A systems approach to understanding the emergence of functional protocells

A Thesis

submitted to

Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

by

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Date: April, 2023

Under the guidance of

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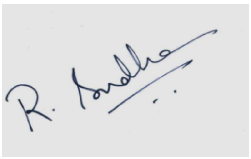
From May 2022 to Mar 2023

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled ‘A systems approach to understanding the emergence of functional protocells’ towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Sahil Sunil Mulewr at Indian Institute of Science Education and Research under the supervision of Dr Sudha Rajamani, Associate Professor, Department of Biology, during the academic year 2022-2023.



Dr Sudha Rajamani

Committee:

Dr Sudha Rajamani

Prof Manickam Jayakannan

This thesis is dedicated to my friends and family for their back
while I take a leap and a space when I fall

Declaration

I hereby declare that the matter embodied in the report entitled 'A systems approach to understanding the emergence of functional protocells' are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr Sudha Rajamani and the same has not been submitted elsewhere for any other degree

Sahil Sunil Mulewar

Date: April, 2023

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Abstract

Life is thought to have originated from a heterogeneous prebiotic soup, wherein various kinds of simple organic and inorganic molecules interacted, increased molecular complexity, and further evolution led to the emergence of life. A systems chemistry approach is required to elucidate the mechanism of the life-forming process and synthesize *in-vitro* life. Under such an approach, it becomes necessary to study interactions between various kinds of biomolecules and the emergent properties of the resulting system. Lipids, amino acids and nucleosides are monomers of life's three fundamental and essential polymers. In this study, we studied two pertinent cross-talks, the first being the covalent interaction between nucleosides and single-chain amphiphiles, yielding the formation of nucleolipids. We synthesized single acyl chain-based nucleolipid molecules and characterized their self-assembly property. The second cross-talk studied is the non-covalent interaction between amino acids and model protocellular membranes. Our results show that amino acids assist and promote the self-assembly of model prebiotic membranes. We further show that the amino acid-membrane interaction affects the physicochemical properties of the membrane. Our results demonstrate that amino acid interaction with the membrane might have been crucial for the origin of the protocell and its evolution.

Acknowledgments

I am indebted to my supervisor Dr Sudha Rajamani who nurtured my passion towards science and giving the opportunity to work with her. My journey of scientific exploration officially started with a reading project with her at the end of my first year of BS-MS. Since then, she has given me a space to learn, be curious, explore, fail, and grow. The independence of curiosity and experimentation in her lab has taught me to design experiments on my own and develop as a better researcher. Her support and trust, whenever any experiment fails, have given me the strength to continue. She has been not only a mentor in science but also in certain aspects of life.

I am thankful to my thesis expert Prof Manickam Jayakannan for his advice and suggestions whenever needed.

I acknowledge Dr Anupam Sawant for his mentorship and help in synthesizing nucleolipids. He taught me the art of organic chemistry.

Dr Manesh Joshi has been my first mentor in the lab. The discovery that oleic acid forms vesicles under amino acid buffers was made by him. In addition to teaching me various techniques, he taught me how to be a better mentor and the importance of patience and consistent efforts.

I am also thankful to Souradeep Das for mentoring me and teaching me techniques. I am learning the importance of having a good work-life balance from him.

I acknowledge the help of Dr Srivatsan's lab members for the synthesis steps involved in our work.

I am thankful to IISER Pune microscopy, NMR, MALDI and HRMS facilities.

Lastly, thanks to all the members of the COoL lab for keeping me motivated and for the pipette tips.

Contributions

Contributor name	Contributor role
Sahil Mulewar, Manesh Joshi, Souradeep Das, Sudha Rajamani	Conceptualization Ideas
Sahil Mulewar, Anupam Sawant, Manesh Joshi, Souradeep Das	Methodology
Sahil Mulewar	Software
Sudha Rajamani	Validation
Sahil Mulewar	Formal analysis
Sahil Mulewar	Investigation
Sudha Rajamani	Resources
Sahil Mulewar	Data Curation
Sahil Mulewar	Writing - original draft preparation
Sudha Rajamani, Anupam Sawant, Souradeep Das	Writing - review and editing
Sahil Mulewar	Visualization
Sudha Rajamani	Supervision
Sahil Mulewar, Sudha Rajamani	Project administration
Sudha Rajamani	Funding acquisition

This contributor syntax is based on the Journal of Cell Science CRediT Taxonomy[1].

[1] https://journals.biologists.com/jcs/pages/author-contributions

Chapter 1 Introduction

Throughout the history of humanity, understanding the enigma called *life* has kindled and puzzled countless poets, philosophers and scientists. An even tougher problem is understanding the origins of life on our planet, tackled by the highly interdisciplinary branch of science called prebiotic chemistry. Prebiotic chemists, such as ourselves, are investigating various ways of synthesising in-vitro life. Erwin Schrodinger, in his book *"What is life?"*(Schrödinger 1992), has described life as an entropically ordered system at the cost of global disorder. Living systems try to remain in disequilibrium and entropically ordered state at the expense of energy and disorder from the environment. Evolving molecular complexity is a fundamental trait of such chemical systems(Phillips 2021; Schrödinger 1992).

The emergence of life on our planet is thought to have happened on Hadean-Archean earth from a prebiotic soup consisting of heterogeneous sets of inorganic and simple organic molecules(Benner et al. 2020). Various kinds of interactions between these molecules led to the synthesis of complex organic molecules and reaction networks, which evolved with time and further led to the transition of such chemistry to biology(Black and Blosser 2016). Both covalent and non-covalent interactions between the molecules would have played a crucial role in increasing the complexity of the prebiotic mixture. Covalent interactions between the molecules leads to the formation of new molecules, and non-covalent interactions, such as self-assembly-driven supramolecular assemblies, can result in crucial emergent properties. In the transition from non-life to life, there would have been a stage representing minimal self-sustained replicating cellular system called protocell(Allen et al. 2003; Schrum, Zhu, and Szostak 2010). Current prebiotic chemistry research aims at synthesising such protocell systems using a bottom-up approach.

In our lab, we are testing whether the protocell stage can be achieved through messy chemistry in a heterogeneous prebiotic milieu. We take a systems chemistry approach to characterise the interactions between various classes of molecules and study the effect of this interaction on the resulting complexity of the system(Ruiz-Mirazo, Briones, and de la Escosura 2014). In the past, Joshi *et al*., from our lab, demonstrated the prebiotic synthesis of single acyl chain-based lipidated amino

acids from a pool of interacting amino acids and lipids(Joshi, Sawant, and Rajamani 2021). They then characterised their membrane-forming properties, underlining the direct consequences it could have had for the formation of functional protocells. In another study, Deshpande *et al.*, have studied the effect of nucleotide-membrane interactions on the physicochemical properties of the protocellular membrane(Data unpublished).

Building upon this work, in this MS project, we are interested in two cross-talks. Firstly, the focus is on characterising the products of the reaction between nucleosides and prebiotic lipids, called nucleolipids, followed by characterizing their supramolecular assembly and their potential to functionalise as a protocell. Secondly, studying the non-covalent interaction between amino acids and prebiotic membranes and its effect on the physicochemical properties of model protocell membranes. Investigating both of these pertinent cross-talks have crucial implications for understanding the emergence of complex protocells from a heterogeneous prebiotic milieu.

A) Design and synthesis of nucleolipid molecules for the functionalisation of protocells

Nucleic acids and membrane-forming amphiphiles are two crucial biomolecules central to all contemporary life. Formation in simulated prebiotic syntheses experiments and their presence in carbonaceous chondritic content, strongly suggest the presence of their basic units, i.e., nucleobases, nucleosides and single acyl chain amphiphiles, in the prebiotic soup(Deamer et al. 2002; Oba et al. 2022; Saladino et al. 2015). These basic units are thought to have interacted, complexified and evolved to give their respective functional polymers that we see in extant biology today. Given this, it is pertinent that there existed a cross-talk between membrane-forming amphiphiles and nucleosides in a heterogeneous prebiotic milieu. A chemical reaction between nucleosides (nucleotides) and lipids can, in principle, give rise to lipid-nucleosides (nucleotides) hybrid conjugates called nucleolipids.

Nucleolipids are hybrid organic molecules consisting of a lipid tail and a nucleobase/nucleoside/nucleotide/oligonucleotide as a head group(Rosemeyer 2005). They are also found in extant processes; for e.g., cytidine diphosphate diacylglycerol is the most ubiquitous naturally occurring nucleolipid. It is an essential intermediate in phospholipid metabolism from which glycolipids and lipoproteins are formed. Many other natural forms of nucleolipids have been discovered, which act as

antibiotics(Rosemeyer 2005). Their natural presence in living organisms makes a strong case for their formation/presence in the prebiotic chemical space and, ergo, for their potential implication for protocell emergence and evolution. Recently, Sutherland et al. have shown the formation of such molecules in methyl isocyanide-based activation chemistry reactions using AMP(Bonfio et al. 2020).

Nucleolipids are especially interesting because they possess the properties of two classes of biomolecules(Baillet et al. 2018). Lipid tails along with polar head groups gives them the amphiphilic nature required for self-assembly, while the nucleoside component of the head group confers them the ability to show molecular recognition. Since their discovery in natural systems, various kinds of nucleolipid molecules containing complex lipid components have been synthesised and studied. These synthetic chemistry studies have also shown the potential of these molecules to self-assemble into vesicles, micelles, hydrogels and organogels(Baillet et al. 2018; Berti et al. 1998; Milani et al. 2007; Moreau et al. 2008). These molecules possess base-specific recognition properties similar to what is seen in nucleic acids. Even though synthesised versions of nucleolipids have been used for drug delivery, the fundamental properties such as self-assembly into vesicles and membrane dynamics of membranes comprising of these molecules remain to be systematicaly investigated.

In this project, we synthesise potentially prebiotically relevant nucleolipids containing single acyl chain lipids as early protocells are thought to have been made of such lipids. Single acyl chain amphiphiles are considered precursors of modern-day complex diacyl phospholipids(Deamer et al. 2002). We set out to characterise the supramolecular assemblies of that result from these nucleolipid molecules and also investigate the functional attributes their presence confer to a protocell.

B) Self-asssembly of protocell membranes in an amino acid milieu

As already mentioned, compartmentalisation is an essential attribute of life. In the process of origin of cellular life on our planet, functional protocells would have directly resulted from the self-assembly of protoamphiphlies and their subsequent evolution(Schrum, Zhu, and Szostak 2010). Early protocell membranes are thought to have been composed mainly of single-chain amphiphiles such as fatty acids and this is due to their potential prebiotic availability as suggested by their presence in

carbonaceous chondrites and formation in prebiotic processes like Fischer-TropschType synthesis(Deamer et al. 2002). Unlike their contemporary diacyl counterparts i.e. the phospholipids, fatty acids self-assemble into membranes in a very narrow pH range, i.e., when the pH of the environment is around the pKa of their head group(Morigaki and Walde 2007). However, this process of protocell membrane assembly would have been affected by various organic and inorganic co-solutes present in a heterogeneous prebiotic milieu. The presence of these co-solutes would have directly influenced the self-assembly, stability, and evolution of protocells(Black and Blosser 2016).

Amino acids are one of the essential classes of biomolecules in contemporary biology. Hence, studying membrane-amino acid interactions becomes directly relevant. Amino acid interaction with phospholipids is a well-studied phenomenon. Multiple studies show that this interaction affects the physicochemical properties of phospholipids membrane(Ishigami, Suga, and Umakoshi 2015; Kanwa et al. 2020). However, not much is known about the influence of amino acids on the self-assembly of prebiotic membranes. Relevantly, Black et al. have demonstrated that amino acids can bind to vesicles of fatty acids and can stabilise them against high salt concentrations(Cornell et al. 2019).

In this aforementioned context, this project aimed to investigate the following objectives:

1) How does the presence of amino acids as co-solutes influence the self-assembly of prebiotic membranes?

