Fatty acid-assisted nonenzymatic synthesis reactions of ribonucleotides

Indian Institute of Science Education and Research, Pune

Thesis submitted in partial fulfillment of the requirements of Five year BS-MS Dual Degree Program

By

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Certificate

This is to certify that this dissertation entitled "Fatty acid-assisted nonenzymatic synthesis reactions of ribonucleotides", 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 Prasenjeet Kawale at IISER Pune under my supervision in the department of biology during the academic year 2014-2015.

Signature of the Supervisor **Date: March 25th, 2015**
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Declaration

I hereby declare that the matter embodied in the report entitled **"Fatty acid-assisted nonenzymatic synthesis reactions of ribonucleotides"** are the results of the investigations carried out by me at the Department of Biology, IISER Pune under the supervision of **Dr. Sudha Rajamani** and the same has not been submitted elsewhere for any other degree.

Prasenjeet Kawale
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Date: March 25th, 2015

Acknowledgement

I would like to express my deep gratitude towards Dr. Sudha Rajamani for her continuous guidance, support and encouragement. Her assistance and untiring efforts have resulted in the successful completion of this project. She has not only been a great mentor and advisor, but also a great teacher for which I'm sincerely thankful. I am grateful for all the intellectual discussions and support that my lab members provded. I would like to thank Aparna, Chaitanya, Dhruv, Niraja, Rajarajeshwari, Sanjana ,Yashaswi and Yovhan for always being people that I could count on and for helping me develop valuable skills. The non-teaching staff contributed significantly to this project as well. For that I would like to thank Vijay Vittal for his aid in microscopy and Naina for carrying out HRMS. Dr. Thomas Pucadyil's useful suggestions have helped hone this project, for which I'm highly grateful. The funding from IISER Pune has made this project possible and I am truly thankful for it.

I am utmost grateful and indebted towards my parents who deserve all the credit for making me who I am today. They have stood by me through all the difficult times and their loving encouragement and support has been invaluable. I thank all the people whose contribution has resulted in the favourable outcome of this project.

Abstract

The vital role of RNA in present day cells and its ability to act as both informational and catalytic entity supports the “RNA World” hypothesis. As chemical polymerization of ribonucleotides is an uphill process, various methods have been used to establish the independent origin of RNA. One such study has demonstrated the polymerization of nucleotides under alternating dehydration and rehydration (DH-RH) conditions in the presence of lipids. The collective effect of temperature and organizing effect brought about by the lipids is thought to overcome the energetic barrier. However, the presence of complex lipids on early Earth is debatable. One solution to surpass this shortcoming is to use simpler prebiotically relevant amphiphiles. In the current project we have analyzed the role of fatty acids in promoting nonenzymatic polymerization of RNA monomers. In addition to DH-RH reactions, polymerization in eutectic phases and clay-catalyzed polymerization was also studied. Overall, the presence of fatty acids did not particularly improve polymerization efficiency in the various scenarios tested. However, in the eutectic phase and DH-RH reactions, the fatty acids had a protective role in reactions that involved adenosine ribonucleotides. However, this was not seen with other ribonucleotides. This study does not corroborate previous findings in which the authors had used phospholipids in their oligomerization reactions. This indicates that the chemical identity of the lipid involved in such nonenzymatic reactions will determine their potential for catalyzing oligomerization of ribonucleotides into RNA-like polymers.

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Introduction

Study on the origin of life is one of the most intriguing and fascinating arena of science which has evoked interest among scientists from varied backgrounds including chemists, biologists and physicists. It however remains an unsolved mystery to date. The basic problem encountered in the study of origin of life is to define the exact transition where the non-living entities are conferred “life-like” properties; the most fundamental of which is informational transfer via replication. An important distinguishing criterion was provided by Leslie Orgel in his book, The Origins of Life: Molecules and Natural Selection, where he proposed the phrase “specified complexity”. The early Earth was composed of inorganic and organic molecules in which the inorganic components lacked complexity, whereas the organic molecules were devoid of specificity or organization. Thus, the acquisition of “specified complexity” in systems is often considered as the first step to the origin of life.There are various theories on how this transition came about, but an unequivocal consensus is yet to be achieved.

The earliest hypothesis which was widely prevalent is the spontaneous generation theory, which states that living entities can originate spontaneously from inanimate objects. But as the years passed by, this theory was strongly contested through well characterized experiments by notable scientists, one of them being Louis Pasteur, which finally led to the rejection of this hypothesis. Also, the theory of Panspermia, proposed by Richter in 1865 has received much attention. This theory states that the cosmic dust carried traces of life from some unknown part of the universe, which deposited here on Earth leading to evolution of life. The strong evidence against this theory is that the extremely harsh conditions of cold, dryness and high intensity ultraviolet ray which exist in space are potentially non-conducive to the survival of living matter.

Currently, the dominant hypothesis is the modern theory of origin of life which was proposed by Alexander Oparin in 1923. This was also endorsed by J.B.S. Haldane in 1928 and is therefore also known as the Oparin-Haldane theory. It deals with three aspects of origin of life as described by Lederberg (Lederberg, 1965)-

1. Chemogeny (Chemical Evolution)
2. Biogeny (Cellular Evolution)
3. Cognogeny (Nature and evolution of archaic life)

The theory of chemical evolution deals with the formation of macromolecules necessary for the origin and evolution of life. This is the aspect that is dealt with in this project.

The first hint of life is suggested to have originated during the Hadean Eon (before 3.9 Ga.) in the form of microorganisms. The atmosphere during that era was hypothesized to contain ammonia, methane and water vapour. Urey-Miller's famous experiment about generation of amino and hydroxy acids from atmospheric molecules laid the foundation for chemical evolution of life. Urey assumed the Earth's atmosphere to be reducing. However, later it was found that the experiment was not a true representation of the prebiotic atmosphere when it was established that Earth's original atmosphere was composed mostly of H_2O, CO_2, and N_2, with trace amounts of CO and H_2. When the Urey Miller experiments are carried out under such conditions, the yields of prebiotically relevant moieties was found to be low. This moved the focus of the origin of life from atmosphere to the hydrosphere (Zahnle et al., 2010). Even though the results of this experiment are not completely valid, it is a landmark in the field of origins of life which led to the development of various other theories of life's origin.

Even within the chemical evolution scenario, there exists a "metabolism first" hypothesis and "Gene first" hypothesis. Both these hypotheses have their advantages and disadvantages.. The metabolism first theory argues that the repeated spontaneous formation of informational molecules is a highly unlikely event without continuous replenishment of reactant supply through metabolism. Primitive metabolism is believed to be a series of cross catalytic reactions which serve to increase complexity. The main opposition to this theory is that such networks are incapable of Darwinian evolution (Vasasa et al., 2010). The gene first approach, on the other hand, takes care of both the problems associated with the metabolism first approach. Genes not only exhibit the property of self replication, but they are also capable of undergoing mutations and therefore lead to diversity and evolution. (Plaxco and Gross, 2006). The leading candidate for such a role is ribonucleic

acid as investigations revealed that it has self replicating ability as well as a catalytic and information storage role. The earliest mention of RNA as the prebiotic genome was by Carl Woese in his famous book, The Genetic Code (Woese, 1967). The idea that RNA could be the precursor to DNA and proteins in the ancient world was later supported by Francis Crick and Leslie Orgel. However, the true impetus to this school of thinking was provided by Nobel Laureate Thomas Cech and Altman who discovered the catalytic role of RNA and called them ribozymes. Finally in 1986, the phrase “RNA World Hypothesis” was christened by Nobel Laureate Walter Gilbert taking into consideration then recent discoveries which highlighted properties of RNA suitable for acting as a pioneer for the origin of life (Robertson and Joyce, 2010).

For a polymer like RNA, the origin of its complex monomers is an interesting question to ask. The origin of these monomers was unknown for a long time before the synthesis of pyrimidine mononucleotides, through a highly complex set of chemical reactions, was demonstrated (Powner et al., 2009). Another progress in this regard was published by Ferus and co-workers that reveal the origin of nucleobases could be during the Late Heavy Bombardment (~4.0 – 3.8 Ga) era by the dissociation products of formamide generated in the impact plasma in an uncatalysed reaction. (Ferus et al., 2015). Another important hurdle in the formation of polymers is joining the monomers together. The formation of biopolymers in modern cells is orchestrated by the activity of enzymes but the existence of such complex molecules on prebiotic Earth is debatable (the chicken and egg paradox). Therefore, in their absence, the formation of polymers is an uphill process. The role of enzymes in the archaic Earth is hypothesised to be fulfilled by certain niches which promote the joining together of monomers through condensation reactions. These niches are thought to be-

1. Mineral surfaces
2. Eutectic phases
3. Hydrothermal pools

One of the niches which favour oligomerization of mononucleotides, as proposed by Bernal and Goldschmidt, is mineral surface eg. Montmorillonite clay. Montmorillonite clay is formed as a result of weathering of volcanic ashes and its prebiotic relevance can also be established by the fact that it has been discovered on Mars (Bishop et al., 1995). The structure of clay consists of tetrahedral SiO4 and octahedral AlO^6 as shown in the Fig 1. The layers bind due to Van der Waals forces and cationic interactions in between the

layers. Their large crystalline surface area (>100m^2/g), multilamellar structure and surface adsorption properties make them ideal for concentrating and organizing the monomers between the layers promoting polymerization(Ferris, 2005).

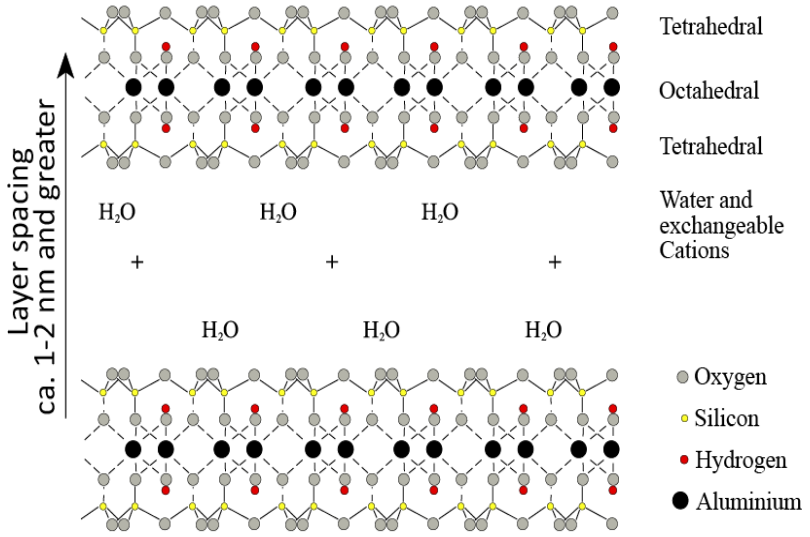

Fig. 1: Structure of montmorillonite clay adapted from Ferris, 2006.

The studies on Montmorillonite clay have been undertaken using a modified nucleotide known as activated nucleotide. Activated nucleotide is formed by replacing one of the OH groups in the phosphate by a better leaving group such as Imidazole. The reaction scheme is depicted in Fig.2.

