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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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4 Hugo Gizardin-Fredon^{1,2}, Paulo E. Santo^{3,4}, Marie-Eve Chagot⁵, Bruno Charpentier⁵, Tiago M. Bandejas^{3,4},
5 Xavier Manival⁵, Oscar Hernandez-Alba^{1,2}, Sarah Cianféroni^{1,2,*}

6

7 ¹ Laboratoire de Spectrométrie de Masse BioOrganique, IPHC UMR 7178, Université de Strasbourg, CNRS,
8 67000 Strasbourg, France

9 ² Infrastructure Nationale de Protéomique ProFI – FR2048, 67087 Strasbourg, France

10 ³ iBET, Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2781-901 Oeiras, Portugal

11 ⁴ Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Av. da República,
12 2780-157 Oeiras, Portugal

13 ⁵ IMoPA, CNRS, Université de Lorraine, Nancy F-54000, France

14

15 *Corresponding author: sarah.cianferani@unistra.fr

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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 in-
25 solution quantification of all coexisting XL species, including sub-complexes and non-specific XL aggregates,
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-established
38 atomic-resolution biophysical techniques such as X-ray crystallography (XRC)¹, nuclear magnetic resonance
39 (NMR)², and electron microscopy (EM)³ have boosted structural biology, several limitations (requirement of
40 highly purified systems, extensive sample preparation protocols, production of high-quality crystals or cryo-
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
43 mass spectrometry approaches nicely complement the classical structural biology toolbox^{4,5}. Structural MS is
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
46 labelling proteomics-based technique that has drastically progressed this last decade from *in vitro*⁷, to
47 proteome-wide⁸ or even *in-situ* and *in-cellulo* applications⁹⁻¹¹. 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.

61 Mass photometry (MP), that has been recently introduced as a single-molecule biophysical technique¹³
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
65 between the scattered light and reflected light of biomolecules in solution upon irradiation with a visible laser
66 light¹⁴⁻¹⁶. As the contrast intensity linearly scales with the mass, MP can serve to estimate masses of
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
69 extensive sample preparation (e.g. buffer exchange)¹⁷, iv) low sample quantity requirements (pM-nM

70 concentration range, compared to μM range for nMS), v) a broad mass range (30 kDa to 5 MDa)^{17,18} and iv)
71 single molecule detection which enables to relatively quantify the detected populations leading to the
72 possibility to estimate affinity constants in the nM- μM concentration range^{19,20}. Although nMS mass accuracy
73 is unmatched compared to MP, nMP has already emerged as a valuable asset to characterize highly
74 heterogeneous complexes²¹, membrane proteins solubilized with different types of membrane mimics^{18,22,23},
75 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
82 reference protein complexes of increasing sizes and complexities as proof-of-concept and dMP performance
83 assessment. We then evaluated our dMP protocol for XL reaction monitoring and benchmarked it against the
84 reference gold standard denaturing SDS-PAGE gel method. Due to its single molecule detection capabilities,
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
95 and quaternary structures²⁷⁻²⁹, along with the H₂O/ACN/FA mix (50/50/1) classically used in intact MS mass
96 measurement in denaturing conditions. The H₂O/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
99 signal using “protein-free” droplets (**Fig. 1A**) by monitoring three output indicators (signal-*Si*, sharpness-*Sh*
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
102 be as low as possible and always <0.05 % to avoid extensive peak broadening¹⁷. *Sh* refers to the level of detail
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 ≥ 5 % at lower concentrations
111 (**Table S1**). Similarly, *Br* increases at lower denaturing agent concentrations with an optimal value of ~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- μ M concentration range, an
116 approximately 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
120 proteasome) either to assess mass precision, accuracy and peak broadening in dMP (BSA) or to further
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 (2σ , 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

140 perfectly superimposable dMP spectra (**Fig. S1 B**)), demonstrating that no significant protein refolding will
141 occur within the timeframe of dMP analysis (typically 1 min).

