Lipid membranes modulate the activity of RNA through sequence-specific interactions

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Abstract

RNA is a ubiquitous biomolecule that can serve as both catalyst and information carrier. Understanding how RNA activity is controlled and how it in turn regulates bioactivity is crucial for elucidating its physiological roles and potential applications in synthetic biology. Here we show that lipid membranes can act as RNA organization platforms, introducing a novel mechanism for ribo-regulation. The activity of R3C ribozyme can be modified by the presence of lipid membranes, with direct RNA-lipid interactions dependent on RNA sequence, structure and length. In particular, the presence of guanine in short RNAs is crucial for RNA-lipid interactions, while double-stranded RNAs further increase lipid-binding affinity. Lastly, by artificially modifying the R3C-substrate sequence to enhance membrane binding we unexpectedly generated a lipid-sensitive riboswitch. These findings introduce RNA-lipid interactions as a tool for developing riboswitches and novel RNA-based lipid biosensors, and bear significant implications for RNA World scenarios for the origin of life.

Introduction

RNA performs diverse functions ranging from information storage, to regulation of other biomolecules, to directly catalysing biochemical reactions. The functional versatility of RNA has implications for understanding plausible scenarios for the origin of life[1–3], and for developing tools in synthetic biology[4–6]. Research aimed at understanding how an RNA World could have emerged has motivated development of ribozymes with functions including RNA ligation[7–10], replication[11,12] and other activities[13–16]. The experimental development of functional RNAs raises the possibility of RNA-based synthetic systems. For both synthetic life and understanding the origin of self-replicating organisms, RNA has intrinsic appeal: it can serve functions of both DNA (information storage) and proteins (enzymes), obviating the need for translation machineries and protein chaperones. Furthermore, RNA does not undergo irreversible denaturation like proteins, lending robustness against a broad range of physical and chemical conditions. In order to design a system based on RNA it is essential to be able to coordinate RNA activity in space and time.

Key to harnessing the functional versatility of RNA is in understanding how to spatially and temporally modulate its properties and to selectively modulate the activity of different RNAs within one system. The physicochemical environment surrounding an RNA molecule is a central determinant of its structure, stability and activity. Spontaneous RNA hydrolysis and ligation, as well as the catalytic RNA activity are sensitive to pH[17], ionic strength[18,19] and RNA concentration changes[11], among other parameters. Similarly, RNA activity can be modulated by interactions with molecules such as ions, proteins, and other nucleic acids[20]. Thus, one approach to determining RNA activity could be via

tunable interactions with binding partners that affect RNA's structure, concentration or chemical microenvironment.

One mechanism for modulating RNA activity could be through direct RNA-lipid interactions[21–23]. Because of their amphiphilic nature, lipids spontaneously self-assemble into membranous structures that can encapsulate RNA into protected and selective microcompartments[24–26]. Alternatively, direct RNA-lipid interactions could localize RNAs to the membrane surfaces, increasing its local concentration and reducing dimensionality for intermolecular interactions[21]. Lastly, localization to a lipid surface brings RNA into a physicochemically unique microenvironment with sharp gradients of hydrophobicity, electrical permittivity, and water activity. Through these effects, RNA-lipid interactions could provide a powerful mechanism for modulating RNA activity.

The first functional RNA-lipid interaction was described more than 40 years ago[27], with subsequent research revealing various factors that facilitate nucleic acid-lipid binding[28–38]. More recently, specific RNA sequences have been generated through SELEX with affinity for fluid membranes comprised of phospholipids and cholesterol[39–41]. Interestingly, mixtures of RNAs were also shown to bind to membranes that are in a solid crystalline (gel) phase[42,43]. These studies revealed that while most randomized mixtures of RNA sequences can bind to gel membranes, there is a relatively small chemical space of oligomers that have affinity for fluid membranes. Thus, conceptually, gel membranes could provide a platform for modulating the activity of a diverse range of RNAs. However, effects of gel membranes on RNA activity and the sequence selectivity of such interactions are relatively unexplored.

This study reports the effect of lipid membranes on RNA catalytic activity. We show that RNA-lipid binding depends on the RNA primary sequence, secondary structure, and length. Using the trans-acting R3C ligase ribozyme we observed that R3C-lipid binding changes ribozyme activity in a concentration-dependent manner. Lipid binding assays show that the interaction of short RNA sequences with gel membranes is sequence-specific and depends on guanine content and presence of double-stranded structures. Lastly, modification of R3C's substrate sequence increased tunability of the R3C based reactions, through a lipid-dependent riboswitch. Our findings prove that membranes can serve as platforms for RNA-activity modulation, which can contribute to the development of RNA-based biosensors and riboswitches. This approach introduces new tools for molecular and synthetic biology and raises the prospect of previously unrecognized roles for RNA-lipid interactions in the origin and evolution of life.

Results

RNA-lipid binding changes probability of RNA-RNA interactions

Building on previous observations that RNA binds to lipid membranes[27,39–42], we hypothesized that RNA-membrane interactions could influence ribozyme activity by changes of RNA local concentrations on the membrane surface and/or influencing RNA conformations. Indeed, RNA oligonucleotides can bind to lipid membranes in the gel phase in the presence of the divalent cations, leading to aggregation into large RNA-lipid assemblies (**Fig. 1a**) [28,36,42,43]. Depletion of divalent ions or increasing temperature above the lipid melting temperature (i.e. producing fluid instead of gel membranes) reduces RNA-membrane binding and aggregation. This reversible aggregation could provide a tunable mechanism to concentrate RNAs. To test this, we asked whether RNAs are in fact concentrated in the presence of gel membranes, and how that influences trans-acting-ribozyme activity.

To determine changes in local RNA concentration upon interaction with lipid membranes, we relied on UV-mediated crosslinking. All membranes in this study were comprised of dipalmitoylphosphatidylcholine (DPPC) which are in a solid crystalline (gel) phase at the chosen experimental temperature of 24 °C. Nucleic acid bases absorb UV light, producing chemical changes that yield base-base covalent bonds in a distance dependent manner, yielding insights into the structure and interactions of nucleic acids[44–46]. We first observed the effect of gel-membranes on the crosslinking of a single defined RNA sequence, the R3C ligase, which predominately forms one structure (R3C ligase, **Fig. 1d**). When subjected to UV, the R3C ligase shows a slight increase of crosslinking in the presence of low lipid concentrations compared to the reaction in the absence of lipids. A further increase of lipid concentration led to a decrease of crosslinking efficiency, possibly through dilution of the RNA species on the surface of the lipid membranes. This effect was inversely correlated with RNA-lipid binding efficiency (**Fig. 1b**). On the other hand, a mixture of RNA oligomers with randomized sequences which is able to create a more diverse range of intermolecular structures (**Fig. 1d**) showed continuously increasing and overall higher UV-crosslinking efficiency in the presence of lipids (**Fig. 1c**). The contrasting effect of crosslinking for R3C and randomized oligomers might be explained by the influence of lipid membranes on RNA interactions: membrane binding could potentially stabilize base-pairing[47] and thereby enhance crosslinking efficiency, whereas the decrease of crosslinking might be caused by a dilution effect at higher membrane surface areas or lipid-based steric hindrance. These observations demonstrate that RNA-membrane interactions can influence RNA-RNA interactions in a manner that is dependent on lipid concentration and RNA sequence diversity. This further suggests that membrane binding could have an effect on trans-acting ribozyme activity derived from base pairing.

RNA-lipid interactions influence RNA catalytic activity

To investigate the functional consequence of RNA-lipid binding, we tested the effect of lipids on the trans-acting R3C ligase ribozyme activity. R3C ligase is a relatively short ribozyme that catalyzes ligation of substrate strands to the ribozyme[10](**Fig. 2a**). Since R3C is also part of an RNA self-replication system [11,12], the effects of lipids on R3C is also interesting with regard to the emergence, evolution and artificial synthesis of autonomous self-replicating systems. The R3C reaction rate in the absence of membranes was 11 pM/min, with reaction constant rate of $k = 6.4 \times 10^{-4}$ min^{-1}**.** Addition of lipid vesicles led to an increase of the reaction rate (14 pM/min, +29%), which could plausibly be due to increased ligase-substrate interaction on the membrane either through increased concentrations at the membrane surface, or through stabilization of base pairing[47]. At the highest lipid concentration the reaction rate dropped to 8 pM/min (-27%) (**Fig. 2b**). A decrease in reaction rate was correlated with further R3C ligase binding to the lipid membranes (>100 lipid/R3C, **Fig. 1b**) and we speculate that this may be caused by steric hindrance from the lipid vesicles. These results show that gel-membranes can influence the reaction rate of a trans-acting ribozyme in a lipid concentration-dependent manner.

