Abiotic nanofluidic environments induce prebiotic condensation in water

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Research Article

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Abstract

A long-standing, central problem in the research on the chemical evolution towards the origin of life is the so-called “water paradox”: Despite life depends on liquid water, key biochemical reactions such as nucleotide condensation are inhibited by it. Current hypotheses addressing this paradox have low prebiotic plausibility when taking the conservative nature of evolution into account. We report spontaneous, abiotic RNA synthesis in water driven by nanouidic effects in temporal nanoconfinements of aqueous particle suspensions. Our findings provide a solution of the water paradox in a multifaceted way: abiotic, temporal nanouidic environments allow prebiotic condensation reaction pathways in water under stable, moderate conditions, emerge in suspensions as a geologically ubiquitous and thus prebiotic highly plausible environment and are consistent with evolutionary conservatism as living cells also work with temporal nanoconfined water.

Main Text

Prebiotic chemistry is facing a serious problem when it comes to the role of water in abiogenesis: Although water is essential for all life as we know it, key biochemical reactions such as the polymerization of nucleotides into RNA are impeded in watery solutions. In aiming to overcome this so-called “water problem” in prebiotic chemistry several hypotheses have been proposed. Among them are concepts of adding condensing agents such cyanamide, using alternative solvents such as formamide, setting high temperatures of about 160°C or designing prebiotic scenarios based on wet/dry cycles. However, when appraising the plausibility of such scenarios some general weaknesses appear. First, life manages the water problem within a stable environment full of water at ambient temperatures and do not rely on conditions as proposed in such scenarios. Second, evolutionary conservatism – the principle that evolution builds on existing pathways – indicates that the same physicochemical effects were involved in the abiotic origin of biopolymers as now being tapped by living systems via complex enzymes. Here we demonstrate how the water paradox in prebiotic chemistry can be solved without facing these weaknesses by considering nanouidic effects on water. According to our results abiotic polymerizations of nucleotides into RNA can occur within aqueous dispersions of polyaromatic hydrocarbons and inorganic minerals in the absence of enzymes or additives. We present the synthesis of various amounts of RNA depending on variations of the experimental parameters and propose an explanatory model based on non-classical effects on water when temporarily confined by suspended particles. The model describes how such nanoconfinements solve the water paradox via altering crucial physico-chemical properties of water and how evolutionary conservatism can link these natural nanouidic reaction vessels to enzymatic biopolymerisation in biological cells.

Living cells contain an intracellular aqueous fluid which is crowded with large, complex biomolecules. In this environment virtually all water exists as interfacial water. When viewing this dense mixture from the perspective of materials science it can be described as an aqueous suspension of highly concentrated nanoparticles. In the vicinity of such particles various nanouidic phenomena emerge in interfacial and...
nanoconfined water. Consequently, such water differs significantly compared to bulk in terms of properties such as, for example, reactivity, H-bonding network dynamics, density, dielectric constant or the quantum state of the protons\textsuperscript{10-12}. From a geochemical point of view, aqueous suspensions of mineral particles of micro-and nanoscopic size can be regarded as a comparable environment which generate nanofluidic effects. Inspired by this similarity, we designed experiments to test the potential of nanofluidic environments within aqueous suspensions of particles for inducing key biochemical reactions in a possibly prebiotic context. As the abiotic synthesis of RNA in water is a common goal of prebiotic chemistry and synthetic biology\textsuperscript{13,14} we chose the polymerisation of nucleotides into RNA as an example reaction. The focus was set on the formation of a pure adenosine-based RNA (poly-A-RNA) since adenosine monophosphate (AMP) is the most common nucleotide in living cells\textsuperscript{15}. Furthermore, poly-A-RNAs are common RNAs in cells in the form of poly-A-tails of messenger RNAs during protein biosynthesis. Thus, we first prepared samples which contained dissolved AMP. In order to create a nanofluidic environment for AMP solutions we selected quinacridone (QAC) as an example of polyaromatic heterocycle particles and graphite as an inorganic suspended particle species as both compounds have well been characterized in terms of inducing nanofluidic phenomena in aqueous suspensions\textsuperscript{16}.

