Acid-Catalyzed RNA-Oligomerization from 3′,5′-cGMP

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1. Supplementary Figures

Figure 1: Oligomeric Gx standards corresponding to the electrophoretic signals of the mixed-sequence RNA ladder. The reference ladder (lane L in main text Figure 1a) used as a size marker is composed of a mixture of a low range ssRNA ladder (50-1000 nt, NEB N0364S) and micro-RNA markers (17, 21 and 25 nt, NEB N2102S). The micro-RNA marker (Lane: Marker) containing defined sequences of all four nucleotides, shows similar electrophoretic mobility as that of pure oligoG sequences (shown in lanes G9-P, G10-P, G12-P, G16-P, G24, oligomers purchased from Biomers.net, P suffix indicates that the oligomer is 5’-phosphorylated) of similar length under the given electrophoretic conditions. G16-P runs similar to the RNA marker of the length of 17 nt. Bands also appear at higher positions in the lanes of oligoG standards, which we attribute to higher-order aggregates of the purchased Gx oligonucleotides. Length distribution of the polymerization products shows a similar pattern to that of the positive control (Lane: +) which is prepared by dissolving and precipitating the oligoG sequences present as impurities in the commercial cGMP-H powder available from Sigma (see section 2.3). The electrophoretic profiles of the positive control and those of polymerized materials shown in the main text exhibit two intense bands: one between the 17 and 21 nt markers and one right above the 25 nt marker. The high propensity of oligoG sequences to form aggregates explains the origin of the smear observed at the top part of the gel.
Figure 2: (a) UV chromatogram for dry-polymerized 3',5'-cGMP-H. (b) UV chromatogram and corresponding MS spectra of 4-6 nt peaks. (c) Column void peak at RT 1.7 min and corresponding MS spectrum. UV chromatogram was acquired with Agilent 1260 Infinity II Diode Array Detector WR at 252 nm ($\lambda_{max}$ of G nucleotide). The chromatographic peaks were identified by their corresponding MS spectra. Other peaks observed before the n-mer peak (at RT ~9 min, ~13.8 min, and ~19 min) have masses corresponding to n-mer–H$_2$O which are attributed to non-covalent aggregates of monomers. Monomers have no interaction with the column with the current method and thus unbound monomers come out in the void volume. During ionization, the monomer aggregates (nx monomer) and the corresponding masses are shown in the spectrum (c).
Figure 3: Weak bases like Tris, NH₄OH, and triethylamine suppress the polymerization of 3’,5’-cGMP-H.

1 mM 3’,5’-cGMP-H solution was mixed with a) Tris, b) NH₄OH or c) Et₃NHOH bases in 0.1-10 molar equivalents before drying at 80 °C for 20h in vacuum. Polymerization of cGMP is inhibited in samples with molar equivalents ≥1 of the base (i.e. at pH>3.3). 1 molar equivalent of ammonia is not inhibitory as the pH is 3.3 before drying. Lanes: 0-10 indicate the molar equivalents of bases added, pH of the resulting solutions is given under the respective lanes; + denotes positive control (see main text Figure 1a and SI Figure 1 for correspondence of the bands of the positive control and those of the marker.)
Figure 4: Computed free energy profile for the chain-extension step of the acid-catalyzed ring-opening polymerization of 3',5'-cGMP using a simplified model (see Section 3.1 for more details) representing the attack of the 3'-OH of a nucleotide at the activated (i.e. positively charged) phosphate moiety of a cyclic nucleotide. TS1, TS2: transition states; IM1: trigonal bipyramidal intermediate. The chain extension step requires an activation energy of 17.9 kcal/mol if two catalytic water molecules are included in the computational model. The role of these water molecules is to mediate the two key proton transfer steps of the pathway, i.e. the proton-shift from the nucleophile to the =O group of the phosphate and the subsequent displacement of the proton from one of the equatorial OH groups of the trigonal bipyramidal intermediate to the axial oxygen. This latter step results in the formation of a new nucleophilic center initiating the next transphosphorylation step. Indeed, clustering of water molecules along the phosphate groups was observed in the newly determined crystal structure of 3',5'-cGMP-H.[1]
2. Experimental details

