Combined role of amphiphiles and clay on nonenzymatic oligomerization reactions of nucleotides

A thesis submitted towards the partial fulfillment of

BS-MS Dual Degree Programme

By

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CERTIFICATE

This is to certify that this dissertation entitled "**Combined role of amphiphiles and clay on nonenzymatic oligomerization reactions of nucleotides**" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by **Rajarajeswari S at Indian Institute of Science Education and Research**, **Pune (IISER Pune)** under the supervision of **Dr. Sudha Rajamani, Asst. Professor, Biology Department** during the academic year 2015-2016.

Dr. Sudha Rajamani

Date: Nov 15th, 2016

DECLARATION

I hereby declare that the matter embodied in the report entitled "**Combined role of amphiphiles and clay on nonenzymatic oligomerization reactions of nucleotides**" are the results of the investigations carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Sudha Rajamani and the same has not been submitted elsewhere for any other degree.

15-11-2016

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Abstract

The “RNA World” hypothesis is aimed at explaining the chemical origins of life on prebiotic Earth. RNA is thought to have performed the dual role of both a catalyst and an information carrier during the early stages of life’s origin on Earth. Before the advent of protein catalysts, RNA would have assisted in its own replication by chemical means to pass on its genetic information. However, the formation of such an RNA oligomer of adequate length is required for it to act as a catalyst. Several studies focusing on chemical RNA polymerization, in simulated prebiotic environments, have been reported. In this context, clay, especially montmorillonite, has been shown to successfully catalyze the oligomerization of activated ribonucleotides that result in long oligomers (up to 40-50 mers). Amphiphilic molecules have also been shown to assist in the oligomerization reactions of non-activated nucleotides by providing favourable microenvironments. Furthermore, montmorillonite has been shown to accelerate the conversion of fatty acid micelles to vesicles while also allowing for adsorption of RNA that can then get encapsulated into these vesicles. In this study, we try to decipher the combined role of amphiphiles and clay, on nonenzymatic oligomerization reactions of nucleotides that might have resulted in oligomers of the RNA World. Role of varying temperatures in these reactions have also been investigated. Different montmorillonite clays have been used to discern their catalytic efficiency. The study indicates that the presence of phospholipid in the reactions along with the clay do not necessarily confer an additive role in the efficiency of clay catalyzed nucleotide oligomerization.

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Introduction

RNA is considered to have existed as the early functional biopolymer on prebiotic Earth and is believed to have played the dual function of acting as a catalyst and also as an information carrier molecule (Gilbert, 1986)(Figure 1). Chemical synthesis of RNA and nonenzymatic replication would have been crucial for the existence of such putative RNA World on prebiotic Earth. The discovery of ribozymes supports this hypothesis. Studies have shown that RNA molecules as small as 5 nucleotides in length could have catalytic activity (Turk et al., 2010). Several nonenzymatic nucleic acid polymerization studies have been reported so far amongst which lipid assisted polymerization, clay catalysis of nucleotide polymerization and polymerization of nucleotides in eutectic phase are discussed here in details.

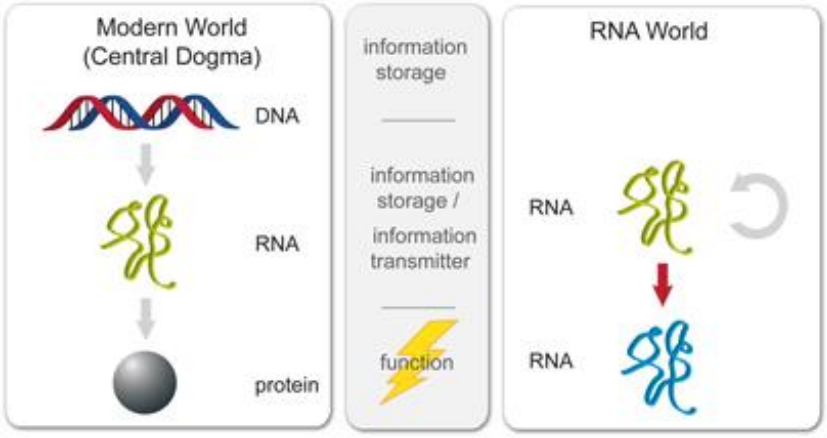


Figure 1: A schematic picture of RNA World

Eutectic phase polymerization of nucleotides:

Addition of salts to water reduces the freezing point of the solution and results in eutectic freezing. As a result, solute molecules get concentrated in one particular space, thereby interact efficiently (Monnard et al., 2003). Eutectic-ice phase facilitates the oligomer formation by concentrating the starting material (Kanavarioti, 2001). Activated nucleotides are nucleotides with a good leaving group attached to phosphate. There are different activating groups available for nucleotide activation. Some of them are shown in Figure 2. Activated nucleotides under eutectic conditions are known to be stable due to the lower hydrolysis rate of the activation group at lower temperature. 3', 5'-phosphodiester bond formation leading to the formation of oligouridylates (5-11 bases long) from activated uridine-5'- phosphorimidazolide monomers has been shown under eutectic conditions (Kanavarioti et al 2001). Formation of RNA oligomers with mixed sequences in presence of catalyst Pb^{2+} and Mg^{2+} under eutectic conditions has also

been reported (Monnard et al., 2003).Template directed polymerization in eutectic phases have been shown using activated nucleotides (Trinks et al., 2005). Uridine monomers elongates using RNA hairpin structure as their templates (Mansy and Szostak, 2009) In addition, Ribozymes have been shown to be functional at subzero temperatures in ice-water matrices (Vlassov et al., 2005).

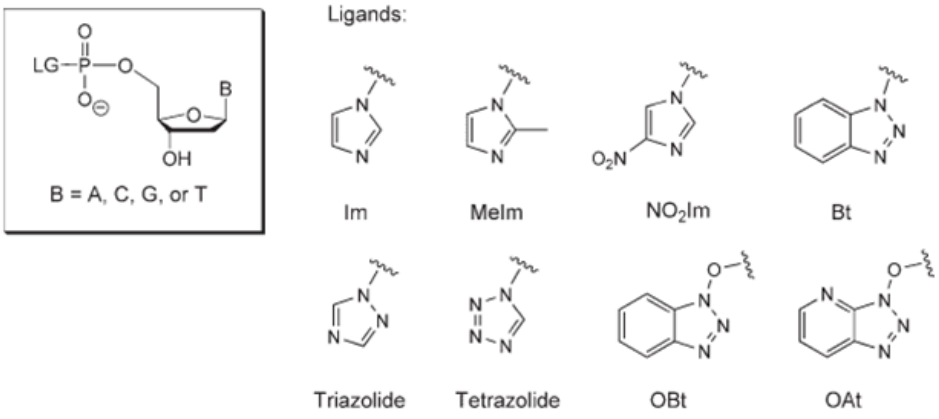


Figure 2: Better leaving groups used for nucleotide activation. Im-Imidazolide, MeIm: 2-methylimidazole, NO_2Im: 4-nitro imidazole, Bt: Benzotriazole, OBt: Oxybenzotriazole, OAt; Oxyazabenzotriazole

(Adapted from Stütz et al, 2007)

Clay Catalysis of nucleotide polymerization:

Montmorillonite (smectite group clay) forms as a result of weathering of volcanic ash. A schematic diagram of the structure of montmorillonite is shown in Figure 2a. Montmorillonite is a sheet of octahedrally coordinated Gibbsite (Al_2 $(OH)_6$) sandwiched between two sheets of tetrahedrally coordinated silicate (SiO_4^{2-}) and is highly efficient in cation exchange. Montmorillonite has been shown to successfully catalyze the oligomerization of activated ribonucleotides that result in longer oligomers (Joshi et al., 2009). Clay catalysis depends on the magnitude of negative charge and the number of interlayer cations present in it. Cation exchange capacity (CEC) of the clays has been exploited to make it catalytic. Montmorillonite needs to be activated prior to catalyzing it (Banin, 1973).The raw montmorillonite has to be treated with dilute HCl to replace the interlayer cations with H^+ and then titration with the desired cation is carried out to make catalytic clay. Non catalytic montmorillonites have higher negative charge mainly because of the natural substitution of tetravalent and trivalent cations (in the octahedral and tetrahedral layers) by divalent and monovalent cations that are of lower valency. The negative charge is then neutralized by the ionic binding of the interlayer cations.

The edge surface of the montmorillonite has an isoelectric point of approximately 7. At pH < 7 the edge surface has positive charge and at pH>7 the edge surface has negative charge. Under acidic conditions, phosphate group of the activated nucleotide bind to the positively charged edge surface of the montmorillonite. However under basic conditions both the planar surface and the edge surfaces of the montmorillonite are negatively charged. Then the phosphate group of the activated nucleotide gets adsorbed to the clay by ligand exchange with the hydroxyl group attached to aluminium ions at the edge surface (Hashizume, 2015).The extent of adsorption on clay is influenced by the acid dissociation constant of the nucleic acid base and the pH of the solution (Hashizume, 2012).

Daily addition of activated nucleotides 5'-phosphorimidazolide of adenine (ImpA) and 5'-phosphorimidazolide of uridine (ImpU) to montmorillonite based reactions yielded longer oligomers of length 30-50 nucleotides (Ferris, 2002). Further, the presence of salts in the reaction mixture has been to shown to help RNA oligomerization (Miyakawa et al., 2006). Smaller cations and anions facilitate the formation of longer RNA oligomers (Joshi and Aldersley, 2013). The protonated or the activated montmorillonite titrated to pH 6-7 acts as a good catalyst for RNA formation whereas those titrated to pH 11 are not catalysts (Joshi et al., 2009). Complete replacement of the protons by the desired cation (saturation of the interlayer) inhibits catalysis. The mechanism of clay catalysis involves general acid/base catalysis and the presence of protons in the interlayer make the imidazole group of the activated nucleotide a better leaving group (Joshi et al., 2012). The proposed mechanism of clay catalysis as shown by Joshi et al 2009 is depicted in Figure 2b. The absence of protons in saturated clay compared with pH 7 montmorillonite, results in the loss of catalytic activity. Further investigation on the mechanism of montmorillonite catalysis has revealed variation in the interlayer spacing upon nucleotide binding (Joshi et al., 2011). Montmorillonite has also been shown to facilitate vesicle formation of fatty acid micelles. Clay along with the adsorbed RNA molecule can then get encapsulated in to vesicles. Such vesicles can grow at the expense of other fatty acids and divide without further dilution. Several cycles of growth and division of these vesicles indicate the role of mineral surfaces in the formation, growth and division of the earliest cell in prebiotic earth (Hanczyc et al., 2003).