2) Do these interactions affect the physicochemical properties of the membranes?

3) Do the different side chains of various amino acids affect the membranes differently?

Specifically, in this study, oleic acid (a C18 fatty acid) was used as the amphiphilic system of interest. Amino acids of choice were glycine, valine, alanine, serine and histidine. These amino acids were chosen either because they are some of the simpler forms of amino acids and/or are thought to have been available in the prebiotic milieu on the Hadean-archean Earth(Miller 1953). Histidine was chosen because we were curious to study effect of aromatic side chain on the interaction. In previous studies investigating membrane-amino acid interactions, the role of amino acid was that of only an interacting partner. We used a different approach to discern

this role by using amino acids as buffering agents. This is because the amino group of amino acids have a pKa in a range of 9 to 10, and when amino acid solutions are used at pH 9.6 as ‘prebiotic buffers’, their capacity to buffer reactions could have interesting implications for the self-assembly processes involving fatty acids. We, therefore, studied the self-assembly of C-18 systems, using amino acids, glycine, valine, alanine, serine and histidine, as buffering agents.

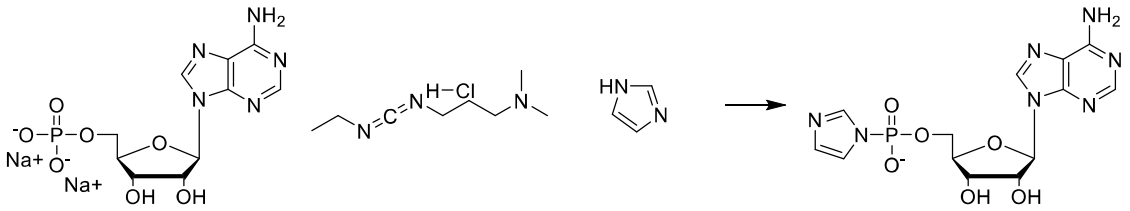
Understating the emergence of protocell from a heterogeneous prebiotic milieu is a systems chemistry problem involving various interactions. A systematic approach towards solving it would be to increase complexity of system step-wise and study the emergent properties. The two projects described above are discerning the emerging complexity of systems involving two different fundamental interactions. Amino acids and nucleosides are fundamental monomers for two essential biopolymers. Hence, the implications of this study would be at the core of the messy chemistry approach to the origin of life research.

Chapter 2 Materials and Methods

A) Design and synthesis of nucleolipid molecules for the functionalisation of protocells

Adenosine-5'-phosphoroimidazolide (ImpA) (2) synthesis

ImpA **2** was synthesised using two reported procedures(Li et al. 2017):



In the first procedure, disodiated salt of AMP **1** (1 eq, 50 mg), EDC (5 eq, 122.6 mg) and imidazole (4 eq, 34.9 mg), were all dissolved in 1 ml of water and the pH was adjusted to 7 and the solution was stirred for 6 hours.

In the second procedure, 100 mg of **1** (1 eq), imidazole (5 eq, 98 mg), triethylamine (4 eq, 116.6 mg), triphenylphosphine (9 eq, 679.9 mg) and DPDS (10 eq, 634.5 mg), were all stirred at RT in 9 ml DMSO for 2 hours. After this, the reaction mixture was added to a pre-chilled mixture of 57.2 ml acetone, 71.5 ml diethyl ether and 8.6 ml triethylamine. Precipitate was observed after addition of 600 ul saturated $NaClO_4$ in acetone to the solution. The solution was then centrifuged at 4000 rpm for 5 min to settle the precipitate. After decanting the supernatant, pellets were washed with acetone (3 ml).

In both the procedures, product **2** was purified by reverse phase flash chromatography. The sample was eluted between a gradient of 0% and 15% acetonitrile in aqueous 25 mM TEAB buffer over 8 column volumes (CV) with a flow rate of 40 ml/min (Fig 1).

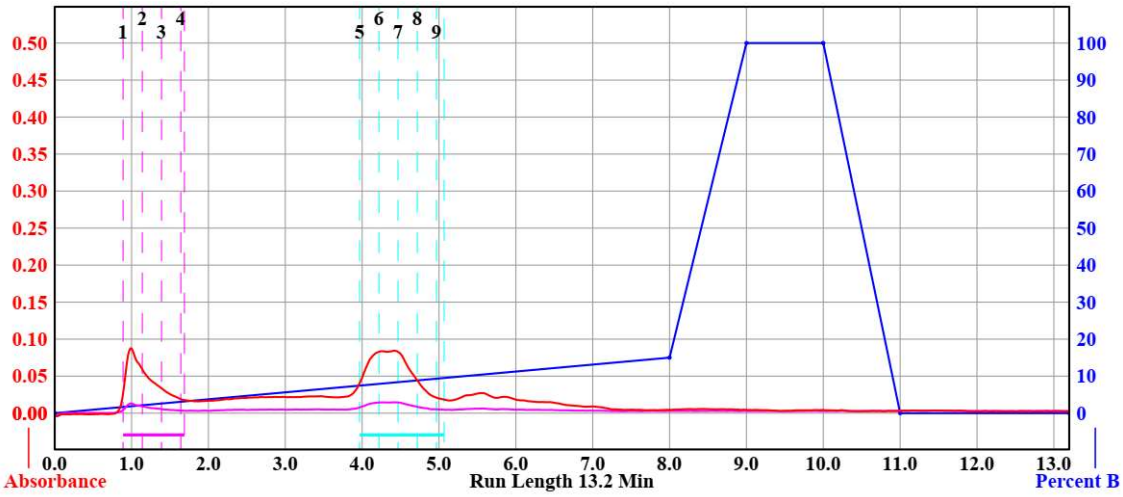


Fig 1: Flash column chromatogram for purification of **2**. First peak indicates elution of AMP. Second peak corresponds with elution of the product ImpA **2**.

Fractions containing the product were concentrated and then lyophilised to yield the product **2**. Product formation was confirmed using H1 and P31 NMR in D2O (Fig 2 & 3), which matched with the previous reports.

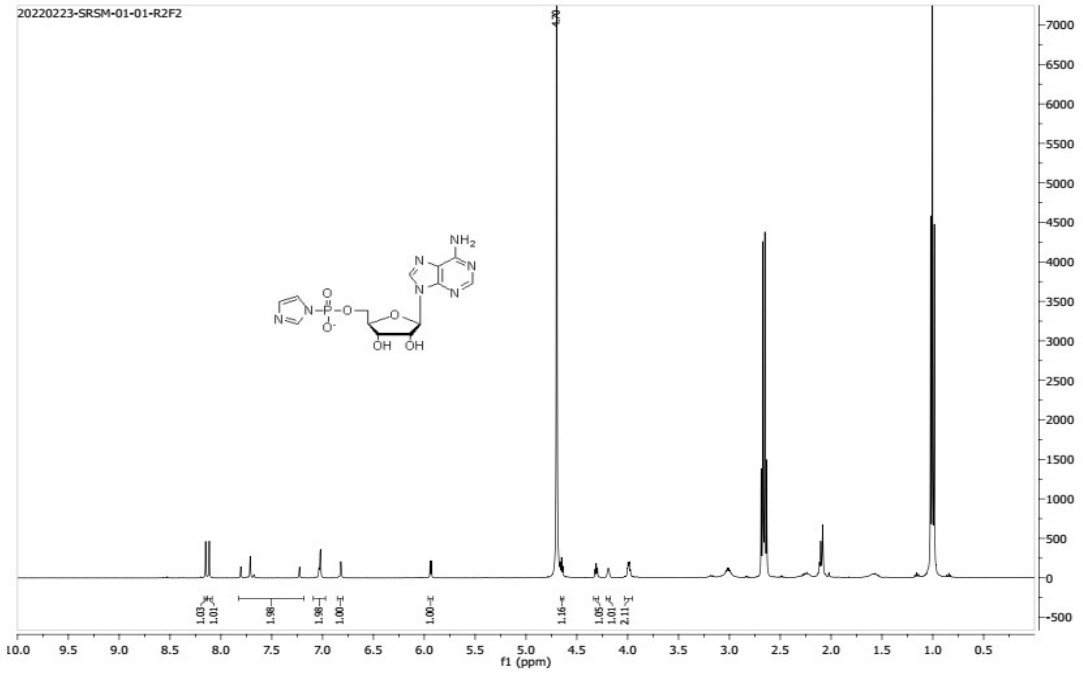


Fig 2: H1 NMR spectrum confirming the formation of **2**.

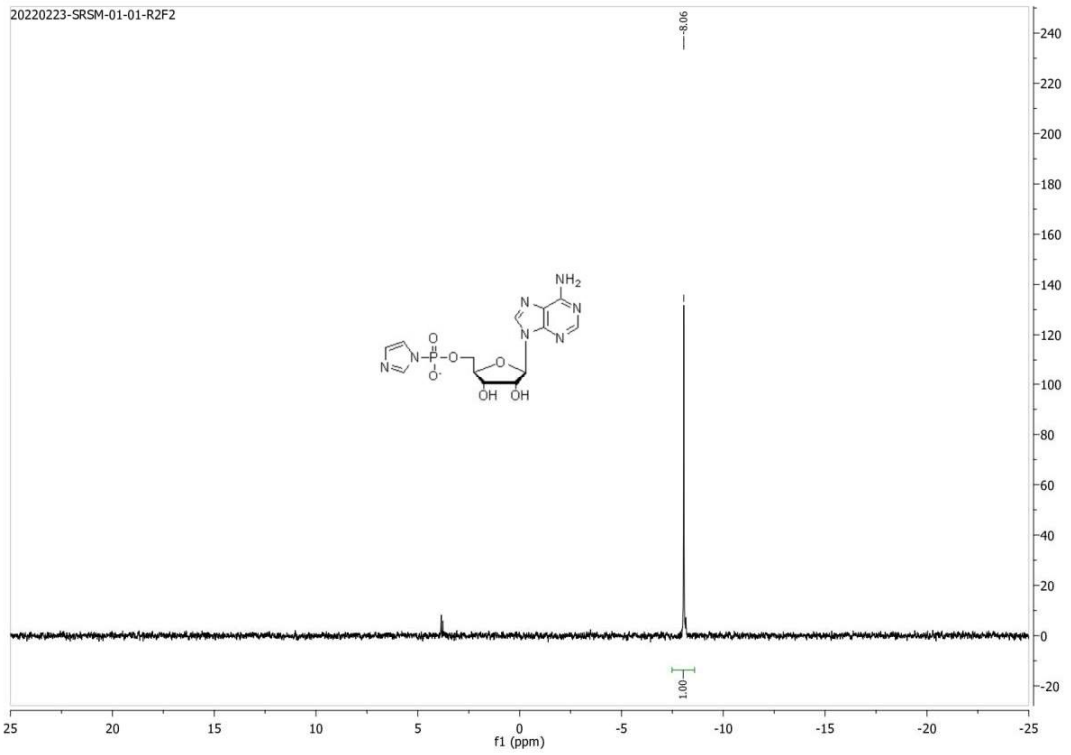
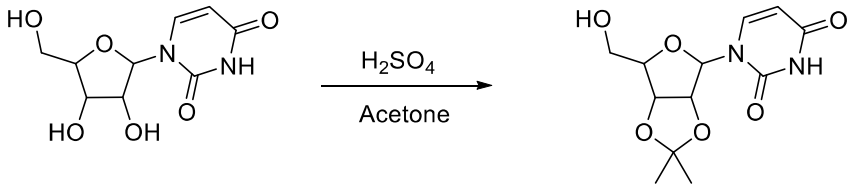


Fig 3: P31 NMR spectrum confirming the formation of **2**.

Synthesis of uridine-2',3'-acetonide 6



6 was synthesised using an earlier reported procedure(Fujihashi et al. 2013). To a stirred solution of 500 mg uridine **5** in 25 ml dry acetone, 250 ul conc. H2SO4 was added dropwise. The reaction mixture was stirred for 2 hrs and then neutralised with triethylamine. Reaction mixture was concentrated and purified using silica gel column chromatography (1-3% MeOH in DCM). Product formation was confirmed using H1 & C13 NMR and MALDI (Fig 4 & 5). MALDI: [M+ Na+]: expected: 307.257, observed: 307.499.

