

1 Crowding-induced phase separation and solidification by 2 co-condensation of PEG in NPM1-rRNA condensates

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6 7 **ABSTRACT**

8 The crowdedness of the cell calls for adequate intracellular organization. Biomolecular condensates, formed
9 by liquid-liquid phase separation of intrinsically disordered proteins and nucleic acids, are important
10 organizers of cellular fluids. To underpin the molecular mechanisms of protein condensation, cell-free
11 studies are often used where the role of crowding is not investigated in detail. Here, we investigate the
12 effects of macromolecular crowding on the formation and material properties of a model heterotypic
13 biomolecular condensate, consisting of nucleophosmin (NPM1) and ribosomal RNA (rRNA). We studied
14 the effect of the macromolecular crowding agent PEG, which is often considered an inert crowding agent.
15 We observed that PEG could induce both homotypic and heterotypic phase separation of NPM1 and NPM1-
16 rRNA, respectively. Crowding increases the condensed concentration of NPM1 and decreases its
17 equilibrium dilute phase concentration, while no significant change in the concentration of rRNA in the
18 dilute phase was observed. Interestingly, the crowder itself is concentrated in the condensates, suggesting
19 that co-condensation rather than excluded volume interactions underlie the enhanced phase separation by
20 PEG. Fluorescence recovery after photobleaching (FRAP) measurements indicated that both NPM1 and
21 rRNA become immobile at high PEG concentrations, indicative of a liquid-to-gel transition. Together, these
22 results shed new light onto the role of synthetic crowding agents in phase separation, and demonstrate that
23 condensate properties determined *in vitro* depend strongly on the addition of crowding agents.

24 25 **STATEMENT OF SIGNIFICANCE**

26 *Liquid-liquid phase separation of proteins and nucleic acids leads to the formation of biomolecular*
27 *condensates. To mimic biomolecular condensates in vitro, polymeric crowding agents, such as PEG, are*
28 *often added. Such crowding agents are considered to make in vitro solutions more physiologically relevant,*
29 *by mimicking the high cellular macromolecule concentrations. However, these crowding agents are*
30 *commonly selected for their commercial availability and solubility in water, and their influence on phase*
31 *separation and the physicochemical properties of condensates are seldom studied. Here we use biophysical*
32 *methods to show that PEG induces phase separation of a model condensate through co-condensation rather*
33 *than volume exclusion. As a consequence, crowding changes the partitioning, concentrations and*
34 *viscoelastic properties of the condensates significantly, which sheds new light onto studies aimed at*
35 *quantifying the material properties of biomolecular condensates.*

36 INTRODUCTION

37 One of the most fascinating aspects of the inner biochemistry of a cell is how it functions in an extremely
38 crowded environment. Typical estimates indicate that cellular biopolymers, including proteins and RNA,
39 occupy 20 to 30 vol% of the cell, limiting intracellular diffusion and making the cell's interior a very
40 crowded place (1-3). To enable effective biomolecular reactions, the cellular organization is of great
41 importance (4,5). Traditionally, membrane-bound organelles have been extensively studied for their roles
42 in organizing biochemical processes. This research has recently entered a new phase, as biomolecular
43 condensates, also referred to as membraneless organelles, were found to be involved in the regulation of
44 several biological processes, including transcription (6-8), cell signalling (9,10) and ribosome biogenesis
45 (11-13).

46 Biomolecular condensates benefit from the absence of a physical membrane barrier, giving these
47 condensates dynamic properties: they can fuse, ripen and wet membranes or become engulfed by other
48 condensates (14). This reflects the molecular nature of these condensates: they are typically enriched in
49 proteins containing intrinsically disordered regions (IDRs) and nucleic acids (11,15). The IDRs are not only
50 disordered, they also contain certain repetitive motifs, for example in the form of weakly charged patches
51 (16,17). Condensation is typically driven by liquid-liquid phase separation (LLPS) of these sticky moieties
52 in the IDRs, but it is not limited to IDRs, as structural domains have been found to act as effective stickers
53 as well (18-20). The balance between intermolecular association being not too strong to avoid turning the
54 condensate into a gel, and not too weak to avoid dissolving it, makes most condensates highly responsive
55 to changes in their environment, such as salinity and crowding, or subtle changes to the molecular
56 constituents, such as enzymatic product formation or post-translational modifications (4,21).

57 Many of the molecular mechanisms underlying condensate formation and phase behavior have been
58 unravelled through *in vitro* (here: cell-free) experiments (22-25). However, the cell-free environment in
59 which proteins are studied is often far from realistic intracellular conditions. Factors that are typically
60 regulated in what are considered to be 'physiological' conditions include ionic strength, pH and temperature
61 (26). In contrast, the high degree of crowding in a living cell is not commonly taken into account in cell-
62 free experiments despite the fact that many studies have shown that crowding can have a significant effect
63 on protein stability, complexation and reactivity (3,4,27,28). When crowding is taken into account, the
64 crowded cellular milieu is often mimicked by the addition of water-soluble polymers such as poly(ethylene
65 glycol) (PEG), Ficoll and dextran (29-34). Although these polymers are highly water soluble, PEG and
66 dextran can also undergo segregative phase separation, ending up in separate phases (35,36). This indicates
67 that they have non-negligible interactions with each other, and suggests that these and other crowding
68 agents could also interact with disordered and structured biomolecules, and affect their phase behavior.
69 Therefore, a systematic study of the effect of crowding agents on commonly studied biomolecular
70 condensates is of great relevance to understand the role of crowding in LLPS.

71 Crowding agents can affect biomolecular condensates in three ways. (i) They could promote (or in
72 theory also suppress) phase separation by enhancing the weak intermolecular interactions through excluded

73 volume effects or co-condensation, thereby shifting the binodal line to lower concentrations (37). (ii)
74 Crowding agents can co-localize into the dense phase, as has been observed for dextran (38). This may be
75 caused by the distinct local chemical environment (for instance nuage bodies are thought to have more
76 hydrophobic interior (22)), but the change in composition could also lead to a further change in the local
77 environment. (iii) Finally, the enhanced intermolecular interactions and altered composition could result in
78 a change of the biophysical properties. For example, the condensed phase could become more viscous or
79 switch to a solid-like state, such as previously observed for FUS (39) and NPM1 (29).

