Thermostability of model protocell membranes

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The earliest cells may have consisted of a self-replicating genetic polymer encapsulated within a self-replicating membrane vesicle. Here, we show that vesicles composed of simple single-chain amphiphiles such as fatty acids, fatty alcohols, and fatty-acid glycerol esters are extremely thermostable and retain internal RNA and DNA oligonucleotides at temperatures ranging from 0°C to 100°C. The strands of encapsulated double-stranded DNA can be separated by denaturation at high temperature while being retained within vesicles, implying that strand separation in primitive protocells could have been mediated by thermal fluctuations without the loss of genetic material from the protocell. At elevated temperatures, complex charged molecules such as nucleotides cross fatty-acid-based membranes very rapidly, suggesting that high temperature excursions may have facilitated nutrient uptake before the evolution of advanced membrane transporters. The thermostability of these membranes is consistent with the spontaneous replication of encapsulated nucleic acids by the alternation of template-copying chemistry at low temperature with strand-separation and nutrient uptake at high temperature.

origin of life | RNA world | synthetic biology | vesicle | prebiotic

We have recently described a laboratory model of a simple protocell that is useful for assessing the interactions and compatibility of the protocell components. We found that protocell membranes composed of fatty acids and related molecules are reasonably permeable to nucleoside phosphorimidazolides and that efficient template directed copying reactions can take place in the vesicle interior after the addition of external activated nucleotides (1). These observations provide strong support for the plausibility of the heterotrophic protocell model but immediately bring to mind several additional questions. Most important, if a genetic polymer is copied inside membrane vesicles, how could the strands of the double-stranded product be separated? The possibility of thermal strand separation, as in PCR, has seemed problematic in view of the presumed thermal instability of fatty-acid-based vesicles (2, 3). A second question is whether there might be environmental conditions that could facilitate nucleotide uptake, allowing more efficient replication or perhaps the utilization of more highly charged substrates such as nonactivated nucleotides, nucleoside polyphosphates, or short oligonucleotides (3). Again, high temperatures would seem likely to help, but this possibility has not been explored because of the assumption that such conditions would disrupt vesicle integrity and lead to the release of contents, including genetic materials, to the environment. The assumption of thermal instability is based on the instability of fatty-acid vesicles under several environmental conditions that do not affect phospholipid vesicles. For example, the critical aggregate concentrations for fatty acids are much higher than for phospholipids (4), and dilution of vesicles below this concentration leads to vesicle dissolution. Also, divalent cations such as Mg^{2+} and Ca^{2+} are very destabilizing to fatty-acid vesicles, because the salts of fatty acids tend to crystallize out of solution (5, 6). Furthermore, vesicles composed solely of fatty acids are only stable within a fairly narrow pH range (4), although stability to high pH can be conferred by admixture with fatty alcohols and fatty-acid glycerol esters (7). However, as we show here, these environmental sensitivities do not extend to temperature, and certain fatty-acid-based vesicles are remarkably thermostable. This observation extends the range of environments that could be tolerated by early cells and opens up ways in which thermal fluctuations could be used to advantage by primitive cells.

Results

Retention of DNA Oligonucleotides at High Temperatures. We initially examined the thermal stability of 2:1 myristoleic acid (MA)/monomyristolein (GMM) vesicles, because this composition leads to vesicles that tolerate a wide range of salt and pH conditions, including the presence of up to 4 mM Mg^{2+} (5, 6). We tested vesicle stability by encapsulating a fluorescein-labeled 10-mer oligodeoxynucleotide (dA_{10}), removing unencapsulated DNA by size-exclusion chromatography (SEC), and then incubating aliquots of the purified vesicles at different temperatures for 1 h. The fraction of the oligonucleotide that was released from the vesicles during the high-temperature incubation was determined by a second round of size-exclusion chromatography, with free and encapsulated DNA measured by fluorimetry. Surprisingly, 2:1 MA/GMM vesicles remained completely stable to 100°C, with no detectable release of the encapsulated oligonucleotide (Fig. 1) or change in vesicle size (measured by dynamic light scattering). However, longer high-temperature incubation times did result in significant leakage (≈20% released after 10 h at 100°C).

