Lipid assisted peptide formation under SIPF conditions

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

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Certificate

This is to certify that this dissertation entitled **"Lipid assisted peptide formation under SIPF conditions"** 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 **Mr. Gaurav Arya at IISER Pune** under the supervision of **Dr. Sudha Rajamani, Asst. professor, biology** during the academic year 2013-2014.

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Declaration

I hereby declare that the matter embodied in the report entitled **"Lipid assisted peptide formation under SIPF conditions"** 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.

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Abstract

Theories on chemical origin of life suggest that amino acids and peptides were among the first biomolecules that were formed on the prebiotic Earth. Salt-induced peptide formation (SIPF) in aqueous solution is one of the simplest models for peptide synthesis on early Earth which forms peptides with the help of copper ions and sodium chloride. We revisited this reaction to determine the catalytic role of lipids in SIPF. From our results, we demonstrate the formation of short L- His peptides in presence of lipids. In L-His based SIPF reactions, carried out in the presence of POPC and under dehydration/rehydration conditions, we were able to detect up to a trimer, which has been shown to date in only two special cases of SIPF. In most other SIPF reactions of L- His in the presence of lipids, which included clay or a catalyst, we could demonstrate the formation of up to a dimer. To date, the best yields of a His-His dimer has been shown to be about 0.3%. Despite our results being qualitative, we can state with reasonable confidence that presence of lipids in the reaction seems to increase the yields of the reaction to some extent. These results point to a potential catalytic role of lipids in SIPF reactions. Delineating the reaction mechanism underlying this process will enable a better understanding of how the two components of the first primitive cell, i.e. the encapsulating membrane and the encapsulated genetic material, might have interacted to allow for the emergence of small peptides on prebiotic Earth.

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Introduction

Origin of life has been one of the greatest mysteries that has evaded scientists for a long time. There are several views about the chemical origin of life and many intriguing scientific ideas that explain how life might have originated on earth, but there is very little that we know for sure. One cannot deny the idea that the chemical origin of life might have had multiple possibilities. People in the sixteenth and early seventeenth centuries extensively believed that life might have transformed from water, stones and other inanimate objects(Oparin, 1961). The idea that maggots in rotting meat, fleas in dung and intestinal worms are generated spontaneously in decaying materials was mostly accepted then. However, as science advanced, the study of nature involved more of the experimental and observational arguments than that of philosophical views, and, hence people who believed that spontaneous generation could occur became less in number. In 1862, Louis Pasteur conducted experiments that rejected the idea of spontaneous generation, even for microbes, and showed that a sterile broth would "spoil" only if microorganisms could invade from the environment. The first concise theory for an origin of life from inorganic chemical building blocks was given independently by two scientists; Alexander Oparin in 1927 (Oparin, 1961) and J.H. Haldane in 1929, who also coined the term 'primordial soup'. According to this theory, the prebiotic Earth contained all necessary components for life to come about in the 'primordial soup', which then finally lead to living systems by a process of chemical evolution.

The primordial Earth formed about 4.6 billion years ago. Few hundred million years after the bombardment of meteorites, it is thought that the very hot Earth's crust cooled down to about the same temperature as that of the boiling point of water(Pascal et al., 2006). The presence of liquid water seems to be the most important condition for life to develop on the early Earth and, therefore, the most basic condition for chemical evolution to have started. Thunderstorms on the prebiotic Earth formed hot primordial

oceans, lakes and lagoons with salts and a variety of small compounds dissolved in them. There was very little atmospheric oxygen so it is thought that there was a reducing atmosphere. Energy sources, such as lightning, volcanic activity, and ultraviolet sunlight, were more intense than what we experience today. One scenario for the origin of life is that it originated in dilute, hot soup of ammonia, formaldehyde, formic acid, cyanide, methane, hydrogen sulfide and organic hydrocarbons either at the ocean's edge, or in hydrothermal deep-sea vents, or someplace else(Plaxco and Gross, 2006). The consensus among researchers is that life arose spontaneously from such early mixtures less than 4 billion years ago. While the way in which this happened remains a puzzle, the curiosity about how the earliest steps happened that eventually led to the origin of life, is still buzzing among the researchers.

Some of the predominant theories on how life may have originated include the following:

1. **Extraterrestrial origin** (panspermia): Life might not have originated on Earth at all; instead, life might have come to Earth from some other planet. This view talks about how origin of life might have taken place in some other part of the universe and was brought to early Earth by meteorites. In our opinion, this only transfers the problem of actually understanding how life might have originated to a different setting!

2. **Spontaneous chemical origin**: Life might have evolved from inanimate matter. In this view, the fundamental forces leading to life was selection and evolution. As changes in molecules increased their stability and made them to persist longer, these molecules could initiate more and more complex associations, eventually leading to the formation of primitive cells. In other words, the first cells might have originated by chemical evolution on a young Earth.

Most scientists understandably favour the hypothesis that life on Earth evolved from nonliving materials that became ordered into aggregates that were capable of self-replication and metabolism. The first building blocks and biological precursor molecules had to be formed in hot and salt rich environment in the presence of strong UV radiation coming from the Sun. Aggregation of these monomers would have led to the assembling of polymers, which would have needed to have the ability to carry

information, to replicate, and to pass on their informational content to the next generation of molecules. Based on our current understanding, the only biologically relevant molecules that can fulfil these requirements, in part or full, are nucleic acids and peptides/proteins.

The theory of chemical evolution rests upon three basic assumptions:

1) The hypothetical primitive atmosphere must have been either reducing or neutral. This means that there was no free oxygen in the atmosphere in the Earth’s distant past.

2) Simple molecules like amino acids, purines, pyrimidines, sugars etc were formed within this atmosphere under the action of various parameters including ultraviolet radiation, electrical discharges, radioactivity, and thermal energy.

3) In the course of time, these molecules might have given rise to precursors of proteins and nucleic acids and other primitive cellular components, which in turn gave rise to the first cellular entities, the protocells, and finally to the living cell.

These assumptions lead to predictions that can be tested in the laboratory. In 1953, for the first time, Stanley Miller and Harold Urey tested the basic hypothesis by recreating conditions in the laboratory that had been postulated for early Earth(Miller, 1953). They filled a flask with the four gaseous components, which were suggested to be part of the prebiotic atmosphere by Oparin, i.e. hydrogen, ammonia, methane and water vapour (a reducing atmosphere). By passing electrical discharges through this flask, to stimulate primordial lightening, Miller was able to show the formation of several amino acids and other relatively complex organic molecules of biological importance, from simpler molecules. There is a huge debate about whether the primitive atmosphere was really reductive. However this great experiment demonstrated the fact that relatively complex biochemicals can be formed from a mixture of very simple components through a chemical pathway that can be regarded as prebiotic.

In the past six decades since the Urey-Miller experiment, a variety of abiotic amino acid synthesis have been shown using different kinds of energy inputs and gas mixtures in a reducing atmosphere, but in 2004 Rode and his colleagues showed the possibility of

formation of several amino acids in a completely neutral atmosphere, consisting only of nitrogen, carbon dioxide and water vapour (Plankensteiner et al., 2004). Miller's reaction was revisited by his student Jeffrey Bada who, with the help of his colleagues, did the experiment once again, in addition to re-analyzing samples from Miller's original experiment. They have indeed demonstrated the synthesis of a range of amino acids with the help of new and improved analytical techniques and were able to come up with better resolved and extensive results as compared to Miller(Johnson et al., 2008).

Paths of chemical evolution:

There are many different models and approaches that researchers have presented in the literature to describe what the origin of life might have been like. Each one of them is very unique and resides on a set of prebiotic assumptions and has a different set of experiments to validate their hypothesis. All of them have their advantages and disadvantages and have been discussed below in brief. One of the major scenario that is accepted by most researchers is the "RNA world" approach and the second is the "metabolism first" approach. There are, however, other alternate approaches that talk about co-evolution of RNA world and peptide world, which are the theories that lay the foundation for our work.

I. RNA world approach

RNA world is largely based on the assumption that RNA came about earlier that DNA and proteins and that DNA and proteins are derived from RNA (Gilbert, 1986; Orgel, 2008). The discovery of the catalytic activity of RNA further solidified the theory of the RNA world (Robertson and Joyce, 2012). The researchers who agree with this concept as the most plausible explanation of the origin of life, question the role of peptides in the origin of life as RNA can act as both as an enzyme and information carrying molecule. Could this RNA World have been the main path for the origin of life? This is currently still an open question. The RNA world scenario seems to be too complex to have arisen without synthesis by a genetic precursor or prior enzyme-less metabolism. It is believed that the RNA world might have been preceded by another system of replicating molecules called the pre-RNA world (Orgel, 2004). A pre-RNA world in which the

backbone of the first genetic material would have been different from the ribose phosphate seems more likely, but the nature of this backbone is unknown (Nelson et al., 2000). Despite this criticism of the prebiotic RNA world, there is a general consensus that RNA was a key part in determining, if not the origin of life, then its early evolution.

II. Metabolism first approach

The metabolism first theory argues for the appearance of primitive self-catalytic metabolic networks as a first step towards the synthesis of replicators. Metabolism here means a cycle of chemical reactions that produce energy in a form that can be harnessed by other processes. This theory suggests that nucleotides, the fundamental unit of nucleic acids like RNA, are too complex to have formed spontaneously again and again. Formation of these would have required self-organized prebiotic processes that would have provided a scaffold for the emergence of genetic molecules. This theory also suggests that once a primitive metabolic cycle was established, it would have begun to produce ever more complex compounds. The metabolic machinery today is a complex network of highly controlled chemical transformations whose specificity and selectivity depend on the catalytic activity of enzymes. Reasonably, the initial metabolic machineries were much simpler, and the network of metabolic reactions were not so orderly and less selective than extant metabolisms

Two other lesser accepted theories of origin include the below two possibilities:

III. Peptide-Nucleic Acid World

Miller's experiment, as mentioned earlier, demonstrated that amino acids are easy to form in prebiotic atmosphere, unlike nucleotides, and these amino acids could have then assembled to form simple peptides. However, many don't subscribe to this idea because proteins cannot copy themselves. Therefore, the potential difficulty with this scenario is that it would need a subsequent process that would have converted the amino acid sequences into genetic information. This leads to an alternate theory with respect to origin of life, which talks about the possibility that the first informational molecules may have evolved with the help of both peptides and nucleic acids (Fig 1). It assumes that there must be a pre-RNA world where peptide nucleic acid (PNA)

molecules were the basis for life , which had a peptide-like backbone and nucleotide bases on the side chains (Nelson et al., 2000).This way, peptides could have played both the role of information carriers and that of catalysts in a pre-RNA world. Unlike RNA, which is known to be pretty unstable, PNA is stable and might have been synthesized by plausible prebiotic means(Nelson et al., 2000). PNA binds DNA and forms double and triple helical structures that are similar to the Watson-Crick helix (Nelson et al., 2000), and recently, it has also been shown to be able to self-replicate (Plöger et al.).

