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1 **TITLE**

2 **Denaturing mass photometry for rapid optimization of chemical protein-protein**
3 **cross-linking reactions**

4
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19

20 **ABSTRACT**

21 Chemical cross-linking reactions (XL) are an important strategy for studying protein-protein interactions (PPIs),
22 including low abundant sub-complexes, in structural biology. However, choosing XL reagents and conditions
23 is laborious and mostly limited to analysis of protein assemblies that can be resolved using SDS-PAGE. To
24 overcome these limitations, we develop here a denaturing mass photometry (dMP) method for fast, reliable
25 and user-friendly optimization and monitoring of chemical XL reactions. The dMP is a robust 2-step protocol
26 that ensures 95 % of irreversible denaturation within only 5 min. We show that dMP provides accurate mass
27 identification across a broad mass range (30 kDa-5 MDa) along with direct label-free relative quantification of
28 all coexisting XL species (sub-complexes and aggregates). We compare dMP with SDS-PAGE and observe that,
29 unlike the benchmark, dMP is time-efficient (3 min/triplicate), requires significantly less material (20-100x)
30 and affords single molecule sensitivity. To illustrate its utility for routine structural biology applications, we
31 show that dMP affords screening of 20 XL conditions in 1 h, accurately identifying and quantifying all coexisting
32 species. Taken together, we anticipate that dMP will have an impact on ability to structurally characterize
33 more PPIs and macromolecular assemblies, expected final complexes but also sub-complexes that form *en*
34 *route*.

35 INTRODUCTION

36 Vast majority of biological processes that drive life depend on formation of specific protein-protein interactions
37 (PPIs). Thus, characterizing PPIs has been one of the cornerstones of structural biology for decades, and many
38 structures of macromolecular assemblies are now available and allow detailed analysis of factors that
39 determine interactions between macromolecules. However, many physiologically relevant PPIs form only
40 transiently, and these have been notoriously difficult to capture and subject to structural analysis. Protein
41 cross-linking (XL) uses chemical reagents to introduce covalent bonds between residues from two or more
42 proteins that are in close proximity, *i.e.* involved in a PPI interface, and is a routinely used strategy to overcome
43 this challenge¹⁻⁶. For example, XL is frequently used before electron microscopy (EM) to stabilize complexes or
44 targeted conformations, and in mass spectrometry workflows (XL-MS) in order to identify regions of proteins
45 that are in close proximity. In general, the first step of the chemical XL protocol is of utmost importance as its
46 success drives the outcome of the biophysical measurement⁷. Therefore, the use of XL requires careful
47 optimization of XL conditions to ensure that PPIs are accurately captured and preserved throughout
48 subsequent structural analysis, and that generation of “biologically non-specific” XL aggregates is minimal⁸.
49 The optimization steps routinely include selection of the XL reagent, among dozens available, and screening XL
50 reaction conditions that are often dependent on the specific PPI under investigation, resulting in time-
51 consuming and fastidious nature of these steps. For both XL-MS⁹ and EM sample preparation, denaturing
52 sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) is recommended to monitor and
53 optimize XL conditions, as it allows visualization of high molecular weight (MW) cross-linked species and
54 concomitant vanishing of the bands corresponding to individual protein partners. Although SDS-PAGE is widely
55 available, easy to use, and affordable, this method: i) allows only rough estimate of MWs; ii) is not suitable for
56 analysis of high MW complexes as they don’t enter the gel; iii) is time consuming (gel casting, sample
57 denaturation, gel migration, staining); and iv) is intrinsically low throughput. Moreover, given these limitations
58 of SDS-PAGE, outcomes of XL reactions are often obtained only after additional biophysical evaluation, further
59 complicating the process. Therefore, more accurate and rapid methods to identify the covalently-stabilized
60 species generated upon chemical XL represent a major need for the field.

61 Mass photometry (MP) is an emerging single-molecule biophysical technique¹⁰ that operates under native
62 conditions (nMP) to allow analysis of protein complexes recalcitrant to native mass spectrometry (nMS), *i.e.*
63 “nMS-resistant” protein complexes¹⁰⁻¹³. MP is based on the principle of the interferometric scattering
64 microscopy (iSCAT) that uses the contrast generated as a result of the destructive interference between the
65 scattered light and reflected light of biomolecules in solution upon irradiation with a visible laser light¹⁴⁻¹⁶. As
66 the contrast intensity linearly scales with the mass, MP can serve to estimate masses of biomolecules after
67 proper calibration with reference molecules. Importantly, MP analysis requires simple sample preparation,
68 takes only minutes to complete using small amounts of sample (100 pM – 100 nM) without prior buffer
69 exchange, displays broad mass range (30 kDa to 5 MDa), and yet allows multiplexing and automation^{11,17}.

70 Additionally, single molecule detection enables relative quantification of detected populations, which can be
71 used to estimate affinity constants in the nM- μ M concentration range^{18,19}. Although nMS mass accuracy
72 remains superior, nMP has emerged as a valuable asset for characterizing highly heterogeneous complexes²⁰,
73 membrane proteins solubilized with different types of membrane mimics^{11,21,22}, ribosomes¹², and viral
74 capsids^{13,23}. In addition to analyzing native-like complexes, nMP was recently used in combination with
75 chemical cross-linking (XL) to verify stabilization of an XL-ed oligomer before EM experiments²⁰. These
76 advantages of nMP and compatibility with chemical XL conditions, suggest that developing a denaturing MP
77 (dMP) technique may offer opportunities for improved and rapid optimization of chemical XL reaction
78 conditions, and overcome current limitations of SDS-PAGE.

79 In this work, we develop an accurate and robust single-molecule protocol to perform MP analysis in denaturing
80 conditions. We first use reference protein complexes of increasing sizes and complexities as proof-of-concept,
81 and for dMP performance assessment. We then evaluate our dMP protocol for XL reaction monitoring and
82 benchmark it against the reference gold standard denaturing SDS-PAGE gel method. Due to its single molecule
83 detection capabilities, dMP is more precise than conventional SDS-PAGE analysis, providing accurate mass
84 identification as well as relative quantification of all coexisting XL-ed species. Thus, dMP represents an
85 improved technique to monitor and optimize XL reactions through large screens, as we illustrate here using
86 XL-MS study of R2SP complex interactions.