To test whether this temperature stability is a general feature of vesicles composed of single-chain amphiphiles or is a unique characteristic of 2:1 MA/GMM vesicles, we examined vesicles made with a variety of amphiphiles and amphiphile mixtures by using the same oligodeoxynucleotide retention assay (Fig. 1). We examined four pure fatty acids at pH 8.5, which is approximately the pH at which half of the fatty acid is ionized and half is protonated, and which corresponds to the pH of maximum stability because every protonated carboxylate can act as a hydrogen bond donor to an adjacent ionized carboxylate (4, 8). Pure myristoleic acid (C14:1) vesicles released small amounts of DNA after 1 h at 50°C, with increasing amounts released at higher temperatures (≈10–20% released after 1 h at 60–80°C). Palmitoleic acid (C16:1) and oleic acid (C18:1) vesicles were completely stable to 90°C, but did show 30–35% leakage of entrapped DNA after 1 h at 100°C (Fig. 1*A*). Thus, increasing chain length leads to increased thermal stability of the bilayer membrane, consistent with membrane stabilization through the entropic effect of water release as the hydrophobic acyl chains are buried in the membrane (9). We also examined linoleic acid (C18:2) vesicles; this lipid leads to more fluid membranes with increased permeability to nucleotides at low temperatures (1). This C18:2 fatty acid behaved similarly to the C18:1 oleic acid, with linoleic acid vesicles being completely stable to 90°C but

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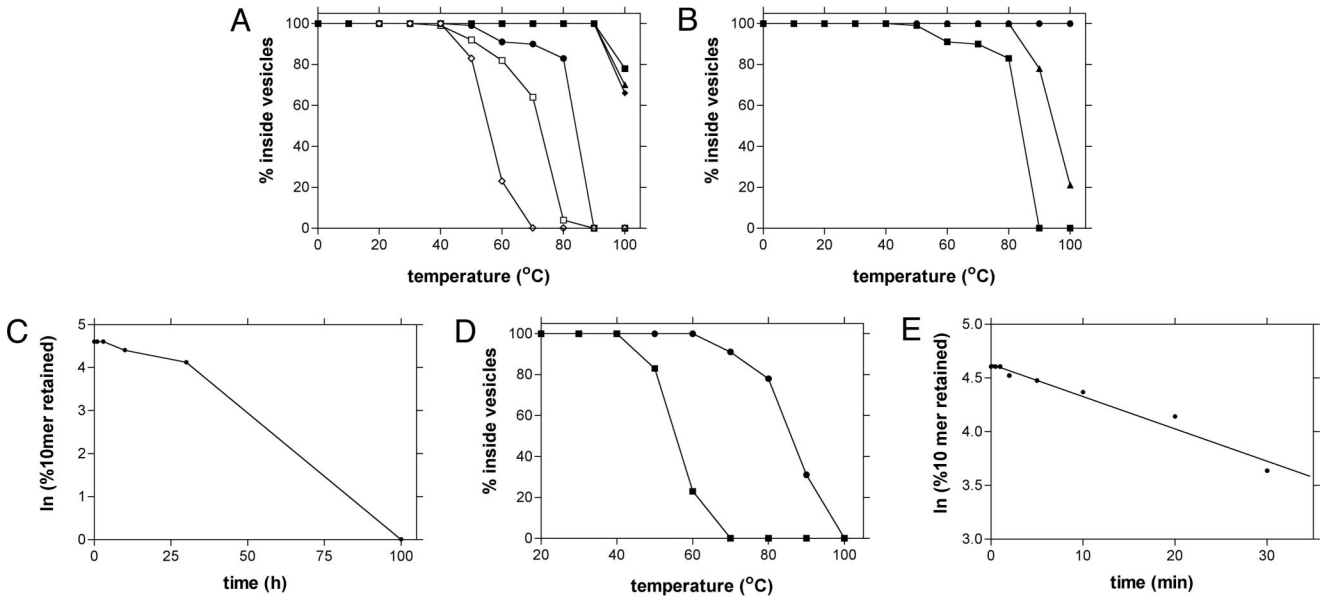

