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Oligoarginine peptides slow strand annealing and assist non-enzymatic RNA replication

Tony Z. Jia[1,2], Albert C. Fahrenbach[1,3], Neha P. Kamat[1], Katarzyna P. Adamala[1†] and Jack W. Szostak[1,2,3,4]*

The non-enzymatic replication of RNA is thought to have been a critical process required for the origin of life. One unsolved difficulty with non-enzymatic RNA replication is that template-directed copying of RNA results in a double-stranded product. After strand separation, rapid strand reannealing outcompetes slow non-enzymatic template copying, which renders multiple rounds of RNA replication impossible. Here we show that oligoarginine peptides slow the annealing of complementary oligoribonucleotides by up to several thousand-fold; however, short primers and activated monomers can still bind to template strands, and template-directed primer extension can still occur, all within a phase-separated condensed state, or coacervate. Furthermore, we show that within this phase, partial template copying occurs even in the presence of full-length complementary strands. This method to enable further rounds of replication suggests one mechanism by which short non-coded peptides could have enhanced early cellular fitness, and potentially explains how longer coded peptides, that is, proteins, came to prominence in modern biology.

RNA has been postulated to be the biopolymer from which early life on Earth evolved, owing to the central role of RNA as a mediator of information transfer between DNA and proteins, and of the ability of RNA to act as both a propagator of genetic information and as a catalyst. Most notably, RNA is the catalyst responsible for the ribosomal synthesis of all coded proteins[1–3], which strongly suggests that RNA-based catalysis preceded the evolution of coded peptide synthesis. Furthermore, recent findings point to a potential prebiotic pathway for the synthesis of ribonucleotides and thus of RNA[4–6]. If RNA was, indeed, the original biopolymer of cellular life, then selective pressures for faster and more accurate RNA replication would probably have led to the evolution of an RNA polymerase ribozyme that could catalyse the replication of increasingly complex RNA genomes. However, prior to the evolution of the first RNA polymerase ribozyme, RNA must have replicated non-enzymatically[3,7]. This conclusion has motivated a long history of efforts to copy RNA templates non-enzymatically, and although the efficient copying of arbitrary template sequences has not yet been demonstrated, recent advances[7–11] suggest that such template copying may well be possible.

Given the potential for non-enzymatic template copying to generate seminal RNA strands, one must then ask what additional steps are required to enable repeated cycles of RNA replication. The non-enzymatic copying of a template strand results in the formation of an RNA duplex, which must then denature to provide templates for the next round of replication. Previously, we showed that the thermal separation of the strands of an RNA duplex is facilitated by the incorporation of a fraction of 2′–5′ linkages in the RNA backbone; these linkages form as a consequence of non-enzymatic template copying and significantly lower the melting temperature of the resulting duplex[12]. However, subsequent rounds of non-enzymatic RNA replication are inhibited by the rapid reannealing of the separated strands after heating and cooling[13], which prevents the weakly binding RNA primers and activated monomers required for polymerization from associating with the template[14] (Fig. 1a). For subsequent rounds of replication to be possible, reannealing of the separated single strands must occur on a timescale that is comparable to or slower than the rate of strand copying. In principle, this kinetic control could be accomplished by operating in a highly dilute regime (≤1 nM RNA)[7,13]; however, 1 nM RNA corresponds to only a few strands per protocell 3–4 μm in diameter. Protocells that contain only a few strands of RNA would not have contained a sufficient concentration of an RNA with catalytic activity to confer a benefit to the protocell[7]. We therefore sought to identify conditions under which RNA strand reannealing at more relevant micromolar concentrations is significantly slowed but with a minimal effect on the template-copying chemistry.

Considerable evidence supports the possibility that peptides and RNA could have been present together[15–17] on the primitive Earth. Atmospheric discharge experiments[18,19], transport from meteors and cosmic dust[20,21], and more recent scenarios for prebiotic amino acid synthesis[16,17] all point towards the existence of amino acids on the Earth's surface shortly after its formation. Although early studies indicated that the basic amino acids arginine and lysine would have been among the least abundant[22,23], recent studies illustrate a prebiotically plausible arginine synthesis from simple precursors in a cyanide-rich reducing environment in the presence of hydrogen sulfide[24]. Arginine is particularly interesting functionally, because arginine-rich oligopeptides are known to bind strongly to both DNA and RNA[25,26]—the DNA condensing protein protamine is mostly composed of arginines[27,28] and Arg_3 has been reported to condense DNA *in vitro* via electrostatic interactions[29]. If arginine-rich peptides could have assembled on the early Earth, it seems probable that they would have interacted

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[1] Department of Molecular Biology and Center for Computational and Integrative Biology, Howard Hughes Medical Institute, Massachusetts General Hospital, 185 Cambridge Street, Boston, Massachusetts 02114, USA. [2] Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138, USA. [3] Earth-Life Science Institute, Tokyo Institute of Technology, 2-12-1-IE-1 Ookayama, Meguro-ku, Tokyo 152-8550, Japan. [4] Department of Genetics, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115, USA; † Present Address: Departments of Biological Engineering and Brain and Cognitive Sciences, MIT Media Lab and McGovern Institute, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA. *e-mail: szostak@molbio.mgh.harvard.edu

