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Supporting Information

Enhanced Ribozyme-Catalyzed Recombination and Oligonucleotide Assembly in Peptide-RNA Condensates

Kristian Le Vay[+], Emilie Yeonwha Song[+], Basusree Ghosh, T.-Y. Dora Tang,* and Hannes Mutschler**

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Supporting Information

Materials

Trizma base (Tris) (Thermo Fisher Scientific), sodium hexametaphosphate ($(NaPO_3)_6$, 611.77 g/mol) (Sigma-Aldrich), formamide (CH_3NO, 45.04 g/mol) (Sigma-Aldrich), ethylenediaminetetracetic acid disodium salt dihydrate (EDTA, $C_{10}H_{14}N_2Na_2O_8{\cdot}2H_2O$, 372.24 g/mol) (Sigma-Aldrich), magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$, 203.30 g/mol) (Merck), sodium hydroxide (NaOH, 39.997 g/mol) (VWR), sodium chloride (NaCl, 58.44 g/mol) (Sigma-Aldrich), urea (CH_4N_2O, 60.06 g/mol) (Carl Roth), hydrochloric acid (HCl, 37 %, 36.46 g/mol) (VWR), boric acid (H_3BO_3, 61.83 g/mol) (Merck), ammonium persulfate (APS, $(NH_4)_2S_2O_8$, 228.20 g/mol) (VWR), acrylamide (19:1 bisacrylamide) (Thermo Fisher Scientific), tetramethylethylendiamine (TEMED, $C_6H_{16}N_2$, 116.21 g/mol) (Carl Roth), SYBR gold stain (Thermo Fisher Scientific), RNA oligomer length standard (20/100 single strand RNA ladder) (IDT).

All peptides were purchased from Sigma-Aldrich and used without further purification (poly-L-lysine hydrobromide (4–15 kDa, 19-72 residues, monomer: 161.67 g/mol), poly-L-lysine hydrobromide (1-5 kDa, 5-24 residues, monomer: 161.67 g/mol). All RNA oligomers were obtained from Integrated DNA Technologies and are listed in Table S1. Fluorescently labelled oligomers were ordered with HPLC purification, whilst unlabelled oligomers were desalted and used without further purification. All RNA oligomers were dissolved in RNAse-free water and stored at -80 ºC.

RNA recombination

All RNA recombination reactions were performed using the same general methodology. RNA oligomers were first mixed in the desired ratio. The following components were then added in order: RNAse-free water (as required to reach the final volume), 10 x Tris.HCl buffer and $MgCl_2$. Finally, poly-L-lysine was added to initiate phase separation, and the reaction mixture was mixed by pipetting. All quoted $(Lys)_n$:RNA mixing ratios are calculated in terms of total monomer concentrations. The samples were then incubated in a PCR cycler with a heated lid to prevent condensation. The samples were not centrifuged prior to incubation. All measurements were performed in triplicate.

The simple recombination assay was performed using the following aqueous RNA concentrations: 1 µM srB23_Cy5, 9 µM srB23, 1 µM srB3_FAM, 9 µM srB3, 10 µM SBS.rec, 10 µM loopB5 and 10 µM loopB3 in 10 mM Tris·HCl pH 8. Final magnesium and poly-L-lysine concentrations were varied, as were the temperature and incubation time.

The direct ligation assay was performed similarly to the recombination assay, with 2',3'-functionalised srB2 replacing srB23 and srB23_Cy5. The 2',3'-cyclic phosphate was synthesised from a 2'/3'-phosphate using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as described previously[62]. The final composition was the following aqueous RNA concentrations: 1 µM srB23_Cy5, 9 µM srB23, 1 µM srB3_FAM, 9 µM srB3, 10 µM SBS.rec, 10 µM loopB5 and 10 µM loopB3 in tris buffer (10 mM, Tris·HCl pH 8) with 0.67:1 $(Lys)_{19\text{-}72}$:RNA and 1 mM $MgCl_2$.

HPz ladder recombination was performed using the following aqueous concentrations: 15 µM St, 15 µM SBS.16mer, 15 µM loopB5, 15 µM loopB3, 10 mM Tris·HCl (pH 8), and 1 mM $MgCl_2$. The magnesium concentration was fixed at 1 mM. The ratio of $(Lys)_{19\text{-}72}$:RNA was 0.75:1. The total RNA monomer concentration was 1.14 mM. The sample was incubated for 24 h at 30 ºC.