1H NMR (400 MHz, MeOD) δ 7.79 (d, J = 8.1 Hz, 1H), 5.83 (s, 1H), 5.65 (d, J = 8.1 Hz, 1H), 4.87 (dd, J = 6.4, 2.8 Hz, 1H), 4.78 (dd, 1H), 4.19 – 4.15 (m, 1H), 3.76 – 3.65 (m, 2H), 1.51 (s, 3H), 1.31 (s, 3H).

13C NMR (101 MHz, MeOD) δ, ppm: 166.20, 152.07, 143.84, 115.11, 102.63, 94.09, 88.34, 85.80, 82.20, 63.04, 27.54, 25.53.

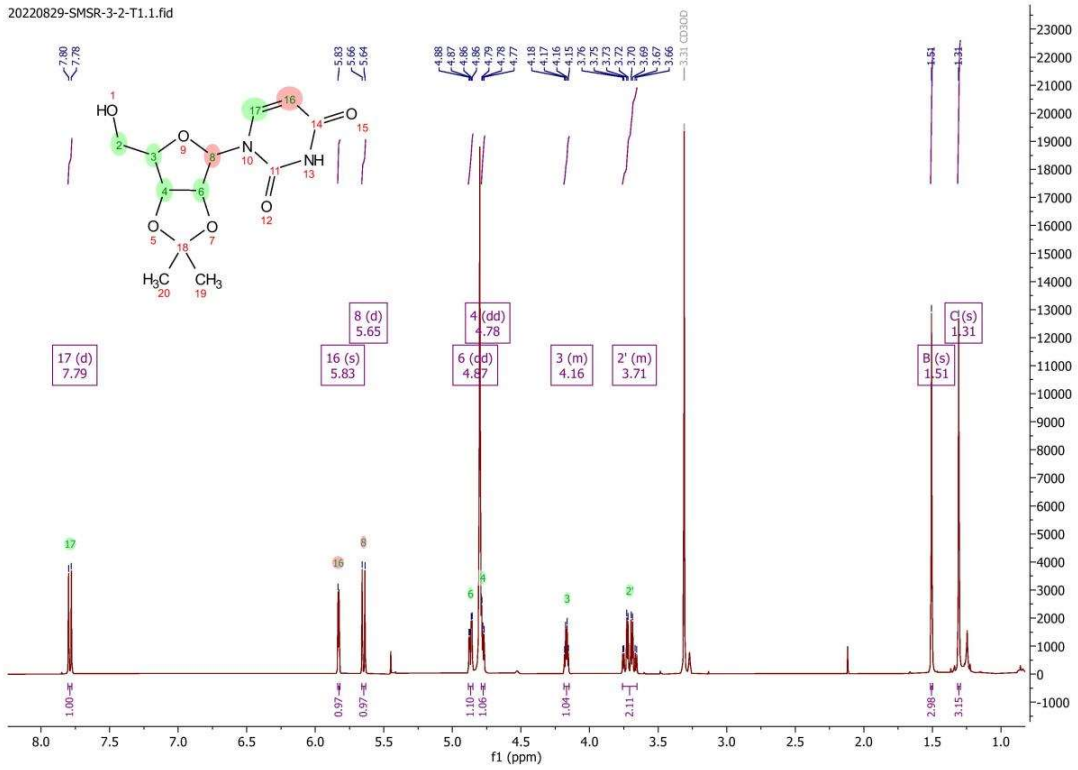


Fig 4: H1 NMR spectrum confirming the formation of product **6**.

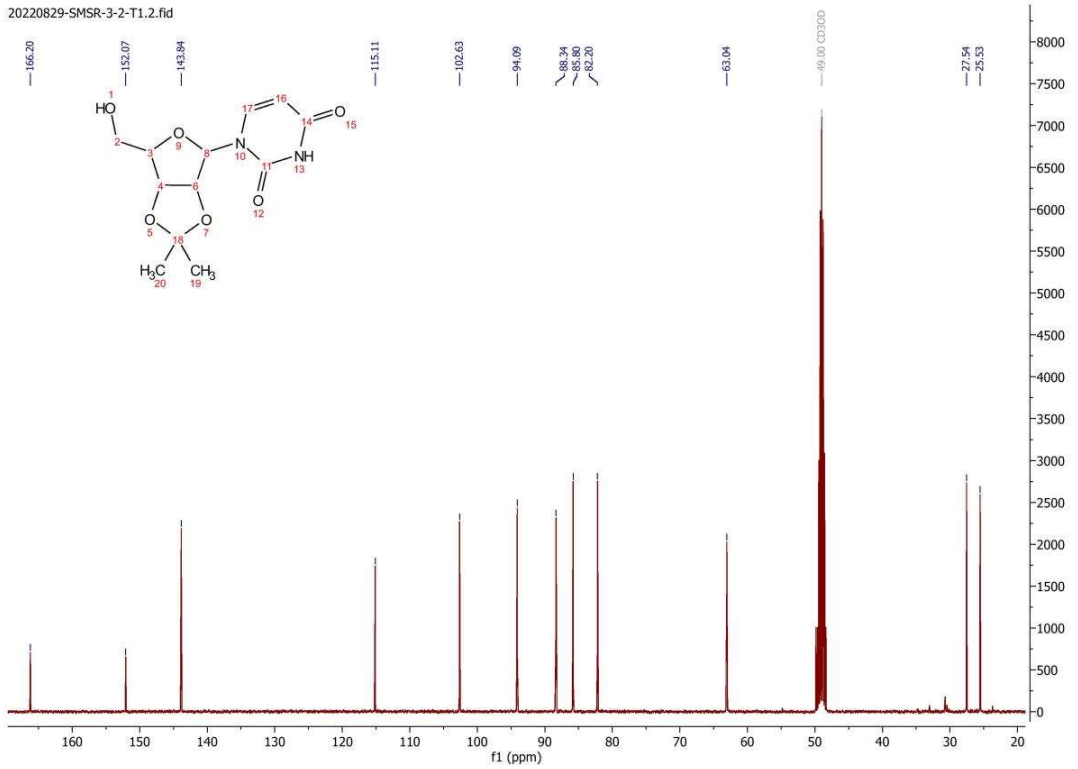
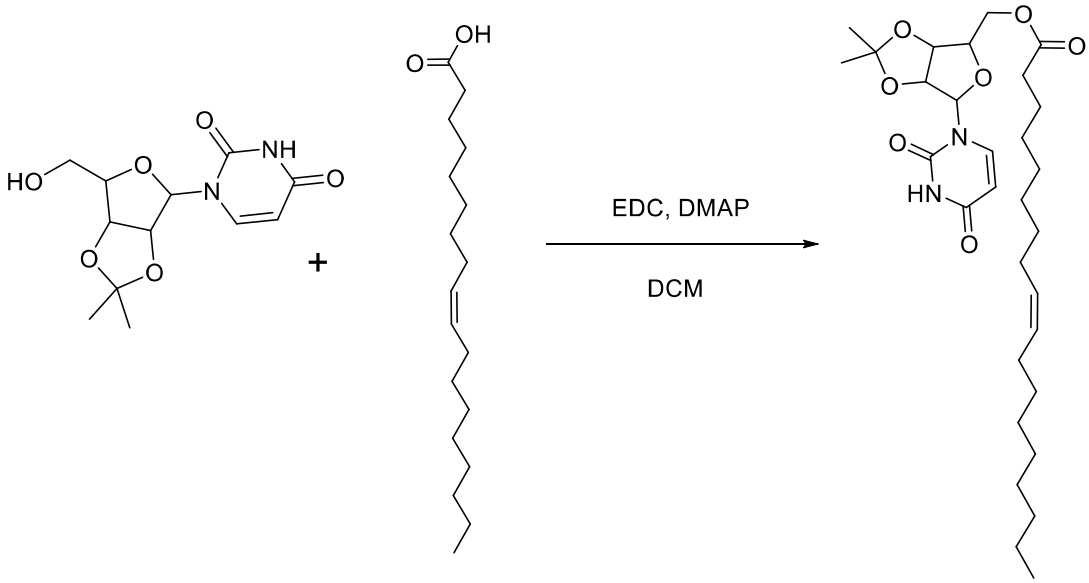


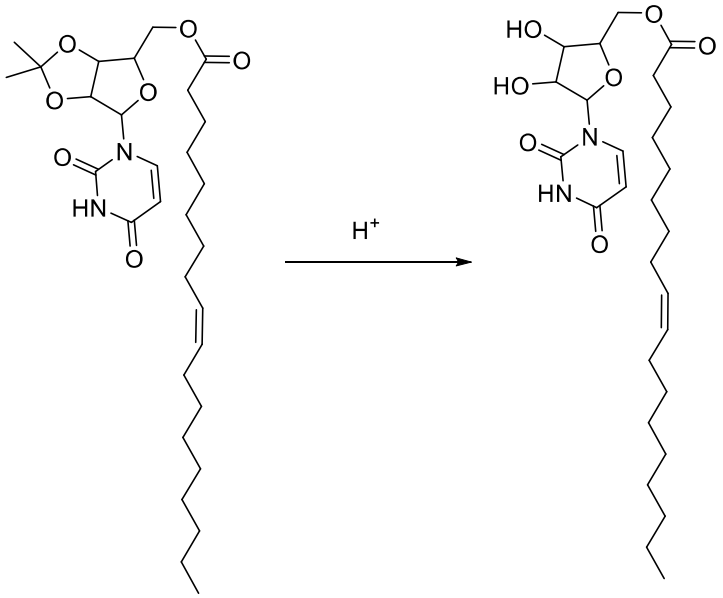
Fig 5: C13 NMR spectrum confirming the formation of product **6**.

Synthesis of 5'-oleyl uridine nucleolipid 8



100 mg of **6** (1 eq), DMAP (0.5 eq, 21.5 mg), oleic acid (1.1 eq, 109.3 mg) and EDC (1.1 eq, 74.18 mg) were stirred overnight at RT in 1 ml anhydrous DCM. The product 5'-oleyl uridine-2',3'-acetonide **7** formation was monitored over TLC. Reaction mixture was diluted in chloroform and extracted using saturated NH4Cl and then with

water. Combined organic layer concentrated and the product was purified using silica gel column chromatography (0.5%-3% MeOH in DCM).



7 was then subjected to acetonide deprotection reaction by dissolving it in 10 ml of 80% acetic acid in water and stirred at 50 deg for 2 days. Reaction mixture was then transferred to a beaker containing 20 ml saturated $NaHCO_3$ and 20 ml ethyl acetate, and stirred until neutralisation. Entire material was transferred to a separating funnel and ethyl acetate layer was extracted. It was further extracted using brine, dried over Na_2SO_4 and concentrated. Product 5'-oleyl uridine **8** was purified using silica gel column chromatography (1-5% MeOH in $CHCl_3$). (yield: 107 mg). Nucleolipid **8** formation was confirmed using HRMS: [M+H+] m/z: expected: 509.3222, observed: 509.3217, H1, C13, H1-H1 COSY, H1-C13 HSQC NMR spectroscopies (Fig 6-9).

1H NMR (400 MHz, $CDCl_3$) δ 10.46 (s, 1H), 7.61 (d, J = 8.1 Hz, 1H), 5.84 (d, J = 3.4 Hz, 1H), 5.73 (d, J = 8.2 Hz, 1H), 5.34 – 5.32 (m, 2H), 4.42 – 4.31 (m, 2H), 4.29 – 4.24 (m, 2H), 4.15 – 4.10 (m, 1H), 2.37 – 2.30 (m, 2H), 2.03 – 1.97 (m, 4H), 1.64 – 1.58 (m, 2H), 1.30 – 1.24 (m, 20H), 0.87 (t, J = 6.8 Hz, 3H).