IV. Rinonucleoprotein World

One other theory in this category talks about the interplay of RNA and small peptides very early on to form complexes called ribonucleoproteins (something like a very primitive ribosome). It is believed that ribozymes evolved into ribonucleoproteins, which gradually lost their RNA components to give way to modern protein enzymes but the problem with this theory is that we don't find any clues in today's world that actually point to this. The idea that life began in a system linking nucleic acid replication and genetically coded peptide synthesis has also been presented (Pascal et al., 2006). There is a growing view in the field that the two pathways cannot be viewed as two separate processes. Given this interesting and realistic possibility, in our lab, we are interested in looking at how small peptides might have arisen on early Earth by relevant abiotic means.

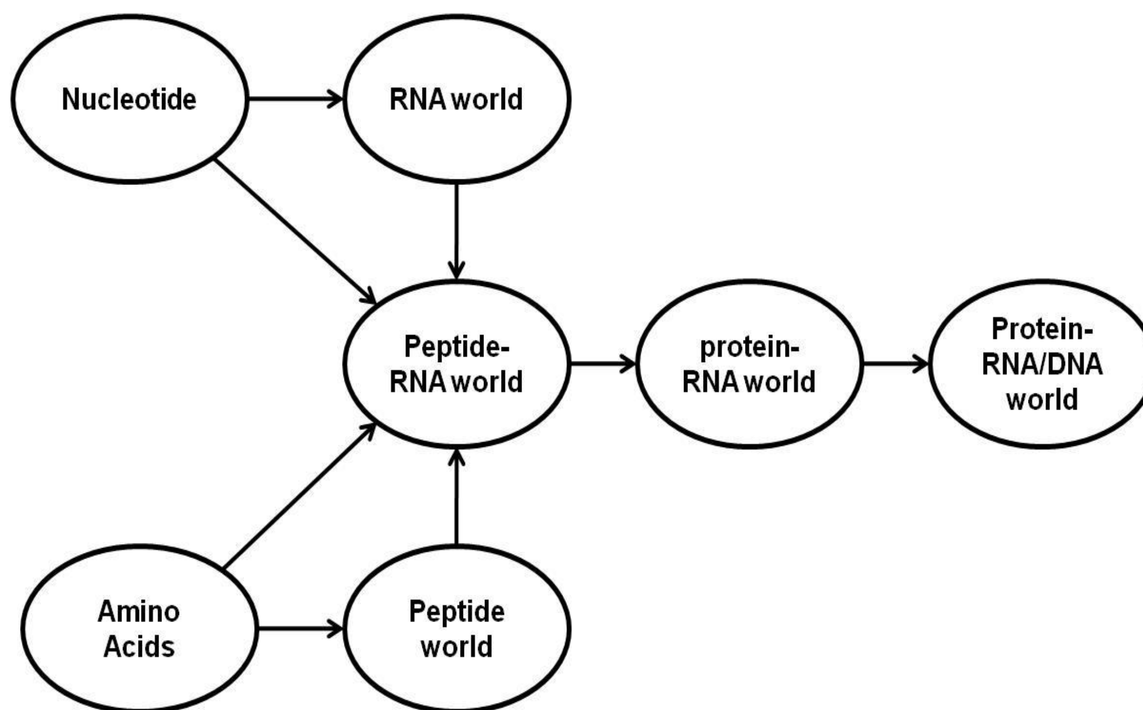


Fig 1: Schematic representation of how chemical origin of life might have come about.

Abiotic peptide synthesis

Abiotic peptide synthesis has been shown to occur under prebiotic conditions by a few different means that include Salt Induced Peptide formation (SIPF) reactions (Schwendinger and Rode, 1992), clay mediated peptide formation (Rode et al., 1998), peptide synthesis using simple elements like hydrogen cyanide, iron and carbonyl sulfide (Huber, 1998). Some amino acids in the latter case form dimers in yields of 0.2–5%, and, primarily, these results demonstrated that amino acids can be activated under geochemically relevant conditions. One other reaction involved carbonyl sulfide (COS), a simple volcanic gas. Results from this study showed that, depending on the reaction conditions, additives used and the interaction between the α-amino acids and COS etc, up to trace amounts of hexapeptide were generated in reasonably short time scales at room temperature (Leman et al., 2004). However, the prebiotic relevance of ambient temperature conditions used in this reaction seems implausible. The SIPF reaction is particularly relevant as, according to most discussions of the prebiotic theory, biological precursor molecules might have formed and evolved in the context of a sodium chloride ocean on primordial Earth (Danger et al., 2012; Rode, 1999; Schwendinger and Rode,

1992; Schwendinger et al., 1995). We hence decided to concentrate on these reactions and are particularly interested in characterizing the potential catalytic role of lipids in the formation of peptides by SIPF.

SIPF reactions

Rode and co-workers found that sodium chloride acts as a dehydrating agent to overcome the thermodynamic barrier of peptide bond formation in aqueous solution. Two scenarios can be thought up for reaching the higher concentrations of salt which is needed for dehydrating action and both of these can be reproduced in experiments. One pertains to large-volume lagoons with constant replacement of the evaporated water by rain and tides, which can be mimicked in constant volume experiments with reflux condensers. The other scenario considers smaller puddles along the shorelines of oceans, where the water would quickly evaporate during the day and then be replaced by rain and tides. Importantly, the reaction involves copper, Cu (II), ions that have the ability to form complexes with amino acids, which brings two reaction partners together and keeps their reactive groups in the neutral state that is required for a peptide bond formation. The precambrian rocks which consist mostly of copper minerals provides a strong evidence for the presence of Cu (II) on prebiotic earth. Another evidence is the modern estimations of the oxygen content of the prebiotic atmosphere, ranging from 10^{15} to 0.1atm of the present atmospheric level, which favours the presence of copper ions in the divalent state (Rode and Suwannachot, 1999). The superiority of copper to accelerate and enhance peptide formation in systems containing high concentrations of sodium chloride has been demonstrated(Rode and Suwannachot, 1999). Cu (II) is thought to act as central ion for the formation of a complex with one chloride ligand, two H_2O molecules and the two interacting amino acids (Fig 2). One of them chelates via its amino N-atom and the carboxylate O-atom, while the other one only coordinates end-on via one of its carboxylate O-atoms.

Exploring more realistic peptide synthesis mechanisms would include a combination of two or more of the aforementioned abiotic synthesis reactions. One such experiment describes a combination in which SIPF was carried out in the presence of clay (Rode et al., 1998). In our experiments, we have included yet another crucial element i.e. lipid

membranes. So, in addition to NaCl and $CuCl_2$, we also used lipids to check its role in catalyzing the forward reaction that favours peptide formation as there is reason to believe that they might have played an active role in catalyzing the formation of peptides on early Earth (Zepik et al., 2007)..

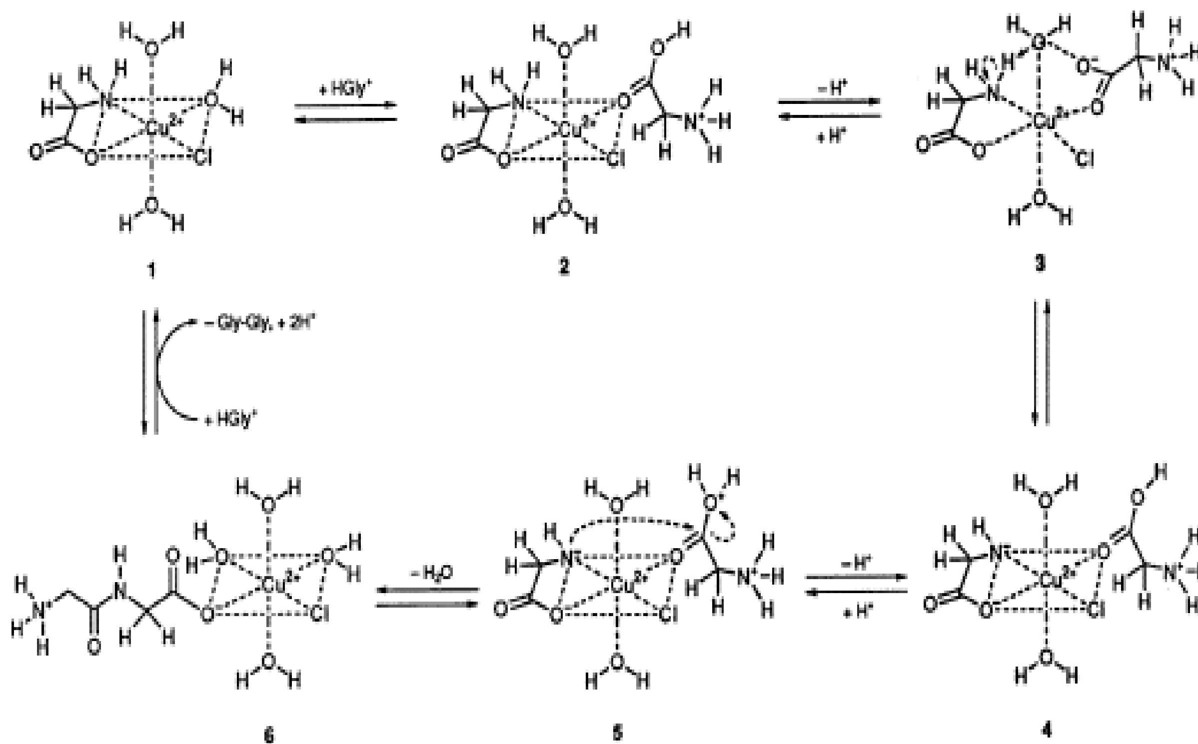


Fig 2: Cu (II) complex formation with two glycines and water. Image reproduced from Rode and Suwannachot(1999).