R3C ligase and its substrate bind differently to the membrane

Previous findings determined effect of lipid-R3C ligase interaction on its activity. To further understand how R3C and substrate membrane binding might contribute to changes in ligation rates, lipid-binding affinities of the ligase and its substrate were determined. To quantify binding to the membranes we calculated lipid – buffer partition coefficients (K). The short 12 nt substrate shows a more than 3 orders of magnitude lower partition coefficient compared with the 84 nt R3C ligase (**Fig. 2c**). Thus, in this system substrate-membrane binding is essentially negligible compared with R3C-membrane binding, suggesting that the increased activity observed in **Figure 2b** was due to an effect of the membrane on

137 the R3C ligase-substrate complex stability, rather than through enhanced substrate-lipid interactions
138 on the membrane surface.
139
140 The large difference in membrane partitioning between the R3C ligase and its substrate raised the
141 possibility that RNA features such as length, base composition or structure could influence membrane
142 binding (**Fig. 2c**). Understanding the features that influence membrane interactions could allow us to
143 engineer RNAs with higher membrane affinity, and thereby potentially enhance activity by
144 concentrating RNAs at the membrane surface. Consequently, we next investigated what features of
145 RNA influence its interaction with lipid membranes.
146
147 **RNA sequence influences binding to gel membranes**
148 Interactions of RNA with gel phase membranes have been previously shown to exhibit low sequence-
149 specificity, based on binding of oligos with randomized sequences[42,43]. However, the large difference
150 in binding for R3C and its substrate suggested to us that RNA-gel membrane interactions might in fact
151 be dependent on RNA composition or structure.
152
153 We first evaluated how the base content influences membrane binding of short RNA species. 19nt
154 oligomers with only one type of nucleotide were tested together with a random sequence as a control.
155 To quantify relative changes of RNA-membrane binding, we calculated the RNA binding efficiency as
156 the percentage of total RNA bound to membranes at a fixed oligo to lipid ratio. Remarkably, while the
157 19xG oligomer binds very efficiently to the gel phase membrane (84% binding efficiency), oligomers of
158 A, C or U showed practically no binding. A mixture of 19-mer RNA with randomized sequences showed
159 moderate binding (33% binding efficiency), as did one with AG-repeats (**Fig. 3a**), most probably due to
160 the introduction of guanine. Guanine could conceivably enhance binding either through direct
161 interactions with the membrane, or alternatively by promoting G≡C base pairing, thereby influencing
162 structure. The enhanced binding of an oligomer with only AG-repeats suggests that membrane binding
163 can be influenced by direct guanine-membrane interactions.
164
165 We next examined the effect of deleting a specific base from mixtures of 40-mer oligos with
166 randomized sequences. Depletion of A or U led to slight decrease of membrane binding, whereas
167 removal of G or C showed a significant binding decrease with highest effect for guanine-depleted
168 oligomers. The decrease in binding affinity in all of the base-depleted oligos indicated that base-pairing
169 may influence membrane binding (**Fig. 3b**). However, the larger effect of G and C depletion indicates
170 that base pairing is not the only factor responsible for membrane binding consistent with our previous
171 observation that G-content alone can influence binding. Finally, by comparing 19-mer and 40-mer
172 randomized oligos, we observe that increasing RNA length also led to enhanced binding, possibly due
173 to increased length and G content (**Fig. 3 a,b**).
174
175 To further determine how guanine content affects membrane binding, lipid-buffer partition
176 coefficients were determined for short oligomers with varying G-content. RNA membrane-buffer
177 partition coefficients indicate how well a particular molecule binds to the membrane by comparing the
178 relative amount of RNA in buffer and on the lipid membrane. The RNA oligos measured in this study
179 had partition coefficients around 10^5, which is in the range of partition coefficients determined for
180 hydrophobic peptides[48]. Remarkably, addition of 2 G's per oligo led to a 5-fold increase of the partition
181 coefficient relative to guanine-depleted RNA. Oligos with 4 guanine residues showed the highest
182 partition coefficient, plateauing with additional guanosines and a small decrease of binding for 7

183 guanine/oligo. This result shows that the presence of guanine affects RNA-lipid binding and even low
184 guanine content significantly increases membrane binding efficiency (**Fig. 3c**).
185
186 **RNA-membrane binding is dependent on RNA structure**
187 Our observations of the effect of base depletion on oligomer binding suggested that base-pairing may
188 be a key factor in membrane binding. To investigate the effect of base-pairing-derived secondary
189 structures on membrane interactions, we estimated binding efficiency for ssRNA and dsRNA
190 (composed of 100% complementary sequences) species. We observed that not only was the
191 membrane binding efficiency higher for dsRNA compared with ssRNA species, but also that only dsRNA
192 promoted vesicle aggregation (**Fig. 4a**). It is interesting to note that the difference in binding efficiency
193 of the two ssRNAs, which have identical base composition but different sequence, is consistent with
194 the importance of sequence as key determinate of membrane binding. We further observed that
195 dsRNA interacts with fewer lipids than ssRNA, implying different models of binding (**Fig. 4b**). Thus,
196 membrane binding efficiency can be dependent on the propensity of an RNA molecule to form intra-
197 or inter-molecular structures through base pairing.
198
199 To gain further insight into the effect of base pairing-dependent intra- and inter-molecular
200 interactions, we estimated the lipid to nucleotide binding ratios of ssRNA and dsRNA (**Fig. 4c**). By
201 calculating the molecular ratio of lipid/nucleotide binding we introduce a normalization factor that
202 allows us to directly compare membrane binding for oligos of varying length. Furthermore, varying
203 lipid to nucleotide stoichiometry reflects changes in the average lipid/nucleotide interaction strength.
204 With this estimate we compared lipid/nucleotide binding for ssRNA and dsRNA alongside oligos with
205 varying guanine content from our previous analysis (**Fig. 3c**)**.** The differences between ssRNA and
206 dsRNA, all containing 9 guanines per oligo reiterate our previous observation that sequence variation
207 (with constant base content) and base pairing can both influence binding. However, the reason for a
208 change in lipid/nucleotide ratio for oligos with varying guanine content was not readily apparent.
209 Analysis of oligos with varying guanine content by native non-denaturing gel revealed that the
210 presence of guanine promotes a larger spread in migration, indicating that guanines promote
211 intermolecular interactions. These observations show that guanine can enhance binding by promoting
212 intermolecular interactions, and further confirm the importance of base pairing in RNA-lipid
213 interactions.
214
215 **Modifying R3C substrate sequence enhances membrane binding and modifies reaction rates**
216 In previous experiments we observed that the binding affinity of the short substrate was >3 orders of
217 magnitude lower than for the R3C ligase and that reaction rates correlated strongly with R3C-
218 membrane binding (**Fig. 2c, d**). We therefore reasoned that by modifying the R3C ligase substrate to
219 enhance membrane binding, we might be able to further modulate ligase activity through increased
220 RNA density at the membrane surface. Having determined that guanine content is a key factor
221 influencing RNA-lipid binding (**Fig. 3**), we modified the R3C substrate sequence to increase lipid
222 membrane binding efficiency by addition of a 5'-overhang with varying amounts of AG repeats (**Fig.**
223 **5a**).
224
225 We first assessed the effect of modifying the R3C ligase substrate on ligation rate in the absence of
226 membranes (**Fig. 5a**). We observed that increasing the guanine content decreased ligase activity,
227 suggesting that 5' modification of the substrate was inhibiting the reaction either through steric
228 hindrance, or promotion of inhibitory inter- or intra-molecular interactions. To determine if AG-rich