Figure 1a shows results of fluorometric RNA concentration measurements of such samples. Ethanol precipitation to isolate possible poly-A-RNAs of 2-10 nt length and isopropanol precipitation to isolate such RNAs longer then 10nt have been applied. The results of both precipitation methods reveal that poly-A-RNA strands have formed in significant amount with respect to the negative controls (Fig. 1c) and that oligonucleotides of more than 10 nt length are abound in such samples.

To cross-check the fluorometric analysis with a totally independent but also highly specific method we performed quantitative Polymerase Chain Reaction after reverse transcription (RT-qPCR). The results (Fig. 2a) show that the fluorescence signal of the sample exceeds the background fluorescence after 7.57 cycles. This indicates a high yield (103-fold) of input RNA in comparison to the respective signal of the positive control miRNA (14.25 cycles). To obtain more information about the length of formed RNA we performed a comparative melting curve analysis. This analysis includes the sample RNA and a positive control miRNA with a length of 23 nt. The comparison reveals that the medium melting point of the sample is at higher temperature than the positive control (Fig. 2b). This results suggests that the sample contains created oligonucleotides with lengths of equally or longer than 23 nt.

For identifying substantial factors that lead to the observed abiotic polymerisation we first analysed the possible role of $\pi$-stacking of nucleoside monophosphates (NMPs) as stacking has been suggested to be an important factor for RNA polymerisation in terms of bringing the monomers in close contact\textsuperscript{17}. To do this analysis, we extended the fluorometric analysis of ethanol precipitated AMP-based samples to CMP- and UMP-based samples and compared the relation of the different RNA concentration results with the relation of respective published stacking equilibrium constants and stacking free energies of NMPs. The comparison reveals that the detected relative concentration of poly-A-RNA is about four times higher than
poly-C-RNA and poly-U-RNA (Fig. 1a) and that this order (poly-A-RNA >> poly-C-RNA ~ poly-U-RNA) correlates to the order of the respective stacking equilibrium constants and stacking abilities as derived from stacking free energy profiles of NMPs. This correlation suggests that in particle suspensions stacking and polymerisation of nucleotides is linked. Therefore, enhancing the stacking ability of a nucleotide should result in a higher RNA concentration. To test this, we selected UMP due to its low self-stacking ability and its comparatively low poly-U-RNA formation and add the amino acid arginine to the sample mixture. Arginine is known for its high stacking ability with aromatic groups. The fluorometric analysis of such UMP/arginine-based samples reveals that arginine increases the formation of poly-U-RNA by more than 100% (Fig. 1a). This observation supports the necessity of π-stacking for the polymerisation in aqueous suspensions and also points to a possibly prebiotic relevant kind of interplay between amino acids and nucleic acids.

However, base stacking cannot be the key factor for RNA formation in the described samples. This becomes evident when taking the water paradox into account and when considering the result of a negative control based on an AMP solution without suspended particles (Fig. 1c): Although AMP has the highest self-stacking constant among all NMPs and is the only NMP with the ability to self-associate in indefinite stacks the negative control shows that in comparison to the poly-A-RNA concentrations reported above only minute amounts can be found when the sample contains no added particles. This result suggests that there is a key factor in promoting both stacking and polymerisation that is closely linked to the particle suspension nature of the samples.

It is known that reducing the dielectric constant of water favours stacking and reducing the activity of water (e.g. via adding alternative solvents or inducing wet/dry cycles) enhances polymerisation. As nanofluidic phenomena emerging in nanoconfined water can reduce both properties simultaneously and watery suspensions of particles give rise to such phenomena we infer that the occurrence of nanofluidic phenomena in our samples is the key factor for the observed nucleotide polymerisation. This implies that the enhancement of nanofluidic phenomena should correlate with an increase of polymerisation reactions.