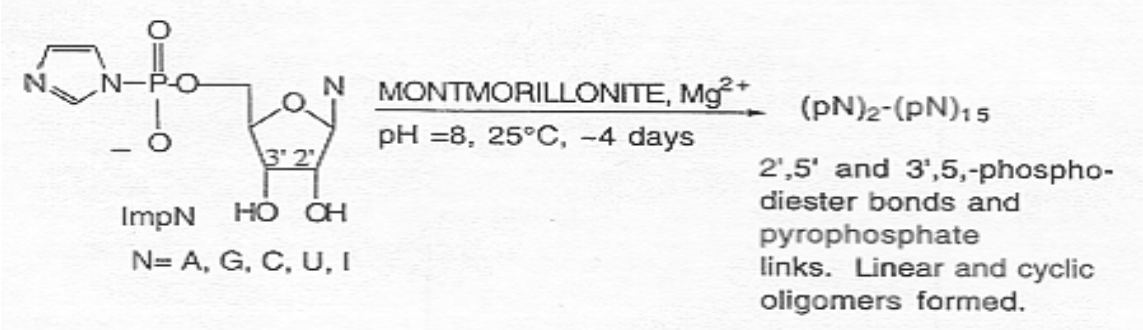

Fig. 2: Imidazole activated reaction scheme (Ertem and Ferris, 1997)

The second catalytic niche, which is not only distinct from the other two niches, but is also largely hypothetical, consists of the eutectic phase. Eutectic phases are solute-studded fluids channels formed between microcrystals of pure ice. These allow the concentration of reactants even in dilute solutions. This niche is said to be hypothetical because early Earth is assumed to be a hot mass. As the temperature distribution of early Earth is not precisely known we cannot rule out the role of eutectic phases in the RNA world (Deamer and Weber, 2010). The formation of RNA-like moieties using the eutectic phases has also been typically carried out using activated nucleotides as the rate of phosphodiester bond formation under such extremely cold conditions is very slow. Without the use of these bond facilitating modifications, it would be extremely difficult to study these reactions. In nature though, the timescale for bond formation is not much of a concern as life originated over a course of millions of years.

The most widely prevalent conditions on early Earth included regions of high volcanic activity. The edge of these volcanoes would have had small pools of liquid water. One of the hypotheses for the origin of macromolecules necessary for life revolves around these niches called geothermal pools. The volcanoes provide high temperature along with acidic conditions. During the day-night cycles, the temperature fluctuation results in alternate dehydration and rehydration of these pools. These niches serve as an excellent niche for driving uphill reactions such as polymerization .The high temperatures also help overcome the thermodynamic barrier with heat catalyzing the condensation process by reducing water activity and concentrating the reactants. In addition, the acidic conditions favour phosphodiester formation in a manner similar to that seen in acid-catalyzed formation of ester bonds. This principle was applied by researchers on amino acids and nucleotides to allow the formation of proteins and oligonucleotides by exposing the reactants to elevated temperatures (Deamer and Weber, 2010). One of the difficulties faced with this approach was that the solid phase formed as a result of evaporation of solvent restricted diffusion and hence inhibited long range interactions. The other problem was that at high temperatures, the nonspecificity of bond formation increased resulting in the formation of tar in extreme conditions. Even with these issues, the use of heat to drive condensation reaction is the most pertinent method of synthesis if the correct range of temperature is chosen. Another advantage of using this process is that continuous cycling between hydrated and dehydrated conditions results in the formation of RNA-like polymers with the use of non-activated nucleotides thereby making the reaction more prebiotically relevant (Deamer and Weber, 2010). Apart from heat, another mechanism which ensures concentration of reactants is compartmentalization. This compartmentalization in an aqueous phase can

be achieved by the presence of amphiphilic substances such as lipids, fatty acids and surfactants. A combinatorial approach was used by Rajamani et al. (2008) where both the concentrating mechanisms of heat and compartmentalization by lipids was used to drive the polymerization reaction forward by alternate dehydration-rehydration cycles . It has also been demonstrated that apart from concentrating the reactants, the lipids also play a role in organizing them in a conformation suitable for polymerization (Toppozini et al., 2013). This organizing effect is the result of multilamellar structures which are formed when lipid bilayers are dehydrated. Whereas on rehydration they form vesicles which encapsulate reaction components and this is considered as a pathway to the primitive cell, known as protocell. Lipid bilayers are liquid crystalline matrices which allow diffusion, do not form polymerized tar and the solid phase barrier that is otherwise produced during dehydration in the absence of lipids. This in turn permits molecules other than the immediate neighbours to interact and therefore increases the probability of bond formation (Deamer, 2012).

Rajamani et al's study demonstrated that heat could be used as a catalyst in the presence of phospholipids but the origin and abundance of phospholipids on prebiotic Earth is questionable. On the other hand, the presence of fatty acids on early Earth has been documented as it was discovered in carbonaceous meteorites and can be formed by Fischer-Tropsch like synthesis (Deamer, 2002). These fatty acids form vesicles at their respective dissociation constants, which are in the basic pH range. As the polymerization reaction requires acidic conditions, a mixture of Oleic acid (OA) and Oleylamine (ON) fatty acids in 1:1 molar ratio was chosen, which forms vesicles even at acidic pH <3. ON becomes protonated at acidic pH, which allows the formation of pseudo diacyl fatty acid chains with OA through hydrogen bonding and leads to vesicle formation (Namani and Deamer, 2008). It was also discovered that solid aromatic macromolecules and gaseous polycyclic aromatic hydrocarbons (PAHs) were abundantly distributed in the cosmos and were also found in meteorites. This, along with their stability and ability to serve as container elements, makes incorporation of PAH in fatty acid vesicles prebiotically relevant as well as an attractive candidate in the early protocell membrane development (Groen et al., 2012). Therefore, the efficiency of these simpler and prebiotically more relevant amphiphiles, in carrying out lipid-assisted oligomerization has been studied in this current research. Additionally, in the various studies carried out thus far, the effect of clay, amphiphiles and temperature, on the oligomerization of RNA, has only been characterized in isolation. In one aspect of these studies, we also analyzed the combined role of clay and fatty acid on the oligomerization of activated mononucleotides and non-activated monomers, using alternate dehydration – rehydration conditions. This unique combination is prebiotically

relevant and has been hypothesized to have led to the formation of primitive protocells (Hanzyc and Szostak, 2003). We therefore aim to characterize the collective effect of these previously studied catalysts on the oligomerization of RNA monomers. Additionally, these reactions were also studied at -18ºC to discern the efficiency of these reactions under eutectic phase conditions.

Materials and Methods

Materials:

Oleic acid was procured from Avanti® Polar Lipids Inc. (Alabaster, AL, USA). Oleylamine, Pyrene, 1-hydroxypyrene, Adenosine 5'-monophosphate (5'-AMP), uridine 5'-monophosphate (5'-UMP), guanosine 5'-monophosphate (5'-GMP) and cytidine 5'-monophosphate (5'-CMP) were purchased as disodium salts from Sigma-Aldrich (Bangalore, India) and used without further purification. Imidazole Adenosine phosphate(ImpA), Imidazole Guanosine phosphate(ImpG), Imidazole Cytosine phosphate(ImpC) and Imidazole Uridine phosphate(ImpU) were obtained from GL synthesis. All other reagents used were of analytical grade and purchased from Sigma-Aldrich (Bangalore, India).

Vesicle preparation:

A mixture of 5mM oleic acid and 5mM oleylamine was prepared in 10mM HCl and bath sonicated for 30 minutes to make a homogenous solution of the fatty acids. The pH was then adjusted to 3 using concentrated HCl and the solutions were incubated overnight at room temperature to allow vesicle formation.

Previous studies suggest that the concentration of Polycyclic Aromatic Hydrocarbon (PAH) which can be incorporated in a fatty acid system is 10mol% (Groen et al., 2012). This concentration was therefore used for pyrene or 1-hydroxypyrene. Oleic acid/Oleylamine mixture containing 10mol% pyrene was prepared in chloroform whereas those containing

10mol% 1-hydroxypyrene were prepared in dimethyl sulfoxide (DMSO). The organic solvents were then evaporated under a constant nitrogen gas flow. Further processing of the mixtures was carried out as mentioned above.

Determination of Critical Vesicle Concentration (CVC) of the fatty acid system:

The CVC of the oleic acid/oleylamine system was determined using the turbidity assay. This method is based on the principle of light scattering by small particles. In a solution, the fatty acids exist independently. But when their concentration increases a threshold value, the fatty acids aggregate to form an organized structure known as a vesicle. This threshold is known as CVC. The vesicles scatter light and decrease percentage transmission which results in increased absorbance of a solution containing vesicles. When formation of vesicles takes place, the absorbance suddenly rises steeply as the scattering species increases drastically (breakpoint). The concentration at which this occurs is representative of the CVC. The determination of CVC by such a method (of recording breakpoint absorbance) is a simple method, however it is not known to be very precise. Two difference methods were used for vesicle preparation, prior to measuring the CVC by this method. These are outlined in the flowchart given below(Fig. 3). The result from the second method was considered more appropriate as the sonication step allows the reaction to start from a relatively homogeneous state.

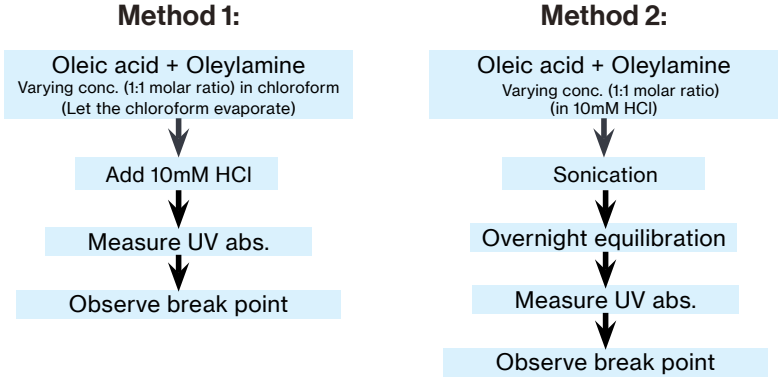

Fig. 3: Flow chart outlining method for CVC determination

Dehydration-Rehydration (DH-RH) Cycling:

Prebiotic conditions, similar to that of volcanic geothermal pools (high temperature acidic environments), were simulated by assembling a bench-top heating block with provision to accommodate reaction vials. To maintain anaerobic environment, the vials were equipped with caps that had Polytetrafluoroethylene (PTFE) septa in them through which PEEK tubing carrying CO_2 was fitted. Another PEEK tube served to release the excess CO_2 and water vapor generated in the reaction.

The total volume for a typical reaction was 300µL which contained the components as mentioned in table 1:

Table 1: Reaction conditions for DH-RH cycling

	5mM Oleic Acid	5mM Oleylamine	10mol% Pyrene	10mol% 1-Hy-droxypyrene	10mg Clay	5mM NMP
Reaction1	+	+	-	-	-	+
Reaction2	+	+	+	-	-	+
Reaction 3	+	+	-	+	-	+
Reaction4	+	+	-	-	+	+
Reaction5	-	-	-	-	+	+
Control	-	-	-	-	-	+

The heating block was maintained at 90°C. After every 30 minutes, when the contents were completely dry, the mixture was rehydrated with 15mM HCl using another PEEK tubing inserted in the PTFE septa. These DH-RH reactions were carried out till 10 cycles and time points were taken after 1, 4, 7 and 10 cycles along with zero time (taken at the start of the reaction).

Clay-catalyzed oligomerization reactions:

Li+-activated Montmorillonite clay was used for carrying out all the reactions with total volume as 200µL. Reactions were carried out for 10 days and time-points were taken after 1, 5 and 10 days with the control time-point collected immediately on addition of the reactants.

The reactions carried out using activated nucleotides contained the components as mentioned in table 2:

Table 2: Reaction conditions for Clay-catalyzed oligomerization reactions

Temperature (°C)	5mM Oleic Acid	10mg Clay	10mM ImpG	1M LiCl
-18, 4, RT	+	+	+	+
-18, 4, RT	-	+	+	+

Amphiphile Extraction:

For carrying out the characterization of the products formed in the reaction, it is necessary to separate fatty acids from the mixture. This is achieved by butanol and hexane. Butanol, being slightly polar, is able to mix with the aqueous phase allowing all the fatty acid molecules to come in contact with the butanol and hence dissolve in it. Hexane on the other hand is completely immiscible with the aqueous phase and serves to remove all traces of butanol remaining in the aqueous phase. The protocol for carrying out the fatty acid extraction is as follows:

- 200µL of reaction mixture was made up to 400 µL using MilliQ water.
- 400µL of butanol was first added and mixed thoroughly. The sample was then centrifuged at 6000 rpm for 5 minutes at 4°C.
- The upper phase (organic) was removed and for a second time, 400 µL of butanol was added, mixed thoroughly and the mixture was then centrifuged at 6000 rpm for 5 minutes at 4°C.
- The organic phase was discarded and 200 µL of hexane was added and mixed thoroughly.
- It was centrifuged again at 6000 rpm for 5 minutes at 4°C.
- The organic phase was discarded.
- The aqueous sample was then kept in speed vac for 15 minutes at 50°C to get rid of any remaining hexane.