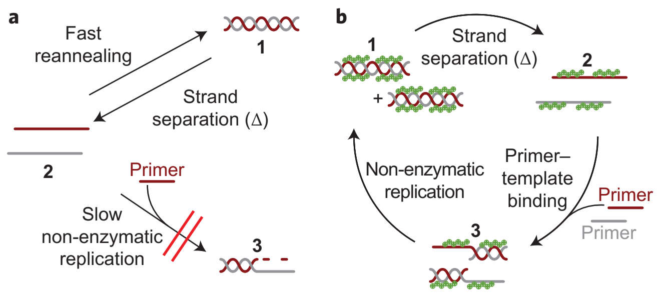

Figure 1 | The reannealing problem and a proposed solution. a, Complete template-directed primer extension results in a full-length duplex **1** (newly synthesized strand in maroon, original template in grey). After strand separation by heating (Δ) to give **2**, subsequent cooling results in rapid reannealing of the newly synthesized complementary strand to the template strand **1**, which prevents primer–template binding, outcompeting the slow process of non-enzymatic RNA polymerization that gives **3** and thereby prevents further rounds of RNA replication. **b**, RNA-binding oligoarginine peptides (green) inhibit strand annealing and promote further rounds of non-enzymatic replication. After an RNA duplex is formed, **1**, the strands are separated by heating (Δ) to give **2**. Subsequent cooling allows the peptide to bind to the separated complementary strands, but not to the shorter RNA primers. This selectivity prevents reannealing of the full-length replicated strands, which allows each strand to act as a template to which shorter primers can then bind **3**. The non-enzymatic polymerization reaction is free to proceed, which results in a complete replication cycle that would not be possible without the peptide.

strongly with RNA and potentially enhanced or inhibited aspects of RNA replication and function. A variety of prebiotically plausible mechanisms for peptide bond formation have also been explored[30–32], which include solution-phase elongation of the peptide chain by the stepwise addition of either *N*-carboxyanhydrides (NCAs) or 2-thiono-5-oxazolidones formed by the reaction of amino acids with the volcanic gases carbonyl sulfide[33] or carbon disulfide[34], respectively. Although most of these processes lack sequence specificity, efficient oligoarginine production via polymerization of the L-arginine-NCA through a unique mechanism that involves a six-membered-ring intermediate has been reported[35]. The polymerization of arginine on acidic mineral surfaces provides an alternative experimentally demonstrated pathway to oligoarginine peptides that could operate in the presence of a complex mixture of amino acids[36].

Given the potential existence of arginine-rich peptides on the early Earth, coupled with the ability of these peptides to bind to RNA, we sought to explore the nature and consequences of such RNA–peptide interactions on non-enzymatic replication. In the course of these investigations, we discovered that short arginine-rich peptides can prevent the annealing of complementary RNA strands in a concentration- and length-dependent manner. In the present work, we simulate a post-replication round of non-enzymatic RNA polymerization by thermally denaturing an RNA duplex, and show that template-directed primer extension proceeds only in the presence of an oligoarginine peptide (Fig. 1b). We also show that the non-enzymatic primer-extension reaction occurs within a phase-separated condensed state, that is a coacervate, formed by the electrostatic binding of oppositely charged RNA and oligoarginine polyelectrolytes. Taken together, these results show that cationic peptides could have enhanced the fitness of an emerging protocell by assisting in multiple rounds of replication.

Results

Binding of RNA to oligoarginine peptides. As a prelude to exploring the functional consequences of the binding of arginine-rich peptides to RNA oligonucleotides, we used a series of independent assays to examine the binding of oligoarginine peptides ($R10NH_2$ and $R9NH_2$; NH_2:C-terminal amide) to short RNA oligonucleotides. Initial gel electrophoresis assays showed that the oligoarginine peptide $R10NH_2$ at concentrations ≥100 μM caused a visible shift in the mobility of an RNA 15-mer (Supplementary Fig. 1). Furthermore, the fluorescence emission intensity of a 2-aminopurine (2AP)-containing RNA 15-mer increased significantly on the addition of 100 μM $R9NH_2$ (Supplementary Fig. 2). As 2AP fluorescence is quenched by the stacking of adjacent bases[37], the increase in fluorescence suggests that peptide binding alters the conformation of single-stranded RNA (ssRNA) such that base stacking is decreased. Finally, a solution that contained a fluorescently labelled RNA 15-mer became turbid in the presence of 10 mM $R9NH_2$ because of the formation of aggregated peptide–RNA complexes (Supplementary Fig. 3). The driving force for this complexation is probably electrostatic, as seen previously for the association of polylysine with DNA[38].

To examine the effect of peptide and RNA length on the concentration dependence of the interaction, we turned to fluorescence anisotropy titrations. We observed that the fluorescence anisotropy of an RNA 15-mer containing 2AP increased with increasing peptide concentrations; an increase in fluorescence anisotropy indicates a decrease in the rate of rotational diffusion, consistent with peptide binding to RNA (Fig. 2a). The presence of $R10NH_2$ resulted in a greater increase in the fluorescence anisotropy compared with that of R7 or R5 at the same peptide concentrations; the addition of R5 did not result in any significant increase in the fluorescence anisotropy. Similarly, the addition of $R10NH_2$ to an RNA 15-mer

