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Visualizing ribonuclease digestion of RNA-like polymers produced by hot wet-dry cycles

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Abstract

Polymerization of nucleotides under prebiotic conditions simulating the early Earth has been extensively studied. Several independent methods have been used to verify that RNA-like polymers can be produced by hot wet-dry cycling of nucleotides. However, it has not been shown that these RNA-like polymers are similar to biological RNA with 3'-5' phosphodiester bonds. In the results described here, RNA-like polymers were generated from 5'-monophosphate nucleosides AMP and UMP. To confirm that the polymers resemble biological RNA, ribonuclease A should catalyze hydrolysis of the 3'-5' phosphodiester bonds between pyrimidine nucleotides to each other or to purine nucleotides, but not purine-purine nucleotide bonds. Here we show AFM images of specific polymers produced by hot wet-dry cycling of AMP, UMP and AMP/UMP (1:1) solutions on mica surfaces, before and after exposure to ribonuclease A.

AMP polymers were unaffected by ribonuclease A but UMP polymers disappeared. This indicates that a major fraction of the bonds in the UMP polymers is indeed 3’-5’ phosphodiester bonds. Some of the polymers generated from the AMP/UMP mixture also showed clear signs of cleavage. Because ribonuclease A recognizes the ester bonds in the polymers, we show for the first time that these prebiotically produced polymers are in fact similar to biological RNA but are likely to be linked by a mixture of 3’-5’ and 2’-5’ phosphodiester bonds.

Introduction

A significant juncture in the history of early life was the transition from prebiotic chemical mixtures into primitive biological systems. A substantial weight of evidence supports the “RNA world” hypothesis that the earliest forms of life passed through a phase in which RNA served to store and transfer genetic information as well as catalyzing biochemical reactions [1-4]. Assuming that RNA did play such a role, a non-enzymatic condensation reaction must have been available to produce polymers resembling RNA.

For this reason, polymerization of nucleotides under prebiotic conditions simulating the early Earth has been intensively studied. For instance, early studies by Verlander et al. [5] observed polymerization of adenosine cyclic 2’-3’-phosphate on polyuridylic acid templates that produced oligomers ranging up to 7 nucleotides in length. Inoue and Orgel [6] reported that guanosine monophosphate activated as an imidazole ester polymerized on a polycytidylic acid template with products averaging between 6 and 10 nucleotides.

Non-activated nucleotides can also be polymerized by wet-dry cycles. Rajamani et al. [7] and DeGuzman et al. [8] showed that small amounts of polymers resembling RNA were synthesized when AMP and/or UMP were exposed

to hot wet-dry cycling. Wet-dry cycles concentrate potential reactants and add the free energy needed to form phosphodiester bonds between nucleotides [9]. These conditions simulate hydrothermal processes that commonly occur in volcanic hydrothermal fields today and were presumably ubiquitous in the prebiotic environment [10]. They showed that the inclusion of an amphiphilic phospholipid as an ordering agent enhanced the reaction and captured the products in lipid vesicles. The addition of monovalent salts as organizing agents greatly improved the yields of polymers [11]. During dehydration, crystallization of the salts concentrated and organized nucleotides in such a way that condensation reactions synthesized ester bonds. However, the synthesized polymers showed very little sensitivity to ribonuclease digestion suggesting that non-biological 2’-5’ phosphodiester bonds dominated the polymer chains.

Hassenkam et al. [12] and Hassenkam and Deamer [13] exposed single nucleotides or 1:1 mole ratio mixture to hot wet-dry cycles on atomically flat mica surfaces. Atomic force microscopy (AFM) revealed polymers as linear strands up to 5 kb in length and as ring structures 30–40 nm in diameter. However, a crucial question remains: Are these polymers similar to biological RNA in that they are linked by 3’-5’ phosphodiester bonds?

Contrary to modern life, in which the enzymatic transcription using DNA templates produces RNA exclusively linked by 3′–5′ phosphodiester bonds, in the absence of complex macromolecular catalysts, prebiotically synthesis of RNA can generate 2′–5′ and 3′–5′ linkages because of the proximity and similar nucleophilicity of the 2′- and 3′-hydroxyls on the ribose moiety (fig. 1). Regioselectivity of the non-enzymatic RNA synthesis is a major challenge to make a robust system for the chemical replication of RNA [14].

