Investigations into α-Hydroxy Acid Oligomer Polymers under Wet-Dry Cycling

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The candidate confirms that the work submitted is his own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others. *Further details of the jointly-authored publications and the contributions of the candidate and the other authors to the work is included below this statement.*

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Covid-19 Impact Statement

Due to the Covid-19 pandemic, my first day of school of chemistry was the day the school was closed. Many difficulties came with the closure of the school and delays in the collection of data. Six months later, the school of chemistry reopened with many restrictions, and the lasting impact is reflected in any equipment training, lab work, and ordering any materials from suppliers that needed more time to process. Details of planned research activities disrupted, including more α-hydroxy acids should be added, and more wet/dry cycling should be processed. Any more complex combinations of reactants and rheological studies of gel were cancelled,

However, one positive effect of pandemics on the project was that we had plenty of time to write our book chapter (chapter 1). During the manuscript's completion, Dr Terence P. kee and Dr Kuhan Chandru provided great assistance and eased my anxiety about not being able to get into the lab for research. It is not doubted that this is an effective and safe way to mitigate our project that was prevented by Covid-19.

All the difficulties we experience will make us stronger.

Abstract

This thesis studied the influence of ions in seawater on the early stage of forming polymers, which have the potential to form membrane-less compartments, based on lactic acid (LA) and similar hydroxy-esters. Two complementary strategies have been examined here; (i) a top-down approach where high molecular weight poly lactic acid (PLA) was tested for hydrolytic stability in the presence of various salts and (ii) a bottom-up protocol where oligomerisation of lactic acid monomer was investigated in the presence of salts, using wet-dry cycling. The ultimate objective was to examine how plausibly prebiotic membranous material could have emerged and behaved under aqueous saline conditions.

Chapter 1 provides a selective review of prebiotic chemistry in relation to abiogenesis and an introduction of the potential role of soft matter in the origin of life and it explains the aims and objectives of this project.

Chapter 2 describes studies on the hydrolysis of poly lactic acid hydrolysis in water, seawater and selected salt-waters. The hydrolysates are analysed using APC, LC-MS and SEM. Up-to 10-mer and down-to 4-mer could be identified and seawater. $NaNO_3$, NaCl and $NaHCO_3$ solutions are favoured with both yield and length distribution in PLA hydrolysis.

Chapter 3 describes how the oligomerisation behaviours of lactic acid differ between water and salt-water, characterised by LC-MS. Up-to 9-mer can be found in NaCl solution; 7-mer is generated in seawater, $NaNO_3$, and $NaHCO_3$ solution, while only 5-mer is detected in Na_2SiO_3 and $MgCl_2$ solutions

Chapter 4 discusses the connection between PLA hydrolysis and LA oligomerisation in the different salt-waters through quantifying the hydrolysate and oligomerisation products quantifying the formation of LA oligomers. Taking water as the reference, it is found that seawater, NaCl, $NaNO_3$, and $NaHCO_3$ solutions will facilitate LA oligomer formation, while Na_2SiO_3 and Mg_2Cl solutions will suppress generation of LA oligomer.

Chapter 5 provides the experimental details for chapters 2, 3 and 4.

Chapter 6 offers a conclusion and future work perspective.

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List of Abbreviations

aegPNA	Aminoethylglycine PNA
APC	Advanced Polymer Chromatography
BET	Brunauer–Emmett–Teller
DKPs	Diketopiperazines
DMF	Dimethylformamide
EDX	Energy dispersive X-ray spectroscopy
GA	Glycolic acid
HT	Hydrothermal
LA	Lactic acid
LC-MS	Liquid Chromatography–Mass Spectrometry
LUCA	Last universal common ancestor
MA	2-Hydroxy-4-methylpentanoic acid
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
OoL	Origins of Life
PA	Phenyllactic acid
PLA	Polylactic acid
PNA	Peptide nucleic acid
rTCA	Reversed-tricarboxylic Acid
SA	2-Hydroxy-4-(methylsulfanyl) butanoic acid
SEM	Scanning Electron Microscope
SHG	Silica hydrogel
SHG	Silica hydrogel
TAN	Threose-based nucleic acid

