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Supporting Information

Electrostatic Localization of RNA to Protocell Membranes by Cationic Hydrophobic Peptides

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Materials and Methods

Materials. Phospholipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Liss Rh-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). For membrane growth studies, the phospholipids N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (NBD PE) and Lissamine rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (Rh-DHPE) were used (Life Technologies, Grand Island, NY). Oleic acid was purchased from Nu Chek Prep (Elysian, MN). RNA was purchased from IDT (Coralville, IA). Peptides were purchased from Peptide 2.0 (Chantilly, VA) and GenScript (Piscataway, NJ)—a list of peptides used appears in Table S1. 2-undecylimidazole was purchased from TCI America (Portland, OR) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Vesicle Preparation. Phospholipid and oleic acid vesicles for FRET, gel filtration, and Zeta potential experiments were prepared by thin film hydration of the lipid or fatty acid in a chloroform solution. The fluorescent lipid, Rhodamine Lissamine PE, was dissolved in chloroform and added to the phospholipid or fatty acid to constitute 0.15 mol % of the total amphiphile for FRET and gel filtration studies. The solution was dried with N_2 on the surface of a glass vial and the solvent was evaporated in a vacuum oven for > 12 h. Phospholipid films were hydrated with a sucrose solution (200 mOsm or equiosmolar to the final expected salt conditions) to a final concentration of 20 mM lipid. The system was heated at 60 ° C for 1 h and briefly sonicated, followed by extrusion through 100 nm polycarbonate membranes (Avanti). Oleic acid films were hydrated with 250 mM Tris-HCl, pH 8, briefly vortexed and tumbled overnight before extrusion. For vesicles containing undecylimidazole, 2-undecylimidazole was prepared in a methanol stock solution and mixed with POPC dissolved in chloroform at various molar ratios of 2-undecylimidazole: POPC. Thin films were prepared from the 2-undecylimidazole + POPC stock solution following the above protocol and later hydrated with a sucrose solution to a final concentration of 20 mM lipid.

Giant unilamellar vesicles (GUVs) were prepared by thin film hydration methods on roughened Teflon as described by Kamat et. al.[2] A hydration solution of fluorescently labeled oligomer (5 μM)

was prepared in 200 mOsm sucrose. For the formation of Oleic acid / POPC (90 /10 molar fraction) blended GUVs, oleic acid and POPC were mixed at the desired molar ratio in chloroform and added to roughened Teflon. A hydration solution of 200 mOsm sucrose with 5 mM Tris-HCl, pH 8, was used for films containing fatty acid to ensure pH regulation.

FRET assay for RNA localization. FRET efficiencies between RNA and vesicles were approximated as $F_a/(F_a+F_d)$, where F_d is the donor fluorescence in FAM-labeled RNA oligomers and F_a is the acceptor fluorescence in Liss Rh-PE-labeled vesicles. Fluorescence was excited at 495 nm, donor emission was measured at 525 nm, and acceptor emission was measured at 580 nm. FRET studies for oligomer localization were conducted with 7.5 mM amphiphile containing 0.15 mol % Liss Rh-PE. FAM-labeled oligomers were added to the outside of empty vesicles at a final concentration of 1 µM. All FRET studies were conducted in 250 mM Tris-HCl (pH 8). The resulting FRET signal was monitored over 10 hours using a fluorescence plate reader (Gemini EM, Molecular Devices, Sunnyvale, CA) and the final FRET signal was reported as a % difference from control samples that contained the same concentrations of RNA, vesicles, and buffer as test samples.

For FRET assays involving peptides, a correction factor, γ, was determined for each peptide to account for the effect of a particular peptide on acceptor fluorophore emission in the absence of the donor fluorophore. FRET efficiency was calculated as,

$$\frac{F_a \cdot \gamma}{F_a \cdot \gamma + F_d},$$

where γ describes the effect of a peptide on acceptor fluorophore emission, in the absence of the donor fluorophore and is calculated as,

$$\gamma = \frac{F_{d,-acceptor,-peptide}}{F_{d,-acceptor,+peptide}}$$

Zeta-Potential Measurements. Zeta-potential measurements were performed in a Malvern Zetasizer Nano ZS (Malvern, UK) with a backscattering detection at a constant 173° scattering angle and with a He-Ne laser (excitation 632 nm) at 25°C. Zetasizer folded capillary cells (DTS 1060, Malvern, UK) were used. POPC samples were prepared at a final concentration of 0.5 mM in 5 mM Tris-HCl buffer, pH 8, for all measurements.