Nanoconfining environments change the behaviour of water especially in terms of its hydrogen bond network dynamics which, in turn, affects the thermodynamic property of water activity. The anomalous behaviour of nanoconfined water results from a highly complex interplay of various nanofluidic phenomena and forces which are related to, for example, surface energy and size of the confining boundaries, shear, molecular structure, electrical double-layer and fluctuations of general order parameter. To cope with this high complexity when assessing a possible synergy between nanofluidic phenomena and polymerisation we chose an experimental approach that allows to focus on a single, quantifiable effect. This approach uses the effect of Organic Solid/Solid Wetting Deposition (OSWD) as a probe. OSWD is the final result of a network of various nanofluidic phenomena on confined water between suspended organic crystals, including double-layer forces, Casimir-like fluctuation induced forces and dewetting induced hydrophobic collapse. It manifests as the adsorption and self-assembly
of insoluble polyaromatic heterocycles at solid/solid interfaces and thus be quantifiable via surface coverage determination.

We quantified the efficiency of OSWD as a function of different biomolecules dissolved in watery suspensions of QAC particles. For the quantification we measured the surface coverage of graphite crystals with QAC monolayer via Scanning Tunnelling Microscopy. The results (Fig. 3) reveal that adding AMP to the particle suspension enhances the coverage with very high statistical significance ($p < 0.0001$) in relation to QAC suspensions based on pure water (w/o, Fig. 3). A significant increase in coverage ($p < 0.01$) also occurs when using arginine as the added biomolecule. By contrast, coverages found in samples based on CMP or UMP are statistically nearly identical among each other and significantly lower with respect to the AMP- and arginine-based samples. Using a mixture of different NMPs leads to no significant increase of coverage but results in some very high single coverage measurements with respect to the CMP and UMP samples (NMPs, dot plot, Fig. 3) possibly caused by clusters of the AMP fraction of the mixture. The comparison of these OSWD-caused coverages with RNA quantities (Fig. 1a) indicates that relative differences in the extent of nanofluidic phenomena between the samples correlate with relative differences in RNA concentrations. This supports the hypothesis of a synergy between nanoconfinement effects and nucleotide polymerisation in aqueous particle suspensions. We thus propose that the emergence of nanofluidic effects on confined water is the key factor in the promotion of both stacking and polymerisation of nucleotides within aqueous suspensions of particles.

So far we used QAC/graphite particle systems to ensure comparability of the results from different experimental approaches. To test if the formation of RNA in such an environment is generalizable, we measured samples prepared by the same protocol but containing particles based on other compounds. As inorganic substitutes we chose magnetite and silica as also being geologically widespread compounds and selected the organic compound anthraquinone as being abundant in carbonaceous meteorites\textsuperscript{24}. Fluorometric measurements of partly or fully substituted samples show poly-A-RNA concentrations which are similar to or substantially higher than the comparable output of the QAC/graphite model system (Fig. 1b). This indicates that the abiotic formation of RNA within nanofluidic environments of aqueous suspensions is of general nature. Our results also imply that the output of RNA can be increased to higher concentrations by identifying appropriate particle suspension systems. The influence of the particle species on the RNA concentration as indicated by Fig. 1b (including results from inert polyethylene particles for comparison) is consistent with the fact that the anomalous behaviour of nanoconfined water is partly determined by the characteristics of the confining surfaces\textsuperscript{11}.

For nanofluidic effects to become dominant the confining surfaces must approach, at a conservative estimate, within the range of 10 nm and below\textsuperscript{11}. To evaluate if stacking of nucleotides within such nanoconfinements is possible in a sufficiently stable and ordered way for being able to prime polymerisation we performed dynamic molecular mechanics calculations. To do these calculations, we modelled a stack of 12 AMPs as an example system referring to the observation that polymerized AMPs of more than 10 nt length are abound in our samples. We arranged this stack in parallel between two nanoscale separated crystals of a QAC/graphite system and added water to the confinement. Figure 4a
shows the condition of the stack at the end of a dynamic simulation modelled with a confinement gap size of about 4 nm. The condition implies that the nucleotide stack remained arranged in such a confinement when surrounded by water (see also Supplementary Video 1). Comparative calculations suggest that such stacks are stable even when the density of water—which can be lower in nanoconfined condition— is decreased by about 60% (Fig. 4b, Supplementary Video 2). It requires a reduction of the gap size to about 3 nm and below to destabilize a stack due to increased interactions with confining surfaces (Extended Data Fig. 3). In sum, these simulations indicate that it is feasible to assume that nucleotides can associate in stable stacks within nanoconfinement gap sizes well below 10 nm for priming polymerisation.