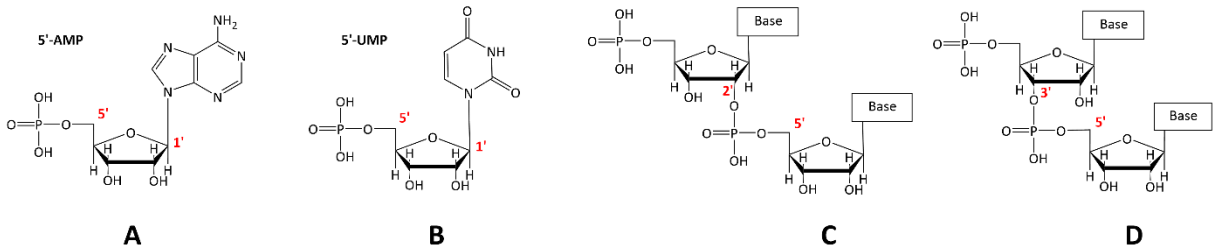

Figure 1: Structures of the starting materials 5’-AMP (A) and 5’-UMP (B) as well as possible products 2’-5’- and 3’-5’-dinucleotides (C and D). The molecules are shown in neutral form with the numbering of the ribose ring given in red for selected carbon atoms.

To determine If the polymers produced by hot wet-dry cycling are similar to biological RNA, we devised an experiment in which polymers were generated by wet-dry cycling of AMP, UMP and 1:1 mole ratio AMP/UMP mixture on freshly cleaved mica surfaces. The surfaces with polymers were then examined by AFM and the same polymers were imaged before and after exposure to ribonuclease A. If the polymers are chemically identical to biological RNA, ribonuclease A is expected to hydrolyze the 3’-5’ phosphodiester bonds between UMP and UMP or between UMP and AMP, but not the 2’-5’ phosphodiester bonds or bonds between AMP and AMP. This is because ribonuclease A is an endoribonuclease that has evolved to preferentially target 3’-5’ phosphodiester bonds at pyrimidine residues, thereby degrading single-stranded RNA [15,16]. The results reported here are consistent with the presence of phosphodiester bonds in the polymers produced by wet-dry cycling.

Materials and methods

Adenosine 5’-monophosphate (AMP, Sigma-Aldrich) and uridine 5’-monophosphate (UMP, Sigma-Aldrich) were dissolved in MilliQ water to obtain a concentration of 10 mM. The nucleotides were in their free acid form, so the pH of the solutions was between 2 and 3. All nucleotide solutions were filtered using Pierce™ Protein Concentrators PES, 3K MWCO, 0.5 mL, centrifuged at 14,000 rpm for 30 minutes. MS2 RNA (3569 nucleotides) from bacteriophage MS2 (Sigma-Aldrich) was used as a control for RNase A treatment. Ribonuclease A (RNase A) (Sigma-Aldrich, from bovine pancreas) preferentially cleaves ssRNA at pyrimidine residues.

Reaction mixtures

Mica plates were purchased from SPI Supplies (Mica grade v-4) and were cleaved using adhesive tape. Freshly cleaved mica (fixed on steel discs) was pre-heated 10 minutes on a temperature-controlled hotplate to raise the temperature of the mica surface to 85°C. Aliquots (30 µL) of filtered solutions of AMP, UMP or 1:1 mole ratio AMP/UMP mixture were placed on the mica surfaces and dried for 30 minutes at 85 °C before being re-hydrated with 30 µL MilliQ water. Such cycles were repeated 5 times to maximize polymerization. At the end of the experiment, the mica plates were carefully flushed with MilliQ water and dried with nitrogen gas.

RNAse A treatments

Images of polymers on the mica surfaces were recorded by AFM. The polymers were subsequently exposed to RNase A by adding a 5 µL RNase A 20 nM droplet that covered the region where the polymers were localized. The

droplet remained in place for 1 minute at room temperature. The droplet was removed by gently flushing the mica surface with 2-5 ml of MilliQ water and dried with nitrogen gas. The same region with polymers was then re-analyzed with AFM. To confirm that the RNase A was active, 0.5 µg of MS2 RNA (0.8 µg/µl) in 50 µl MilliQ water was mixed with 20 nM RNase A and incubated at room temperature for 1 minute. The hydrolysis products were analyzed by capillary electrophoresis (CE).