Fig. 1. Thermostability of model protocell membranes. The leakage of a fluorescein-labeled dA_{10} oligonucleotide from vesicles of the indicated composition was monitored as a function of time. (*A*) Influence of the acyl chain on vesicle stability. ◇, 2:1 decanoic acid/decanol; ●, MA; ▲, palmitoleic acid; ◆, oleic acid; ■, linoleic acid; □, 2:1 MA/farnesol. (*B*) Influence of the head group on C14:1 vesicle stability. ■, MA; ▲, 2:1 MA/MA-OH; ●, 2:1 MA/GMM. Identical results were given for 2:1 palmitoleic acid/monopalmitolein and 2:1 oleic acid/monoolein as for 2:1 MA/GMM (*C*) Time-dependent leakage from 2:1 MA/GMM vesicles at 100°C. (*D*) Influence of the head group on C10:0 vesicle stability. ■, 2:1 decanoic acid/decanol; ●, 4:1:1 decanoic acid/decanol/monocaprin. (*E*) Time-dependent leakage from 4:1:1 decanoic acid/decanol/monocaprin model prebiotic vesicles at 100°C. Solution conditions were 0.2 M sodium bicine, pH 8.5.

exhibiting ≈20% release of encapsulated DNA after 1 h at 100°C.

We then examined the effect on vesicle stability of a series of additives to pure MA. As noted above, MA/GMM (2:1) vesicles are stable during prolonged incubation at 100°C, presumably because of the ability of the glycerol headgroup to provide two hydrogen bond donors that can interact with the ionized carboxylates of MA. Consistent with this argument, myristoleyl alcohol (MA-OH), which can provide one hydrogen bond donor per molecule, also stabilizes MA vesicles, but less effectively than GMM. To determine whether the thermostability of vesicles composed of longer-chain fatty acids could be similarly improved, we examined mixtures of palmitoleic acid and oleic acid with their corresponding glycerol monoester derivatives. As expected, in both cases the addition of the glycerol monoester lipid conferred stability at 100°C, and no leakage of encapsulated DNA was observed (Fig. 1*B*). Thus, vesicle thermostability can be enhanced either by strengthening head group interactions (e.g., adding GMM or M-OH to MA) or acyl chain interactions (e.g., increasing chain length from 14 to 16 to 18 carbons). In contrast, the highly branched isoprenoid alcohol farnesol decreased vesicle stability, presumably by weakening acyl chain interactions (Fig. 1*A*).

We examined the stability of vesicles composed of short-chain (C10) saturated amphiphiles (Fig. 1*D*) because these molecules are more prebiotically plausible than longer-chain unsaturated amphiphiles. Pure capric acid (C10:0, decanoic acid) vesicles, as previously reported (4) are only stable at ≥50 mM total amphiphile concentration; these vesicles were too unstable to purify by size-exclusion chromatography (10), and therefore, we were unable to measure their ability to retain dA_{10} at elevated temperatures. However, synthesis by modified Fischer–Tropsch Type (FTT) chemistry is likely to yield a mixture of hydrocarbons, alcohols, aldehydes, and acids (11, 12), and glycerol esters of fatty acids could form in a typical ''drying lagoon'' scenario (13). Therefore, we examined mixtures of capric acid with some of these related compounds (Fig. 1*D*). Vesicles of 2:1 capric acid/decanol were somewhat more thermostable, but started to leak encapsulated oligonucleotide at 50–60°C. Vesicles of 2:1 capric acid/monocaprin could be observed by microscopy and were capable of retaining an entrapped oligonucleotide, as evidenced by dialysis and microscopy, but were not stable enough to survive gel filtration chromatography and gradually crystallized overnight at room temperature. This instability may reflect the large ratio of head group size to acyl chain length for monocaprin. To decrease the average head group size, we explored ternary mixtures of capric acid with decanol and monocaprin. Interestingly, these exhibited significantly increased thermostability and retained oligonucleotides for 1 h at 60–70°C and could tolerate shorter periods of high temperatures up to 100°C for 1 min with no detectable loss of encapsulated oligonucleotide (Fig. 1*E*). These experiments show that amphiphile mixtures of the kind that may have been present on the early earth could encapsulate nucleic acids and retain these at least for brief periods at high temperature.