RPR4 recombination was performed using a 1.25-fold excess of each 3' strand relative to its 5' binding partner, as reported previously. The final aqueous composition was: 1 µM RPR4.1t, 1.26 µM SBS.RPR4.1, 1.58 µM RPR4.2t, 2 µM SBS.RPR4.2, 2.48 µM RPR4.3t, 3.1 µM SBS.RPR4.3, 3.88 µM RPR4.4, 8 µM loopB5, 8 µM loopB3, 10 mM Tris·HCl (pH 8), 8 mM $MgCl_2$ and 1.03 mM $(Lys)_{19\text{-}72}$ (0.8 $(Lys)_{19\text{-}72}$:RNA). Samples were incubated for 8 – 24 h at 30 ºC.

Denaturing urea polyacrylamide gel electrophoresis (PAGE)

The progress of all recombination reactions was assessed by denaturing urea-PAGE. All reactions were quenched by the sequential addition of one volume of 5 µL of 5 M NaCl, 5 µL of 1.25 M hexametaphosphate (HMP) and then four volumes of RNA gel loading buffer (formamide, EDTA (10 mM, pH 8.0), bromophenol blue (0.025 % w/v). Samples were denatured at 80 ºC for 5 minutes, cooled to room temperature then centrifuged for 5 minutes before gel loading. Quenched samples were stored at -80 ºC if not run immediately, and all samples were quenched before storage.

1 – 2 µL of quenched sample were loaded onto pre-warmed 15-20 % acrylamide maxi gels (20 cm x 20 cm x 1 mm). Urea-PAGE gels were run in 1x TBE buffer at a constant power of 30 W, until the blue loading dye band reached the end of the gel. Fluorescence imaging was performed using a Typhoon FLA9500 (GE Healthcare Life Sciences). Band intensities were measured using ImageQuant 8.2, and plotted using GraphPad Prism 9. For the simple recombination assay, ligation and cleavage yields were calculated as simple ratios of the intensities of the cleaved and uncleaved bands. For RPR4 ligation, full length yield was calculated as the ratio the full-length product band intensity to the summed total intensities of observed bands.

Microscopy

Microscopy was performed on a Leica Thunder inverted widefield microscope equipped with an sCMOS camera Leica DFC9000 GTC using a 63x / NA 1.47 objective. Fluorescence channels were Ex 484 - 496 nm / Em 507 - 543 nm for FAM, and Ex 629 - 645 nm / Em 669 - 741 nm for Cy5. The sample stage was warmed to 30 ºC. Samples were loaded into clear bottomed 384 well plates or microchannel slides (Ibidi µ-Slide VI 0.4) 5 minutes after the addition of poly-L-lysine and mixing, then imaged after 2 h. All slides and plates were passivated using Pluronic F-68 to prevent droplet wetting and adhesion.

Electrospray Ionisation Mass Spectrometry

Electrospray mass spectrometry (ESI-MS) analysis was performed on a Maxis HD QTOF Mass Spectrometer (Bruker Daltonics GmbH, Bremen, Germany). The samples were diluted to 5 mM in 40 % acetonitrile (ACN), 0.05% fumaric acid (FA) or H_2O for $(Lys)_{5\text{-}24}$ and $(Lys)_{19\text{-}72}$ respectively. 1 µL of the diluted sample was injected and analyzed by continuous flow injection over 3 minutes (Agilent Infinity 1290, buffer A: 0.1 % FA in H_2O, buffer B: 80 % ACN, 0.1 % FA in H_2O, 250 µl min^{-1}, 70% B isocratic flow). All solvents were LCMS grade. The mass spectrometer was operated in positive mode at 4500 V capillary voltage, 500 V endplate offset, nebulizer N_2 pressure of 1.5 bar and dry gas flow of 10 L min^{-1} at 200 °C. Mass spectra were acquired at a mass range of m/z = 200 to m/z = 3000 at 10000 resolution and a rate of 1.0 Hz. The data were analyzed using Bruker Compass DataAnalysis 5.2 software.

Table S1. RNA oligomers used in this study. All RNA oligomers were ordered from IDT. Unlabelled oligomers were desalted, whilst labelled oligomers were purified by HPLC.