Atomic Force Microscopy

We used a Cypher from Asylum research (now Oxford instruments) equipped with a standard AC240 silicon tip from Olympus with a spring constant around 2 nN/nm and a resonant frequency around 70 kHz. Images were at least 512 × 512 pixels. The scanning was performed in ambient conditions at 1 Hz. Igor Pro software was used for image acquisition and data analysis. The AFM images were flattened and the thickness of the polymers was measured using the section analysis tool in the Igor Pro control software for the AFM.

Capillary electrophoresis

Capillary electrophoresis (CE) was performed using a temperature–controlled Agilent 7100 electrophoresis system equipped with UV–Vis diode array detector operating between 190 and 600 nm. Separation was made on standard bare fused-silica capillaries (48.5 cm × 50 µm id). The capillary was activated before use with 1 M NaOH for 10 min followed by 10 min with the MilliQ water at 930 mbar pressure. The separation buffer consisted of 10 mM phosphate buffer at pH 6.3. The temperature of the capillary cassette was 20 °C and the vial holder

temperature was maintained at 16 °C. The voltage was 20 kV in positive polarity mode with a typical current of 5 µA. Prior to each run, the capillary was rinsed with 0.1 M NaOH for 3 min followed by the separation buffer for 3 min. Samples were injected at 50 mbar for approximately 10 s followed by injecting separation buffer at 50 mbar for approximately 10 s before starting the separation. Instrument control and data acquisition was performed using ChemStation software (Agilent Technologies).

Results

Polymers of single nucleotides

Figure 2 shows AFM images of polymers produced after five wet-dry cycles at 85 °C of a pure UMP solution (fig. 2A) and a pure AMP solution (fig. 2C) on mica, so that the only polymers generated would be pure polyU or polyA. As in the previous study [13], linear polymers of a few hundred nanometers in contour length were observed as well as tangles of polymers with contour lengths stretching several micrometers covering the surface. Because the spacing of each nucleotide in the strand is about 0.6 nm [17], ssRNA-like polymers of more than 2 kb in length were observed. In general, the thickness of the polymers came in two main categories. The first structure had an average thickness of around 0.3 nm that is consistent with single stranded polymers, while the second category included polymers with thicknesses from 0.6 to 1 nm. The polymers in figure 2A observed by AFM had an average thickness of 0.3 nm and completely disappeared after treatment with RNase A (fig. 2B). There are no segments or particles, but instead there were patchy structures around 1.5-2 nm thick. We did a similar experiment with AMP, and examples of polymers appearing after wet-dry cycles of the AMP solution on mica are shown in figure 2C. In this case, we observed two different types of polymers: 0.3 nm and 0.6-1 nm thick, but in

contrast to the previous experiments we could not detect any substantial changes to the polymers after RNase A treatment (fig. 2D). There were a few cases of repositioning of the polymers consistent with parts of polymers detaching and reattaching to new spots on the surface during the RNase A treatment, but no cleavage or removal was detected.