Membrane Growth assay. POPC vesicles were prepared with 0.1 mol % of the FRET dyes NBD PE and Rh-DHPE. Growth from the addition of peptides was assessed by adding each peptide to a vesicle solution and monitoring the resulting change in FRET signal. Fluorescence was excited at 463 nm, donor emission was measured at 517 nm, and acceptor emission was measured at 580 nm. The FRET signal was converted into relative surface area, through a standard curve correlating mol % of FRET dyes in the membrane to FRET signal. The change in surface area after a10 h incubation with the tested reagent was reported. Fluorescence measurements were performed using a plate reader (Gemini EM, Molecular Devices, Sunnyvale, CA).

Vesicle stability assays. Vesicle stability assays for small molecule leakage were performed as previously described.[5] The ratio of calcein fluorescence of the vesicle sample to total fluorescence, achieved after sample lysis with 0.1 % Triton X-100, was reported.

Microscopy with GUVs. POPC vesicles prepared by thin-film methods and containing a sucrose-based solution were mixed with an equiosmolar solution of 125 mM Tris-HCl, pH 8, and imaged in Lab-Tek II Coverglass (Thermo Fischer Scientific, Waltham, MA) chambers that were pre-blocked with a 1% BSA solution. For studies with oleic acid/POPC blended vesicles, glass chambers were blocked with a 500 µM oleic acid solution, and giant vesicles were diluted into a 500 µM solution of oleic acid (200 mOsm) to maintain the concentration of fatty acid above the critical aggregation concentration of oleic acid. GUVs were imaged on a Nikon A1R MP Confocal (Melville, NY) and processed in ImageJ.[6]

For studies of RNA localization to the outside of vesicle membranes with peptide, RNA and the peptide were pre-mixed at a ratio of 2: 1 peptide: RNA for 10 min. Giant vesicles were then added to the peptide/RNA mixture so that the final concentration of each component was 40 µM vesicles, 10 µM peptide, and 5 µM RNA. After a 30 minute incubation, 10 µL of the vesicle/peptide/RNA mixture was diluted 50 fold into either 125 mM Tris-HCl buffer or 500 µM oleic acid (200 mOsm) and imaged. The RNA used was 5′ AlexaFluor647 -GCG UAG ACU GAC UGG-3′.

For studies of the localization of encapsulated RNA, GUVs containing a 5′-FAM-U_{15} RNA oligomer solution were added to a Lab-Tek II Coverglass chamber and allowed to settle. Small unilamellar vesicles (SUVs) containing 40 mol% undecylimidazole and 0.15 mol % Liss Rh PE, or peptide in a water solution, were added to GUVs and imaged after one hour of incubation. The final concentration of each component was 40 µM vesicles and 10 µM undecylimidazole or peptide to match the molar ratios of each component used in FRET studies monitoring RNA localization.

Table S1. Summary of peptides

Peptide Name	Sequence (NH2 – COOH)	Source
RF	RF	Peptide 2.0
RF2	RFF	Peptide 2.0
R3F2	RRRFF	Peptide 2.0
R3F3	RRRFFF	Peptide 2.0
RF3	RFFF	Peptide 2.0
R5I2	RRRRRII	Peptide 2.0
R3I3	RRRIII	Peptide 2.0
R5W2	RRRRRWW	Peptide 2.0
R3W3	RRRWWW	Peptide 2.0
HHWWW	HHWWW	GenScript
HWW	HWW	GenScript