	5' functionalisation	Sequence	3' functionalisation	Description
loopB5	-	ACGUGAGAAACACUGCAG	-	5'-fragment of HPz loop B used in all ligation experiments
loopB3	-	CUGCAGGUACAUUACCACGUA	-	3'-fragment of HPz loop B used in all ligation experiments
SBS.rec	-	GGAAGUGAAGUAAU	-	Substrate binding strand (SBS) for recombination assay
sub	-	GUACAUUACAGGGCUUCCUA	-	Unlabelled 20 nt substrate for recombination assay
subC	-	GUACAUUACAGGGCUUCCUA	Cy5	3'-Cy5 labelled 20 nt substrate for recombination assay
3frag		GUACAUUACA	3' phosphorylation	Substrate for direct ligation to srB3. Requires activation with EDC to form 2',3'-cyclic phosphate
5frag	-	GGGCUUCCUA	-	Unlabelled 10 nt substrate for recombination assay
5fragF	-	GGGCUUCCUA	6-FAM	3'-FAM labelled 10 nt substrate for recombination assay
St	-	GUCCUGCCCAAUGACAAUCCUG	-	Substrate for HPz recombination ladder with cleavable 6 nt 3'-tail (yellow)
SBS.16mer	-	GGGCAGAGAAGUCA	-	HPz SBS for HPz recombination ladder
RPR4.1t	6-FAM	GUCAUUGAAAAAAAAAGACAAAUCUGCCCUCAGAGCUUG AGAACACAGUCGUGUG	-	RPR4 fragment 1 with cleavable 8 nt 3'-tail (yellow) used for assembly from inert oligonucleotides. Contains a 5'-6-carboxy-fluorescein
RPR4.2t	-	GUCGUGUGCAGAGGAGGCAGCCUUCGGUGGCGCGAUAGC GCCAACGUUCUCAACAGUCGCCC	-	RPR4 fragment 2 with cleavable 7 nt 3'-tail (yellow)
RPR4.3t	-	GUCGCCCAAUACUCCCGCUUCGGCGGGUGGGGAUAACAG UCGACGA	-	RPR4 fragment 3 with cleavable 8 nt 3'-tail (yellow)
RPR4.4	-	GUCGACGAAAAGGCGAUGUUAGACACGCCCGACUCAUAA UCCCCGGAGCUUCGGCUCC	Cy5	RPR4 fragment 4 used for direct ligation and for assembly from inert oligonucleotides.
SBS.RPR4.1	-	CUGCACACAGAAGUGUUCUC	-	HPz SBS used for ligation of RPR4 fragment 1 and 2
SBS.RPR4.2	-	AUUGGGCAGAAGUUGAGAAC	-	HPz SBS used for ligation of RPR4 fragment 2 and 3
SBS.RPR4.3	-	CUUUUCGUCAGAAGUUAUCCC	-	HPz SBS used for ligation of RPR4 fragment 3 and 4