To this extent, we are examining the SIPF reactions in presence of different amphiphiles to characterize the role of the latter in potential catalysis of SIPF reactions. We carry out these reactions under simulated hydrothermal pool like conditions which might have existed on prebiotic Earth near the edge of volcanoes. Typical reactions involved cycling of various amino acids monomers in the presence/absence of different lipid vesicles by subjecting them to repeated dehydration-rehydration (DH-RH) cycles at high temperatures under both aerobic and anaerobic conditions. Lipids can form multilamellar structures that have the potential to organize and concentrate monomers in a given reaction. When dehydrated, lipid vesicles form multilamellar structures with bilayers stacked and fused into multilamellar phases. The other solutes which are

present in the reaction are trapped and concentrated between the lipid head groups of these phases(Ben-eli and Deamer, 1999). Since the bilayers are liquid crystals, the solute present in the reaction has some diffusional mobility, and these scenarios promote condensation reactions at high temperatures unlike in a solid material(Deamer and Weber, 2010). In addition, multilamellar lipid structures have been shown to facilitate polymerization of mononucleotides (Rajamani et al., 2008). This result demonstrated that the chemical potential available in anhydrous conditions is sufficient to drive an uphill reaction like polymerization as long as the monomers are concentrated and organized in an environment that permits diffusional mobility. Studies combining lipid and SIPF reactions are important to consider because lipids would have allowed for encapsulation of primitive replicating moieties, which is an essential step for kick-starting prebiotic evolution.

Materials & Methodology

Materials

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1, 2-dilauroyl-sn-glycero-3-phosphocholine (DLPC), were bought from Avant Polar Lipids, Inc. Sodium chloride (NaCl), copper chloride ($CuCl_2$), L-Glutamic acid (Glu), L- Alanine (Ala), L-L- His(His), L and D-Methionione (Met), L-Lysine (Lys), L-Glycine (Gly), L-Proline (Pro), tetraethylammonium bromide (TEAB),acetonitrile (ACN), ortho-phthalaldehyde (OPA), β-mercaptoethanol (βME), trifluoroacetic acid (TFA), butanol, glacial acetic acid, hexane, sodium acetate (NaOAc), ethylenediaminetetraacetic acid (EDTA), sodium tetraborate decahydrate (Borax), methanol, alpha-Cyano-4-hydroxycinnamic acid (CHCA),sodium hexanesulfonate, potassium dihydrogen phosphate, phosphoric acid, disodium hydrogen phosphate, sodium azide andtridecafluoroheptanoic acid (TDFHA),were all of bought from Sigma Aldrich.C-18 ZipTips were bought from Millipore.

Methodology

1. Preparation of lipid vesicles:

To get final working stock concentration of 25mM, 4.23 ml of POPC and DLPC were taken from their respective 25mg/ml stocks in chloroform, dispensed into a 50ml beaker and kept at room temperature till the chloroform completely evaporated. Subsequently, 10ml of MilliQ water was added to the beaker to dissolve the dried lipids.

1.1. Extrusion of the Lipid:

In order to start with a homogeneous sample of the lipid vesicles, both POPC and DLPC were extruded through 800nm membrane using an extruder (Fig 3) bought from Avanti Polar Lipids, Inc.

Fig 3: Extruder, Avanti Polar Lipids, Inc

2. Preparation of amino acids stock solutions:

The working stock concentration of all amino acids was 225mM which was prepared by dissolving appropriate weight of different amino acids into 5ml of MilliQ water. The stocks made were stored at 4ºC till further use.

3. Preparation of buffers:

A working stock of 5M NaCl was made, which was prepared by dissolving 1.461gm of NaCl in 5ml of MilliQ water.

A working stock of 4M $CuCl_2$ was made, which was prepared by dissolving 3.409gm of $CuCl_2$ in 5ml of MilliQ water.

4. Cycling reaction:

SIPF reaction conditions were, for most part, similar to the ones described by Rode et al. The main difference was the addition of lipids in our reactions to test for their catalytic role. Final concentrations of lipids, amino acids, NaCl and $CuCl_2$in our reactions were 12.5mM, 75mM, 500mM and 40mM, respectively. The above mixtures were subjected to repeated cycles of dehydration-rehydration on a hot plate at 90ºC (Fig 4), to simulate prebiotic hydrothermal pools that existed at the edges of volcanoes, The lipid based reactions were carried out mostly under CO_2conditions to prevent potential lipid oxidation while non-lipid reactions were done under air (similar to Rode et al.'s reactions). The mixtures were rehydrated with MilliQ water during every rehydration

stage and seven time points of the reaction (each at ~24 hrs) were taken. Table 1 carries the list of all the L-His reactions done to date.

Fig 4: System used to simulate prebiotic hydrothermal pool like conditions to carry out dehydration-rehydration cycles under CO_2/Air

Table 1: List of L-His reactions

Sr.No.	**Rxn components**	**Rxn volume (ml)**	**Temp ºC**	**No. of cycles**	**Time per cycle**	**Environmental condition**
Reactions subjected to DH-RH cycles						
1	L-His + POPC + NaCl + $CuCl_2$	2	90ºC	7	24 hr	CO_2
2	L-His + DLPC + NaCl + $CuCl_2$	1	90ºC	7	24 hr	CO_2
3	L-His + DLPC + NaCl + $CuCl_2$	1	90ºC	7	24 hr	Air
4	L-His + DLPC + NaCl + $CuCl_2$ + L-Gly(15mM)	1	90ºC	7	24 hr	CO_2

Sr.No.	Rxn components	Rxn volume (ml)	Temp ºC	No. of cycles	Time per cycle	Environmental condition
5	L-His +DLPC+NaCl+$CuCl_2$ +L-His+Na^+-Montmorillonite	1	90ºC	7	24 hr	CO_2
6	L-His + DLPC + NaCl + $CuCl_2$ + Li^+-Montmorillonite	1	90ºC	7	24 hr	CO_2
Reactions not subjected to DH-RH cycles						
7	L-His + DLPC + NaCl + $CuCl_2$	1	90ºC	7	24 hr	CO_2
8	L-His + NaCl+$CuCl_2$	1	90ºC	7	24 hr	Air

5. Extraction of lipid from reaction time points:

At the end of the experiment, lipids were extracted from the mixtures, twice using n-butanol (2:1volume) followed by hexane treatment (1:1) volume, to remove excess butanol). 50µl of original mixture was kept aside for microscopic analysis of lipids. After the extraction was done, all aqueous samples were made to equal final volumes by adding MiliQ water, as lipid extraction results in volume variability resulting from some of the water also getting pulled out along with the lipid.

5.1. Extraction of clay samples in clay containing SIPF reactions

Aqueous phase of sample aliquots from clay based reactions were pipetted out after centrifugation at 13000 rpm for 5min and kept separately. The clay pellet was then subjected to four rounds of extraction using 50uL of 0.1M NaCl in 30%ACN (2 x 2 hr, 1 x overnight and 1 x 1 hr), to remove any residual reaction mixtures that might still be sticking to the clay. The extracted material was centrifuged and supernatants from the four rounds were mixed together and used for analysis.

6. **TLC analysis**

TLC analyses were carried out on Silica Gel 60as well as C-18 plates with mobile phase solvent as butanol - glacial acetic acid – water (4:3:3) which had 0.4% ninhydrine.

7. **High Performance Liquid Chromatography (HPLC)**

HPLC is a separation technique that involves the injection of small volumes of liquid sample in a tubular column packed with tiny particles, 3 to 5 micron (μm) in diameter, called the (stationary phase). Individual components of the sample are moved down the column with a liquid (mobile phase), forced through the column at high pressure that is delivered by a pump. The sample components are separated from one another by the column packing that involves various chemical and/or physical interactions between the sample molecules and the packing particles.

In our case we used a particular type of separation mode in HPLC which is Reverse-phase HPLC (RP-HPLC). In an RP-HPLC, the stationary phase is non-polar (e.g. silica, C18, C8, C3, phenyl, etc.) and the mobile phase is water (buffer) + water-miscible organic solvent (e.g. methanol, acetonitrile) (Fig 5).By this technique one can separate non-polar, polar, ionizable and ionic molecules.

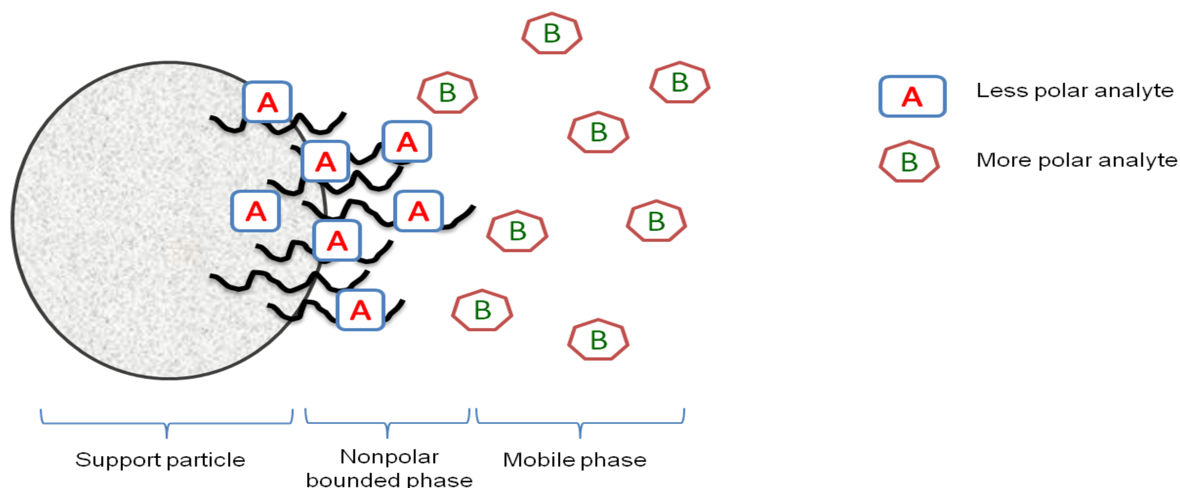


Fig 5: Representation of RP-HPLC mechanism

7.1. Method development for HPLC analysis

Many different HPLC columns, solvent systems and gradients were used for standardizing the HPLC conditions required for analyzing our reaction mixtures (details mentioned in results). A typical solvent for HPLC was made by mixing appropriate amounts of the required buffer components in water and filtering it, followed by degassing the solvent. Solvent B in most cases was pure ACN which was filtered and degassed prior to use.