Clay extraction:

Clay acts as a catalytic surface and therefore extraction of products from the clay is extremely important. This is achieved by using 30% acetonitrile (ACN) and 0.1M sodium chloride NaCl. The sodium ions, being positively charged, help elute the negatively charged RNA, which then dissolves in ACN.

In reactions which contained both lipid and clay, the samples were first subjected to butanol-

hexane treatment to remove the fatty acid. The clay pellets, separated after fatty acid extraction, were treated with 0.1 M NaCl in 30% ACN for 1 hour at 4°C. The supernatant obtained was kept aside and the pellets were again subjected to the aforementioned treatment for 1 hour. Then the clay pellets were again treated with 0.1 M NaCl in 30% ACN but this time it was incubated overnight at 4°C. The final treatment of the resultant clay pellets was done for 1 hour again with 0.1 M NaCl in 30% ACN. (2 x 1 hr, 1 x overnight and 1 x 1 hr).

Thin Layer Chromatography (TLC)

Thin-layer chromatography (TLC) is a separation technique applied to characterize non-volatile analytes. Like all forms of chromatography, it has a stationary phase and a mobile phase. The stationary phase is most often an adsorbent sheet and the mobile phase is drawn by this sheet via capillary action. Separation is achieved by difference in retention factor. In our experiments, Silica gel 60 was used as the stationary phase with mobile phase solvent as chloroform: methanol: water in volumetric ratio of 65:25:4. This was mainly used for fatty acid analysis to check if their chemical identity remained intact over the course of the reaction.

Microscopy:

The formation of vesicles was confirmed by carrying out Differential Interference Contrast (DIC) microscopy on Zeiss Imager.Z1 Apotome. DIC separates polarized light into two perpendicular parts and the interference produced by these parts upon recombination depends on the optical path difference, which is the product of refractive index and geometric path length. As the refractive index of vesicle differs from water, DIC uses this property to generate contrast in the image and allows observation of vesicles. This in turn enables us to check the stability of the vesicles at different time-points as the reaction progresses.

High Performance Liquid Chromatography analysis:

High Performance Liquid Chromatography (HPLC) is an analytical technique which allows separation of solutes based on their differential affinities towards specific stationary and mobile phases. It employs a column tightly packed with a matrix of small particles which constitutes the stationary phase. The fluid, called as the mobile phase, is passed continuously through this column at high pressure. The stationary and mobile phases have different chemical and physical properties, which leads to the preferential elution of a solute due to higher affinity for one of the phases. The physicochemical interactions that determine separation vary with the different forms of HPLC techniques used. In this study, separation was achieved using ion exchange HPLC chromatography. In this technique, charge on the molecule determines its separation efficiency. The number of charges and their location in the molecule govern the affinity to the stationary phase and in turn affect the time of elution.

Anion exchange chromatography was carried out on the Agilent 1260 Infinity series (Agilent Technologies, Santa Clara, CA, USA) using a DNAPac PA200 column (4 mm × 250 mm) from Dionex (Thermo Scientific, Sunnyvale, CA, USA). DNAPac PA200 column is positively charged due to 130 nm quaternary amine functionalized NanobeadsTM packing and therefore it attracts negatively charged solutes. The mobile phase is a mixture of solvent A (2mM Tris pH 8.0) and solvent B (2mM Tris + 0.4M $NaClO_4$). Separation is only possible if the pKa of the mobile phase is intermediate to the pKa or pI of the solute to be separated and the column support. As the support is highly basic and the solute is acidic, the mobile phase is designed to have a pH of 8.0. The incorporation of salt ($NaClO_4$) facilitates elution of moieties on the basis of their charge. As the salt concentration increases linearly, the affinity of the least charged molecule to the solid substrate decreases and it elutes first and with further increase in the salt concentration the other components elute in the order of their increasing charge.

Different salt gradients are required to separate molecules of interest as the chemical and physical interactions of solute components differ. In this current project, polymerized nucleic acids are formed from activated and non activated nucleotide monomers. The reaction by polymerization of non-activated nucleotides is carried out at acidic pH whereas in case of activated nucleotides, it is at neutral pH. This different initial pH of the reaction mixture affects the separation of the oligomers formed, on the column and therefore the following two gradients were used for their separation, as depicted in Table 3 and Table 4.

Table 3: HPLC gradient for reaction with non-activated nucleotides

Time (minutes)	% Solvent A	% Solvent B
0	100	0
2	100	0
12	50	50
13	0	100
18	0	100
20	100	0
25	100	0

Table 4: HPLC gradient for reaction with activated nucleotides

Time (minutes)	% Solvent A	% Solvent B
0	100	0
3	100	0
6	93	7
8	87	13
10	25	75
12	60	40
14	0	100
18	0	100
20	100	0
23	100	0

High Resolution Mass Spectrometry (HRMS):

The predicted identity of the peaks obtained in HPLC analysis needs to be confirmed, which is most often by the mass of the molecule. In mass spectrometers, the mass to charge ratio of ionized molecules is used to separate them. It also serves the dual role of aiding in the identification of molecules and estimating their relative abundance. Molecules are identified based on their characteristic fragmentation patterns. The main advantage of using high resolution mass spectrometry (HRMS) is that it gives a much more accurate estimation of the mass as it does not assume the elemental masses to be integers and provides resolution up to a tenth of a millimass unit.

HRMS analysis was done using the Acquity UPLC+ system from Waters, with an Alltima C18 column (2 µm, 2.1 mm × 50 mm) using water/acetonitrile gradient containing 0.1% formic acid. Positive ion mode with SYNAPT G2 Mass Spectrometry (Waters, Milford, CT, USA) was used for determining the mass. The predicted mass of the molecules of interest was obtained through Chemdraw Ultra 12.0 ©. The mass observed from HRMS was assumed to correspond with the predicted mass if they are within 5% ppm error.

Results and Discussions

Optimization of fatty acid systems:

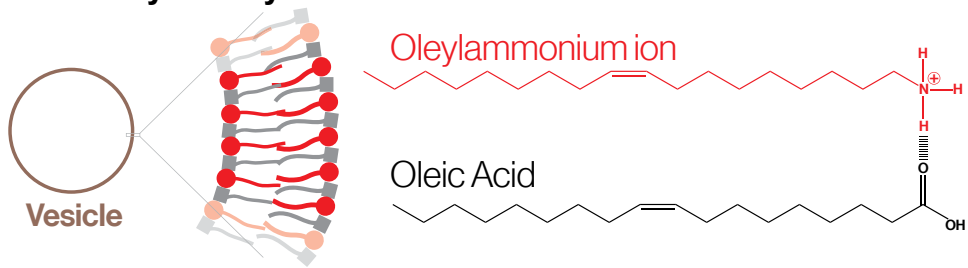

Fig. 4: Schematic of vesicle formation by oleic acid/oleylamine adapted from Namani and Deamer, 2012.

As mentioned earlier in the introduction, the choice of OA/ON system was based on the ability of the mixture to form vesicles at acidic pH as shown in the figure 3. The CVC was found to be around 250 µM and 10µM, respectively, using the two different vesicle preparation method (Fig. 5 and Fig.6). This variation in CVC observed on using two preparation methods was probably because the fatty acid system was not very homogenous to begin with in method 1. It has been observed in a previous study that different vesicle preparation methods lead to different CVC values, particularly for vesicles composed of oleic acid (Maurer et al., 2009). Additonally, to our knowledge, there are no previous articles in the literature which report the CVC of oleic acid/oleylamine system at pH 2 to compare our results with. Therefore, to characterize the CVC of our mixed fatty acid system beyond doubt, the experiment needs to be carried out using a different assay such as Merocyanine 540 dye assay. Also, the DH-RH reaction regime uses extreme conditions like high temperature and low pH. As we were unsure whether the fatty acids would survive this treatment, we anyways decided to use a higher concentration of the fatty acids (> 5mM).

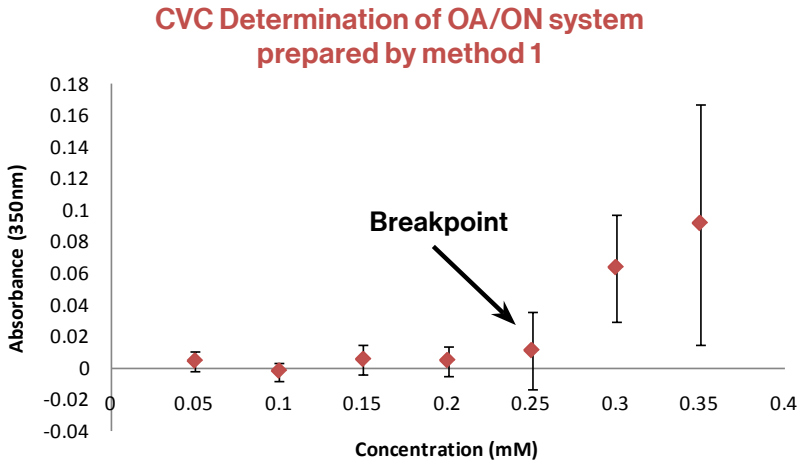

Fig. 5: Determination of CVC by method 1. Error bars reflect standard deviation of data collected from two independent experiments.

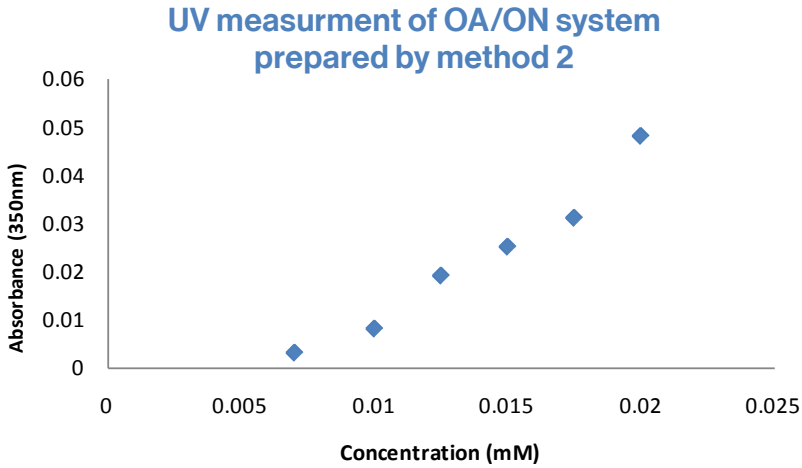

Fig. 6: Representative data for determination of CVC by method 2.

Microscopy of fatty acid concentrations equal to and above 5mM showed multilamellar vesicles in large number which is favorable for the reaction, whereas lower concentrations did not show distinct vesicles. Therefore, it was decided that 5mM concentration of the fatty acids would be incorporated in the reaction. In addition, a variation of the fatty acid system containing a combination of OA/ON and PAH (either pyrene or 1-hydroxypyrene) was prepared. The main aim of including PAH in the reaction was to verify whether it has a stabilizing effect on the vesicles and therefore the vesicle forming ability of the fatty acids was investigated after carrying out 10 dehydration-rehydration cycles. However, when phase contrast microscopy of this system was carried out there was no significant visual difference in the vesicle characteristics as compared to the OA/ON fatty acid system.