80 Here, we investigate the presence of these three effects in a well-studied condensate model of NPM1-
81 rRNA using the most prevalent crowding agent found in studies of protein phase separation, PEG. We show
82 that PEG induces both homotypic and heterotypic phase separation, and quantified the changes in the dilute
83 and condensed phase. We observed the strongest crowding effect for NPM1, while there was no significant
84 effect on rRNA in the dilute phase. Through confocal microscopy we were able to prove that PEG weakly
85 partitions into the condensed phase, where it causes a differential increase in the NPM1 and rRNA density,
86 thereby altering the condensate composition. Finally, using fluorescence recovery after photobleaching
87 (FRAP) we measured the viscoelastic properties of both NPM1 and rRNA, which showed a rapid decrease
88 of the mobile fraction in the presence of PEG.

89

90 **MATERIALS AND METHODS**

91 **Materials**

92 Unless otherwise stated all chemical were purchased from Sigma-Aldrich. All aqueous solutions were
93 prepared in Milli-Q water (18.2 M Ω cm), except for the rRNA stock solution which was dissolved in
94 nuclease free water (Invitrogen).

95

96 **Protein expression and purification**

97 *E.coli* BL21 (DE3) were transformed with pET28a(+)-NPM1-wt. Bacterial cell cultures were grown in Luria
98 Broth (LB-broth) supplemented with 50 μ g L⁻¹ kanamycin at 37 °C till OD₆₀₀ reached 0.6-0.8 before
99 expression was induced with 1 mM IPTG. Protein expression was carried out overnight at 18 °C and pelleted
100 through centrifugation. Pellets were either stored at -80°C or directly used for purification. For purification,
101 pellets were thawed on ice and resuspended in lysis buffer (10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 20
102 mM imidazole) supplemented with 5 mM β -mercaptoethanol, 1 \times protease inhibitor (Roche), and 10 mM
103 PMSF. Cell suspensions were either lysed by sonication (Sanyo Soniprep 500) or French Press
104 homogenizer (Homogenizing Systems LTD). The lysate was cleared through centrifugation at 20,000 g at
105 4 °C for 30 minutes in a Beckman JA25.50 rotor. The supernatant was loaded on an equilibrated 5 mL His-
106 trap column (GE healthcare/Cytiva) at 4 °C. The loaded column was washed with 10 column volumes (CV)
107 lysis buffer and eluted with 3 CV elution buffer (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5 mM β -
108 mercaptoethanol, 500 mM imidazole). Protein containing fractions were dialysed overnight against SEC-

109 buffer (10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM DTT) and concentrated to <5 mL. The concentrated
110 protein sample was loaded on a Superdex 200 16/600 (GE-healthcare) size exclusion column connected to
111 an AKTA Basic FPLC (GE Healthcare) in SEC-buffer. Elution was carried out at room temperature at 1
112 mL/min and monitored at 205 nm, 254 nm and 280 nm. Fractions of the main peak were pooled, and
113 concentrated. The concentration was determined using the NanoDrop One^C (Thermo Scientific), and
114 aliquots were snap frozen in liquid nitrogen and stored at -80 °C.

115

116 **NPM1-Alexa488 labelling**

117 NPM1-wt was labelled using AlexaFluor488 C5 maleimide dye (Thermo Fisher) according manufacturer's
118 protocol. In short, 100 µM NPM1 was dialysed against 10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM
119 TCEP. Using a Millipore spin filter (MWCO: 10 kDa), the excess TCEP was removed, and 200 µM
120 AlexaFluor488 C5 maleimide dye was added, and incubated overnight at 4 °C. Excess dye was removed
121 through dialysis (Millipore, MWCO 3.5 kDa) against SEC buffer, and the concentration was determined
122 using the NanoDrop One^C.

123

124 **Ribosomal RNA isolation**

125 *E. coli* (DE3) pLysS cells were grown at 37 °C in LB-broth till OD₆₀₀ reached 1.2, and pelleted through
126 centrifugation (5,000 g at 4 °C for 15 minutes). Pellets were washed twice in S30 buffer A (50 mM Tris-
127 HCl, pH 7.7, 60 mM potassium glutamate, 14 mM magnesium glutamate, 2 mM DTT), and resuspended
128 in S30 buffer A (1 mL buffer to 1 gram cell pellet). This cell suspension was then homogenized through
129 sonication (Sanyo Soniprep 150) and cleared through centrifugation (15,000 rpm at 4 °C for 25 minutes) in
130 a Beckman JA25.50 rotor. Ribosomes were isolated by ultracentrifugation for 3 hours at 50,000 rpm
131 (Beckman-Coulter Optima-90, with a fixed angle 90-Ti rotor). The glassy rough ribosome pellets were
132 dissolved overnight in S30 buffer B (5 mM Tris-HCl, pH 8.2, 60 mM potassium glutamate, 14 mM
133 magnesium glutamate, 2 mM DTT) at 4 °C. The ribosomal RNA (rRNA) was isolated from the ribosomes
134 through standard phenol chloroform extraction using phenol:chloroform:isoamyl alcohol (PCI,
135 49.5:49.5:1). The final rRNA concentration was determined using the NanoDrop One^C, where 1 OD₆₀₀ =
136 40 µg mL⁻¹ RNA and stored at -80 °C.