7.2. Derivatization of amino acids for HPLC analysis

In order to increase the delectability of the amino acid monomers and resultant higher mers in our reactions, online derivatization was carried out prior to analysis by HPLC as most of the amino acids do not have absorbance of their own. Derivatization helps in the formation of a cyclic product which has florescence absorbance (Fig 6). Derivatization enhances detector response, vastly improves analyte resolution, improves analyte peak shape, and also reduces the polarity thus increasing its retention time in reversed-phase chromatography.

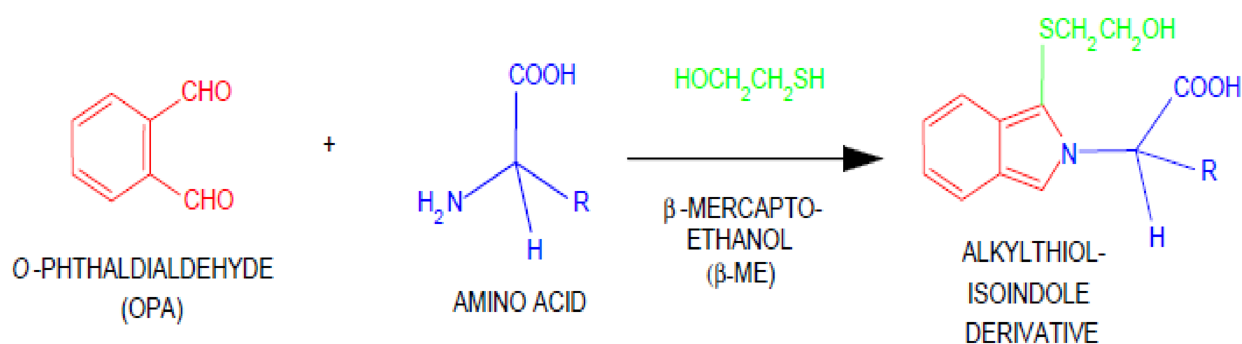
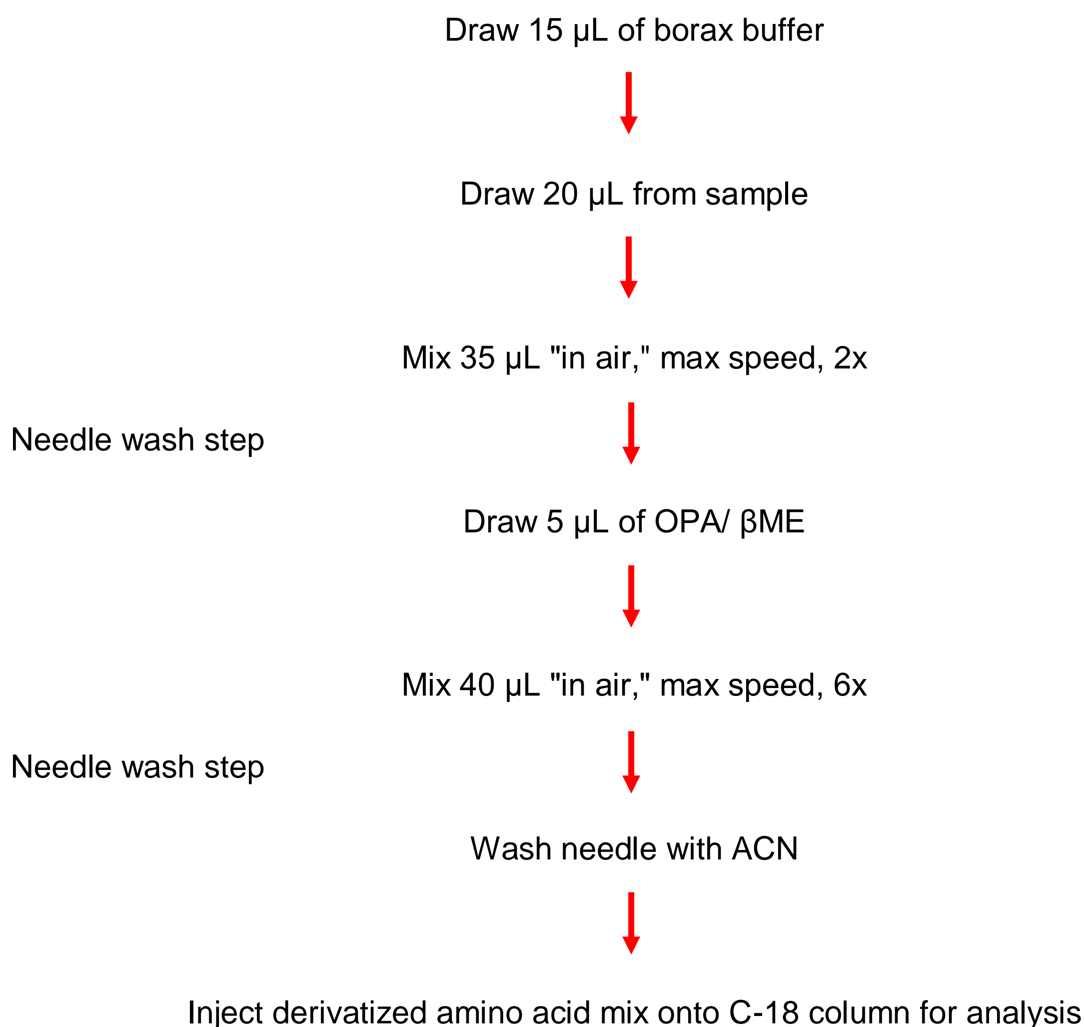


Fig 6: Derivatization mechanism for amino acids using OPA-βME.

7.3. Preparation of derivatizing agent

100mM stock of sodium tetraborate decahydrate (Borax) buffer was prepared using 572 mg of Borax powder and dissolving it in 15ml of MilliQ water. Ortho-phthalaldehyde (OPA) solution was prepared using13.5 mg of OPA, which was dissolved in 500 µl of methanol. To this solution, 2.5 µl of β-mercaptoethanol (βME) was added. Where required, working stocks of the derivatizing agent was prepared by mixing 2.5 ml of OPA/ βME stock with 7.5ml of Borax buffer. In most cases, the below online derivatization protocol was used.

7.4. Online derivatization protocol using HPLC software Chemstation ODS:



8. **Microscopy:**

Phase contrast microscopy of samples containing POPC and DLPC was performed using Apotome microscope (Olympus Inc.) to check for the ability of the lipids to form vesicles as the reactions are subjected to high temperature cycling conditions for several days that could potentially be harsh. In typical analysis, 5μl of the butanol phase sample obtained after lipid extraction was placed on a slide and allowed to dry out; it was then rehydrated using MilliQ water and observed under the microscope for the presence of vesicles.

9. **MALDI -TOF mass spectrometry :**

MALDI (Matrix-assisted laser desorption/ionization) is an ionization method used for the analysis of biomolecules and synthetic polymers. The matrix used assists in desorption and ionization of the analyte and the mass is obtained with the help of a detector, The basic concept of TOF mass analyzer (Time-of-flight) is that the ions are separated based on the time it takes for the ion to drift down the flight tube to the detector. Lighter ions have higher velocities than heavier ions and reach the detector first.

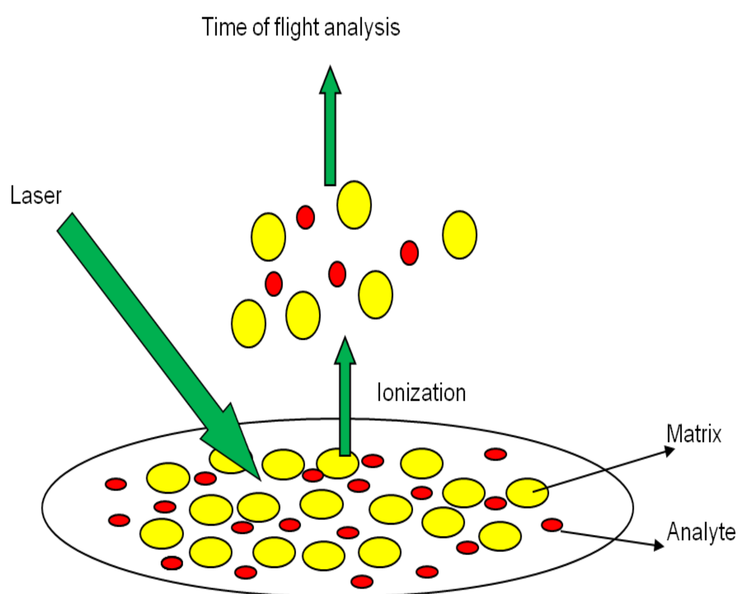


Fig 7: MALDI working description (http://paperedu.org/docs/index-16393.html)

9.1. **Interpreting Mass spectra:** Below is an example of a MALDI spectrum followed by a table that provides an understanding of how MALDI spectra are interpreted.