13C NMR (101 MHz, $CDCl_3$) δ 173.55, 164.09, 151.39, 140.07, 130.18, 129.77, 102.60, 90.71, 82.12, 74.93, 70.23, 63.36, 34.23, 32.02, 29.89, 29.83, 29.65, 29.45, 29.44, 29.36, 29.27, 29.25, 27.35, 27.30, 24.96, 22.81, 14.24.

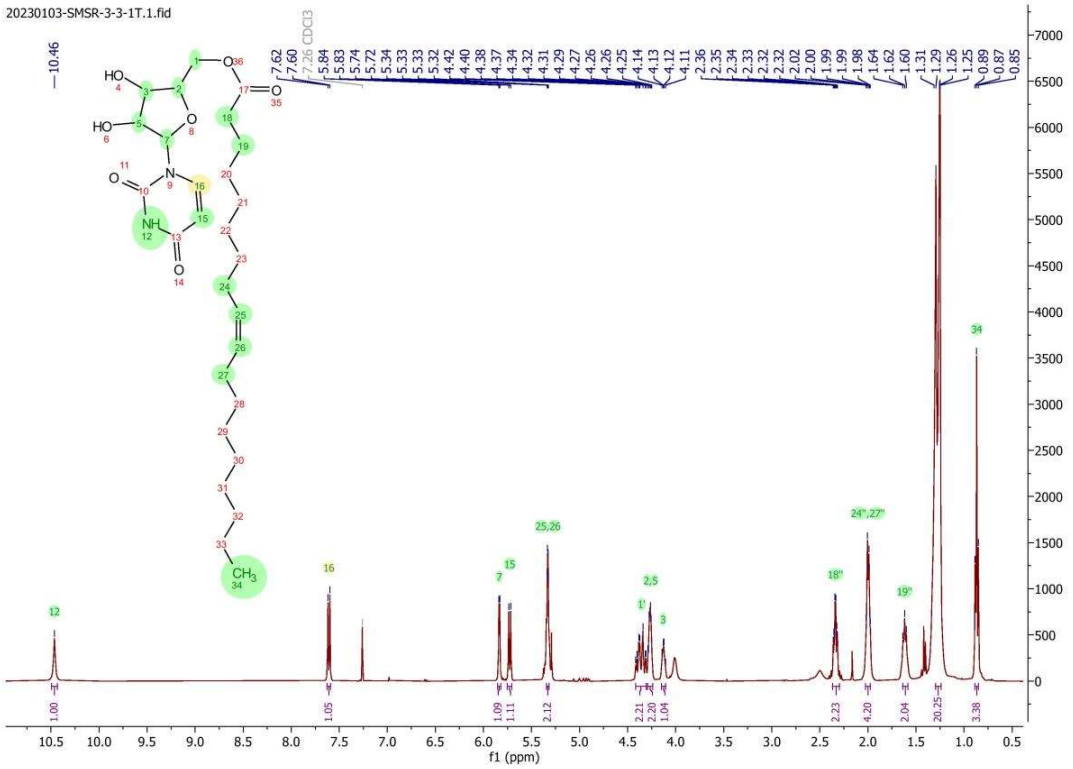


Fig 6: H1 NMR spectrum confirming the formation of the nucleolipid **8**.

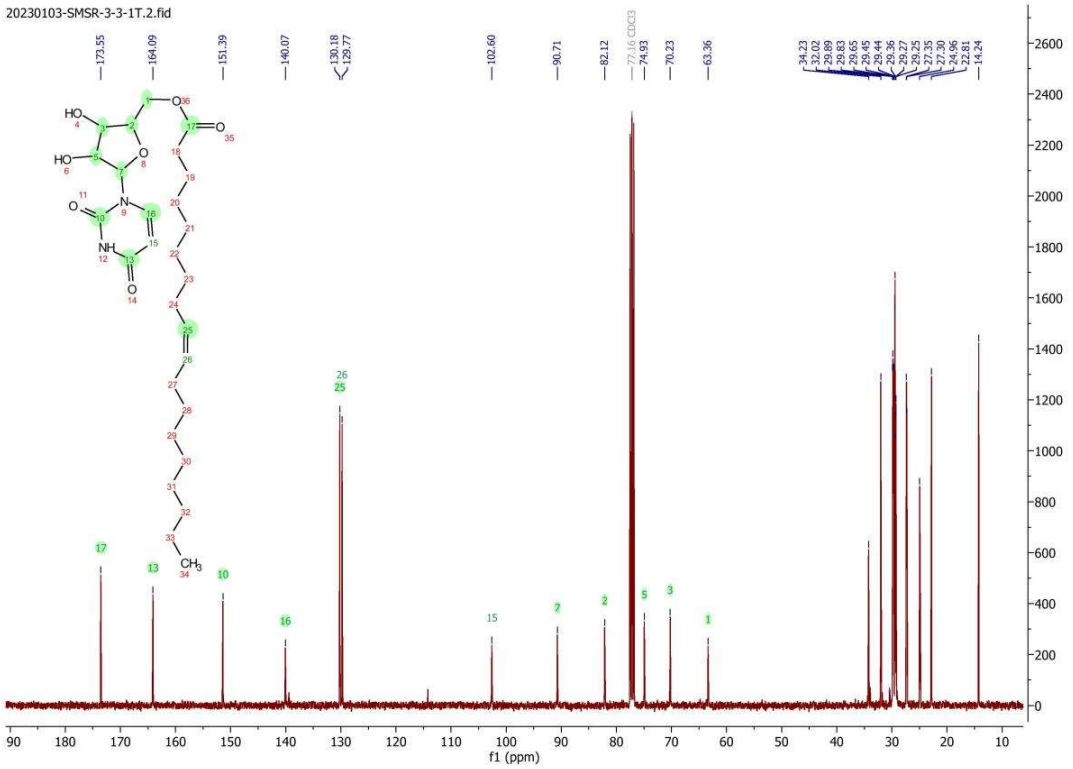


Fig 7: C13 NMR spectrum confirming the formation of the nucleolipid **8**.

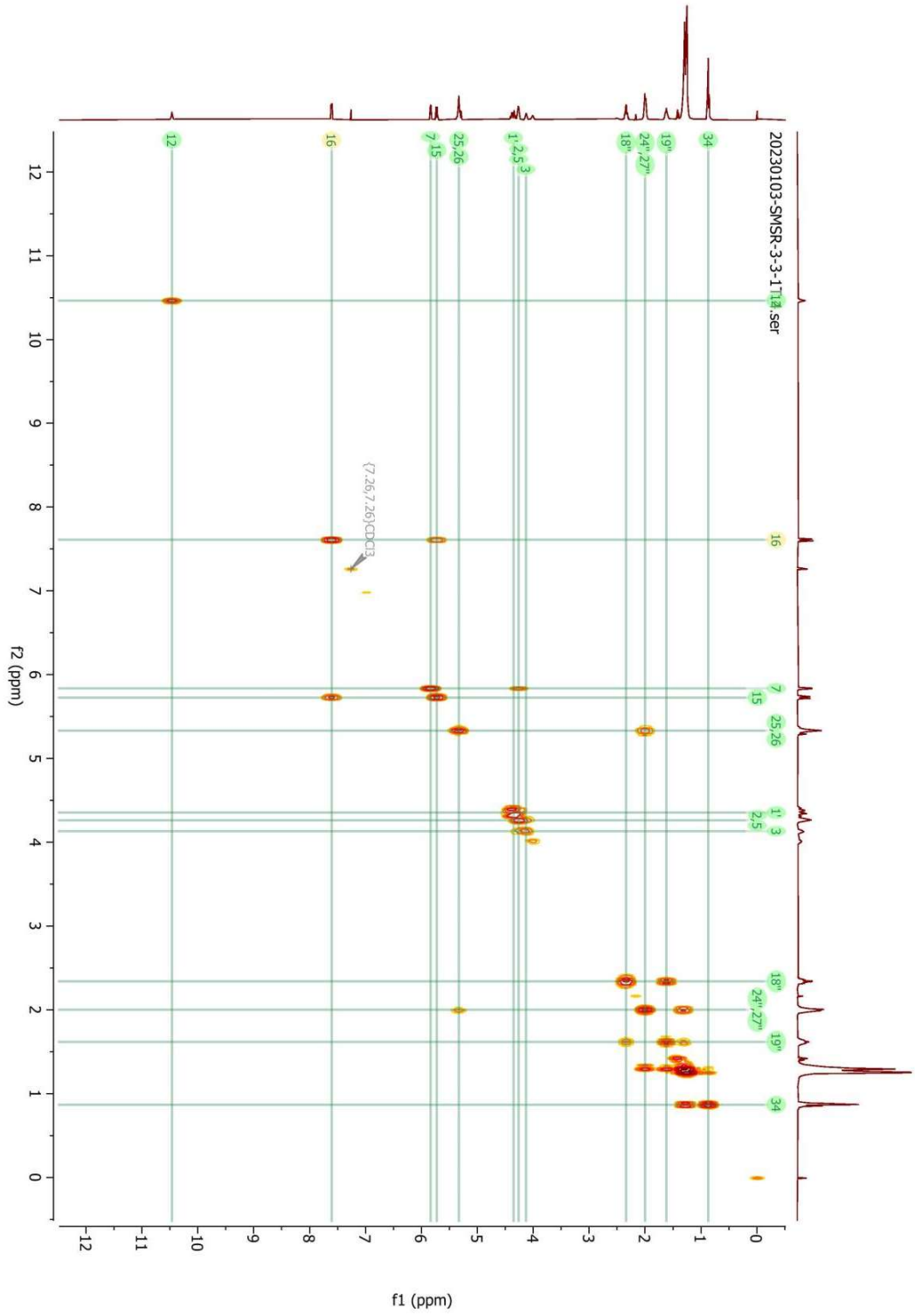


Fig 8: H1-H1 homonuclear correlation spectroscopy (COSY) NMR spectrum of nucleolipid **8**.