137

138 **RNA-Alexa647 labelling**

139 The '3-end of the rRNA was labelled with AlexaFluor647-hydrazide using a periodate oxidation reaction
140 (40-42). In short, to 80 µL of rRNA (3.4 mg mL⁻¹), 7 µL of nuclease free water, 3.33 µL of 3 M sodium
141 acetate (pH 5.2), and 10 µL of 25 mM sodium periodate (freshly prepared in water on the day) was added.
142 The mixture was incubated on ice for 50 minutes. Subsequently, 20 µL of 3 M sodium acetate (pH 5.2) and
143 80 µL nuclease free water was added. The activated RNA was then precipitated by addition of 400 µL
144 isopropanol through cooling it on ice for at least one hour. The RNA was then spun down (14,000 g at 4 °C
145 for 15 minutes). The supernatant was removed and 150 µL of ice-cold ethanol was added to the pellet

146 without mixing. After another centrifugation step, and removal of the supernatant, the RNA was mixed into
147 the reaction buffer (100 mM sodium acetate, pH 5.2, 25 nmol Alexa-647 hydrazide). The reaction was left
148 over 48 hours, after which the labelled RNA was isolated through a isopropanol and ethanol precipitation.
149 The rRNA-A647 was then redissolved in 80 μ L of nuclease free water. The concentration was determined
150 using the NanoDrop One^C.

151

152 **Preparation of NPM1-rRNA condensates**

153 NPM1-AlexaFluor488 (NPM1-A488) stock solutions were prepared at 200 μ M with 1:9 molar ratio
154 AlexaFluor488 labelled NPM1 to unlabelled NPM1 in 10 mM Tris-HCl (pH 7.5), 300 mM NaCl. NPM1-
155 A488 aliquots of 20 μ L were snap frozen and stored at -80 °C.

156 The order of components for a typical experiment consists of first mixing the PEG (10 kDa, from
157 a 35 wt% stock), with the buffer (from a 4x stock of 40 mM Tris-HCl (pH 7.5), 600 mM NaCl) and then
158 diluted to the required final volume, often 30 μ L at room temperature. 15 minutes prior to measuring the
159 NPM1-A488 and rRNA were added to the premixed diluted buffer.

160

161 **Quantification of the dilute phase**

162 A typical sample of 30 μ L was prepared in 10 mM Tris (pH 7.5), 150 mM NaCl with varying concentrations
163 of PEG, NPM1/NPM1-A488 (1:9 molar ratio labelled), and rRNA-A647 (only labelled) as described above.
164 After incubating for 15-20 minutes at room temperature, the condensed phase was separated from the dilute
165 phase by centrifugation at 21,000 g for 20 minutes at room temperature. The dilute phase was then
166 transferred to a 384-well plate (Nunc, flat bottom) and the fluorescence intensity was measured on a plate
167 reader (Tecan Spark M10) at 485/535 nm for NPM1-A488, and 620/680 nm for rRNA-A647.
168 Concentrations of the dilute phase were calculated based on calibration curves (Fig. S1).

169

170 **Preparation of modified glass coverslips**

171 Ibidi 18-well chambered slides (#1.5) were first cleaned using oxygen plasma and directly afterwards
172 incubated in a 0.01 mg/mL solution of PLL(2)-g[3,5]-PEG(2) (SuSoS AG, Switzerland) in 10 mM HEPES
173 buffer (pH 7.4) for at least 1 hour at room temperature. Ibidi chambers were rinsed several times with
174 MilliQ water and dried with pressurised air. Modified glass slides were stored at -20 °C.

175

176 **Quantification of the condensed phase by confocal microscopy**

177 Images for partitioning were acquired on a Leica Sp8x confocal inverted microscope (Leica Microsystems,
178 Germany) equipped with a DMi8 CS motorized stage, a pulsed white light laser, and 2 x HyD SP GaAsP
179 and 2x PMT detectors. Images were recorded using the LAS X v.3.5 acquisition software, using a HC PL
180 APO 100x/1.40 oil immersion objective.

181 Samples were prepared as described above. After incubation of NPM1-rRNA condensates for 15
182 minutes, the sample was transferred to the modified Ibidi-18 well chambers and the condensates were
183 allowed to settle to the bottom of the chamber for 10 minutes. Partitioning coefficients were analysed by
184 MATLAB, the background was subtracted by measuring non-fluorescent condensates at the same settings
185 as for the fluorescent images. The partitioning coefficient was then calculated using: $K_p = (I_{condensate} -$
186 $I_{background}) / (I_{dilute} - I_{background})$ where $I_{condensate}$ is the average intensity of all condensates in one frame and I_{dilute}
187 the average intensity of the area without condensates. Standard deviations were determined of at least three
188 sets of K_p values derived from three different images.

189

190 **FRAP analysis**

191 For FRAP analysis time lapse videos were recorded at room temperature on a CSU X-1 Yokogawa spinning
192 disc confocal unit connected an Olympus IX81 inverted microscope, using a x100 piezo-driven oil
193 immersion objective (NA 1.3) and a 488, 561 or 640 nm laser beams. Emission was measured with a 200
194 ms exposure time at a rate of 120 frames per minute, using an Andor iXon3 EM-CCD camera. The acquired
195 images have a pixel size of 141 nm and a field of $72 \times 72 \mu\text{m}^2$. For bleaching, a small region of interest (ROI)
196 was selected in the middle of a condensed droplet. The 488 nm or 640 nm laser line was set to at 100%
197 laser power using 75 pulses of 200 μs . The recovery was then imaged at reduced laser intensity with a time
198 interval of 500 ms.

199 Recovery profiles were analysed using ImageJ, the normalized intensities were fitted to a 2D-diffusion
200 with a fixed boundary (43). Using the resulted exponential decay equation $I_{normalized} = A(I - e^{-bt}) + C$, from
201 which we obtained the parameters A , b and C . The recovery half-life was then determined at $t_{1/2} = \ln(2)/b$.

