurements qualities as those reached in PBS droplets (Si ~0.03 %, Sh ~5 %, Br ~73 %), we used the 107 "buffer-free" focusing mode to directly analyze protein-free droplets containing decreasing concentrations of 108 urea or guanidine HCl (from 5.4 to 0.4 M). Of note, focusing the image on the glass surface was tedious for 109 droplets concentrations higher than 1.75 M urea or guanidine HCl. Independently of the denaturing agent, Sh 110 slightly increases progressively from 1-3% at 5.4 M of urea/guanidine HCl to \geq 5 % at lower concentrations 111 (Table S1). Similarly, Br increases at lower denaturing agent concentrations with an optimal value of \sim 67 % 112 obtained at 0.8 M urea/0.4 M guanidine HCl. Si values were < 0.05 % for all tested concentrations lower than 113 5.4M. Altogether, our "protein-free" blank MP acquisitions allowed establishing the optimal concentrations 114 of denaturing agents (< 0.8M of urea or guanidine/HCl) in the PBS droplet in order not to compromise MP 115 measurements quality. As XL reactions are typically conducted in the nM-µM concentration range, an 116 approximatively 10x dilution in Phosphate Buffer Saline (PBS) is preconized prior to dMP measurements. That 117 means that the initial concentration of the chaotropic agent during the denaturing reaction can be set at 5.4 118 M of urea and 6 M of guanidine without overcoming the 0.8 M concentration limit in the final droplet.

119 To further optimize our dMP protocol, we next used reference protein complexes (BSA, ADH, GLDH, 20S proteasome) either to assess mass precision, accuracy and peak broadening in dMP (BSA) or to further 120 121 optimize the denaturation step (ADH, GLDH and 20S proteasome). After Gaussian-fitting of MP histograms, 122 the mean mass (μ) and half-height peak width (2 σ , FWHM) of the Gaussian fits were used to evaluate mass 123 accuracy and peak broadening, respectively. Considering dMP triplicate measurements, the measured mass 124 of BSA oligomers denatured in urea and guanidine HCl did not highlight any major differences compared to 125 nMP measurements (Table S2). In addition, our denaturation protocol does not alter MP repeatability 126 between replicates with SDs \leq 3 kDa and \leq 5 kDa for BSA monomers and dimers, respectively. Finally, 127 denaturation only slightly affects peak broadening (FWHM between 8-10 kDa and 8-14 kDa, compared to 8 128 kDa in nMP for monomers and dimers, respectively), demonstrating that dMP measurements characteristics 129 are similar to those obtained in classical nMP conditions.

130 In order to develop a fast denaturing protocol, we next optimized the duration of the denaturation step on 131 proteins of increasing sizes and complexities (ADH, GLDH and 20S proteasome). Incubation in urea and 132 guanidine HCl were carried out for 5 min to 16 h at room temperature (Fig. 2). After 5 min of urea 133 denaturation, an almost complete denaturation is observed in dMP for all systems with > 95 % of the detected 134 species being monomers (compared to 46 %, 19 % and 51 % of monomers for ADH, GLDH and 20S proteasome 135 in nMP, respectively). Conversely, denaturation in guanidine HCl proved to be less efficient after 5 min for ADH 136 (~34 % monomers) while being equivalent for GLDH and 20S proteasome, suggesting urea as a more 137 ubiquitous denaturing agent. In order to ensure that no protein refolding occurs in the PBS droplet prepared 138 for dMP measurements, and in the timeframe of the analysis, we mimicked our optimized denaturing protocol 139 in a tube (Fig. S1 A)). As expected, only GLDH monomer (61 ± 6 kDa) is detected even after 10 min in PBS, with perfectly superimposable dMP spectra (Fig. S1 B)), demonstrating that no significant protein refolding will
 occur within the timeframe of dMP analysis (typically 1 min).