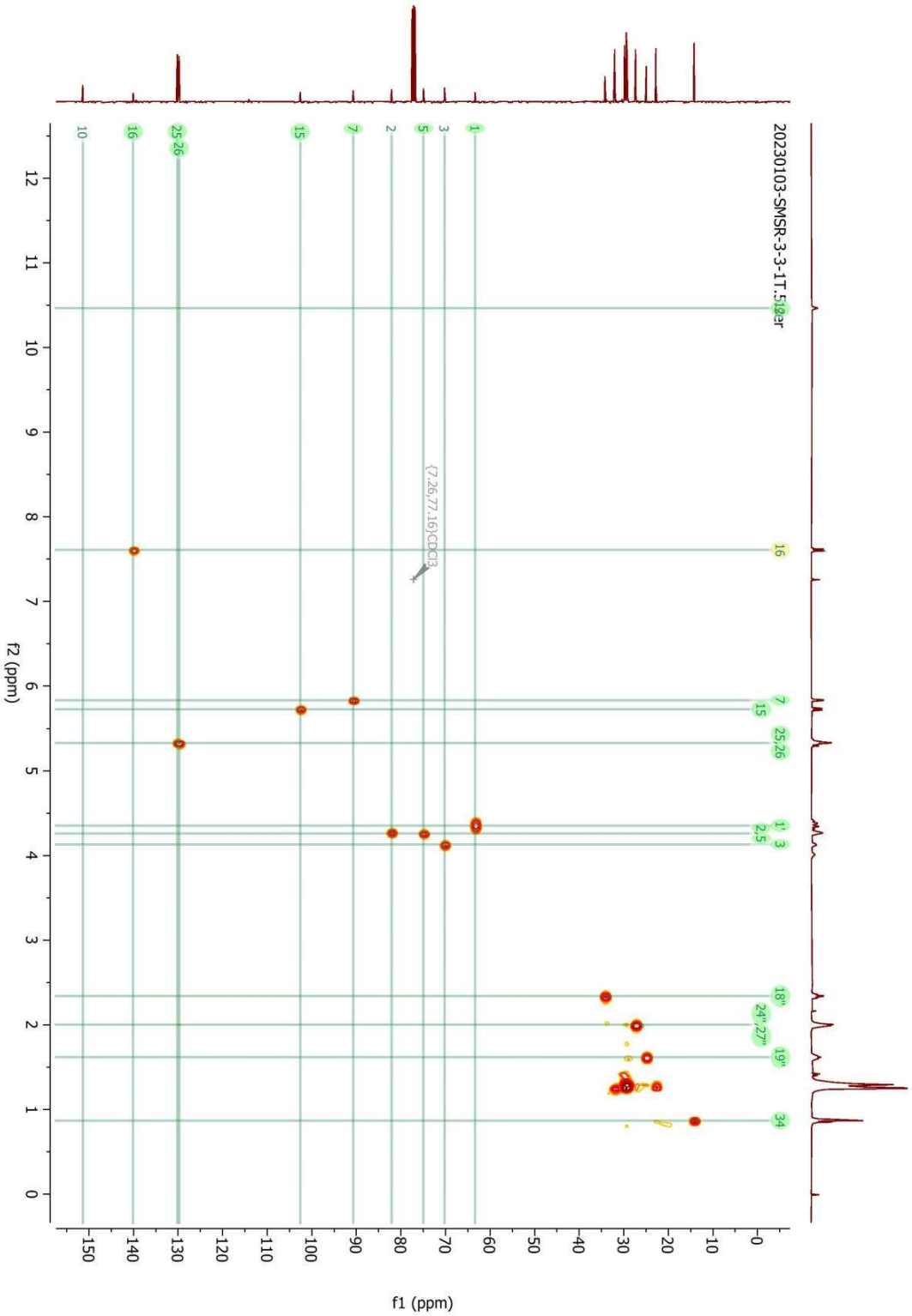


Fig 9: H1-C13 Heteronuclear Single Quantum Coherence spectroscopy (HSQC) NMR spectrum of nucleolipid **8**.

B) Self-assembly of protocell membranes in an amino acid milieu

Buffer Preparation

200 mM buffer of pH 9.6 was prepared in the following manner, 200 mM solution of each amino acid and of CHES was made by adding an appropriate amount of the solute in filtered MilliQ water. The pH of the solution was then brought to pH 9.6 by adding the required amount of NaOH. The pH of the buffer was also checked after adding oleic acid to it, which remained unchanged. This confirmed the buffering ability of amino acid solutions under our experimental conditions.

Vesicle Suspension Preparation

Vesicle suspensions were made using the thin-film method(Zhu, Budin, and Szostak 2013). The lipid film was made by taking an appropriate amount of lipid solution from a 10mg/ml stock of oleic acid in methanol and subsequently evaporating methanol in a vacuum desiccator for 3 hrs. The obtained lipid film was then rehydrated with an appropriate volume of buffer to make the required concentration of oleic acid suspension. The suspension was then kept on thermomixer for 3 hrs at 50 deg C at 350 rpm, and then kept for overnight equilibration at RT.

Microscopy Analysis

3 mM oleic acid suspensions, made in the respective amino acid solutions and in CHES buffer (control), were visualised using Differential Interference Contrast (DIC) microscopy and epifluorescence microscopy. Both the microscopy imaging were done on an Axio Imager A1, Carl Zeiss microscope at 40X magnification (NA = 0.75). For Epifluorescence microscopy, samples were stained with 3 uM Nile red dye and were imaged using a DsRed filter. 8 µL of the sample was placed on the glass slide and covered using a glass coverslip. The coverslip was sealed at the corners using nail polish, and the samples were imaged soon thereafter.

Estimation of Critical Vesicular Concentration (CVC)

DPH (1,6-Diphenyl-1,3,5-hexatriene) fluorescence assay was used to estimate the CVC of oleic acid in the presence of various amino acid buffers. DPH is a hydrophobic fluorescent dye whose intensity of fluorescence depends on its local environment. When present in a hydrophobic environment, it shows a hyperchromic effect on fluorescence emission. In this case, an inflection in the fluorescence intensity of DPH potentially indicates vesicle formation, which also needs to be

confirmed using optical microscopy methods(Sarkar et al. 2020). Different suspensions of varying oleic acid concentrations were made in respective buffers, and an appropriate amount of DPH was added to each sample to make its final concentration to 2 uM. Samples were vortexed and centrifuged before loading on to a 96-well plate. The plate was then kept on a thermomixer at 40 deg C at 300 rpm for 15 minutes before fluorescence measurement was performed using a Tecan multimode plate reader. Samples were excited at 350 nm, and the emission was recorded at 452 nm.

Laurdan fluorescence assay

Laurdan is a non-polar fluorescent solvatochromic probe that shows a red shift with increasing polarity in its environment(Gunther et al. 2021). It has a hydrocarbon chain, which makes it preferably partition into the bilayer, with its ring structure present closer to the head group of the amphiphile. All these properties make laurdan a preferable choice for studying membrane properties. Appropriate volume of 200uM laurdan stock was added to the lipid suspensions, so as to keep its concentration at 3uM in the final solution. The samples were then incubated at RT for a minimum of 15 minutes before subjecting to fluorescence spectroscopy using a fluorescence spectrophotometer. Each sample was subjected to a steady-state fluorescence assay.

Redshift of laurdan was calculated by the parameter generalised polarity (GP), which is defined as

$$GP = (I_{450} - I_{510)} / (I_{450} + I_{510})$$

Each sample was excited at 350 nm, and the emission spectrum and anisotropy were recorded.

Pyrene fluorescence assay

Pyrene excimer formation was used to estimate bilayer content in each sample using the protocol previously standardised in our lab(Sarkar, Dagar, and Rajamani 2021). 3 uM pyrene was added in each vesicle suspension and this was incubated for at least 15 minutes at RT before subjecting it to fluorescence spectroscopy. Each sample was excited at 335 nm, and the emission spectra was obtained between 350 and 600 nm. The maximum intensity of the broad excimer peak was observed around 470 nm (I_{ex}). The excimer intensity Iex was normalised with intensity at 372

nm (I1), yielding I_{ex}/I_1. I_{ex}/I_1 is inversely proportional to the vesicle content in the suspension.

Dynamic light scattering

Vesicle sizes were estimated using dynamic light scattering (DLS). In DLS, when a monochromatic light beam passes through a solution containing macromolecules, the light beam is scattered in all directions. The intensity of scattered light changes with time due to Brownian motion of macromolecules. In DLS, intensity fluctuations of scattered light is analysed to obtain the diffusion coefficient, which correlates with the hydrodynamic size of the macromolecule(Stetefeld, McKenna, and Patel 2016).

Each lipid suspension was subjected to DLS using a 633 nm red laser from Malvern instruments. In each experiment, 800 uL of the lipid suspension was taken in a polycarbonate cuvette and analysed, which was followed by vesicle size estimation on a Zetasizer Nano ZS90. Three replicates were performed for each sample and the average value is plotted.

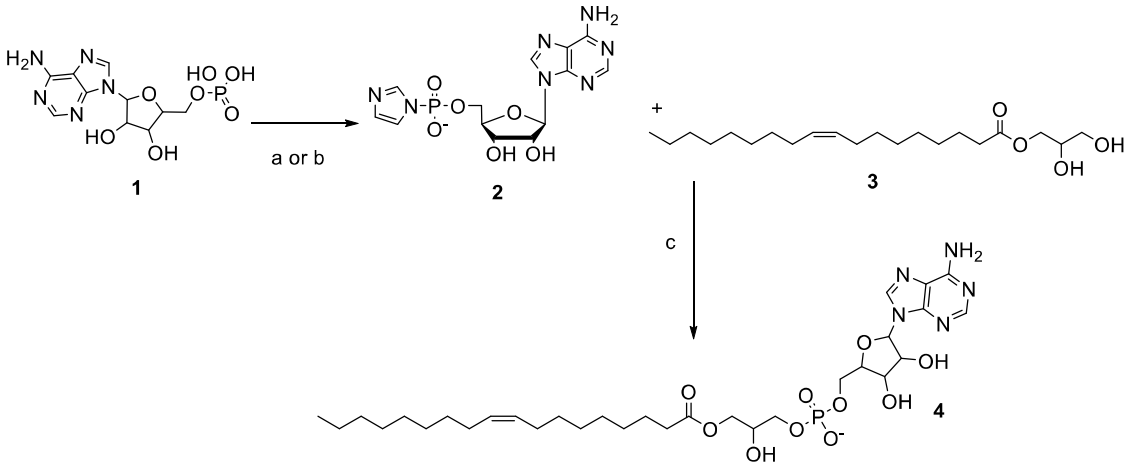
Chapter 3 **Results**

A) Design and synthesis of nucleolipid molecules for the functionalisation of protocells

Design and synthesis of nucleolipids

We aimed to synthesize single acyl-based nucleolipids of uridine and adenosine. Scheme 1 shows nucleolipid containing 1-oleyl-lysophosphatidic acid based nucleolipid and scheme 2 shows oleyl linked nucleolipid.

In scheme 1, we started with activating the nucleotide by attaching an imidazole ring, which is a prebiotically relevant activating agent. To this activated nucleotide **2**, we attempted nucleophilic attack of glycerol monooleate **3**. We tried various temperature and solvent conditions, however, the hydrolysis product of **2** dominated over the desired reaction. No product formation was observed using TLC and mass analysis. This step is being optimized to improve the product yield.

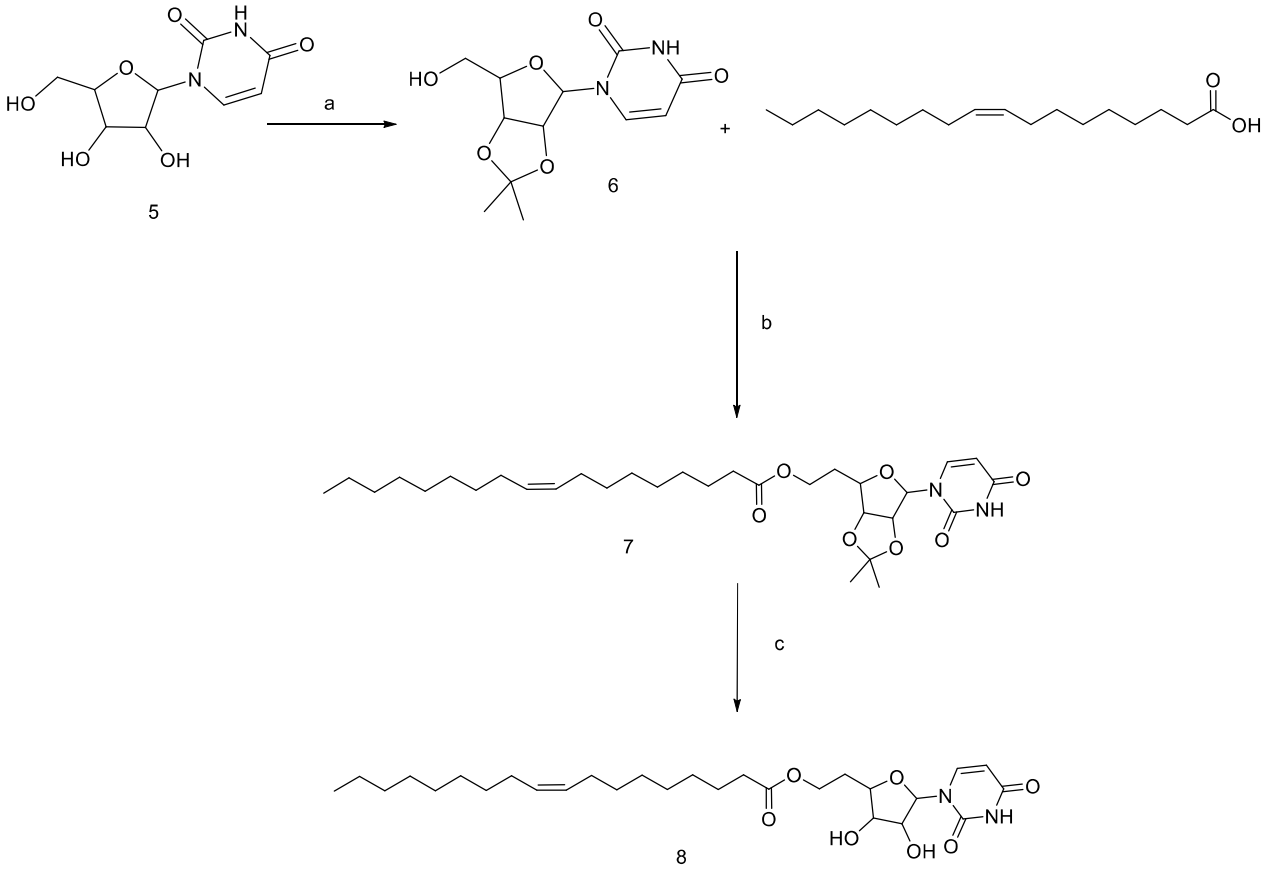


Conditions: **a)** EDC.HCl, Imidazole, Water pH 7, RT 6hrs (Yield: 20%); **b)** Imidazole, DPDS, PPh3, TEA, DMSO, RT 2hrs (Yield: 45%); **c)** MeOH: H2O (1:5) RT 1 to 21 days