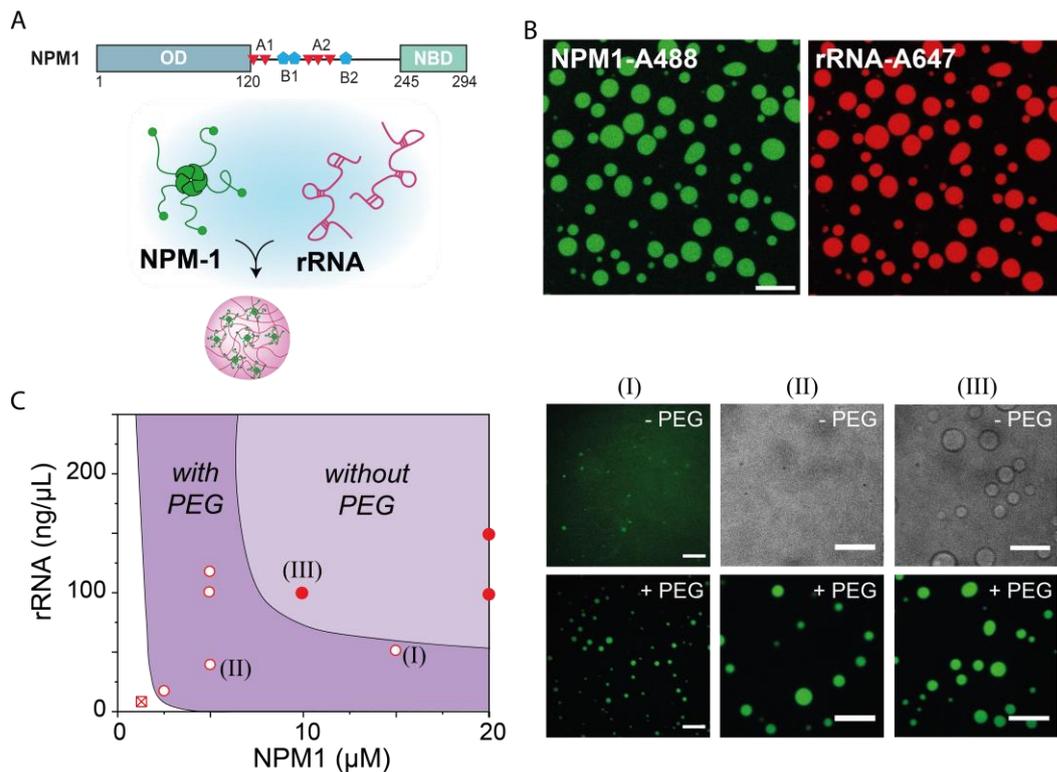
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203 **RESULTS AND DISCUSSION**

204 **PEG shifts the phase diagram of NPM1-rRNA**

205 Inspired by the numerous membraneless organelles that contain both proteins and RNA, we chose a
206 heterotypic system consisting of nucleophosmin (NPM1) and ribosomal RNA (rRNA) as model
207 condensate. This system is illustrated in Fig. 1A, and forms liquid droplets under ‘physiological’ conditions
208 (here defined as physiological salt by 10 mM Tris, 150 mM NaCl), even without crowding. By labelling
209 both NPM1 and rRNA with fluorophores we could observe that indeed the rRNA co-localizes into the dense
210 NPM1 droplets (Fig. 1B), in agreement with our previous study (40). We found a maximum degree of
211 phase separation, as inferred from microscopy analysis, at a NPM1:rRNA ratio of 20 μM NPM1 : 150 ng
212 μL^{-1} rRNA (19,40). This corresponds roughly to one pentamer NPM1 interacting with 110 nucleotides of
213 rRNA. When keeping this ratio constant, we could decrease the protein concentration to a lower limit of 10
214 μM and still observe liquid condensates (Fig.1C, S2).

215 When we added polyethylene glycol (PEG), a commonly used crowding agent, to the mixtures of
 216 NPM1 and rRNA, we observed phase separation at lower NPM1 concentrations. For 2 wt% PEG, we could
 217 observe phase separation for concentrations down to 2.5 μM NPM1 and 19 $\text{ng } \mu\text{L}^{-1}$ rRNA (Fig. 1C, S2),
 218 which suggests that PEG enhances the association between protein and RNA. Moreover, we found that at
 219 2 wt% PEG, NPM1 could also phase separate without RNA at 10 μM protein concentrations and higher
 220 into apparently homotypic droplets (Fig. 1C, S3). These findings are in agreement with a previous study of
 221 Kriwacki and co-workers, who showed that no second component, such as arginine-rich peptides or rRNA,
 222 is needed for phase separation of NPM1 under PEG-based crowding conditions (29,30). These findings
 223 suggest that crowding by PEG not only enhances associative interactions between NPM1 and rRNA, but it
 224 enhances the self-interactions of NPM1 even more. Assuming that volume exclusion by the crowding
 225 agents is responsible for enhancing the intermolecular interactions, the observed effects could be explained
 226 by the structural differences between NPM1 and rRNA: the protein NPM1 has a more globular shape and
 227 a larger effective radius than the polymeric rRNA and will therefore experience stronger volume exclusion
 228 due to crowders. Crowding by PEG will thus likely affect the concentrations of NPM1 and rRNA in both
 229 the dilute and the dense (condensate) phase in a non-trivial way. Therefore, we next sought to quantify the
 230 NPM1-rRNA phase diagram under crowding conditions.