Chapter 1 Introduction and Background of soft matter abiogenesis

Prebiotic chemistry often focuses on the presumed re-construction of biomolecules, biopolymers, and subsequent assemblies common to contemporary biological cells. Considerable effort is given to building such molecules within some sort of cellular (or rather proto-cellular) environment in order to provide a molecular context to explain the Origins of Life (OoL). While this is logical, it can overlook the fact that prebiotic chemistry produces much chemistry that may have limited relevance to the emergence of biological organisms. Recently, prebiotic chemists have sharpened focus on behaviours of chemical compounds that may have played a potentially valuable role in pre-biology, but which do not necessarily involve specific molecules used by contemporary biochemistry. The view is that some primitive systems, unlike those we can infer from present-day life, may have played a role in the development of living systems, or perhaps have acted to scaffold primitive living systems towards biological life-as-we-know-it. The aim of this chapter is to summarise some of the inherent problems in prebiotic chemistry which result from adopting a contemporary biology-centric approach, provide a brief overview of non-biological chemistry published to date in relation to abiogenesis, and emphasise how soft-matter science may potentially have a role in OoL studies.

1.1 Introduction

Prebiotic chemistry has long sought to bridge early earth geology and contemporary biology (1). Collectively, there has been tacit agreement that the end-goal of prebiotic chemistry is the formation of a primitive, functional cellular system of some description. Such a system would be responsive to its environment, but that responsiveness would be mediated by some form of internal periodic machinery contained within the “organism” itself. It seems reasonable to imagine that such a proto-cell might not be exactly the same in terms of composition and function as contemporary biological cells, although there are likely to be similarities, including the ability to change their phase behaviour in response to chemical or physicochemical signals (Figure 1.1) (2).

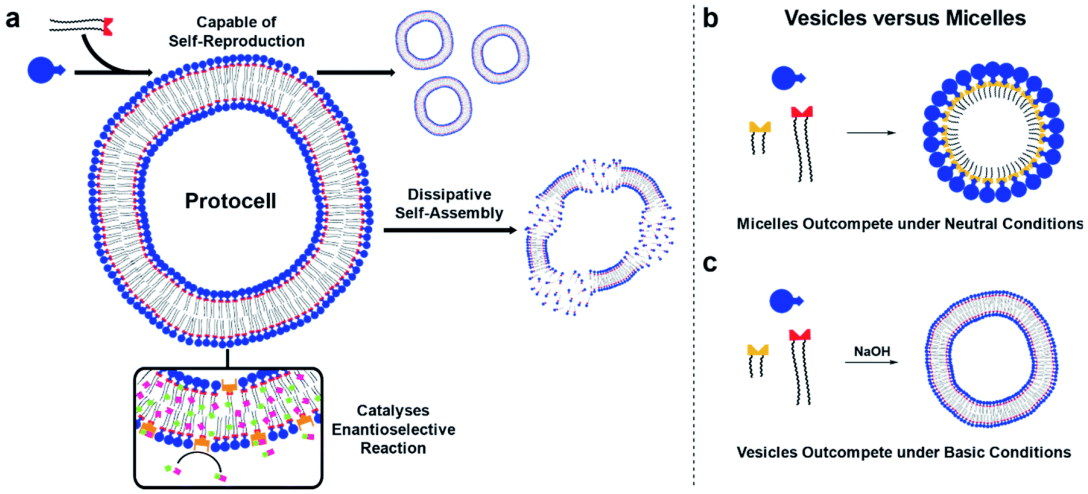

Figure 1.1 (A) example image of some putative protocellular arrangement similar to a lipid vesicle with specific functions such as self-reproduction, self-assembly and self-catalysis. (B) and (C) illustrate how in principle, different environmental conditions could legislate for different emergent behaviours (2).

Many authors have attempted to paint pictures of where the similarities may lie in terms of information-containing molecules (3), chemical reactions (4), autocatalysis and creating a distinction between the inside and outside of a cell. This is not really the place to discuss proto-cells and compartmentalisation in general as much has already been contributed to this field over the years (5, 6). However, as an illustration of the type of thinking on primitive biological systems, the Ganti-Chemoton model encompasses key biological motifs including (a) information transfer sub-systems, (b) metabolic (chemical reaction) sub-systems and (c) a cellular membranous container (Figure 1.2) (7).