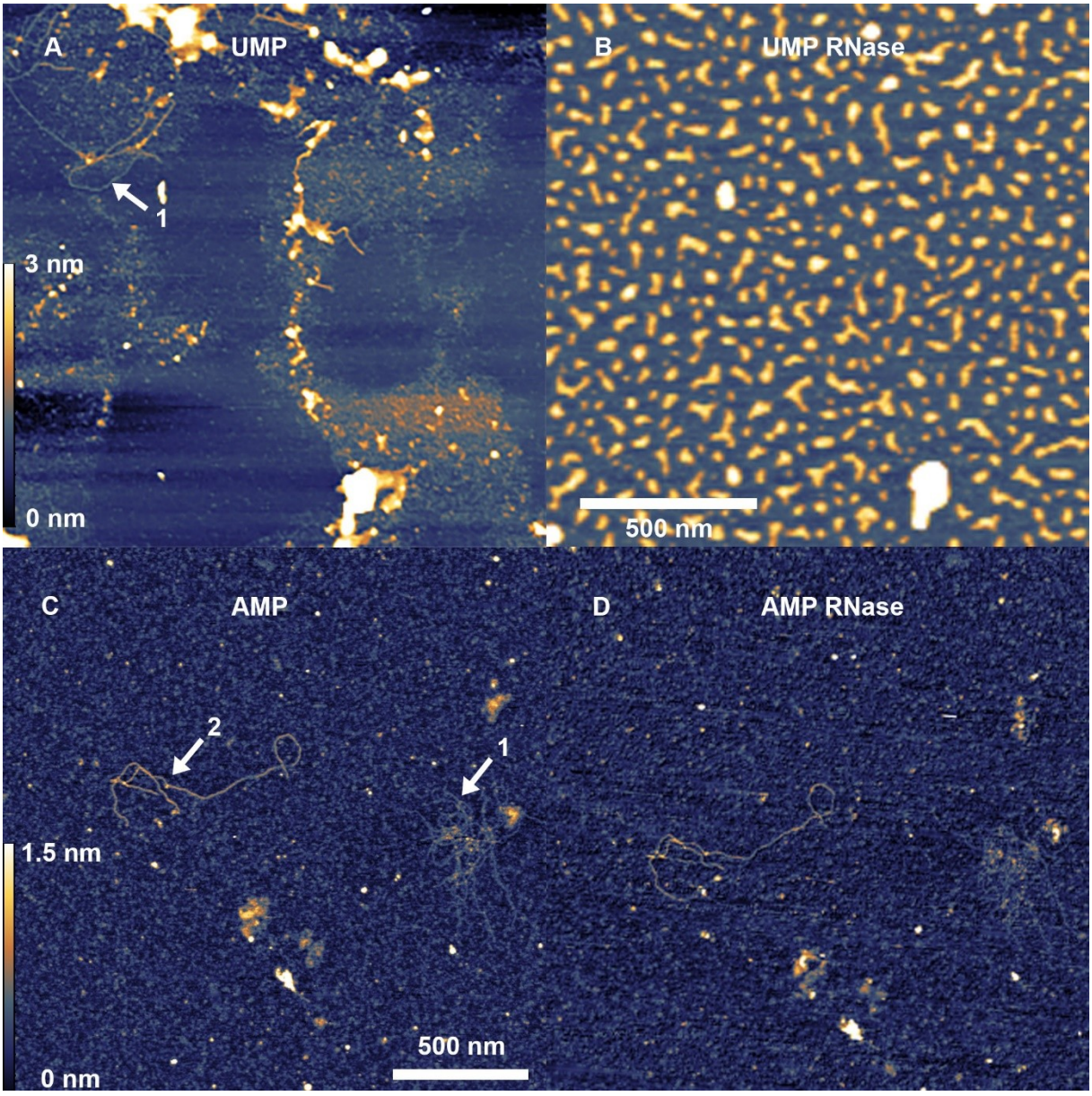

Figure 2 A and B shows AFM images of UMP polymers before (A) and after (B) RNase A treatment. Figure 2C and D shows AFM images of AMP polymers before (C) and after (D) RNase A treatment. Both were exposed to 5 wet-dry cycles on mica surfaces at 85 °C and imaged before and after 20 nM RNase A was added at

room temperature. Arrowheads 1 show 0.3 nm thick filaments and arrowhead 2 shows 0.6-1 nm thick filaments.

Polymers from nucleotides mixture

We continued with an experiment using an AMP/UMP (1:1) solution exposed to five wet-dry cycles at 85 °C on mica. Figures 3A and 3C show examples of polymers imaged by AFM. Here again, linear polymers of a few hundred nanometers and tangles of polymers of several micrometers were observed. 0.3 nm and 1 nm thick strands are apparent in figure 3A and indicated by arrows. Figure 3B illustrates how RNase A treatment affects the polymers and the region surrounding them. Some polymers remained nearly intact with a slight change in thickness. An example of this is shown in figure 3A and 3B where isolated long polymers (~1 µm and ~500 nm in length), indicated by the arrowhead, appear to be slightly affected by the RNase A treatment. An apparent decrease in polymer thickness from around 1 nm to 0.6 nm was observed, with some segments maintaining the 1 nm thickness (fig. 1 suppl. info).