142 To conclude, we have developed and optimized a fast (5 min), efficient (> 95 % denaturation) and non-
143 reversible (in the timeframe of dMP measurements) denaturation protocol compatible with MP analysis,
144 which will be further referred to as “denaturing MP protocol” (dMP). This workflow consists of a first step of
145 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
146 right before dMP analysis (**Fig. 1B**). Obtained dMP measurements quality along with mass accuracies and peak
147 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 ≥ 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.

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

176 aggregates. Altogether dMP, that allows single-molecule sensitivity, proved to be more precise, quantitative
177 and 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)
183 that gains popularity for both *in vitro* and *in vivo* XL-MS studies^{6,30}. For GLDH, obtained dMP mass distributions
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-oligomeric 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**)
212 complex constituted of RuvB-Like1 without the DII_{ext} (R1ΔDII, R1) and RuvB-Like2 without the DII_{ext} (R2ΔDII,
213 R2)³¹, SPAG1₆₂₂₋₉₂₆ (S') and PIH1D2₂₃₁₋₃₁₅ (P')^{32,33}. We compared 4 amine-reactive cross-linkers⁶, including PhoX
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
217 a progressive stabilization of sub-complexes with increasing cross-linker concentration of PhoX (Eff_{XL} ~31-35%,
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).
225 Altogether, our results demonstrate that dMP affords unmatched performances for fast and rational screening
226 for optimal XL conditions (e.g. reagent, reaction length, temperature, buffer, etc.). Our dMP results highlight
227 that DSBU appears to outperform DSAU, PhoX, and even DSSO for inter-protein XL on our biological systems.
228 This reagent provides the best compromise between stabilization of the covalent-XL-assembly of interest (SF_{XL}
229 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
244 chemical reagents drive the stabilization of XL-species and iv) MS-cleavable DSBU and DSSO might be best
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”
247 yield of the chemical XL reaction ($< 10\%$)³⁰, often correlated to the low number of identified XL dipeptides
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
252 the generation of non-specific XL resulting from “forced pairing” of distal amino acid sequences that might not
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
263 better XL efficiencies/stabilizations (Eff_{XL} , SF_{XL}) than other tested reagents, which is consistent with the 12.5 Å
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 data³⁴⁻³⁶.
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.

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

275

276 **MATERIALS AND METHODS**

277 **Stocks, reagents, and instruments.**

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

280

281 **Sample preparation and cross-linking reactions.**

282 Bovine Serum Albumin (BSA, Sigma, Saint-Louis, USA), Alcohol dehydrogenase from baker's yeast (ADH, Sigma,
283 Saint-Louis, USA) and L-Glutamate dehydrogenase from bovine liver (GLDH, Sigma, Saint-Louis, USA) were
284 diluted to 1mg/mL in Gibco™ phosphate buffer saline (PBS, Life technologies Corporation, NY, USA), pH 7.4.
285 Human 20S proteasome (20S, South Bay Bio, San Jose, USA) was diluted to 1mg/mL in 50 mM HEPES, 100 mM
286 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
288 ratio of 1:4, respectively. The mixed complexes were incubated over night at 4 °C and the formed R2SP
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,
291 pH 8³¹ prior to cross-linking. For cross-linking (XL) reactions, aliquots of XL reagents were freshly diluted in
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.

298 XL reactions were carried for all samples at room temperature (18°C) for 45 min, and quenched with Tris HCl
299 (15 mM final concentration) for 20 min. A 1.5 µg aliquot of each non-XL control and XL sample was kept for
300 SDS-PAGE migration.

301

302 **SDS-PAGE separation of cross-linked samples.**

303 All cross-linked proteins and complexes were migrated on in-house 12% acrylamide denaturing SDS-PAGE gels
304 (1.5 mm thickness). Volume corresponding to 1.5 µg of each XL sample (and non-XL controls) was diluted (1:1)
305 with 2x concentrated Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.01% bromphenol blue
306 and 0.125 M Tris HCl) and incubated 5 min at 95°C. After sample loading, gels were migrated at 50 V for 20
307 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 %
308 phosphoric acid, 50 % ethanol), washed 3x20min with milli-Q water and stained overnight with Coomassie
309 Brilliant Blue (G250, Sigma, Saint-Louis, USA). They were finally rinsed 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 µm, 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 (CultureWell™, 50-3 mm DIA x 1mm Depth, 3-10 µL, 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 µL 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 Denaturing MP (dMP): 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 µL 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 MP Data processing: 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.

$$344 \quad \text{Global XL efficiency} = \left(\frac{\sum S_{oligomers > 1}}{\sum S} \right) * 100 = \% \pm SD$$

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}).