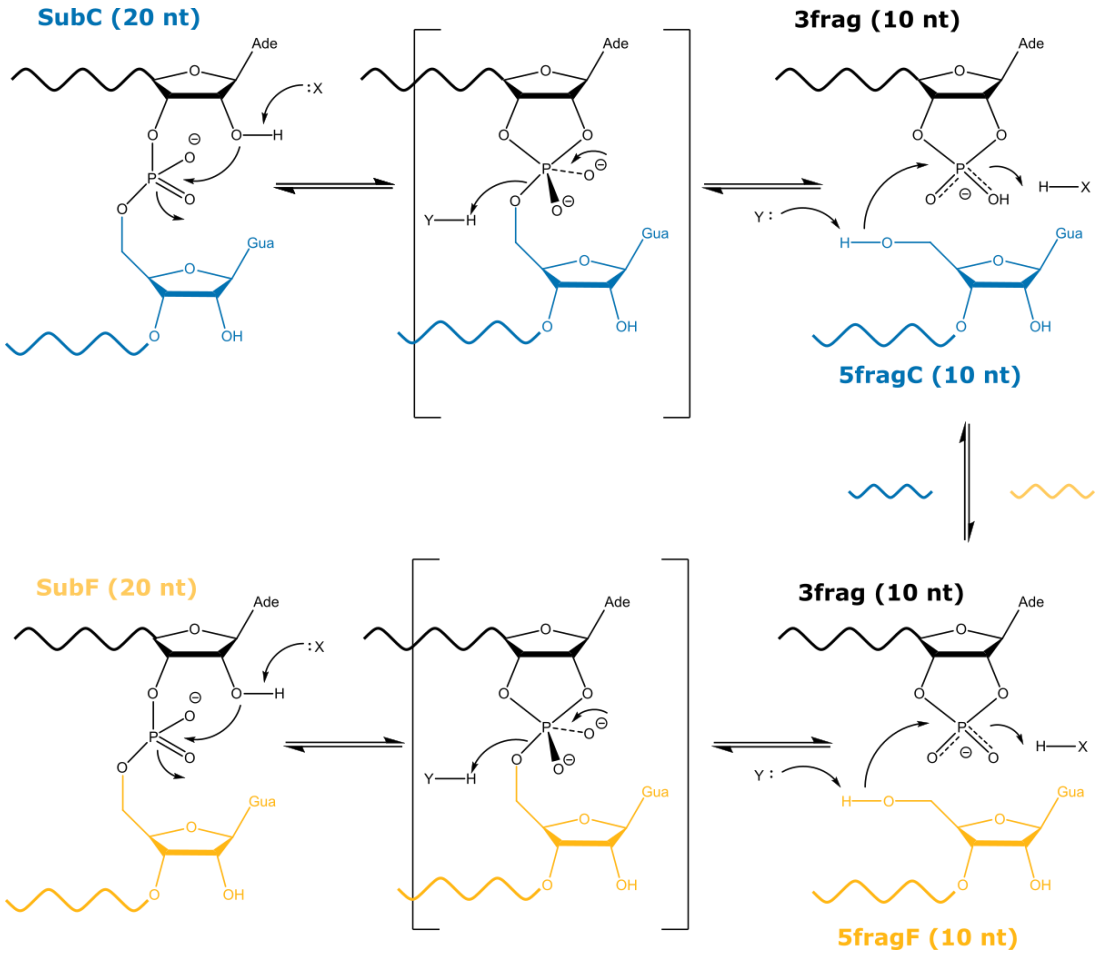

Figure S1. Mechanistic overview of the recombination process. First, the subC substrate strand is cleaved in a transesterification reaction which proceeds via S_N2 attack by the adjacent 2'-OH, resulting in a 3frag strand that is functionalised with a 2',3'-cyclic phosphate. Strand dissociation and reannealing may occur, resulting in exchange of RNA fragments with different 3'-fluorophores (5fragF or 5fragC). Ligation occurs when the 5'-OH of either 5fragC or 5fragF attacks the cyclic phosphate, and is the reverse of the cleavage process.

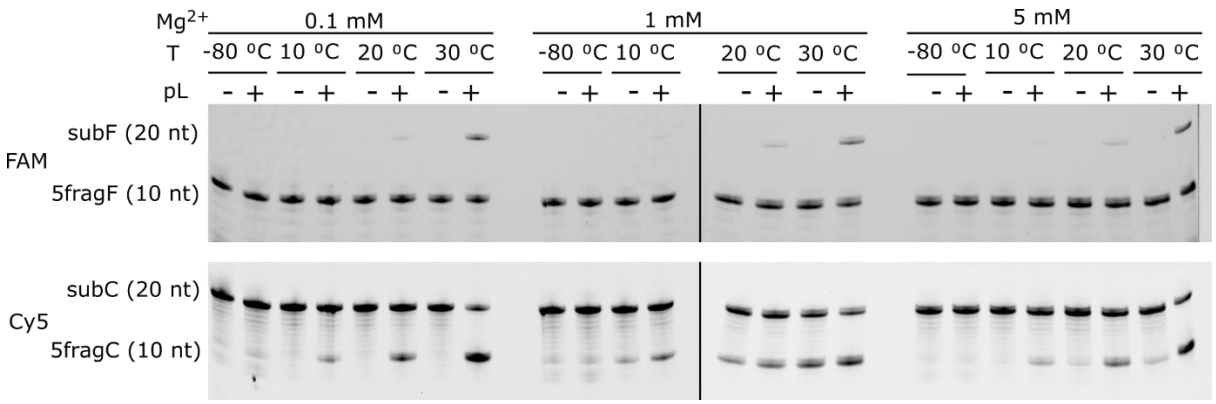

Figure S2. HPz recombination system activity under various conditions. An example urea PAGE gel showing the products of the HPz recombination system with and without $(Lys)_{19\text{-}72}$ (0.67:1 $(Lys)_{19\text{-}72}$:RNA), after 24 h at various temperatures and magnesium concentrations. Initial cleavage of the subC substrate to form 5fragC is monitored in the Cy5 channel, whilst recombination of 5fragF substrate to form subF is monitored in the FAM channel.

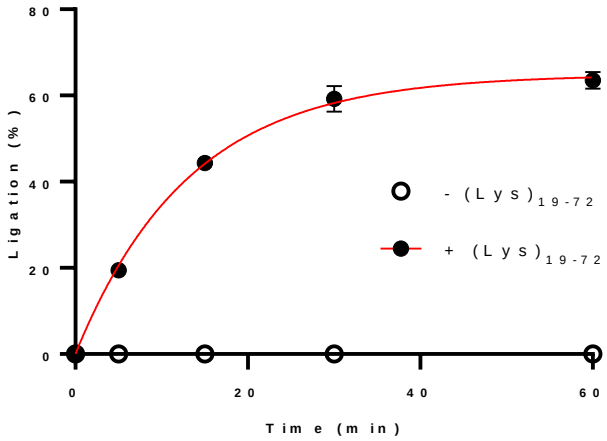

Figure S3. Ligation kinetics of pre-activated substrates. The 2',3'-cyclic phosphate functionalized 3frag oligomer is directly ligated to the FAM-labelled 5fragF oligomer by the HPz ribozyme. The reaction was performed in solution (open circles) or with 0.67:1 $(Lys)_{19\text{-}72}$:RNA (filled circles) at pH 8, 30 ºC and 1 mM $MgCl_2$. The ligation in the presence of poly-L-lysine was fitted with a single exponential ($R = 0.995$, red trace), yielding an apparent ligation rate of $k_{lig} = 0.076 \pm 0.004$ min^{-1}. No ligation was observed in the absence of $(Lys)_{19\text{-}72}$ up to 100 mM $MgCl_2$.