- To conclude, we have developed and optimized a fast (5 min), efficient (> 95 % denaturation) and nonreversible (in the timeframe of dMP measurements) denaturation protocol compatible with MP analysis, which will be further referred to as "denaturing MP protocol" (dMP). This workflow consists of a first step of denaturation (5 min in 5.4M urea) followed by dilution of 2 μ L of the denatured sample to a 18 μ L PBS droplet right before dMP analysis (**Fig. 1B**). Obtained dMP measurements quality along with mass accuracies and peak width were comparable with those obtained in classical nMP analysis.
- 148

149 dMP outperforms SDS-PAGE gel analysis for XL reaction monitoring

150 We next benchmarked our dMP protocol against SDS-PAGE, the gold standard for XL reaction optimization, 151 on our reference systems (ADH, GLDH, 20S proteasome), using the MS-cleavable cross-linker disuccinimidyl 152 dibutyric urea (DSBU) as proof of concept. For a 25:1 DSBU:ADH ratio (Fig. 3A), monomers, dimers, trimers 153 and tetramers are observed in dMP (50, 25, 14, 10 % of total counts, respectively), in good agreement with 154 SDS-PAGE (Fig. S2). Tetrameric species are the most abundant from DSBU molar excesses \geq 100 with up to 53 155 % of total counts. No non-specific high-mass aggregates were detected neither using SDS-PAGE nor dMP, both 156 methods suggesting optimal conditions around 100:1 DSBU:ADH. For GLDH (Fig. 3B), dMP allows to visualize 157 that increasing XL concentration progressively stabilize higher oligomeric states, with a reduction of 158 intermediary sub-complexes (23 to 9 % from 100 and 400 DSBU molar excess) at the expense of the hexamer 159 (57 to 79 % from 100 and 400 DSBU molar excess). At both 100:1 and 400:1 DSBU:GLDH ratios, only a small 160 proportion of dodecamers (6 %) is formed, suggesting non-specific XL aggregates. Conversely, regardless the 161 XL condition, only a broad band in the loading-well is observed on the SDS-PAGE gel at high masses, 162 highlighting that GLDH high mass oligomers do not enter the gel, along with remaining free monomers still 163 observed for all DSBU molar excesses. This impairs a proper assessment of stabilized oligomerization states 164 using this technique. For cross-linked 20S proteasome (28 subunits), SDS-PAGE is even less useful, as only a 165 slight decrease in the free subunits intensity can be seen upon cross-linker concentration increase (Fig. 3C). 166 Additional masses between 100-200 kDa are also stabilized, but, in all cases, SDS-PAGE does not allow to 167 visualize the ~700 kDa 20S proteasome assembly. Conversely, dMP mass range revealed 20S proteasome 168 covalent stabilization even at 25:1 DSBU:20S ratio (16 %) with a maximal 20S cross-linking at 400:1 DSBU:20S 169 (25 %), while avoiding non-specific aggregation.

Thanks to its single molecule detection capabilities along with higher mass accuracies and increased mass range (30 kDa-5 MDa) compared to SDS-PAGE (10 kDa-300 kDa), dMP allowed fast in-solution mass measurements and relative quantification of all co-existing single molecules detected. This is of utmost advantage for high mass and high-heterogeneity samples for which SDS-PAGE clearly fails to give the needed output for a rational XL optimization. In addition, dMP highlights that the use of high XL reagent concentrations usually not recommended (400 molar excesses) only leads to a very limited amount of non-specific XL

aggregates. Altogether dMP, that allows single-molecule sensitivity, proved to be more precise, quantitativeand provides less arbitrary optimization than SDS-PAGE.

178

179 dMP offers unmatched rapidity for straightforward quantitative screening of optimal XL conditions

180 We next evaluated the versatility of dMP to monitor XL reaction using a variety of chemical reagent : two MS-181 cleavable compounds, DSBU (disuccinimidyl dibutyric urea, linker size ~ 12.5 Å) and DSAU (disuccinimidyl 182 diacetic urea, linker size ~7.7 Å) and the less-flexible IMAC-enrichable cross-linker PhoX (linker size ~5.5 Å) that gains popularity for both *in vitro* and *in vivo* XL-MS studies^{6,30}. For GLDH, obtained dMP mass distributions 183 184 are similar for PhoX and DSAU cross-linking, but clearly differ from DSBU (Fig. 4a): while GLDH hexamers and 185 monomers are detected as main components at 25:1 DSBU:GLDH ratio, monomers, dimers and trimers are 186 formed in similar conditions with DSAU and PhoX. This trend is even more obvious at 100:1 and 400:1 cross-187 linker:GLDH ratios, with an almost complete stabilization of GLDH hexamers using DSBU when only low 188 abundant hexameric species are detected with PhoX and DSAU. Similar behaviors were also observed in dMP 189 profiles of cross-linked ADH and 20S proteasome, with a significantly higher stabilization of intact complexes 190 with DSBU (Fig. S3 A) and B)). To go deeper into cross-linker comparison, we defined two metrics for rational 191 and quick comparison of different XL conditions (reagent, reaction time, pH etc): i) the global inter-protein XL 192 reaction efficiency Eff_{xL} (Material and method Eq. 1), as an indicator of the relative amount of inter-protein 193 XL-stabilized species (all cross-linked stabilized species except monomers); and ii) a XL-stabilization factor SF_{XL} 194 (Materials and Methods Eq. 2), as an estimate of the amount of "expected XL-stabilized complex".