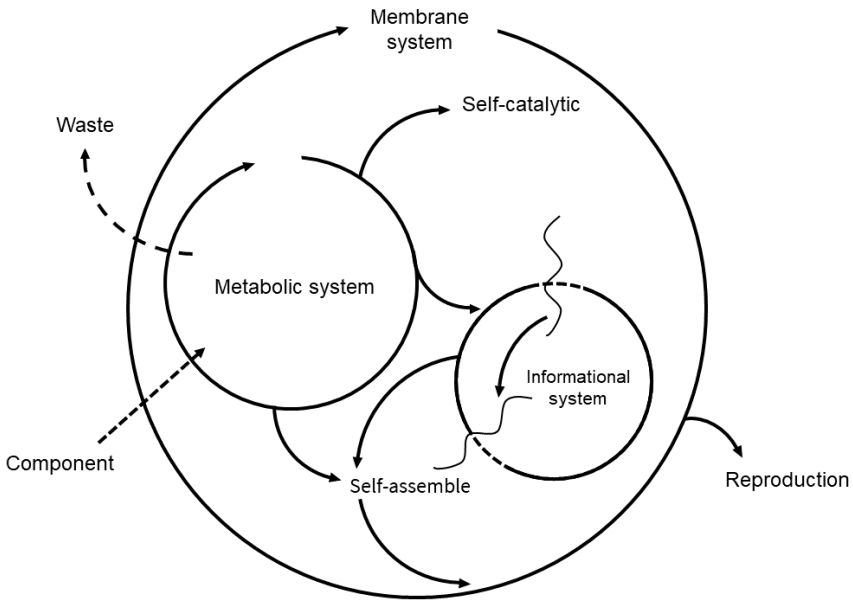

Figure 1.2 Representation of Tibor Ganti's Chemoton Model of possible proto-cellular environments in which three, inter-connected cycles loosely map onto metabolic, informational and membrane compartment systems of contemporary biological cells.

From a biological perspective, many of these investigations have focused on defining a last universal common ancestor (LUCA) (8). Such an organism is anticipated to be quite complex from a biochemical perspective with a ribosomal apparatus and primitive abilities to transfer and transpose molecular-based information into functional protein macromolecules (9). This approach has proved highly fruitful in many different ways. For example, phylogenetic analyses have suggested that the most primitive biological organisms may most probably have been anaerobic and thermophilic in nature with an ability to fix CO_2 and dependent upon reduction using H_2 as an electron source (10). It has helped focus attention on certain metabolic processes, which have been found through phylogenetic analyses to be ancient and which could conceivably lie within the chemical capabilities of primitive cellular entities (11). These have in turn allowed chemists to look into ways in which molecular building blocks could have been constructed within putative primitive geological environments.

From a chemical perspective, there has been a logical focus on molecules which are fundamental within contemporary biology; proteins, peptides, amphiphiles, nucleic acids, sugars etc… This is in no small part due to the ubiquitous nature of these families of molecular species across biological organisms and a growing body of knowledge as to both what functions they

perform within the organism and how they accomplish this. However, it is also recognised that not only are there question marks over how such anatomically complex molecular species may have emerged under prebiotic environments, but that there is no independent evidence to suggest that such molecules were the first and only such molecular tools constructing a route to what we now consider biological. Certain gaps exist and arguably some of these gaps might prove fatal to discovering precisely how biological life emerged on earth. For example, while we continue to know more each year, we still do not know what sort of geological environment the first cellular life emerged within (12). We do not know whether the most primitive proto-cells, those that must logically have preceded LUCA, employed proteins, nucleic acid informational systems and a ribosomal-like apparatus to connect the two classes of macromolecules. In essence, we do not know much about the specific: geological environment(s), chemical compositions or functions of the most primitive cellular life. Many have of course speculated and on very reasonable, logical grounds for each of these features (13-15). The question as to whether any of these are right or wrong in terms of being the actual routes to biological life has yet to be determined and it is indeed very possible that we will get some answers to these questions, but it is arguably more likely that we will learn more about possible emergence pathways to biology than that we will identify the historically correct ones (16, 17). In this context, there has been a growing interest in moving away from the specific molecular systems used by contemporary biology to explore non-biological but prebiotic molecules which nevertheless have potential to act in certain ways similar to the way biological cells act (14). There has been a slight shift in emphasis from aiming at specific biological molecules to aiming for less “functionally-evolved” molecules (perhaps molecules that may have found it easier to emerge within prebiotic environments) which are nevertheless capable of acting in certain ways, for example as informational molecules or cellular components. Ultimately, this approach has a more general focus on functional activity rather than contingent composition and may bring new modes of thinking to the field of abiogenesis.