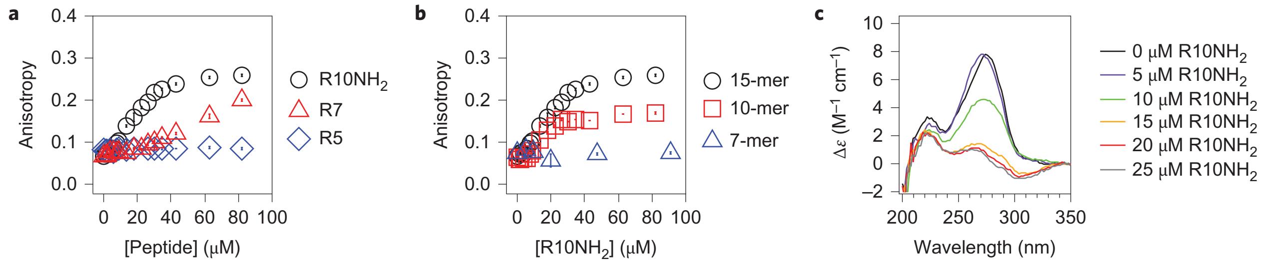

Figure 2 | RNA–peptide binding measured by fluorescence anisotropy and circular dichroism (CD). All experiments were performed in annealing buffer (see Methods). **a**, Fluorescence anisotropy of a 2AP-containing RNA 15-mer (5′-CC(2AP)GUCAGUCUACGC-3′, 10 μM) in the presence of three oligoarginine peptides of different lengths (R5, R7 and $R10NH_2$; NH_2:C-terminal amide) over increasing peptide concentrations. Higher peptide concentrations and increasing peptide lengths lead to increased fluorescence anisotropy. **b**, Fluorescence anisotropy of 2AP-containing RNAs of increasing lengths (7-mer, 10-mer and 15-mer, 10 μM; see Methods for sequences) with increasing $R10NH_2$ concentrations. Longer RNAs show a greater increase in anisotropy. **c**, CD traces of an RNA 15-mer (5′-CCAGUCAGUCUACGC-3′, 5 μM) with increasing concentrations of $R10NH_2$. The initial spectrum is characteristic of an A-type helical conformation[40]. The molar CD ($\Delta\varepsilon$) of the 270 nm peak decreases with increasing peptide concentration (up to 25 μM). Error bars: ±1 s.e.m.

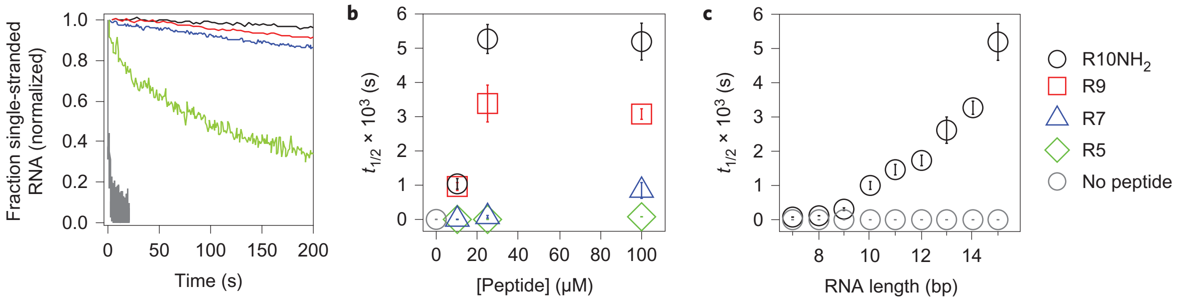

Figure 3 | RNA-annealing rates in the presence of peptides. Initial second-order annealing half-lives ($t_{1/2}$) were obtained for an RNA 15-mer (5′-GCGUAGACUGACUGG-3′) and its 2AP-containing complement (5′-CC(2AP)GUCAGUCUACGC-3′) in annealing buffer (see Methods) at RNA concentrations of 1 μM (see Supplementary Methods for fitting parameters and Supplementary Table 1 for a list of all conditions tested). **a**, Kinetic traces show the annealing of the two RNA 15-mers in the absence (grey) or presence of 100 μM R5 (green), R7 (blue), R9 (red) or R10NH$_2$ (black). **b**, $t_{1/2}$ for RNA 15-mers with increasing concentrations of R5, R7, R9 and R10NH$_2$. **c**, $t_{1/2}$ for RNA of different lengths (7-mers to 15-mers on the *x* axis (see Supplementary Methods for sequences)) annealing to the 2AP-containing 15-mer with or without 100 μM R10NH$_2$. Longer RNAs exhibit slower annealing kinetics in the presence of the peptide. Error bars, ±1 s.e.m; bp, base pairs.

resulted in a greater increase in the fluorescence anisotropy compared with that of the addition of R10NH$_2$ to a 10-mer, whereas R10NH$_2$ addition to a 7-mer did not result in any significant increase in the fluorescence anisotropy (Fig. 2b). These data indicate that longer peptides and RNA bind more tightly to each other than do their shorter counterparts, a result that would be expected from association driven by electrostatic interactions[39].

To explore further the possibility of a conformational change in the RNA on peptide binding, we monitored the circular dichroism (CD) spectrum of a ssRNA 15-mer in the presence of increasing concentrations of R10NH$_2$ (Fig. 2c). The spectrum in the absence of peptide is characteristic of an A-type helical conformation[40], but the 270 nm peak decreases in intensity with increasing peptide concentration, which indicates that the global helical structure of the ssRNA is significantly disrupted on peptide binding. Finally, we measured the effect of the addition of Mg^{2+} to a preformed RNA–peptide complex. Both fluorescence anisotropy (Supplementary Fig. 4) and CD (Supplementary Fig. 5) measurements show that added Mg^{2+} displaces the peptide in the RNA–peptide complex, presumably by disrupting the electrostatic interactions between the peptide and RNA.