195 Eff_{xL} estimation is thus an interesting indicator for XL conditions screening but lacks in precision as it takes into 196 account both expected specific and non-specific XL species, which is not the case of SF_{xL} that focuses on one 197 species. The SF_{xL} factor should be maximized to get close to a value of 1 for a complete XL-stabilization of the 198 non-covalent complex : SF_{xL} values > 1 means stabilization of expected complex's abundance along with a 199 decrease in generation of transient XL-sub-complexes assemblies, that might occur over the XL reaction time 200 (30 min to 1 hour). For homo-oligometric samples (ADH and GLDH), DSBU showed much higher Eff_{xL} values (60-201 70 %) compared to DSAU (~45 %-48 %) and PhoX (45-55 %) (Fig. 4B, bar charts), suggesting overall better XL 202 efficiencies for DSBU. For the hetero-multiprotein 20S proteasome, trends are different: i) the Eff_{xL} is 203 decreased (max. ~35 %) and ii) DSAU and DSBU are the most potent cross-linkers over PhoX. For all reference 204 systems, SF_{xL} values : i) increase as a function of cross-linker:complex ratio, suggesting increased stabilization 205 of expected covalent assemblies at 400:1 cross-linker:complex ratio and ii) are higher for DSBU compared to 206 PhoX and DSAU that exhibit the lowest SF_{xL} values (Fig. 4B, solid dots). Low SF_{xL} values combined with good 207 Eff_{xL} (26-48 %) translate DSAU abilities to generate more sub-complexes but low amounts of expected XL-208 stabilized ADH/GLDH/20S proteasome tetramers/hexamers/28-mer. Of note, it appears that SF_{xL} values 209 obtained for ADH and GLDH tend to plateau with increasing DSBU molar excess, which is not the case for the 210 20S proteasome, constituted of a higher number of subunits (28) compared to AHD (4) and GLDH (6).

211 Finally, we applied dMP for *ab initio* fine-tuning of R2ΔIIS'P' (here called R2SP) cross-linking, ~540kDa (Fig. S4) complex constituted of RuvB-Like1 without the DII_{ext} (R1ΔDII, R1) and RuvB-Like2 without the DII_{ext} (R2ΔDII, 212 R2)³¹, SPAG1₆₂₂₋₉₂₆ (S') and PIH1D2₂₃₁₋₃₁₅ (P')^{32,33}. We compared 4 amine-reactive cross-linkers⁶, including PhoX 213 214 (5.5 Å), DSAU (7Å), DSSO (10.3 Å), DSBU (12.5 Å), each at 5 different molar excesses (25/50/100/200/400) plus 215 the control non-XL sample (see Fig. S5 for SDS-PAGE). This 24-conditions screen was realized with 216 unprecedented rapidity (~1.5 hours), including calibration and dMP triplicates measurements. Despite a dMP a progressive stabilization of sub-complexes with increasing cross-linker concentration of PhoX (Eff_{XL}~31-35%, 217 218 max. SF_{XL} ~0.1) and DSAU (Eff_{XL} ~31-35 %, SF_{XL}~0, **Fig. 5E**), none of these reagents are able to significantly 219 stabilize the ~540 kDa R2SP detected in nMP (Fig. 5A and 5B). In contrary, both DSSO (Eff_{xL} ~31-35 %, max. 220 SF_{xL}~0.4) and DSBU (Eff_{xL}~31-35 %, max. SF_{xL}~0.7) at high molar excesses (> 100:1 DSSO/DSBU:R2SP) yield to 221 stabilization of covalent ~540 kDa R2SP, without significant non-specific aggregate formation or sub-complex 222 stabilization (Fig. 5C-E). From dMP, a 400:1 DSBU:R2SP ratio (max. Eff_{xL}~77 ± 6 %) would be selected as optimal 223 XL condition for further XL-MS analysis (Table S3), which confirmed identification and validation of a higher 224 proportions of inter-XL peptides (from 45 % for 25:1 DSBU:R2SP to 55 % for 400:1 DSBU:R2SP).