87

88 RESULTS

89 Development of a denaturing mass photometry (dMP) protocol

90 To develop a fast, efficient and non-reversible denaturation protocol while maintaining good quality MP signal
91 intensities, we optimized several sample preparation parameters. First, we focused on the choice of the
92 denaturing agent and examined effects of urea and guanidine hydrochloride (GdnHCl), two well-known and
93 widely used protein denaturants²⁴⁻²⁶, as well as H₂O/ACN/FA mix (50/50/1), which is classically used in intact
94 MS mass measurement under denaturing conditions. We observed that H₂O/ACN/FA was not compatible with
95 stabilization of MP droplets, and was not pursued further. We next assessed the impact of urea/GdnHCl
96 solutions on the quality of MP signal using “protein-free” droplets (**Fig. 1a**) by monitoring three output
97 indicators (signal-*Si*, sharpness-*Sh* and brightness-*Br*) that reflect the quality of the MP images/frames. In
98 general, *Si* reports on the level of activity in each frame, which can be due to either protein binding, or
99 contaminants/salts/surfactants presence, and its values should be as low as possible (<0.05 %) to avoid
100 extensive peak broadening¹⁷. *Sh* refers to the level of detail visible in each frame, which impacts the ability to
101 find and maintain the good focusing position, and its value should be as high as possible. Finally, *Br*
102 characterizes the amount of light available in images, and its value should be maximized to avoid peak
103 broadening. With the aim to reach similar MP signal quality as the one reached in Phosphate Buffer Saline
104 (PBS) droplets (*Si* ~0.03 %, *Sh* ~5 %, *Br* ~73 %), we used the “buffer-free” focusing mode to directly analyze

105 protein-free droplets containing decreasing concentrations of urea or GdnHCl (from 5.4 to 0.4 M).
106 Independently of the denaturing agent, *Sh* increased progressively from 1-3 % at 5.4 M of urea/GdnHCl to ≥ 5
107 % at lower concentrations (**Supplementary Table 1**). Similarly, *Br* increased at lower denaturing agent
108 concentrations with an optimal value of ~ 67 % obtained at 0.8 M urea/0.4 M GdnHCl. *Si* values were < 0.05 %
109 for all tested concentrations lower than 5.4 M. Altogether, our “protein-free” blank MP acquisitions allowed
110 establishing the optimal concentrations of denaturing agents (< 0.8 M of urea or GdnHCl). As XL reactions are
111 typically conducted in the nM- μ M protein concentration range, these samples need to be diluted
112 (approximately 10x dilution in PBS) prior to dMP measurements. That means that the initial concentration
113 of urea or GdnHCl during the denaturing reaction can be set at 5.4 M and 6 M, respectively, without exceeding
114 the 0.8 M concentration limit in the final droplet.

115 To further optimize our dMP protocol, we used reference protein complexes (BSA, ADH, GLDH, 20S
116 proteasome) to either assess mass precision, accuracy and peak broadening in dMP (BSA), or to further
117 optimize the denaturation step (ADH, GLDH and 20S proteasome). After Gaussian-fitting of MP histograms,
118 the mean mass (μ) and half-height peak width (2σ , FWHM) of the Gaussian fits were used to evaluate mass
119 accuracy and peak broadening, respectively. Considering dMP triplicate measurements, the measured mass
120 of BSA oligomers denatured in urea and GdnHCl was the same as the one obtained using nMP measurements
121 (**Supplementary Table 2**). In addition, our denaturation protocol does not alter MP variability between
122 replicates, with SDs ≤ 3 kDa and ≤ 5 kDa for BSA monomers and dimers, respectively. Finally, denaturation
123 only slightly affected peak broadening (FWHM between 8-10 kDa and 8-14 kDa, compared to 8 kDa in nMP for
124 monomers and dimers, respectively), demonstrating that dMP performance was comparable to nMP.

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

137 To conclude, we have developed and optimized a fast (5 min), efficient (> 95 % denaturation) and non-
138 reversible (in the timeframe of dMP measurements) denaturation protocol compatible with MP analysis,
139 which will be further referred to as “denaturing MP protocol” (dMP). This workflow consists of a first step of
140 denaturation (5 min in 5.4M urea) followed by 10x dilution of the denatured sample right before dMP analysis

141 (Fig. 1b). Obtained dMP measurements are of comparable quality with respect to mass accuracy and peak
142 width, as those obtained in classical nMP analysis.

143

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

145 We next benchmarked our dMP protocol against SDS-PAGE, the gold standard for XL reaction optimization,
146 using our reference systems: ADH tetramer (~145 kDa), GLDH hexamer (~313 kDa), and 20S proteasome 28-
147 mer (~700 kDa, see **Supplementary Figure 2** for nMP). We used disuccinimidyl dibutyric urea (DSBU; linker
148 size ~ 12.5 Å), one of the most used MS-cleavable cross-linker in XL-MS workflows as proof of concept. We
149 tested several XL:protein concentration ratios (25:1, 100:1, 400:1, 800:1 and 1000:1), as is commonly done
150 during XL reaction optimization. Comparing SDS-PAGE and dMP results for ADH side by side (**Fig. 3a**), showed
151 that SDS-PAGE provides only a rough visualization of the products and yields of the cross-linking reaction. In
152 contrast, dMP was able to detect monomers, dimers, trimers and tetramers at all concentration ratios, as well
153 as allow quantification based on the relative abundance of each species. We observed that the relative yields
154 of different assemblies (dimer vs. trimer vs. tetramer) varies with different amount of XL reagent used, as
155 expected (**Fig. 3a, 3b, Supplementary Figure 3**). The tetrameric species formation maxed out at about 70 ± 11
156 % of total counts at 800 molar excesses, although even at 100:1 DSBU:ADH yields of tetramer approached this
157 limit. No non-specific high-mass aggregates were detected either using SDS-PAGE or dMP, and both methods
158 suggested that optimal conditions were around 100:1 DSBU:ADH.