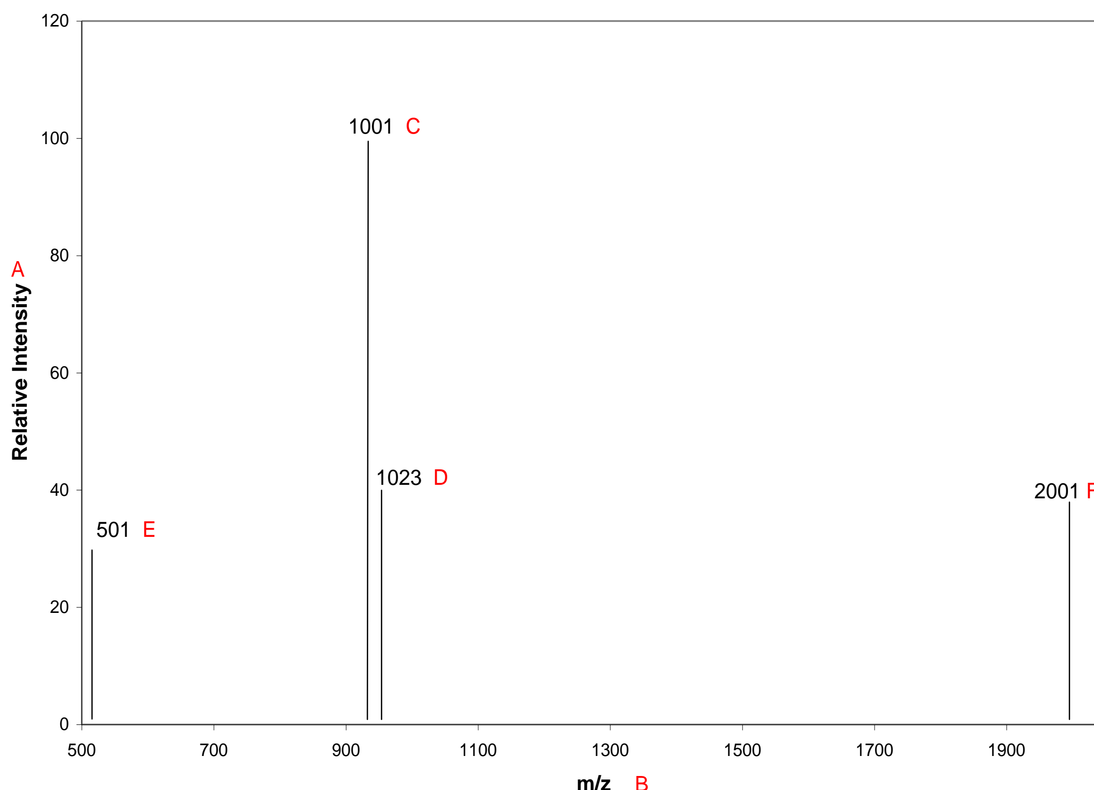


Fig 8: Interpreting mass data (http://paperedu.org/docs/index-16393.html)

A	This is the intensity relative to the tallest peak in the spectrum with the tallest peak set to 100%.
B	m/z, mass divided by charge
C	base peak: the tallest peak in the spectrum
D	Sodium adduct: 1000+23
E	Double charge: (1000+2)/ 2
F	Dimer: 2001

9.2. Matrix preparation

The matrix used in our MALDI was alpha-Cyano-4-hydroxycinnamic acid (CHCA). Fresh saturated matrix solution was prepared by dissolving CHCA powder in 50% acetonitrile/water and 0.1% TFA. The solution was then vortexed for the powder to get dissolved and centrifuged to remove the undissolved matrix from the solution. The top clear matrix was used for MALDI sample preparation.

9.3. MALDI sample preparation

Reaction sample aliquots were completely lyophilized and redissolved in 20 µl MilliQ water. 5 µl from that solution was taken separately for mass analysis and the remaining 15 µl went through ZipTip process to reduce the amount of salt in the samples. ZipTip is a micro pipette tip with a C-18 matrix placed at its tip that is used to also concentrate samples prior to mass analysis.

9.3.1. Sample preparation for ZipTip process

1 µl of neat TFA was mixed with 15 µl of reaction aliquots resulting in ~7% of final TFA concentration. The recommended concentration is in the range of 0.1% to 1% (http://www.millipore.com/catalogue/module/c5737). However, with these TFA concentrations we could not achieve considerable salt removal. Hence, we used more than the recommended amounts to make our desalting procedure more robust since our reaction itself is driven by high salt concentrations. Solutions required for the ZipTip procedure:

Wetting solution – 100% ACN

Equilibration solution – 0.1 % TFA in Water

Wash solution – 0.1 % TFA in water

Elution solution – 0.1 %TFA in 50% ACN/Water

9.3.2. ZipTip protocol

Wetting of the ZipTip matrix with wetting solution

Equilibration of the ZipTip matrix with equilibration solution

Sample is pipetted 10-12 times through ZipTip matrix

Washing of the ZipTip matrix with wash solution

Elution of the material from the ZipTip matrix

Samples from both wash and elute steps were collected for mass analysis. Finally, 1 µl of this sample is mixed and loaded with 1 µl of CHCA matrix on the MALDI plate for final analysis.

Results and Discussion

Microscopy

Phase-contrast microscopy was done to look for the presence of lipid vesicles after each cycle. Both, POPC and DLPC retained the capability to form vesicles in L-His based SIPF reactions even after 5 dehydration-rehydration cycles (corresponding to 5 days) at 90ºC. Representative images from reactions involving L-His + NaCl + $CuCl_2$+ POPC is shown in Fig 9a and 9b, for 0^{th}cycle and 5^{th}cycle respectively. There have been no reports in the past that show vesicle formation even after prolonged periods of time under such extremes conditions.

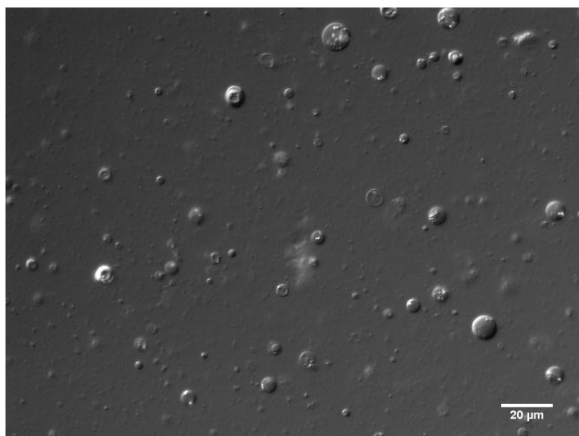


Fig 9a: L-His 0^{th}Cycle

Fig 9b: L-His 5^{th}Cycle

TLC analysis

Initial reaction products were analyzed using TLC. The results obtained were not very reliable for making any conclusions because we did not observe clear separation of the product bands. Also, the presence of high concentration of salt hampered the movement of the solutes across the stationary phase.

HPLC

HPLC, as mentioned earlier, is extensively used in the separation and analysis of polar, hydrophilic and hydrophobic solutes. However, one constraint with HPLC of single amino acids is that most of them do not have any absorbance of their own, excepting for some of them which have a ring structure (e.g. tryptophan, phenylalanine etc). Therefore, in our case we started with analyzing L- His based reaction samples and began by looking at the absorbance at 210 nm (using a Diode Array Detector), at which L- His and peptide bond, both have absorbance. This exercise was not really successful as many different solvents, including acetonitrile (ACN), which is one of our HPLC reagents, absorb at this wavelength. ACN is one of the main solvents used for RP-HPLC analysis of amino acids. The absorbance of L- His is very less in comparison to ACN and due to this there were a lot of peaks at 210nmin the HPLC chromatogram of the reaction samples, which we could not designate confidently to our molecules of interest.

To overcome these problems we decided on derivatizing L- His control and our reaction samples with the help of ortho-phthalaldehyde (OPA) and β-mercaptoethanol (βME), a procedure that is used extensively to detect single amino acids and very short peptides. The protocol and mechanism for the derivatization of amino acids is discussed in detail in the methods section(Schuster, 1988). We looked for florescence absorbance after derivatization at 230 nm. The derivatization worked for the control L-His sample (Fig 10) as we can see a clear single peak in the HPLC profile. However, our reactions have lots of salts present (NaCl and $CuCl_2$), which seems to have some effect on derivatization process. This becomes apparent in the case where a control L-His sample was mixed with salt (same concentration as in our reaction), prior to derivatization. We can now see more than one peak in the HPLC profile (Fig 11), which is otherwise not the case if salts (NaCl and $CuCl_2$) were not added to the sample. These salt-related peaks were of potential concern while analysing our reaction mixtures as the yields of the SIPF reactions, to begin with, are known to be very low.