Altogether, our results demonstrate that dMP affords unmatched performances for fast and rational screening for optimal XL conditions (e.g. reagent, reaction length, temperature, buffer, etc.). Our dMP results highlight that DSBU appears to outperform DSAU, PhoX, and even DSSO for inter-protein XL on our biological systems. This reagent provides the best compromise between stabilization of the covalent-XL-assembly of interest (SF_{XL} values close to 1 while minimizing covalent sub-complexes stabilization or non-specific aggregate formation.

230

231 DISCUSSION

232 We report here on the development of a MP protocol allowing analysis in denaturing conditions, thereby 233 addressing the demand for versatile tools for XL reaction monitoring to better understand biologically relevant 234 PPIs. The dMP approach consists in a fast and efficient denaturing protocol that does not alter the quality of 235 the MP measurement, resulting in > 95 % denaturation of the sample within 5 minutes (using urea 5.4 M 236 followed by dilution of 2 µL in a classical PBS droplet). The applicability of the dMP protocol is illustrated to 237 fine tune XL reaction conditions. Our results highlight that dMP outperforms SDS-PAGE analysis in XL-MS 238 workflows, allowing faster (few minutes) and more precise mass measurements along with relative 239 quantification of all the different single-molecule resolved cross-linked species. We propose dMP as an 240 empowered technique alternative to SDS-PAGE analysis to screening for best XL conditions before MS analysis. 241 Several lessons can be learned from this pilot dMP study, as our results challenge a number of XL-MS well-242 established rules: i) chemical XL reactions are not necessarily low efficiency; ii) a large molar excess of cross-243 linkers does not necessarily generate artefactual XL non-specific aggregates; iii) size and flexibility of the chemical reagents drive the stabilization of XL-species and iv) MS-cleavable DSBU and DSSO might be best 244 245 adapted compared to PhoX or DSAU for *in vitro* intra-protein XL workflows.

246 Thanks to its unique single-molecule detection capabilities, dMP allowed addressing the question of the "low" yield of the chemical XL reaction (< 10 %)³⁰, often correlated to the low number of identified XL dipeptides 247 248 (low abundant XL dipeptides compared to linear non-XL peptides). Our dMP results reveal that significant 249 stabilization efficiency of covalent XL-assemblies (> 50 %) can be achieved and easily approximated through 250 SF_{xL} calculations (target $SF_{xL} \sim 1$). Another fundamental point raised by our pilot dMP study concerns the dogma 251 in the XL-MS community to perform XL reactions at a relatively low excess of cross-linkers (50-200x), to avoid the generation of non-specific XL resulting from "forced pairing" of distal amino acid sequences that might not 252 253 be biologically relevant. Surprisingly, our dMP results rather highlight that: i) low molar excess of XL reagents 254 (100-200x) would rather stabilize sub-complexes while ii) a high excess (400x) would be required to stabilize 255 expected covalent XL assemblies as a whole.

256 dMP results also strengthen the importance of the XL reagent choice for successful XL-MS analysis. The choice 257 of the XL reagent is guided by several properties ranging from the amino acids to be targeted (homo-versus 258 hetero- reagents), the length of the XL reagent (that defines the reachable distances) but also by the chance 259 to identify XL peptides by MS/MS (MS cleavable versus non cleavable reagents). MS-cleavable cross-linkers 260 (such as DSSO, DSAU and DSBU) along with IMAC-enrichable reagents (PhoX) are mostly used because they 261 allow more straightforward MS/MS identification of the XL-peptides. Our dMP results suggest that inter-262 protein XL efficiency/stabilization depends on the length and flexibility of the spacer arm. DSBU thus provides better XL efficiencies/stabilizations (Eff_{xL}, SF_{xL}) than other tested reagents, which is consistent with the 12.5 Å 263 264 highly flexible DSBU increased potential to access a higher number of potential K-K pairs over a wider distance 265 range compared to less flexible and smaller sized DSAU and PhoX, in agreement with published data^{34–36}. 266 Conversely, less flexible XL reagents with smaller sized spacer arms might rather lead to stabilization of sub-267 complexes instead of "expected native complexes". PhoX could finally be more suited for intra-protein XL.