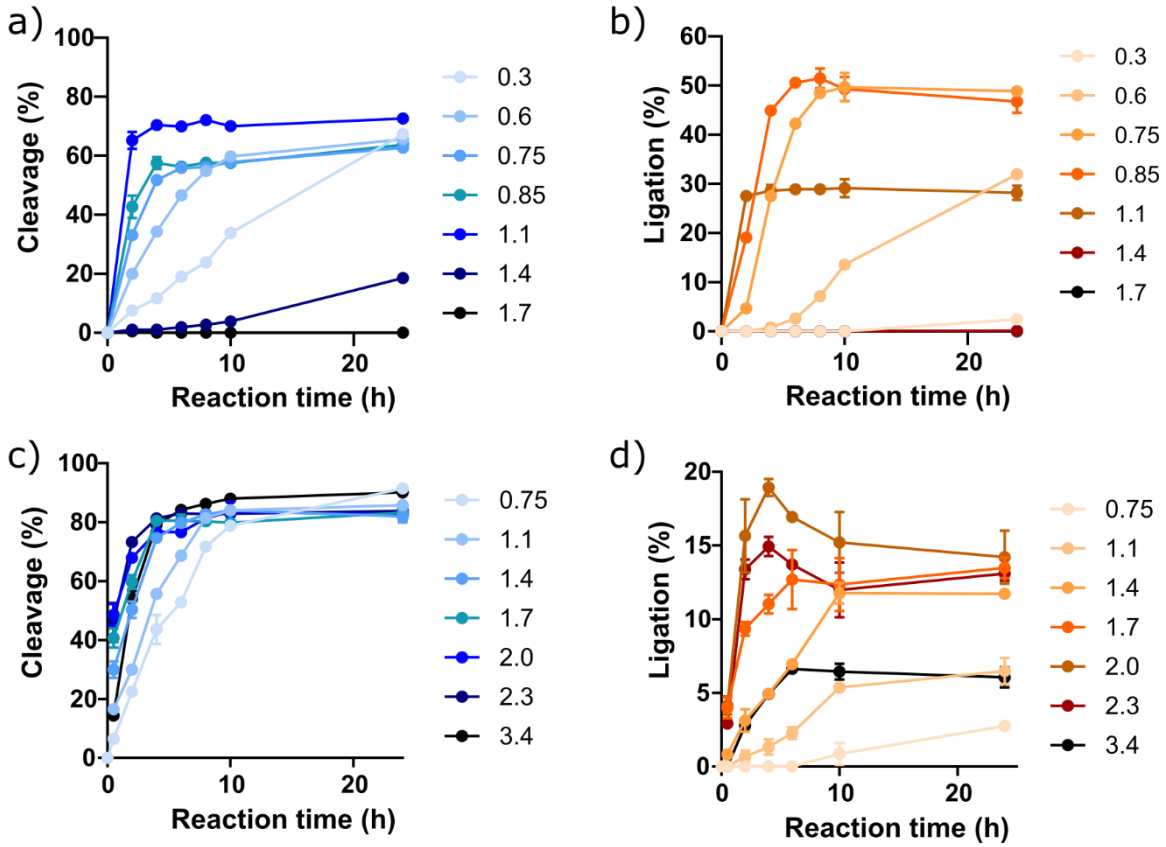

Figure S4. Influence of $(Lys)_n$ length and $(Lys)_n$:RNA mixing ratio on HPz recombination kinetics. HPz recombination activity was assayed with varying ratios of either $(Lys)_{19\text{-}72}$ (a, b) or $(Lys)_{5\text{-}24}$ (c, d). Cleavage of the Cy5-tagged subC to form 5fragC substrate is shown in a and c, whilst recombination of the FAM-tagged 5fragF to form subF is shown in b and d. The reaction was performed using 1 mM $MgCl_2$, pH 8 at 30 ºC.