We wondered whether observed temperature stability extends to multiple thermocycles or whether rapid and large changes in temperature result in vesicle disruption. To answer this question, we subjected MA/GMM (2:1) vesicles to 20 cycles of 2 min at 20°C and 2 min at 90°C, and we quantified the loss of entrapped oligonucleotide. After 20 cycles, no loss of nucleic acid was observed. We then examined the response of prebiotic-model vesicles [decanoic acid (DA)/decanol (DOH)/glycerol monoester of decanoic acid (GMD)::4:1:1] to a similar thermocycling regime (20 cycles of 30 s at 25°C and 30 s at 90°C). In this case, 10–20% of the labeled DNA contents did leak out over 20 cycles, corresponding to a loss of 0.5–1.0% of contents per cycle. The stability of vesicles to multiple thermocycles is an important characteristic because it demonstrates that vesicles composed of simple single-chain amphiphiles are capable of surviving environments such as hydrothermal vents and hot springs that are candidates for sites of early evolution (14).

DNA Strand Separation within Vesicles. We used an oligonucleotide FRET pair strategy to demonstrate that vesicle thermostability

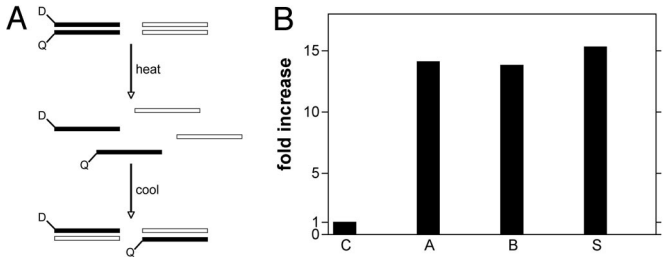

Fig. 2. Intravesicular DNA strand melting. (*A*) Schematic representation of the experimental setup. Black bars represent DNA molecules either modified with a fluorophore (D) or quencher (Q). White bars represent unlabeled strands of DNA. (*B*) Increase in fluorescence arising from intravesicular DNA strand melting and annealing. C, control; A, 2:1 MA/GMM; B, 4:1:1 decanoic acid/decanol/monocaprin; S, solution reaction (that is, not inside vesicles). The control reaction was of the same composition but not subjected to a thermocycle.

can be exploited for DNA strand separation and reannealing within vesicles. Complementary 19-mer oligodeoxynucleotides that were labeled with a fluorophore and a quencher at their 5′ and 3′ ends, respectively, were annealed and mixed with an excess of unlabeled oligonucleotide duplex of exactly the same sequence (Fig. 2). Heating above the T_m of the dsDNA (75°C) leads to strand separation, and subsequent cooling allowed random reannealing of labeled and unlabeled oligonucletides, thus yielding an increase in fluorescence. This assay was applied to both 2:1 MA/GMM and 4:1:1 capric acid/decanol/monocaprin vesicles with a 1-min incubation at 90°C, followed by cooling to 20°C for reannealing. After size-exclusion chromatography to ensure that only encapsulated DNA was monitored, both vesicle compositions gave rise to the increased fluorescence signal that is diagnostic of strand separation and reannealing. The measured fluorescence increase was similar to control reactions in solution and consistent with complete strand melting and reannealing as a result of thermocycling within vesicles.