Nucleotide polymerisation into RNA is a water-releasing condensation reaction which faces a thermodynamic barrier as the change in the Gibbs free energy of such a reaction is positive. Consequently, nucleotide polymerisation in water is a thermodynamically uphill reaction which is extremely inefficient to occur spontaneously under ambient conditions. However, if the entropic part ($\Delta S$) of the Gibbs free energy change ($\Delta G = \Delta H - T \Delta S$) gets very positive the reaction can become exergonic ($\Delta G < 0$) and thus favourable. As the entropic part is very positive when the activity of water is low the thermodynamic barrier can be overcome by reducing water activity. Against this background, we propose that our observed abiotic formation of RNA within aqueous particle suspensions can be explained by the rise of anomalous properties of water when getting temporarily confined between suspended particles: Nanoscale confinements change, among others, the vapour pressure and hydrogen-bond network dynamics of water. This can reduce water activity as a function of gap size and the characteristics of the confining surfaces. We suggest that in comparison with other ways to circumvent the thermodynamic barrier of such condensation reactions the exergonic impact of nanofluidic phenomena in aqueous particle suspensions on RNA polymerisation and stabilization is of high relevance for prebiotic plausibility as it does not require non-physiological conditions such as temperatures well above 100 °C, drying events or alternative solvents.

Our results indicate that abiotic temporal nanoconfinements of water can serve as natural reactions vessels for prebiotic RNA formation. The findings can solve the water paradox in such a way that nanofluidic effects in aqueous particle suspensions open up an abiotic route to biopolymerisation and polymer stabilization under chemical and thermodynamic conditions which are also prevalent within the crowded intracellular environment of living cells. The fact that polymerase enzymes also form temporal nanoconfined water clusters inside their active site implies that the same physico-chemical effects are tapped for nucleotide condensation in water both by biochemical pathways and our reported abiotic route. These aspects indicate that our model is consistent with evolutionary conservatism stretching back to the era of prebiotic chemical evolution and the origin of cellular life. The consistency is further supported by the fact that water is not trapped by nanoconfinements within the polymerase core but can exchange with the surrounding intracellular fluid—a situation which is also prevalent in nanofluidic environments within aqueous particle suspensions. Our experimental finding that under the reported conditions an amino acid catalyses the abiotic polymerisation of nucleotides may give a hint to a
nanofluidic origin of cooperation between amino acids and nucleotides evolving to the interdependent synthesis of proteins and nucleic acids in living cells\textsuperscript{30}.

Abiotic RNA polymerisation in temporal nanoconfined water does not depend on highly specific mineralogical and geological environments: Now as then in the prebiotic world, watery suspensions of micro- and nanoparticles are virtually ubiquitous\textsuperscript{31}. They exist, for example, in the form of sediments with pore water\textsuperscript{32}, hydrothermal vent fluids containing precipitated inorganic and polyaromatic particles\textsuperscript{33,34} or dispersed aggregates inside water-filled cracks in the crust of the earth and possibly of icy moons such as Enceladus\textsuperscript{35}.