Vesicle stability:

The stability of the vesicles needs to be assessed as their solute organizing effect is important in promoting the oligomerization of RNA mononucleotides (Deamer, 2012). This stability was evaluated by phase-contrast microscopy to ensure the presence of fatty acid vesicles after every dehydration-rehydration cycle. The OA/ON system, in 1:1 molar ratio,formed vesicles at pH 3 but individually neither OA nor ON seemed to be forming vesicles at that low pH. This capability of OA/ON system to form vesicles at low pH was retained even after 10 dehydration-rehydration cycles (corresponding to 5 hours) at 90°C. When PAH incorporated OA/ON system was analyzed for stability, intact vesicles were still observed after prolonged exposure (5hours) to 90°C and pH 2. Representative images from DH-RH reactions involving 5'-AMP +OA/ON and 5'-AMP+OA/ON+ pyrene are shown in Fig.7. To our knowledge, this is the first time where this particular fatty acid system has been shown to form stable vesicles under such extreme conditions of high temperature and low pH. Additionally the system retained its vesicle forming capability despite repeated cycling under geothermal conditions. As the vesicles were stable, we used these fatty acid systems for carrying out dehydration-rehydration reactions.

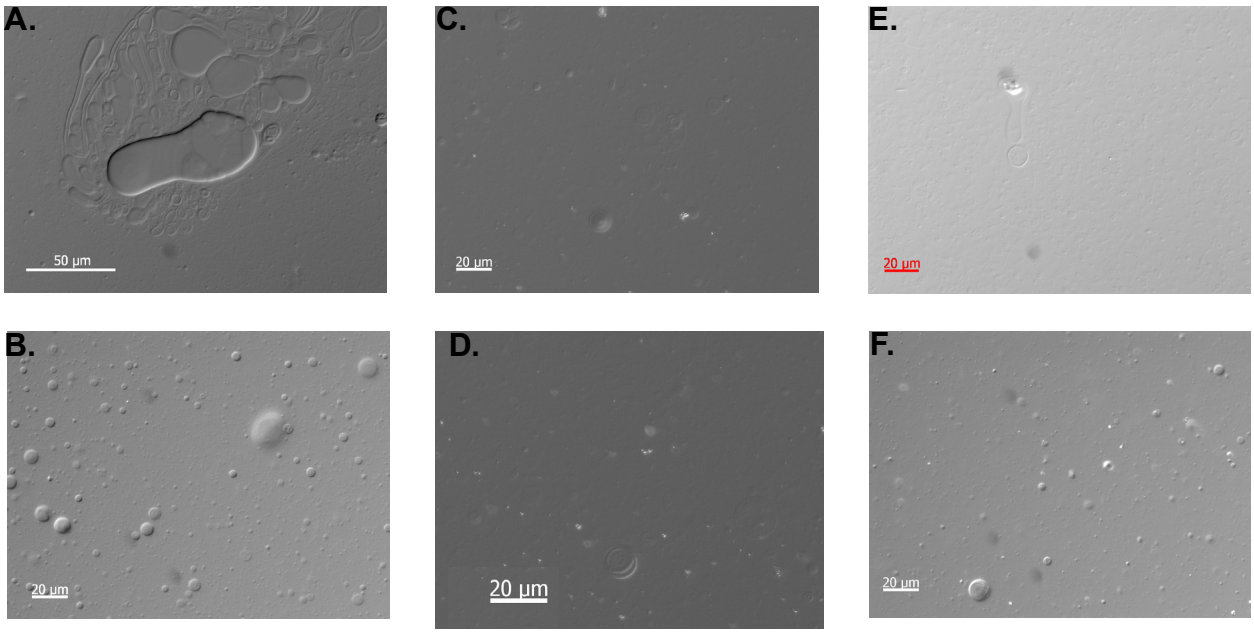

Fig. 7: (A) 0^{th} timepoint OA/ON, (B) 10^{th} timepoint OA/ON, (C) 0^{th} timepoint OA/ON +pyrene, (D) 10^{th} timepoint OA/ON +pyrene, (E) 0^{th} timepoint OA/ON +hydroxypyrene, (F) 10^{th} timepoint OA/ON +hydroxypyrene

Optimization of the number of DH-RH cycles:

Even though the vesicles were found to be stable, it is important to check whether the individual components of the fatty acid system retain their chemical identity after repeated DH-RH cycling. Thus presence of OA and ON after each cycle was analyzed using TLC. Separation of both the components was clearly visible after the plate was stained with iodine. When observed visually, the intensity of iodine staining reduces over cycles for oleic acid but was maintained in case of oleylamine as we can see in figure 8. This indicates that the oleic acid concentration was reducing over cycles which could be due to the effect of high temperature and low pH conditions. But as the vesicle forming ability is maintained, this slight degradation of OA did not lead to observable detrimental changes. Even though the concentration of fatty acids decreases slightly, for obtaining measurable yield of oligomerized products, the polymerization reaction has to be carried out for more number of cycles. Therefore to find a middle ground between the polymerization yield and degradation of fatty acids, 10 DH-RH cycles was chosen for carrying out the experiment.

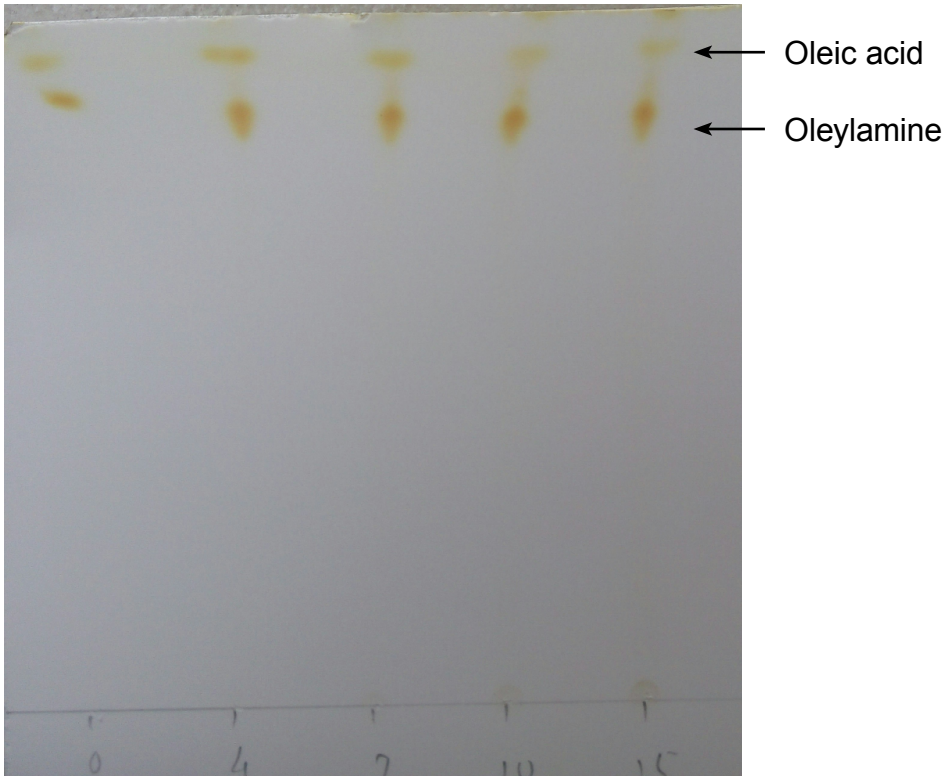

Fig. 8: Thin Layer Chromatography of 0, 4, 7, 10 and 15 timepoints of DH-RH reaction containing 5mM oleic acid/oleylamine with 15mM HCl as rehydrating agent (From left to right) and the solvent being chloroform: methanol: water in volumetric ratio of 65:25:4.

Optimization for Rehydrating Agent:

DH-RH reactions with lipids, for the polymerization of RNA monomers, have been optimized previously in our lab (Mungi & Rajamani, 2015). It was found that polymerization is most efficient at pH 2. At this pH, the nucleophilic attack on the phosphate group in 5'-NMP, which exists in protonated state, is favored by a neighboring nucleotide's 2'-OH or 3'-OH.This was used as guideline for carrying out the rehydration dehydration cycles with fatty acids. The most efficient rehydrating agent in their study was H_2SO_4. However, it was found that H_2SO_4 did not support vesicle formation with oleic acid/ oleylamine system. This puzzling result does not appear in any previous studies and it is unclear why this might br the case as phospholipids readily assemble into vesicles in the presence of ~2 µmoles of H_2SO_4 (Mungi & Rajamani, 2015). We therefore used HCl instead as a rehydrating agent in which the mixed fatty acid system's vesicles formed readily. As the system contains oleylamine, which is basic in nature and gets protonated, the concentration of mineral acid needed to make a pH 2 solution was greater to compensate for the basicity of this fatty acid. The concentrations of HCl tested initially were 10mM, 25mM, 50 mM and 66mM. The resultant HPLC analysis revealed that though the yield was higher when more concentrated HCl was used, the degradation of the RNA by depurination (breakdown) was also quite high, as shown in Fig. 9

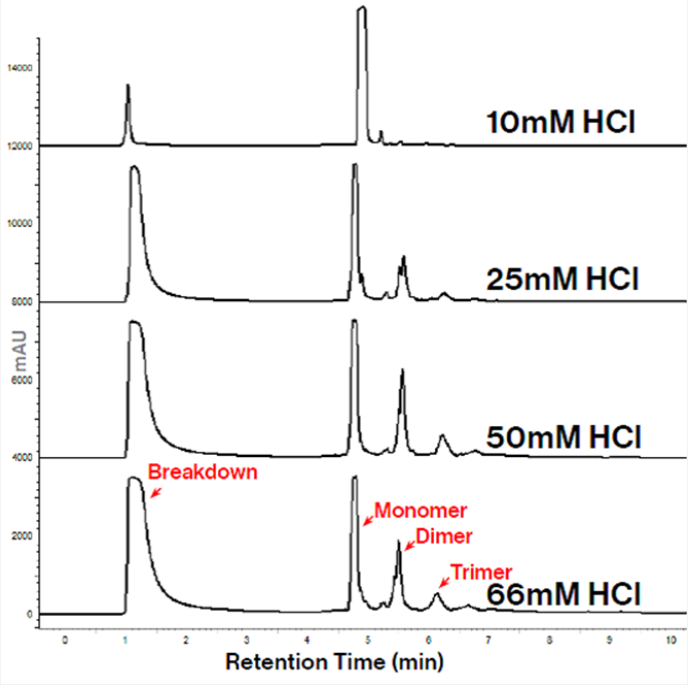

Fig. 9: HPLC chromatogram of 7th timepoint of DH-RH reaction using 5mM adenosine 5'-monophosphate with 5mM oleic acid/ oleylamine rehydrated with 10mM, 25mM, 50mM and 66mM HCl.

Using a lower concentration was advantageous in terms of conservation of the RNA, but it did not give sufficient yield. Therefore, the range of standardization was changed to 15mM and 20mM. Addition of 15mM HCl and 20mM HCl resulted in a pH of 1.5-2 which remained constant throughout the experiment for all concentrations of HCl (Fig. 10). Though the extent of polymerization and breakdown were not significantly different with the HCl concentrations used, the breakdown however was slightly less with 15mM HCl as indicated by the chromatograms and the graph (Fig. 11(A), 11(B), 12) Although visually the difference between the breakdown is not noticeable, the graph of area % indicates a slight increase in breakdown in case of 20mM HCl and therefore 15mM HCl was chosen as the rehydrating agent.