159 For GLDH, the difference in performance between SDS-PAGE and dMP was even more pronounced as only
160 one broad band in the loading-well was observed on the SDS-PAGE gel at high masses regardless of the amount
161 of DSBU used (**Fig. 3c and 3d**). This highlights the key disadvantage of SDS-PAGE as high-mass oligomers of
162 GLDH do not enter the gel, and as a consequence, neither clear identification nor quantification of GLDH
163 degree of oligomerisation is possible from SDS-PAGE results. On the other hand, dMP clearly resolved all GLDH
164 coexisting oligomeric forms and allowed quantitative monitoring of the progressive stabilization of higher
165 oligomeric states as a function of increasing XL concentrations. We observed that, as expected, as hexamer
166 abundance increased from at 17 ± 5 % to 61 ± 11 %, the concentration of intermediary sub-complexes
167 (combination of 2-mer, 3-mer, 4-mer, 5-mer) decreased from 35 ± 5 % to 7 ± 1 % from low (25x) to high (1000x)
168 DSBU molar excesses, with the small amount of dimer of hexamer (12-mer) forming at a 1000 DSBU:GLDH
169 ratio (4 ± 1 %). Lastly, the superior performance of dMP was even more obvious for cross-linked 20S
170 proteasome (28 subunits), as SDS-PAGE was not able to detect the final product, unlike dMP that showed clear
171 formation of the 20S proteasome (**Fig. 3e and 3f**). Thus, dMP unambiguously detected the ~700 kDa covalently
172 stabilized intact 20S proteasome under all XL conditions (12 ± 1 % of total abundance at 25:1 DSBU:20S ratio,
173 to 44 ± 7 % at 800:1), as well as very low abundance ~170 kDa 7-mer (~1% abundance in all conditions). Taken
174 together, these results indicate that dMP outperforms SDS-PAGE in terms of mass accuracy, resolution and

175 broader mass range (30 kDa-5 MDa for our MP instrument). Furthermore, dMP allowed accurate relative
176 quantification of all coexisting species.

177

178 **dMP for quantitative evaluation of XL reaction performances**

179 We next evaluated the versatility of dMP to screen for optimal chemical XL reaction using a variety XL reagents
180 used in XL-MS workflows or for EM grid preparations. To enable quantitative assessment of the method, we
181 defined two performance parameters: i) total inter-protein XL reaction efficiency Eff_{XL} (Material and method
182 **Eq. 1**), as an indicator of the total amount of inter-protein cross-linking that occurred (all cross-linked species
183 beyond monomers); and ii) “specific” XL factor SF_{XL} (Materials and Methods **Eq. 2**), as an estimate of the
184 amount of the “specific” (*i.e.* expected main product) XL complex”. Eff_{XL} is thus an indicator for overall yield of
185 the XL reaction and accounts for both specific and non-specific XL species, whereas SF_{XL} focuses only on one
186 specific species that is the desired product (when already known). In general terms, Eff_{XL} would be a preferred
187 metric for analysis of samples where the exact stoichiometry of the final complex might be unknown, while
188 SF_{XL} would be more appropriate for complexes with known stoichiometries.

189 We first evaluated our dMP method for NHS-ester chemical reagents classically used in XL-MS experiments:
190 DSBU, DSAU (disuccinimidyl diacetic urea, linker size ~ 7.7 Å), and PhoX (linker size ~ 5.5 Å; less-flexible IMAC-
191 enrichable cross-linker that gained popularity for both *in vitro* and *in vivo* XL-MS studies^{1,27}). For GLDH, dMP
192 mass distributions were similar between PhoX and DSAU, both of which failed to yield hexamers even at higher
193 concentrations (**Fig. 4a**). Conversely, GLDH hexamers and monomers are detected as main components at
194 25:1 DSBU:GLDH ratio, and this trend is even more obvious at 100:1 and 400:1 XL:GLDH ratios (**Fig. 4a**). Similar
195 behaviors were also observed in dMP profiles of cross-linked ADH and 20S proteasome, with a significantly
196 higher yield of expected oligomers with DSBU (**Supplementary Figure 4**). For homo-oligomeric protein
197 complexes (ADH tetramer and GLDH hexamer), DSBU showed much higher Eff_{XL} values (60-70 %) compared to
198 DSAU (45-48 %) and PhoX (45-55 %) (**Fig. 4b**, bar charts), suggesting overall better XL efficiencies for DSBU.
199 For the large hetero-multiprotein 20S proteasome, trends were different as the Eff_{XL} decreased (max. ~ 35 %),
200 and none of the XL reagents performing as well. In all examples studied, SF_{XL} values increased as a function of
201 XL:complex ratio, with DSBU outperforming PhoX and DSAU (**Fig. 4b**, solid dots). Low SF_{XL} values combined
202 with good Eff_{XL} (26-48 %) translate DSAU abilities to generate more sub-complexes and lower amounts of
203 expected XL-stabilized ADH, GLDH, 20S proteasome tetramers, hexamers, 28-mer. Off note, it appears that
204 SF_{XL} values obtained for ADH and GLDH tend to plateau with increasing DSBU molar excess, which is not the
205 case for the 20S proteasome, composed of a higher number of subunits (28) compared to AHD (4) and GLDH
206 (6). Our dMP results on different biological systems highlight that DSBU efficiency to stabilize expected
207 complexes/oligomers is significantly higher (SF_{XL} values close to 1) than PhoX and DSAU. These increased
208 efficiencies go along a low abundance of sub-complexes and potential non-specific higher-masses
209 stabilization. Importantly, the screening of the 9 different XL conditions was achieved within 30 min using only
210 1.8 μ g of proteins.

211 Given that SDS-PAGE is also the gold standard for monitoring XL reaction before EM and cryoEM applications²⁸,
212 we tested a common XL reagent used for cryo-EM purposes, formaldehyde (FA, **Supplementary Figure 5**)³¹⁻³³.
213 We carried out XL experiments of GLDH and 20S proteasome with increasing concentrations of FA (0.05 % to
214 1 %, ~20-360 mM). Our results showed that FA starts to cross-link GLDH into hexamers at 0.2 % ($16 \pm 2\%$ of
215 total counts) and 20S proteasome 28-mer at 0.05 % ($12 \pm 1\%$ of total counts). FA maximally cross-links GLDH
216 and 20S proteasome at 1 % concentration with $25 \pm 2\%$ and $40 \pm 9\%$ of total counts, respectively. The
217 experiment took ~25 minutes to complete per complex, therefore highlighting how dMP can facilitate rapid
218 assessment of XL reaction yields prior to cryo-EM analysis. Taken together, these studies illustrate the power
219 of dMP to evaluate performance of different XL reagents and reaction conditions using small amounts of
220 sample, rapidly (in 40 minutes or less) and accurately (with mass resolution sufficient to distinguish between
221 a range of different XL species), which represents a major improvement over the performance of SDS-PAGE.