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**Methods**

**Compounds**

Adenosine-5’-mono-phosphate (AMP, Sigma, disodium salt, CAS-Nr. 61-19-8); Uridine-5’-mono-phosphate (UMP, Alfa Aesar, disodium salt, CAS-Nr. 58-97-9); Cytidine-5’-monophosphate (CMP, Alfa Aesar, disodium salt, CAS-Nr. 6757-06-8); Guanosine-5’-mono-phosphate (GMP, Alfa Aesar, disodium salt, CAS-Nr. 85-32-5); L-Arginine (Alfa Aesar, CAS-Nr. 74-79-3); Quinacridone (Clariant GmbH, CAS-Nr. 1047-16-1); Anthraquinone (Fluka, CAS-Nr. 84-65-1); Carbon nanopowder (<50 nm (TEM); Sigma Aldrich, CAS-Nr. 7440-44-0); Carbon (mesoporous, Sigma Aldrich, CAS-Nr. 1333-86-4); Iron (II, III) oxide (Sigma Aldrich, CAS-Nr. 1317-61-9); Silicon dioxide (-325mesh, Sigma Aldrich, CAS-Nr. 60676-86-0); Polyethylene (<400 micron, Alfa Aesar, CAS-Nr.9002-88-4).

**Suspension preparation**

3ml aqueous suspensions were made of each 0.1 g/ml inorganic substrate (e.g. graphite powder) and/or organic pigment with a total nucleoside monophosphate concentration of 50mM. Samples were incubated over night at 60°C while mixed horizontally at 300 rpm, to avoid sedimentation of substrates and pigment. After incubation, samples were centrifuged at 8000x g, 1 min, at room temperature (RT). The supernatant was transferred to a new collection tube.

**Precipitation**
Precipitation was carried out with 0.2 M NaCl and 3.5 volumes of EtOH for isolation of short RNAs (2–10 nt), and with 0.3 M NaOAc (pH 5.2) and 0.7 volumes of isopropanol for isolation of longer RNAs (> 10 nt). After addition of the regarding amount of salt-solution and alcohol, samples were mixed by inverting the tubes 5 times and precipitation reactions were incubated over night at -20°C. Afterwards the samples were subsequently centrifuged at 14.000x g, 1h, at room temperature. Supernatant was discarded, leaving about 20µl of it inside the reaction tube, in addition to any formed gel pellet. Formed gel pellets were dried at 37°C for 20min and resuspended with an appropriate amount of H₂O dest.

**Concentration measurements**

Concentrations of miRNA suspensions were measured using a Qubit® 3 Fluorometer (Invitrogen™), and the Qubit® microRNA Assay Kit (Invitrogen™) or the Qubit® RNA HS Assay Kit (Invitrogen™) due to its high specificity and reliability. Concentrations were calculated by the chosen miRNA or RNA program of the Qubit® 3 Fluorometer. Standard curves and samples were prepared, following the manuals. Concentrations were calculated and normalized regarding the respective volume of each sample.

**Quantitative RT-qPCR**

For RT-qPCR selected samples were reverse transcribed using the TaqMan™ Advanced miRNA cDNA Synthesis Kit (Thermo Fisher) following the manual. As a positive control the synthetic miRNA hsa-miR-134-3p (5´-phosphorylated) (eurofins) with an oligonucleotide length of 23 nt was used. As a negative control nuclease-free water was used. For qPCR the Master Mix reaction was composed of 10µl QuantiTect SYBR® Green (Qiagen), 2µl amplification primer mix from the TaqMan™ Advanced miRNA cDNA Synthesis Kit (Thermo Fisher), and 8µl of diluted sample. qPCR was ran on a LightCycler® 480 Instrument II with the following settings: 15min 95°C, 40x: 15s 94°C – 30s 60°C – 30s 72°C – Single Data Acquisition, 72°C 2 min, Melting curve.

**Scanning tunnelling microscopy**

For Scanning Tunnelling Microscopy (STM) investigations aqueous suspensions were prepared of each 2% w/w of quinacridone pigment and a nucleotide monophosphate or amino acid final total concentration of 25mM, if required. Suspensions were drop cast to graphite substrates to induce OSWD and dried overnight. The samples were subsequently covered with dodecane to achieve a defined, stable environment between STM tip and substrate. Each sample was prepared twice. From each replicate 15 images of 200nm x 200nm size were generated from 3 different spots. Images were acquired with a self-build STM combined with a SPM 100 control system, supplied by RHK Technology. The scans settings were: bias = 1 V, tunnel current 300 = pA, line time = 50 ms. Further, the voltage pulses used to improve
the scan quality were applied within the range of 4.3 V to 10 V. All STM measurements were performed under ambient conditions.