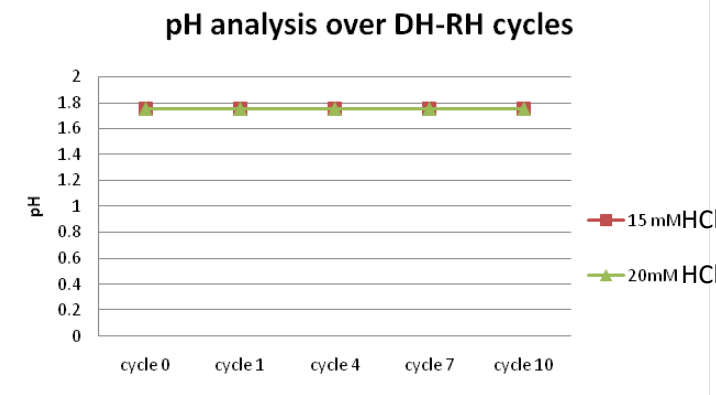

Fig. 10: Representative data for pH analysis of the DH-RH reaction carried out with 15mM and 20mM HCl over the duration of the experiment

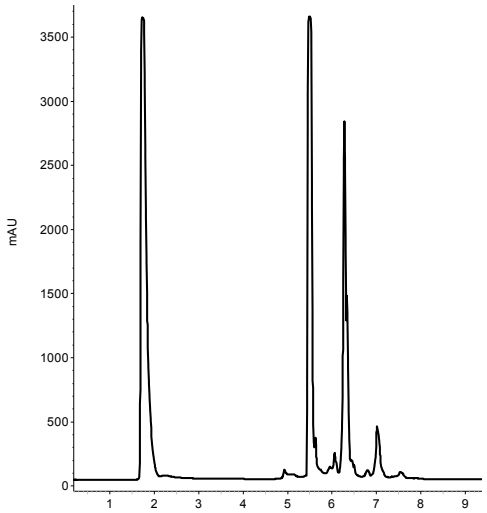

Fig. 11(A): HPLC chromatogram of 10th timepoint of DH-RH reaction using 5mM guanosine 5'-monophosphate with 5mM oleic acid/oleylamine rehydrated with 15mM HCl

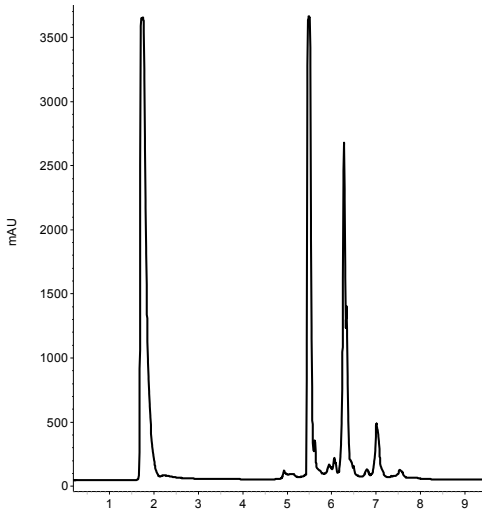

Fig. 11(B): HPLC chromatogram of 10th timepoint of DH-RH reaction using 5mM guanosine 5'-monophosphate with 5mM oleic acid/oleylamine rehydrated with 20mM HCl

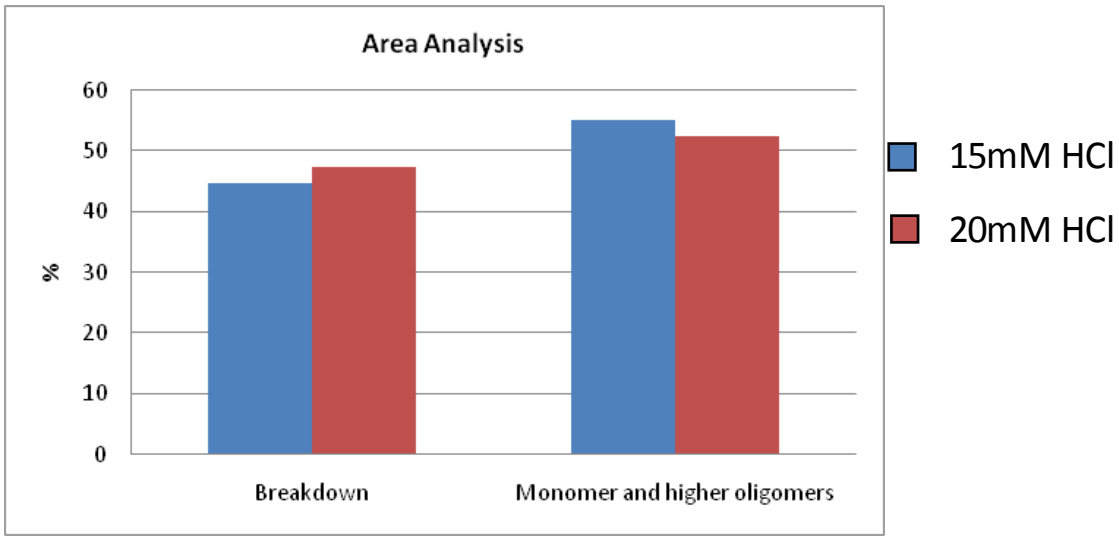

Fig. 12: Representative data for area percentage analysis of the HPLC chromatogram for DH-RH reactions with 15mM and 20mM HCl as rehydrating agent

Analysis of the products formed in the DH-RH reaction:

The DH-RH reactions were carried out in three parts:

1. Reaction with OA/ON fatty acid system
2. Reaction with PAH containing OA/ON fatty acid system
3. Reaction with OA/ON fatty acid system in the presence of clay

1. Reaction with OA/ON fatty acid system:

The DH-RH reaction was carried out at 90°C with 5'-GMP and OA/ON fatty acid system(Fig. 13). For the control, fatty acids were completely omitted from the reaction and the reaction contained only 5'-GMP. The other controls were such that the reaction contained only one of the fatty acids from OA/ON system.

Neither the yield, nor the breakdown in case of the control without any fatty acid was found to be significantly different from that observed when both OA/ON were present in the reaction. Slightly more breakdown was observed when either of the fatty acids was incorporated individually in the reaction as depicted in the graph (Fig. 14). As the difference in the breakdown between the control reactions (without fatty acid) and in reactions containing OA/ON was observed to be minimal, the fatty acid system does not necessarily seem to have an important role in protecting the nucleotides from degradation.

The yield in the control was also found to be higher than the OA/ON system and therefore the use of fatty acids does not seem to be favorable in the reaction. As the control does not contain any basic moiety unlike the Oleylamine present in OA/ON system, the addition of same volume of HCl will result in slightly lesser pH in case of the control. This could explain higher yield of the control as acidic pH promotes polymerization. This reaction was carried out with 5'-GMP as the mononucleotide. To verify if this effect is just specific to 5'-GMP or is valid for all the ribonucleotides, the same reaction was carried out with 5'-AMP, 5'-CMP and 5'-UMP. Surprisingly, when pyrimidine nucleotides- 5'-CMP and 5'-UMP were used in the reaction, no polymerization was observed (Fig. 13). The exact reason for this observation is unclear, but the stacking of purine nucleotides could positively contribute to the organization thus increasing molecular interactions. This could promote the polymerization in purine nucleotides. As pyrimidine nucleotides do not exhibit stacking, in the absence of this promoting effect they would not polymerize as efficiently.

However, when the same reaction was carried out using 5'-AMP, the addition of fatty acids exhibited reduced breakdown as compared to the control (without fatty acid) only in one replicate, as illustrated in the HPLC chromatograms (Fig. 15) and the % area analysis graph (Fig 16). Even though OA/ON systems retains their vesicle forming capacity at such harsh conditions, they may not able provide any advantages to the reaction.

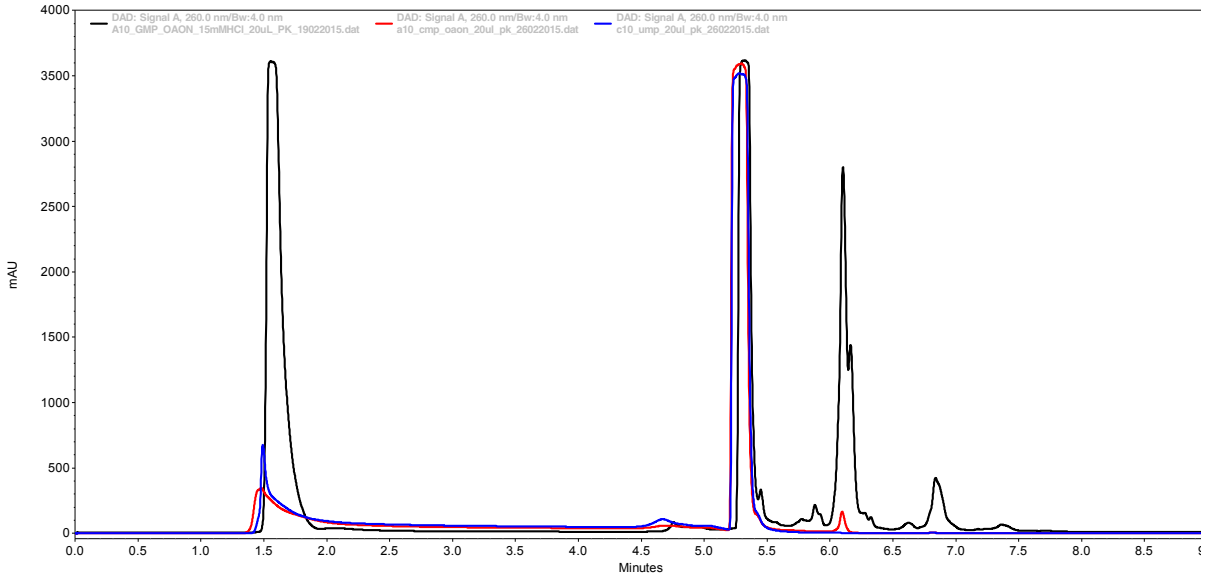

Fig. 13: HPLC chromatogram of 10th timepoint of DH-RH reaction using 5mM guanosine 5'-monophosphate(Black), 5mM cytosine 5'-monophosphate(Red) and 5mM uridine 5'-monophosphate(Blue) with 5mM oleic acid/Oleylamine rehydrated with 15mM HCl

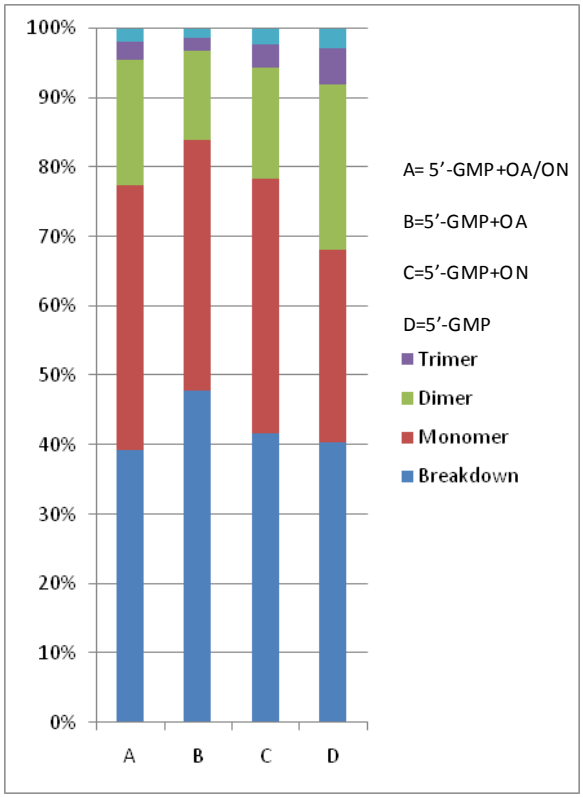

Fig. 14: Representative data for comparison of products formed in DH-RH reaction with 5'-GMP in presence and absence of fatty acids.