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

356
$$\text{Complex stabilization factor} = \left(\frac{S_{\text{complex}}(XL)}{\sum S(XL)} / \frac{S_{\text{complex}}(CT)}{\sum S(CT)} \right)$$

357 **Equation 2.** S_{complex} is the integrated number of counts corresponding to the complex in both the cross-linked dMP XL
358 sample (XL) and nMP non-XL sample (CT); $\sum S$ is the sum of all integrated oligomeric populations in the cross-linked dMP
359 XL 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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372 AUTHOR INFORMATION

373 Corresponding Author

374 *Sarah Cianféroni. Phone : +33 (0)3 68 85 26 79 ; Email: sarah.cianferani@unistra.fr; Fax: +33 (0)3 68 85 27

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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

380 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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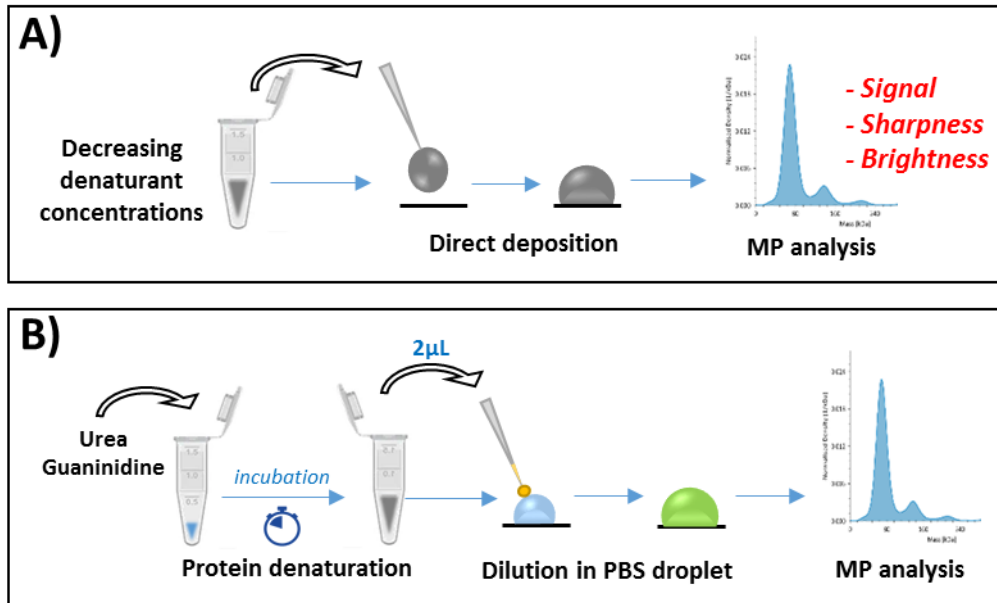
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464
465
466