Surface coverage determination

To determine coverage, meaning how much area of graphite is covered by supramolecular QAC adsorbate structures (Extended Data Fig. 1), black/white histograms were created. For this purpose, depicted QAC adsorbate structures were masked out in the image (white) while setting the background (graphite without QAC adsorbate structures) to black (Extended Data Fig. 2). Black/white histogram values were calculated using “histogram” within the “analyse” menu of the software ImageJ. The mean values given in the histogram data box were then converted to percentages of the graphite area covered with QAC adsorbate structures, whereby a histogram value of 0 means 0% of the graphite surface is covered with adsorbate and a histogram value of 255 means 100% of the graphite surface is covered. Note that AMP itself does not form monolayer on graphite due to its 3D-structure. For statistical analysis of the coverage results t-test with Welch’s corrections was used since the samples show unequal distribution variance. The calculations were performed using Excel, giving the results of two sided unequal variances t-test.

Computer simulations

All molecular mechanics calculations were performed with the Materials Studio package (Accelrys). The force field used was Dreiding\textsuperscript{39} where partial charges of atoms within the molecule are calculated with the Gasteiger method\textsuperscript{40}. Geometry optimization calculations were calculated where convergence tolerance regarding to energy was 1.0e-4kcal/mol, to force was 0.005kcal/mol/Å and to displacement was 5.0e-5 Å. The Smart algorithm was used which is a concatenation of steepest descent, Newton-Raphson and quasi-Newton methods to get a better behaviour for the different stages of downstream minimization. Dynamics calculations were performed for a NVE ensemble, with a temperature of 298 K, with random values assigned for the initial velocities of the atoms and with a time step of 0.1fs for the integration algorithm.

A chain of 12 AMP molecules was constructed starting with building an AMP dimer. The dimer itself was built by duplicating an AMP and performing an 180° rotation related to the axis lying in the plane of the adenine molecule and passing through the centres of the hexagon and pentagon. Afterwards, the rotated AMP was translated normal to the plane of the adenine by a distance of 3.5 Å. This dimer was stacked 6 times in a row such that all planes of the adenine molecules were parallel each by a distance of 3.5 Å. This artificially constructed model was finally optimized related to geometry.

The entire system is modelled within a supercell containing two graphene layers, on top in some distance a chain of AMP molecules and finally two layers of an QAC crystal above the AMP chain. The entire system was filled with water molecules for different concentrations. The initial position of the AMP chain
related to the graphene layers and the QAC crystal layers is stated below for the different calculations performed. The vertical dimension of the supercell has been chosen to be safe that an interaction of the graphene layers and the QAC crystal layers can be neglected (gap is 43 Å). The rectangular in-plane dimensions of the supercell have been determined to minimize the distortion of the periodically structure of the QAC crystal layers. The deviation of the unit cell vectors of the QAC layers are almost zero in one direction (0.1%) but quite large in the other direction (5.6%). However, because the outer layer of the QAC crystal is always kept fixed, the adjacent free movable QAC layer was observed stable throughout all simulations. This observation was the reason why this significant deviation was accepted for all calculations. The graphene layers are not distorted with regard to the optimal unit cell dimensions. Dimensions of the supercell are: plane \( x, y \): 80.956 Å, 56.58 Å, \( z \): 103.4 Å.

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**Declarations**

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**Author contributions**
A.G.H. designed and performed experiments and data analysis, T.M. designed and performed computer simulations, F.T. conceived the project and directed the research, F.T and A.G.H. wrote the manuscript.

Competing interests

The authors have no competing interests.

Additional Information

Supplementary information is available for this paper.

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