Oligoarginine peptides interfere with RNA annealing. The formation of charge-neutralized RNA–peptide complexes suggested that peptide binding might increase the rate of annealing of complementary strands; on the other hand, the apparent change in RNA conformation on peptide binding hinted that the rate of annealing might be decreased. To address these hypotheses we used stopped-flow fluorometry to measure the effect of oligoarginine peptides on the second-order rate of annealing of complementary RNA 15-mers (Fig. 3a). Each RNA strand was incubated separately with the peptide at room temperature for at least one hour to ensure maximum peptide–RNA binding before annealing. A single 2AP residue present in one RNA strand was employed as a reporter of the fraction of RNA in duplex form, as 2AP fluorescence is quenched on duplex formation[37]. RNA melting analysis showed that replacing one A residue in one of the strands with 2AP does not compromise the duplex stability at room temperature (Supplementary Fig. 6). We observed that the $t_{1/2}$ (initial second-order annealing half-life) values for two RNA 15-mers increased from 0.9(1) s in the absence of peptides up to a maximum of 5.2(5) × 10^3 s (s.e.m. values in parentheses) in the limit of increasing concentrations and lengths of oligoarginines (Fig. 3b). For the most-effective peptide, R10NH$_2$, no further inhibition of annealing was observed above a concentration of ~15 μM (see Supplementary Table 1 for the annealing $t_{1/2}$ for all conditions tested), perhaps because of the formation of a phase-separated coacervate at concentrations >15 μM R10NH$_2$ (Supplementary Fig. 7). To confirm that this annealing inhibition was caused by the cationic nature of the oligoarginine peptides, we tested the anionic peptide GDGEGDGEGD, which did not result in a significant increase in the RNA annealing $t_{1/2}$ (Supplementary Fig. 8).

As the fluorescence anisotropy results (Fig. 2b) indicate that R10NH$_2$ binds to longer RNAs more strongly than to shorter RNAs, we investigated whether R10NH$_2$ could selectively inhibit the annealing of longer RNAs. Indeed, we observed that after R10NH$_2$ addition the annealing rate of RNA decreased more for the longer RNA oligonucleotides (Fig. 3c). Remarkably, a 15-mer anneals roughly two orders of magnitude more rapidly to a 7-mer ($t_{1/2}$ = 71(7) s) than to its full-length complementary strand ($t_{1/2}$ = 5.2(5) × 10^3 s) in the presence of 100 μM R10NH$_2$. Next, we asked whether the ions and ionic compounds required for non-enzymatic replication[9] would reduce the degree of peptide-conferred annealing inhibition. In the presence of 100 μM R10NH$_2$, the addition of Mg^{2+} (up to 50 mM (Supplementary Fig. 9)), the activated monomer cytidine 5′-phosphor-2-methylimidazolide (2-MeImpC, up to 50 mM (Supplementary Fig. 10)), cytidine monophosphate (up to 50 mM (Supplementary Fig. 11)) and sodium citrate (up to 25 mM (Supplementary Fig. 12)) all significantly increased the annealing rate of two RNA 15-mers (by up to two orders of magnitude). High concentrations of Tris-Cl buffer (up to 500 mM) also increased the annealing rate of two RNA 15-mers in the presence of R10NH$_2$, but to a lesser extent (Supplementary Fig. 13). These observations are consistent with the binding of oligoarginine peptides to RNA by electrostatic interactions, which are disrupted by monovalent and divalent cations. Finally, we also confirmed that the ability of R10NH$_2$ to slow RNA annealing is not affected significantly by pH within the range pH 6–9 (Supplementary Fig. 14). We suggest that the binding of oligoarginine to RNA changes the RNA structure significantly and contributes to annealing inhibition. At sufficiently high concentrations, added oligoarginine results in the formation of a condensed phase (Supplementary Fig. 7) driven by charge neutralization, not unlike those observed for ATP in the presence of polyvalent cations[41,42].

Oligoarginine-assisted non-enzymatic RNA replication. The observation that R10NH$_2$ greatly slows the annealing of two RNA 15-mers with little effect on the rate of annealing of a 7-mer to a 15-mer led us to hypothesize that, in the presence of R10NH, a short primer would still be able to anneal to a longer template even when the complementary strand to the template is present, and so allow primer extension to occur. Traditionally, high