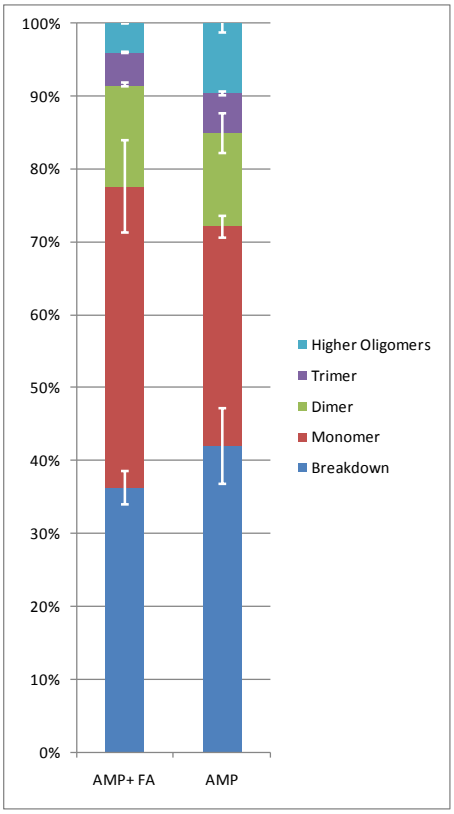

Fig. 16: Comparisonomparison of products formed in DH-RH reaction with 5'-AMP in presence and absence of fatty acids. Error bars reflect standard deviation of data collected from two independent experiments.

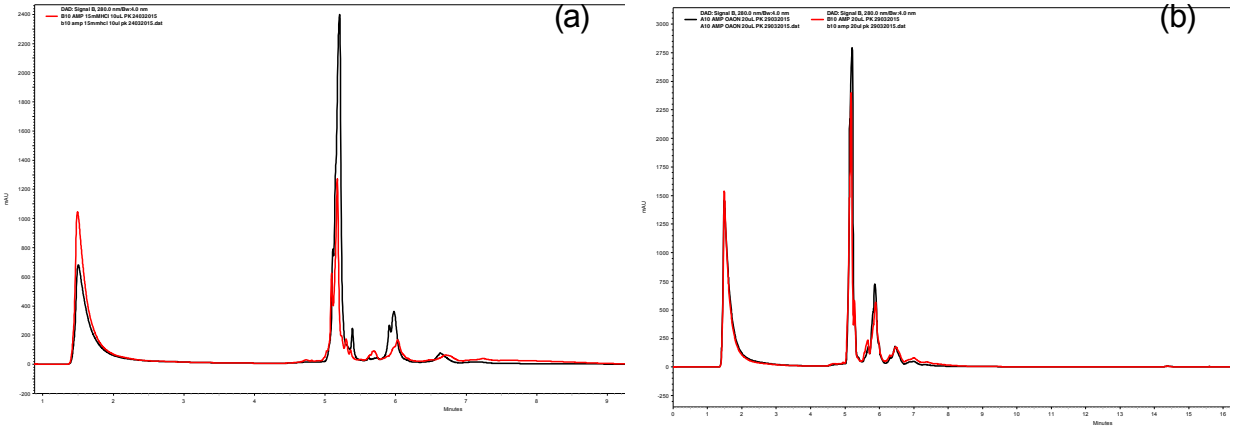

Fig. 15(a, b): HPLC chromatogram of 10th timepoint of DH-RH reaction using 5mM Adenosine 5'-monophosphate with 5mM oleic acid/oleylamine (Black) and without fatty acid (red) rehydrated with 15mM HCl. (a) is from data set 1 and (b) is from data set 2.

2. Reaction with PAH containing OA/ON fatty acid system:

To analyze the role played by PAH in the polymerization of RNA monomers, the DH-RH reaction was carried out at 90°C with either 5'-GMP or 5'-AMP in the presence of PAH containing OA/ON fatty acid system. The control reaction did not contain the fatty acid system and comprised of only the nucleotide.

HPLC analysis of the products that were formed revealed that in case of 5'-GMP, the breakdown in OA/ON containing reaction was higher than the control in all cases i.e. both in the case of the reaction when only OA/ON system was present and when PAH was incorporated in the OA/ON system. The yields were also lower than the control. Similarly, reactions containg 5'-AMP showed no significant differences in the percentage of breakdown seen even in OA/ON containing reaction across all cases (Fig 17). This demonstrates that neither fatty acids nor PAH offer any benefit to the polymerization reaction involving 5'-GMP or 5'-AMP. A part of this observation is contrary to earlier studies which used only phospholipids as the amphiphile in the DH-RH reactions (Mungi and Rajamani, 2015). To understand this difference stemming from the nature of the amphiphile used in more accurate manner, one has to study the organization of the system at elevated temperature by performing structural studies using techniques like x-ray diffraction.

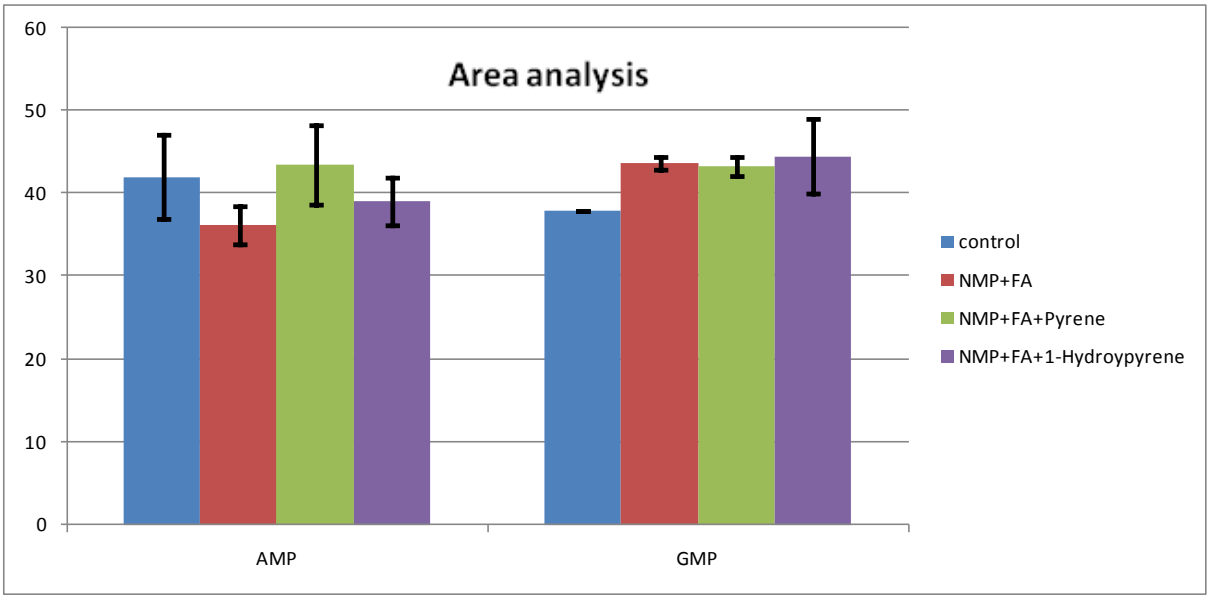

Fig. 17: Comparison of % breakdown between 5'-AMP and 5'-GMP in PAH containing vesicles. Error bars reflect standard deviation of data collected from two independent experiments.

3. Reaction with OA/ON fatty acid system in the presence of clay:

For this subset, the DH-RH reaction was performed at 90°C with ribonucleotides, Montmorillonite clay and OA/ON fatty acid system. Fatty acids were excluded from the reaction in case of the control. After the completion of the reaction, the sample processed by fatty acid extraction and clay extraction was subjected to HPLC analysis. This analysis of the products formed in the reaction showed an interesting trend. When 5'-AMP was used in the reaction with fatty acids, the breakdown observed was less throughout the duration of the experiment. But in case of 5'-GMP with fatty acids, initially the breakdown was less compared to the control, whereas in later cycles the control reaction showed lesser breakdown. This corroborates what we saw in Fig. 17. However, it is not yet clear what the underlying reason for this might be.

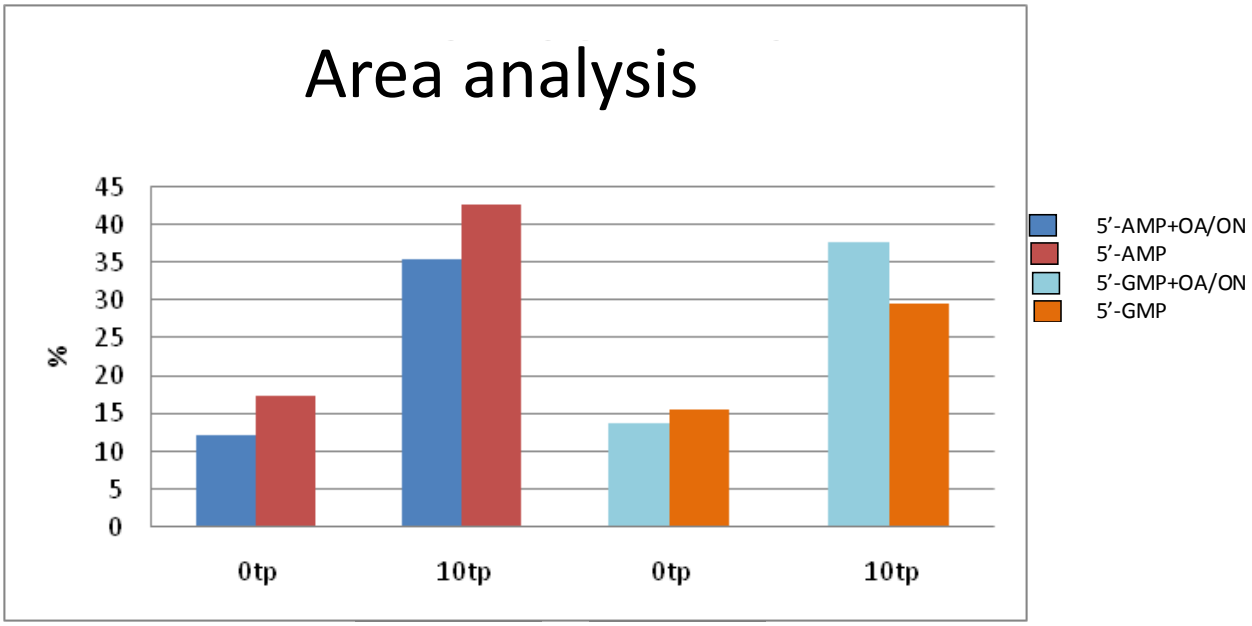

Fig. 18: Representative data for comparison of % breakdown between 5'-AMP and 5'-GMP carried out with vesicles in presence of clay.

The yield of the reaction containing 5'-AMP with clay and fatty acids on the other hand was negligible as there was no polymerization seen during HPLC analysis as shown in the figure 19. The breakdown of the nucleotides increased over DH-RH cycles without leading to formation of oligomerized product. Similar results were obtained in the reaction containing 5'-GMP.