2.1. Materials and Chemicals
An aqueous solution of the free acid form of 3’5’ cyclic guanosine monophosphate (3’,5’-cGMP-H) at 1 mM concentration was purchased from Biolog Life Science Institute (G001H). It was custom made from 3’,5’-GMP-Na(G001) via cation exchange and was neither precipitated nor dried in the manufacturing process, therefore preserving its monomeric state. All the experiments with 3’,5’-cGMP-H were conducted using this batch of H-form material from Biolog. As a positive control, 3’,5’-cGMP-H in dried powdered state was purchased from Sigma Aldrich (G7504). This material is known to contain oligonucleotides.[3] Ethanol (9065.4), NaCl (P029.2), HCl (K025.1), NaOH (K021.1), Tris (4855.2), MgCl2 (KK36.2), NH4(aq.) (6774.1) and triethylamine (X875.1) were purchased from Carl Roth. KCl (409316), glycogen from Oysters (G8751), and ammonium acetate (A7262) were purchased from Sigma Aldrich. Aqueous solutions were prepared in Ambion™ nuclease-free water (AM9932) from ThermoFisher Scientific.

2.2. Polymerization experiments
For a typical polymerization experiment (unless otherwise stated in the text), 1 mL of 1 mM 3’,5’-cGMP-H solution was used. To test the effect of various ions and pH, to this 1 mL nucleotide solution calculated amount of prepared aqueous stock solutions of ions/salts (10 mM, 100 mM, or 1M) was added to obtain the required molar equivalents of the test ion. For example, to test the effect of Na+ ions, to 1 mL 1 mM 3’,5’-cGMP-H, 25, 50 and 100 µL of 10 mM NaCl stock solution was added to have 0.25, 0.5 and 1 µmoles of Na+ in the reaction mixture. Similarly, for 5 and 10 µmoles of Na+, 50 and 100 µL of 100 mM NaCl solution was added, while for 50, 100 and 200 µmoles of Na+, 50, 100 and 200 µL of 1 M NaCl solutions were added. The solutions were then dried in a centrifugal vacuum evaporator (Genevac EZ-2 Elite) at 80°C for 20 hours. The drying time was found to be ca. 2 hours for 1 mL solution, thus the 20 hours of vacuum-drying includes ca. 2 hours of drying followed by ca. 18 hours of incubation in dry state. The dry products were then dissolved in 100 µL of nuclease-free water such that the final cGMP concentration would be 10 mM. All reactions were done in unbuffered solutions unless specific buffer interactions were being studied. This was done to limit the interactions of buffers and other ions apart from the test ion with cGMP. The resulting solutions were solubilized by intermittent heating at 60°C and vigorous vortexing and immediately used (without precipitation) for electrophoresis on a polyacrylamide gel (see below).

2.3. Ethanol precipitation of long oligonucleotides.
To maintain a consistent positive control, the dried form of 3’,5’cGMP-H was dissolved and long oligomers were precipitated using ethanol. To a 10 mM cGMP-H solution (prepared by dissolving dried cGMP-H), 20 µg of glycogen and 500 mM ammonium acetate (1/10 volume, 5 M) were added. To the resulting solution, 3 volumes of cold 100% ethanol were added and incubated at 4°C for 18 hours. Precipitated nucleotides were isolated by centrifugation at 15000 rpm for 30 minutes at 4°C. The supernatant was discarded and the pellets were washed with cold 70% ethanol and centrifuged again at 15000 rpm for 30 minutes at 4°C. The final supernatant was discarded and the pellet containing oligonucleotides was dried. The dried pellets were stored at 4°C and dissolved in nuclease-free water at 1/10 of starting volume prior to use. Samples for LC-MS analysis were also prepared similarly. After the 18h drying step, the samples were re-dissolved to 10 mM concentration and precipitated as described above. The pellets were re-suspended in nuclease-free water prior to injection into the HPLC-MS. Resuspension volumes are given in the main text.
2.4. Polyacrylamide gel electrophoresis