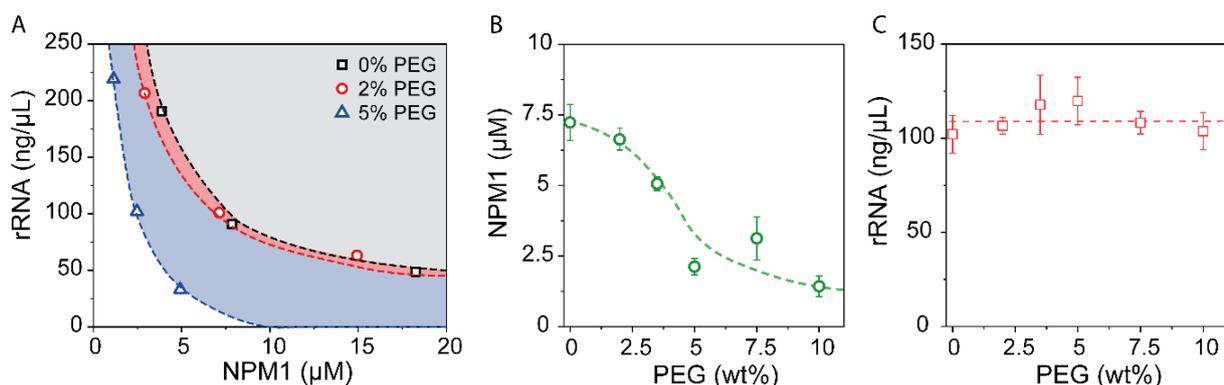
231

232 **Figure 1: PEG induced phase separation of NPM1-rRNA.** (A) Schematic illustration of nucleophosmin (NPM1)
 233 protein domains and the formation of condensates with rRNA. NPM1 encodes for a structural N-terminal
 234 oligomerization domain (OD) and a C-terminal nuclear binding domain (NBD) linked by an intrinsically disordered
 235 region with two acidic tracts (A1 and A2) and two weak basic tracts (B2 and B2). Pentamers of NPM1 phase separate
 236 with rRNA into condensates in vitro. (B) Fluorescent microscopy images of NPM1-rRNA condensates at 10 μM
 237 NPM1-Alexa488; 150 $\text{ng } \mu\text{L}^{-1}$ rRNA-Alexa647 (in 10 mM Tris pH 7.5, 150 mM NaCl). Scale bar, 10 μm . (C)
 238 Schematic representation of the shift in phase diagram of NPM1-rRNA liquid-liquid phase separation, and three
 239 conditions in the presence or absence of 2 wt% PEG: (I) 15 μM NPM1, 50 $\text{ng } \mu\text{L}^{-1}$ rRNA, (II) 5 μM NPM1, 37.5 ng
 240 μL^{-1} rRNA, (III) 10 μM NPM1, 75 $\text{ng } \mu\text{L}^{-1}$ rRNA. Scale bars, 10 μm .

241 PEG reduces only NPM1 concentrations in the dilute phase

242 Excluded volume theory predicts that crowding enhances the effective attractions between macromolecules
243 by increasing the entropy of the crowders upon complexation of the macromolecules. For our phase
244 separating system, enhanced attraction could result in phase separation at lower concentrations, as
245 suggested by the measurements in Figure 1C. To obtain a quantitative understanding of the effect of
246 crowding on phase separation between NPM1 and rRNA, and how this affects their concentrations in the
247 dilute and condensed phase, we determined the compositions of the droplets and supernatant as function of
248 the amount of crowding agent PEG.

249 First, we examined the concentrations of NPM1 and rRNA in the dilute phase. The condensates were
250 separated from the dilute phase by centrifugation (Fig. S4). We quantified the concentration of NPM1 and
251 rRNA in the dilute phase for three different overall mixing ratios. In all cases, the addition of PEG reduced
252 the concentration of NPM1 in the dilute phase, while the rRNA remained approximately constant or even
253 increased (Fig. 2A). It is clear that this is not the expected behavior for a classical crowding agent that
254 enhances the attraction between NPM1 and rRNA. In that case, the saturation concentration of both NPM1
255 and rRNA should decrease with increased crowding, although the relative degree of decrease might depend
256 on the overall mixing ratio. Instead, we found that the rRNA saturation concentration remained constant
257 for all mixing ratios. Crowding thus seems to leave the interaction between NPM1 and rRNA unchanged,
258 while it enhances the interactions of NPM1 with itself, as the saturation concentration of NPM1 decreased
259 significantly. Our results regarding the NPM1 concentrations in the dilute phase corroborate what has
260 previously been observed for homotypic NPM1 condensation (29): crowding decreased the solubility of
261 NPM1. However, it was also observed that the ratio between the arginine-rich SURF6 peptide (S6N) and
262 NPM1 remained constant with increasing crowding concentrations, probably because the short peptide
263 partitions as a client into the homotypic NPM1 droplets.



264

265 **Figure 2: PEG reduces NPM1 concentration and not the rRNA concentrations in the dilute phase.** (A)

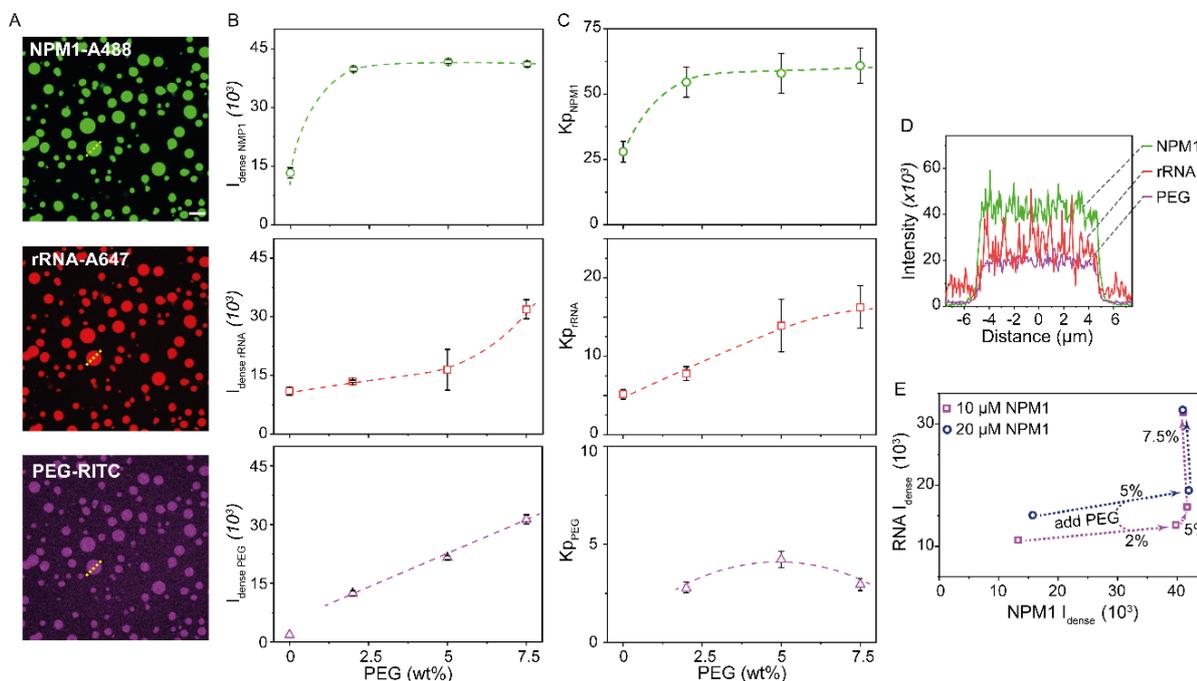
266 Concentrations of NPM1 plotted against rRNA in the dilute phase for three fixed NPM1:rRNA ratios: 5:150, 10:100,
267 20:50 (μ M NPM1: ng μ L⁻¹ rRNA), at three different PEG concentrations. (B-C) Addition of PEG to 10 μ M NPM1,
268 100 ng μ L⁻¹ rRNA reduces the concentration of NPM1 in the dilute phase (B) but not for rRNA (C). The errors in
269 these figures are standard deviations from triplicate measurements, and the dashed lines are present to guide the eye.