Supporting Figures

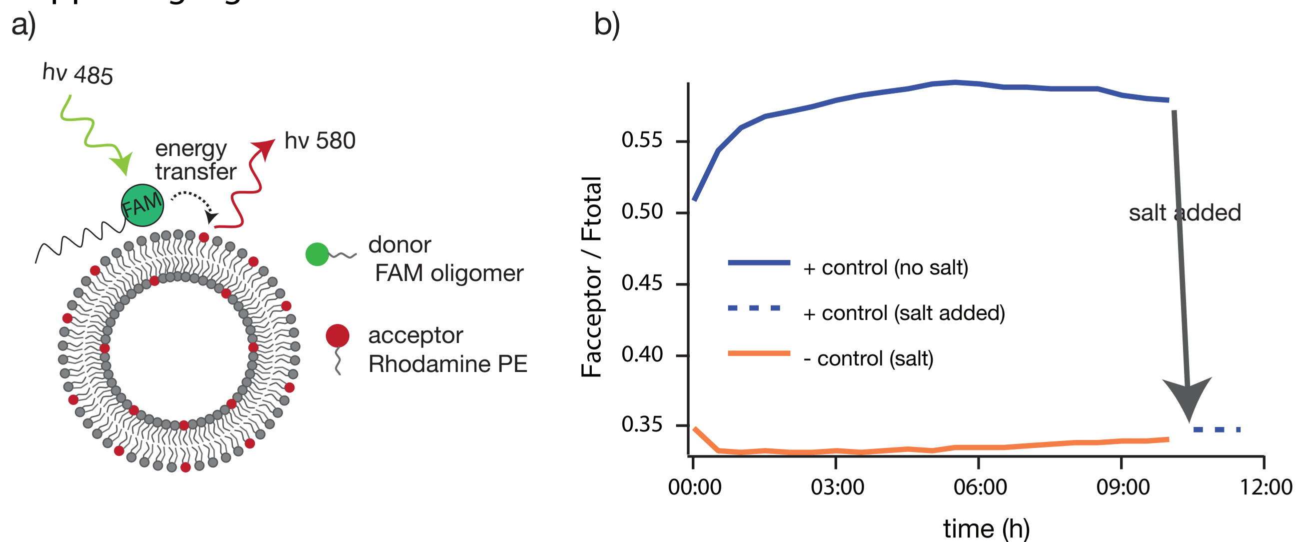

Figure S1. FRET assay for RNA association to vesicle membranes

(a) Schematic of the FRET assay in which a 5′ FAM-labeled oligonucleotide serves as the FRET donor and a Lissamine Rhodamine lipid serves as the FRET acceptor. The proximity of the two molecules was monitored through the FRET efficiency, E, the ratio between the acceptor emission intensity and the total emission intensity ($I_{580}/(I_{580} + I_{525})$). All FRET studies were conducted with 1 μM FAM-labeled oligonucleotide and 7.5 mM vesicles containing 0.15 mol % Liss Rh-PE, maintaining the overall concentrations of FRET acceptor and donor in all samples. (b) The accuracy of the FRET assay was tested with a positive control, in which RNA was completely bound to vesicles, and a negative control, in which RNA did not exhibit association with the membranes. The positive control was achieved in a low salt condition resulting in complete binding of RNA to zwitterionic vesicle membranes (SI Figure 2). This sample yielded an increased FRET signal with respect to the negative control. Once the RNA was removed from the vesicle surface through the addition of salt, the FRET signal was correspondingly reduced to the negative control value.