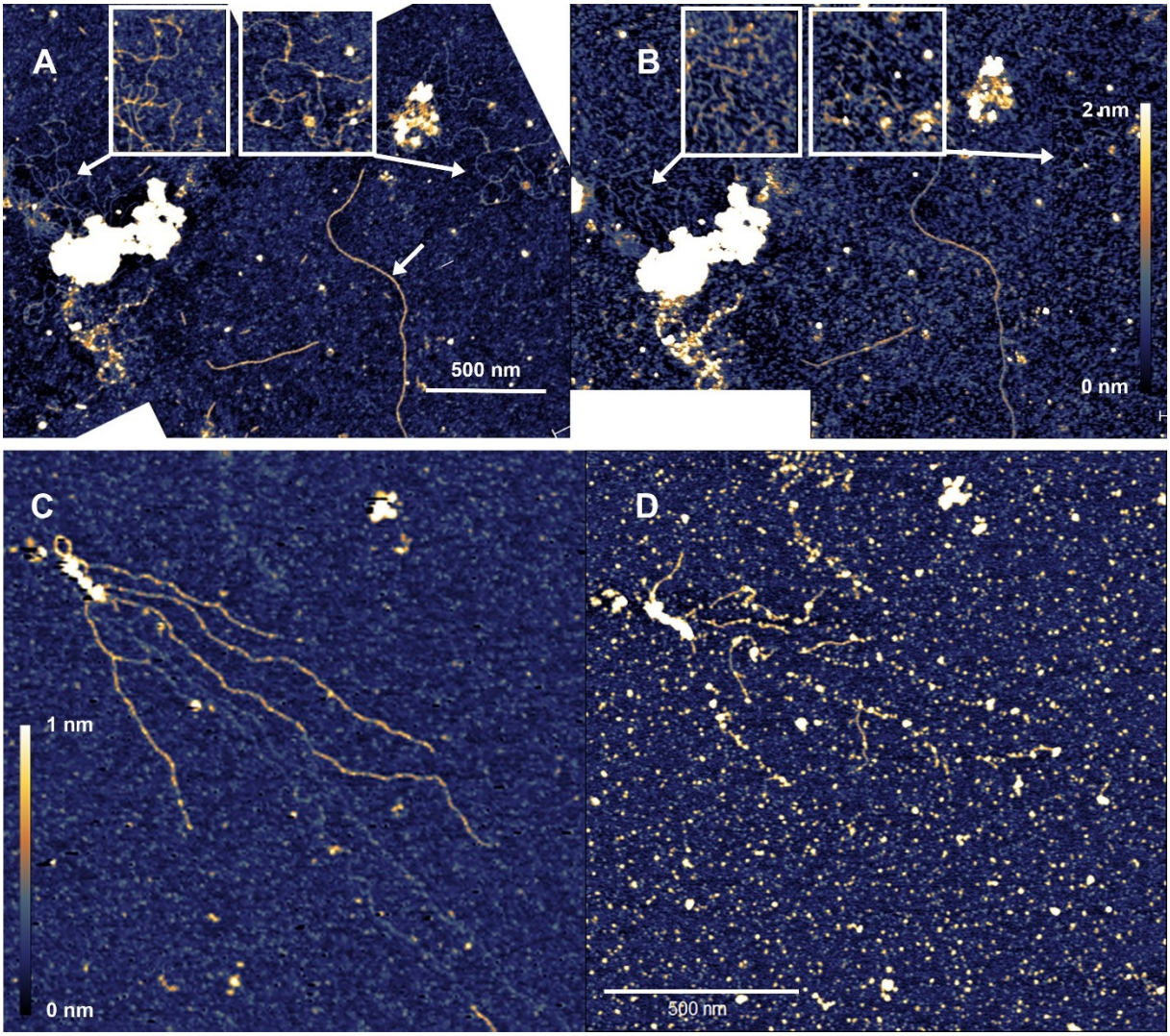

Figure 3 shows AFM images of 1:1 mixture of 5 mM of AMP and UMP exposed to 5 wet-dry cycles before (A and C) and after (B and D) RNase A treatment. Arrowhead shows 1 nm thick filaments. The insets in A and B show magnified views of the 0.3 nm thick filaments.

Other polymers were highly sensitive to the RNase A (fig. 3C and 3D). Some 0.3 nm thick polymers disappeared entirely, while others had numerous cleavage sites. The general outline of the original polymers network in figure 3C is still visible, presumably as small fragments of the original polymers that were not cleaved. In figures 3A and 3B, 0.3 thick polymers also show the same

sensitivity to RNase A digestion (see insets). Some structures near the limit of resolution (0.1 nm thick) were observed and interpreted as polymers embedded in monolayer of nucleotides that covered the surface (fig. 3C). The roughness of the mica surface here is high and suggests a 0.3 thick monolayer of nucleotides. Those structures in figure 3C completely disappeared after RNase A treatment, suggesting that they might either have been removed by the RNase A treatment or by flushing of the mica with water after the treatment (fig. 3C and 3D).