229 substrates are forming intra-molecular structures[49] and/or exhibiting reduced binding with the R3C
230 ligase, electrophoresis in native conditions with and without ligase was conducted (**Fig. 5b**). Firstly, the
231 non-modified short substrate migrated as one band and was fully bound to the R3C ligase. Secondly,
232 4x(AG) 20 nt substrate variant migrates as two bands, which suggests that part of the substrate
233 molecules have some folding, which might bias binding to the R3C ligase – in the presence of ligase
234 30% of the substrate was non-bound, which correlated with a 27% reaction rate drop. For the longest
235 substrate variant (31 nt) electrophoretic mobility suggested higher folding, since only one band is
236 present and migrates half-way between the non-folded 12 nt and 20 nt substrates. 70% of the 9x(AG)
237 substrate is not bound to the ligase, which correlates with a R3C reaction rate decrease. Lastly, the AG-
238 rich oligomer does not co-migrate with the R3C ligase, thus the inhibition effect is not based on non-
239 specific substrate-ligase interactions (not shown). These observations show that increasing guanine
240 content reduced R3C ligase-substrate binding, likely accounting for decreased activity.
241
242 We next determined whether varying guanine content through 5' modification of the substrate
243 influenced membrane binding and, consequently, ligation activity in the presence of membranes. As
244 expected, the longer guanine-containing R3C substrates showed significantly higher membrane
245 binding compared with the shorter variant (**Fig. 5c**). To determine if binding of the substrates to the
246 membrane enhanced ligase activity, R3C reaction rates were determined in the presence of the gel
247 lipid membranes (**Fig. 5d**). For modified substrates, the reaction rates increased significantly to the
248 level of the unmodified substrate. Interestingly, the low-G substrate (2G) shows no significant reaction
249 increase with the introduction of membranes, indicating that the rescue of activity of the other
250 guanine variants is not due to increased substrate-ligase interactions through enhanced binding to the
251 membrane, but rather due to interaction of the membrane with 5' overhangs. Thus, substrate-
252 membrane interaction increases activity by rescuing the inhibitory effect of the guanine-rich 5'
253 overhangs (**Fig. 5e**).
254
255 In summary, by modifying the R3C ribozyme substrate with an additional short sequence containing
256 guanine residues, we increased membrane affinity. The addition of guanines, however, also introduced
257 an inhibitory effect on ligation activity that was reversed through the introduction of gel-membranes
258 to the reaction. Thus, we unexpectedly produced a lipid-sensitive riboswitch-like system that could be
259 tuned in a lipid concentration-dependent manner.
260

261 ## Discussion

262
263 We demonstrated that RNA-gel membrane interactions are dependent on the primary sequence and
264 secondary structure of ribonucleic acids. Varying guanosine content of RNAs changed the membrane-
265 buffer partitioning by several orders of magnitude. Additionally, double stranded RNAs exhibited
266 higher membrane partitioning than single stranded RNAs, indicating that secondary structure is a
267 critical factor in RNA-membrane interactions. Although gel-membranes are not widely observed in
268 living cells, RNA-gel membrane interactions can be employed for ribo-regulation in synthetic biological
269 systems. Gel membranes also plausibly accumulated in ancient prebiotic scenarios, potentially serving
270 as an organizational scaffold, as has been proposed for "ribofilms" on mineral surfaces[50]. Furthermore,
271 understanding the factors involved in RNA-gel membrane interactions can provide insight for designing
272 RNA sequences with affinity for physiologically-relevant fluid membranes. Indeed, our finding that
273 guanosine is a key factor in promoting RNA-gel membrane partitioning is consistent with previous

speculation that guanosine residues might play a role in the binding of RNA aptamers for fluid membranes[39]. Taken together, the sequence and structural dependence of RNA-gel membrane interactions shown here provides the necessary framework to design synthetic RNAs with varying membrane affinities.

Our observation of riboswitch-like behavior suggests a novel mechanism for regulation of RNA through membrane interactions. Riboswitches are RNAs that have regulatory effects that are derived from sensitivity to physicochemical conditions or through binding with an interaction partner. Riboswitch regulation through temperature changes, interaction with nucleic acids and small molecules have been described previously[51–55]. However, lipid sensitive riboswitches have not been documented. It is now plausible that spontaneously self-assembling lipid membranes in ancient environments could have served as an organizational scaffold regulating the activity of RNAs in a sequence and structure dependent manner. Furthermore, the importance of guanosine in membrane binding suggests a possible mechanism for its selection as one of the canonical nucleic acids. In extant organisms, synthetic or naturally occurring RNAs exhibiting sensitivity to lipids could potentially act as biosensors or riboswitches. By demonstrating that RNA-gel membrane interactions can induce riboswitch-like behaviors, we raise the possibility that such functionalities could be an ancient mechanism of ribo-regulation and may be present among the myriad of uncharacterized small RNAs in modern cells.

RNA-membrane interactions provide a basis for selection of RNAs based on sequence and structure. The induction of RNA-membrane interactions allows spatial segregation or co-localization of RNAs based on differential membrane affinities, affording an additional degree of control over localization of RNAs in cell-free synthetic systems. RNA-membrane localization in a gel membrane system could be controlled by shifting the temperature above and below the membrane gel-liquid transition temperature, thereby turning on and off RNA-membrane binding. Membrane localization could also be regulated dynamically through enzymatic modification[56–58] of RNA. Lipid-dependent sorting and aggregation of RNAs could have played a role in primordial scenarios, where one of the biggest challenges was to increase concentration of molecules. The sequence and structural selectivity of RNAs for membranes could also be employed in the design of novel RNA membrane biosensors. Selective RNA-membrane interactions would also influence the sequence space explored by evolving ribozymes and may give rise to novel functions. Looking forward, RNA-membrane interactions can provide tools for selective ribo-regulation in synthetic systems, development of membrane biosensors, and might facilitate the emergence of novel functions through artificial and natural evolution of ribozymes in the presence of membranes.

In conclusion, we reveal that lipids, which are present in every modern cell and were plausibly part of a prebiotic world[59–61], can interact with RNA and change its activity. These findings have significant application in fields such as synthetic biology where merging the selective affinities of aptamers with ribozyme activity (aptazymes) is currently a developing field[62–64]. Our results provide a foundation for exploring the basis for membrane-RNA interactions with physiologically relevant fluid membranes exhibited by synthetic aptamers[16,65,66] thereby providing new regulatory mechanisms in synthetic biology through the specific and anchor-free localization of RNA molecules within lipid compartments. Nonetheless, insights from the present study can already be implemented in bioengineering applications such as in improving mRNA drug delivery mechanisms and introducing lipid sensitive ribo-regulation to synthetic ribozyme networks. Furthermore, the interaction between RNA and lipid membranes could have provided an evolutionary pressure in ancient times through selection for base

composition or secondary structural features. Our observations show that RNA-lipid interactions have applications in designing ribozyme-based metabolic circuits, lipid-based riboswitches, and provide new mechanisms for evolution of function in an RNA World.

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References

1. Pace, N. R. & Marsh, T. L. RNA catalysis and the origin of life. *Origins Life Evol B* **16**, 97–116 (1985).

2. Gilbert, W. Origin of life: The RNA world. *Nature* **319**, 618–618 (1986).

3. Rich, A. On the problems of evolution and biochemical information transfer. *Horizons in biochemistry* 103–126 (1962).

4. Breaker, R. R. Engineered allosteric ribozymes as biosensor components. *Curr Opin Biotech* **13**, 31–39 (2002).

5. Wittmann, A. & Suess, B. Engineered riboswitches: Expanding researchers' toolbox with synthetic RNA regulators. *Febs Lett* **586**, 2076–2083 (2012).

6. Isaacs, F. J., Dwyer, D. J. & Collins, J. J. RNA synthetic biology. *Nat Biotechnol* **24**, 545–554 (2006).

7. Bartel, D. & Szostak, J. Isolation of new ribozymes from a large pool of random sequences. *Science* **261**, 1411–1418 (1993).

8. Ekland, E., Szostak, J. & Bartel, D. Structurally complex and highly active RNA ligases derived from random RNA sequences. *Science* **269**, 364–370 (1995).

9. Robertson, M. P., Hesselberth, J. R. & Ellington, A. D. Optimization and optimality of a short ribozyme ligase that joins non-Watson–Crick base pairings. *Rna* **7**, 513–523 (2001).

10. Rogers, J. & Joyce, G. F. The effect of cytidine on the structure and function of an RNA ligase ribozyme. *Rna* **7**, 395–404 (2001).

355 11. Paul, N. & Joyce, G. F. A self-replicating ligase ribozyme. *Proc National Acad Sci* **99**,
356 12733–12740 (2002).

357 12. Lincoln, T. A. & Joyce, G. F. Self-Sustained Replication of an RNA Enzyme. *Science* **323**,
358 1229–1232 (2009).