222 223 **Application of dMP in integrative structural biology: the R2SP case study**

224 To examine utility of dMP in a real-life scenario, we applied dMP to optimize XL conditions before MS analysis
225 of R2SP, a multicomponent protein complex that includes two hexamers of 3 AAA+ ATPases RuvB-Like1 (R1)
226 and 3 RuvB-Like2 (R2)³¹ each, 1 SPAG1 (S) molecule, and 1 PIH1D2 (P) molecule^{32,33}. The first nMP
227 measurement of the R2SP preparation allowed us to identify R1R2 hexamers at 513 ± 1.1 kDa ($42 \pm 10\%$ of
228 total counts, over triplicates, **Supplementary Figure 6**) coexisting with R2SP complexes at 549 ± 4 kDa ($28 \pm 10\%$
229 % of total counts, over triplicates – expected mass ~540 kDa, **Fig. 5**). We next used dMP to screen XL reaction
230 conditions for further XL-MS analysis. We screened four different cross-linkers, PhoX, DSAU, DSSO
231 (disuccinimidylsulfoxide, 10.3 Å linker length) and DSBU at 5 different molar excesses (25/50/100/200/400)
232 plus the control non-XL sample (see **Supplementary Figure 7** for complete dMP profiles). The complete
233 analysis of 20-conditions was completed within 1 hour using dMP (including triplicate measurements), in
234 comparison to SDS-PAGE which required 20 hours using in-house made SDS-PAGE (including gel casting,
235 sample preparation, migration, fixation, staining, and unstaining, **Supplementary Figure 8**). Furthermore, dMP
236 used lower amounts of biological materials than SDS-PAGE (~3 µg in total compared to ~24 µg, respectively).
237 Importantly, mass precision of dMP allowed us to completely resolve all the cross-linked oligomeric
238 populations, including the low abundant ones, which allowed us to map finer differences between different
239 XL reagents. Thus, longer/more flexible reagents (DSSO and DSBU) performed better than PhoX and DSAU,
240 resulting in high yields of ~540 kDa R2SP complex (> 100 molar excess, **Fig. 5a and 5b**), with $\text{Eff}_{\text{XL}} \sim 55-63\%$,
241 $\text{max. SF}_{\text{XL}} \sim 0.4$ for DSSO and $\text{Eff}_{\text{XL}} \sim 40-77\%$, $\text{max. SF}_{\text{XL}} \sim 0.7$ for DSBU (see **Fig. 5e**), with no significant over-XL
242 species formation. In contrast, PhoX and DSAU did not yield to R2SP stabilization (Fig. 5c and 5d), although
243 they did form sub-complexes with increasing concentrations of the XL reagent ($\text{Eff}_{\text{XL}} \sim 50-60\%$, $\text{max. SF}_{\text{XL}} \sim 0.1$
244 for PhoX, and $\text{Eff}_{\text{XL}} \sim 30-60\%$, $\text{SF}_{\text{XL}}=0$ for DSAU, **Fig. 5e**). Of note, performing a nMP measurement after XL
245 reaction confirmed that no significant denaturation resulted from the XL reaction (**Supplementary Figure 9**).

246 To conclude, dMP allowed unmatched rapidity for XL reaction condition screening in the real-life scenario
247 using R2SP assembly as an example.

248

249 To pursue characterization of R2SP structure further we used XL-MS, using DSBU as the XL reagent based on
250 the results of our screening experiments. Currently, no structures of R2SP complex exist, primarily due to the
251 difficulties in stabilizing this assembly. Thus, we conducted XL-MS experiments in triplicate on R2SP complex
252 at 25, 100, 400 molar excesses of DSBU. In general, the XL-MS experiments capture all cross-linked peptides,
253 both those resulting from intra- and inter-subunit cross-linking reactions. As shown in **Fig. 6**, we identified
254 similar numbers of “unique XL” (defined as those XLs present in at least 2 out of the 3 replicates) at 25:1 (94)
255 and 100:1 (97) molar excess ratios. The number of unique XLs identified at 400:1 molar excess ratio decreased
256 to 71, which might be explained by decreased digestion efficiencies due to lysine sites congestion and
257 difficulties for softwares to identify those highly modified peptides³⁴. Nevertheless, the reproducibility
258 (measured as the number of unique XL peptides detected in at least 2 out of 3 replicates) within all three
259 datasets was acceptable and varied from 52% at 25:1 to 61% for 400:1 DSBU:R2P molar ratio. In terms of
260 prevalence of intra- vs. inter-XLs, we observed that the relative % of unique inter-XLs identified increased with
261 increasing molar ratio of DSBU, from 43% at 25:1 to 51% at 400:1 (**Fig. 6a-6c**). Next, we mapped the identity
262 of validated unique XLs identified using different XL conditions onto different components of the R2SP
263 structure (**Fig. 6d-6g**). Overall, the overlap of validated unique XLs between the three conditions represents
264 52 XL peptides (41 % of total unique XLs, **Fig. 6g**), among which 50 % are inter-XLs. We also identified several
265 inter-XL specific to each condition: 4 RuvBL2-SPAG1 XLs in the 25:1 condition; 1 RuvBL1-RuvBL2 XL and 3
266 RuvBL2-SPAG1 XL peptide in the 100:1 condition; and 3 more RuvBL1-RuvBL2 inter-XLs at 400:1 DSBU:R2SP.
267 These results highlight the complementarity of XL interactions captured at low and high reagent
268 concentrations, as already reported in a proteome-wide study³⁵. Finally, when combining the results from all
269 three datasets, we identified 127 unique XLs (see **Supplementary Table 3**) that correspond to 57 inter-XL (41
270 R1-R2, 5 R1-SPAG1, 11 R2-SPAG1, 45 % of total XL peptides) and 70 intra-XL peptides (26 R1-R1, 21 R2R2, 23
271 SPAG1-SPAG1, 55 % of total XL peptides). In particular, our results suggest that the RPAP3 domain of SPAG1
272 interacts with both the DI domain of RuvBL1 and the DIII domain of RuvBL2. Additionally, the TPR3 domain of
273 SPAG1 appears to be in close proximity to the DIII domain of RuvBL1. We also plotted interactions between
274 R1 and R2 we identified in our analysis onto the available X-ray structure of the R1R2 complex (PDB:2XSZ), and
275 observed excellent agreement between measured and structure-predicted XLs, with 93% (11 intra-XLs, 14
276 inter-XLs) satisfying the maximal C α -C α distances of 30 Å (accepted range for DSBU). Taken together, the R2SP
277 example showcases how dMP can facilitate structural biology studies by rapidly screening cross-linking
278 reaction conditions and reagents. Given that many complexes in biology are transient and/or difficult to isolate
279 and stabilize for structural characterization, we expect that dMP-enabled XL reaction condition screening and
280 optimization will accelerate progress in this area.