270 To analyze the effect of PEG in more detail, we focussed on one ratio of NPM1:rRNA (10 μM NPM1
 271 with 100 ng μL^{-1} rRNA), and looked at several concentrations of PEG (Fig. 2B and C). The dilute phase
 272 NPM1 concentration gradually decreased from 7.5 μM without crowding to 2.5 μM NPM1 at 10 wt% PEG
 273 (Fig. 2B). We did not observe a plateau at high crowding, despite the fact that the condensates had turned
 274 into gel-like structures with very little relaxation already at 2 wt% PEG (see also section: Crowding reduces
 275 condensate fluidity), but rather a gradual decrease that becomes asymptotic towards zero, in agreement with
 276 simple theoretical predictions for crowding-induced phase separation. For rRNA, we observed no clear
 277 change in the concentration in the dilute phase, as was found for other mixing ratios as well: it remained
 278 constant around 100 ng μL^{-1} (Fig. 2C).

279

280 PEG partitions into NPM1-rRNA condensates and increase local concentrations

281 We next analyzed how crowding affects the composition of condensates by studying the condensed phase
 282 in more detail. The tiny combined volume of the condensate droplets ($< 0.1 \mu\text{L}$) made an equivalent analysis
 283 of the absolute concentrations by fluorescence spectroscopy impossible. We therefore analyzed the relative
 284 changes in the composition of the condensates using fluorescence microscopy (Fig. 3A), despite the
 285 intrinsic limitations of quantitative fluorescence at high concentrations (44,45). Here, we used labelled
 286 NPM1 and rRNA at low enough concentrations that the fluorophores are further apart than their typical
 287 homo-FRET distance (46).



288

289 **Figure 3: Confocal microscopy analysis of NPM1-rRNA condensates under crowded conditions.** (A)
 290 Fluorescence images of NPM1-Alexa488, rRNA-Alexa647, and PEG-rhodamine, indicating PEG co-localizes into
 291 the condensed phase (20 μM NPM1, 150 ng μL^{-1} rRNA, 5 wt% PEG). Scale bar, 10 μm . (B-C) Average fluorescence
 292 intensities (B) and partitioning coefficients (K_p) (C) of NPM1, rRNA, and PEG plotted against the concentrations of
 293 PEG. (D) Intensities profile across one single condensate (dashed line in A). (E) Graph representing the influence of
 294 crowding on the intensities (and therefore concentration) of NPM1 and rRNA within condensates. The errors in this
 295 figure are standard deviations from triplicate measurements, and all dotted lines and arrows are to guide the eye.

296 Confocal microscopy images show a three-fold increase in fluorescence intensity of NPM1 (Fig. 3B)
297 upon addition of 2 wt% PEG. The partitioning coefficient (K_p) was determined based on this fluorescence,
298 and as a consequence the K_p for NPM1 (Fig. 3C) is showing the same trend. Further increasing the
299 concentration of PEG did not lead to additional increase in NPM1 fluorescence, and most likely in
300 concentration. An increase in NPM1 concentration upon crowding is expected if the crowders enhance the
301 association between NPM1 proteins, although the magnitude of the observed increase is higher than
302 expected for a crowder at approximately 5 vol% (47). Moreover, in the case of pure excluded volume
303 interactions between crowder and the phase separating biomolecules, the local concentration of
304 biomolecules inside the condensates and thereby their density is expected to continue increasing when the
305 crowder concentration is increased further, since increasing the crowder concentration leads to further
306 enhancement of the association strength between the biomolecules, which translates into a higher binodal
307 condensate concentration. The fact that we do not observe a further increase in density of the NPM1
308 concentration inside the condensates suggests that the interactions between NPM1 and PEG may not be
309 limited to volume exclusion.

310 For rRNA, we found that addition of PEG did not affect the concentration in the dilute phase. In
311 general, rRNA showed a much lower partitioning into the condensates ($K_p = 5$) than NPM1 in the absence
312 of PEG, and the rRNA concentration increased only slightly upon addition of PEG (Fig. 3B). At low (0-5
313 wt%) PEG concentration, the rRNA concentration increased about 25%, whereas the local NPM1
314 concentration increased threefold. However, at the highest PEG concentration (7.5 wt%), the rRNA
315 concentration and partitioning increased about twofold, while the NPM1 concentration did not increase
316 further at this PEG concentration (Fig. 3E), and also the rRNA concentration in the dilute phase did not
317 change. The slight increase in rRNA in the condensed phase at low PEG concentrations is in agreement
318 with our observation that the dilute phase concentration did not increase: PEG mostly enhances NPM1 self-
319 interaction, which causes the condensed phase to become denser, but the interaction between NPM1 and
320 rRNA is not strongly affected. Moreover, PEG is known to enhance folding of RNA into more compact
321 states (48,49), which could explain the higher concentration of rRNA inside the condensates. The strong
322 increase in rRNA concentration at the highest PEG concentration suggests that the condensates may no
323 longer be pure liquids governed by LLPS at that point, but that they are kinetically trapped in a gel state, as
324 we will discuss further below.