To conclude, the developed single molecule dMP strategy not only provides an unmatched increase in the speed and quality of screening for optimal XL conditions, but also provides identification and relative quantification of all single molecule coexisting cross-linked species, including sub-complexes and non-specific XL aggregates, previously not clearly identified by SDS-PAGE. dMP uniquely provides evidence for the rational quantitative selection of best-adapted chemical XL conditions based on XL stabilization/efficiency indicators. Altogether, we anticipate single-molecule dMP to be a high-impact game-changer go-to method to leverage the quality and reliability of XL-MS datasets by providing direct snapshots of all coexisting species.

276 MATERIALS AND METHODS

277 Stocks, reagents, and instruments.

The complete list of reagents and instruments used in this study are listed in Materials section in the Supplementary Information.

280

281 Sample preparation and cross-linking reactions.

Bovine Serum Albumin (BSA, Sigma, Saint-Louis, USA), Alcohol dehydrogenase from baker's yeast (ADH, Sigma,
Saint-Louis, USA) and L-Glutamate dehydrogenase from bovine liver (GLDH, Sigma, Saint-Louis, USA) were
diluted to 1mg/mL in Gibco[™] phosphate buffer saline (PBS, Life technologies Corporation, NY, USA), pH 7.4.
Human 20S proteasome (20S, South Bay Bio, San Jose, USA) was diluted to 1mg/mL in 50 mM HEPES, 100 mM
NaCl, pH 7.4 prior to cross-linking.

287 R2SP complex complex was formed by incubating pure RuvBL1/RuvBL2 with excess pure SPAG1/PIH1D2 at a ratio of 1:4, respectively. The mixed complexes were incubated over night at 4 °C and the formed R2SP 288 289 complex was separated from excess SPAG1/PIH1D2 using a Superose 6 16/60 XK (GE Healthcare), see 290 Supplementary Information for detailed protocol. It was diluted to 1mg/mL in 20 mM Hepes, 150 mM NaCl, pH 8³¹ prior to cross-linking. For cross-linking (XL) reactions, aliquots of XL reagents were freshly diluted in 291 292 DMSO. Following reagents were used: PhoX (Disuccinimidyl Phenyl Phosphonic Acid. Bruker): DSAU 293 (Disuccinimidyl diacetic urea, CF Plus Chemicals, Brno-Řečkovice, Czech Republic); DSSO (Disuccinimidyl 294 sulfoxide, Thermo Fischer Scientific, Rockford, IL, USA); DSBU (Disuccinimidyl dibutyric urea, CF Plus Chemicals, 295 Brno-Řečkovice, Czech Republic). BSA, ADH, GLDH and 20S samples were each split in six aliquots and 296 incubated with 25, 100, or 400 molar excess of each reagent. R2SP stock solution was split into aliquots 297 subsequently reacted with PhoX, DSAU, DSSO, DSBU at molar excesses of 25, 50, 100, 200, 400.

XL reactions were carried for all samples at room temperature (18°C) for 45 min, and quenched with Tris HCl
(15 mM final concentration) for 20 min. A 1.5 µg aliquot of each non-XL control and XL sample was kept for
SDS-PAGE migration.

301

302 SDS-PAGE separation of cross-linked samples.

All cross-linked proteins and complexes were migrated on in-house 12% acrylamide denaturing SDS-PAGE gels (1.5 mm thickness). Volume corresponding to 1.5 µg of each XL sample (and non-XL controls) was diluted (1:1) with 2x concentrated Læmmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.01% bromphenol blue and 0.125 M Tris HCl) and incubated 5 min at 95°C. After sample loading, gels were migrated at 50 V for 20 min, 100 V until the 2/3 of the gel and 120 V until the end. After migration, gels were fixated for 20 min (3 % phosphoric acid, 50 % ethanol), washed 3x20min with milli-Q water and stained overnight with Coomasie Brillant Blue (G250, Sigma, Saint-Louis, USA). They were finally rinced 3x20 min with milli-Q water.