In this piece, we will show how this mode of thinking is now making its way in the OoL field by first highlighting certain problems in prebiotic chemistry where a “messy” prebiotic chemistry is given consideration. We will then add another layer of perspective, soft-matter chemistry — where we will reason ways it could have played a significant role during the early stages of the OoL.

1.2 Problems of selection in prebiotic chemistry

1.2.1 The vast landscape of chemical

While the name itself, prebiotic chemistry, suggests this field to be concerned with chemical processes which may have logically preceded biology, one still needs to consider where to draw the line at what could be considered to be biological. Researchers have shown that prebiotic compounds or prebiotic precursor compounds can participate in synthetic reactions resulting in the formation of biomolecules under primitive geochemical environments (4). Examples of this include the classic Miller-Urey experiments and hydrothermal vents experiments among others. Other more directed approaches to study the OoL, e.g., using synthetic chemistry methods to arrive at particular biomolecules-of-desire using prebiotic compounds, have also been extensively explored. Chief among these involves the synthesis of polynucleic acids (and their components) (18, 19) and, more recently, the components of the reversed-tricarboxylic Acid (rTCA) cycle (20). While these directed synthesis approaches show the simplest route or a plausible prebiotic mechanism to a desired class of biomolecule, they do so without considering the true premise of prebiotic chemistry — a premise which is essentially “messy” (21). By “messy” in this context we mean that many more chemical transformations are occurring simultaneously or contiguously than are relevant or useful in the context of a putative biological organism. This becomes an increasingly complex issue when one considers that the chemical processes “necessary” to a primitive biological organism do not spontaneously make themselves known as such in a prebiotic context; we have needed to work backwards from contemporary biochemistry. Moreover, it is not clear which (if any) chemical processes not themselves integral to putative biological organisms are nevertheless key components in the establishment of chemical networks which are. This is understandable considering the constraints on studying such systems given the limitation of analytical chemistry in studying complex chemical systems in their entirety without some form of data compression/suppression or selection. Nevertheless, while showing direct prebiotic routes are significant within their own right, the idea of having to deal with “messy” prebiotic chemistry is becoming more embedded in the OoL field. Chemical reactions which produce multiple products have long been considered to be a problem in prebiotic chemistry. For example, some prebiotic chemistry experiments were found to form “tar,” or unresolved complex mixtures when using just a few starting chemicals (22, 23). By a

similar token, the Miller-Urey experiment is also known to produce tar (24), the products of poorly constrained or un-controlled chemical reactions when large amounts of energy were inputted into chemical systems. The same can be said of much organic material found within natural meteorite materials (25). With advances in analytical chemistry, more compounds are capable of being detected. For example, the Miller-Urey experiment led to the detection of only 23 amino acids (26) whereas a re-analysis of the same samples using modern analytical tools showed that (at least) 40 amino acids were present (27, 28). However, when a non-targeted mass spectrometry technique (MS) was applied to the samples, a wider variety of chemicals (including amino acids) was identified (29) emphasising that particular families of chemicals are present as part of a wider collective. Similarly, a non-targeted analysis of the Murchison meteorite revealed the presence of over 14000 individual compounds in the extract, and this number would be expected to rise if regio-isomerism and stereoisomerism were to be considered (25). More recently, a theoretical paper has shown how, starting with only six plausibly prebiotic compounds (H_2O, HCN, NH_3, CH_4, H_2S and N_2), 36603 distinct non-biomolecules and only 82 biomolecules appeared after seven generations of computer simulated chemical reactions (30), based on many prebiotic reaction classes (e.g., additions, eliminations) including specific synthesis related to the OoL (e.g., acrylonitrile synthesis, methanethiol formation, etc.).

All of these recent works highlight that the prebiotic world is best viewed as a chemically rich inventory of materials potentially available for further manipulation and complexification. Furthermore, we might logically also expect that the vast majority of the chemicals available within this inventory have not been used in biochemistry and may be considered as prebiotically available but not necessarily selected for use by primitive biological organisms. This does not however, mean that, just because they have no recognised role in contemporary biology, they may never have played roles in developing nascent biology (31). Indeed, while one can acknowledge that the molecular machinery central to contemporary biology is finely tuned to perform the functions that it does, there is no a priori evidence that those same molecules performed the same functions in the most primitive biological organisms.