359 13. Saragliadis, A., Krajewski, S. S., Rehm, C., Narberhaus, F. & Hartig, J. S. Thermozymes:
360 synthetic RNA thermometers based on ribozyme activity. *Rna Biol* **10**, 1009–1016 (2013).

361 14. Ferretti, A. C. & Joyce, G. F. Kinetic Properties of an RNA Enzyme That Undergoes Self-
362 Sustained Exponential Amplification. *Biochemistry-us* **52**, 1227–1235 (2013).

363 15. Lam, B. J. & Joyce, G. F. Autocatalytic aptazymes enable ligand-dependent exponential
364 amplification of RNA. *Nat Biotechnol* **27**, 288–292 (2009).

365 16. Janas, T., Janas, T. & Yarus, M. A membrane transporter for tryptophan composed of
366 RNA. *Rna* **10**, 1541–1549 (2004).

367 17. Pino, S., Ciciriello, F., Costanzo, G. & Mauro, E. D. Nonenzymatic RNA Ligation in Water*.
368 *J Biol Chem* **283**, 36494–36503 (2008).

369 18. Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N. & Altman, S. The RNA moiety of
370 ribonuclease P is the catalytic subunit of the enzyme. *Cell* **35**, 849–857 (1983).

371 19. Anella, F. & Danelon, C. Prebiotic Factors Influencing the Activity of a Ligase Ribozyme.
372 *Life* **7**, 17 (2017).

373 20. Dai, X., Zhang, S. & Zaleta-Rivera, K. RNA: interactions drive functionalities. *Mol Biol Rep*
374 **47**, 1413–1434 (2020).

375 21. Müller, U. F. & Bartel, D. P. Improved polymerase ribozyme efficiency on hydrophobic
376 assemblies. *Rna* **14**, 552–562 (2008).

377 22. Anella, F. & Danelon, C. Prebiotic Factors Influencing the Activity of a Ligase Ribozyme.
378 *Life* **7**, 17 (2017).

379 23. Anella, F. & Danelon, C. Reconciling Ligase Ribozyme Activity with Fatty Acid Vesicle
380 Stability. *Life* **4**, 929–943 (2014).

381 24. Chen, I. A., Salehi-Ashtiani, K. & Szostak, J. W. RNA Catalysis in Model Protocell Vesicles. *J*
382 *Am Chem Soc* **127**, 13213–13219 (2005).

383 25. Mansy, S. S. *et al.* Template-directed synthesis of a genetic polymer in a model protocell.
384 *Nature* **454**, 122–125 (2008).

385 26. Bianconi, G., Zhao, K., Chen, I. A. & Nowak, M. A. Selection for Replicases in Protocells.
386 *Plos Comput Biol* **9**, e1003051 (2013).

387 27. Budker, V. G., Kazatchkov, Yu. A. & Naumova, L. P. Polynucleotides adsorb on
388 mitochondrial and model lipid membranes in the presence of bivalent cations. *Febs Lett* **95**,
389 143–146 (1978).

390 28. McManus, J. J., Rädler, J. O. & Dawson, K. A. Does Calcium Turn a Zwitterionic Lipid
391 Cationic? *J Phys Chem B* **107**, 9869–9875 (2003).

392 29. Kharakoz, D. P., Khusainova, R. S., Gorelov, A. V. & Dawson, K. A. Stoichiometry of
393 dipalmitoylphosphatidylcholine-DNA interaction in the presence of Ca2+: a temperature-
394 scanning ultrasonic study. *Febs Lett* **446**, 27–29 (1999).

395 30. Gromelski, S. & Brezesinski, G. Adsorption of DNA to zwitterionic DMPE monolayers
396 mediated by magnesium ions. *Phys Chem Chem Phys* **6**, 5551–5556 (2004).

397 31. Uhríková, D. *et al.* The structure of DNA–DOPC aggregates formed in presence of calcium
398 and magnesium ions: A small-angle synchrotron X-ray diffraction study. *Biochimica Et*
399 *Biophysica Acta Bba - Biomembr* **1713**, 15–28 (2005).

400 32. Gromelski, S. & Brezesinski, G. DNA Condensation and Interaction with Zwitterionic
401 Phospholipids Mediated by Divalent Cations. *Langmuir* **22**, 6293–6301 (2006).

402 33. McLoughlin, D. *et al.* Surface Complexation of DNA with Insoluble Monolayers. Influence
403 of Divalent Counterions. *Langmuir* **21**, 1900–1907 (2005).

404 34. Michanek, A. *et al.* RNA and DNA Association to Zwitterionic and Charged Monolayers at
405 the Air–Liquid Interface. *Langmuir* **28**, 9621–9633 (2012).

406 35. Michanek, A., Kristen, N., Höök, F., Nylander, T. & Sparr, E. RNA and DNA interactions
407 with zwitterionic and charged lipid membranes — A DSC and QCM-D study. *Biochimica Et*
408 *Biophysica Acta Bba - Biomembr* **1798**, 829–838 (2010).

409 36. Marty, R., N'soukpoé-Kossi, C. N., Charbonneau, D. M., Kreplak, L. & Tajmir-Riahi, H.-A.
410 Structural characterization of cationic lipid–tRNA complexes. *Nucleic Acids Res* **37**, 5197–
411 5207 (2009).

412 37. Suga, K., Tanabe, T., Tomita, H., Shimanouchi, T. & Umakoshi, H. Conformational change
413 of single-stranded RNAs induced by liposome binding. *Nucleic Acids Res* **39**, 8891–8900
414 (2011).

415 38. Lu, D. & Rhodes, D. G. Binding of phosphorothioate oligonucleotides to zwitterionic
416 liposomes. *Biochimica Et Biophysica Acta Bba - Biomembr* **1563**, 45–52 (2002).

417 39. Khvorova, A., Kwak, Y.-G., Tamkun, M., Majerfeld, I. & Yarus, M. RNAs that bind and
418 change the permeability of phospholipid membranes. *Proc National Acad Sci* **96**, 10649–
419 10654 (1999).

420 40. Vlassov, A. & Yarus, M. Interaction of RNA with Phospholipid Membranes. *Mol Biol+* **36**,
421 389–393 (2002).

422 41. Vlassov, A., Khvorova, A. & Yarus, M. Binding and disruption of phospholipid bilayers by
423 supramolecular RNA complexes. *Proc National Acad Sci* **98**, 7706–7711 (2001).

424 42. Janas, T., Janas, T. & Yarus, M. Specific RNA binding to ordered phospholipid bilayers.
425 *Nucleic Acids Res* **34**, 2128–2136 (2006).

426 43. Pannwitt, S., Slama, K., Depoix, F., Helm, M. & Schneider, D. Against Expectations:
427 Unassisted RNA Adsorption onto Negatively Charged Lipid Bilayers. *Langmuir* **35**, 14704–
428 14711 (2019).

429 44. Quarless, S. A. & Cantor, C. R. Analysis of RNA structure by ultraviolet crosslinking and
430 denaturation gel electrophoresis. *Anal Biochem* **147**, 296–300 (1985).

431 45. Behlen, L. S., Sampson, J. R. & Uhlenbeck, O. C. An ultraviolet light-induced crosslink in
432 yeast tRNA phe. *Nucleic Acids Res* **20**, 4055–4059 (1992).

433 46. Nejedlý, K., Kittner, R., Pospíšilová, Š. & Kypr, J. Crosslinking of the complementary
434 strands of DNA by UV light: dependence on the oligonucleotide composition of the UV
435 irradiated DNA. *Biochimica Et Biophysica Acta Bba - Gene Struct Expr* **1517**, 365–375 (2001).

436 47. Lengyel, A., Uhríková, D., Klacsová, M. & Balgavý, P. DNA condensation and its thermal
437 stability influenced by phospholipid bilayer and divalent cations. *Colloids Surfaces B*
438 *Biointerfaces* **86**, 212–217 (2011).

439 48. White, S. H., Wimley, W. C., Ladokhin, A. S. & Hristova, K. [4] Protein folding in
440 membranes: Determining energetics of peptide-bilayer interactions. *Methods Enzymol* **295**,
441 62–87 (1998).

442 49. Lee, J. S., Evans, D. H. & Morgan, A. R. Polypurine DNAs and RNAs form secondary
443 structures which may be tetra-stranded. *Nucleic Acids Res* **8**, 4305–4320 (1980).