Nucleotide Permeability at Elevated Temperatures. We have previously shown that activated nucleotides can spontaneously diffuse across the fatty-acid-based bilayer membranes, so that nucleotides added to the outside can enter vesicles and take part in template-directed primer-extension reactions on the inside of the vesicle (1). Nucleotide permeation appears to operate via a concerted flipping of an amphiphile-solute complex as opposed to packing defects arising from gel to liquid phase transitions, as seen for dimyristoyl phosphatidylcholine membranes (15). When we realized that these vesicles are stable to elevated temperatures, we wondered whether nucleotide permeability would be significantly enhanced at higher temperatures. By encapsulating radiolabeled nucleotides, incubating at high temperatures, followed by SEC to resolve retained and leaked nucleotides, we were able to observe rapid permeation of activated nucleotides, with uridine-5′-phosphorimidazolide equilibrating across 2:1 MA/GMM membranes within 30 s at 90°C. Because our previous low-temperature data demonstrated that the permeability of nonactivated nucleotide monophosphates was significantly slower than activated nucleotides (1), we sought to determine whether high temperatures would also increase the permeability of unactivated nucleoside monophosphates. Unactivated NMPs are much less permeable than activated NMPs because the phosphate bears two negative charges instead of one. We observed that the permeability of the more polar nonactivated nucleoside monophosphates dramatically increased, with release of encapsulated nucleotides from 100-nm MA/GMM vesicles reaching completion within 10 min for AMP, GMP, CMP, UMP, and deoxyadenosine-5′-monophosphate (dAMP) (Fig. 3*A*). Under the same solution conditions but at 23°C, <10% of nucleoside monophosphates crossed the membrane over a 24-h period (1). We further probed the influence of charge by measuring the permeation of ADP and ATP (Fig. 3*B*). In the absence of Mg^{2+}, ADP crossed the membrane more slowly than AMP (≈20 min for complete equilibration of ADP vs. <10 min for AMP); however, in the presence of Mg^{2+}, ADP permeated more rapidly than AMP (complete equilibration in <3 min), consistent with the higher affinity of ADP for Mg^{2+} (16) and consistent with previously observed trends at 23°C (1). ATP permeation was much slower (>1 h for completion) and could not be fit to a single exponential curve. Simple prebiotic model membranes are clearly more robust than previously appreciated, allowing for conditions, such as elevated temperatures, that facilitate the uptake of critical nutrients without the loss of larger entrapped material such as oligonucleotides.

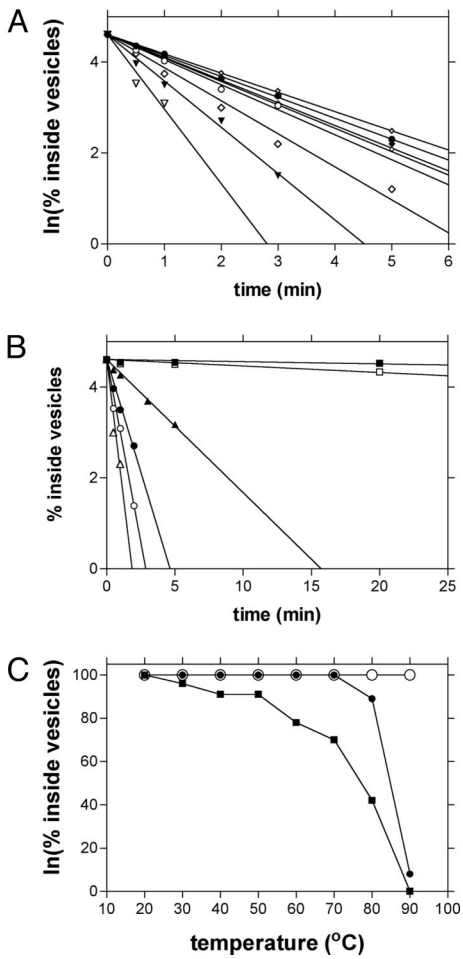

Fig. 3. High-temperature nucleotide permeability. (*A*) Nucleotide monophosphate permeability of 2:1 MA/GMM vesicles at 90°C. ▼, AMP; ▽, AMP + $MgCl_2$, ●, CMP; ○, CMP + $MgCl_2$, ◆, GMP; ◇, GMP + $MgCl_2$, ■, UMP; □, UMP + $MgCl_2$. (*B*) Influence of 5′ phosphates on 2:1 MA/GMM vesicle permeability at 90°C. ●, AMP; ○, AMP + $MgCl_2$; ▲, ADP; △, ADP + $MgCl_2$; ■, ATP; □, ATP + $MgCl_2$. (*C*) Oligomer permeability of 2:1 MA/GMM vesicles after a 1-h incubation at each indicated temperature. ■, NAD; ●, AAA; ○, AAAA.