325 Finally, we also looked at the distribution of the crowding agent PEG over the two phases (Fig. 3). In
326 the case of ideal excluded volume interactions linked to a first-order phase transition of a solute that is
327 driven by a crowding-enhanced association, and ignoring the contribution of the crowders to the osmotic
328 pressure, the concentration of crowders in the dilute and condensed phase is predicted to be the same. More
329 generally, the high local concentration of biomolecules in the condensates exclude volume for the crowder
330 molecules, and therefore, we expect that crowders are weakly depleted from the condensates. However,
331 when we measured the distribution of PEG over both phases via fluorescence microscopy, we found that
332 PEG was enriched in the condensed phase by a factor of 3 (Fig. 3B-C). To minimize the effect of the dye,

333 less than 0.1% of the PEG was labelled in this study. Interestingly, the enrichment was independent of
334 crowding concentration, and suggests that PEG exhibits associative interactions with NPM1 or rRNA or
335 both. Therefore, the classical picture of PEG as an inert crowding agent that interacts via excluded volume
336 interactions with biomolecules is not accurate. Instead, PEG seems to co-condense with NPM1 and/or
337 rRNA in a form of ternary associative phase separation.

338 Partitioning of crowding agents was also observed by a few other groups. Hammer and co-workers
339 observed similar partitioning of their small molecular weight dextran (4.4 kDa) in condensates made of the
340 LAF1 RGG domain as in our PEG-based crowding studies (38). Moreover, they observed decreasing
341 partitioning of dextran when its size was increased. In a systematic study on coacervates made of spermine
342 and polyuridylic acid (polyU), Keating and co-workers observed a similar effect of PEG (8 kDa) and Ficoll
343 (70 kDa) as crowding agents on the induction of phase separation. In contrast to our study, they observed
344 a weak exclusion of PEG, even though its size was similar, but an enrichment of Ficoll into spermine-polyU
345 coacervates (50). Their results indicated that for spermine-polyU coacervates, PEG behaves more like an
346 inert crowder. However, in both cases (crowding agent exclusion and inclusion) a promotion of phase
347 separation was observed, which they contributed to an enhancement of the favourable base-stacking of
348 polyU. If the exclusion of PEG by polyU-based coacervates is taken as an indication that there is no specific
349 association between PEG and RNA in the condensed phase, we can infer that in our system of NPM1-
350 rRNA, PEG is mostly associated with the NPM1. This suggests that PEG-induced phase separation of
351 homotypic NPM1 droplets is likely driven by a co-condensation of PEG and NPM1 (Fig. S3). The same
352 may be the case for a significant number of other homo- and heterotypic IDP-based condensates that have
353 been reported in the presence of PEG (4), for example a recent preprint by Knowles and co-workers
354 indicated PEG interacts with FUS-protein condensates (51).

355 Finally, our results show that the ratio between NPM1 and rRNA in condensates is altered upon
356 increasing PEG concentration, in contrast to the NPM1-S6N condensates by Kriwacki and co-workers,
357 which rely on electrostatic interactions (29). Our results indicate that the co-condensation with PEG, driven
358 by weak associations between the PEG and NPM1, is responsible for altering the condensate composition,
359 and increasing the overall macromolecular concentration within the condensed phase. By extension, a
360 similar mechanism may play a role in the crowded environment in the cell: besides excluded volume
361 interactions, there may be many (weak) soft repulsive or attractive forces (3,52) between all the crowders
362 and phase separating IDRs, which could result in promotion of phase separation through co-condensation
363 or segregation (53). This might also lead to changes in the material properties of condensates formed in the
364 presence of crowding agents, as we investigated next.

365

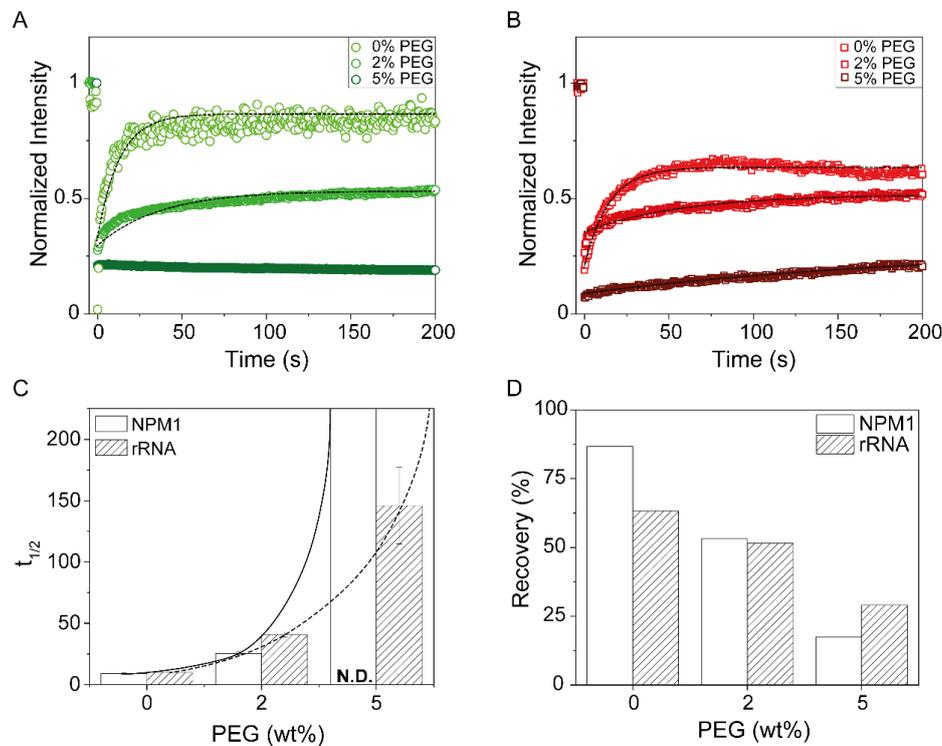
366 **Crowding reduces condensate fluidity**

367 Liquid-liquid phase separation is characterized by the formation of liquid droplets that typically exhibit
368 rapid recovery of fluorescence after photobleaching (FRAP). Indeed, our model system shows full and fast

369 recovery after photobleaching in the absence of PEG (Fig. 4). The mobile fraction correlates to the recovery
370 percentage, and for NPM1 in buffer this is almost 90%, and for rRNA around 65% (Fig. 4D). This indicates
371 that NPM1 shows almost a full recovery, since the total recovery was not corrected for the size of bleached
372 area. From the recovery curves we were able to determine the recovery half-time ($t_{1/2}$), which was for both
373 NPM1 and rRNA approximately 10 seconds. Thus, although NPM1 and rRNA have similar characteristic
374 recovery times, a larger fraction of the rRNA in condensates was immobile compared the NPM1. We
375 attribute this to possible RNA-RNA interactions (40).