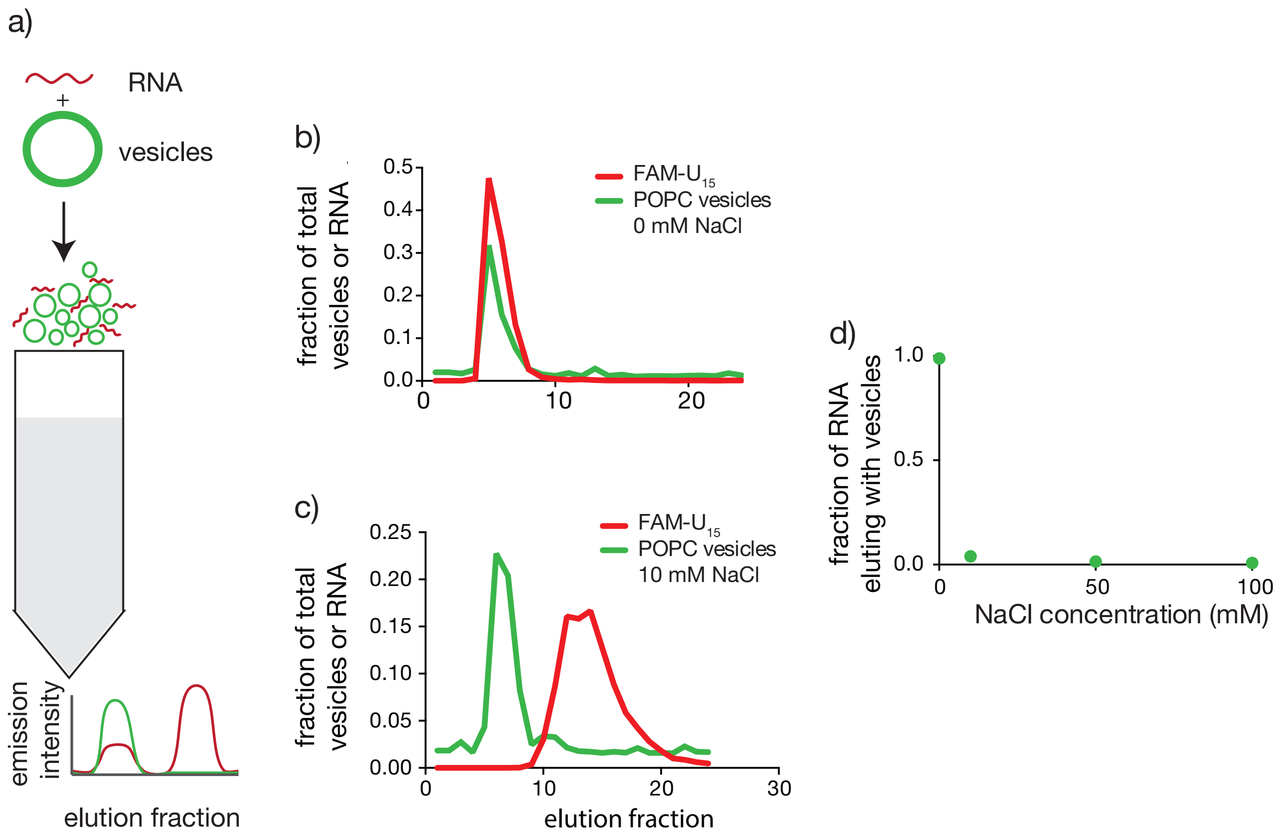

Figure S2. Gel filtration assay for RNA-liposome binding

(a) RNA association to vesicles can be determined through size exclusion gel-filtration. RNA bound to vesicle membranes will co-elute with vesicles in the void volume of a Sepharose 4B column.[7] RNA and vesicle membranes are detected in elution fractions by using fluorescently labeled RNA and vesicle membranes (with 0.15 mol % Liss Rhodamine-PE incorporation). RNA that is not bound to vesicles elutes in later fractions. (b) In low salt conditions (< 5 mM Tris buffer or monovalent salt, pH 8), all RNA is bound to POPC membranes. (c) The addition of salt, 10 mM NaCl, results in the disruption of interactions and the RNA and vesicles elute separately. (d) The fraction of RNA bound to vesicle membranes is calculated as a function of salt concentration. Below 10 mM NaCl, RNA-membrane associations are sufficiently strong to co-elute in the gel filtration assay and this condition provides a positive control for complete RNA binding to POPC membranes.

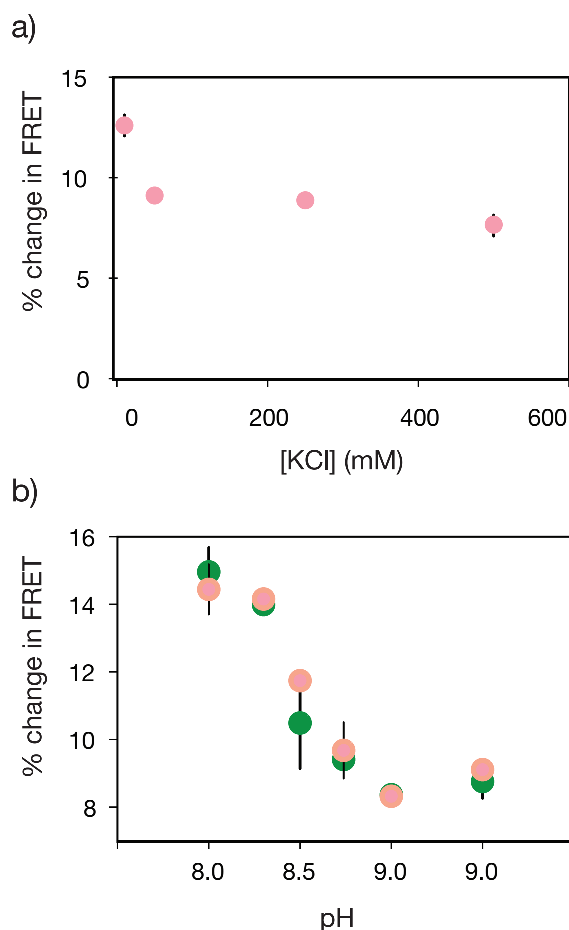

Figure S3. Effect of salt and pH on undecylimidazole-mediated FAM-U_{15} RNA-membrane association