Fig 10: Scheme1 showing pathway for synthesis of adenosine based nucleolipid **4.**

In order to synthesize oleyl linked nucleolipids, scheme 2 was employed. First, 2' and 3' -OH groups of uridine **5** were protected using acetonide protection. Then, the protected uridine **6** was esterified with oleic acid under alkaline conditions to yield **7**. 5'-oleyl uridine-2',3'-acetonide **7** was further deprotected under acidic conditions to

yield the desired product 5'-oleyl uridine nucleolipid **8**. Final product **8** was obtained a white crystalline solid at room temperature.



Conditions: a) Conc. H2SO4, acetone, RT 2 hrs; b) DMAP, EDC, DCM, RT overnight; c) 80% acetic acid in water, 50 deg overnight.

Fig 11: Scheme 2 showing pathway for the synthesis of 5'-oleyl uridine nucleolipid **8**.

B) Self-assembly of protocell membranes in an amino acid milieu

Amino acids assisted self-assembly of vesicles

Oleic acid, which has a pKa=8.5, self-assembles into vesicles when the pH of the medium is between 8 to 9. As expected, in a biological goods buffer such as CHES at pH 9.6, it does not form vesicles. However, surprisingly, in the case of amino acid buffers at pH 9.6, oleic acid readily formed vesicles (Fig 12). This suggests that amino acids are assisting in vesicle assembly in some manner. This is pertinent because both these entities would have been present as co-solutes in the prebiotic soup, and their interactions could have led to a new behaviour like that of oleic acid

vesicle formation at pH 9.6, which is at a pH that is one order of magnitude above the pKa of the carboxylic acid head group of the oleic acid.

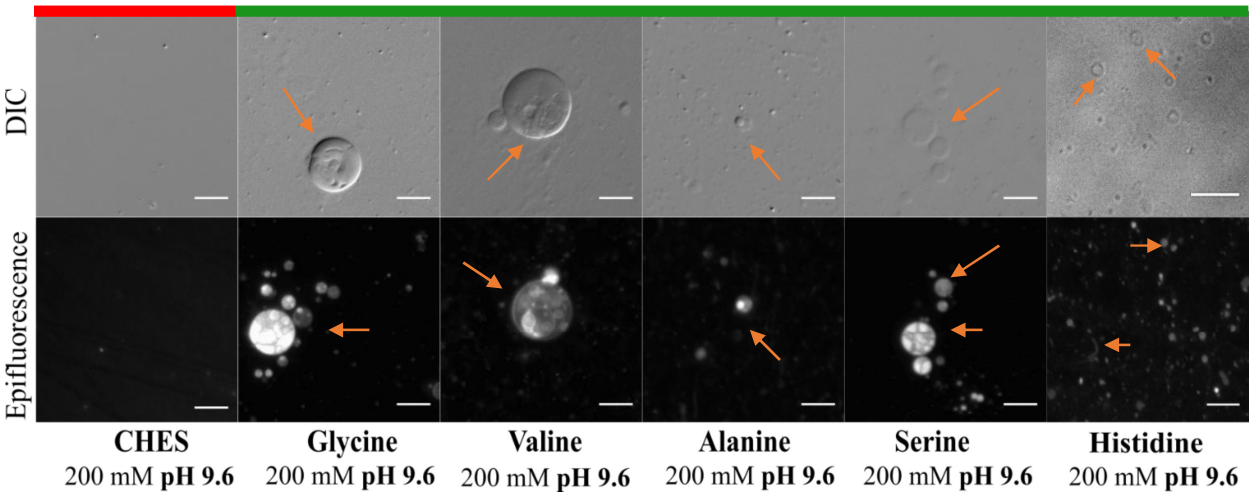


Fig 12: Microscopy images showing presence of vesicles of oleic acid (3mM) as indicated by orange arrows in various amino acid buffers. Scale: 10 microns, Dye used: 3 uM Nile red. N=4.

Estimation of Critical Vesicular Concentration (CVC)

After the microscopy observation, we wanted to study the effect of amino acid interaction on the self-assembly parameters of the membrane. To begin with, we evaluated the CVC of oleic acid vesicles in each amino acid buffer using the fluorescent probe DPH.

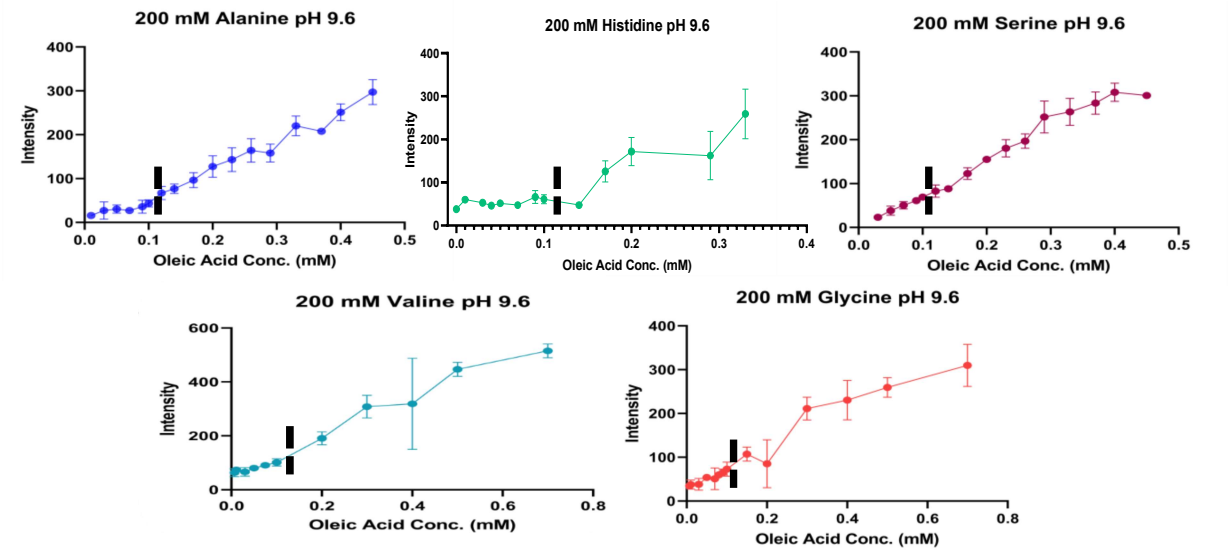


Fig 13: CVC estimation: Graphs represent DPH intensity across various oleic acid concentrations. Black lines show inflexion in intensity indication formation of vesicles at that concentration. N=3.

Even though the inflection points are not clearly apparent in most of the analysis (except in histidine-based reaction), at a preliminary level it seems that no appreciable change was observed in the CVC of oleic acid in the different amino acid buffers. Overall, the CVC seemed to be around 0.1 to 0.12 for most of the amino acids.

Physicochemical properties of oleic acid membranes in the amino acid milieu

In order to study the effect of amino acid interactions on the physicochemical properties of the membrane, we probed various parameters such as membrane polarity, fluidity and bilayer content using fluorescent membrane probes laurdan and pyrene, as described in the methods section. We also compared these properties with oleic acid vesicles that were formed at the usual pH of 8.5 in bicine buffer. The significance of statistical comparison was done using one-way ANOVA. Each sample was compared with every other sample, and the significance was calculated. For ANOVA: p-value = 0.12 (ns), 0.033 (*), 0.002 (**), <0.001 (***).

Membrane polarity

Membrane polarity or water content of the membrane was probed using the GP value of laurdan. The lower the GP value, the more the membrane polarity.

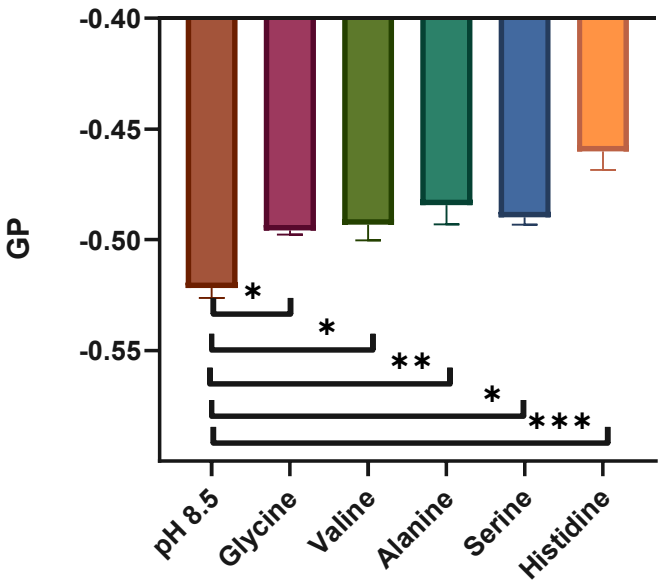


Fig 14: Laurdan GP value analysis to probe membrane polarity. Comparison is done with respect to pH 8.5 bicine buffer sample. N=3

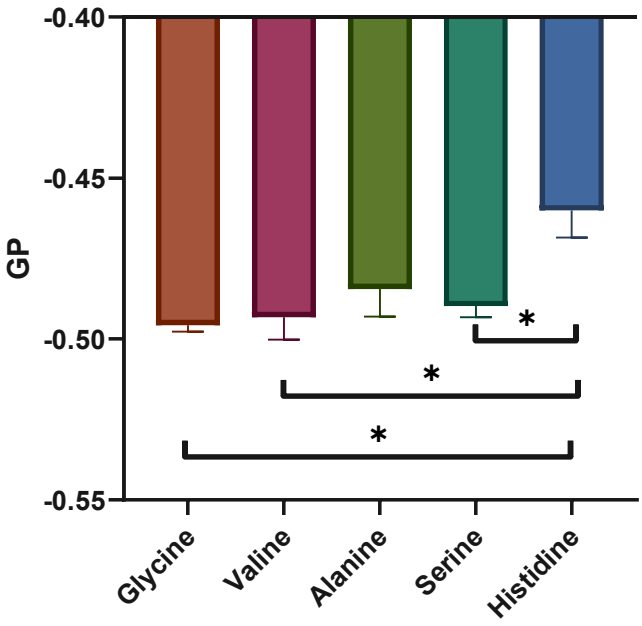


Fig 15: Laurdan GP value analysis to probe membrane polarity. In this data set, each sample was compared with every other sample. Only significant comparisons are shown, rest are non-significant. N=3

We observe that when compared to vesicles formed in pH 8.5 buffer, vesicles in amino acid buffers have a higher GP value. The change is significant for all the samples with the most significant one being for vesicles that formed when histidine and alanine were used as buffers (Fig 14). This indicates that amino acid interaction is decreasing the water content of the membrane, with histidine and alanine interaction resulting in the most observable decrease.