FIGURES



467

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

470

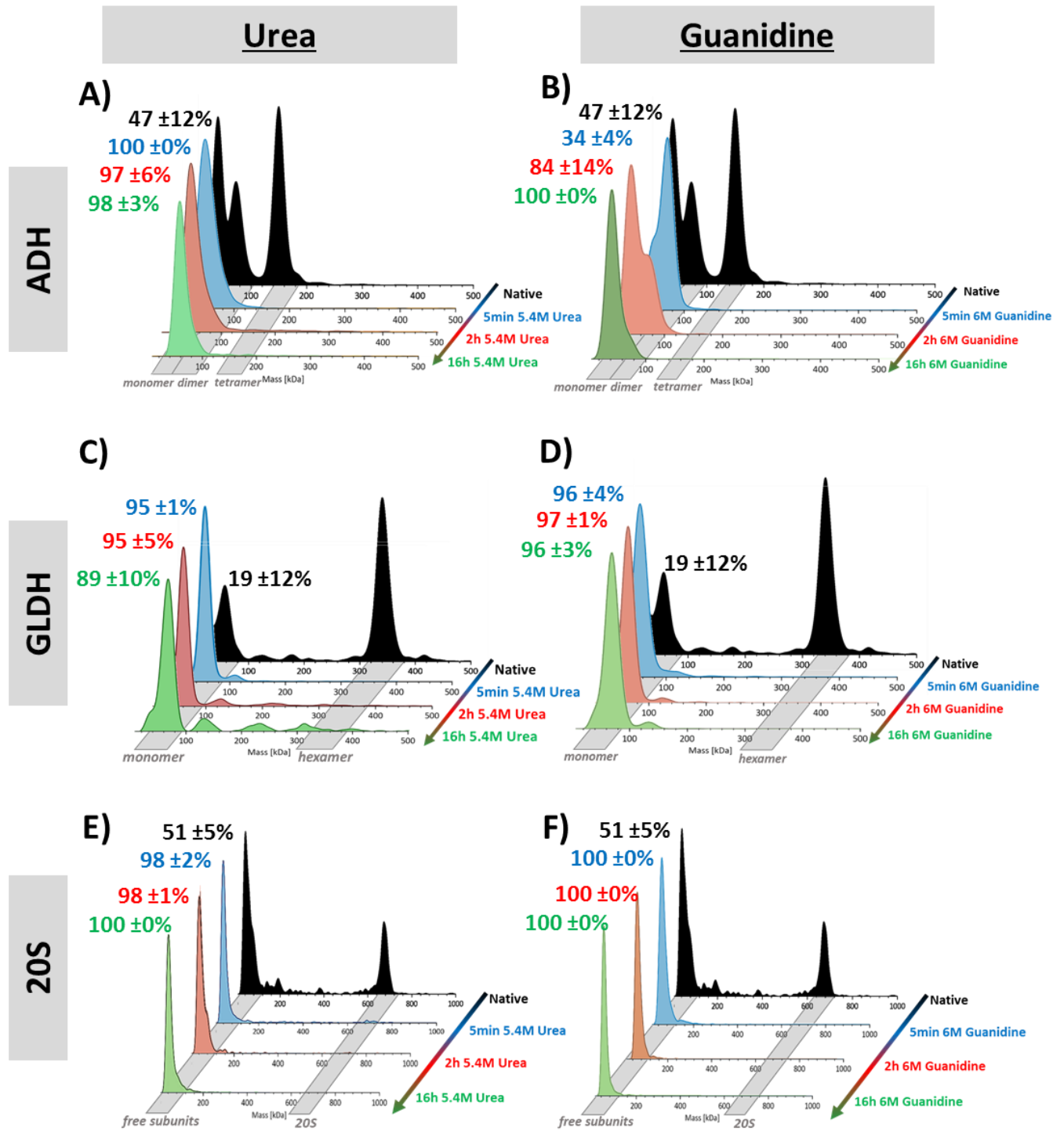
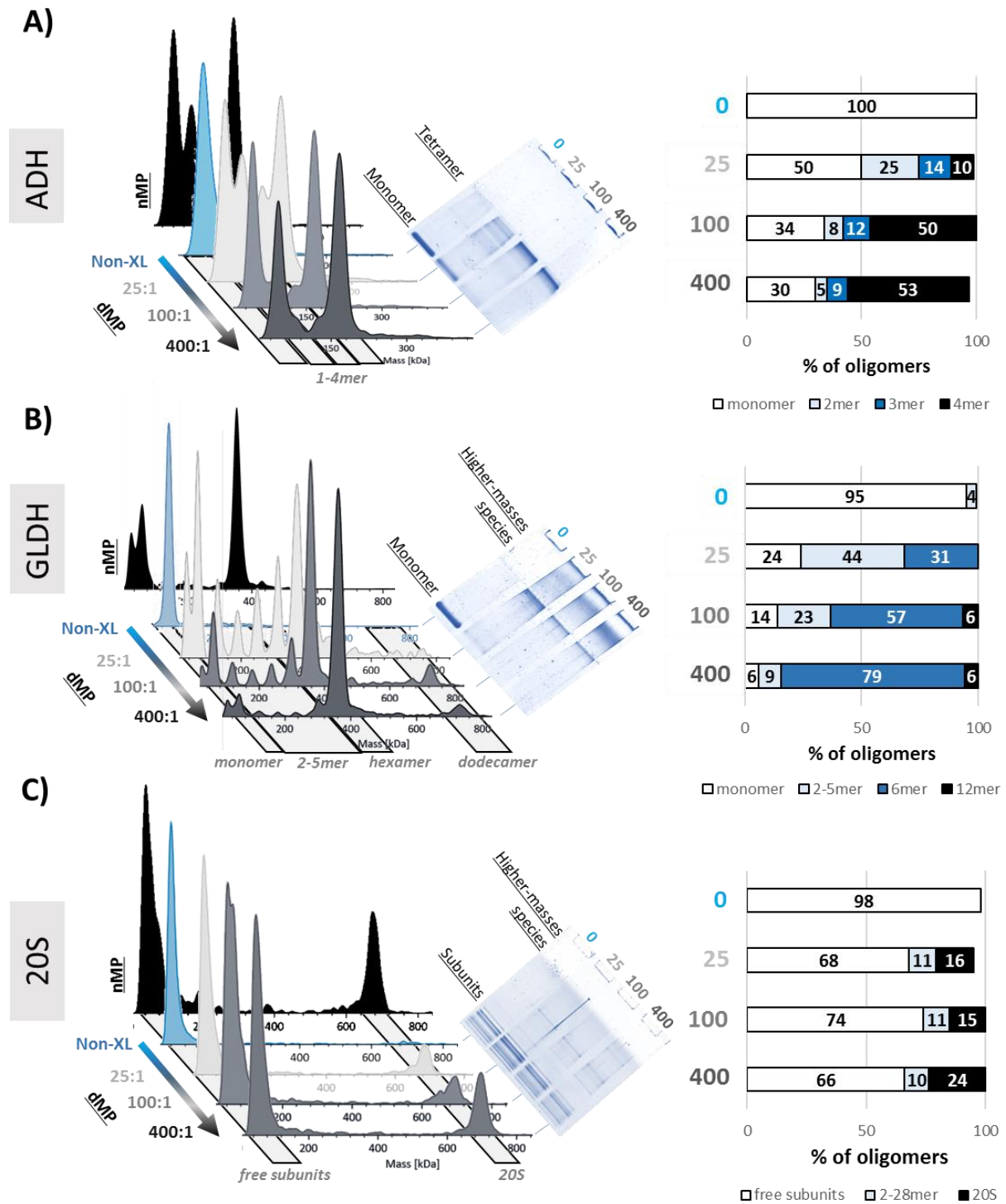