Separation of the oligonucleotides formed in a typical polymerization experiment as described in section 2.2. was performed using polyacrylamide gel electrophoresis. All the required solutions for gel electrophoresis were purchased from Carl Roth. 25-26% polyacrylamide stock solution was prepared by adding 50 wt% of urea to 40% 29:1 acrylamide-bisacrylamide (ROTIPHORESE Gel 40 (29:1)) solution and the required volume of the 10X TBE buffer containing 50 wt% (ROTIPHORESE sequencing gel buffer concentrate) and diluting with 1X TBE buffer (ROTIPHORESE sequencing gel diluent) as required. For making the gel, to 5 mL of this stock solution, 25 µL of 10% ammonium peroxydisulphate (APS) and 2.5 µL of N,N,N',N'-tetramethylethlenediamine (TEMED) were added. The gels were cast using Bio-rad MiniPROTEAN hand-cast systems and were allowed to polymerize. The polymerized gel was then loaded into the electrophoresis chamber containing 1X TBE Buffer (100 mM Tris-Borate pH 8.3 and 2 mM EDTA). The gel was pre-warmed before loading to +45 °C by preheating the buffer and running the empty gel for 1h at 400V. Samples were prepared by adding 1 µL of the test solution to 4 µL of water and 5 µL of 2X Orange G loading dye (0.05 wt% Orange-G, 5 vol% glycerol, and 50 µM EDTA in formamide). The resulting solution was then heated to 80°C for 5 minutes and rapidly cooled to 4 °C before loading on the gel for denaturing the oligonucleotides. For approximate size estimation, a mixture of low range ssRNA ladder (50-1000 nt) (New England BioLabs Inc.) and a microRNA Marker (17, 21, and 25 nt) (New England BioLabs Inc.) was used (see Supporting information Figure 1 for comparison of the electrophoretic profiles of oligoG standards with that of the marker). 10 µL of each prepared sample and the reference ladder was then loaded into the wells of the gel. The loaded material was allowed to settle into the wells at 100V for 10 minutes and then it was electrophoresed at 400V for 40 minutes or till the dry front was out of the gel. The gel was then stained in 1X SYBR-Gold staining solution (prepared by diluting the 10,000X SYBR-Gold stock solution in 1xTBE buffer, pH 7.5) for 5 minutes and rinsed with 1xTBE buffer. The stained gel was visualized in the BIORAD ChemiDoc Gel imaging system at 488nm.

2.5. Ion-Pairing Reverse Phase HPLC-MS

For the detection and quantification of oligonucleotides of <10nt length, Ion-Pairing Reverse Phase HPLC (Agilent 1260 Infinity II LC System) coupled with a time-of-flight MS (6230B Time of Flight LC/MS) was used. The chromatography was conducted on an Agilent AdvanceBio Oligonucleotide 4.6x150 mm column with particle size 2.7 µm. The following eluents were used: bottle A: water with 8 mM triethylamine (TEA) and 200 mM hexafluorosopropanol (HFIP) and bottle B: 50 v% methanol in water with 8 mM TEA and 200 mM HFIP. The HPLC protocol had a flow rate of 1 mL/min and began with an isocratic flow of 1 %B for 2 minutes (column void volume = 1.7 mL) which was followed by a gradient of 1-30 B% in 22.5 minutes, followed by 30-40 B% in 15 minutes. Time-of-flight mass spectrometry was performed in negative ion mode (ion source: Dual AJS ESI; gas temperature: 325 °C, gas flow: 8 L/min; nebulizer: 45 psig; sheath gas temperature: 400 °C; sheath gas flow: 11 L/min, VCap: 3500; nozzle voltage: 2000V; fragmentor: 175; skimmer: 65; OctopoleRFPeak:750). The chromatograms for the oligonucleotides were analyzed using the Agilent MassHunter Qualitative Analysis Navigator. The CSVs of the chromatograms were extracted and the integrated ion counts and concentrations were calculated using a program written in LabView which can be provided upon request.
2.6. Terminal phosphorylation and polyacrylamide-gel electrophoresis of the polymerization products formed in the experiment shown in Figure 3a, main text

Dry products of the polymerization reaction described in Figure 3a, main text were dissolved in 15 µL of H₂O and labeled at the 5’-end with radioactive phosphate [³²P] in a polynucleotide kinase (PNK) reaction: the labeling reaction mixture contained the polymerization products dissolved in 15 µL water, 2.0 µL 10x PNK buffer, 1.0 µL (10 µCi) γ[³²P]-ATP and 1.0 µL (10 units) PNK (NEB, Lithuania). The reaction was carried out at 37 °C for 60 min and terminated by the addition of 180 µL H₂O and 200 µL phenol-chloroform-isoamyl alcohol (25:24:1) mixture. Nucleic acids were then extracted into the water phase by vortexing and centrifugation in an Eppendorf tube (12 000 rpm, 1 min). The upper phase was removed and mixed with chloroform-isoamyl alcohol (24:1) mixture and extracted. Nucleic acids were precipitated with 3 volumes of ice-cold absolute ethanol in the presence of glycogen (1 µg). After incubation at -20 °C for 2 hours, the precipitate was sedimented by centrifugation (12 000 rpm, 5 min). The sediment was washed with absolute ethanol and air-dried.