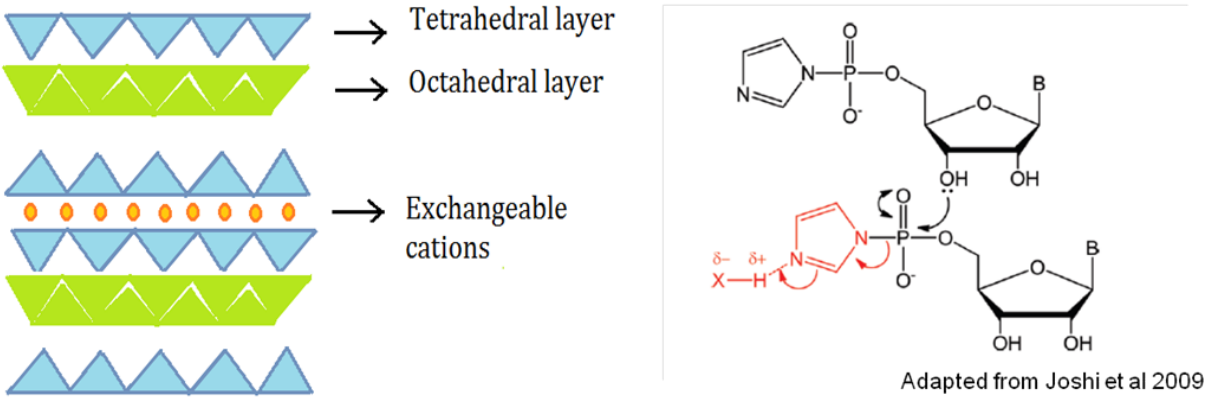


Figure 2a) A Schematic diagram of montmorillonite clay b) Proposed phosphodiester bond formation on montmorillonite, XH: undifferentiated protic species within clay

Lipid assisted polymerization of nucleotides:

Amphiphilic molecules contain both hydrophobic and hydrophilic parts. Biomolecules such as lipids (e.g. fatty acids, phospholipids etc.) are amphiphiles that form membranes in modern cells. Amphiphiles assemble into micelles or vesicles based on the hydrophobicity, pH of the solution and solvent properties. Compared to contemporary cells containing lipids, simpler fatty acid systems are thought to have been present on the primitive Earth and are thought to have formed the membrane-like structures of the early cells (protocells).

Encapsulation of materials by vesicles helps them to get concentrated and thus to react. A pioneering study by Mansy et al. has demonstrated template-directed primer extension, encapsulated inside fatty acid vesicles (Mansy et al., 2008). Amphiphiles have also been shown to assist in oligomerization process. Studies on dehydration – rehydration cycles of nucleotides in presence of phospholipid molecules at low pH resulted in RNA like oligomers (Rajamani et al., 2008). On dehydration, lipid molecules form liquid crystalline multilamellar matrix (Figure 3) that effectively arranges the solute molecules between the lamellae (Deamer, 2012), thus aiding in the polymerization. Formation of RNA like oligomers with abasic sites through lipid assisted nonenzymatic synthesis has also been recently reported from our lab (Mungi and Rajamani, 2015).

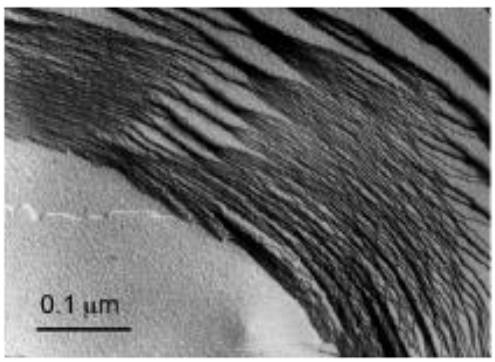


Figure 3: Liquid crystalline multilamellar matrix formed by the lipids. Each line in the image represents a lipid bilayer of 5nm thickness (Adapted from Deamer, 2012)

Figure 4: Modified Joshi et al reaction

Scheme depicting oligomerization of ImpA in presence of montmorillonite (catalyst) and POPC vesicles (potential micro environment provider and a potential catalyst)

In this study we are interested in delineating the combined role, if any, of lipids along with clay in nonenzymatic oligomerization of nucleotides in presence of salts. Nucleotides activated by imidazole have been used for our study, like in several previous studies that have looked at nonenzymatic reaction (Ferris. 2002; Joshi et al., 2009) This is useful as the activating group is a good leaving group, thus facilitating oligomerization of the nucleotides in detectable yields. Initial characterization has been done using ImpG nucleotides as it tends to stack better than the pyrimidines which might be helpful in enhancing the inherently slow rate of nonenzymatic polymerization reactions. Lithium Li^{+}–montmorillonite (clay) (Joshi and Aldersley, 2013) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), as lipid are used in our study to understand their combined role on RNA oligomerization. To start with, we have modified Joshi et al's reaction as shown in Figure 4, to carry out our study. The reaction time has been varied from 3 days to 1 month. Reactions under different temperature also have been investigated, to understand the role of temperature in this scenario. Additionaly, preliminary investigations on catalytic efficiency of different clays on oligomerization reactions has also been carried out.

Materials:

Guanosine disodium hydrate (GMPdss.H_2O), Adenosine mono phosphate (AMP), Uridine mono phosphate (UMP), Cytidine mono phosphate(CMP), Trizma base, Hydrochloric acid (HCl), Sodium Chloride (NaCl), Sodium perchlorate ($NaClO_4$), Butanol, Hexane, Acetonitrile(ACN), Lithium hydroxide (LiOH), Lithium Chloride (LiCl), Montmorillonite K-10 , Dowex Monosphere 550 A Hydroxide were purchased from Sigma Aldrich.1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine(POPC) was purchased from Avanti Polar Lipids. Guanosine 5ˈ-phosphorimidazolide (ImpG), Uridine 5ˈ-phosphorimidazolide (ImpU) was purchased from GLSynthesis Inc. Worcester. Dimer (5ˈP-AA) and Trimer (5ˈP-AAA) were purchased from Dharmacon part of GE Healthcare and used without further purification. Volclay, C-bed clay and F-bed clay were gifts from Dr. Prakash C Joshi of RPI, New York.

Methods:

1. **Activation of montmorillonite:** Montmorillonite K 10 from Sigma (12gm) was treated with 0.5 M HCl (50 ml) by continuously stirring at 4°C for 30 minutes. Subsequently, centrifugation was carried out at 3500 rpm at 4°C to remove the excess acid present in the falcon. The excess acid was discarded after pH analysis. This HCl treatment was repeated 3 more times. The H^+-montmorillonite from this stage was then washed with deionized water in order to remove the chloride ions. The supernatant collected after each round of centrifugation was discarded after pH analysis. The same procedure (of treatment with deionized water) was repeated four times. In order to remove excess of Cl^- , 1000 mL of deionized water was added to H^+-montmorillonite slurry and treated with 45 mL wet anion exchange resin (Dowex Monosphere 550 A Hydroxide form) for about 2 hrs with continuous stirring. Lyophilization of the resultant H^+-montmorillonite which was at pH 3 was carried out for 24 hours, to get the activated montmorillonite as a dry powder.

2. **Preparation of catalytic montmorillonite:** The activated H^+-montmorillonite was converted into “catalytic” lithium montmorillonite (Li^+ -M) by titrating it with LiOH. Typically, 100 ml of MilliQ was added to 1 gm of the activated clay. Titration was carried out until the pH of the solution became pH 7. The catalytic, Lithium montmorillonite is referred to as montmorillonite/clay, throughout this study, unless mentioned otherwise.

3. **Reactions using Li^+-montmorillonite and POPC:** To start with, the ratio of POPC to activated nucleotide was maintained as 1:12 in the reaction and the total volume of the reaction was varied from 100 µl to 500 µl. The reactions were allowed to proceed at 24° C, 4° C and -30° C. The time duration of each of the reaction set, were varied from 3

days to 1 month and the time points were taken for further analysis. The details of the various reactions carried out are mentioned below.

Reaction Conditions:

1) Room temperature (RT) reactions of ImpG:

ImpG reactions under RT were set up as per the details from table 1. Reaction volume was 200 µl and the time points were taken after 0 hr, 1 day, 2 days, 3days and analyzed.

Table1: ImpG Reaction carried out in presence of LiCl at RT

Reactions	**Clay (Li^+-montmorillonite, pH =7)**	**POPC (30 mM)**	**ImpG in 1 M LiCl solution (100 mM, pH=7)**	**LiCl solution(1 M, pH=7)**
ImpG	-	-	24 µl	176 µl
ImpG +POPC	-	6.63 µl	24 µl	169.3 µl
ImpG+ Clay	10 mg	-	24 µl	176 µl
ImpG+ Clay+ POPC	10 mg	6.63 µl	24 µl	169.3 µl

2) Stability reactions of ImpG and ImpU:

ImpG and ImpU nucleotides (60mM) were incubated in 1M LiCl solution (pH =7),and followed up to 4 days, at RT.

3) Long term Reactions of Activated nucleotides:

Reactions of imidazole activated nucleotides were carried out at two different temperatures: 4˚C and -30˚ C and followed up to 30 days. A second set of the same reaction with slight modification was also carried out. The details of the reaction are given in table 2.Reaction volume was 100 µl and the time points were taken after 0 hr, 1 day,5days,10 days and 15 days and analyzed.

Similar reactions were carried out with ImpA and ImpC, however due to technical difficulties with HPLC we could not analyze the time points

Table 2: ImpN (ImpG/ImpU) long term Reaction carried out in presence of LiCl at -30˚ C and 4˚C

Reactions	Clay (Li^+-montmorillonite, pH =7)	POPC (30 mM)	ImpN in 1 M LiCl solution (100 mM, pH=7)	LiCl solution (1 M, pH =7)
ImpN	-	-	12 µl	88 µl
ImpN +POPC	-	3.3 µl	12 µl	84.7 µl
ImpN + Clay	5 mg	-	12 µl	88 µl
ImpN + Clay+ POPC	5 mg	3.3 µl	12 µl	84.7 µl

4) Extractions:

4a) **Extraction of lipids:** The time points of the reactions were subjected to butanol-hexane extraction in order to remove the lipids. In every case, butanol extraction was carried out twice followed by one hexane extraction. The ratio of butanol and hexane used in the extraction was 1:1:0.5 with respect to the sample volume. The samples were then subjected to drying, in a vacuum centrifuge to remove any remaining hexane from the sample. Finally, the aqueous phase was collected and analyzed by High Performance Liquid Chromatography (HPLC) to characterize the products formed in the reactions.

4b) **Extraction of clay:** 0.1 M NaCl in 30% ACN is used to extract reaction products that might be attached to the clay. The clay is dissolved in this solution and kept at 4˚C for (2 hrs x 2, overnight x 1, 2 hrs x1). Extraction of samples containing only POPC was carried out using butanol-hexane procedure (refer 4a). Samples with only clay were extracted using 0.1M NaCl in 30% ACN. While the samples with both POPC and clay were first processed using butanol-hexane treatment to remove the lipid and then were subjected to extraction with 0.1 M NaCl in 30% ACN.

5) High Performance Liquid Chromatography (HPLC) Analysis: DNAPac PA200 column (anion exchange column) from Dionex was used for analysis of the aqueous phase samples of all reactions to identify the formation of oligomers in the same. 2mM Tris (pH 8) (Solvent A) and 0.4M $NaClO_4$ in 2mM Tris (Solvent B) were used as HPLC solvents. A gradient of 40 % B was used for 25 minutes. DNAPac PA200 column offers single nucleotide resolution. In our case, dimer and higher oligomers elute after the monomer as their retention is greater than that of a monomer.

5a) **Details of the HPLC columns used**: Different columns were used for aqueous phase analysis and peak collection of the sample.

- DNAPac PA 200: analytical column with dimensions (4*250mm),
- DNAPac PA 200: Semi-Prep column (9*250mm),
- C18 Semi-Prep (Eclipse XDB-C18, 9.4*250mm) (Solvents: 25mM TEAA, 70% ACN)

Gradients used for each column are mentioned in table 3.