(a) The effect of salt on undecylimidazole-mediated RNA localization was studied by monitoring FRET efficiency between a FAM-U_{15} RNA oligomer and empty POPC vesicles containing 0.15 mol % Liss Rh-PE and 0.4 mol fraction 2-undecylimidazole: POPC. The Tris-HCl buffer (pH 8) was reduced to 10 mM for this study to better analyze the effects of salt. The effect of increasing KCl concentration on the FRET signal is reported with respect to a control sample that contained no undecylimidazole in the membrane. Increasing the salt concentration decreased RNA localization, however RNA localization was not completely reduced to control values even in the presence of 500 mM KCl. (b) RNA localization (5′-FAM-U_{15}) to POPC membranes containing an undecylimidazole analogue (undecylimidazole modified with an adenosine monophosphate group) is pH dependent and increases with decreasing pH with respect to control samples measured at each pH (n=3, error bars represent standard deviation (s.d.)).

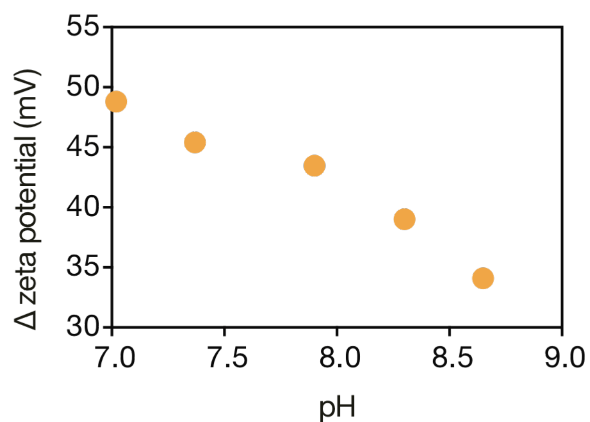

Figure S4. Zeta potential measurements of vesicle membranes

POPC vesicles containing 0.53 mol % 2-undecylimidazole display increased zeta potential with decreasing solution pH. Data is presented as a change in zeta potential from control samples measured at each respective pH, but that do not contain 2-undecylimidazole.