The radioactively labeled oligomers in the pellet were dissolved in the electrophoretic buffer containing 100% formamide and bromophenol blue and separated on a 15% polyacrylamide [(acrylamide: bisacrylamide = 45:1) supplemented with 50 % (w/v) urea] gel [Hoefer scientific (USA) apparatus]. Size markers were G₉ and G₁₉ RNA oligomers (from Biomers) radioactively labeled with γ[³²P]-ATP as above. The gel running conditions were as follows: 1x TBE electrophoretic buffer, 399V, 55 °C, 35 cm glass length, and a 5-hour run length. The radioactivity was scanned using a Phosphorimager (Typhoon 7300, GE Healthcare, England) and bands were visualized by the ImageQuant (GE Healthcare) software. Exposure times to phosphor screens were typically 15-30 min.

2.7. Scanning electron microscopic (SEM) analysis

The SEM imaging of the dried 3’,5’-cGMP-H and -Na samples were performed in the R&D laboratories of TESCAN Brno, s.r.o., Brno, Czech Republic. The samples were not treated with any conductive coating and were observed in the original state on the microscopic glass. The TESCAN CLARA FEG (field emission gun) SEM in high-vacuum mode with 2kV acceleration voltage, 10 pA beam current and 5 mm working distance was used. Images were acquired by an Everhart-Thornley (secondary electrons) detector, 1 µs/pixel dwell time and stacked from 250 frames using built-in DCFA (drift corrected frame accumulation) algorithms.

3. Theoretical calculations

3.1. Computational details.

Computations were carried out in vacuum using the TPSS density functional[3] combined with the TZVP basis set of atomic orbitals[4,5]. In the calculations, we have used a simplified model of the reaction complex described in Ref. [6]. The simplification is necessary because the reaction is water-assisted and interaction of the added two water molecules with the nucleobase part of the full nucleotide-model used in Ref. [6] would falsify the results and would make the transition state search practically impossible. We are confident that this simplification does not affect the energetic data derived from the calculations to a significant extent. Thus, the computed model consists of (i) a 1,3-propanediol-cyclic phosphate protonated at the =O of the fully protonated neutral phosphate moiety, representing the substrate and (ii) a methanol molecule in the role of the attacking nucleotide, as well as (iii) two catalytic water molecules. The role of these water molecules is to mediate the proton shuffle from the attacking hydroxyl of the nucleophile to the leaving oxygen of the phosphate in a two-
The video in the Supporting information explains the correspondence of the simplified model to the full model consisting of four nucleotides used in Ref. [6].

Wherever it was possible, we used full geometry optimizations (i.e. no geometrical constraints were imposed). Transition state structures were verified with frequency calculations. Free energies of the studied compounds (G) were calculated from the total electronic energy (E_{tot}) and from the thermal and entropic correction terms to the Gibbs free energy (δG) derived from frequency calculations performed in the harmonic approximation at 298 K: \( G = E_{tot} + δG \). All calculations were performed with the Gaussian09 computer code [7].

### 3.2. Cartesian coordinates for the optimized geometries shown in Figure 4, SI.

**methanol + H2O complex, component 1**

- **initial state, E(TPSS/TZVP)=−192.2518759**
  - O: 0.780996, -0.736959, -0.03339
  - C: 1.563024, 0.452800, 0.02826
  - H: 2.607861, 0.143961, 0.12036
  - H: 1.465636, 1.068500, -0.88065
  - H: 1.311294, 1.078539, 0.89998
  - H: -0.158732, -0.474928, -0.10284
  - O: -2.006259, 0.059346, -0.03456
  - H: -2.385379, 0.816419, -0.50652
  - H: -2.416719, 0.071613, 0.84372

**protonated 1,3 propanediol cyclic phosphate, component 2**

- **initial state, E(TPSS/TZVP)=−837.921321731**
  - P: 0.198159, 0.820497, -0.098139
  - O: 1.751774, 0.869156, -0.285917
  - O: -0.436168, 2.124371, 0.611735
  - O: 0.466399, -0.341265, 1.037562
  - O: -0.704050, 0.331895, -1.314944
  - C: -1.981227, -0.389166, -1.048891
  - O: -1.231172, -1.004602, 1.260018
  - H: 1.192593, 0.053985, -2.037278
  - H: -2.701940, 0.352582, -0.690710
  - H: -0.247659, 2.122702, 1.570002
  - H: -2.39983, -0.35520, -0.207480
  - C: -1.783854, -1.523983, 0.058355
  - H: -1.09378, -1.805434, 1.965110
  - H: -1.912842, -0.287644, 1.732527
  - H: -2.755761, -1.999480, 0.121210
  - H: -1.114444, -2.279873, -0.476526
  - H: 3.382148, -1.691653, 0.641278
  - O: 0.303014, -1.354913, -0.198671
  - H: 3.731233, -1.471203, -0.860791