Table 3: Gradients used for a) DNAPac Analytical column b) DNAPac Semi-Prep column c) C18 Semi-Prep column

a) DNAPac Analytical gradient, flow rate: (0.8 ml/min)

Time (min)	%A	%B
0.00	100.0	0.0
3.60	100.0	0.0
15.60	93.0	7.0
20.60	87.0	13.0
23.00	75.0	25.0
25.40	60.0	40.0
27.80	0.0	100.0
35.00	0.0	100.0
37.40	100.0	0.0
42.20	100.0	0.0

b) DNAPac Semi-Prep gradient (flow rate: 3ml/min)

Time (min)	%A	%B
0.00	100.0	0.0
5.00	100.0	0.0
17.00	60.0	40.0
20.00	0.0	100.0
25.00	0.0.	100.0
28.00	100.0	0.0
33.00	100.0	0.0

c) C18 Semi-Prep Gradient (Flow rate: 3 ml/min)

Time (min)	% A	%B

0.00	100.0	0.0
7.00	100.0	0.0
17.00	50.0	50.0
20.00	0.0	100.0
25.00	0.0	100.0
28.00	100.0	0.0
33.00	100.0	0.0

6) **High Resolution Mass Spectrometry (HRMS):** This is a high resolution mass spectrometric technique. The column used is ACQUITY UPLC BEH C18 Column, 1.7 µm (2.1 mm X 50 mm). The temperature of the column is maintained at 30˚ C. The solvents are 0.1% Formic Acid in water (Solvent A) and Acetonitrile (Solvent B). 5ul of the sample was used for HRMS analysis. HRMS gradient is shown in table 4

Table 4: Gradient used for HRMS analysis:

Time	Flow ml/min	% A	% B
0	0.4	90	10
0.4	0.4	70	30
2	0.4	20	80
3.2	0.4	10	90
3.5	0.4	90	10
5	0.4	90	10

7) **Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS or HPLC-MS):** This includes two techniques, the physical separation of the compounds (by HPLC) and subsequent mass analysis (by Mass Spectrometer). C18 column is used here. As the material pass through the column, the solvent is removed and the material is ionized using electrospray ionization (ESI) technique. HPLC helps to differentiate the isomers present in the sample. The solvent system used was 70% Acetonitrile and MilliQ with 0.1% TFA.

LC-ESI-MS analysis for the samples was carried out in collaboration with Dr. Ajeet Singh at CAMS Venture Centre at NCL Innovation Park.1260 Infinity Nanoflow

LC was used for high resolution separation and 6540 UHD Accurate Mass QTOF was used for mass spectrometry.

RESULTS AND DISCUSSION

Control runs: HPLC was used as a preliminary analytical technique to monitor the reaction progress. Towards this extent, anion exchange column was used. The elution times of the nucleotides differ based on their charges. To start with, monomer controls were loaded on HPLC column to find out the elution times and thus to compare it with the reactions done. It was observed that the ImpG elutes faster as compared to GMP. Spiking experiments, of activated nucleotide sample, spiked with the corresponding non-activated nucleotide followed by analysis on HPLC column, were carried out. The chromatograms of these spiked controls are shown in Figure 5a and 5b. Dimer and Trimer of Adenosine nucleotide were also analyzed by HPLC (Figure 6) to get a general idea of elution times for dimer and trimer species. In principle GG dimer and GGG trimer should also elute in a similar manner, as the separation is based on charges.

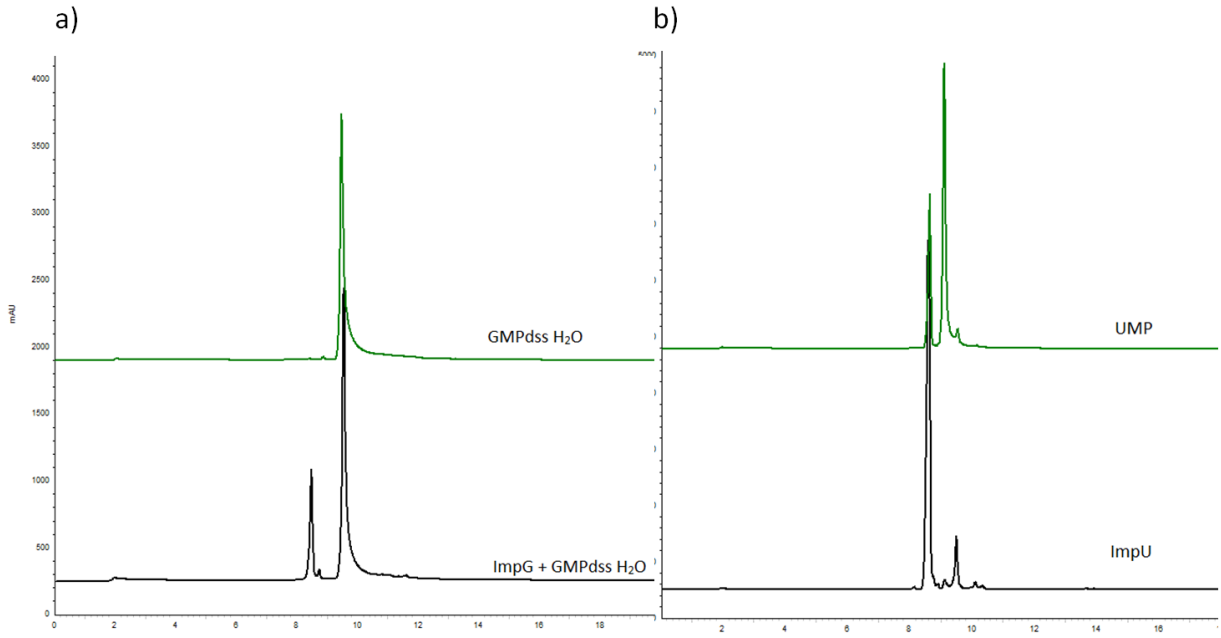


Figure 5a) HPLC chromatogram of GMPdss sample and the same spiked with ImpG sample.
b) HPLC chromatogram of ImpU and the same spiked with UMP sample. A gradient of 40 % B was used for 25 minutes (refer Table 3a).

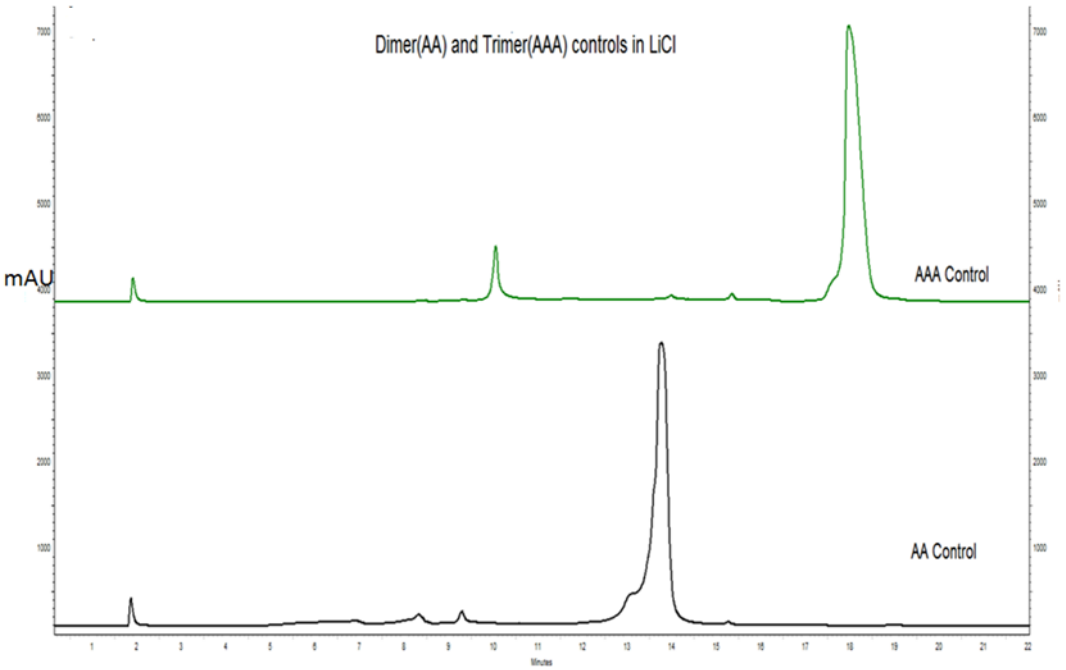


Figure 6: HPLC chromatogram of Dimer (5'P-AA) and Trimer (5'P-AAA) controls. The elution time of dimer is in between 13-14 minutes and the trimer elutes at 17.5 -19 minutes whereas the monomer AMP elutes at 9.4 minutes. A gradient of 40 % B was used for 25 minutes (refer Table 3a).

1) Stability of Activated Nucleotides in 1M LiCl (pH=7) solution

Previous studies have shown oligomerization of nucleotides in presence of salts (Joshi and Aldersley, 2013). However, to our knowledge the stability of the activated nucleotides in salt solution has not been reported to date. In order to ascertain the stability of activated nucleotides in salt solution, ImpG and ImpU were incubated in 1M LiCl solution (pH=7.0) at 24° C in a thermomixer and followed up to 4 days. The molarity of the activated nucleotide in the reaction mixture was 60mM. The time points of the reactions were immediately loaded on HPLC column (DNAPac analytical) and analyzed to estimate the half life of the activated nucleotides in above mentioned conditions. A representative chromatogram of ImpG and ImpU in 1 M LiCl along with solvent B ratio is shown in Figure 7a. Graphical representation of area under each of the peaks (as indicated in the chromatograms) for ImpG and ImpU reactions in LiCl are depicted in Figure 7b and 7c.

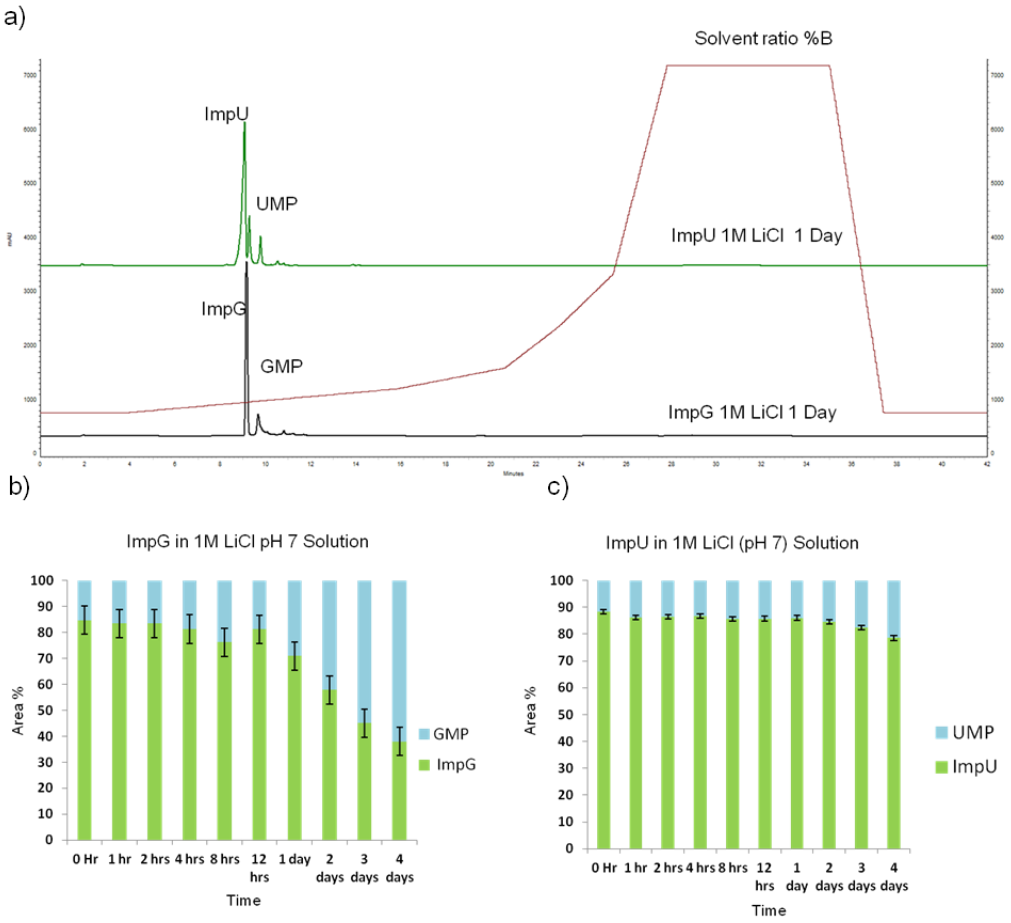


Figure 7a) Representative chromatogram of the 24 hour time point of ImpG and ImpU in 1 M LiCl along with solvent B ratio. Graphical representation of area percent of b) ImpG and GMP peaks c) ImpU and UMP peaks at different time points taken from 0 hour to 4 days (n=2).