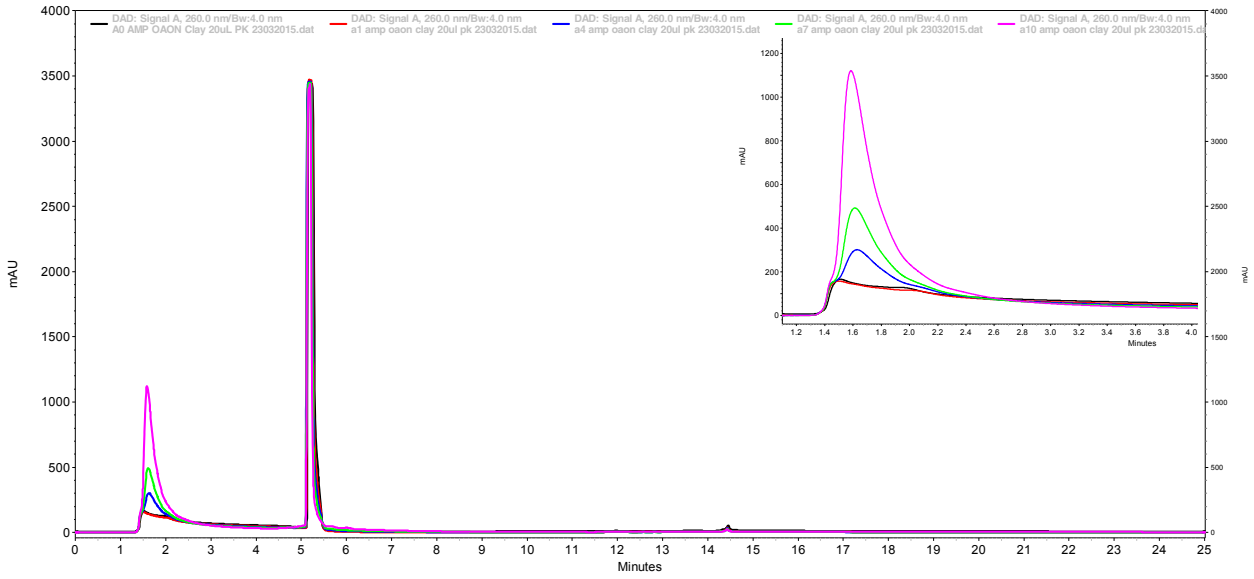

Fig. 19: HPLC chromatogram of 0th (black), 1st (red), 4th (blue), 7th (green) and 10th(pink) timepoint of DH-RH reaction using 5mM Adenosine 5'-monophosphate with 5mM oleic acid/oleylamine with montmorillonite clay rehydrated with 15mM HCl. Inset shows a zoomed version of breakdown.

Mass analysis of oligomerized products:

To confirm the identity of products, mass analysis was carried out using HRMS technique. The kinds of products that we suspected could be formed in the reaction, based on Mungi and Rajamani 2015, are illustrated in fig. 20.

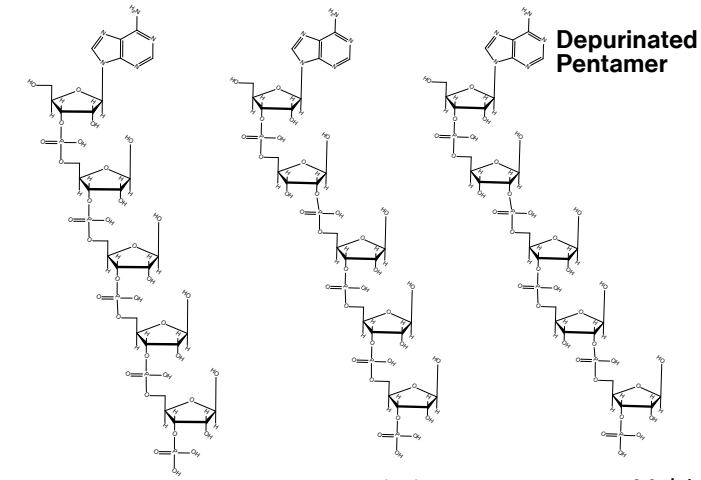

Fig. 20 : Suspected products formed in DH-RH reaction with 5'-AMP

As we subject the RNA to extreme temperature and pH conditions, the adverse effect that occurs is that the nucleotides lose their nitrogenous base. This was observed in the HRMS profiles obtained and are shown below in Fig 21.

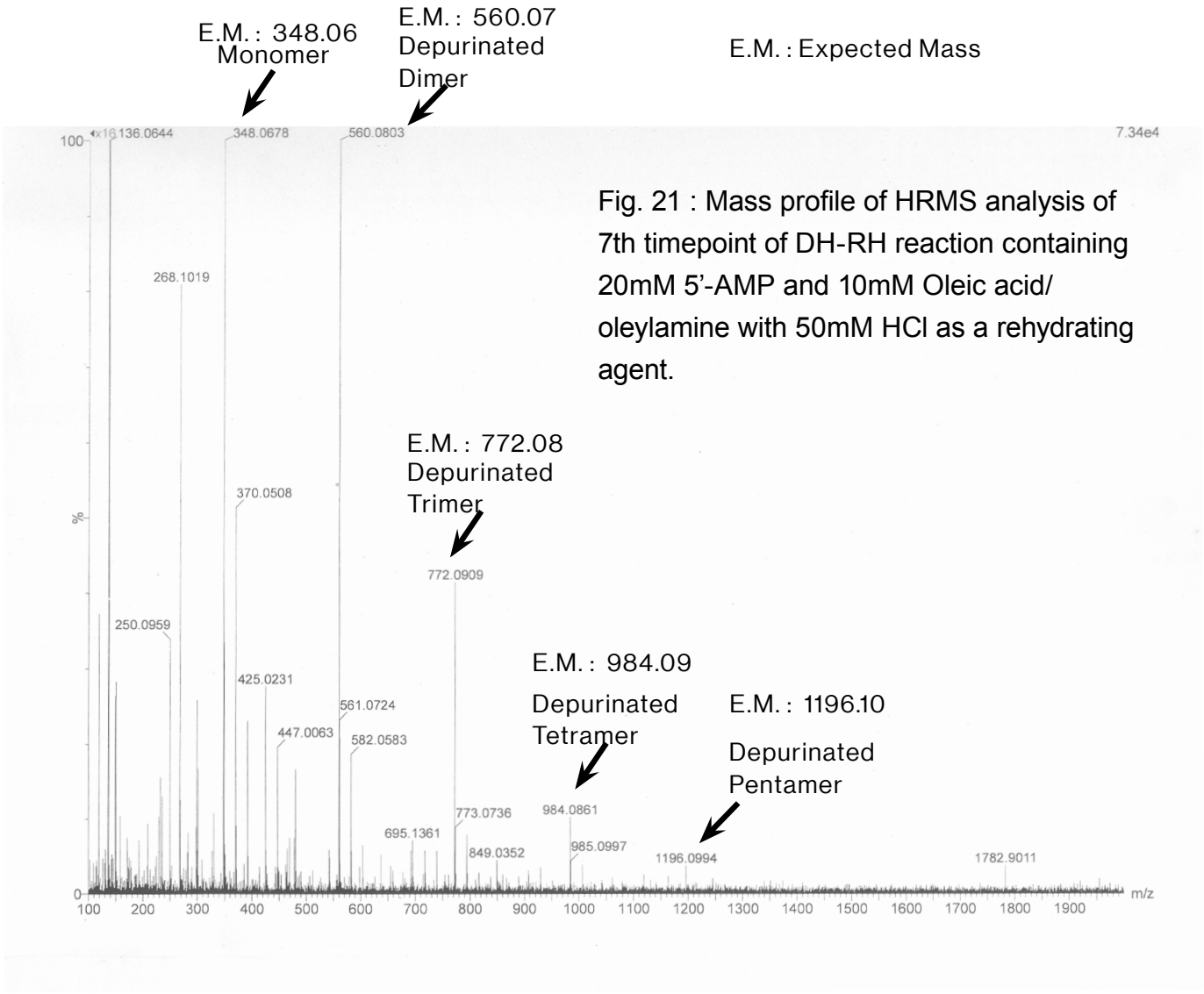

Fig. 21 : Mass profile of HRMS analysis of 7th timepoint of DH-RH reaction containing 20mM 5'-AMP and 10mM Oleic acid/ oleylamine with 50mM HCl as a rehydrating agent.

Table 5 outlines the results of DH-RH reaction involving 5'-AMP obtained through mass analysis which conform to the results acquired through HPLC analysis. The mass analysis of 5'-GMP could not be performed as the precipitation of 5'-GMP hindered the detection process. We are working to find a way around this.

Table 5 : Assignment of chemical identities to peaks from HRMS profile based on expected and observed mass numbers

Chemical species	Expected Mass	Observed Mass
Adenine	136.0617	136.0644
AMP	348.0703	348.0678
AMP Dimer - 1 Base	560.0789	560.0803
AMP Trimer -2 Bases	772.0875	772.0909
AMP Tetramer - 3 Bases	984.0961	984.0861
AMP Pentamer - 4 Bases	1196.1047	1196.0994

Analysis of the products formed in the clay catalyzed reaction:

Preliminary studies were undertaken to look at the combined role of lipid and clay on oligomerization of imidazole activated ribonucleotides. The eutectic phase reaction was carried out with activated mononucleotides, clay and oleic acid at -18°C. As a control, fatty acid was omitted from the reaction mixture. The same reactants were also incubated at room temperature and at 4°C to assess the effect of eutectic phase on fatty acid-assisted polymerization. The breakdown in case of all the conditions was similar but the yield of polymerized product was higher in case of-18°C as compared to 4°C, which was in turn higher than the room temperature reaction (Fig. 22, 23). This could be attributed to the fact that, at lower temperature, dissociation of imidazole group of activated nucleotide is less. As the nucleotide exists in the activated state for a longer time, it is capable of carrying out more number of polymerization reactions, leading to an increase in the yield of oligomerized product. This also explains higher yield in the reaction carried out at 4°C as compared to the reaction carried out at room temperature.

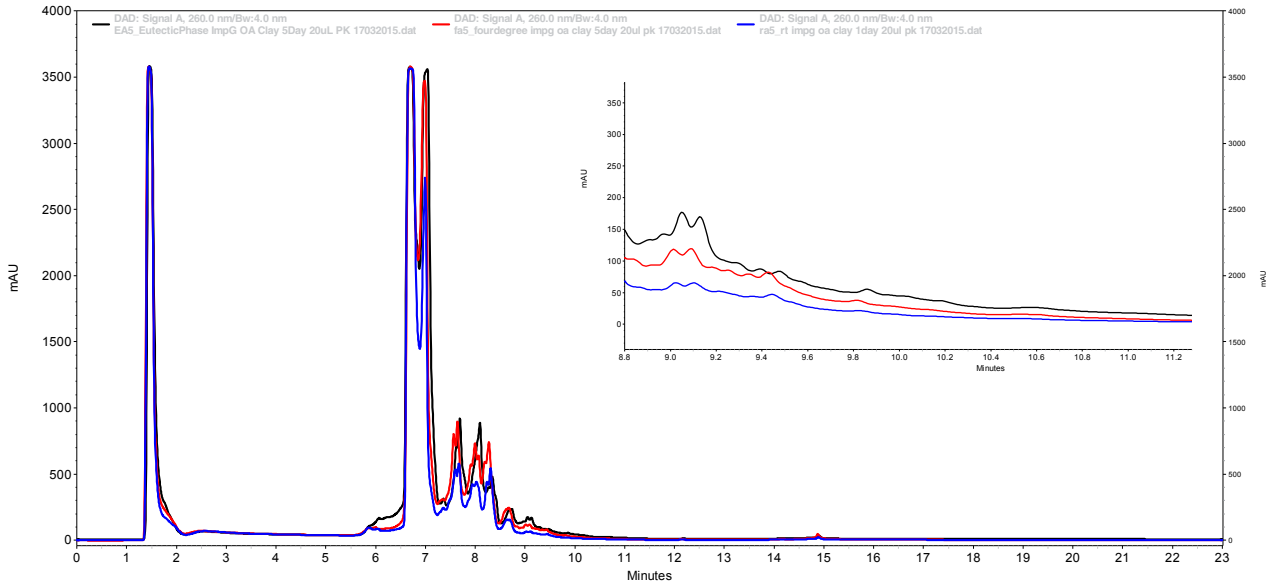

Fig. 22: HPLC chromatogram of 5th day time point of clay catalyzed reaction containing 10mM imidazole guanosine phosphate (ImpG) with 5mM oleic acid at eutectic phase temperature (Black), 4oC (Red) and room temperature (Blue). The inset represents a zoomed in image of the same chromatogram to demonstrate higher yields in the reactions carried out at eutectic phase temperature (black).