Although the vesicle stability and nucleotide permeability characteristics of these membrane compositions are clearly compatible with PCR, we were unable to successfully reconstitute PCR within fatty-acid vesicles, most likely due to the strong inhibition of DNA polymerases by even low concentrations of fatty acids (17). PCR within phospholipid vesicles has been previously documented, but because of the impermeability of the

membrane, the nucleotides had to be encapsulated during vesicle formation rather than delivered across the membrane (18).

Permeability of Oligonucleotides. The permeation of mononucleoside diphosphates and triphosphates, but not a 10-mer oligonucleotide, suggested that short oligonucleotides might cross the membrane at elevated temperatures. Therefore, we measured the retention of a series of oligomers within 100-nm MA/GMM (2:1) vesicles at 90°C. In the absence of Mg^{2+}, dinucleoside diphosphates (NppN) required 4 min for 50% leakage from the vesicles, whereas trinucleotides (pNpNpN) leaked out more slowly, with 50% loss after 37 min. Tetranucleotides were completely retained after 1 h at 90°C (Fig. 3*C*). Thus, protocell replication schemes that invoke the sequential templated ligation of oligonucleotide substrates are only feasible for dinucleotides and trinucleotides, assuming that the substrates are taken up from the external environment. Alternatively, the intracellular generation of substrate oligonucleotides of length ≥4 could be used to generate a pool of trapped substrates for replication, while entrapped molecules smaller than a tetramer would be rapidly lost to the environment at 90°C.

Discussion

The high thermal stability of the model protocell membranes that we have studied has significant implications for the origin of cellular life. At the most basic level, our results suggest that even very primitive, self-assembled protocell structures could survive in environments with large and/or frequent temperature fluctuations. Thus, consideration of appropriate environments for the origin of the first cells need not be limited to sheltered subsurface locales, but can also reasonably include surface and near-surface locations subject to diurnal temperature variations, as well as locations near heat sources such as hydrothermal vents and hot springs. Perhaps more interesting than the simple ability of early cells to tolerate temperature fluctuations is the possibility that primitive cells could take advantage of thermal cycling to facilitate both strand separation of replicated genomic templates and the rapid uptake of nutrients during high-temperature excursions. For example, one could imagine a primitive "cell cycle," in which replication of the genetic material and growth of the vesicle compartment proceed at low to moderate temperatures, interrupted by high-temperature interludes that lead to strand separation and the influx of additional nucleotides. Cell division, perhaps triggered by environmental shear forces, would lead to random assortment of genetic molecules into daughter cells.

Diurnal temperature fluctuations are frequently invoked in prebiotic scenarios as a means of cyclic concentration by evaporative drying followed by redissolution, or as a means of driving chemical transformations within evaporated materials. Such temperature fluctuations could also drive periodic strand separation and nutrient uptake in vesicles, but the length of the diurnal cycle, even on the early earth, would lead to significant leakage of genetic polymers from primitive cells unless the cell membranes were composed of fairly long-chain fatty acids. Another attractive possibility for an environment that would subject primitive cells to large thermal fluctuations is provided by the possibility of convection cells near or within surface hot springs or hydrothermal vents. Rayleigh–Bernard convection cells have been shown, in the laboratory, to allow for the rapid cycling of DNA molecules between moderate and high temperatures, leading to amplification by PCR (19, 20). On the early earth, such convection cells might subject an adjacent fluid reservoir containing a population of replicating protocells to brief periods of high temperature, sufficient for strand separation and enhanced nutrient uptake separated by long and random interludes at lower temperatures.