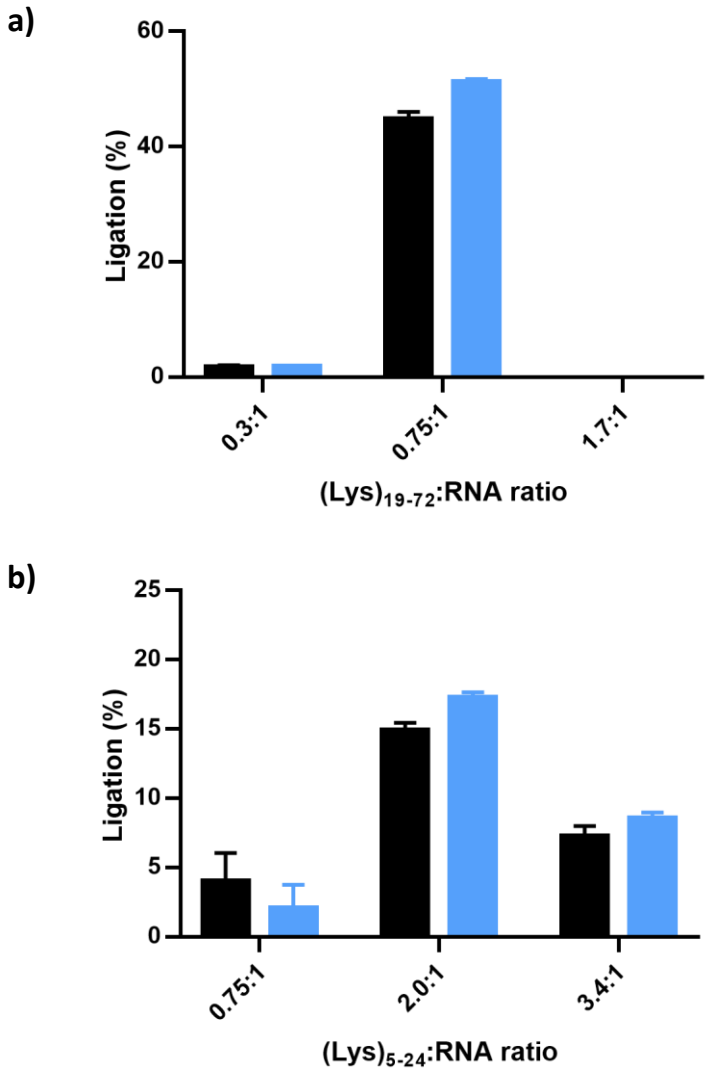

Figure S5. Comparison of recombination yields for reactions with and without Cy5 tagged substrate at various charge ratios. Reactions were performed varying mixing ratios of (a) $(Lys)_{19-72}$ or (b) $(Lys)_{5-24}$ (1 mM Mg^{2+}, pH 8, t = 24 h). Reactions with and without Cy5-tagged sub are shown in blue and black respectively.