It was observed that, in the presence of salt (1M LiCl), both the nucleotides tend to have small breakdown peak, from very early on. The half life of the activated nucleotide ImpG in presence of 1M LiCl was found to be in between 1 to 2 days at room temperature (RT). However, on the other hand, the half life of ImpU was found to be greater than 4 days under similar conditions. This assures availability of sufficient amounts of intact ImpU for polymerization reactions, over longer duration.

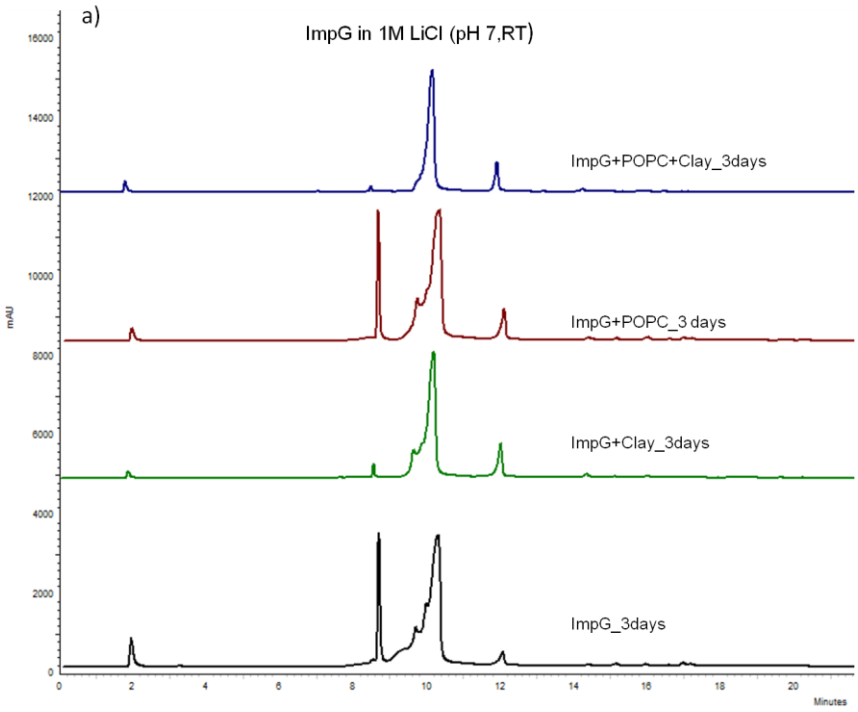
2) Role of temperature in nonenzymatic oligomerization of activated nucleotides

Table 5: Reactions carried out at different temperatures with various clays

No:	REACTION	REACTION VOLUME	TEMPERATURE	REACTION TIME
1	ImpG	200 µl	RT	3 Days
2	ImpG + POPC	200 µl	RT	3 Days
3	ImpG + Clay	200 µl	RT	3 Days
4	ImpG + POPC + Clay	200 µl	RT	3 Days
5	ImpG	500 µl	4˚C and -30˚C	30 Days
6	ImpG + POPC	500 µl	4˚C and -30˚C	30 Days
7	ImpG + Clay	500 µl	4˚C and -30˚C	30 Days
8	ImpG + POPC + Clay	500 µl	4˚C and -30˚C	30 Days
9	ImpU	500 µl	4˚C and -30˚C	30 Days
10	ImpU + POPC	500 µl	4˚C and -30˚C	30 Days
11	ImpU + Clay	500 µl	4˚C and -30˚C	30 Days
12	ImpU + POPC + Clay	500 µl	4˚C and -30˚C	30 Days
13	ImpG	100 µl	-30˚C	30 Days
14	ImpG + POPC	100 µl	-30˚C	30 Days
15	ImpG + Clay	100 µl	-30˚C	30 Days
16	ImpG + POPC + Clay	100 µl	-30˚C	30 Days
17	ImpU	100 µl	-30˚C	30 Days
18	ImpU + POPC	100 µl	-30˚C	30 Days
19	ImpU + Clay	100 µl	-30˚C	30 Days
20	ImpU + POPC + Clay	100 µl	-30˚C	30 Days
21	ImpG	100 µl	RT	3 Days
22	ImpG + POPC	100 µl	RT	3 Days
23	ImpG + C-bed	100 µl	RT	3 Days
24	ImpG + POPC + C-bed	100 µl	RT	3 Days

25	ImpG	100 µl	RT	3 Days
26	ImpG + POPC	100 µl	RT	3 Days
27	ImpG + F-bed	100 µl	RT	3 Days
28	ImpG + POPC + F-bed	100 µl	RT	3 Days
29	ImpG	100 µl	RT and -30˚C	5 Days
30	ImpG + POPC	100 µl	RT and -30˚C	5 Days
31	ImpG + Clay	100 µl	RT and -30˚C	5 Days
32	ImpG + POPC + Clay	100 µl	RT and -30˚C	5 Days
33	ImpG	100 µl	RT and -30˚C	5 Days
34	ImpG + POPC	100 µl	RT and -30˚C	5 Days
35	ImpG + Volclay	100 µl	RT and -30˚C	5 Days
36	ImpG + POPC + Volclay	100 µl	RT and -30˚C	5 Days

Polymerization of phosphorimidazolide activated nucleotides in eutectic ice–water phase in presence of salts such as Pb^{2+}, Mg^{2+} has been reported by (Monnard et al., 2003). The temperature in the reported study was -18˚C. Phosphodiester bond formation, which is the condensation reaction, is not favoured in aqueous solutions due to presence of bulk water phase. However in the eutectic phase, the monomers get concentrated in the brine channels. The hydrolysis rate of the activated nucleotides is also slowed down at lower temperature. This enables them to interact and facilitates the phosphodiester bond formation. Polymerization of activated nucleotides in presence of clay has only been reported at RT thus far (Joshi et al., 2007). We were interested to understand the role of temperature on the nonenzymatic oligomerization reaction of activated nucleotides in the presence of clay as well as lipid. Reactions were carried out at three different temperatures: 24˚C (RT), 4˚C and -30 ˚C (refer Table 5). Considering the higher rate of breakdown of the activated nucleotide at RT, the room temperature reactions were followed only up to 3 days (72 hrs), whereas the lower temperature reactions were followed for a longer duration (1 Month). A representative chromatogram is shown in Figure 8a. Graphical representation of the area percent of the peaks (as seen in chromatograms) for the different temperature is depicted in Figure 8b, 8c, 8d.



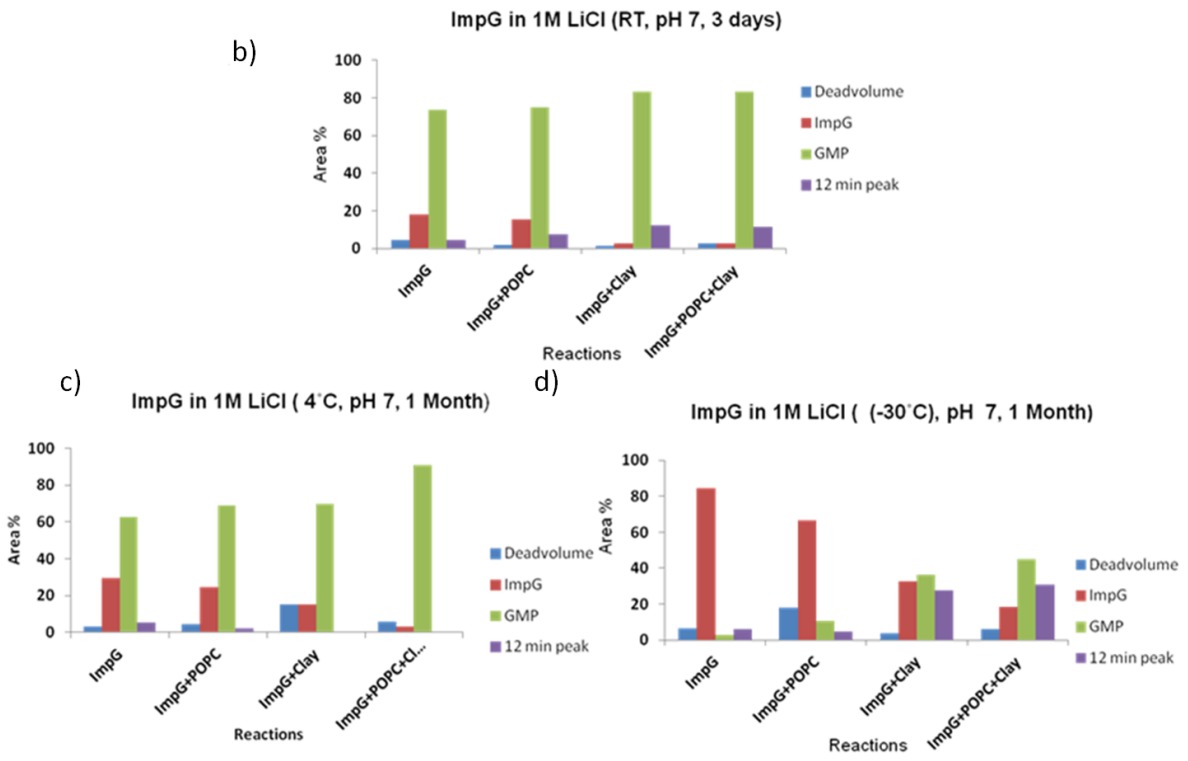


Figure 8: a) Representative chromatogram of ImpG reaction in 1M LiCl, under RT (3 days). Area analysis of the ImpG reaction in 1M LiCl solution (pH 7) at different temperature b) 3 days time point at RT. c) 1 Month time point at 4˚ C. d) 1 Month time point at -30˚ C.

It was observed that, considerable amounts of ImpG stays intact even after 30 days at -30˚ C, increasing the possibility of formation of oligomers at this temperature. Reactions at 4˚C also show the presence of ImpG at the end of 30 days. ImpG at RT is mostly converted to GMP within 3 days of the reaction. A peak at 12 minutes increased over time in -30˚ C reactions. Though the elution time is different from that of the linear dimer, we suspect this peak to potentially be a cyclic dimer, as dimers with different linkages, are known to have different retention times on the DNAPac PA200 column. 12 minute peak was also found to be present in RT reactions and increased over time. Mass characterization of this peak is necessary to identify the chemical nature of the species. Importantly, presence of the 12 minute peak was not significant in the reactions carried out at 4˚C even after 1 month.

The chromatograms in the figure are obtained from the aqueous phase of the reaction sample. For reactions involving clay, it is required to extract the molecules bound to the clay surface, using a specified protocol (mentioned in Materials and Methods, refer section 4b). Extractions were done for both ImpG and ImpU reactions. The samples were analyzed on HPLC column. A representative HPLC chromatogram of the extracted phase samples is shown (Figure 9). Graphical representation of the area under each peak for the extracted phase samples of these reactions at both 4 ˚C and -30 ˚C are shown in Figure 10.