310

311 Mass photometry measurements.

312 MP measurements were performed with a TWO^{MP} (Refeyn Ltd, Oxford, UK) at room temperature (18 °C). 313 Microscope slides (24x50 mm, 170±5 μ m, No. 1.5H, Paul Marienfeld GmbH & Co. KG, Germany) were cleaned 314 with milli-Q water, isopropanol, milli-Q water and dried with a clean nitrogen stream. Six-well reusable silicone 315 gaskets (CultureWellTM, 50-3 mm DIA x 1mm Depth, 3-10 μ L, Grace Bio-Labs, Inc., Oregon, USA) were carefully 316 cut and assembled on the cover slide center. After being placed in the mass photometer and before each 317 acquisition, a 18 μ L droplet of PBS was put in a well to enable focusing on the glass surface.

318 Contrast-to-mass calibration: To allow MP mass measurements, contrast-to-mass calibration was performed 319 twice a day by measuring a mix of Bovine Serum Albumin (66 kDa), Bevacizumab (149 kDa), and Glutamate 320 Dehydrogenase (318 kDa) in PBS buffer, pH 7.4. The distributions of scattering events (given as contrast) were 321 Gaussian-fitted using DiscoverMP (Fig. S6 A)). Contrasts values are converted into masses using linear relation 322 between the contrast and the mass of the binding object. Calibrations were accepted for R2>0.995 (Fig. S6 B). 323 Native MP (nMP): Samples were first diluted with their native buffer to 100-400 nM. Finally, 2 µL of the stock 324 solution are finally drop-diluted and carefully mixed to 10-40 nM in a 18 µL PBS droplet¹⁷. Three movies of 325 3000 frames were recorded (60 s) for each sample using the AcquireMP software (Refeyn Ltd, Oxford, UK).

326 <u>Denaturing MP (dMP)</u>: Denaturing MP experiments were carried out by incubating first the samples to a 327 protein concentration of 100-400 nM in 5.4 M Urea (Sigma, Saint-Louis, USA) or 6 M Guanidine (Sigma, Saint-328 Louis, USA). For non-crosslinked samples incubation times evaluated ranged from 5 min to 16 hours at room 329 temperature (18°C). After incubation and right before MP measurements, 2 μL of the solution were quickly 330 drop-diluted¹⁸ in an 18 μgL PBS droplet to 10-40 nM. All measurements were done immediately following the 331 droplet dilution. For the final optimized dMP protocol, denaturation was done in Urea 5.4 M for 5 min.

332 <u>MP Data processing</u>: Data were processed using the DiscoverMP software (Refeyn Ltd, Oxford, UK). Obtained 333 distribution histograms represent the number of counts per contrast value (or per mass after calibration). To 334 obtain the average masses, peak width and number of counts for each mass distribution, a Gaussian fitting 335 was performed by integrating each distributions at its half-height. Relative amounts of each oligomer were 336 calculated using the number of counts under the Gaussian fit curve of each distribution. For figures, Kernel 337 Density Estimate (KDE) was applied to transform the histogram into a curve.

338

339 Calculation of the global inter-protein cross-linking reaction efficiency (Eff_{XL}).

- 340 Global inter-XL efficiency was calculated using number of counts after Gaussian fitting of each oligomeric
- 341 state distribution (example of calculation in **Table S4**). This value represent the efficiency of XL reaction to
- 342 stabilize inter-protein interactions, i.e. all oligomeric states > 1 remaining after denaturation. Inter-XL
- 343 efficiency does not discriminate specific interactions of unspecific aggregation.
- 344 $Global XL efficiency = \overline{\left(\frac{\sum S_{oligomers > 1}}{\sum S}\right) * 100 = \% \pm SD}$

345 Equation 1. $\sum S_{oligomers>1}$ is the sum of all populations with oligomeric states > 1; $\sum S$ is the sum of all counts for masses > 346 30 kDa

347

348 Calculation of the complex stabilization factor (SF_{XL}).

The non-XL native measurements were used as a reference to obtain the proportion represented by the complex to be XL-stabilized in the sample. Then, we similarly calculated the proportion of this complex among total counts of the cross-linked denatured sample. Using these two values, the complex stabilization factor can be calculated (example of calculation in Table S5). This value expresses the amount of native complex that could effectively be XL-stabilized in XL samples (Eq. 2). A factor value of 1 correspond to the stabilization of all the native complex after XL reaction. Value > 1 expresses an enrichment of the complex upon XL reaction. Stabilization factor should be ideally \ge 1.