Numerous particles of around 0.8 nm in height appeared on the mica surface after RNase A treatment (fig. 3D). It is interesting that similar small particles with a height just short of 1 nm are connected to the polymer fragments, while other particles appear to bind to the ends. This is consistent with the particles being individual RNase A molecules adhering to the mica surfaces or more likely as clusters. To confirm that the observed particles could indeed be RNase A sticking to the mica, we exposed a clean mica surface to the RNase A solution. Similar particles having 0.8 nm height were apparent (fig. 2 suppl. info).

Our assumption has been that wet-dry cycles of AMP/UMP mixture generate RNA-like polymers containing random sequences of AMP and UMP linked either by 2’-5’ or by 3’-5’ phosphodiester bonds. However, we sometimes observed pure segments of apparent AMP polymers and UMP polymers, in which case only UMP polymers would respond to the RNase A treatment. Evidence for this can be seen in Figure 3A which shows long, relatively featureless strands (arrow) and tangles of thin polymers (insets). The thin strands are highly sensitive to RNase A suggesting they are mostly composed of long homopolymers (polyU) but the thicker strands are slightly sensitive, suggesting the digestion of the pyrimidine strand in a double-stranded RNA-like polymer (fig. 3B).

Control for RNase A activity

To verify that the RNase A did in fact cleave biological RNA under our conditions we used MS2, a known linear RNA composed of 3569 nucleotides. We attempted to image the RNA adsorbed to the mica but the RNA appeared to coil up into large particles rather than strands (fig. 3 suppl. info). As an alternative, we prepared a solution of the MS2 RNA and then, analyzed the RNA solution by capillary electrophoresis before and after adding RNase A (20 nM, 25°C for 1 minute). The red line in figure 4 shows the capillary electrophoretic separation of intact MS2, the blue line shows the pure UMP monomers used as standard and the black line shows the MS2 after being exposed to RNase A. The MS2 RNA appears as a single peak at around 10 minutes that decreases after RNase A treatment but also appears to spread out. This suggests an increase in various lengths of the remaining undigested RNA or possibly the presence of RNase A clusters, which would be consistent with the observations from the AFM images. A new peak next to that of UMP monomers corresponds to hydrolysis products, confirming that RNase A was active, but hydrolysis did not go to completion in the 1 minute allowed for the reaction.