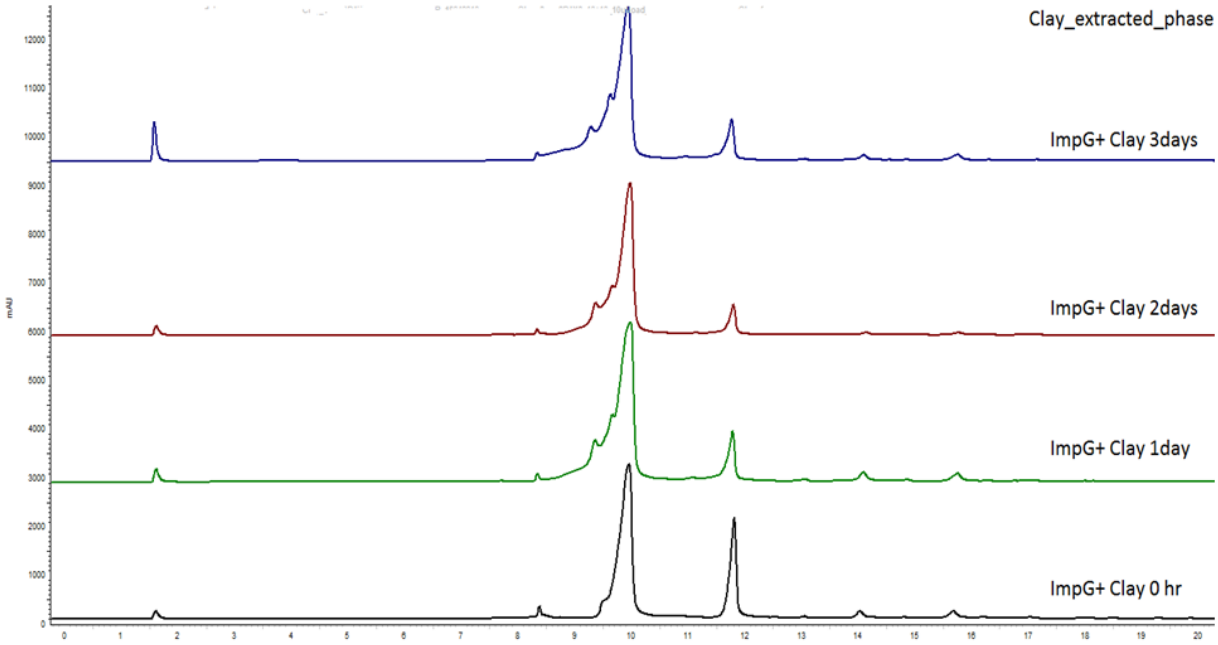
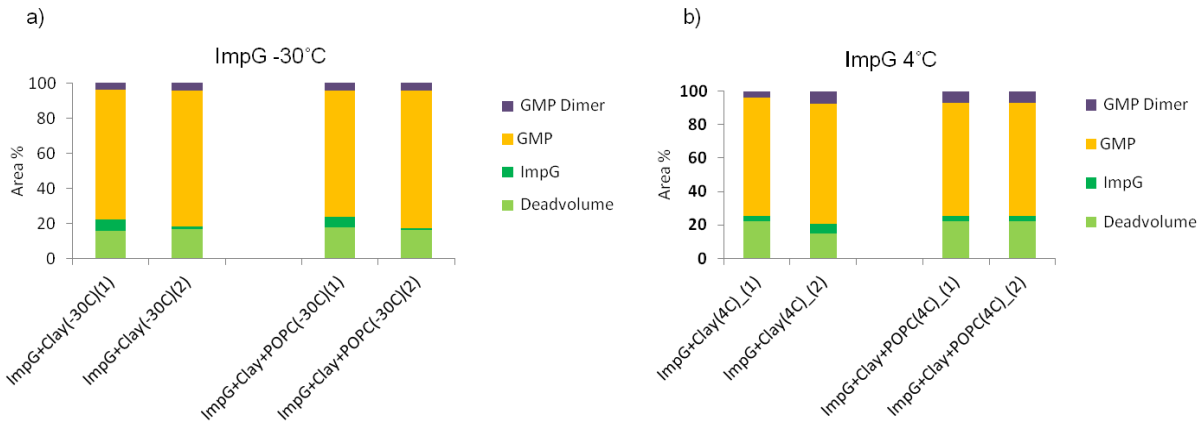


Figure 9: A representative HPLC chromatogram of the clay extracted phase sample of the reaction ImpG along with montmorillonite, at RT over different time points.



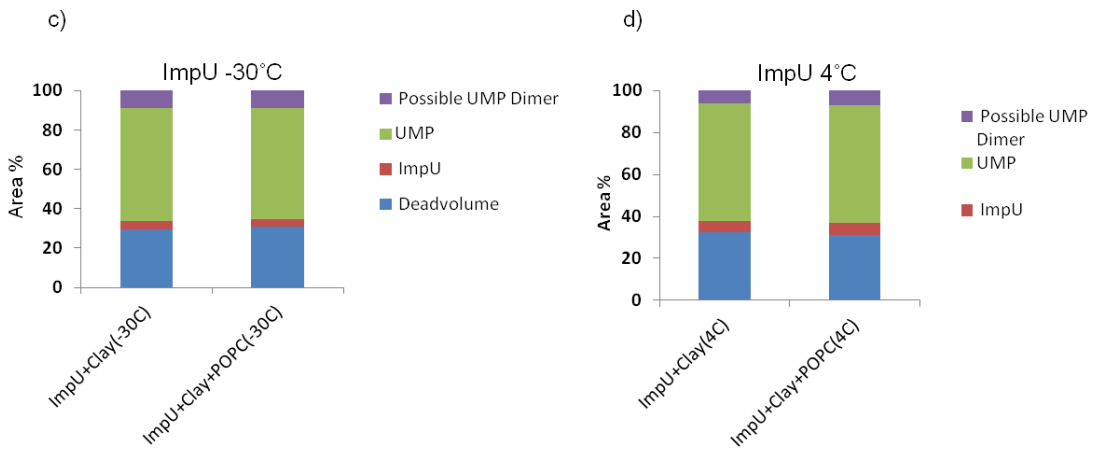


Figure 10: Graphical representation of area analysis of extracted phase samples from clay in a) ImpG reaction at -30˚C b) ImpG reactions at 4˚C c) ImpU reaction at -30˚C d) ImpU reaction at 4˚C.

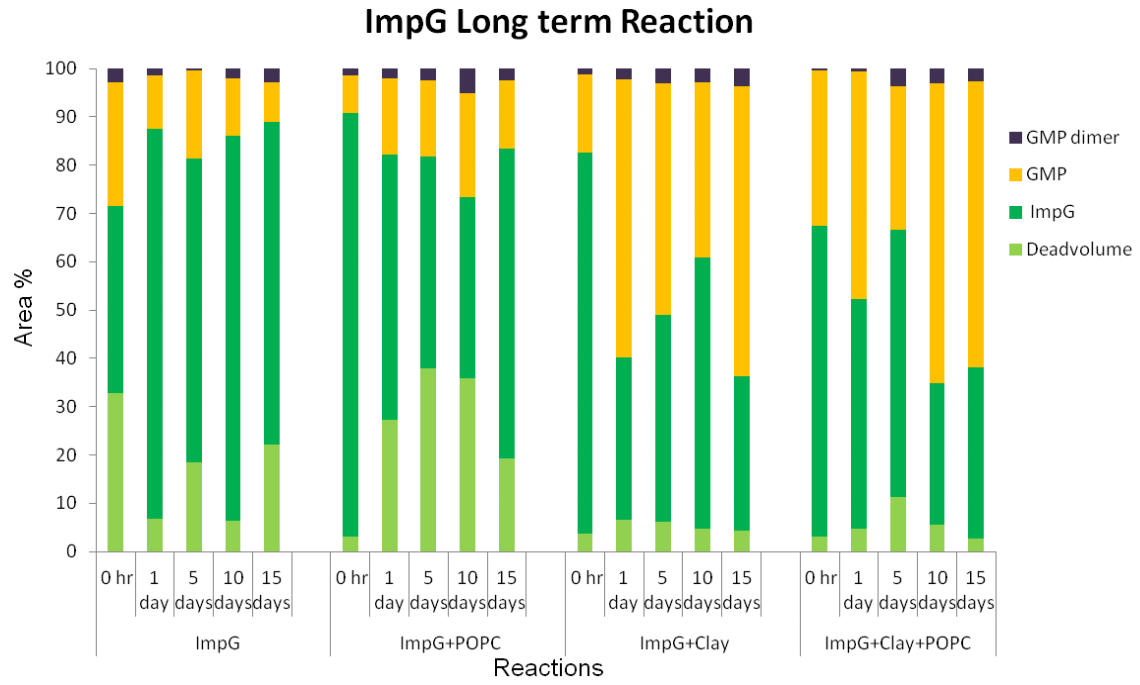
It is evident that monomers are bound to the clay surface. Overall the major species present in the clay extracted phase samples is the non-activated nucleotides (GMP / UMP). 12 minute peak along with certain higher oligomers seems to reduce over time as per the chromatogram of extracted phase sample. Activated nucleotides are also observed in traces. Dimer (which was characterized by LC-ESI-MS, will be discussed later) are observed in all the clay extracted phase samples at both 4 ˚C and -30˚C. This suggests that clay adsorbs monomers; which potentially help them to interact with each other to form oligomers. In addition, no significant difference was observed when lipid was present along with clay in the reaction mixtures.

Since -30˚C reactions favoured 12 minute peak formation along with the least breakdown rate of the activated monomers over long duration of time; we decided to focus at this temperature for further characterization of the oligomerization reactions.

The second set of long term (30 days) -30˚C reaction for ImpG and ImpU is ongoing. The time points up to 15 days of these long term reactions have been analyzed. Area analysis of the respective chromatograms has been carried out to analyze the products from the reactions mentioned. Figure 11 shows the graphical representation of the area percent of the compounds present in the samples over a time duration of 15 days.