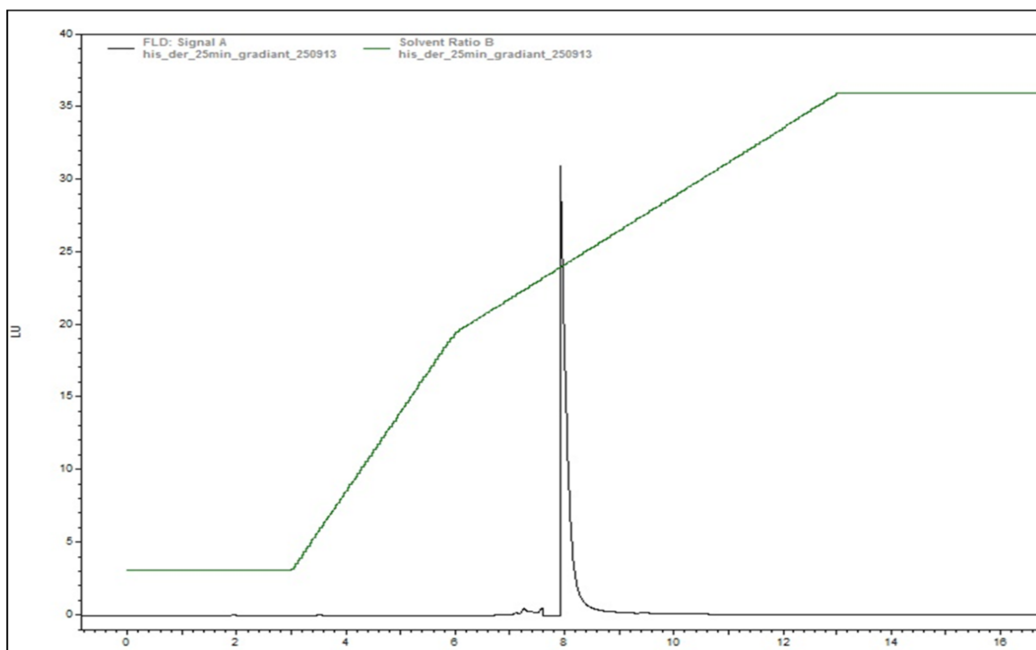


Fig 10: HPLC of L-His control post derivatization with OPA-βME

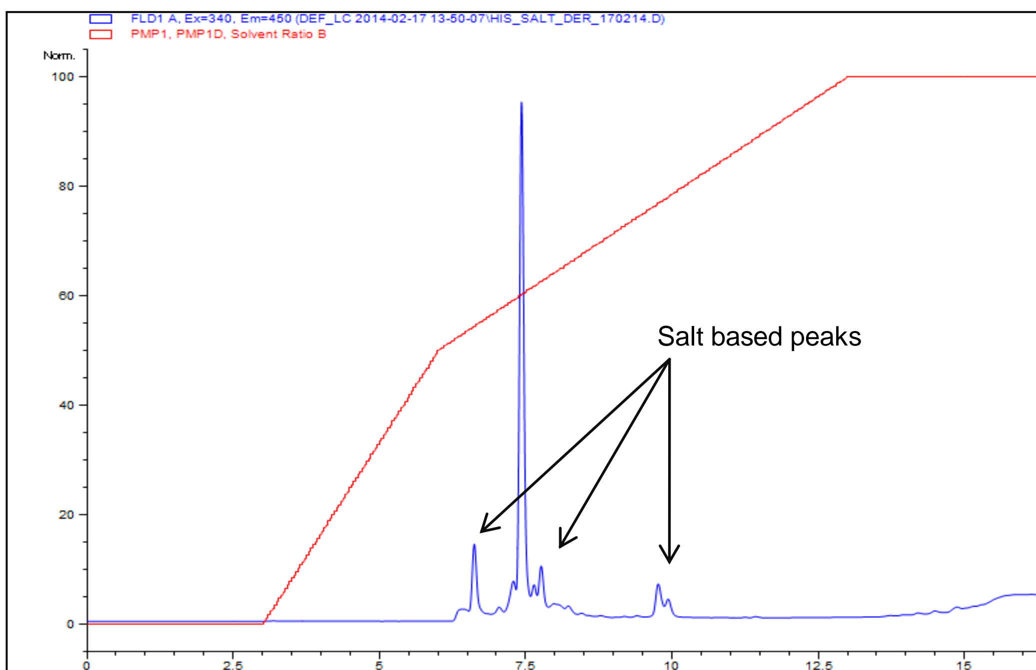


Fig 11: HPLC of OPA-βME derivatized L- His control which contains salts (NaCl and $CuCl_2$)

HPLC method development:

Given that our main detection method was going to be HPLC, we spent a good amount of time on narrowing down the conditions that worked best for our reaction mixtures' analyses. Many different HPLC columns, solvent systems and gradients were used for standardizing the best HPLC conditions, a list of which is mentioned in Table 2. This was a non-trivial task given that single amino acid/very small peptide analyses are inherently tricky, which got compounded by the large amounts of salt in our reactions. An important thing to note is that all of these analyses involved online derivatization, whose protocol is already detailed in the methods in section 7.4.

Table 2: List of all HPLC conditions

Sr. No.	HPLC Instrument	HPLC column	Solvent system	Gradient
1	Dionex ICS 3000	Phenominex C18 column (5 µm, 4.6 * 250 mm).	Solvent A – 30 mM NaOAc, $3H_20$, 0.2 mM EDTA, 0.2% THF Solvent B – 100 mM NaOAc, $3H_20$, 0.1mM EDTA, 80% ACN	0 to 50% B in 10 min followed by 50 to 100% B in 0.5 min at 0.5 ml/min flow rate
2	Agilent 1206 infinity	Eclipse Zorbax C18 column (5 µm, 4.6 * 250 mm).	Solvent A – 25 mM TEAB Solvent B – 70% ACN	0 to 50% B in 5 min followed by 50 to 100% B in 25 min at 1 ml/min flow rate
3	Agilent 1206 infinity	Eclipse ZorbaxC18 column (5 µm, 4.6 * 150 mm).	Solvent A- 0.5 mM TDFHA in water Solvent B- 100% ACN	0 to 50% B in 3 min followed by 50 to 100% B in 7 min at 1 ml/min flow rate

Sr. No.	HPLC Instrument	HPLC column	Solvent system	Gradient
4	Agilent 1206 infinity	Eclipse Zorbax C18 column (5 µm, 4.6 * 150 mm).	Solvent A - 50 mM Potassium dihydrogen phosphate, + 7.2 mM sodium hexane sulfonate, pH 2.5 adjusted byPhosphoric acid Solvent B – 100% ACN	0 to 30% B in 4 min followed by 30 to 50% B in 2 min followed by 50 to 70% B in 1 min followed by 70% B for 5 min at 1 ml/min flow rate
5	Agilent 1206 infinity	Eclipse Zorbax C18 column (5 µm, 4.6 * 150 mm).	Solvent A- 0.5 mM TFA in water Solvent B- 100% ACN	0 to 50% B in 3 min followed by 50 to 100% B in 7 min at 1 ml/min flow rate
6	Agilent 1206 infinity	Poroshell C18 column (2.7 µm, 4.6 * 50 mm).	Solvent A- 0.5 mM TFA in water Solvent B- 100% ACN	0 to 100% B in 8 min at 0.4 ml/min flow rate
7	Agilent 1206 infinity	Eclipse Zorbax C18 column (5 µm, 4.6 * 150 mm).	Solvent A- 0.1% TFA in water Solvent B- 100% ACN	0 to 50% B in 3 min followed by 50 to 100% B in 7 min at 1 ml/min flow rate

Sr. No.	HPLC Instrument	HPLC column	Solvent system	Gradient
8	Agilent 1206 infinity	Eclipse Zorbax C18 column (5 µm, 4.6 * 150 mm).	Solvent A - 10 mM Disodium hydrogen phosphate, 10 mM sodium tetra borate decahydrate, 0.5 mM Sodium azide Adjust to approximately pH 8.2. Solvent B – ACN : methanol: water (45:45:10 by volume)	0 to 50% B in 3 min followed by 50 to 100% B in 7 min at 1 ml/min flow rate
9	Agilent 1206 infinity	Propac SAX-10Analytical(4* 250mm)	Solvent A – 20 mM NaCl Solvent B – 20 mM NaCl in 100 mM tris pH adjusted to 8.5	0 to 50% B in 3 min followed by 50 to 100% B in 7 min at 1 ml/min flow rate
10	Agilent 1206 infinity	Eclipse ZorbaxC18 column (5 µm, 4.6 * 150 mm).	Solvent - 10 mM sodium hexane sulphonate acidified with phosphoric acid to pH~ 2.5 Solvent B- 100% ACN	0 to 30% B in 4 min followed by 30 to 50% B in 2 min followed by 50 to 70% B in 1 min followed by 70% B for 5 min at 1 ml/min flow rate

The HPLC conditions mentioned in row number 5 in the above table (highlighted in green) was most appropriate for our analyses as it gave the best separation among all different conditions that were tried.

Given below are some of the representative HPLC chromatograms from our reaction mixtures which show the presence of both monomers of L-His and some higher oligomers (Fig 12 to Fig 14). Two different lipids have been used in the SIPF reactions. We started with POPC, which has two 18-hydrocarbon chains, one saturated and one unsaturated. Due to non-availability of POPC, the next sets of reactions were done using DLPC, which has two 12-hydrocarbon chains that are both saturated. We hoped that this small difference would not affect the reaction parameters and this was confirmed by similar trends in the HPLC chromatograms for the two lipids (Fig 12 and Fig 14).

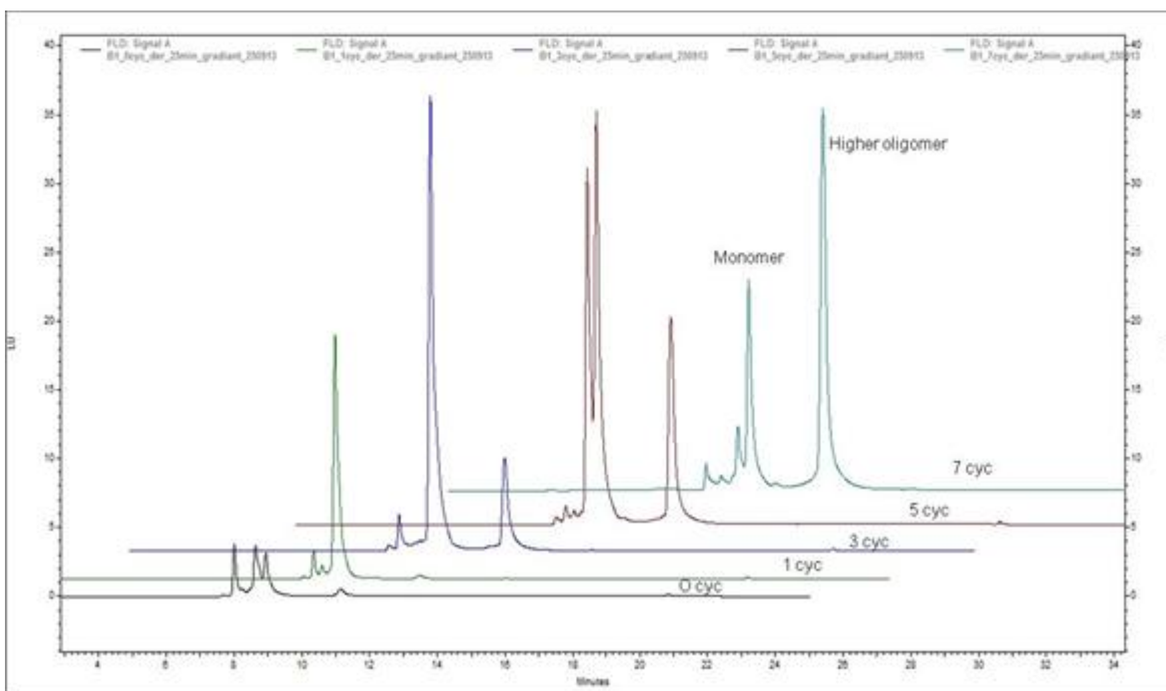


Fig 12: Overlaid HPLC chromatograms of reactions that involve L-His + POPC + NaCl+ $CuCl_2$ under CO_2 at different cycle time points as indicated in the figure.