356

$$Complex stabilization factor = \overline{\left(\frac{S_{complex}(XL)}{\sum S(XL)} / \frac{S_{complex}(CT)}{\sum S(CT)}\right)}$$

357Equation 2. $S_{complex}$ is the integrated number of counts corresponding to the complex in both the cross-linked dMP XL358sample (XL) and nMP non-XL sample (CT); $\sum S$ is the sum of all integrated oligomeric populations in the cross-linked dMP359XL sample (XL) and nMP non-XL reference sample (CT).

360

361 DATA AVAILABILITY

362 XL-MS datasets are deposited in the ProteomeXchange Consortium via the PRIDE³⁷ partner repository under 363 the number PDX....

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377 AUTHOR CONTRIBUTIONS

- 378 H.G.F performed the experiments. H.G.F., O.H, S.C conceived the study. S.C supervised the work. H.G.F., O.H,
- 379 S.C wrote the manuscript. M.E.C. carried out the cloning of the domains of SPAG1 and PIH1D2. P.E.S. carried

- out the expression and the purification of R2SP complex. P.E.S, M.E.C., B.C., T.M.B. and X.M. proofread the
- 381 manuscript.

382 COMPETING INTERESTS

- 383 The authors declare no competing financial interest.
- 384
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464

- 465
- 466 FIGURES



467

Figure 1. Schemes representing two MP-based assays used during dMP method development. A) Evaluation of of
 denaturing agents compatibility with MP measurements, B) Optimized general workflow for dMP analysis.



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Figure 2. Optimization of denaturation step for dMP. Mass distributions, represented as probability density (KD), show the evolution of oligomeric states abundances. ADH denaturation in urea A) or in guanidine HCl B) ; GLDH denaturation in urea C) or in guanidine HCl D) ; 60S proteasome denaturation in urea E) or in guanidine HCl F). For each protein and condition, the % of monomeric species are indicated with similar color-code as their respective time point.

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Figure 3: Benchmarking of dMP vs SDS-Page for cross-linking optimization. Mass distributions are represented as probability density
 (KD). Following samples were cross-linked with increasing molar excesses of DSBU: A) nMP of non-cross-linked ADH, dMP and SDS PAGE results of ADH cross-linking. B) nMS of non-cross-linked GLDH, dMP and SDS-PAGE results of GLDH crosslinking C) nMS of non cross-linked 20S proteasome, dMP and SDS-PAGE results of 20S proteasome crosslinking. Bar plots show calculated dMP-calculated
 percentages of oligomeric populations for each concentration of DSBU.

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Figure 4: dMP screening of cross-linking conditions on ADH, GLDH and 20 proteasome. A) *Effect of cross-linking reagent (size, flexibility) on oligomeric states stabilized, measured in dMP*: presented results are probability densities (KD) of GLDH samples crosslinked with increasing molar ratios of PhoX, DSAU, DSBU. B) *dMP-based quantitative results of XL condition screening for ADH, GLDH and 20S complexes.* Bar charts represent the dMP-calculated global inter-XL efficiency (% ± SD) for each complex and XL condition (25/100/400:1 cross-linker:complex molar ratio). Plain dots represent the complex stabilization factor for each complex and XL condition. The black dash line corresponds to the stabilization factor value of 1 indicating a complex abundance similar to the native sample.



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Figure 5: dMP results of XL reaction optimization for R2SP complex. dMP profiles of R2SP cross-linked with increasing molar excesses of A) PhoX, B) DSAU, C) DSSO, D) DSBU. E) Quantitative results of R2SP XL optimization. Bar charts represent the dMP-calculated global inter-XL efficiency (% ± SD) for each complex and XL condition. Plain dots represent the complex stabilization factor for each complex and XL condition. The black dash line corresponds to the stabilization factor value of 1 indicating a complex abundance similar to the native sample.

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