The -30˚C reaction of both ImpG and ImpU have a dead volume peak from 0 hour time point and it seems to vary over the various reaction time points. The reaction conditions in these reactions are vastly different from those that are known to result in loss of nitrogenous base, which results in a dead volume peak. Given this we suspect in -30˚C reaction, the dead volume peak could be nucleotides which are unable to interact with the column due to presence of high amounts of salts.

a) ImpG long term reaction -30˚C



b) ImpU long term reaction -30˚C

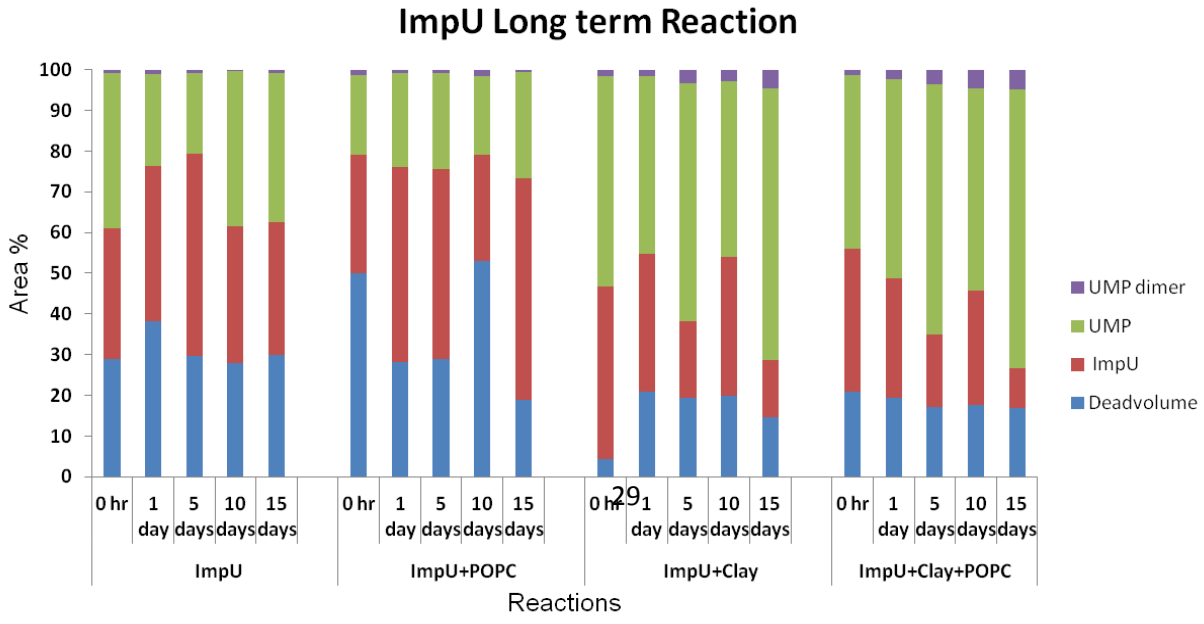


Figure 11: Long term reactions of a) ImpG reaction set at -30˚C (n=1) b) ImpU reaction set at -30˚C (n=1). X axis shows the different types of reactions with different time points analyzed and along the Y axis is the area percent of the compounds present in the aqueous phase of the reaction

Similar to RT reaction -30˚C reaction of ImpG and ImpU also have a dead volume peak. As alluded to earlier this could be coming from nucleotides not interacting with the column matrix. As seen in Figure 11 this varies from time to time. It seems to vary over the various reaction time points. However we have observed that the dead volume peak is less when the reaction mixture contained montmorillonite. This observation indicates the adsorption capacity of the clay. In both reaction sets, ImpG and ImpU, it was observed that the HPLC chromatograms of the time points of the reactions with clay and that of the reactions containing clay along with lipid appeared to be similar. This suggests that the presence of lipid (POPC) does not necessarily confer any additive role to the nucleotide oligomerization in presence of clay. Interestingly, analysis of only aqueous phase samples, indicate presence of high amounts of GMP and UMP in reactions involving montmorillonite (yellow and light green bars in Figure 11a and 11b respectively). It is potentially indicative of high hydrolysis rate of activated nucleotides in presence of montmorillonite. Alternatively this also could result from differential adsorption of activated and non-activated nucleotides by montmorillonite. The area percent shown here is from the HPLC chromatograms of the aqueous phase samples. The clay samples are yet to be extracted from the aforementioned reactions involving clay to separate the molecules that are bound to the clay surface via their negative charges. Analysis of the molecules bound to the clay surface is ongoing.

3a) Mass characterization of Long term Reaction at -30˚ C containing clay

HRMS was used as the analytical method. First, the control dimer sample (5'P-AA) was analyzed on HRMS and the corresponding spectrum is shown in Figure 12. However satisfactory results were not obtained for the reaction time points primarily due to the problems in ionization of sample. One of the main reasons for this could be the presence of high amounts of salts in the samples. Steps such as 1) Lyophilization of the samples, to concentrate them 2) Passing the samples through a C18 column to remove salts were taken to resolve this problem. Unfortunately, despite this HRMS analysis of the sample using in-house facility has been largely unsuccessful.

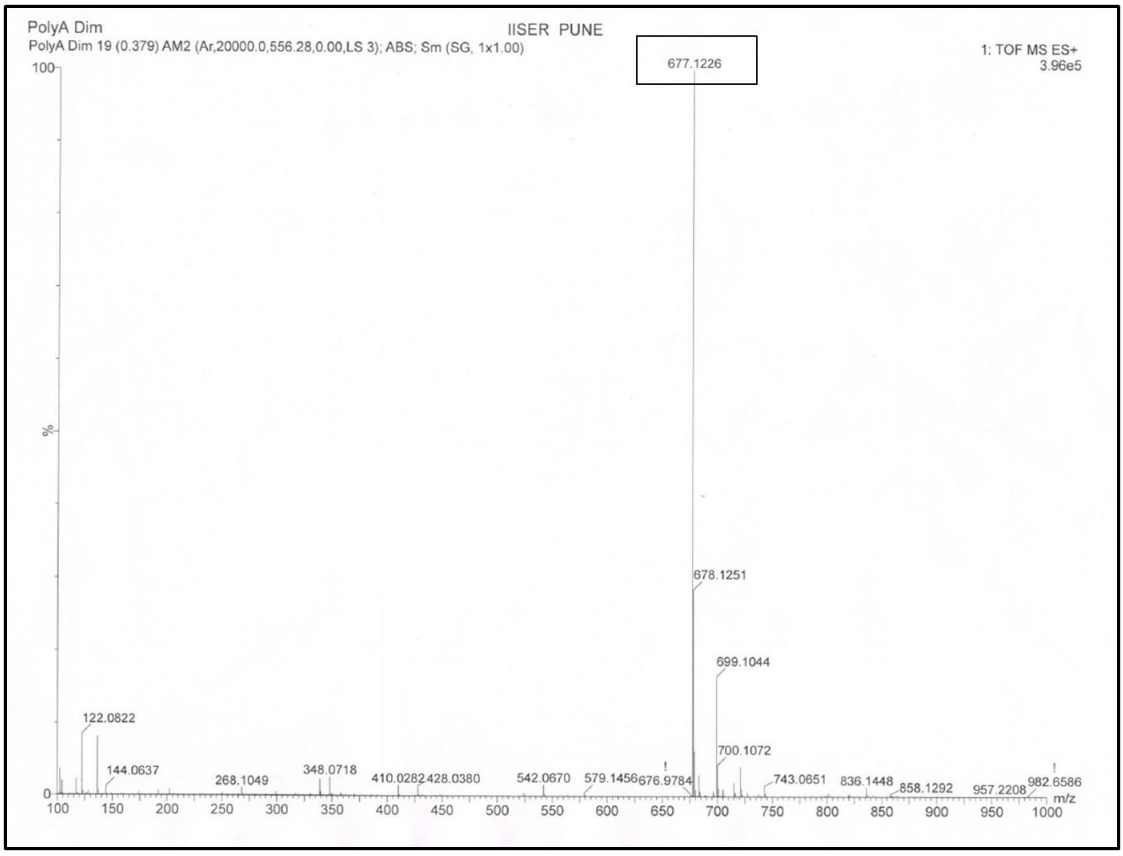


Figure 12: HRMS Spectra of Poly A Dimer (5'P-AA) control. The expected mass of AA dimer in HRMS is 677.1228 and the observed mass is 677.1226

Due to the aforementioned problems, we went on to collaborate with Dr. Ajeet Singh at NCL Innovation Park for mass characterization using ESI-LC-MS

From the -30˚ C ImpG reaction set, 27 day old ImpG + montmorillonite reaction was selected for mass analysis. The peaks were collected from multiple sample injections on DNAPac Semi-Prep Column. The chromatogram obtained in DNAPac Semi-Prep column for the above mentioned sample is shown in Figure 13**.** The regions indicated using red lines corresponds to the different peaks collected from the sample during each HPLC run. Subsequently each of those samples were lyophilized and re-dissolved in a lower volume of buffer (2mM Tris). Importantly, the samples were subsequently passed through C18 column multiple times to remove salts. The samples thus collected were lyophilized again prior to submitting for LC-ESI-MS mass analysis.

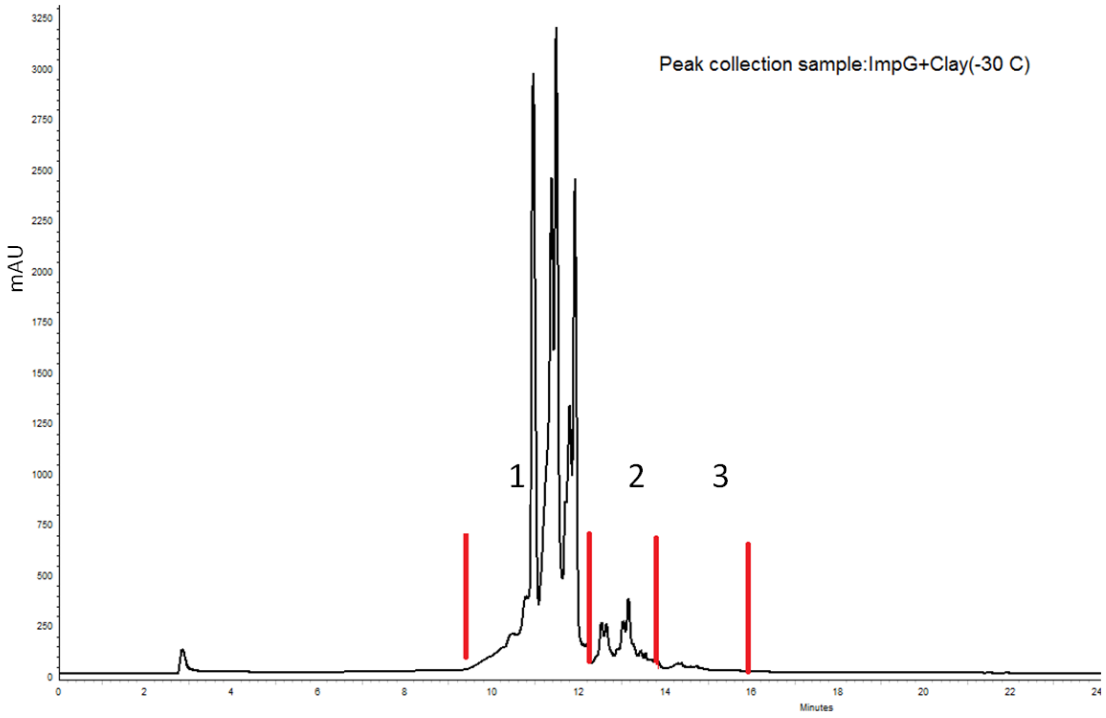


Figure 13: The HPLC chromatogram of the sample: ImpG + montmorillonite -30˚C (27 days) as appeared in DNAPac Semi-Prep column. The red lines indicate the peak/ peak clusters collected separately for mass analysis

3b) Results from LC-ESI-MS Analysis:

a) Peak 1: 9.5-12 minute's peak

Table 6: Species detected in the peak 1 sample with mass error

Target Name	Target Score	Reference mass	Observed mass	Mass error (ppm)
GMP	96.21	363.058	363.0558	-6.09
GMP Dimer	96.38	708.1054	708.1039	2.1
GMP Cyclic	71.54	345.0474	345.0487	3.74