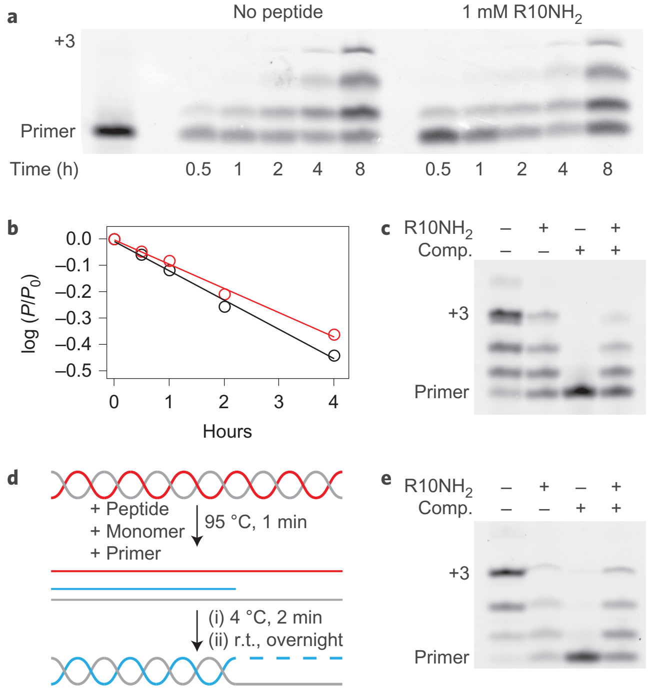

Figure 4 | Non-enzymatic RNA polymerization. Reactions performed with 10 mM $MgCl_2$, 250 mM Na-HEPES pH 8 and 10 mM 2-MeImpG. **a**, Polyacrylamide gel of non-enzymatic additions of 2-MeImpG to a 5′-cyanine 3 (Cy3)-labelled primer (5′-Cy3-CAGACUGG-3′, 2 μM) on a C4 template (5′-AA**CCCC**CCAGUCAGUC-3′, 2.5 μM) with or without 1 mM $R10NH_2$ (cytosines in bold represent 2-MeImpG binding sites on the template). **b**, Fraction (log) of unreacted primer for gel lanes in **a** versus time, with (red) or without (black) $R10NH_2$. The slope of the lines (linear fit, $R^2 = 0.99$) represents the pseudo-first-order rate constant, k_{obs}, per hour for the respective reactions. With 1 mM $R10NH_2$, $k_{obs} = 0.084(5)\ h^{-1}$. With no peptide, $k_{obs} = 0.110(5)\ h^{-1}$; s.e.m. in parentheses, $n = 5$. **c**, Polyacrylamide gel of an overnight non-enzymatic primer-extension experiment with 1.2 μM primer and 1.25 μM template (incubated with or without 1.5 mM $R10NH_2$) after the addition of 0 or 2 μM complementary (Comp.) strand to the template (5′-GACUGACUGGGGGGUU-3′) separately incubated with or without 1.5 mM $R10NH_2$. **d**, Post-replication round of non-enzymatic primer extension. The template (grey) and its complement (red) were annealed, then peptide, monomer and primer (blue) were added and the mixture was heated briefly to 95 °C. After cooling to 4 °C to allow primer–template binding, the system was left to warm to room temperature (r.t.). **e**, Polyacrylamide gel of the overnight primer-extension experiment described in **d** with 0.95 μM primer, 1 μM template and with or without 1.2 μM complementary strand and 1.5 mM $R10NH_2$, respectively.

concentrations (≥50 mM) of Mg^{2+} and activated monomer are used in non-enzymatic replication experiments[9]. However, because both Mg^{2+} and the activated monomers disrupt the electrostatic interactions between $R10NH_2$ and RNA, we performed all non-enzymatic polymerization studies at lower concentrations of Mg^{2+} and monomer (10 mM each). The annealing of complementary 15-mers in buffer conditions simulating a non-enzymatic primer-extension experiment (10 mM $MgCl_2$, 10 mM 2-MeImpC and 250 mM Na-HEPES pH 8) is still effectively slowed by 100 μM $R10NH_2$ ($t_{1/2} = 8.5(4) \times 10^2$ s). As $R10NH_2$ alters the conformation of ssRNA, to establish whether $R10NH_2$ would inhibit non-enzymatic primer extension we measured the rate of primer extension on a 16-mer C4 template using guanosine 5′-phosphor-2-methylimidazolide (2-MeImpG). We found that the rate of primer extension decreased by only ~30% in the presence of 1 mM of the $R10NH_2$ peptide (Fig. 4a,b and Supplementary Fig. 15)—in contrast to the increase of more than three orders of magnitude in the annealing $t_{1/2}$ of two RNA 15-mers in the presence of 100 μM $R10NH_2$ (Fig. 3). To our satisfaction, a promising result was obtained when the complementary strand to a template, pre-incubated with $R10NH_2$, was added to a preformed primer–template complex, also pre-incubated with peptide (Fig. 4c). Under these conditions, it appears that the peptide effectively inhibited the annealing of the complementary strand to the template and still allowed the primer-extension reaction to proceed, whereas in the absence of peptide, the addition of the complementary strand resulted in complete inhibition of the primer-extension reaction.

We then turned our attention to simulating a post-replication round of non-enzymatic template-directed primer extension by starting with an RNA duplex, that is, a template strand already bound to its complement. We added the other components of the non-enzymatic primer-extension reaction (primer, Mg^{2+} and 2-MeImpG) and briefly heated the sample to 95 °C to melt the duplex, followed immediately by cooling on ice. Rapid cooling is necessary (Supplementary Fig. 16), most probably to allow peptide–RNA binding to occur before the annealing of complementary RNA strands. The primer-extension reaction was then allowed to proceed as the reaction mixture warmed to room temperature overnight (Fig. 4d). In the absence of the oligoarginine peptide, no primer extension was observed, as expected from rapid annealing of the complementary strand to the template. In the presence of peptide, however, the primer-extension reaction did proceed (Fig. 4e); we propose that the thermally separated template and complement each tightly bind to the peptide, which prevents them from reannealing. However, the shorter primer binds more weakly to the peptide and is still able to hybridize to the template, thus allowing non-enzymatic primer extension to proceed under conditions that approximate a post-replication round of RNA polymerization.

Primer extension within the RNA–peptide condensed phase. As we observed the formation of phase-separated droplets when $R10NH_2$ was added to both ssRNA (Fig. 5a) and duplex RNA (Fig. 5b), we suspected that the primer-extension reaction occurred in the highly concentrated RNA–peptide coacervate rather than in the very dilute aqueous phase. To begin to test this hypothesis, we used confocal fluorescence microscopy to monitor the localization of a 5′-cyanine 3 (Cy3)-labelled RNA 8-mer primer in the presence of $R10NH_2$ and a complementary RNA 15-mer. As expected, the 8-mer and 15-mer phases separate together into large (~5–10 μm) globular structures (Fig. 5c); this phase-separation phenomenon is dependent on a variety of factors, which include the presence of peptide and a complementary RNA (Supplementary Fig. 17), the salt concentration (Supplementary Figs 18 and 19) and the length of the RNA itself (Supplementary Figs 20 and 21). A quantitative ultraviolet spectroscopic analysis also revealed that in the presence of $R10NH_2$ and a complementary 15-mer, roughly 97% of a native RNA 7-mer resides in the condensed phase (Supplementary Figs 22 and 23 and Supplementary Table 2). These observations suggest that the primer-extension reaction occurs within the coacervate phase because that is where most of the primer is localized.