**TS1, E(TPSS/TZVP)=−1030.2008367**

- P: 0.188377, 0.107655, 0.308428
- O: -0.534708, 1.459018, -0.002352
- O: -0.496593, -0.738930, 1.493810
- O: 1.596245, 0.639532, 0.921161
- O: 0.574810, -0.866367, -0.885156
- C: 1.774245, -1.747200, -0.763112
TS2, E(TPSS/TZVP)=−1030.2066703

P  -0.186865  0.030985  0.112405
O  -0.736887  1.385702  -0.362221
O  -0.401253  -0.546668  1.626726
O  1.436228  0.764482  0.593311
O  0.415443  -0.546668  1.626726
C  1.752171  -2.410652  -1.608367
H  1.545234  -2.456848  0.162367
H  0.222959  -0.121172  2.241554
H  0.224358  2.456848  -0.162367
H  0.222959  -0.121172  2.241554

Product, E(TPSS/TZVP)=−1030.229336

P  -0.724319  -0.271694  -0.035526
O  -1.210648  1.180369  -0.475650
O  -0.085431  -0.402944  1.355021
O  2.100756  0.704178  1.258965
O  0.369056  -0.536715  -1.188293
C  1.524435  -1.433644  -1.000894
C  3.107917  -0.205187  0.663553
H  1.584226  -1.984170  -1.940340
H  1.327576  -2.134700  -0.183264
H  1.117436  0.192588  1.389011
H  0.483052  2.498792  -0.584677
C  2.807249  -0.632772  -0.771484
H  4.055401  0.332847  0.721183
H  3.139679  -1.054160  1.349451
H  3.649054  -1.271523  -1.072152
H  2.816091  0.230126  -1.446707
O  -2.021756  -1.187185  -0.196792
C  -2.189236  -2.453492  0.533707
H  -1.689207  -3.250525  -0.020218
H  -1.787867  -2.356736  1.543642
3.3. Calculations on the pH of reaction with Tris Buffer.

As per the protocol described by Costanzo et al. 2012,[8], the cGMP-H is dried and dissolved in Tris-HCl buffer (20 mM, pH 8.5).

Prior to the addition of cGMP-H, at pH 8.5, the buffer contains both Tris base and protonated TrisH$^+$ ion in equilibrium. Considering the pKa of 8.1 of Tris, the initial concentrations (before the addition of cGMP-H) of the TrisH$^+$ ($\text{TrisH}^+$) and Tris-base ($\text{Tris}$) can be calculated using the Henderson-Hasselbalch equation.

\[ \text{pH} = \text{pKa} + \log \left( \frac{\text{Base}}{\text{Acid}} \right) \]

\[ 8.5 = 8.1 + \log \left( \frac{\text{Tris}}{\text{TrisH}^+} \right) \]

\[ \text{Tris} = 10^{0.4}\text{TrisH}^+ \]

\[ \text{As TrisH}^+ + \text{Tris} = 20 \text{ mM} \]

\[ (3.512)\text{TrisH}^+ = 20 \text{ mM} \]

\[ \text{TrisH}^+ = 5.695 \text{ mM and Tris} = 14.305 \text{ mM} \]

Where, Base and Acid are the concentrations of the conjugate acid-base pair in the buffer. TrisH$^+$ is the concentration of the conjugate acid of Tris base and Tris is the concentration of the base in the solution.

The phosphate of cGMP-H has a low pKa ~1, thus it would completely deprotonate at the pH of 8.5. The released protons would neutralize the equivalent amount of the Tris base. As per the protocol,[8] 150 µL of 1 mM cGMP-H was dried and concentrated to 10 mM in the Tris-HCl buffer. Upon neutralizing the Tris base with 10 mM cGMP-H the final concentrations Tris and TrisH$^+$ are:

\[ \text{Tris} = 4.305 \text{ mM and TrisH}^+ = 15.695 \text{ mM} \]

With these concentrations of TrisH$^+$ and Tris base, the pH at the equilibrium can be calculated as follows:

\[ \text{pH} = \text{pKa} + \log \left( \frac{\text{Base}}{\text{Acid}} \right) \]

\[ \text{pH} = 8.1 + \log \left( \frac{4.305}{15.695} \right) \]

\[ \text{pH} = 8.1 + \log(0.274) = 8.1 - 0.562 = 7.538 \]

Thus, the pH of the solution would already be reduced to 7.538 prior to heating and drying. As demonstrated by Reinecke et al.,[9] the pH of Tris buffer is decreased with increasing temperature. Good et al.,[10] report the $\Delta$pKa/°C of Tris as -0.031 1/°C. Thus heating from 20 to 60 °C would further lower the pH from 7.538 to 5.676.
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