444 50. Baross, J. A. & Martin, W. F. The Ribofilm as a Concept for Life's Origins. *Cell* **162**, 13–15
445 (2015).

446 51. Johansson, J. *et al.* An RNA Thermosensor Controls Expression of Virulence Genes in
447 Listeria monocytogenes. *Cell* **110**, 551–561 (2002).

448 52. Winkler, W., Nahvi, A. & Breaker, R. R. Thiamine derivatives bind messenger RNAs
449 directly to regulate bacterial gene expression. *Nature* **419**, 952–956 (2002).

450 53. Morita, M. T. *et al.* Translational induction of heat shock transcription factor ς32:
451 evidence for a built-in RNA thermosensor. *Gene Dev* **13**, 655–665 (1999).

452 54. Schilling, O., Langbein, I., Müller, M., Schmalisch, M. H. & Stülke, J. A protein-dependent
453 riboswitch controlling ptsGHI operon expression in Bacillus subtilis: RNA structure rather
454 than sequence provides interaction specificity. *Nucleic Acids Res* **32**, 2853–2864 (2004).

455 55. Nahvi, A. *et al.* Genetic Control by a Metabolite Binding mRNA. *Chem Biol* **9**, 1043–1049
456 (2002).

56. Boo, S. H. & Kim, Y. K. The emerging role of RNA modifications in the regulation of mRNA stability. *Exp Mol Medicine* **52**, 400–408 (2020).

57. Edwards, A. M., Addo, M. A. & Santos, P. C. D. Extracurricular Functions of tRNA Modifications in Microorganisms. *Genes-basel* **11**, 907 (2020).

58. Zhou, W., Karcher, D. & Bock, R. Importance of adenosine-to-inosine editing adjacent to the anticodon in an Arabidopsis alanine tRNA under environmental stress. *Nucleic Acids Res* **41**, 3362–3372 (2013).

59. Patel, B. H., Percivalle, C., Ritson, D. J., Duffy, C. D. & Sutherland, J. D. Common origins of RNA, protein and lipid precursors in a cyanosulfidic protometabolism. *Nat Chem* **7**, 301–307 (2015).

60. Rushdi, A. I. & Simoneit, B. R. T. Lipid Formation by Aqueous Fischer-Tropsch-Type Synthesis over a Temperature Range of 100 to 400 °C. *Origins Life Evol B* **31**, 103–118 (2001).

61. Brady, R. C. & Pettit, R. Mechanism of the Fischer-Tropsch reaction. The chain propagation step. *J Am Chem Soc* **103**, 1287–1289 (1981).

62. Robertson, M. P. & Ellington, A. D. Design and optimization of effector-activated ribozyme ligases. *Nucleic Acids Res* **28**, 1751–1759 (2000).

63. Debiais, M., Lelievre, A., Smietana, M. & Müller, S. Splitting aptamers and nucleic acid enzymes for the development of advanced biosensors. *Nucleic Acids Res* **48**, 3400–3422 (2020).

64. Stifel, J., Spöring, M. & Hartig, J. S. Expanding the toolbox of synthetic riboswitches with guanine-dependent aptazymes. *Synthetic Biology* **4**, ysy022- (2019).

65. Vlassov, A., Khvorova, A. & Yarus, M. Binding and disruption of phospholipid bilayers by supramolecular RNA complexes. *Proc National Acad Sci* **98**, 7706–7711 (2001).

66. Janas, T. & Yarus, M. Visualization of membrane RNAs. *Rna* **9**, 1353–1361 (2003).

494 # Materials and methods

495

496 ## Materials

497 Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids and was used without
498 further purification. HEPES, $MgCl_2$ and $CaCl_2$ were purchased from CarlRoth and were at least >99%
499 pure. All solutions were prepared in MiliQ water, Millipore.

500

501 R3C ligase ribozyme was expressed using pRZ plasmid and T7 RNA polymerase and purified using
502 denaturing PAGE and electroelution. 5' 6-FAM labelled R3C substrates as well as short oligomers used
503 in binding assays were obtained from Integrated DNA Technologies and used without further
504 purification.

505

Sequence name	**Sequence (5' – 3')**
R3C	GAGACCGUAAUGAGUAGUACUUAUUAUGCUCGAUUGUUCGUAAG AACAGUUUGAAUGGGUUGAAUAUAGUGAGGAUCCGCUAGC
Short substrate	6_FAM_CGACUCACUAUA
4xAG substrate	6_FAM_A**G**A**G**A**G**A**G**CGACUCACUAUA
9xAG substrate	6_FAM_A**G**A**G**A**G**A**G**A**G**A**G**A**G**A**G**A**G**ACGACUCACUAUA
2G substrate	6_FAM_HHHHHHHH**G**HHH**G**HHHHHHCGACUCACUAUA
0G	HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH
2G	HHHHHHHHHHHHHHHH**G**HHH**G**HHHHHHHHHHHHHHHH
4G	HHHHHHHHHHHH**G**HHH**G**HHH**G**HHH**G**HHHHHHHHHHH
5G	HHHHHHHHHH**G**HHH**G**HHH**G**HHH**G**HHH**G**HHHHHHHHHH
7G	HHHHHH**G**HHH**G**HHH**G**HHH**G**HHH**G**HHH**G**HHH**G**HHHHHH
9G	HH**G**HHH**G**HHH**G**HHH**G**HHH**G**HHH**G**HHH**G**HHH**G**HH
40xN	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
40xN - G	HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH
40xN - A	BBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBB
40xN - C	DDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDD
40xN - U	VVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVV
19xG	**GGGGGGGGGGGGGGGGGGG**
19xA	AAAAAAAAAAAAAAAAAAA
19xC	CCCCCCCCCCCCCCCCCCC
19xU	UUUUUUUUUUUUUUUUUUU
19xN	NNNNNNNNNNNNNNNNNNN
AG repeats	6_FAM_AGAGAGAGAGAGAGAGAGA
ssRNA (ACUG)	ACUGACUGACUGACUGACUGACUGACUGACUGACUG
ssRNA (CAGU)	CAGUCAGUCAGUCAGUCAGUCAGUCAGUCAGUCAGU

506

507 **B** – C, G or U
508 **D** – A, G or U
509 **H** – A, C or U
510 **V** – A, C or G

511 **Methods**
512 All of the RNA incubations were prepared in Sarstedt DNA low bind tubes at the constant temperature
513 of 24 °C in buffer comprised of 10 mM HEPES pH 7, 5 mM $CaCl_2$, and 5 mM $MgCl_2$. RNA concentration
514 was measured by measuring absorbance at 260nm (A_{260}, SPARK 20M, TECAN). Before every incubation
515 RNA was preheated in SafeLock Eppendorf tubes (90 °C, 5 minutes) to ensure unfolded structures.
516 Samples after UV crosslinking and R3C activity assays were ethanol precipitated.
517
518 **Lipid preparation**
519 DPPC chloroform stock was pipetted into a glass vial and briefly evaporated under a steady flow of
520 nitrogen gas. To remove organic solvent residues, a lipid film was dried under vacuum overnight. Lipid
521 film hydration (in reaction buffer) method was used to obtain multi layer DPPC vesicles (MLV DPPC).
522 Buffer-lipid mixtures were shaken at 60 °C, well above the melting temperature of DPPC (T_m = 42 °C),
523 for one hour. A cloudy liposome suspension was freeze and thawed 10x and extruded 17x through 100
524 nm polycarbonate filters (Millipore) to reduce multi-lamelarity and achieve a consistent size
525 distribution of vesicles. Lipid vesicle stocks were kept in 4 °C. Stock lipid concentrations were confirmed
526 using a phosphate assay.
527
528 **Dynamic light scattering**
529 The size distribution of DPPC vesicles was estimated using a ZetaSizer Nano in 173° backscatter with
530 polydisperse solution calculation multi-mode. Final concentration of lipids was 10 uM and RNA amount
531 was kept at a ratio of 10 lipids / nucleotide. Results were plotted as the size distribution in number of
532 detected species function (NumberPSD).
533
534 **UV-crosslinking**
535 RNA was incubated for 10 minutes in the reaction buffer with or without lipids followed by 10 minutes
536 incubation under UV-B light (300 mW LEUVA77N50KKU00 LED 305 nm) from a distance of 1.5 – 2 cm.
537 Samples were analysed on denaturing PAGE with SybrGold poststaining.
538
539 **R3C activity assays**
540 5 pmol of R3C ligase was mixed with 0.5 pmol of the substrate to ensure saturation of the system
541 (pseudo 1st order reaction) and decrease any batch-to-batch differences coming from R3C purification.
542 Incubation was conducted at various concentrations of DPPC vesicles and samples were ethanol
543 precipitated after 30, 60, 90 and 120 minutes. Reactions were quantified on denaturing PAGE using
544 FIJI software. Product and substrate intensities (I_P and I_S, respectively) were quantified and the
545 concentration of product at time t was calculated:
546
547 $$[\boldsymbol{P}]_{\boldsymbol{t}} = [\boldsymbol{S}]_{\mathbf{0}} * \left(\frac{\boldsymbol{I_P}}{\boldsymbol{I_P} + \boldsymbol{I_S}}\right)$$
548
549 Concentration of product was plotted as a function of time and then fitted with a linear function (fixed
550 [0;0] point) to determine reaction rates.
551
552 **RNA-lipid binding assays**
553 Fig. 3a,b binding assays were conducted using liposome-coated magnetic beads. Fig. 2b,c, Fig. 4
554 binding assays were conducted using an ultracentrifugation method.
555