To show directly that the observed primer-extension reaction occurred in the coacervate droplets, we centrifuged a solution that contained the peptide and all of the components of a non-enzymatic primer-extension experiment and separated the supernatant from the pellet containing the coacervate phase. We then allowed the primer-extension reaction to proceed separately in both the supernatant and the coacervate phase. Subsequent analysis of the reaction products confirmed that, in the presence of peptide, a majority of the

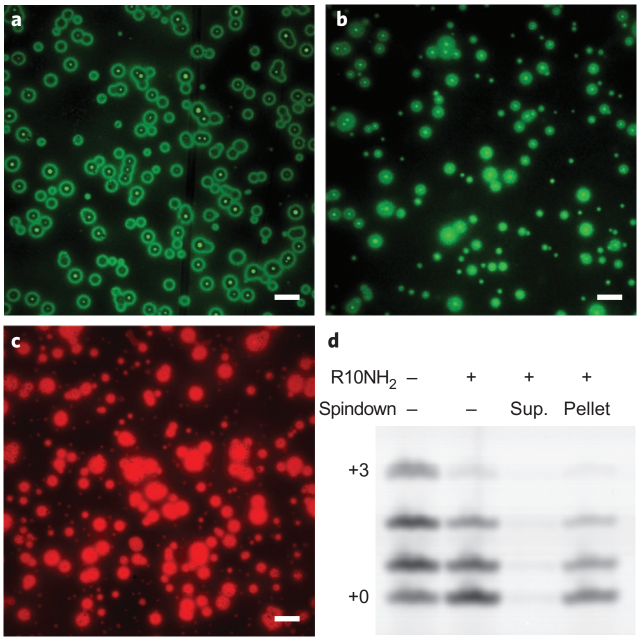

Figure 5 | Condensed phase of RNA. a, Confocal fluorescence microscopy image (488 nm excitation, 525 nm emission) of a 5′-6-carboxyfluorescein (FAM)-labelled RNA 15-mer (5′-FAM-CCAGUCAGUCUACGC-3′, 5 µM) with 1 mM R10NH$_2$ in annealing buffer (see Methods). **b**, Image of the sample from **a** but with an added complementary RNA 15-mer (5′-GCGUAGACUGACUGG-3′, 5 µM). **c**, Confocal fluorescence microscopy image (561 nm excitation, 595 nm emission) of a 5′-Cy3-labelled RNA 8-mer (5′-Cy3-CAGACUGG-3′, 10 µM) with a complementary RNA 15-mer (5′-CCAGUCAGUCUACGC-3′, 10 µM) and 1 mM R10NH$_2$, 100 mM Tris-Cl pH 8 and 10 mM MgCl$_2$. **d**, Non-enzymatic primer extension experiment after four hours with the Cy3-labelled RNA primer (1.25 µM) and template (1.4 µM) in Fig. 4, 10 mM 2-MeImpG, 10 mM MgCl$_2$, 250 mM Na-HEPES pH 8 and with or without 1 mM R10NH$_2$. A sample that contained the peptide was immediately centrifuged and the supernatant (Sup.) isolated from the condensed-phase pellet; the reaction was then allowed to proceed separately in the supernatant and in the pellet. Scale bars, 10 µm.

primer-extension reaction occurred within the isolated coacervate phase (Fig. 5d). We suggest that in the presence of peptide, shorter RNA strands, that is primers, that bind weakly to the peptide are still able to diffuse freely, and hence are still able to hybridize to the template strands in the condensed phase and undergo polymerization.

Discussion

One of the seldom-addressed problems with the RNA world hypothesis is that for multiple generations of non-enzymatic RNA replication to occur, the new single-stranded templates generated by melting the duplex product of template copying must reanneal on a timescale comparable to or slower than the timescale of template copying. In practice, this is a formidable challenge because the reannealing of complementary RNA strands at reasonable concentrations (~1 µM) is extremely fast ($t_{1/2} \approx 1$ s), whereas RNA-copying chemistry, at least in the current state of the art, is quite slow (occurring over hours to days)[10]. We have demonstrated here that the binding of oligoarginine peptides to complementary strands of RNA selectively slows down strand reannealing by up to several thousandfold. This kinetic property allows template-directed non-enzymatic RNA polymerization to occur in the presence of a full-length strand complementary to the template (Fig. 4e). Surprisingly, this non-enzymatic primer-extension reaction occurs in a condensed coacervate phase formed as a consequence of the electrostatic association of the oligoarginine peptides with RNA oligonucleotides (Fig. 5). Within the coacervate phase the annealing of longer RNA oligomers is selectively inhibited (Fig. 3), which thus allows time for primer extension to occur before strand annealing is complete. Further exploration and optimization of the selective inhibition of strand annealing has the potential to allow multiple rounds of RNA replication to occur.

In light of previous research that showed the potential plausibility of prebiotic arginine synthesis and peptide-bond formation[30], the possibility that arginine-rich peptides facilitated continuous cycles of RNA replication in a prebiotic setting should be considered. The prebiotic synthesis of arginine-rich peptides would be enhanced by the existence of local environments with an ambient chemistry that favours arginine synthesis[24]; another potentially important factor is that the oligomerization of arginine specifically, even in the presence of other amino acids, could be templated by negatively charged surfaces such as minerals[36], fatty-acid membranes or even RNA itself[43]. Assuming the simultaneous existence of arginine-rich peptides and RNA on the early Earth, a primitive cell would gain a considerable evolutionary advantage if it were able to synthesize arginine-rich peptides internally, perhaps foreshadowing the evolution of coded translation and the later appearance of the arginine-rich DNA condensing proteins such as protamine[27,28] and histones[44] found in modern cells. However, the concentrations of oligoarginine peptides used in this study for the successful non-enzymatic template-directed primer extension in the presence of a competing complementary strand are quite high (1.5 mM) and it is unclear how such concentrations could be reached in a prebiotic system or whether lower concentrations of peptide could have had the same effect. As it is known that nucleic acids electrostatically bind to many cationic polymers[45], the inhibition of RNA annealing could very well have been a result of the strong binding of RNA to any cationic polymer, some of which may be more effective than oligoarginine. Thus, we cannot discount the possibility that a presently unknown cationic prebiotic polymer, potentially with a simpler structure and/or synthesis than oligoarginine, for example a polyamine[42,46], could also efficiently slow annealing. In addition, the recent discovery of complex macrostructures that result from self-assembling cationic tripeptides[47] suggests that the supramolecular assembly of simple prebiotic peptides to form a cationic RNA-binding matrix should also be considered as a future avenue of investigation.