1.2.2 Non-biological compounds with potential value to prebiotic chemistry

In this section we shall review some examples of molecular systems which do not necessarily play a role within contemporary biochemistry but nevertheless, for logical chemical and potentially logical functional reasons, have been considered possible precursors to modern molecular machinery. We do not intend this selection to be exhaustive, but rather a summary of some chemical exemplars in the field.

1.2.2.1 Peptide nucleic acid (PNA)

Though Peptide nucleic acids (PNAs) contain elements of contemporary biomolecules, most notably nucleobases and a covalently linked macromolecular backbone, they can be considered overall as non-biological compounds considering that their composition is unlike the common polynucleotide used in biology (DNA and RNA) (32) (Figure 1.3). The principal molecular differences lie in the nature of the backbones that use amide backbone instead of sugar-phosphates one.

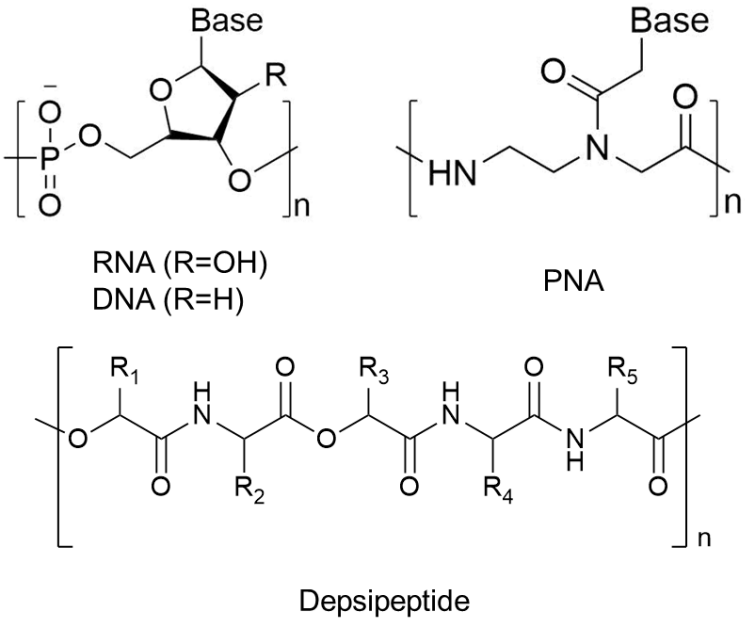

Figure 1.3 Chemical structure of DNA, RNA and PNA

Nevertheless, PNAs have been considered as an interesting genetic analogue of RNA in prebiotic chemistry for the following reasons: 1) their diverse polyamide backbone structures (such as aminoethylglycine PNA or

aegPNA) (33) have been used for connecting together sequences of nucleobases; 2) these molecules are capable of forming hybridised structures through Watson–Crick base-pairing (9, 34, 35); 3) the backbones of PNAs have greater hydrolytic stability than RNA, which might suggest that PNAs may be easier to accumulate under a prebiotic environment than RNA; 4) the building blocks of PNAs are more chemically accessible, under putative early earth environments, than those of RNA. This might especially be considered to be the case for aegPNA which can potentially be synthesised in early Earth environments (36). Despite lacking the sugar-phosphate backbone, it has been demonstrated that aegPNA can pair with high fidelity to complementary RNA, DNA in both parallel and antiparallel orientations (33, 37, 38). Information can be transferred between PNAs and DNA or RNA through sequence-specific template-directed reactions (39), which suggests that different forms of genetic molecules, including PNAs, can be useful as prebiotic information media (see for example Figure 1.4). However, a potential limitation associated with PNAs in particular is that its six-atom backbone unit can easily cyclise upon chemical activation to become a six-membered piperazinone or diketopiperazine (Figure 1.4) (40).