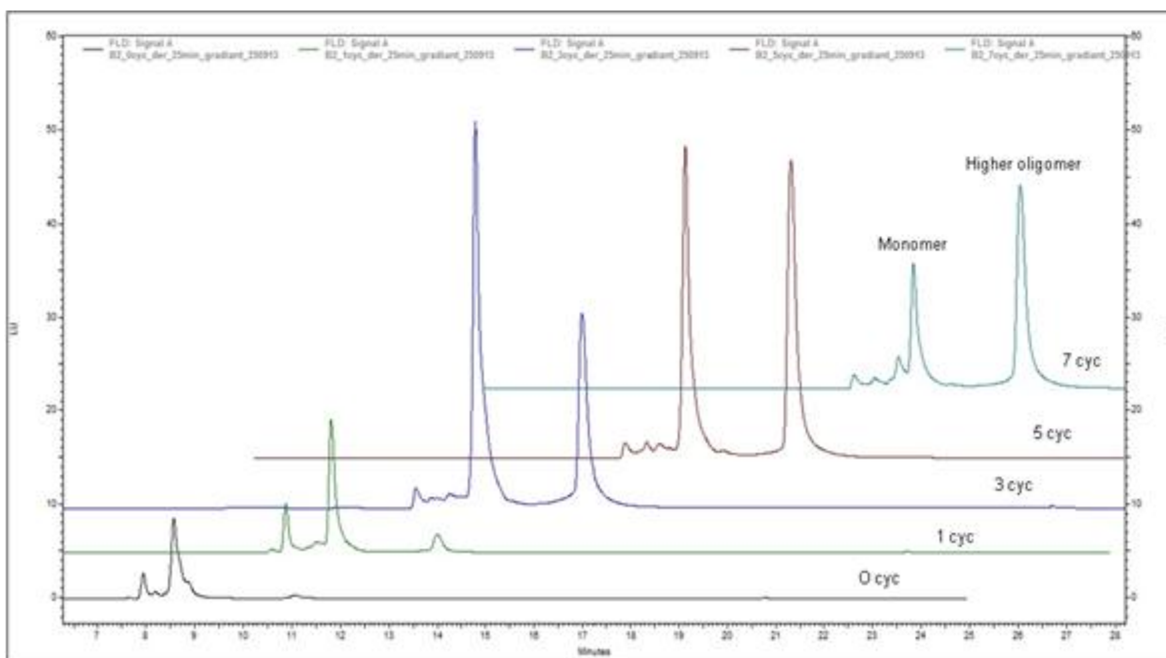


Fig 13: Overlaid HPLC chromatograms of reactions that involve L-His + NaCl+ $CuCl_2$ under air (no lipid control) at different cycle time points as indicated in the figure.

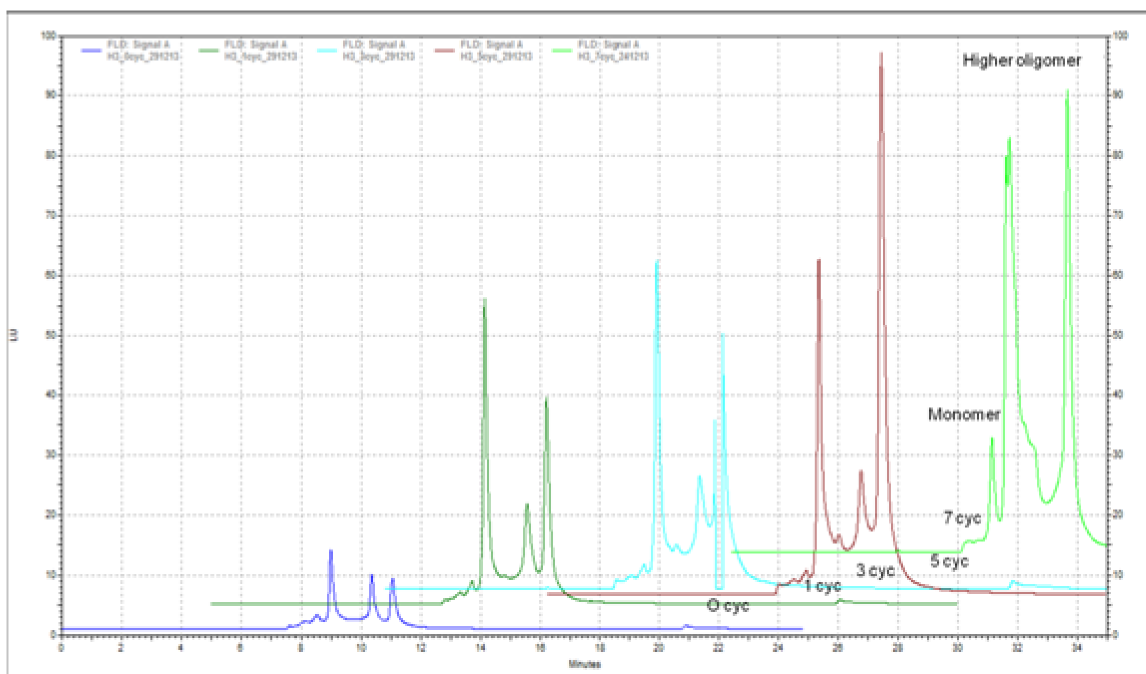


Fig 14: Overlaid HPLC chromatograms of reactions that involve L-His + DLPC + NaCl+ $CuCl_2$ under air at different cycle time points as indicated in the figure.

Because of having to use online-derivatization protocol, it was not easy to discern the different L-His based species in the spectrum due to contribution of peaks from the salt's interaction with the derivatizing agent. Therefore, the only way to confirm the presence of higher oligomers in our reaction products was to carry out MALDI TOF analysis, subsequent to which we could assign a monomer peak and a higher oligo peak (as depicted in the chromatograms), whose details will be discussed in the below section.

MALDI

MALDI TOF analyses were done to confirm the presence of different L-His species present in the reaction mixtures. The analysis of reaction samples where done using alpha-Cyano-4-hydroxycinnamic acid (CHCA) as matrix. As mentioned in the methods section (9.3.1), to overcome salt-related problems, the individual time points were lyophilized and re-dissolved in a really small volume of water and then subjected to ZipTip treatment using higher concentration of TFA. The effect of salt was reduced to some reasonable extent with increased TFA concentration. This resulted in better MALDI profiles in comparison to the samples which had not undergone this ZipTip process.

Thus, there were 2 important constraints that had to be accounted for during the analysis of the MALDI profiles.

1) Peaks contributions from the CHCA matrix itself, and
2) The peaks that resulted from formation of salt adduct both with the matrix as well as with reaction sample themselves.

There are peaks that come just from the CHCA matrix and this is well documented in the mass bank website: http://www.massbank.jp. Some of the most common peaks are379.09 $[2M+H]^+$, 234.00 $[M-H+2Na]^+$, 212.02 $[M+Na]^+$, 190.04 $[M+H]^+$, 172.04 $[M-H_2O+H]^+$, 146.06 $[M-CO_2+H]^+$, where M is the Matrix, H is hydrogen, Na is sodium, H_20 is water and CO_2 is carbon dioxide. During our data analysis, we accounted for these and other matrix-associated peaks, and eliminated them while concerning ourselves with only those peaks that were related to L-His associated species. In addition, due to

the presence of high concentration of salts in our reactions, even after the ZipTip process was carried out, some residual salt remained in the reaction samples which formed adducts with the matrix as well as oligomers. This made the analysis of MALDI profiles challenging, and, it was not possible to do robust quantitative determination of the yields of higher oligomers.

Given below are two representative MALDI profiles (Fig 15 and Fig 16). The numbers boxed in red are the peaks contributed by the CHCA matrix; the numbers boxed in blue are the peaks formed due to salt adducts and the peaks boxed in green are from higher oligomers (monomer, dimer and trimer).

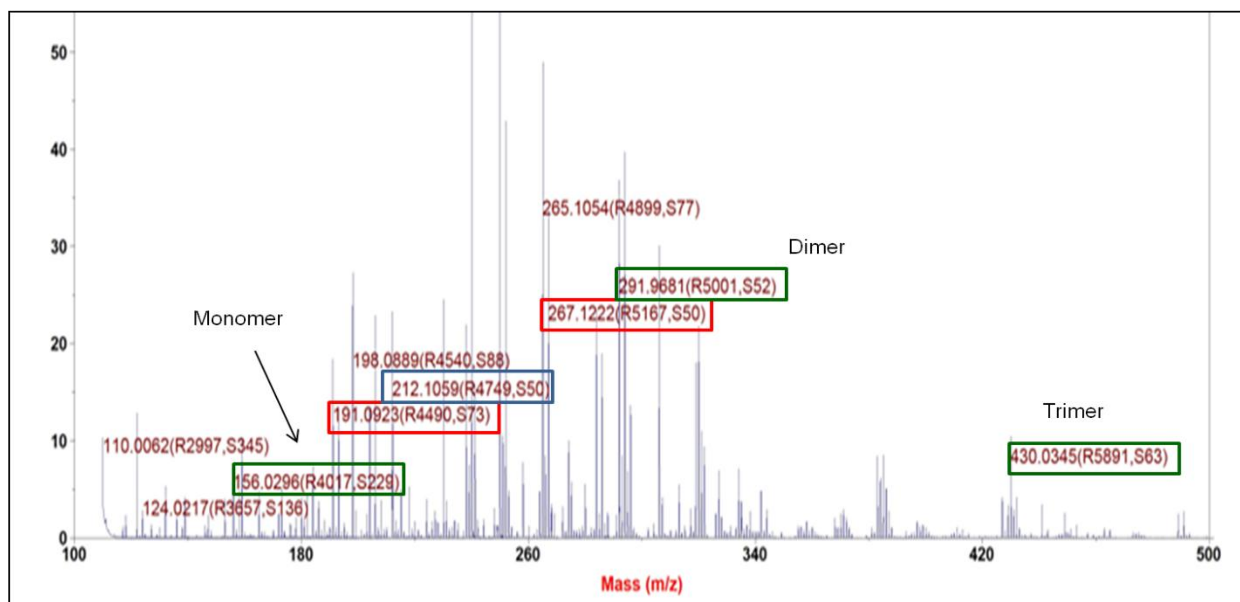


Fig 15: MALDI spectrum representing all different kinds of peaks, including matrix-related peaks, salt adducts and higher oligomer peaks.