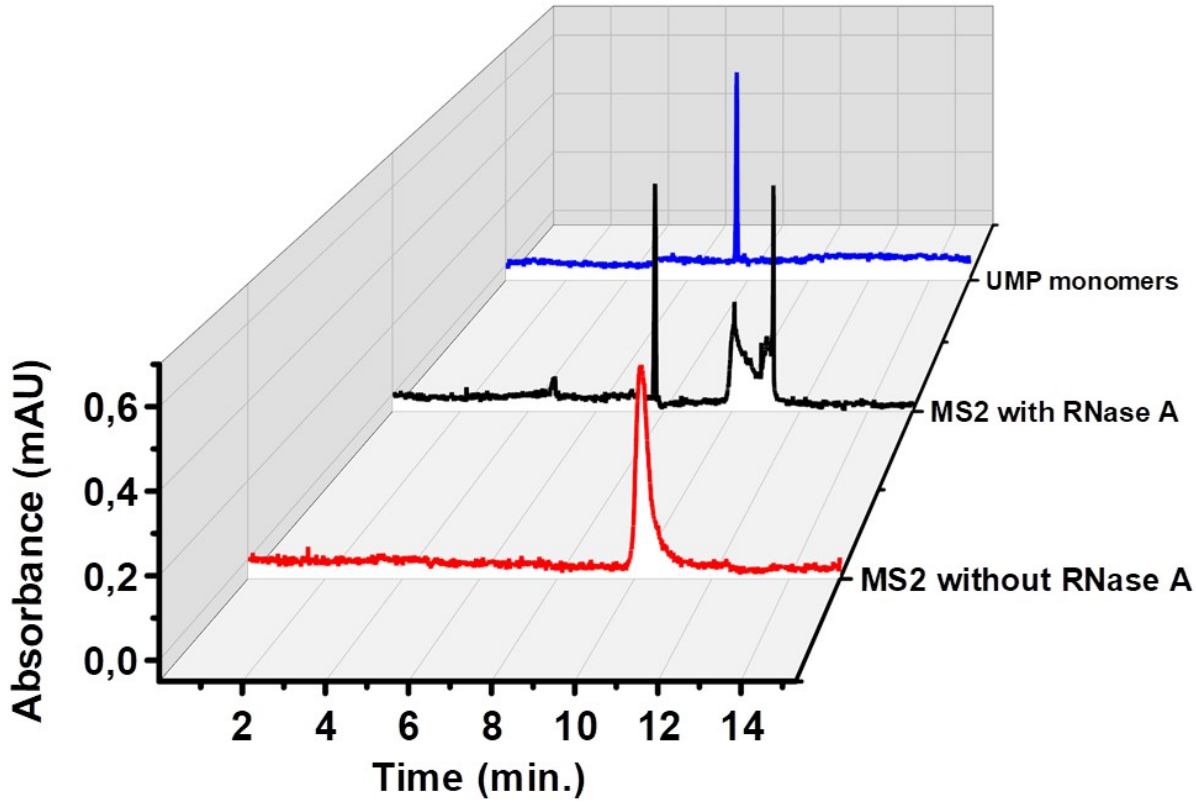

Figure 4: Electropherograms from CE analysis of MS2 RNA before (red) and after 20 nM RNase A treatment (black) at room temperature. UMP monomers alone were used as a control (blue). The absorbance was measured at 260 nm.

Discussion

The most significant results reported here are that polymers synthesized from UMP by wet-dry cycling disappear when exposed to RNase A, even though they are probably a mixture of 3’-5’ and 2’-5’ ester bonds that cannot be attacked by the enzyme. The most likely explanation is that the enzyme does hydrolyze most of the 3’-5’ bonds, producing fragments that are too short to adhere to the mica surface. The fact that the polymers synthesized from AMP are not attacked by RNase A is consistent with the known property of the enzyme which cannot hydrolyze ester bonds between purine nucleotides. An alternative to hydrolysis of the UMP polymers is that the enzyme somehow releases polyU

strands from the mica surface without breaking ester bonds. The fact that the AMP polymers are not affected by the RNase A argues against this possibility.

We also confirmed previous results showing that after three wet-dry cycles at 85 °C on a mica surface, nucleotides polymerize into long polymers. Two main categories of products were observed by AFM imaging. First, the presence of polymers with an average thickness of 0.3 nm is consistent with covalently linked single-stranded products. Indeed, the distance separating two phosphate groups when nucleotides are linked by ester bonds is around 0.3 nm. Thicker polymers (0.6-1 nm thick) are also observed, indicating base-paired duplex secondary structures. It is significant the two thicknesses (0.3 and 0.6-1 nm) were only observed in polymers synthesized by wet-dry cycling of AMP/UMP mixture and pure AMP, which is consistent with the presence of single-stranded polymers and double-stranded secondary structures. In the experiments performed with pure UMP, we could only measure 0.3 nm thick polymers consistent with single-stranded products.

Polymers from AMP and UMP mixture: Response to RNase A

The results with the AMP/UMP mixture are somewhat puzzling. Why do we observe only apparent partial digestion of the synthesized polyAU in figure 3D? In principle, there should be six types of covalent bonds in a polymer with both UMP and AMP. The (biological) 3’-5’ phosphodiester bonds between UMP-UMP, AMP-UMP or AMP-AMP, and/or the corresponding 2’-5’ bonds. If we assume an even split between all six bonds and that the RNase A only cleaves 3’-5’ bonds between UMP-UMP and AMP-UMP, there will be 33% chance that any given bond will be cleaved. In addition, there is the possibility of secondary/duplex structures that would hinder the activity of RNase A which would reduce the likelihood of cleavage even further. Therefore, in the AMP/UMP mixture experiment, based on

our controls, it would be safe to assume that there will be pure AMP segments, mixed AMP-UMP segments and pure UMP segments. RNase A should only cut some of the AMP-UMP and UMP-UMP ester bonds, leaving the AMP-AMP bond untouched. This means that there could be an increased likelihood for long polymers or segments to remain that are not affected by RNase A, which increases the likelihood of the sequence being long enough to adhere to the mica surface and be resolved. This is consistent with the polyU and polyA experiments and may be the reason why we can still see polymers on the surface in the AMP/UMP mixture experiment after RNase A treatment.