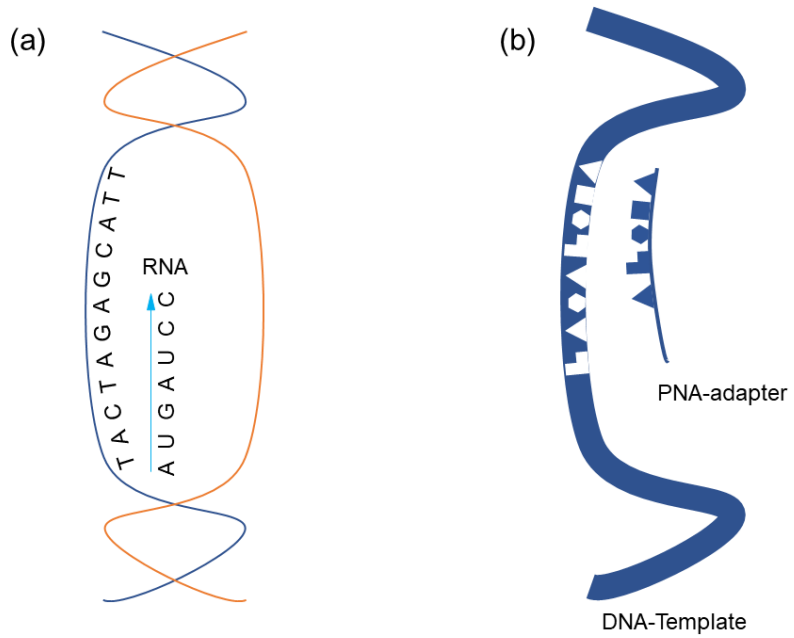

Figure 1.4 The process of transferring information. (A) information transfers from DNA to RNA. (B) information transfers from DNA to PNA.

Other variations of nucleotides, where the ribose phosphate backbone of RNA was replaced with backbones from different sugars have also been

reported. For example, threose-based nucleic acid (TNA) is made by a four carbon based sugar that can form stable Watson-Crick double helices among themselves and also with RNA and DNA (41). These TNAs were speculated as having the ability to play a role in the OoL (42), specifically during the hypothesised pre-RNA world, where information-containing molecules with simpler chemical structures may have dominated (43, 44).

1.2.2.2 Depsipeptides

Alexander Rich suggested in 1971 that the OoL may have been facilitated by prebiotic polymers that are not necessarily based on the peptide linkage (45, 46). By recognising that alpha-hydroxy acids and alpha-amino acids were both formed by the Miller-Urey experiments, Rich demonstrated that ester bond formation can be catalysed by the ribosome that typically catalyses peptide-bond formation (45). This indicates that the ribosome is not restrictively specific in distinguishing amide or ester linkages which in turn may suggest a primordial remnant of primitive ribosomes. Rich also suggested that ester bonds may have been involved in primordial polymerisation during the OoL (46).

It is also well known that there are several issues associated with peptides being accessed prebiotically: 1) amino acid dimers have a known propensity to form diketopiperazines (DKPs) that inhibit polymerisation from a kinetic perspective (47); and 2) peptide formation is an energetically uphill reaction (48, 49), i.e., it requires more energy to make relative to ester bonds (see next section for details). To circumvent this problem, depsipeptides have been suggested to be plausible proto-peptide structures which can be accessed from mixtures of amino acid and hydroxy acids under conditions that have long been considered available to prebiotic geological environments such as under low temperature wet/dry cycles (50).

More potential varied combinations of amino acids and hydroxy acids were also explored, and these combinations reflect various efficiencies and sequences (51). For example, compared with other amino acids, L-proline with a secondary amine structure shows the highest amino acid content in depsipeptides because it is more nucleophilic than others (52). Although L-proline depsipeptides have the highest amino acid content, the amino acid content of all combinations generally increases when subsequent wet/drying cycles are performed. Beyond this, the sequence diversity would have increased over time and cycles, offering abundant proto-peptide libraries.

More recently, Frenkel-Pinter et al (53) have shown that depsipeptides with cationic moieties when prepared with cationic depsipeptides would enable noncovalent assemblies based on stabilising electrostatic interactions between protomers of peptide and RNA. This resembles the interconnectedness between RNA and peptides, which is regarded as perhaps the most important element in modern biology.