281

282 **DISCUSSION**

283 We report here on the development of a denaturing MP (dMP)-based protocol for rapid and reproducible
284 screening of XL reaction conditions. The dMP approach consists of a fast and efficient denaturing protocol that
285 results in > 95 % denaturation of the chemically cross-linked sample within 5 minutes without altering the
286 quality of the MP measurements or affecting the cross-links. This step is followed by the MP data acquisition
287 that captures the mass distribution of the cross-linked species in the matter of minutes. The mass resolution
288 of dMP is sufficient to capture not only the final complex, but all the sub-complexes that form *en route* to the
289 ultimate one. Furthermore, the method yields information about the relative quantities of the multimers that
290 form during the cross-linking reactions, thus enabling more precise reaction monitoring and quantification.
291 Overall, when compared to SDS-PAGE, which is a preferred method for XL reaction quality control, dMP
292 provides more accurate mass estimations across a broader mass range (30 kDa to 5 MDa), and with
293 significantly higher sensitivity, thus enabling unambiguous detection of even low-abundance species. In
294 addition, dMP is much faster (20 XL reaction conditions can be screened in triplicate in 1 hour, compared to
295 several hours (2.5 to 20 hours) needed per single run of precasted or in-house made SDS-PAGE gels⁷), and uses
296 20 to 100-times less material (<1 ng, 0.1 pmol at 5 nM as routine conditions per triplicate) than SDS-PAGE (1-
297 5 µg, single run).^{7,37} Taken together, dMP strategy provides an unmatched increase in the speed and quality
298 of screening of XL conditions, as well as enables identification and relative quantification of all coexisting cross-
299 linked species with a single molecule sensitivity.

300 As cross-linking reactions are widely used in structural biology to stabilize complexes prior to their biophysical
301 and structural characterization, we expect that dMP will significantly expand the number and type of samples
302 that can be analyzed, thanks to its high sensitivity, high speed, and high mass-accuracy. We used dMP to
303 rapidly screen multiple XL reaction conditions and XL reagents, and used two new type of metrics to assess XL
304 efficiencies/stabilizations (Eff_{XL} , SF_{XL}). We showed that longer and more flexible XL reagents, such as DSBU,
305 facilitate cross-linking more effectively compared to less flexible and smaller DSAU and PhoX³⁸⁻⁴⁰. Conversely,
306 less flexible XL reagents with smaller sized spacer arms lead to stabilization of sub-complexes instead of intact
307 complexes/oligomers. In addition, we showed that dMP-based method for XL reaction condition screening,
308 rapidly identified optimal conditions for cross-linking of R2SP, a protein complex that has eluded structural
309 characterization. Using dMP in combination with XL-MS, yielded new insights into inter-subunit contacts
310 between components of R2SP, including a previously unknown interface between RPAP3 domain of SPAG1
311 and DI domain of RuvBL1 and the DIII domain of RuvBL2, as well as the TPR3 domain of SPAG1 and DIII domain
312 of RuvBL1. Although these insights remain to be validated using orthogonal strategies, they illustrate the
313 power of dMP to accelerate XL-MS and cryo-EM workflows, and illuminate previously inaccessible features of
314 macromolecular complexes.

315

316 **METHODS**

317 **R2SP expression and purification.**

318 RuvBL1ΔT127-E233 (R1ΔDII, further named R1) carries an N-terminal 6x His-tag followed by thrombin cleavage
319 site, while RuvBL2ΔE134-E237 (R2ΔDII, further named R2) carries an C-terminal Flag+FH8 tag preceded by
320 HRV-3C cleavage site (Flag was used for detection). The RuvBL1ΔT127-E233- RuvBL2 ΔE134-E237 complex was
321 expressed in *Escherichia coli* (DE3) (Novagen, 71400), with 100 μM IPTG overnight at 18 °C. The complex was
322 immobilized in a 5 ml HisTrap™ HP (GE Healthcare) and eluted with 300mM imidazole. Peak fractions
323 collected from the HisTrap were incubated with 5mM CaCl₂ during 1 h and loaded onto an HiPrep™ Octyl FF
324 16/10 column (GE Healthcare). Bound proteins were eluted using 5mM EDTA. To remove the FLAG_FH8 tag
325 the collected samples were incubated 18 h at 4 °C with 1 % (w/w) HRV-3C protease (Thermo Fisher Scientific).
326 A final Superose 6 equilibrated in buffer 20 mM Tris-HCl pH 8.0, 150mM NaCl, 5% glycerol, 2mM MgCl₂ and
327 0.5mM TCEP, was used to separate a stable dodecameric peak, from the HRV3C protease and cleaved tags.
328 The pooled dodecamer was concentrated to 37.75 mg/ml using a 30 kDa Cut-off Amicon Ultra centrifugal filter
329 (Millipore).