Although we have shown that oligoarginine binding to RNA slows complement–template annealing, the mechanism by which this occurs remains to be elucidated. In principle, the relevant RNA–peptide interaction could be controlled either thermodynamically or kinetically. In the thermodynamically controlled extreme, the addition of the peptide to double-stranded RNA (dsRNA) would destabilize the duplex to form peptide–ssRNA complexes over time. In this scenario, binding of oligoarginine to a single strand of RNA must be much stronger than binding to the duplex, so the peptide would cause the RNA duplex to dissociate. A short primer, being less strongly bound to the peptide than to the full-length RNA[39], could then hybridize to the template–peptide complex and subsequently take part in template-directed primer extension. However, we did not observe primer-extension products without a heating and immediate cooling step (Supplementary Fig. 16), which strongly argues for a kinetically controlled mechanism in which the reannealing of longer strands is inhibited selectively after strand separation. This retardation of hybridization could occur if, for example, peptide binding causes a conformational change in the RNA that prevents the nucleation of base pairing required for an efficient annealing[48,49]. As we have shown, the peptide binds only weakly to short primer strands, which allows the primer to bind to the template and thus permits the primer-extension reaction to proceed. We also propose that a rapid cooling step immediately after strand separation is necessary as it promotes peptide–RNA binding on a timescale competitive with the reannealing of two longer strands. Thus, the prebiotic accessibility of a steep temperature gradient is critical for successful replication in the system we studied. Thermal convection and thermophoresis within the porous rocks of hydrothermal vents[50] and hydrothermal circulation generated by hydrothermal vents in ponds or lakes[51] are two prebiotically plausible environments that would allow biomolecules to access both very hot and very cold aqueous environments on a timescale fast enough to promote successful non-enzymatic replication.

In a search for further evidence that would distinguish between thermodynamically and kinetically controlled mechanisms, we consider the differences between the CD spectra of ssRNA versus dsRNA 15-mers on the addition of R10NH$_2$. ssRNA in the absence of peptide exists predominantly in an A-form helical conformation, but this conformation is disrupted after binding to R10NH$_2$, as evidenced by the disappearance of the 270 nm peak in the CD spectrum (Supplementary Fig. 5). In contrast, the 270 nm peak in the CD spectrum of duplex RNA does not disappear after the addition of R10NH$_2$ (Supplementary Fig. 24); disappearance of the 270 nm peak would have indicated a possible destabilization and/or denaturation of the duplex by peptide binding. Instead, the 270 nm peak shifts to a maximum at 310 nm, consistent with a conformational change. These spectral data provide evidence that strands of the 15-mer RNA duplex do not separate on the addition of R10NH$_2$, which supports a mechanism of peptide-mediated primer extension that is not thermodynamically controlled. Indeed, the CD spectrum may suggest specific binding between an RNA duplex and R10NH$_2$; further investigations into the nature of this complex are ongoing.

It was shown recently that a variety of phase-separated systems can be encapsulated inside model protocells[41]. The assembly of a fatty-acid vesicle around an oligolysine–RNA coacervate has also been reported[52], which is significant as lysine oligomers are also able to slow strand annealing, albeit less effectively than oligoarginine (Supplementary Fig. 25). These results, along with the observations that arginine-rich peptides can localize RNA to[53] and are compatible with (Supplementary Fig. 26) fatty-acid membranes, suggest that oligoarginine–RNA coacervates may also be encapsulated within model protocell membranes that are fatty-acid based. The encapsulation of coacervate compartments within a model protocell is particularly interesting as it could have been a mechanism by which primitive cells organized and concentrated compounds and functionality, perhaps resulting in the earliest organelles[42,54]. Remarkably, modern eukaryotic cells also utilize phase-separated RNA–protein granules for a variety of cellular functions, which include the segregation and concentration of RNA and proteins[55–57]. It is unknown whether these modern coacervates are a relatively recent innovation or reflect an ancient evolutionary origin.

To better understand the origin of life, we aim to design and construct a model protocell system[9]—for example, a fatty-acid vesicle that contains templates, primers, activated mononucleotides, peptides and catalytic metal ions—in which multiple rounds of non-enzymatic primer-extension reactions can be induced by thermal cycling and can be iterated ad infinitum. Multiple rounds of replication would bring us one step closer to emulating the first primitive cells that were able to grow, divide and evolve under early Earth conditions.

Methods

Peptides, RNA and nucleotides. Peptides (R5, R7, R9, R9NH$_2$ and R10NH$_2$; NH$_2$: C-terminal amide) were purchased from either NeoBioLab or GenScript at >95% purity as trifluoroacetic acid salts. DNA and RNA were purchased from Integrated DNA Technologies (IDT) and were used without purification unless otherwise noted (see Supplementary Methods for a list of all the other chemicals and their suppliers as well as for the synthesis of the GDGEGDGEGD peptide (Supplementary Fig. 8)). The activated ribonucleotide monomers 2-MeImpG and 2-MeImpC were synthesized according to published procedures[58] with minor modifications (Supplementary Methods).