1.2.2.3 Polyesters

Considering the prebiotic diversity, and the thermodynamic facility of ester bonds formation (ΔG = ~ 0 kcal mol-1) (54) compared to amide bonds ΔG = ~ 3.5 kcal mol-1 at physiological conditions (55), ester-bonds may have been a preferred choice of the first prebiotic polymers (31, 47). Even though such ester linkages are less thermodynamically and kinetically robust than amide bonds, if they were capable of performing some of the important functions (whatever those might have been) to primitive cells, it is conceivable that their study may have potential in prebiotic terms. To show this, Chandru et al (56) showed that by using a contiguous mixture of alpha hydroxy acid under a wet/dry cycle over a period of 24 hours, a combinatorial library of polyesters can be formed, in which approximately 4300 polyesters were identified. When these hydroxy acids were dried for 1-week, gel-like substances were formed that can phase-transition to become cellular-like droplets capable of differentially segregating and compartmentalising several dyes, RNA's and proteins, and also allowing lipid to scaffold around these droplets (57). Each specific combination or individual alpha hydroxy acid's microdroplets can be shown to be selective in choosing which analyte it can compartmentalise. This ultimately suggests that, given the reversible nature of esters, many combinations can be formed and hydrolysed, and formed again, that may have facilitated specific capacity for concentrating many different kinds of other prebiotic compounds (31). For example, microenvironments created by compartmentalisation may enhance specific molecular function depending on solvent polarity, concentrations of metal ions or co-factors (58, 59). This compartment may offer a non-polar environment with some specific advantages for functionalisation, synthesis, and accumulation of molecules. Moreover, in this system with different multi-

phases character, each molecule has a different affinity for each phase (57).