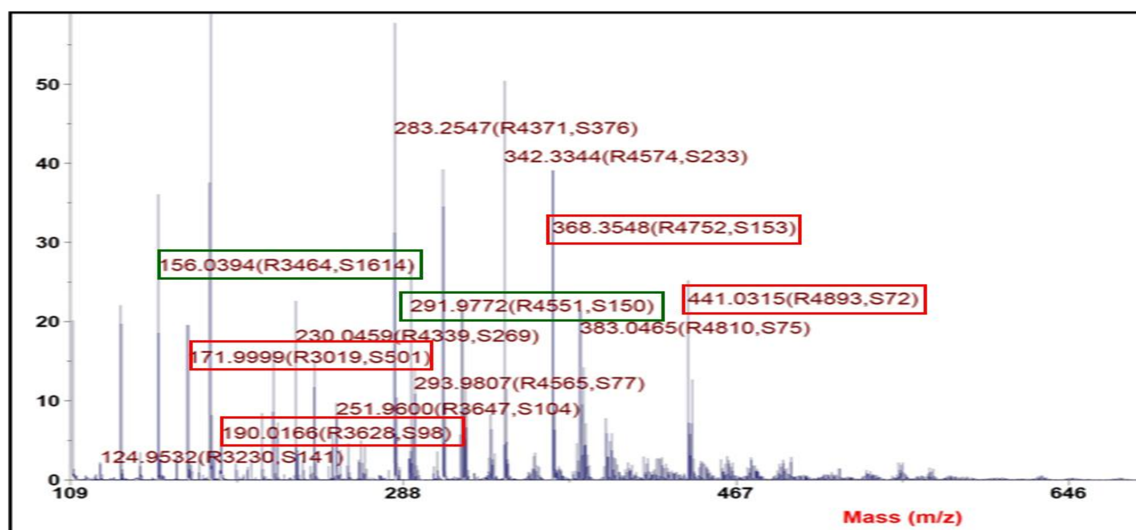


Fig 16: MALDI spectrum representing matrix-related peaks. Also seen are monomer and dimer peaks.

Multiple repeats of MALDI were done for all the L-His reactions to increase the confidence of our analysis. Mass of oligomers up to a trimer was conclusively observed depending on the reaction. In addition, we could find many different salts adducts coming from the oligomers. Table 3lists the expected mass for L-His oligomers and their salt adducts, along with the masses that were observed during MALDI analysis. Given the persistent presence of salt, some anomalies show up due to the samples not being able to ionize and 'fly' well. For e.g., we do not see Na^+ or Cu^+ based adducts for the His-His dimer. This is a limitation of the method as we cannot control beyond a point where the laser actually hits the sample on a MALDI sample plate, and some regions of the sample spot could have more salt while some others could have less. This could however be minimized by trying to completely getting rid of the salt.

Table3: Expected and observed mass of L-His oligomers and its salt adducts

Sr. No.	Oligomers and there salt adducts	Expected mass	Observed mass
1	His	155.0694	156.0303
	His + Na^+	178.0591	178.0031
	His + Cu^+	218.6154	217.9476
2	His + His	292.1388	292.0450
	His + His + Na^+	315.1285	-
	His+ His +Cu^+	355.6848	-
3	His + His + His	429.2082	430.0409
	His + His + His + Na^+	452.1979	452.0483
	His + His + His + Cu^+	492.7542	492.0457

Detailed in Table 4 is a comprehensive analysis of the masses observed by MALDI for different reactions of L-His reactions. The results qualitatively confirmed the presence of L-His monomer and higher oligomers.

Table 4: Represents the presence of higher oligomers of L- His by MALDI analysis

Sr. No.	Reaction	Mass observed in MALDI analysis		
		Monomer	Dimer	Trimer
Reaction were subjected to DH-RH cycles				
1	L-His + POPC + NaCl+ $CuCl_2$ + CO_2	✓	✓	✓
2	L-His + NaCl + $CuCl_2$ + Air	✓	-	-
3	L-His + DLPC + NaCl + $CuCl_2$ + CO_2	✓	✓	-
5	L-His + DLPC + NaCl + $CuCl_2$ + Air	✓	✓	-
6	L-His + DLPC + NaCl + $CuCl_2$ + L-Gly(15mM) + CO_2	✓	✓	-
7	L-His + DLPC + NaCl + $CuCl_2$ + Na^+ -Montmorillonite + CO_2	✓	✓	-
8	L-His + DLPC + NaCl + $CuCl_2$ + L-His + Li^+ -Montmorillonite + CO_2	✓	✓	-
Reactions were not subjected to DH-RH cycles				
9	L-His + DLPC + NaCl + $CuCl_2$ + CO_2	✓	✓	-
10	L-His + NaCl + $CuCl_2$ + Air	✓	✓	-

Based on preliminary analysis, we can say that in reactions that included POPC, we were able to detect trace amounts of a trimer in three separate trials, which has been seen in only two other SIPF-based reactions. In ongoing work, we are scaling up these reactions to clearly detect the presence of a trimer (and potentially even higher mers), even by HPLC. In most other L-His SIPF reactions that were carried out in the presence of lipids, including the ones that had clay or a catalyst, we could discern the formation of a dimer. In reactions which included DLPC, L-His, NaCl and CuCl2 (under CO_2), oligomers up to only dimers were observed (see Table 4), unlike in the POPC reaction. More studies need to be undertaken to determine if the saturation state of a lipid's hydrocarbon chain actually has a role to play in these reactions.

Despite our results not being quantitative, we can state with reasonable confidence that presence of lipids in the reaction does seem to increase the yields of the reaction products. If anything, our preliminary yield estimates from MALDI data (results not shown) seem to be underestimating the dimer and trimer yields due to the salt-based problems plaguing our MALDI sample preparations. In ongoing work, we are using control L-His dimers and trimers to quantify our reaction yields. It is relevant to point out here that till date, the best yields of a His-His dimer under SIPF conditions has been shown to be around 0.3% as shown in Table 5 (Rode et al., 1997). Dimers have been detected in almost all of Rode et al's reactions while trimers were observed only in few reactions pertaining to L-Ala and L-Gly(Rode et al., 1998), in which a catalyst like L-Gly or clay were included (Jakschitz and Rode, 2012). However, their overall dimer yields are very less as is clear from Table 5. The highest dimer yield that they have reported to date is for L-Met based SIPF reaction (2.79 % after 3 cycles)(Li et al., 2008).

Finally, we also did a couple of reactions where each sample time point was kept in a separate vial and the reaction mixtures were not subjected to dehydration-rehydration cycles; just like how Rode el al have carried out their reactions (row 9 and 10 in Table 4). We did these no-cycling controls to check for the effect of DH-RH cycling on SIPF reactions. One important observation was the absence of dimers in the minus-lipid controls, when subjected to cycling under air. This might be because of dimer hydrolysis during the rehydration step as addition of water favours back-reaction. Dimers were however present in the minus-lipid control reaction that was not subjected to DH-RH cycling (this is our Rode-like control reaction), which corroborates results from Rode et al's studies. It seems that DH-RH cycling might only be advantageous when the SIPF reactions included lipids, where lipids protect the growing oligomers by encapsulating a major subset during the rehydration stage. More studies are needed to state this with greater confidence.

Table 5: Yield % of dimer from Rode et al's reactions, adapted from(Rode et al., 1997)

↓A,B→	Gly	His	Ala
Gly	6.57	0.54	0.91
His	0.25	0.33	0.32
Ala	1.20	0.85	1.86

In conclusion, addition of lipids to SIPF reactions seems to increase the efficiency of polymerization to some extent. However, quantitative effects of the lipid-assisted catalysis can be confidently determined only after the resolution of salt-related sample preparation problems. In spite of several attempts, issues remain which pertain to our inability to fully exclude the presence of salts in the samples prior to molecular characterization. Work is ongoing to improve our de-salting methods in order to get cleaner HPLC and MALDI profiles. Unfortunately, the extremely small size of our oligomers excludes the use of dialysis as a desalting procedure, which is a well-known method to remove high amounts of salt from samples. We also undertook reactions involving some other amino acids like L- and D-Met, L-Gly, L-Ala, L-Lys and L-Pro as detailed in Table 6. Work is ongoing to fist sort the salt-related issues before analysis and characterization of these reaction mixtures can be carried out. Also, we subsequently are hoping to characterize all the reaction products with a more sensitive mass analysis technique such as High resolution-mass spectrometry (HRMS)

.

Table 6: Reactions done with other amino acids.

Sr. No.	Rxn components	Rxn volume (ml)	Temp ºC	No. of cycles	Time per cycle	Environmental condition
1	L-Ala + POPC + NaCl + $CuCl_2$	2	90ºC	7	24 hr	CO_2
2	L-Ala + NaCl + $CuCl_2$	2	90ºC	7	24 hr	Air
3	L-Ala + DLPC + NaCl + $CuCl_2$	1	90ºC	7	24 hr	CO_2
4	L-Ala + DLPC + NaCl + $CuCl_2$	1	90ºC	7	24 hr	Air
5	L-Gly + DLPC + NaCl + $CuCl_2$	1	90ºC	7	24 hr	CO_2
6	L-Gly + NaCl+$CuCl_2$	1	90ºC	7	24 hr	Air
7	L-Lys + POPC + NaCl + $CuCl_2$	2	90ºC	7	24 hr	CO_2
8	NaCl + $CuCl_2$ + L-Lys	2	90ºC	7	24 hr	Air
9	L-Met + POPC + NaCl + $CuCl_2$	2	90ºC	7	24 hr	CO_2
10	L-Met + NaCl + $CuCl_2$	2	90ºC	7	24 hr	Air
11	L-Met + DLPC + NaCl + $CuCl_2$	1	90ºC	7	24 hr	CO_2
12	L-Met + NaCl + $CuCl_2$ + L-Met	1	90ºC	7	24 hr	Air
13	L-Met + DLPC + NaCl + $CuCl_2$ + L-His(15mM)	1	90ºC	7	24 hr	CO_2
14	L-Met + DLPC + NaCl + $CuCl_2$ + L-Met + Na^+-Montmorillonite	1	90ºC	7	24 hr	CO_2

Sr. No.	Rxn components	Rxn volume (ml)	Temp ºC	No. of cycles	Time per cycle	Environmental condition
15	D-Met + DLPC+ NaCl + $CuCl_2$ + D-Met	1	90ºC	7	24 hr	CO_2
16	L-Val + DLPC + NaCl + $CuCl_2$	1	90ºC	7	24 hr	CO_2
17	L-Val + NaCl + $CuCl_2$	1	90ºC	7	24 hr	Air
18	L-Val + DLPC + NaCl + $CuCl_2$ + L-His(15mM)	1	90ºC	7	24 hr	CO_2
19	L-Val + DLPC + NaCl + $CuCl_2$	1	90ºC	7	24 hr	CO_2
20	L-Pro + DLPC + NaCl + $CuCl_2$	1	90ºC	7	24 hr	CO_2

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