556 **Magnetic beads binding assay**

557 Magnetic beads (DynaBeads streptavidin T1) were coupled with DPPC liposomes doped with 0.5 mol%
558 biotynoyl cap PE (Avanti Polar Lipids). Lipid concentration on non-diluted beads was estimated using a
559 phosphate assay and a theoretical calculation and came out to be 800 uM. 25 pmol of 19nt or 5 pmol
560 of 40nt RNA species were incubated with 2x diluted magnetic beads for 30'. After incubation the
561 supernatant was separated from beads using magnetic field. The amount of RNA was estimated either
562 using absorbance or Qubit miRNA quantification kit (ThermoScientific).
563
564 **Ultracentrifugation binding assay**

565 RNA was incubated with various concentrations of DPPC vesicles for 30'. After incubation
566 Ultracentrifugation was conducted to separate vesicles from solution (125 000 x G, 40', 24 °C). 9 uL of
567 supernatant (out of 30 uL reaction mix) was pulled and incubated with 1.5 uL of 0.5 M EDTA (90 °C, 5').
568 The amount of RNA was measured by Qubit miRNA quantification kit using a plate reader (SPARK 20M,
569 TECAN).
570
571 Measured fluorescence intensity values were plotted as a function of lipid/oligomer or lipid/nucleotide
572 ratios (we assumed that RNA interacts only with the outer membrane leaflet) and fitted using Hill's fit
573 (OriginLab software):
574

575 $$f(x) = Start + (End - Start)\frac{x^n}{x^n + k^n}$$

576 Where Start and End are function plateau values, k is inflection point of curve and n is cooperativity
577 index. Inflection point of the curve (k) is a parameter which we used for further data analysis.
578

579 **RNA-lipid binding partition coefficient determination**

580 RNA was incubated with high excess of DPPC vesicles (5 mM, 2.5 mM outer leaflet) for 30 minutes.
581 After incubation ultracentrifugation was conducted to separate vesicles from solution (125 000 x G,
582 40', 24 °C). 10 uL out of 30 uL was taken as a supernatant sample, and the rest of the supernatant was
583 discarded to leave only the pellet. RNA from supernatant and pellet samples was ethanol precipitated
584 and analyzed using denaturing PAGE. The partition coefficient K was calculated according to following
585 formula:
586
587
588
589

$$K = \frac{\frac{[R_L]}{[R_L] + [L]}}{\frac{[R_w]}{[R_w] + [W]}}$$

590 **Where:**

591 **[R_L] – RNA bound to the membranes**

592 **[R_W] – non-bound RNA**

593 **[L] – outer lipid concentration**

594 **[W] – water concentration**

595

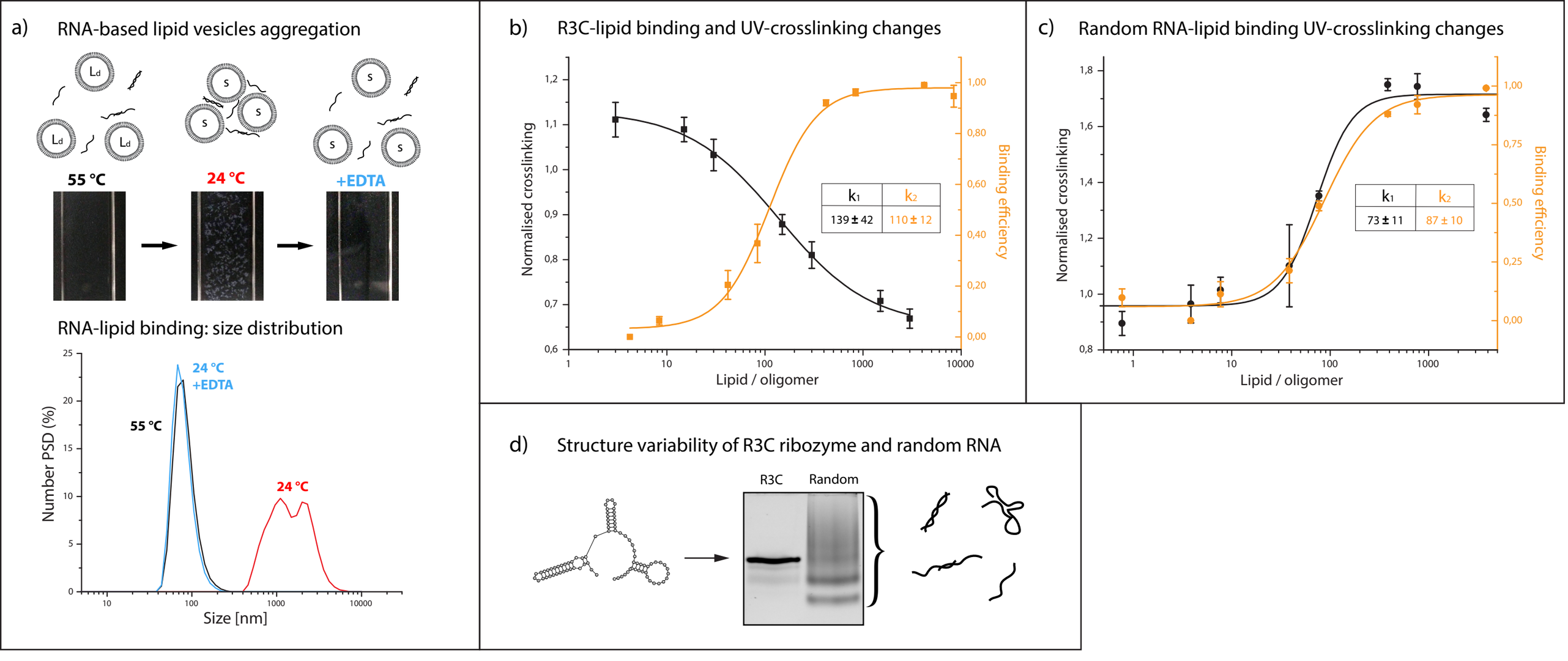
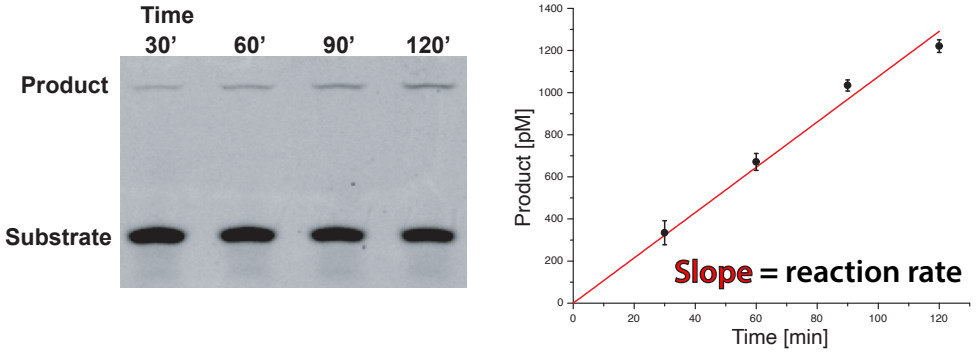
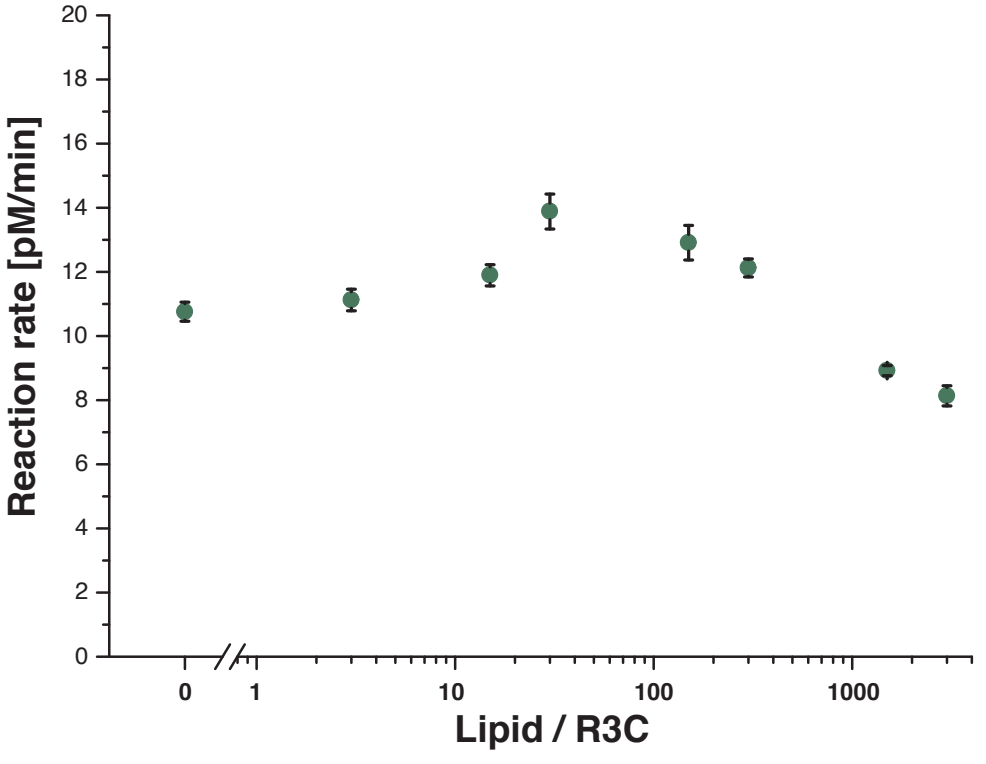