376 The addition of PEG reduced the mobile fraction of both NPM1 and rRNA in the condensed phase
377 (Fig. 3) significantly: 2% PEG decreased the mobile fraction of both NPM1 and rRNA to 50%, while the
378 recovery half-time increased to 25 seconds and 40 seconds respectively. When we increased the PEG
379 concentration further to 5%, both NPM1 and rRNA became completely immobile, indicating that the
380 condensates are no longer liquid droplets governed by LLPS, but have turned into gels.

381 The solidifying effect of PEG on both NPM1 and rRNA is interesting compared to our previous results.
382 For instance, our lab showed that physiological concentrations of Mg^{2+} was only affecting the rRNA
383 diffusion in NPM1-rRNA condensates (40) by enhancing interactions between the rRNA molecules.
384 Kriwacki and co-workers demonstrated that the addition of the arginine rich domain SURF6 could liquefy
385 homotypic NPM1-NPM1 condensates (29). These newly formed NPM1-SURF6 condensates depend on
386 oppositely electrostatic interactions, while NPM1-rRNA condensation is considered to rely on the RNA
387 recognition motifs (RRMs) present in NPM1.



388

389 **Figure 4: PEG changes the viscoelastic properties of NPM1 and rRNA.** (A-B) FRAP recovery curves of NPM1
390 (A), and rRNA (B) upon increasing PEG crowding. The dashed lines represent the model fit for which the $t_{1/2}$
391 and total recovery were determined. (C) Calculated half-life ($t_{1/2}$) of NPM1 and rRNA at different concentrations of PEG.
392 The lines are there to guide the eye. (D) Mobile fraction from the total recovery determined from the FRAP curves.

393 Taking into account that PEG is present within the condensed phase (Fig. 3) we hypothesize that co-
394 condensation of PEG is solidifying NPM1-rRNA droplets *in vitro*. Since PEG is not fulfilling the classical
395 crowding model of an inert macromolecule, we observe that PEG likely binds to NPM1 without affecting
396 the ability of NPM1 to bind rRNA via the RRM. The co-condensation of PEG and NPM1 strongly
397 increases the NPM1 content, but also reduces their diffusion. The rRNA remains condensed with the NPM1
398 via binding to the RRM, but its concentration is not increased as much as NPM1 due to weakly
399 unfavourable interactions with the PEG, as also suggested by the work of Keating and co-workers (50).
400 Nevertheless, the higher local concentration of NPM1 also decreases the diffusivity of rRNA, as it is more
401 likely to be bound by multiple RRM of NPM1. Above a threshold PEG concentration, the local interactions
402 between NPM1-NPM1 and NPM1-PEG become too strong and their mobility decreases sharply, which is
403 reflected by the absence of NPM1 recovery in Fig. 4C.

404

405 CONCLUSIONS

406 In conclusion, we reported that the crowding agent PEG could induce and enhance phase separation of a
407 model biomolecular condensate consisting of NPM1-rRNA. By quantifying the compositions of both the
408 condensed and dilute phase, we could deduce that only the protein component NPM1 is depleted from the
409 dilute phase and enriched in the condensed phase. Although the concentration of rRNA remained constant
410 in the dilute phase, the condensed phase showed a slight PEG-dependent enrichment, possibly due to a
411 more condensed state of the dense phase. Through fluorescent labelling of the crowding agent, we found
412 that, surprisingly, PEG is also enriched in the condensed phase, suggesting that it enhances phase separation
413 by co-condensing with NPM1 rather than through excluded volume interactions. These results also indicate
414 that partitioning of “crowding agents” can change condensate compositions and material properties, and
415 that even crowders that are widely believed to be inert can have significant interactions with biomolecules
416 that undergo liquid-liquid phase separation. This is relevant to the crowded environment of the cell as well:
417 it is very likely that an important fraction of the macromolecules present in the cell exhibit weak repulsive
418 or attractive interactions with the components of membraneless organelles, which results in altered
419 composition and material properties compared to their *in vitro* reconstituted analogues. Finally, our results
420 also put *in vitro* condensates formed in the presence of common crowding agents in a new perspective and
421 suggest that addition of common crowding agents could affect *in vitro* phase separation systems and
422 therefore should be selected with care.

423 AUTHOR INFORMATION

424 Author Contributions

425 Alain A.M. André: conceptualization, methodology, investigation, writing. N. Amy Yewdall:
426 methodology. Evan Spruijt: conceptualization, software, writing, supervision funding acquisition.

427 Declaration of interests

428 The authors declare no competing financial interest.

429

430 ACKNOWLEDGEMENTS

431 The authors thank Prof. Richard Kriwacki (St. Jude Children's Research Hospital) for providing us with
432 the NPM1-GFP plasmid, Aafke Jonker and Frank Nelissen (Radboud University) for providing protocols
433 to isolate ribosomes and labelling RNA, Merlijn van Haren (Radboud University) for providing a
434 MATLAB script to determine partitioning coefficients of the microscopy data and Dr. Ioannis Alexopoulos
435 (Radboud University, now Justus Liebig University) for his guidance in confocal microscopy. We would
436 like to thank Prof. Wilhelm Huck (Radboud University) and Prof. Allen Minton (NIH) for fruitful
437 discussions. This work was financially supported by the Netherlands Organization for Scientific Research
438 (NWO).

439

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