The synthesized polyU appears to be fully degraded in the AFM image (fig. 2B), which is consistent with its increased sensitivity to RNase A because only pyrimidine nucleotides are present. Here the likelihood of bond cleavage is in principle 50% assuming an even distribution of 2’-5’ and 3’-5’ bonds. PolyU molecules are essentially unstructured linear polymers [18] and cannot assemble into double strands. Therefore, RNase A activity is unhindered, which increases the likelihood of cleavage compared to the AMP-UMP polymers. The increased amount of cleavage would result in shorter polymer segments/oligomers that would be more easily released from the mica surface. This accounts for the fact that we do not see any polymers remaining in the UMP experiment after RNase A treatment but rather patchy structures (around 1.5-2 nm thick) that might be oligomers/monomers aggregates. The polyA is not cleaved by RNase A so we would expect polyA to be mostly unaffected by the RNase A treatment, which is consistent with our observations in the AFM data (fig. 2D).

After RNase A treatment, the 0.6-1 nm thick polymers synthesized from AMP/UMP mixture show a slight response to RNase A with a decrease in the polymers’ thickness that might be explained by the digestion of the pyrimidine strands in duplex molecules. Only the single-stranded polymers (with an average

thickness of 0.3 nm), synthesized from AMP/UMP mixture or pure UMP showed a good sensitivity to the digestion by the enzyme. This supports the claim that at least a significant proportion of the linkages in the synthesized polymers are indeed natural 3’-5’ phosphodiester bonds.

Potential contamination by biological nucleic acids

It was important to minimize the possibility that the polymers we observe are traces of nucleic acids present in the nucleotides or water. There was no evidence of nucleic acid contaminants in our previous AFM study [13]. In the present research we used the same experimental protocol, so contamination is unlikely. In addition, our inability to adsorb biological RNA on a mica surface in order to recreate the polymers observed with wet-dry experiments supports the notion that the source of the observed polymers was indeed a product of wet-dry cycling and not simple contamination.

Depurination

Degradation of nucleotides by depurination or deamination should be considered in terms of limiting the yields and lengths of polymers synthesized in the laboratory, as well as the stability and information content of prebiotic polymers. Moreover, the synthesis of RNA-like polymers under simulated hydrothermal conditions occurs in acidic pH between 2 and 3, at elevated temperature (85°C). The hydrolytic cleavage of the N-glycosyl bond of purine nucleotides and to a lesser extent of pyrimidine nucleotides occurs at a certain rate in these conditions [19].

Although depurination of ribonucleotides in solution does occur [20] the reaction is relatively slow and intact AMP is still present even after multiple cycles. It is interesting that the addition of lipids markedly reduces depurination

rates, and preliminary results suggest that the presence of adenine also has a protective effect, perhaps by promoting repurination.

Implications and role of mineral surfaces

Could RNA-like polymers with mixed 2'-5' and 3'-5' linkages be functional in an emerging RNA world? This is not necessarily a significant limitation. Engelhart et al. [21] showed that backbone heterogeneity (involving 10-25% of 2’-5’-phosphodiester bonds) is compatible with RNA folding into defined 3D structures that are necessary for RNA to have ribozyme activity. Mariani and Sutherland [22] also demonstrated that non-enzymatic chemistry can convert 2'-5'-linkages into 3'-5'-linkages through iterative degradation and repair.

A recent study showed that a nucleoside triphosphate (ATP) in the presence of rock glasses formed oligomers that could be fully hydrolyzed by ribonucleases, confirming that biological 3’-5’ phosphodiester bonds can be synthesized non-enzymatically [23]. Mineral surfaces could act as a regioselective catalyst and favor the 3’-5’-phosphodiester bonds over the unnatural 2’-5’-phosphodiester bonds. It is likely that future studies of hydrothermal metamorphic minerals may reveal unexpected catalytic properties that could have promoted polymerization reactions related to the origin of nucleic acids.

Author contributions

Laura Da Silva: Writing – original draft, Conceptualization, Methodology, Investigation.

Simon Holm Jacobsen Eiby: Writing – review & editing, Conceptualization, Methodology, Investigation.

Morten Jannik Bjerrum: Writing – review & editing, Conceptualization, Methodology, Investigation.

Peter Waaben Thulstrup: Writing – review & editing, Conceptualization, Methodology.

David Deamer: Writing – review & editing, Conceptualization, Methodology.

Tue Hassenkam: Writing – review & editing, Conceptualization, Methodology, Investigation.

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