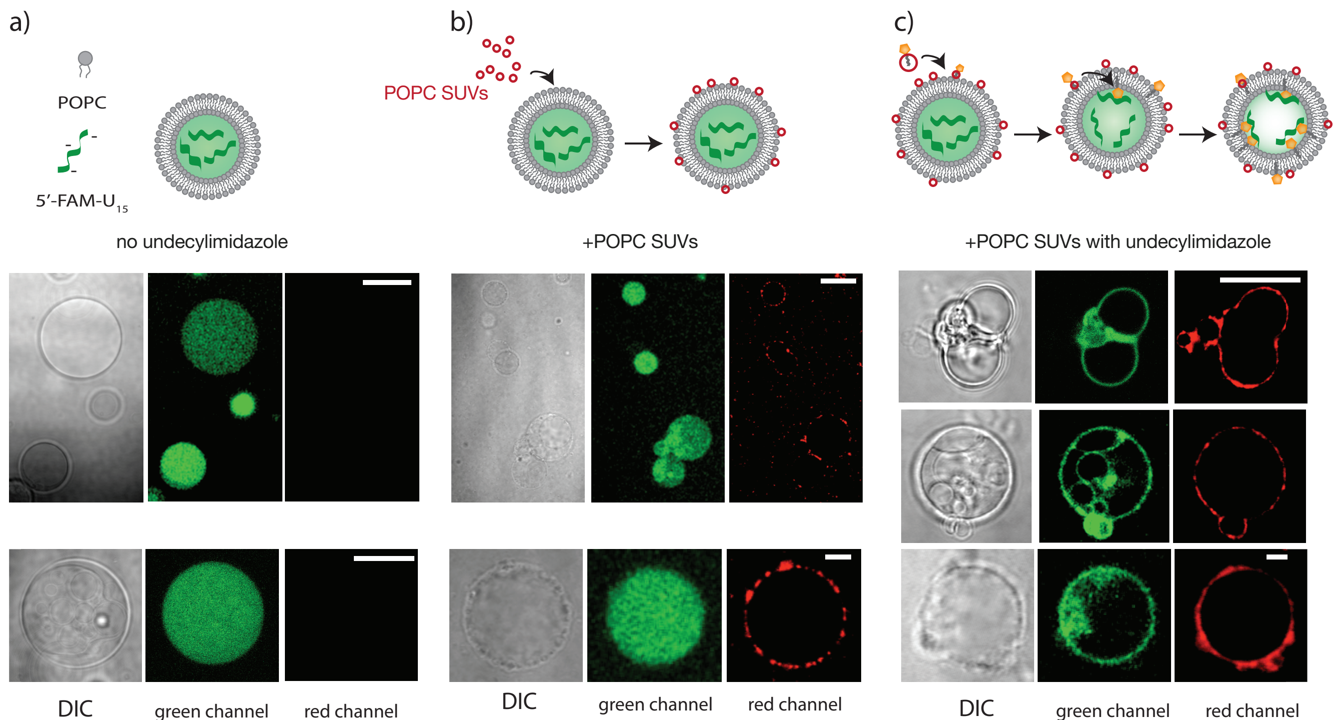

Figure S5. Microscopy of encapsulated RNA localization to POPC membranes with 2-undecylimidazole

Confocal images of 5′ FAM-U_{15} RNA (green) association with giant POPC vesicles membranes in the presence of 2-undecylimidazole. (a) RNA appears uniformly distributed in the interior of POPC GUVs. (b) The addition of SUVs containing 0.15 mol % Liss Rh PE leads to SUV aggregation and association with the giant vesicle membranes, but RNA (green) remains uniformly encapsulated in the vesicle interior. (c) The addition of SUVs containing 0.15 mol % Liss Rh PE and 40 mol % 2-undecylimidazole leads to SUV association with GUV membranes and RNA (green) localizes to the vesicle surface. The localization of encapsulated RNA to the inner vesicle membrane is expected to occur via movement of the undecylimidazole molecules between membranes and across the GUV bilayer. The final concentration of each component was 40 μM vesicles and 10 μM undecylimidazole. Scale bar is 20 μm.