Figure 2. Optimization of denaturation step for dMP. Mass distributions, represented as probability density (KD), show the evolution of oligomeric states abundances. ADH denaturation in urea **A)** or in guanidine HCl **B)** ; GLDH denaturation in urea **C)** or in guanidine HCl **D)** ; 60S proteasome 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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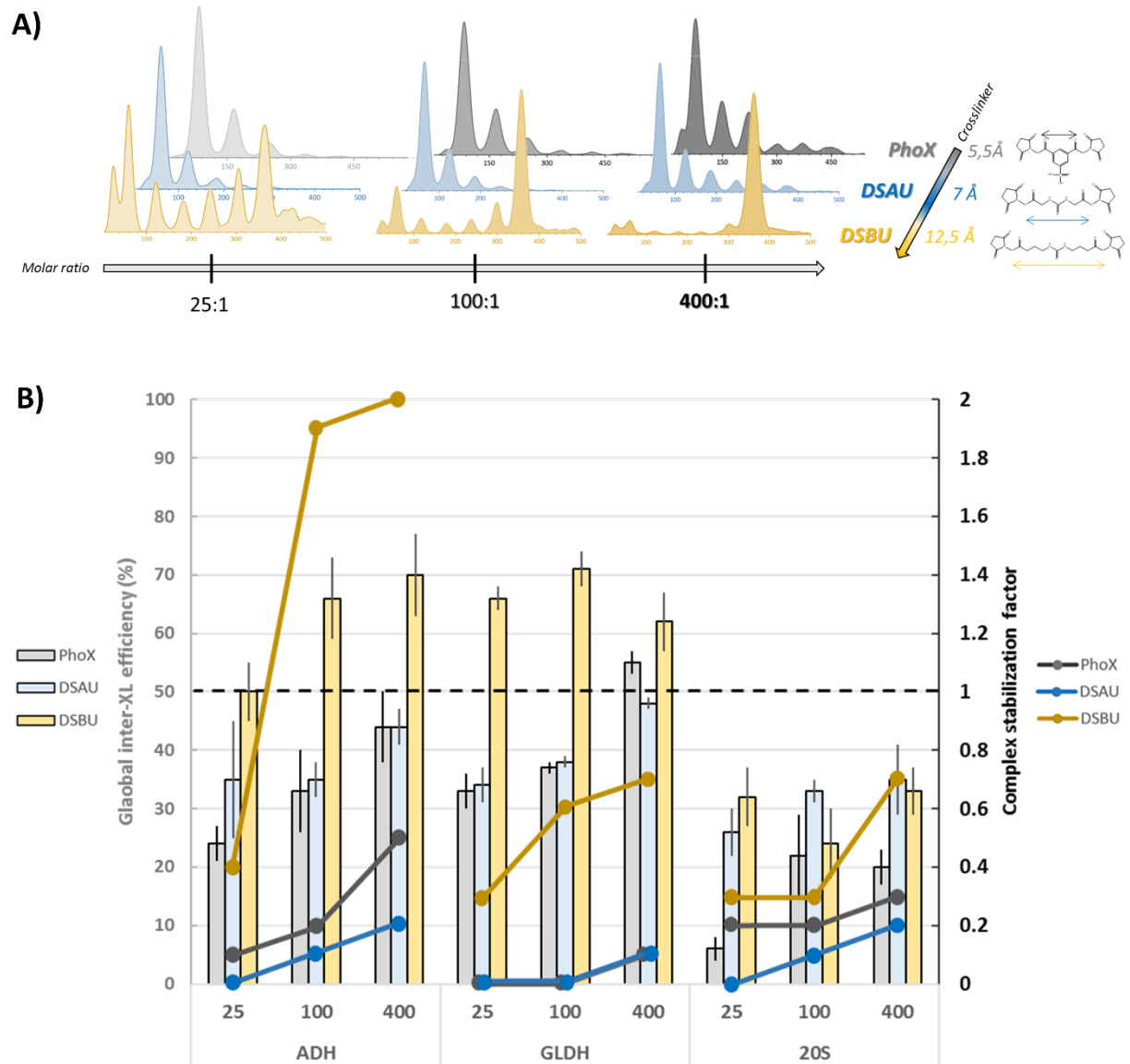