A third significant and surprising outcome of our experiments is that mixtures of amphiphiles seem to form membranes with more desirable characteristics than membranes composed of single, pure amphiphiles. Previous studies have shown that the addition of fatty alcohols and fatty-acid glycerol esters to pure fatty acids lead to membranes that are more tolerant to the presence of divalent cations (5), and which show increased permeability (1) to sugars and nucleotides. Our current work shows that such mixtures form membranes with dramatically enhanced thermostability, compared with pure fatty-acid membranes. Because prebiotic chemical reactions undoubtedly generated complex mixtures of lipids, rather than a single lipid species, it is plausible that vesicles assembled from such prebiotic mixtures might have had greater thermostability, ion tolerance, and permeability than more homogeneous laboratory model vesicles. This behavior is in striking contrast to the situation with genetic polymers, where the availability of a relatively homogeneous set of initial nucleotides is generally regarded as essential to the synthesis of useful oligonucleotides. For example, stereochemical purity is important for RNA replication, as the presence of contaminating incorrect enantiomers leads to the strong inhibition of further polymerization (21).

Materials and Methods

Materials. Fatty acids, fatty alcohols, and the glycerol monoesters of fatty acids were obtained from Nu Chek Prep. All other chemicals were obtained from Sigma–Aldrich.

Vesicle Preparation. Fatty-acid vesicles were prepared by oil dispersion in buffered solutions as previously described (22, 23). For vesicles composed of mixtures of amphiphiles, the oils were mixed before dispersion in aqueous solution. All vesicle preparations were extruded 11 times through 100-nm pore-size polycarbonate filters with an Avanti miniextruder (Avanti Polar Lipids). For the encapsulation of molecules, amphiphiles were resuspended in the presence of the encapsulant. Separation of entrapped and unencapsulated material was by gel filtration with Sepharose 4B resin (Sigma–Aldrich) in which the running buffer contained the same amphiphile composition as the vesicles at a concentration above their critical aggregate concentration. Vesicle size was measured by dynamic light scattering with a PDDLS/CoolBatch 90T (Precision Detectors).

Nucleotide Permeability. Nucleotide permeability measurements were made in 0.2 M sodium bicine, pH 8.5. Radioactive nucleotides (0.1 mM) were encapsulated, and the vesicles were purified by gel filtration (Sepharose 4B). Vesicles were incubated at different temperatures in a Bio-Rad DNA Engine Peltier thermal cycler. Samples were then loaded on a gel filtration column, collected with a Gilson FC 203B fraction collector, and analyzed by scintillation counting with UniverSol scintillation fluid (MP biomedicals) on a Beckman Coulter LS 6500 multipurpose scintillation counter. Isotopes were either ^{14}C or ^{3}H and were obtained from Moravek Biochemicals and Radiochemicals.

Vesicle Stability and Oligonucleotide Permeability. The assay was as described for nucleotide permeability except that oligonucleotides were used in place of mononucleotides. Oligonucleotide sequences were poly(A), except for the dimer, and were either synthesized at the Massachusetts General Hospital DNA core facility or the Yale University Keck facility (New Haven, CT). The 10-mer had a covalently attached 5′-fluorescein. The dimer was ^{3}H-labeled NAD (Moravek Biochemicals and Radiochemicals). Trimer and tetramer oligonucleotides were 5′-labeled by using ^{32}P-γ-ATP and T4 polynucleotide kinase (New England Biolabs). Fluorescence was measured with a SpectraMAX GeminiEM fluorescence plate reader (Molecular Devices).

Strand Melting Assay. Fluorescently labeled oligodeoxynucleotides were obtained from Integrated DNA Technologies; 5′-ATGCGCCCGGCCTAGGGCC-3′ was synthesized with a 5′ tetrachlorofluorescein modification, and 5′-GGCCCTAGGCCGGGCGCAT-3′ was synthesized with a 3′ black hole quencher-1 (BHQ-1) modification. MA/GMM (2:1) samples were incubated at 20°C for 2 min, 90°C for 1 min, and 20°C for 2 min before measurement on SpectraMAX GeminiEM fluorescence plate reader (Molecular Devices) with excitation and emission at 518 and 539 nm, respectively.

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