Experiments, data analysis and figures. All experiments were performed in triplicate or greater. Curve fitting was performed using MATLAB and figures were prepared with Igor Pro (Wavemetrics) and Adobe Illustrator.

Fluorescence anisotropy. A 2AP[37]-containing RNA 15-mer (5′-CC(2AP) GUCAGUCUACGC-3′), 10-mer (5′-CC(2AP)GUCAGUC-3′) or 7-mer (5′-CC(2AP)GUCA-3′) was diluted to 10 μM in annealing buffer (10 mM Tris-Cl, 50 mM NaCl and 1 mM EDTA in nuclease-free water at pH 8) to a total volume of 100 μl in a sample cuvette (10 mm pathlength, Starna Cells, Inc.). We increased the concentration of peptide (R10NH$_2$, R7 or R5) by adding small volumes (~1 μl) of concentrated solutions (100 μM–10 mM) to the sample cuvette and obtained data at each point (303 nm excitation, 370 nm emission) on a Cary Eclipse fluorescence spectrometer (Agilent Technologies). We modulated the magnesium and sodium concentration in the same way (Supplementary Fig. 4; see Supplementary Methods for further details).

CD. An RNA 15-mer (5′-GCGUAGACUGACUGG-3′) (5 μM) or a 15-mer duplex (5′-GCGUAGACUGACUGG-3′ and its complement) (5 μM) in annealing buffer (10 mM Tris-Cl, 50 mM NaCl and 1 mM EDTA in nuclease-free water at pH 8) was prepared in a 300 μl quartz cuvette (10 mm pathlength, Starna Cells, Inc.). Data were obtained using an Aviv 202 CD spectrometer (Aviv Biomedical, Inc.) from 200 to 350 nm with a 2 nm step size and an 8 nm bandwidth (see Supplementary Methods for further details).

Stopped-flow annealing experiments. A typical stopped-flow annealing experiment consisted of mixing two RNAs, for example, one 15-mer (5′-GCGUAGACUGACUGG-3′) and one fully complementary 2AP-containing 15-mer (5′-CC(2AP)GUCAGUCUACGC-3′). All 2AP bases were deoxyribonucleotides, as provided by IDT. Each RNA strand (1 μM) was diluted in annealing buffer (10 mM Tris-Cl, 50 mM NaCl and 1 mM EDTA in nuclease-free water at pH 8) and incubated separately at room temperature for at least one hour either in the presence of or the absence of peptide (R5, R7, R9 or R10NH$_2$, up to 100 μM). The peptide length and concentration, as well as the length of one of the strands of RNA, was varied as indicated in Fig. 3. The two solutions were injected into the mixing chamber of an SX20 stopped-flow spectrometer (Applied Photophysics), and the spectral data were immediately recorded (303 nm excitation, WG 320 nm emission filter) (see Supplementary Methods for further details).

Non-enzymatic primer extension. Non-enzymatic RNA primer-extension reactions were performed with 2-MeImpG (see Supplementary Methods for further details).

Confocal fluorescence microscopy. All samples were imaged using a Nikon A1R confocal microscope (×100, 1.49 normal aperture Apochromat TIRF oil-immersion objective) at 561 nm (pinhole 0.5 Airy units) (see Supplementary Methods for further details).

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Author contributions

T.Z.J., A.C.F. and J.W.S. conceived the experiments and wrote the manuscript. N.P.K. performed the vesicle-leakage assays and T.Z.J. performed all the other experiments. K.P.A. contributed intellectually.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to J.W.S.

Competing financial interests

The authors declare no competing financial interests.

Retraction: Oligoarginine peptides slow strand annealing and assist non-enzymatic RNA replication

Tony Z. Jia, Albert C. Fahrenbach, Neha P. Kamat, Katarzyna P. Adamala and Jack W. Szostak

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We the authors are retracting this Article because our efforts to repeat and follow up on the results have been unsuccessful. Specifically, we have been unable to reproduce observations suggesting that arginine-rich peptides allow the non-enzymatic copying of an RNA template in the presence of its complementary strand (Fig. 4e). We originally dismissed variability in these experiments as resulting from variability in the snap cooling of samples following thermal denaturation. However, we now understand that the data reported in the published article are the result of false positives that arose from an incorrectly designed experiment in which random errors, including transfer and concentration errors, affected the ratio of the concentrations of the RNA template and its complementary strand. This resulted in false positives that were misinterpreted as template copying in the presence of a complementary strand, where in reality these reactions did not contain enough complementary strands to completely inhibit the reaction.

Subsequent experiments suggested that arginine-rich peptides may not slow the reannealing of complementary strands (Fig. 3), and that what we had previously interpreted as a decrease in annealing rate was actually an artefact due to slow coalescence or strand exchange between droplets of RNA–peptide coacervate, as well as droplet coalescence and settling that led to decreased fluorescence intensity. Similarly, the changing circular dichroism spectra shown in Figure 2c, which were originally interpreted to be the result of a change in the global helical structure of RNA upon peptide binding, may also be an artefact due to, for example, loss of signal or light scattering. Although the binding of arginine-rich peptides to RNA does form condensed-phase droplets, and although most of the RNA does reside within the condensed phase, follow-up experiments to confirm that non-enzymatic RNA polymerization occurs within these coacervate droplets have been inconclusive (Fig. 5d).

The experiments showing that vesicles are stable in the presence of arginine-rich peptides (Supplementary Figure 26, by N. Kamat), and the failure of acidic peptides to condense RNA (Supplementary Figure 8, by K. Adamala) have been reproduced. However, since the main conclusions of our paper are incorrect, all of the authors are now retracting the Article. The authors would like to thank Dr Tivoli Olsen for her extensive efforts to unravel the errors in our Article and we apologize to the scientific community for any confusion arising from our publication.