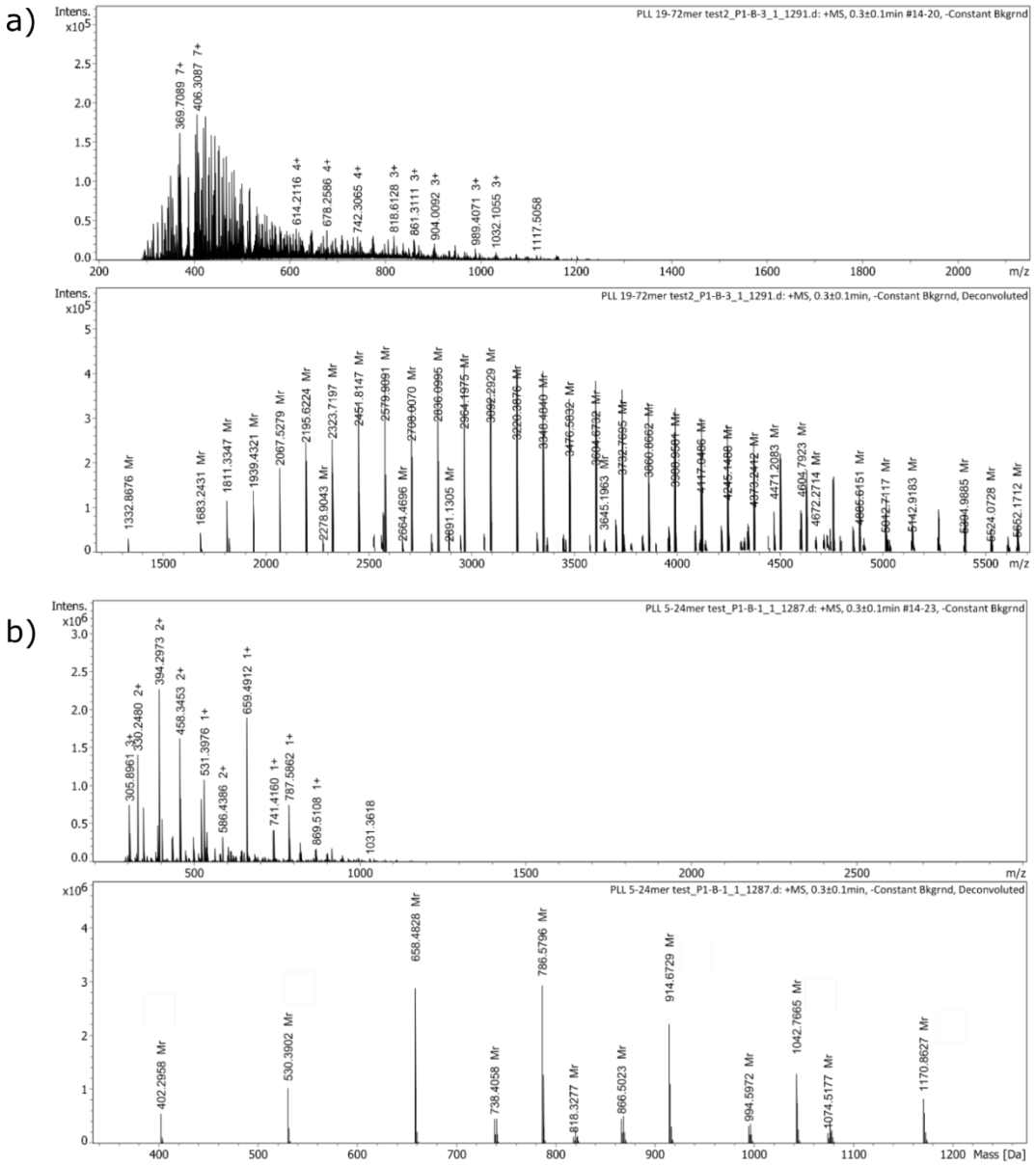

Figure S6. ESI mass spectra and deconvoluted ESI mass spectra of a) $(Lys)_{19\text{-}72}$ and b) $(Lys)_{5\text{-}24}$. Electrospray mass spectrometry (ESI-MS) was performed on a Maxis HD QTOF Mass Spectrometer (Bruker Daltonics GmbH, Bremen, Germany), and the data were analyzed using Bruker Compass DataAnalysis 5.2 software.

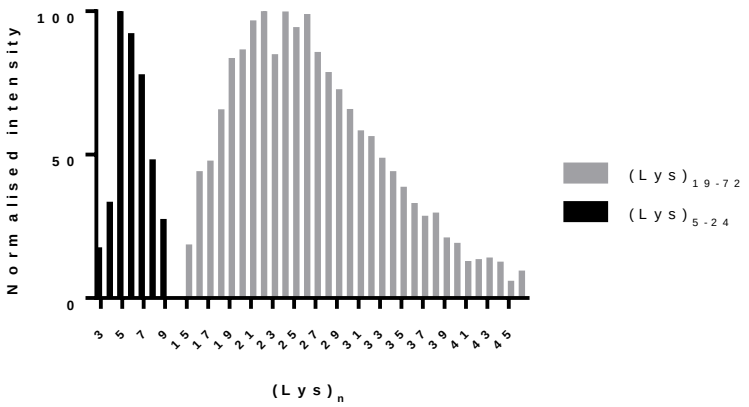

Figure S7. L-lysine oligomer distributions of $(Lys)_{19-72}$ and $(Lys)_{5-24}$ determined by ESI mass spectrometry. Distributions were produced using Bruker Compass DataAnalysis 5.2 software. Higher oligomers may be present in the samples at low concentrations, but fall below the detection threshold due to the high abundance of shorter material.