When vesicles formed in the different amino acid buffers were compared across each other, we find that comparison between vesicles formed in glycine, valine and serine with respect to histidine, was relatively the most significant. All other comparisons were non-significant (Fig 15). This seems to indicate that side chain of amino acid is also potentially playing a role in affecting the membrane polarity.

Membrane fluidity

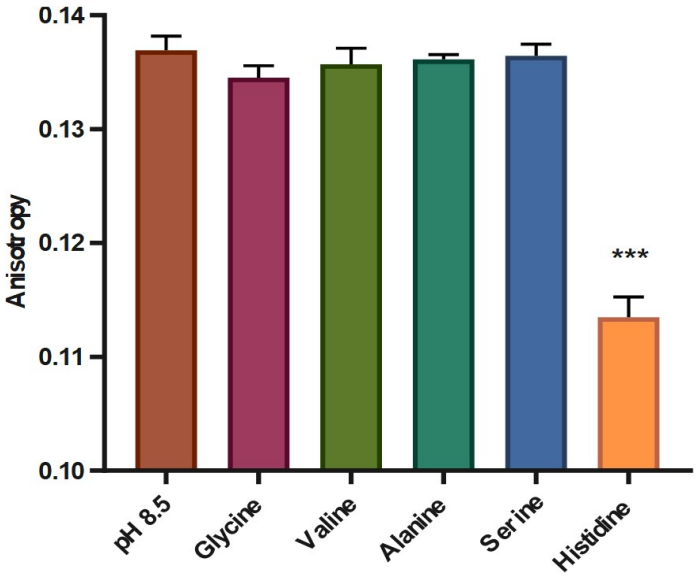


Fig 16: Graph showing anisotropy of Laurdan in vesicles in various buffers. Anisotropy is significantly less in vesicles in histidine buffer than in other buffers. N=3, error bars: std dev.

Fluidity of the membrane was calculated using the anisotropy of laurdan. The lower the anisotropy, the higher is the fluidity of the membrane. Fig 16 shows that the anisotropy was significantly lower for oleic acid vesicles in histidine than in any other sample. All other comparisons were non-significant. This suggests that the membranes formed in the presence of histidine are somehow more fluid than the ones formed in the presence of any other amino acid.

Bilayer content

Pyrene fluorescence emission was used to estimate bilayer content in each lipid suspension. As described in the methods section, I_{ex}/I_1 of pyrene is inversely proportional to the bilayer content.

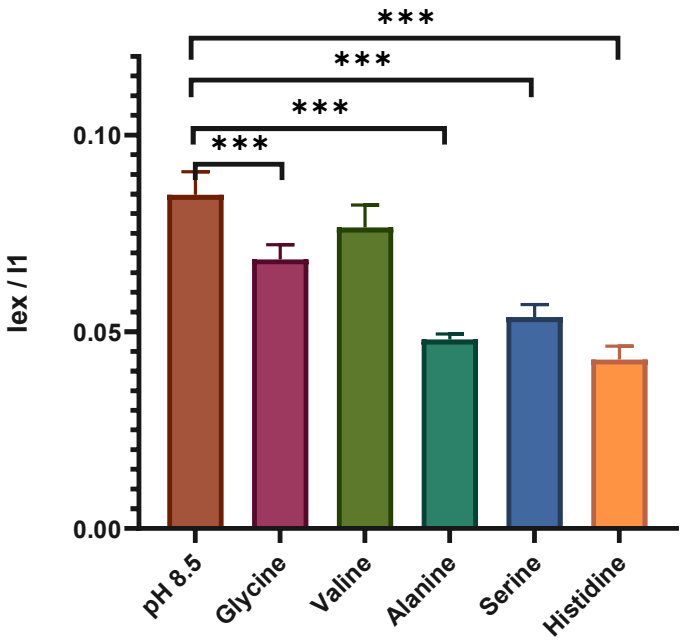


Fig 17: Pyrene excimerization to probe bilayer content in various buffers. Samples with amino acid buffers are compared wrt sample with pH 8.5 bicine buffer. N=3, error bars= std dev.

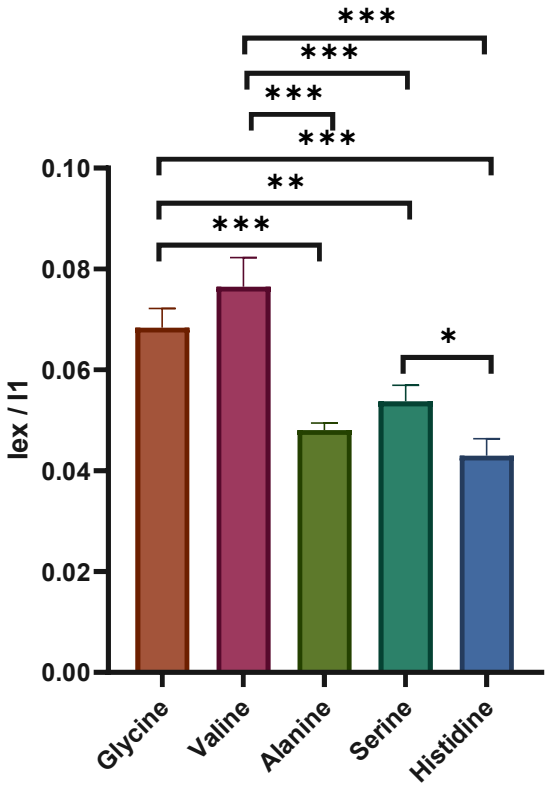


Fig 18: Pyrene excimerization to probe bilayer content in various buffers. Each amino acid buffered sample is compared with every other buffered sample. N=3, error bars= std dev.

Compared to vesicles in pH 8.5 buffer, vesicles in amino acid buffers have a lower Iex/I1. The change is significant for all the samples except for valine (Fig 17). This indicates that bilayer content of the resultant oleic acid vesicles is higher in amino acid buffers than in pH 8.5 bicine buffer, excepting for valine. When vesicles formed in the different amino acid buffers were compared across each other, multiple significant comparisons were observed (Fig 18). This indicates that side-chain interaction plays a prominent role in affecting bilayer content. As the complexity in side chain is increasing, bilayer content is increasing. From these results, vesicle content is highest in alanine and histidine, followed by serine, and then by glycine and valine.

Estimation of vesicle size

Dynamic light scattering (DLS) was used to estimate the size of vesicles formed in each of the amino acid buffers when compared to those formed in pH 8.5 bicine buffer.

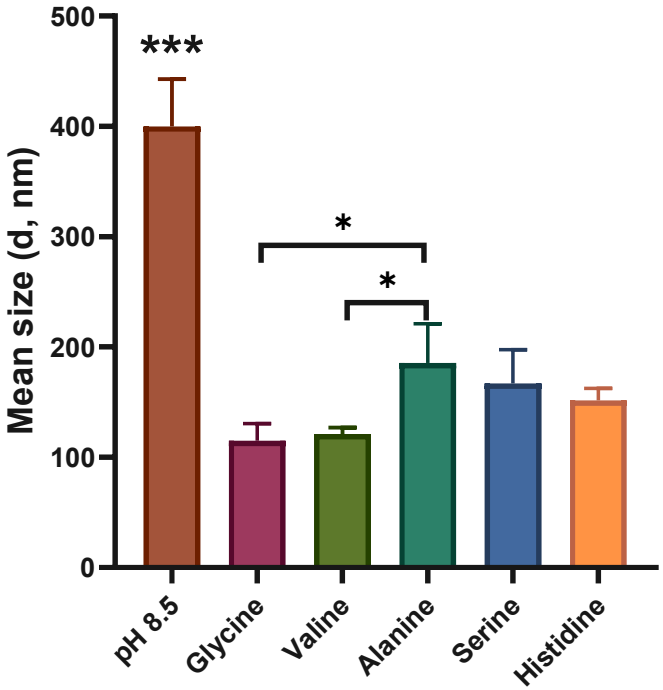


Fig 19: Mean sizes of oleic acid vesicles in various buffers. Vesicles in amino acid buffers have smaller size than compared to vesicles in pH 8.5 bicine buffer. N=3. Error bars: std dev.

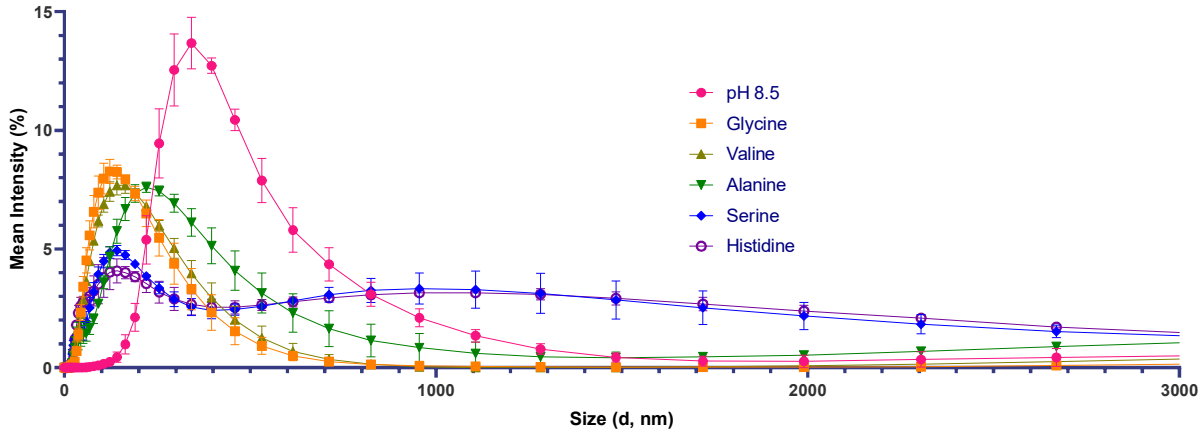


Fig 20: Mean intensity of particles at different sizes in oleic acid suspension in various buffers. Vesicles in amino acid buffers have smaller size than compared to vesicles in pH 8.5 bicine buffer. N=3. Error bars: std dev.

We find that oleic acid vesicle size in pH 8.5 buffer is significantly higher than the ones that resulted in amino acid buffers. At pH 8.5, the mean size of the vesicles is around 400 nm, whereas the once that resulted in the amino acid buffers were predominantly in the size range of 100-200 nm (Fig 19). When vesicles formed in the different amino acid buffers were compared across each other, only glycine v/s alanine and valine v/s alanine came out to be significant. All other comparisons are non-significant. Further investigations are required to elucidate the underlying reason behind smaller vesicle sizes in amino acid buffers than compared to standard bicine buffer.

In the serine and histidine buffered samples, in the plot of mean intensity v/s size, we also saw a broad, less intense second peak at 1000 nm (Fig 20). This broad peak was absent in other samples. At this point, the nature of moieties that result in this broad peak is unknown. Whether it could be vesicles or any other higher order aggregate, arising due to amino acid-oleic acid interaction, can be discerned only with further investigation that would shed light on the nature of these particles.

Chapter 4 Conclusions and Discussion

A) Design and synthesis of nucleolipid molecules for the functionalisation of protocells

We have synthesized an oleyl chain containing uridine-based nucleolipid molecule in high yields. Our ongoing work involves synthesis of adenosine based nucleolipid. Future research directions will involve charactering its self-assembly behaviour and physicochemical properties. Since these molecules have a nucleoside head group, having them as a protocell membrane component have very relevant implications for the function of a protocell. Pertinently, a protocell membrane having nucleolipid molecules in their headgroup, would possess molecular recognition potential because of these nucleobases. Such protocells might easily interact with free nucleotide monomers, potentially concentrating them around a protocell. Such vesicles could act as efficient catalysts for reactions involving nucleotides such as nucleic acid polymerization and replication. These molecules might also hold a potential towards facilitating membrane-nucleic acid interactions via base-stacking and hydrogen bonding (Fig 21). Overall, these molecules could play a key role towards combining lipid and RNA worlds, in the broader area of prebiotic chemistry research.