330 SPAG1(622-926) (SPAG1) carries an C-terminal Flag-tag without a cleavage site, while PIH1D2(231-315)
331 (PIH1D2) has a C-terminal StrepTag II preceded by a Human Rhino Virus 3C cleavage site (HRV-3C). SPAG1(622-
332 926)/PIH1D2(231-315) were co-expressed in *Escherichia coli* (DE3*) (Novagen, 71400), with 50 μM IPTG
333 overnight at 18 °C in a New Brunswick™ (Innova®) 44R Shaker at 150 rpm. The SP_mini complex was
334 immobilized in a 5 ml StrepTactin XT HC (IBA life sciences), and eluted with 50 mM Biotin. Peak fractions
335 collected from the StrepTactin XT were injected in a Superdex 200 16/60 XK equilibrated in Buffer 20 mM
336 Hepes pH 8, 300 mM NaCl, 0.5 mM TCEP, allowing the isolation of a heterodimer. Collected fractions from the
337 main peak were diluted to reduce the concentration of NaCl to 50 mM, and further polished in Resource™
338 Q (GE), and eluted with a linear gradient, allowing the separation of a major peak corresponding to the intact
339 complex (w/o degradation) at approximately 170 mM NaCl. Collected peak fractions were supplemented with
340 20 mM imidazole and tag removal was performed by incubating 18 h at 4 °C with 1% (w/w) HRV-3C protease
341 (Thermo Fisher Scientific). Digested sample was injected in a 5ml StrepTactin XT in tandem with 1ml HisTrap.
342 The collected flow was concentrated to 14.7 mg/ml using a 3 kDa Cut-off Amicon Ultra centrifugal filter
343 (Millipore). All purification steps were carried out at room temperature and were monitored by NuPAGE Bis-
344 Tris gels (Invitrogen, NP0302).

345 **Sample preparation.**

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

350 NaCl, pH 7.4 prior to cross-linking (see **Supplementary Table 4** for composition and molecular weights of
351 different assemblies)
352 R2SP complex was formed by mixing pure RuvBL1(Δ T127-E233)/RuvBL2(Δ E134-E237) with excess pure
353 SPAG1(622-926)/PIH1D2(231-315) complex at a ratio of 1 : 4 (considering RuvBLs dodecameric, and
354 SPAG1/PIH1D2 heterodimeric) over night at 4 °C. Formed R2SP complex was separated from free excess
355 SPAG1/PIH1D2 using a Superose 6 16/60 XK (GE Healthcare) previously equilibrated in 20 mM Hepes pH 8,
356 150 mM NaCl, 2mM MgCl₂, and 0.5 mM TCEP. The eluted peak was concentrated to 7.5 mg/ml using a 3 kDa
357 Cut-off Amicon Ultra centrifugal filter (Millipore). It was diluted to 1mg/mL in 20 mM Hepes, 150 mM NaCl, pH
358 8³¹ prior to cross-linking.

359 **Cross-linking reactions.**

360 For NHS-ester-based cross-linking (XL) reactions, all aliquots of XL reagents were freshly diluted in anhydrous
361 DMSO (Invitrogen™ by Thermo Fisher Scientific, Rockford, IL, USA) . Following reagents were used: PhoX
362 (Disuccinimidyl Phenyl Phosphonic Acid, Bruker); DSAU (Disuccinimidyl diacetic urea, CF Plus Chemicals, Brno-
363 Řečkovice, Czech Republic); DSSO (Disuccinimidyl sulfoxide, Thermo Fisher Scientific, Rockford, IL, USA); DSBU
364 (Disuccinimidyl dibutyric urea, CF Plus Chemicals, Brno-Řečkovice, Czech Republic). BSA, ADH, GLDH and 20S
365 samples were each split in six aliquots and incubated with 25, 100, or 400 molar excess of each reagent. All
366 samples were additionally cross-linked with 800 and 100 molar excesses of DSBU. For R2SP cross-linking, stock
367 solution was split into 20 aliquots subsequently reacted with PhoX, DSAU, DSSO, DSBU at molar excesses of
368 25, 50, 100, 200, 400.

369 For formaldehyde cross-linking, GLDH and 20S proteasome at 1 mg/mL were incubated by adding a
370 formaldehyde 37% stock solution (Merck KGaA, Darmstadt, Germany) diluted to 0.05%, 0.1%, 0.2%, 0.5% and
371 1% (vol/vol final) for 20 min at room temperature⁴¹.

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

375

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

377 All cross-linked proteins and complexes were migrated on in-house 12 % acrylamide denaturing SDS-PAGE gels
378 (1.5 mm thickness). Volume corresponding to 1 μ g of each XL sample (and non-XL controls) was diluted (1:1)
379 with 2x concentrated Læmmli buffer (4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol, 0.01 % bromphenol
380 blue and 0.125 M Tris HCl) and incubated 5 min at 95°C. After sample loading, gels were migrated at 50 V for
381 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
382 % phosphoric acid, 50 % ethanol), washed 3x20 min with milli-Q water and stained overnight with Coomassie
383 Brilliant Blue (G250, Sigma, Saint-Louis, USA). They were finally rinsed 3x20 min with milli-Q water.

384

385 **Mass photometry measurements.**

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

392 Contrast-to-mass calibration: To allow MP mass measurements, contrast-to-mass calibration was performed
393 twice a day by measuring a mix of Bovine Serum Albumin (66 kDa), Bevacizumab (149 kDa), and Glutamate
394 Dehydrogenase (318 kDa) in PBS buffer, pH 7.4. The distributions of scattering events (given as contrast) were
395 Gaussian-fitted using DiscoverMP (**Supplementary Figure 10a**). Contrasts values are converted into masses
396 using linear relation between the contrast and the mass of the binding object. Calibrations were accepted for
397 $R^2 > 0.995$ (**Supplementary Figure 10b**).

398 Native MP (nMP): Samples were first diluted with their native buffer to 100-400 nM. Finally, 2 µL of the stock
399 solution are finally drop-diluted and carefully mixed to 10-40 nM in a 18 µL PBS droplet¹⁷. Three movies of
400 3000 frames were recorded (60 s) for each sample using the AcquireMP software (Refeyn Ltd, Oxford, UK).