474

475 **Figure 3: Benchmarking of dMP vs SDS-Page for cross-linking optimization.** Mass distributions are represented as probability density
 476 (KD). Following samples were cross-linked with increasing molar excesses of DSBU: **A)** nMP of non-cross-linked ADH, dMP and SDS-
 477 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-
 478 cross-linked 20S proteasome, dMP and SDS-PAGE results of 20S proteasome crosslinking. Bar plots show calculated dMP-calculated
 479 percentages of oligomeric populations for each concentration of DSBU.

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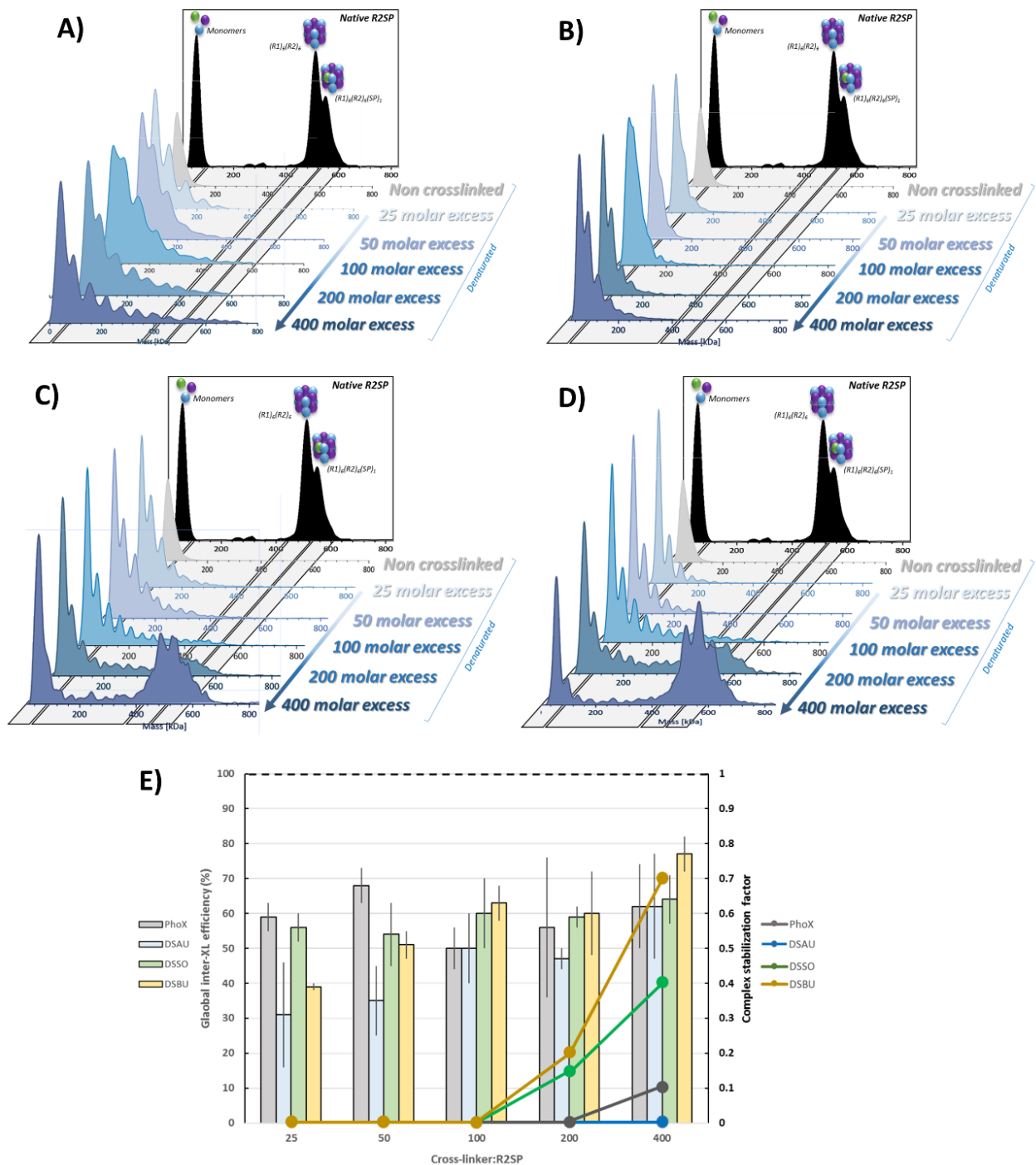
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483 **Figure 4: dMP screening of cross-linking conditions on ADH, GLDH and 20 proteasome. A)** Effect of cross-linking reagent (size,
 484 flexibility) on oligomeric states stabilized, measured in dMP: presented results are probability densities (KD) of GLDH samples cross-
 485 linked with increasing molar ratios of PhoX, DSAU, DSBU. **B)** dMP-based quantitative results of XL condition screening for ADH, GLDH
 486 and 20S complexes. Bar charts represent the dMP-calculated global inter-XL efficiency (% ± SD) for each complex and XL condition
 487 (25/100/400:1 cross-linker:complex molar ratio). Plain dots represent the complex stabilization factor for each complex and XL
 488 condition. The black dash line corresponds to the stabilization factor value of 1 indicating a complex abundance similar to the native
 489 sample.

490



491

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

497

498