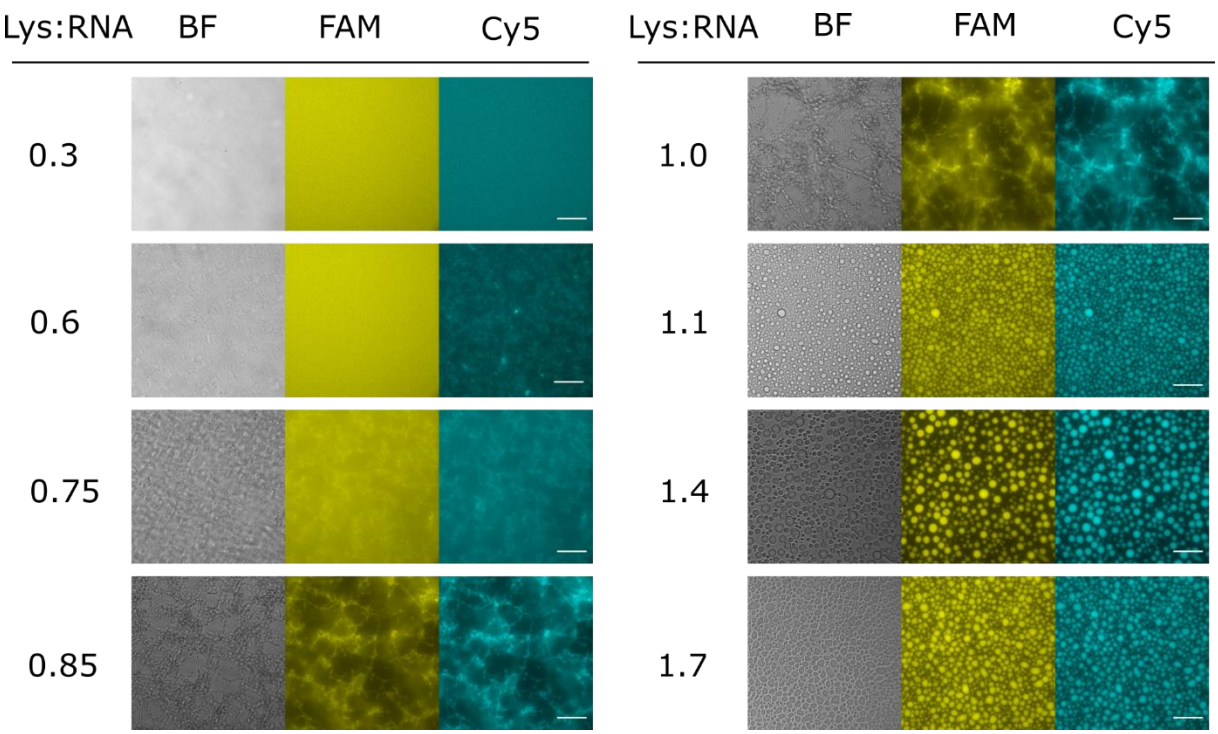

Figure S8. Fluorescence microscopy images of $(Lys)_{19\text{-}72}$:RNA condensates at various ratios. Brightfield imaging is shown in grey, FAM fluorescence (5fragF / subF) in yellow, and Cy5 fluorescence (subC / 5fragC) in cyan. Scale bars = 20 µm.

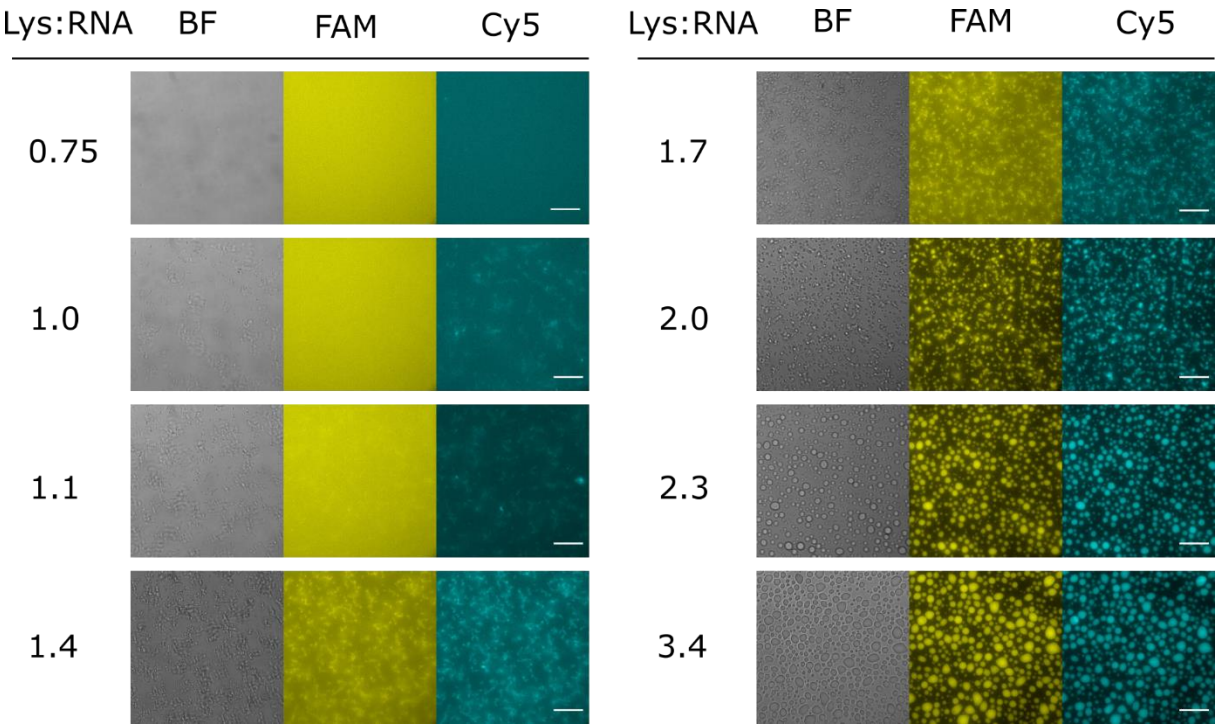

Figure S9. Fluorescence microscopy images of $(Lys)_{5\text{-}24}$:RNA condensates at various ratios. Brightfield imaging is shown in grey, FAM fluorescence (5fragF / subF) in yellow, and Cy5 fluorescence (subC / 5fragC) in cyan. Scale bars = 20 µm.