Fig. 1 **RNA-gel membrane binding has different phenotypes**. Introduction of a randomized pool of RNA oligos to gel lipid membranes promotes vesicle aggregation a divalent cation-dependent process **(a)**. R3C ligase (84 nt) shows a small increase in UV-crosslinking at low lipid concentration, whereas crosslinking efficiency decreases at higher lipid concentration. Binding and UV crosslinking are inversely correlated and show similar inflection points with respect to lipid concentration **(b)**. Random RNA oligos (75-85 nt) show increasing crosslinking efficiency in the presence of lipids with a slight decrease at the highest lipid concentration; crosslinking and lipid binding are closely correlated **(c)**. The R3C ligase forms primarily one structure, whereas randomized RNA forms intermolecular complexes **(d)**.

a) R3C ligase activity determination



b) R3C ligase reaction rate in function of lipid content



c) Lipid binding and characteristics of R3C reactants

Oligo	log(K)	Nucleotide %				Length
		G	A	C	U	
R3C	**6.74 ±** 0.13	27	28	13	31	84 nt
Substrate	**3.39 ±** 0.15	8	33	33	25	12 nt

Fig. 2 Lipid membranes influence R3C ligase activity through lipid-ligase interactions. R3C ligase reaction rates were quantified by measuring the amount of product within 2 hours incubation time **(a)**. R3C reaction rate is dependent on the presence of gel membranes - increased activity is observed for lower lipid concentration followed by drop in activity at higher lipid concentration **(b)**. The 12 nt R3C substrate shows several orders of magnitude lower membrane-buffer partition coefficient compared with the R3C ligase, which could derived from different compositional or structural characteristics of both RNA species **(c)**.

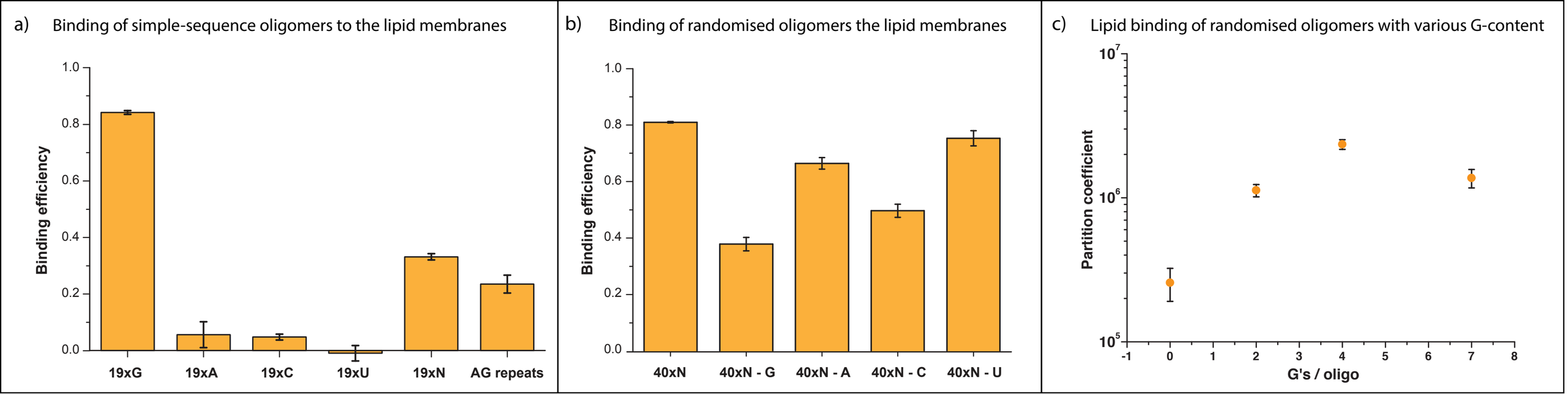


Fig. 3 **RNA-gel membrane binding is RNA sequence-dependent.** Short 19 nucleotide oligomers show sequence-dependent binding to the gel membranes, with highest binding observed for pure G oligomer followed by oligos with randomized sequence or AG repeats. Oligomers containing only A, C or U bases show negligible binding to the lipid membranes **(a)**. Increase in oligonucleotide length leads to a further increase in binding efficiency (compare **(a)** 19xN and **(b)** 40xN). Depletion of one type of nucleotide within oligomers caused a significant decrease in binding efficiency. Lack of guanine and cytidine decreased binding by 43 and 31 percentage points, respectively, whereas lack of adenine or uracil showed a less pronounced effect **(b)**. Oligonucleotides with increasing G content showed higher lipid – buffer partition coefficients compared with G-deficient oligo with the highest value observed for the 4xG oligomer (0.9 log unit) **(c)**.