401 Denaturing MP (dMP): Denaturing MP experiments were carried out by incubating first the samples to a
402 protein concentration of 100-400 nM in 5.4 M Urea (Sigma, Saint-Louis, USA) or 6 M Guanidine (Sigma, Saint-
403 Louis, USA). For non-crosslinked samples incubation times evaluated ranged from 5 min to 16 hours at room
404 temperature (18°C). After incubation and right before MP measurements, 2 µL of the solution were quickly
405 drop-diluted¹¹ in an 18 µL PBS droplet to 10-40 nM. All measurements were done immediately following the
406 droplet dilution. For the final optimized dMP protocol, denaturation was done in Urea 5.4 M for 5 min.

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

413
414 **Calculation of the total inter-protein cross-linking reaction efficiency (Eff_{XL}).**

415 Total inter-XL efficiency (1) was calculated using number of counts after Gaussian fitting of each oligomeric
416 state distribution (example of calculation in **Supplementary Table 5**). This value represent the efficiency of XL
417 reaction to form inter-protein interactions, i.e. all oligomeric states > 1 remaining after denaturation. Inter-XL
418 efficiency does not discriminate between specific interactions and non-specific aggregation.

419 (1) Total XL efficiency = $\left(\frac{\sum S_{oligomers > 1}}{\sum S} \right) * 100 = \% \pm SD$

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

422 **Calculation of the specific XL factor (SF_{XL}).**

423 Specific factor is defined as the specific/intended product of the XL reaction, i.e. the stabilization of native
424 complex. We first use non-XL native measurements as a reference to obtain the proportion represented by the
425 complex to be XL-stabilized in the sample. Then, we similarly calculated the proportion of this complex among
426 total counts of the cross-linked denatured sample. Using these two values, the complex stabilization factor can
427 be calculated (example of calculation in **Supplementary Table 6**). This value expresses the amount of native
428 complex that could effectively be XL-stabilized in XL samples (2). A factor value of 1 correspond to the
429 stabilization of all the native complex after XL reaction. Value > 1 expresses an enrichment of the complex upon
430 XL reaction. Stabilization factor should be ideally ≥ 1 .

431 (2) Specific XL factor = $\left(\frac{S_{complex(XL)}}{\sum S(XL)} / \frac{S_{complex(CT)}}{\sum S(CT)} \right)$

432 **Equation 2.** $S_{complex}$ is the integrated number of counts corresponding to the complex in both the cross-linked dMP XL sample (**XL**)
433 and nMP non-XL sample (**CT**); $\sum S$ is the sum of all integrated populations (monomer included) in the cross-linked dMP XL sample
434 (**XL**) or nMP non-XL reference sample (**CT**).

435

436 **Cross-linking mass spectrometry.**

437 R2SP complex was cross-linked in triplicates at each 25, 100, 400 molar excesses of DSBU (45 min, 18°C), before
438 quenching reaction with 15 mM Tris HCl (20 min). Samples were reduced by adding DTT to a final
439 concentration of 5 mM and incubation at 60°C for 30 min. The alkylation was done by adding Iodoacetamide
440 to a final concentration of 15 mM (1-hour incubation step in the dark). Samples were then processed with
441 overnight digestion with Trypsin/Lys-C (Promega, Madison, USA) at a 50:1 substrate:enzyme ratio (w/w) at
442 37°C overnight. The digestions were finally quenched with 1 % TFA.

443 Peptides were cleaned up by using the AssayMAP Bravo platform (Agilent Technologies; Santa Clara,
444 California) with 5 μ L C18 cartridges (Agilent). Cartridges were primed with 100 μ L 0.1 % TFA in 80 % ACN and
445 equilibrated with 50 μ L 0.1 % TFA in H₂O. 180 μ L of digested peptides diluted in equilibration buffer were
446 loaded on the cartridges and washed with 50 μ L equilibration buffer. Peptides were eluted with 50 μ L 0.1 %
447 TFA in 80 % ACN and stored at -80°C prior to the mass spectrometry analysis. Cross-linked peptides were dried
448 in a SpeedVac concentrator and resuspended in 2 % ACN/0.1 % formic acid.

449 NanoLC-MS/MS analysis was performed using a nanoAcquity UPLC (Waters, Milford, USA) hyphenated to a Q
450 Exactive HF-X mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nanoSpray
451 source. After trapping on a NanoEase M/Z Symmetry pre-column (C18, 100 Å, 5 μ m, 180 μ m x 20 mm; Waters),
452 samples were separated on a NanoEase M/Z BEH column (C18, 130 Å, 1.7 μ m, 75 μ m x 250 mm; Waters)

453 maintained at 60°C. A gradient of 102 min was applied: mobile phases A (0.1% v/v formic acid in H₂O) and B
454 (0.1% v/v formic acid in ACN). The following conditions were applied: 3 % B for 3 min, 3–40 % B for 90 min,
455 40–90 % B for 1 min, 90 % B for 5 min, 90–1 % B for 2 min and finally 1 % B maintained for 2 min (flow rate of
456 350 nl/min). Acquisition in Data Dependant Acquisition mode (Top 10 precursor ions) was done using
457 following parameters: MS resolution of 120.000 (AGC target 3e6), MS/MS resolution of 30.000 (AGC target of
458 2e5), 3-7 charge states enabled, HCD stepped collision energy (27, 30, 33 % normalized collision energy). Raw
459 data were directly processed with Thermo Proteome Discoverer 2.5.0.400 (Thermo Scientific) using the XlinkX
460 node for identification of crosslinks and the Sequest HT node for the identification of linear peptides. For linear
461 peptides identification a database containing R2SP sequences and common contaminants was used. As R2SP
462 complex purity was high, a reduced database containing R2SP subunit sequences (**Supplementary Figure 11**)
463 was used for XL identification. For both linear and cross-linked peptides searches, Cystein
464 carbamidomethylation was set as fixed modification. Methionine oxidation, N-term acetylation, tris-quenched
465 mono-links and water-quenched mono-links were set as dynamic modifications. Trypsin was set as the
466 cleavage enzymes with minimal length of 7 amino acids, 2 (linear peptides) and 3 (cross-linked peptides)
467 missed cleavages were allowed, respectively for proteomics and XL identifications. Mass accuracies for both
468 XL and linear peptides search were set to 10 ppm for MS1 and 0.05 Da for MS2. To increase confidence,
469 identification were only accepted for Maximal XlinkX scores > 40 and $\Delta score > 4$. A 1% false discovery rate was
470 applied for both linear and XL-peptides, and XLs were further manually curated. Out of the three replicates
471 performed for each molar excess of DSBU, unique cross-link sites were validated only when present in at least
472 2 out of 3 replicates.