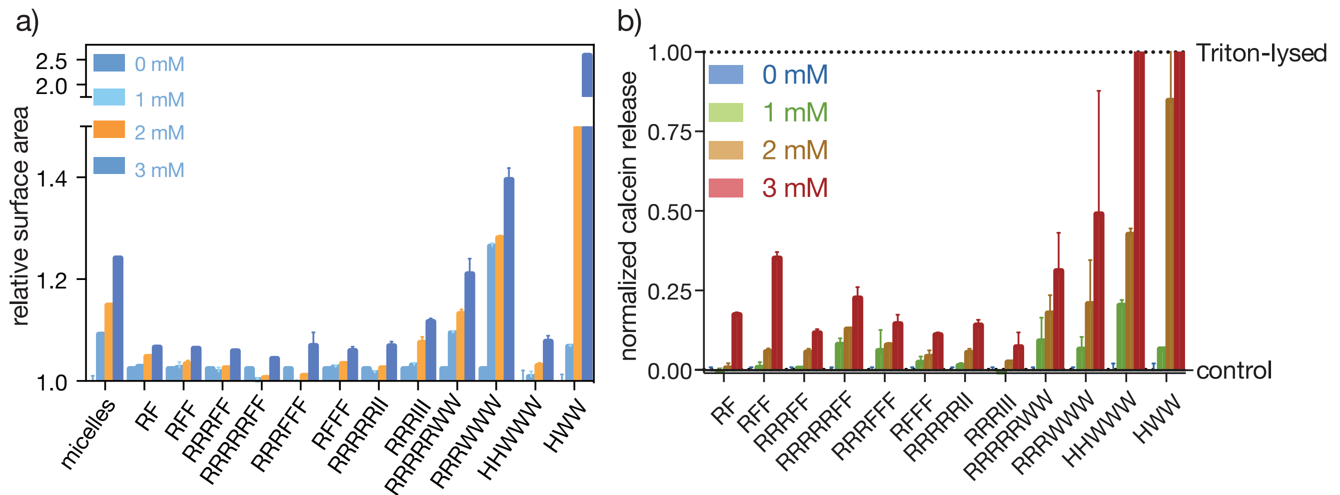

Figure S6. Screening peptides for membrane interaction

(a) Change in surface area of POPC vesicles (7.5 mM) mixed with different peptides or oleate micelles, measured by a FRET membrane growth assay 10 hours after incubation. Vesicles show growth in a concentration-dependent manner with all the peptides. A positive control of oleate micelles, previously shown to induce vesicle growth,[8] is also shown on the graph. (b) The effect of peptides on membrane permeability after a 10 h incubation was assessed by monitoring leakage of a small molecule, calcein. Vesicles were prepared with 20 mM calcein dye, extruded, and unencapsulated dye was removed on a size exclusion column. Vesicles (7.5 mM) were incubated with peptide and leakage was calculated by quantifying the amount of encapsulated/total dye. Samples were lysed with triton after a 10 hour incubation to determine the fluorescent signal of fully lysed samples and data presented is the ratio Fv/Ft where Fv is the calcein fluorescence in the vesicle and Ft is the calcein fluorescence upon complete vesicle lysis. n=2, error bars represent standard deviation (s.d.)

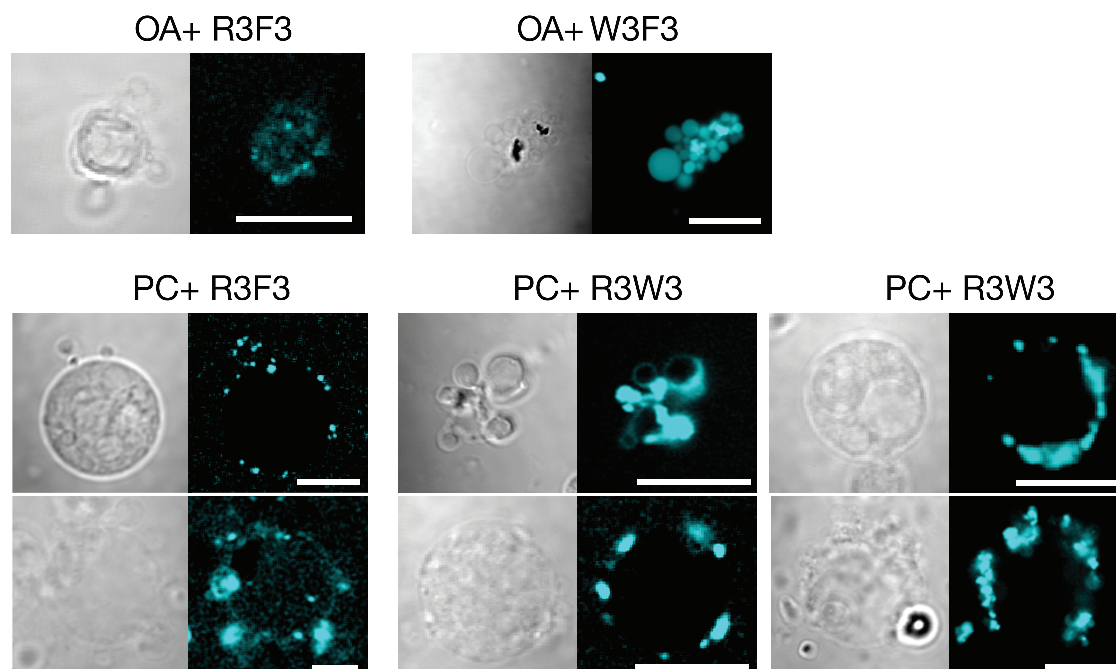

Figure S7. Microscopy of RNA-membrane localization with R3F3 and W3F3 peptides
Confocal images of an AlexaFluor647 labeled RNA oligonucleotide (cyan) association with giant vesicles composed of (top panel) 90% oleic acid and 10% POPC and (bottom panel) 100% POPC membranes in the presence of two peptides, R3F3 or R3W3. (Top, left) RNA associates with 90% oleic acid membranes in punctate aggregates on the vesicle surface in the presence of the R3F3 peptide. (Top, right) In the presence of the W3F3 peptide, most vesicles showed no RNA association, but small aggregate structures were also observed that contained RNA encapsulated in their interior that might result from the absorption of oleic acid monomers onto peptide-RNA aggregates. Bottom panels show typical images of RNA association with 100% POPC membranes.

References

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