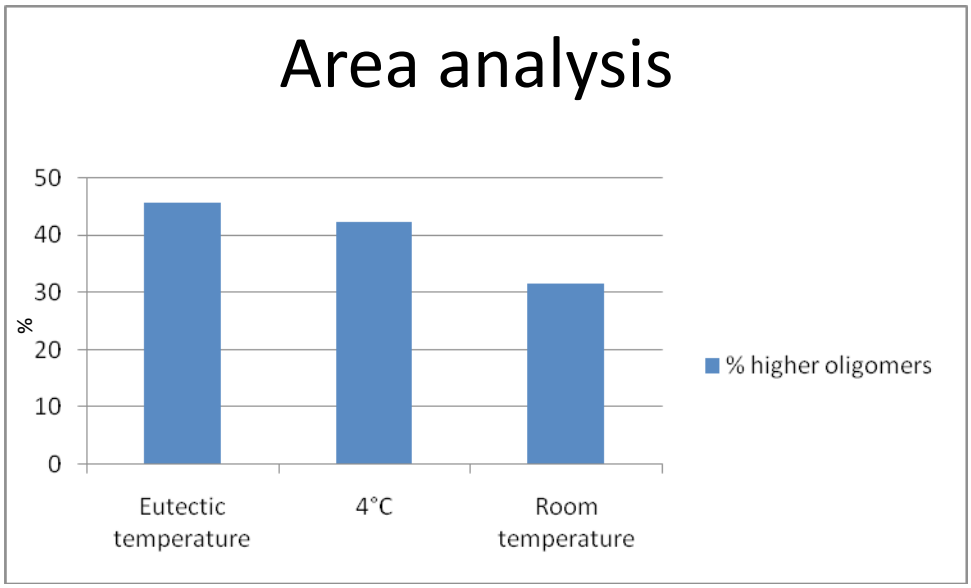

Fig. 23: Area % of higher oligomers formed at different temperature in the reaction containg 10mM imidazole guanosine phosphate (ImpG) with 5mM oleic acid

There is hardly any difference in the breakdown in the presence or absence of fatty acids. However, the yield of higher oligomers appeared to be more in the presence of fatty acids(Fig. 24). Oleic acid was expected to form vesicles at the pH of this reaction, which is 7. But the microscopic studies revealed that the oleic acid vesicles do not form. Thus, the absence of organized fatty acid structure could account for this similarity in the breakdown and yield. Therefore, again the contribution of fatty acids in promoting polymerization of activated nucleotides is questionable.

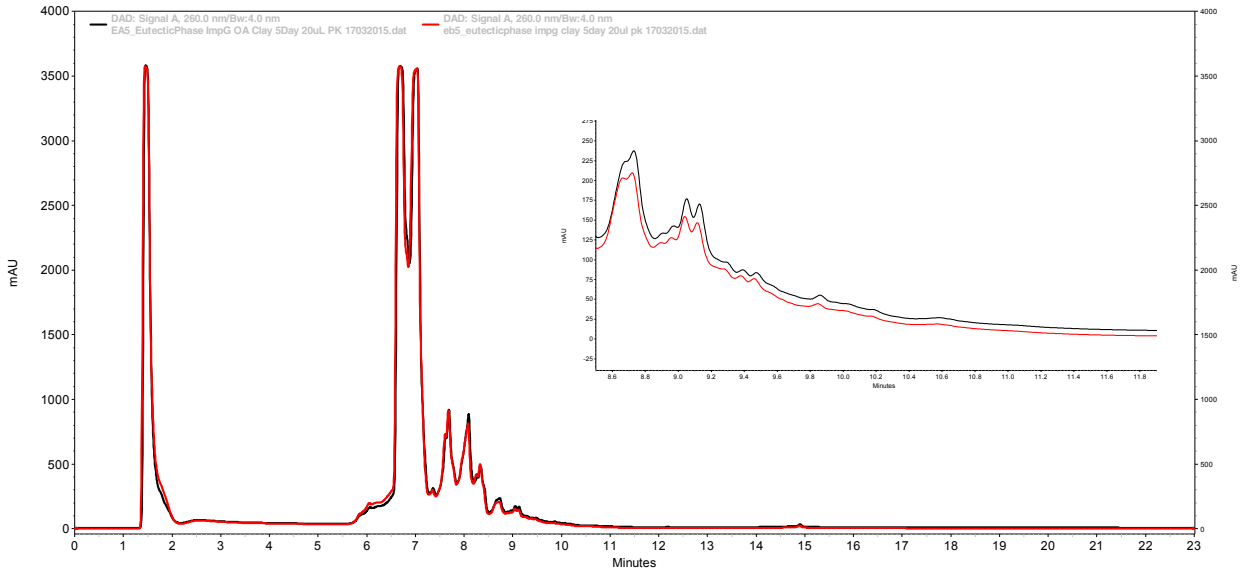

Fig. 24: HPLC chromatogram of 5th day time point of clay catalyzed reaction containing 10mM imidazole guanosine phosphate (ImpG) with 5mM oleic acid (Black) and without oleic acid (Red) at eutectic tempurature. The inset contains a zoomed in image of the same chromatogram to highlight that in the presence of fatty acids the absorbance of oligomerized products is higher, indicating slightly higher yield.

A higher concentration of activated nucleotides (depicted in the table 6, 7) was used for carrying out the reaction for 12 days at room temperature to analyze its effect on the extent of polymerization. As indicated in Table 6, 7, the concentration of ImpU and ImpC used in the experiment was 15-16mM respectively. Due to an error, different concentrations of activated nucleotides were used for purines and pyrimidines. Therefore, we can only compare among themselves and not purine vs pyrimidine. In case of purines, the yield was greater for ImpG as compared to ImpA, and ImpG also showed lesser breakdown.

Among the pyrimidines, the reaction with ImpC shows lesser breakdown and a better yield in comparison to the reaction with ImpU, as shown in the fig. 25. Studies are ongoing with respect to using the same concentration for all activated nucleotides to see if there is a difference in the polymerization potential under similar conditions.

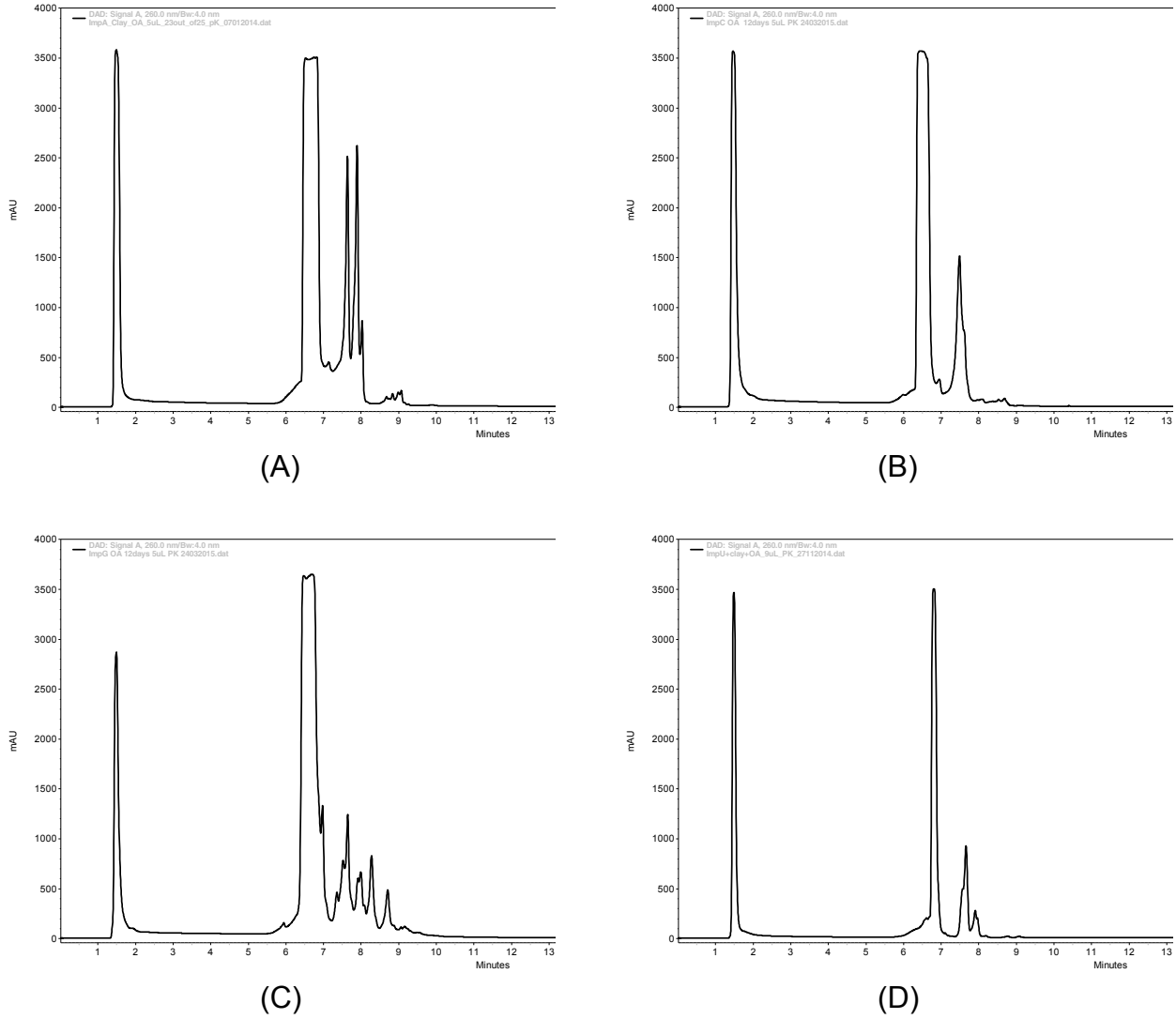

Figure 25: HPLC chromatograms of 12 day timepoint of clay catalyzed reactions with 3.5mM oleic acid at room temperature containing (A) 25.75mM imidazole activated adenosine phosphate (ImpA), (B)15mM imidazole activated cytosine phosphate (ImpC), (C)25.35mM imidazole activated guanosine phosphate (ImpG) and (D)16.25mM imidazole activated uridine phosphate (ImpU).

Table 6: Analysis of reaction containing activated purine nucleotide

Sr. No	Activated Nucleotide in the reaction	Concentration (mM)	Breakdown	Yield %
1	ImpG	25.35	12.947	20.1778
2	ImpA	25.75	21.9551	15.8523

Table 7: Analysis of reaction containing activated pyrimidine nucleotide

Sr. No	Activated Nucleotide in the reaction	Concentration (mM)	Breakdown	Yield %
1	ImpU	16.25	29.9349	12.5042
2	ImpC	15	26.6507	16.5372

In conclusion, our studies conclude that fatty acids do not promote efficient oligomerization of ribonucleotides. They also do not confer any protection on the newly formed oligomers, which is in contrast to what phospholipids have been shown to do (Mungi and Rajamani, 2015). Additionally, preliminary data from our studies that look at the collective role of catalysts (e.g. clay and lipids) did not reveal any added effect when combinations of catalysts were used to drive nonenzymatic polymerization. This is different from the outcome of related studies in the lab, where in we are looking at the combined role of phospholipids and clay on nonenzymatic polymerization. Our hypothesis for this differential effect, stemming possibly from the chemical identity of the lipid (fatty acids vs phospholipid), is that these two amphiphiles form very different structures under dehydration conditions. This can be demonstrated conclusively with targeted biophysical studies (e.g. Small Angle X-ray Scattering studies [SAXS]) that could shed light on the nature of the matrices resulting from fatty acids vs phospholipids. In one recent study, SAXS was used to characterize the matrices formed on dehydration of1, 2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) vesicles. The study concluded that the lamellae formed by dehydrated DMPC vesicles could align reacting solutes in orientations that favour uphill reactions like polymerization. In future studies we aim to characterize potential biophysical differences as this has implications for understanding the role of prebiotic amphilies in polymerization of ribonucleotides into RNA-like oligomers.

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