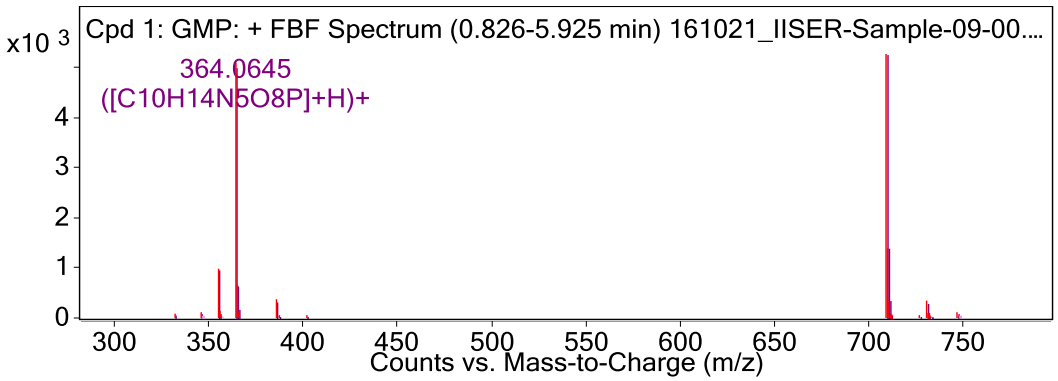


Figure 14: MS zoomed spectrum of sample collected from Peak 1. X axis shows the m/z ratio whereas y axis shows the abundance.

b) Peak 2: 12-14 minutes peak

Table 7: Species detected in peak 2 sample with mass error

Target name	Target score	Reference mass	Observed mass	Mass error (ppm)
GMP Dimer	95.36	708.1054	708.1038	-2.37
ImpG GMP	73.27	758.1323	758.1335	1.51

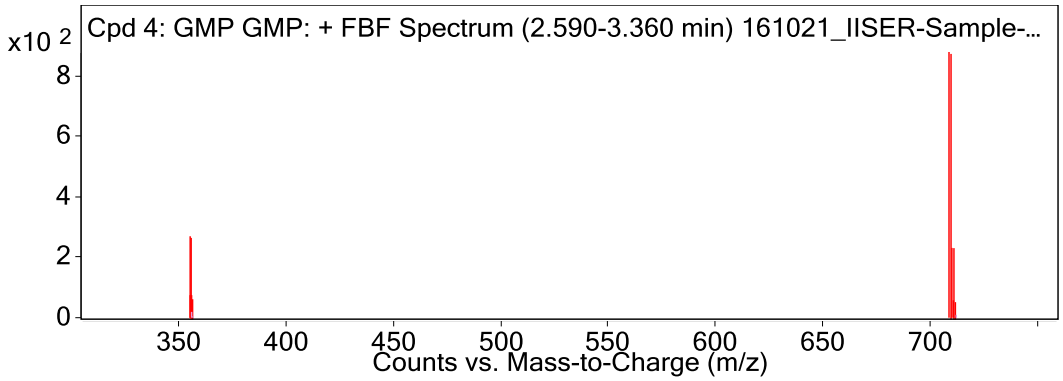


Figure 15: MS zoomed spectrum of sample collected from peak 2

Table 8: MS Spectrum peak list of Peak 2 sample with ion/isotope form and respective abundance

Observed m/z	Charge	Abundance	Formula	Ion/Isotope
355.0594	2	269.01	C20H26N10O15P2	(M+2H)+2
355.5601	2	65.59	C20H26N10O15P2	(M+2H)+2
709.1109	1	875.07	C20H26N10O15P2	(M+H)+
710.1137	1	234.34	C20H26N10O15P2	(M+H)+
711.1146	1	56.12	C20H26N10O15P2	(M+H)+

c) Peak 3 did not show any of the possible compounds, potentially because of low amount of sample material in it, hence needs to be collected again and reanalyzed.

From the LC-MS data obtained, it is clear that the Peak 1 which is collected from 9.5 to 12 minutes mostly contains the non-activated nucleotide (GMP) and also the GMP cyclic species. GMP is known to stack well. The detection of GMP dimer in this region corresponds to the stacked dimer containing GMP and GMP cyclic molecule that has formed from loss of one water molecule from GMP, [with the formula: [(2M+H) + (-H_2O)], where M denotes the monomer, GMP (C10H14N5O8P)]. This indicates that the 12 minute peak observed so far in the reactions could be the stacked dimer of GMP with GMP cyclic molecule, as opposed to the suspected cyclic dimer species. The Peak 2(12

to14 minutes peak) shows the presence of GMP Dimer (two molecules of GMP) with high abundance value (target score). The GMP dimer observed in 12-14 minutes peak has been taken as a reference to assign the dimer region of the nucleotides while doing the area analysis. Mass characterization of other samples is ongoing. The peak present in the dead volume also needs to be collected separately and mass characterized to confirm its chemical identity.

4a) Nucleotide oligomerization studies using different clays

From the aforementioned results, which were obtained from the clay montmorillonite K10 from Sigma has poor catalytic activity. To discern the catalytic efficiencies of different clays in oligomerization of nucleotides, other clays like C-bed and F-bed clay have been used for the reactions. These clays belong to the montmorillonite family, but differ in their source. Both these clays were a kind gift from Dr. Prakash Joshi at RPI, New York. The structural properties of these clays are currently unavailable. The representative chromatograms of the aqueous phase of the reactions containing different clays (viz. C-bed, F-bed or montmorillonite) are shown in the Figure 16.

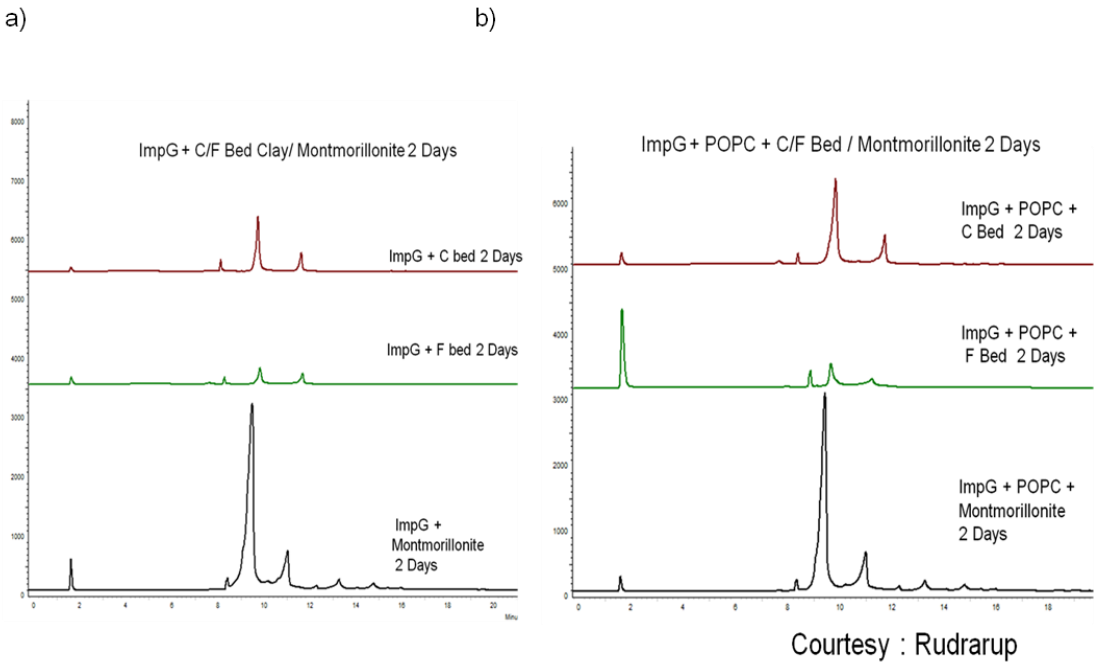


Figure 16a) HPLC chromatograms of 2 days reaction sample containing ImpG + C-bed/ F-bed/ montmorillonite clay b) HPLC chromatogram of 2 days reaction sample containing ImpG + POPC + C-bed/ F-bed/ montmorillonite clay.

These aqueous phase chromatograms allude to the fact that the adsorption capacity of different clays differ. Particularly, in the F-bed clay, most of the monomers and other reaction products formed, appear to have gotten adsorbed on the clay surface and hence were not present in the aqueous phase analyzed by HPLC. The aforementioned clays seems to differ in their adsorption capacity.

4b) Oligomerization reaction of ImpG using Volclay

In a final attempt, we carried out oligomerization reaction using special catalytic clay, namely Volclay (Wyoming), which is known to be an excellent catalyst in the oligomerization of activated nucleotides (Joshi et al., 2009). Volclay too was a kind gift from Prakash C. Joshi of RPI, New York. Since we had very little amount of the clay only ImpG reaction was set up. The reaction conditions were kept similar to those

previously mentioned. Control reactions using montmorillonite clay were also carried out at the same time. Reactions were carried out at RT and -30˚C for 5days. Time points viz.0 hour, 5days (RT) and 5 days -30˚C were analyzed on anion exchange HPLC column**.** Figure 17a and 17b shows the HPLC chromatograms of the reactions containing ImpG along with POPC and Volclay and the same with montmorillonite over different time points with insets for 5 days RT reaction. Figure 18 shows the graphical representation of the area analysis done for both volclay and montmorillonite reactions.

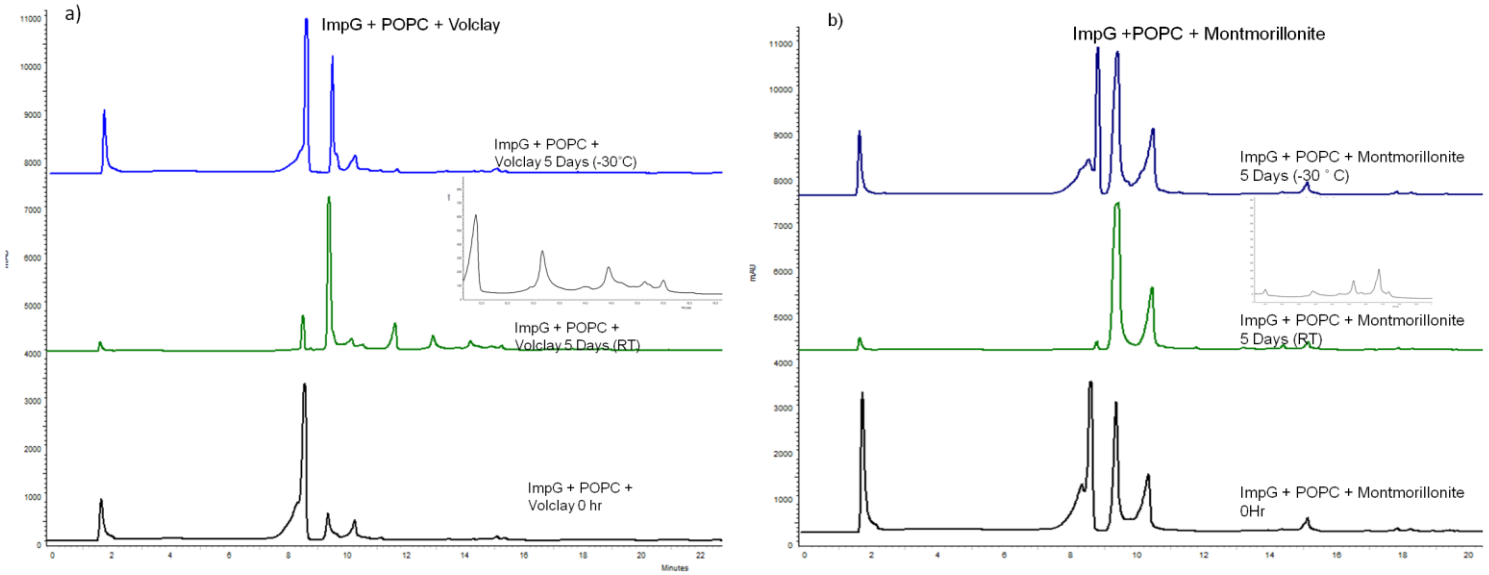
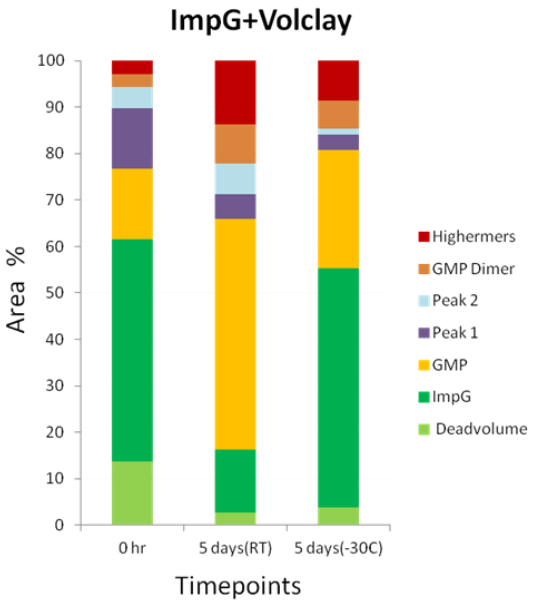
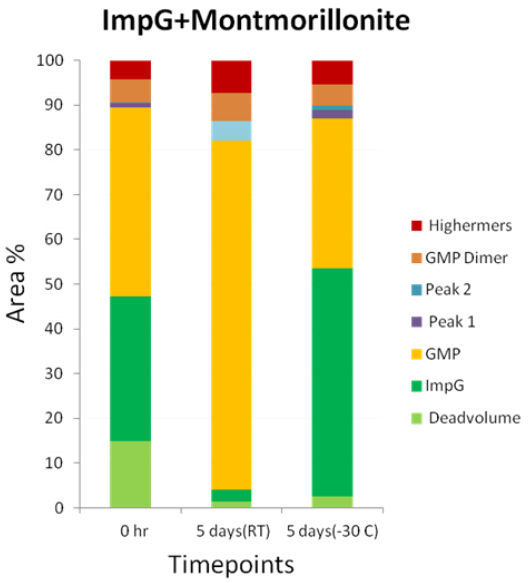