473 Data were visualized using xiVIEW webserver (www.xiview.org), to produce circular interaction network and
474 represent XL sites on protein sequences^{36,42}. Finally, validated cross-links (2/3 file threshold) were plotted on
475 2XSZ X-ray diffraction structure³¹ corresponding to the R1R2 complex. The PyMol Molecular Graphics System
476 (version 2.5.4, Schrödinger, LLC) was used as well as xiVIEW server to visualize and measure XLs C α -C α
477 distances on structure. Corresponding distances were only validated if within ≤ 30 Å threshold.

478 **DATA AVAILABILITY**

479 Source data are provided with this paper. The XL-MS dataset (R2SP with 25, 100, 400 molar excesses of
480 DSBU) generated in this study, including experimental settings and XL identification results, has been
481 deposited on the ProteomeXchange Consortium via the PRIDE⁴⁴ repository with the dataset identifier
482 PXD042549 [<https://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PX042549>] (R2SP cross-
483 linking mass spectrometry). The Mass Photometry raw and treated files generated in this study will be made
484 fully available upon request.

485

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584

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592

593 **AUTHOR CONTRIBUTION STATEMENT**

594 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,
595 S.C wrote the manuscript. M.E.C. carried out the cloning of the domains of SPAG1 and PIH1D2. P.E.S. carried
596 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
597 manuscript.

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602 **COMPETING INTEREST STATEMENT**

603 The authors declare no competing financial interest.

604

605 **FIGURE LEGENDS/CAPTIONS (for main text figures)**

606 **Figure 1. Schemes representing two MP-based protocols used during dMP method development.** **a** Scheme
607 of the assay for the evaluation of denaturing agents' compatibility with MP measurements, **b** Optimized
608 general workflow for dMP analysis.

609

610 **Figure 2. Optimization of denaturation step for dMP using ADH, GLDH and 20S proteasome.** Mass
611 distributions, represented as probability density, show the evolution of monomer abundances, with
612 measurement replicates (n=3) shown as overlapping curves in shades of grey. MP profiles have been measured
613 after 5 min, 2 hours and 6 hours denaturation for: ADH **a** in 5.4 M urea or **b** in 6 M guanidine HCl; GLDH **c** in
614 5.4 M urea or **d** in 6 M guanidine HCl and 60S proteasome **e** in 5.4 M urea or **f** in 6 M guanidine HCl. Scatter
615 plots represent the monomer abundance (mean \pm SD) after ADH, GLDH and 20S denaturation with **g** urea and
616 **h** guanidine. Standard deviations come from measurements replicate (n=3). Source data are provided as a
617 Source Data file.

618

619 **Figure 3. Benchmarking of dMP vs SDS-Page for the DSBU cross-linking optimization.** Comparison of dMP
620 and SDS-PAGE experiments after **a** ADH, **c** GLDH, **e** 20S proteasome cross-linking. Mass distributions are
621 represented as probability density with overlapping curves in lighter shades, showing the measurement
622 replicates (n=3). Relative abundances of different oligomeric states of the corresponding complex are shown
623 as scatter plots (mean \pm SD) for **b** ADH, **d** GLDH, **f** 20S proteasome, with error bars represent the standard
624 deviation related to measurement replicates (n=3). Source data are provided as a Source Data file.

625

626 **Figure 4. dMP screening of cross-linking conditions on ADH, GLDH and 20 proteasome.** **a** Effect of cross-
627 linking reagent (size, flexibility) on oligomeric states stabilized, measured in dMP: presented results are
628 probability densities (KD) of GLDH samples cross-linked with increasing molar ratios of PhoX, DSAU, DSBU,
629 from measurement replicates (n=3). **b** dMP-based quantitative results of XL condition screening for ADH, GLDH
630 and 20S complexes. Bar charts represent the dMP-calculated global inter-XL efficiency (mean \pm SD) for each

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631 complex and XL condition (25/100/400:1 cross-linker:complex molar ratio), from measurement replicates
632 (n=3). Plain dots represent the complex stabilization factor for each complex and XL condition. The black dash
633 line corresponds to the stabilization factor value of 1 indicating a complex abundance similar to the native
634 sample. Source data are provided as a Source Data file.

635

636 **Figure 5. dMP results of XL reaction optimization for R2SP complex.** nMP profiles (yellow) and dMP profiles
637 (grey) of R2SP cross-linked with 0, 25, 100, 400 molar excesses of **a** PhoX, **b** DSAU, **c** DSSO, **d** DSBU, shown as
638 probability densities. Measurement replicates (n=3) are shown as overlapping curves in shades of yellow or
639 grey. **e** Quantitative results of R2SP XL optimization at 25, 50, 100, 200, 400 molar excesses of reagents. Bar
640 charts represent the dMP-calculated total inter-XL efficiency (mean \pm SD) for each complex and XL condition,
641 from measurement replicates (n=3). Plain dots represent the specific factor for each complex and XL condition.
642 The black dash line corresponds to the stabilization factor value of 1 indicating a complex abundance similar
643 to the native sample. Source data are provided as a Source Data file.

644

645 **Figure 6. Description of the XL-MS dataset of R2SP cross-linking in triplicates with 25, 100, 400 molar**
646 **excesses of DSBU.** Proportional Venn diagrams show the reproducibility of cross-linking reaction across
647 biological replicates (n=3), at **a** 25, **b** 100, **c** 400 molar excesses of DSBU. The identified cross-links are
648 represented in circular views³⁶ along RuvBL1, RuvBL2 and SPAG1 sequences at **d** 25, **e** 100, **f** 400 molar
649 excesses of DSBU. In yellow are highlighted the XLs unique to the condition. Venn diagram **g** shows the overlap
650 of validated XLs (file threshold 2/3) between the 3 datasets. **h** we finally mapped identified XLs in dashed lines,
651 on the R2SP structure (2XSZ.pdb), RuvBL1 is shown in light grey, RuvBL2 is shown in light purple.

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