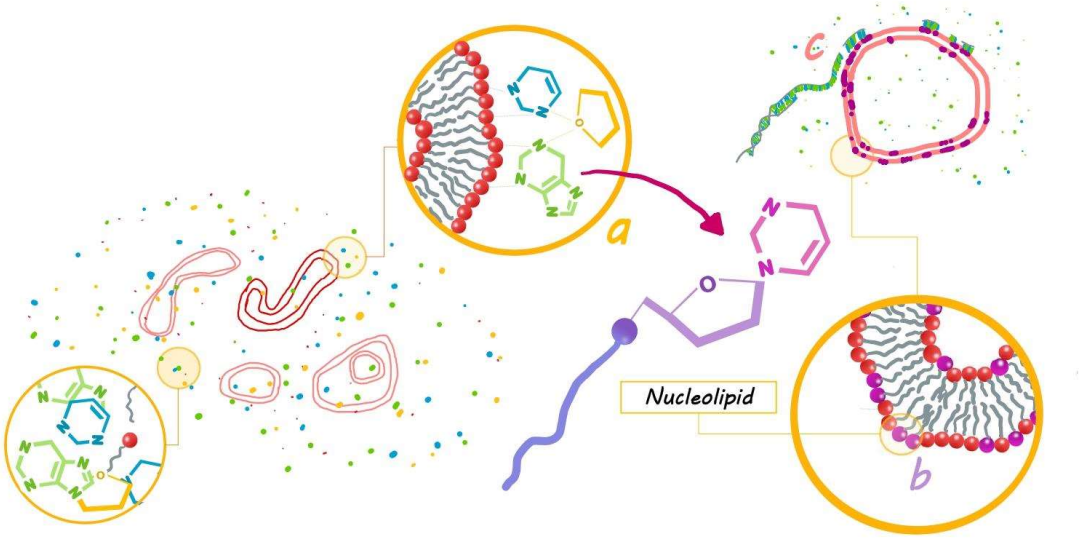


Fig 21: An artistic illustration depicting the a) putative synthesis of nucleolipids in a heterogeneous prebiotic milieu, b) formation of hybrid membranes containing nucleolipids and c) surface catalysis of nucleic acid formation. (Illustration by Sudeepta Sarkar, IISER Pune)

B) Self-assembly of protocell membranes in an amino acid milieu

Fatty acids are considered model candidates for protocell membrane formation. However, fatty acid membranes are very sensitive to various environmental parameters that play a fundamental role in shaping prebiotic processes, including pH, temperature, type and concentration of metal ions etc(Morigaki and Walde 2007; Sarkar et al. 2020). They form vesicles at a narrow pH regime. However, a broad pH regime would have been required for prebiotic reactions to occur that would have eventually resulted in the emergence of a protocell. Fatty acid alone cannot sustain stable membranes under such scenarios. Our result show that amino acid assist oleic acid vesicle formation at a pH, where they cannot self-assemble into vesicles independently. This has important ramifications especially in the aforementioned

context. In addition to helping in vesicle assembly, amino acids also seem to significantly increase the bilayer content in the resultant higher ordered structures. These results show that co-solute interactions in a prebiotic soup context, could indeed have provided robustness to the what is usually thought of as, for e.g., a sensitive membrane forming system in the case of fatty acids. Earlier studies have shown that intermolecular hydrogen bonding plays a prominent role in the membrane-amino acid interactions(Cardenas et al. 2015). Given this, we would like to venture a reasonable guess that such intermolecular hydrogen bonding between amino acids and oleic acid, might have potentially been the facilitating force that assists vesicle formation at a higher pH regime. This, however, needs to be systematically discerned using other experimental and even theoretical studies.

Our studies also show that amino acid-membrane interactions affect the physicochemical parameters of the vesicle. Amino acid interaction with the membrane seems to result in the exclusion of water molecules from the membrane. Water exclusion becomes most prominent with the aromatic amino acid histidine. Interestingly, the interaction with histidine also seems to be making the membrane more dynamic. This indicates that the aromatic ring of histidine might possibly be the prominent interacting group in this scenario. It possible that the aromatic ring might be getting embedded in the bilayer, causing the expulsion of water molecules and affecting the original membrane packing, which results in an increase in lipid mobility within the membrane. Our results show that the side chains of amino acids, in general, seems to play a prominent role in the interaction, affecting the physiochemical properties of the membrane.

These studies, along with the result from Black *et al.,* that showed that amino acid interaction increases the robustness of prebiotic membranes against higher salt concentration(Cornell et al. 2019), argues for the importance of membrane-amino acid interaction in the origin and evolution of cellular life in a heterogeneous prebiotic milieu.

References

Allen, J. F., J. A. Raven, William Martin, and Michael J. Russell. 2003. 'On the Origins of Cells: A Hypothesis for the Evolutionary Transitions from Abiotic Geochemistry to Chemoautotrophic Prokaryotes, and from Prokaryotes to Nucleated Cells'. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 358(1429): 59–85.

Baillet, Julie et al. 2018. 'Lipid and Nucleic Acid Chemistries: Combining the Best of Both Worlds to Construct Advanced Biomaterials'. *Advanced Materials* 30(11): 1705078.

Benner, Steven A. et al. 2020. 'When Did Life Likely Emerge on Earth in an RNA-First Process?' *ChemSystemsChem* 2(2): e1900035.

Berti, Debora et al. 1998. 'Base Complementarity and Nucleoside Recognition in Phosphatidylnucleoside Vesicles'. *The Journal of Physical Chemistry B* 102(1): 303–8.

Black, Roy A., and Matthew C. Blosser. 2016. 'A Self-Assembled Aggregate Composed of a Fatty Acid Membrane and the Building Blocks of Biological Polymers Provides a First Step in the Emergence of Protocells'. *Life* 6(3): 33.

Bonfio, Claudia et al. 2020. 'Activation Chemistry Drives the Emergence of Functionalised Protocells'. *Chemical Science* 11(39): 10688–97.

Cardenas, Alfredo E., Rebika Shrestha, Lauren J. Webb, and Ron Elber. 2015. 'Membrane Permeation of a Peptide: It Is Better to Be Positive'. *The Journal of Physical Chemistry B* 119(21): 6412–20.

Cornell, Caitlin E. et al. 2019. 'Prebiotic Amino Acids Bind to and Stabilize Prebiotic Fatty Acid Membranes'. *Proceedings of the National Academy of Sciences* 116(35): 17239–44.

Deamer, David et al. 2002. 'The First Cell Membranes'. *Astrobiology* 2(4): 371–81.

Fujihashi, Masahiro et al. 2013. 'Substrate Distortion Contributes to the Catalysis of Orotidine 5′-Monophosphate Decarboxylase'. *Journal of the American Chemical Society* 135(46): 17432–43.

Gunther, German et al. 2021. 'LAURDAN since Weber: The Quest for Visualizing Membrane Heterogeneity'. *Accounts of Chemical Research* 54(4): 976–87.

Ishigami, Takaaki, Keishi Suga, and Hiroshi Umakoshi. 2015. 'Chiral Recognition of L-Amino Acids on Liposomes Prepared with l-Phospholipid'. *ACS Applied Materials & Interfaces* 7(38): 21065–72.

Joshi, Manesh Prakash, Anupam A. Sawant, and Sudha Rajamani. 2021. 'Spontaneous Emergence of Membrane-Forming Protoamphiphiles from a Lipid–Amino Acid Mixture under Wet–Dry Cycles'. *Chemical Science* 12(8): 2970–78.

Kanwa, Nishu, Soumya Kanti De, Avijit Maity, and Anjan Chakraborty. 2020. 'Interaction of Aliphatic Amino Acids with Zwitterionic and Charged Lipid Membranes: Hydration and Dehydration Phenomena'. *Physical Chemistry Chemical Physics* 22(6): 3234–44.

Li, Li et al. 2017. 'Enhanced Nonenzymatic RNA Copying with 2-Aminoimidazole Activated Nucleotides'. *Journal of the American Chemical Society* 139(5): 1810–13.

Milani, Silvia, Francesca Baldelli Bombelli, Debora Berti, and Piero Baglioni. 2007. 'Nucleolipoplexes: A New Paradigm for Phospholipid Bilayer−Nucleic Acid Interactions'. *Journal of the American Chemical Society* 129(38): 11664–65.

Miller, Stanley L. 1953. 'A Production of Amino Acids Under Possible Primitive Earth Conditions'. *Science* 117(3046): 528–29.

Moreau, Louis et al. 2008. 'Real Time Imaging of Supramolecular Assembly Formation via Programmed Nucleolipid Recognition'. *Journal of the American Chemical Society* 130(44): 14454–55.

Morigaki, Kenichi, and Peter Walde. 2007. 'Fatty Acid Vesicles'. *Current Opinion in Colloid & Interface Science* 12(2): 75–80.

Oba, Yasuhiro et al. 2022. 'Identifying the Wide Diversity of Extraterrestrial Purine and Pyrimidine Nucleobases in Carbonaceous Meteorites'. *Nature Communications* 13(1): 2008.

Phillips, Rob. 2021. 'Schrödinger's What Is Life? At 75'. *Cell Systems* 12(6): 465–76.

Rosemeyer, Helmut. 2005. 'Nucleolipids: Natural Occurrence, Synthesis, Molecular Recognition, and Supramolecular Assemblies as Potential Precursors of Life and Bioorganic Materials'. *Chemistry & Biodiversity* 2(8): 977–1063.

Ruiz-Mirazo, Kepa, Carlos Briones, and Andrés de la Escosura. 2014. 'Prebiotic Systems Chemistry: New Perspectives for the Origins of Life'. *Chemical Reviews* 114(1): 285–366.

Saladino, Raffaele et al. 2015. 'Meteorite-Catalyzed Syntheses of Nucleosides and of Other Prebiotic Compounds from Formamide under Proton Irradiation'. *Proceedings of the National Academy of Sciences* 112(21): E2746–55.

Sarkar, Susovan, Shikha Dagar, and Sudha Rajamani. 2021. 'Influence of Wet–Dry Cycling on the Self-Assembly and Physicochemical Properties of Model Protocellular Membrane Systems'. *ChemSystemsChem* 3(5): e2100014.

Sarkar, Susovan, Shikha Dagar, Ajay Verma, and Sudha Rajamani. 2020. 'Compositional Heterogeneity Confers Selective Advantage to Model Protocellular Membranes during the Origins of Cellular Life'. *Scientific Reports* 10(1): 4483.

Schrödinger, Erwin. 1992. *What Is Life?: With Mind and Matter and Autobiographical Sketches*. Cambridge: Cambridge University Press.

https://www.cambridge.org/core/books/what-is-life/A876185F2DB06FF5C2CC67C9A60DAD7F (April 1, 2023).

Schrum, Jason P., Ting F. Zhu, and Jack W. Szostak. 2010. 'The Origins of Cellular Life'. *Cold Spring Harbor Perspectives in Biology* 2(9): a002212.

Stetefeld, Jörg, Sean A. McKenna, and Trushar R. Patel. 2016. 'Dynamic Light Scattering: A Practical Guide and Applications in Biomedical Sciences'. *Biophysical Reviews* 8(4): 409–27.

Zhu, Ting F., Itay Budin, and Jack W. Szostak. 2013. 'Chapter Twenty - Preparation of Fatty Acid or Phospholipid Vesicles by Thin-Film Rehydration'. In *Methods in Enzymology*, Laboratory Methods in Enzymology: Cell, Lipid and Carbohydrate, ed. Jon Lorsch. Academic Press, 267–74. https://www.sciencedirect.com/science/article/pii/B9780124200678000209 (May 14, 2022).

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