Figure 17a) HPLC chromatograms of Volclay reaction with ImpG and POPC b) HPLC chromatogram of montmorillonite reaction with ImpG and POPC

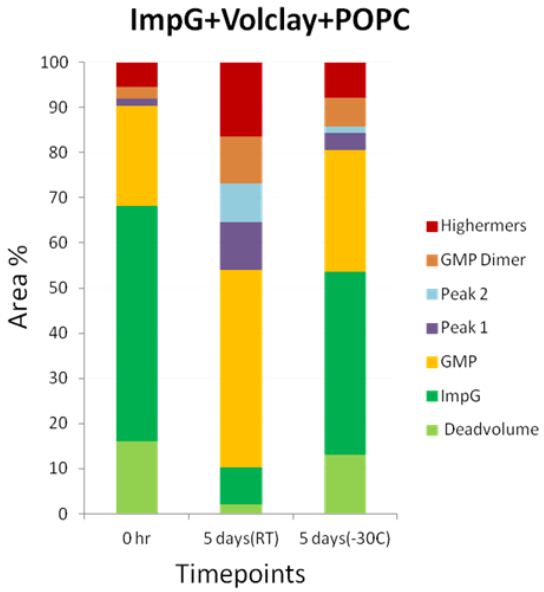
a)


ImpG+Volclay
Area %
100
90
80
70
60
50
40
30
20
10
0
0 hr
5 days(RT)
5 days(-30C)
Timepoints
Highermers
GMP Dimer
Peak 2
Peak 1
GMP
ImpG
Deadvolume

b)


ImpG+Montmorillonite
Area %
100
90
80
70
60
50
40
30
20
10
0
0 hr
5 days(RT)
5 days(-30 C)
Timepoints
Highermers
GMP Dimer
Peak 2
Peak 1
GMP
ImpG
Deadvolume

c)


ImpG+Volclay+POPC
Area %
100
90
80
70
60
50
40
30
20
10
0
0 hr
5 days(RT)
5 days(-30C)
Timepoints
Highermers
GMP Dimer
Peak 2
Peak 1
GMP
ImpG
Deadvolume

d)

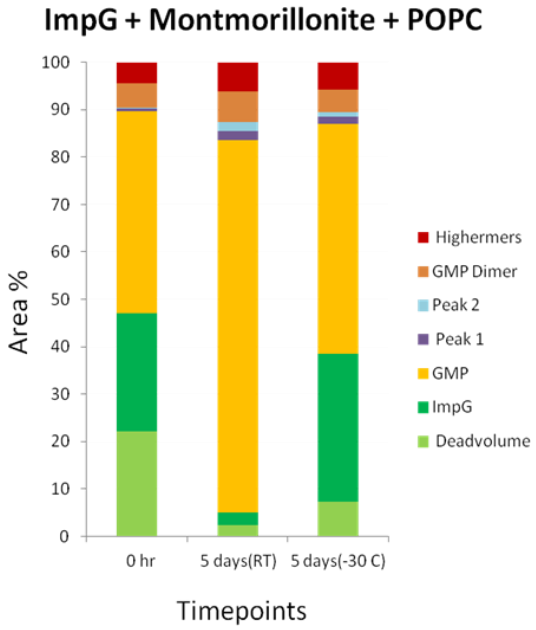

ImpG + Montmorillonite + POPC
Area %
100
90
80
70
60
50
40
30
20
10
0
0 hr
5 days(RT)
5 days(-30 C)
Timepoints
Highermers
GMP Dimer
Peak 2
Peak 1
GMP
ImpG
Deadvolume

Figure 18: Graphical representation of the area percent of the compounds in the chromatograms over different time points of ImpG reaction in presence of a) Volclay b) Montmorillonite c) POPC and Volclay d) POPC and Montmorillonite.

The reaction with volclay differs from that with montmorillonite. In both the reaction sets, carried out at RT, most of the activated nucleotide is broken down to non-activated nucleotides due to hydrolysis. ImpG is stable at -30˚C in both these reaction sets. Interestingly Volclay reactions done under RT has more higher oligomers present in it compared to other reactions, as shown in the insets of Figures 17a and 17b. In between the assigned monomer and dimer region, two other peaks appeared constantly for different Volclay containing samples and have been labeled as Peak 1 and Peak 2 (need to be mass characterized). Both these peaks increase with time in the case of Volclay reaction done at RT. From the preliminary results, it seems that Volclay is a more efficient catalyst at room temperature rather than at -30˚C in presence of salts.

Volclay has the most number of higher oligomers in comparison to montmorillonite. Although in these reactions the fraction of intact ImpG monomer is higher at -30˚C, after 5 days, the extent of higher oligomers formed at -30˚C are similar those formed at RT after 5 days. In both the reaction sets, volclay and montmorillonite, the chromatograms of the reactions with clay and the reactions with clay and lipid (POPC) do not have a considerable difference. Hence it seems that the presence of lipid does not have any additive effect and clay along with salts helps oligomerization of nucleotides to the same extent irrespective of the presence or absence of the phospholipid molecules.

Discussion:

From our initial studies assessing the stability of activated nucleotides in 1M LiCl solution (pH=7, RT), we found that the ImpG is less stable in presence of salt (LiCl) as compared to ImpU. This suggests the higher chances of ImpU reactions yielding oligomers at RT in presence of 1M LiCl. However mass characterization is ongoing, which will shed light on this. Since Guanine based nucleotides are known to stack better and have been shown to oligomerize to a better extent, we decided to concentrate on characterizing ImpG based oligomerization reaction. Furthermore GC-template based reactions are more capable of replication and polymerization in comparison to UA-template based reactions.

One of the focuses of this project was to understand the role of varying temperatures in the nonenzymatic oligomerization of nucleotides. This was studied at three temperatures RT, 4˚C and -30˚C. It was observed that the activated nucleotide is stable even after 30 days at -30˚C. Reduced rate of hydrolysis along with the concentration of the monomers in -30˚C reactions might result in the prominent 12 minute peak (which seems to be the stacked dimer of GMP and GMP cyclic molecule) over time. This peak was also observed in RT reactions, but the stability of activated nucleotides is compromised to a greater extent at this temperature. From LC-MS characterization of -30˚C reaction of ImpG + Clay, the presence of GMP dimer was found out. It closely matches with the elution time of A dimer as hypothesized earlier. From the LC-MS data, it seems that the 12 minute peak could be a stacked dimer of GMP which is formed by the stacking interaction of GMP molecule with the GMP cyclic molecule. No significant formation of oligomers was observed at 4˚C. This might be due to reduced rate of monomer interaction at lower temperature. Also no means of monomer concentration is available at 4˚C unlike that at -30˚C, further reducing the chances of oligomer formation. Or this could be due to the lower concentration of the starting material; hence the formation of higher oligomers is lower and is below the range of detection. The reaction needs to be scaled up in concentration and analyzed. However, as the external synthesis is not cost effective, in-house synthesis of nucleotides are being planned.

From clay extractions of the samples of the reactions at -30˚C and 4˚C, it is seen that clay adsorbs most of the species and is the reason for the variability of chromatograms within different time points. The extent of adsorption differs between clays (Hashizume, 2015). We suspect the rate of desorption will also affect the efficiency of the oligomerization process. Clay extraction followed by biochemical analysis at regular intervals is crucial to infer about the adsorption and desorption rates of different clays. Such studies on adsorption and desorption properties of the different clays available in our lab are being planned and is one of the future goals of this project.

It has been reported that, montmorillonite facilitates the conversion of fatty acid micelles into vesicles (Hanczyc et al., 2003). The clay particles along with adsorbed RNA could then get entrapped in these vesicles resulting in a microenvironment favourable for the forward reaction. These could have been the primitive protocell. Given this we wanted to look at the additive role of lipids on clay catalyzed nonenzymatic oligomerization in the presence of salts. However analysis of the chromatograms (Figure10, 11, 18) of both aqueous phase and extracted phase samples obtained from the clay phase of reactions with clay and clay+ POPC lipid are not vastly different, under conditions tested, indicating that there might not be any additive role provided by the lipid studied (POPC) in the oligomerization process.

Different types of montmorillonite clays were used to understand the catalytic efficiency. Montmorillonite purchased from Sigma has been a poor catalyst in our study. C-bed and F-bed clays used here showed differences in their adsorption capacity. This is reflective in the oligomerization pattern seen in the HPLC chromatograms. Detailed adsorption and desorption studies of various clay samples are currently under consideration and one of the future directions and it has implications for catalytic efficiency of the clay.

Volclay, best known catalytic clay was used in one set of reactions to compare its efficiency with montmorillonite. As alluded to earlier it was of limited availability. From the preliminary results, it appears that Volclay is more efficient catalyst at RT in presence of salts compared to -30˚C. Volclay comparatively has lesser cations in the interlayer at pH 7 compared to montmorillonite (Joshi et al., 2009).The binding efficiency of the monomers depends on the number of cations and interlayer charge which would

be different in both of these clays. This has been hypothesized in the past for the difference in the catalytic efficiency of different clays (Joshi et al., 2009). Apart from this, structural differences between the two clays could also be a reason for their variable catalytic efficiencies. Therefore the clays can be further analyzed using X-ray diffraction, to gain insights into such structural differences. Furthermore among the volclay and montmorillonite based reaction, those reactions involving clay and those that involves both clay and lipid do not differ. This further strengthens the possibility that lipid used in the study (POPC) does not confer any additive role in the aforementioned reactions.

Mass characterization and long term reaction samples from both ImpG and ImpU based reactions are ongoing.

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