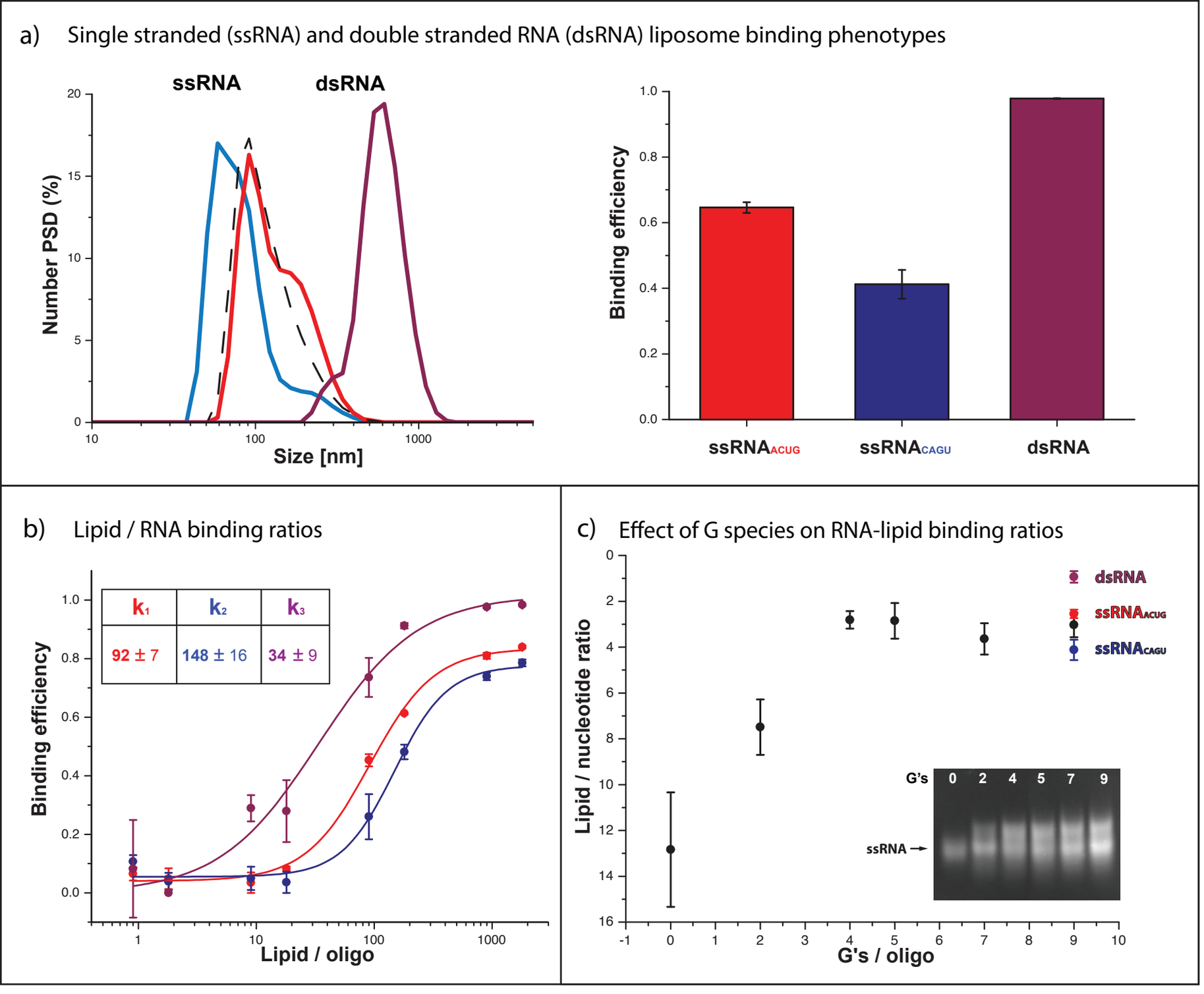


Fig. 4 **RNA-gel membrane binding depends on RNA structure.** The membrane binding of RNA's which can't create any inter- and intramolecular structures (36 nucleotide ACUG or CAGU repeats) was examined together with 100% complementary double stranded structures (mixed ACUG and CAGU oligos). Single stranded RNAs do not cause lipid vesicle aggregation despite moderate membrane binding, whereas double stranded RNA species show both high binding and lipid aggregation **(a)**. RNA:lipid binding ratios are different for single stranded RNAs compared to double stranded species **(b)**. Oligomers with different nucleotide composition show different lipid/nucleotide binding stoichiometry. Analysis of oligomers by native PAGE gel indicates that the presence of guanosine promotes inter-molecular interactions, suggesting, that base-pairing enhances the average strength of nucleotide-lipid interactions **(c)**.

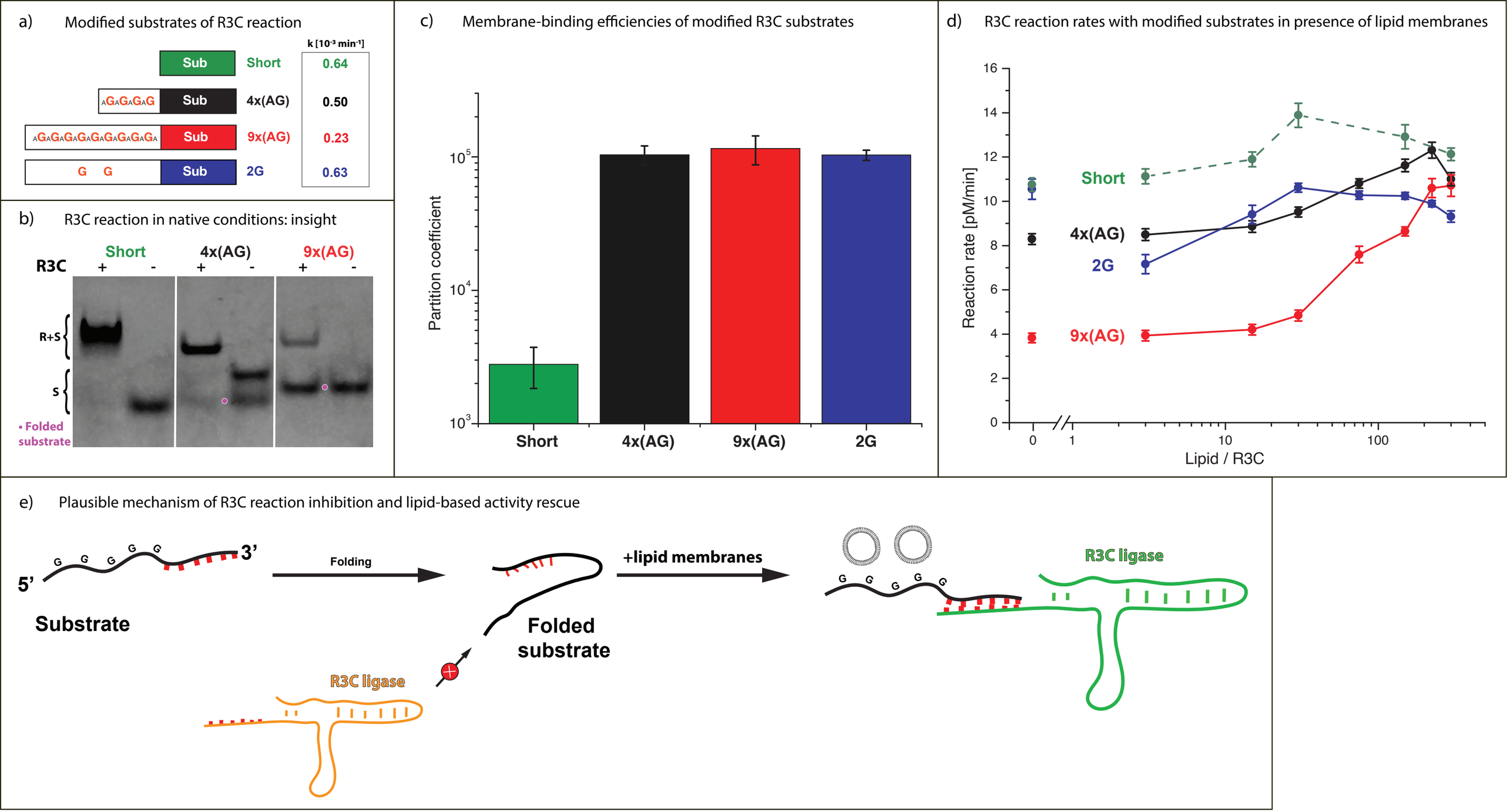


Fig. 5 **Modification of R3C substrate sequences enables tunability of ligation rate.** Modified R3C reaction substrates were synthesized in order to increase binding to the membrane. The non-catalytic 5′ end of the molecule was amended with tails with varying G content **(a)**. Reactions for all AG-modified substrates, but not 2xG, showed lower reaction rate constants compared with the unmodified substrate **(a, table)**. The mechanism for inhibition of activity was evaluated by analysis of R3C reactants using native PAGE **(b)**. The unmodified short substrate was entirely bound to the ligase, whereas AG-modified substrates show mobility abnormalities, which suggest substrate folding **(b, magenta points)**. Modification of the substrate sequence through addition of a 5′ overhang increased binding to the membrane **(c)**. Addition of lipid membranes to reactions with the G-rich substrates increased reaction rates to the level of the unmodified substrate, except for the 2xG substrate which showed no change **(d)**. Decrease of the R3C reaction rates in the presence of G-rich substrates might be based on the inhibition of the catalytic part of the substrate by the 5′ G-rich overhang **(e)**.