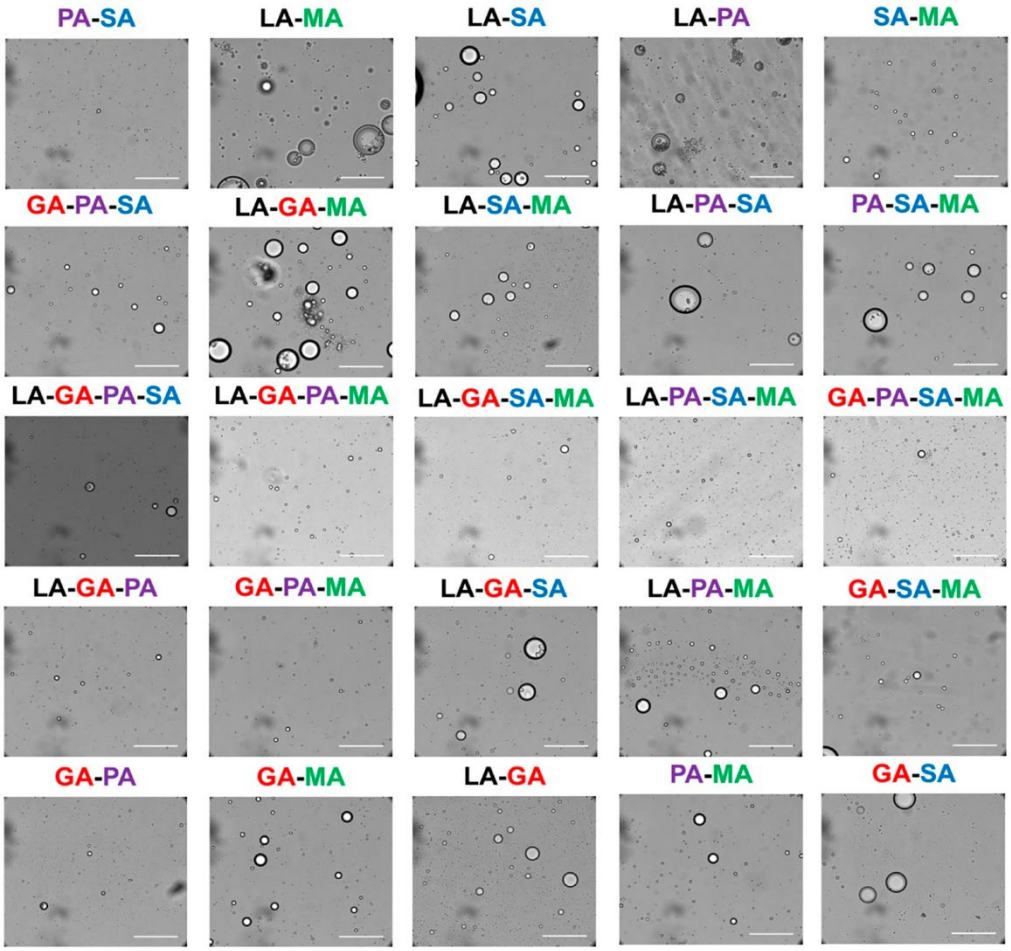

Figure 1.5 Showing how different combination can form different variety of microdroplets (31) LA = lactic acid, GA = glycolic acid, PA = phenyllactic acid, SA = 2-hydroxy-4-(methylsulfanyl) butanoic acid, MA = 2-hydroxy-4-methylpentanoic acid.

1.3 Water and the origins of life

All known biological life requires water in order to function (60, 61). Accordingly, astrobiology missions and the hunt for habitable exoplanets observe this “follow-the-water” rule in the search of extraterrestrial life (62). It is no surprise that most OoL experiments (although it should be said not all; for example (63)) use aqueous solution as a solvent to run prebiotic experiments to show the plausible formation of primitive biomolecules, biomacromolecules or bio-reactions, depending on which geological scenario the experiment subscribes to. For example, the Miller-Urey experiment assumes that a primitive ocean existed that was used to trap the organic compounds that were formed by reducing gases reacting under

simulated lighting, and most RNA world experiments use water as a solvent to prime their reaction pathways towards forming an RNA or any of its constituents.

However, there are several notable problems in relation to (bulk) water being used in prebiotic chemistry. First, the condensation reactions that enable long biopolymers to form are sluggish under aqueous conditions especially without activating agents of some sort (31). Second, assuming prebiotic polymers are readily available, water easily hydrolyses ester and peptide bonds (52), and by extension prebiotically formed nucleotides, peptides and even high-energy bond molecules (64, 65), i.e., thioesters and pyrophosphates, thus obfuscating potential continuity in chemical evolution. Third, a free aqueous medium poses a concentration problem (66). Considering that the synthesis of most prebiotic bio-macromolecules is thermodynamically unfavourable in bulk water, it is expected that their yield will be low, and they have the nature, unless in some way constrained, to diffuse within geological water bodies (e.g., thermal pool, ocean, etc.) thus preventing biomacromolecules from doing “stuff” that may promote complex chemistries.

In contrast, the behaviour of water in a cell differs from bulk water. Water in a cell consists of both bulk water and layered water (or structured water) (67, 68). Cellular water which is so structured has the ability to behave differently from bulk water, both in physico-chemical and chemical reactivity terms, and such water behaves like a liquid crystal phase, i.e., more ordered than bulk water, in the form of lattices, due to its adsorption to cytolytic biomacromolecules (e.g., structural proteins, sugars, etc.) (69, 70). This ultimately lowers water activity in cells (when compared with bulk water), thus retarding the rates of non-enzymatic hydrolysis. The layered water adsorbed to proteins prevents cells from desiccation and freezing when cells are exposed to drying and cold temperatures (beyond 0°C), respectively, ensuring the cell remains stable (69) (Figure 1.6).

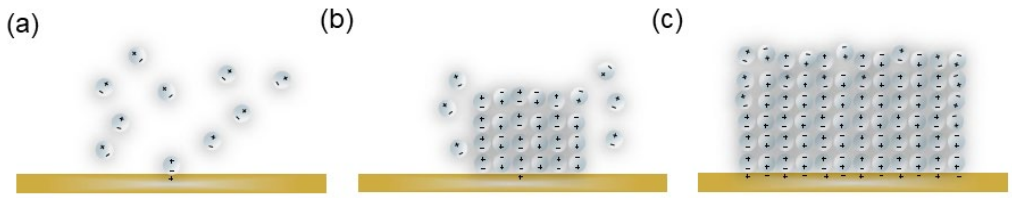

Figure 1.6 Water lattice formation near a protein's surface. (A) water dipole adsorbed on a charged protein site. (B) Adsorbed dipoles then facilitate additional dipoles to further adsorption to form layers. (C) Further reinforcement and extension of the dipole layers on a charged protein's surface.

In most, if not all, prebiotic experiments performed in the laboratory, the aqueous phase is seen predominantly as a dispersant medium and effectively homogeneous. Even in those situations where water itself is seen as an active reactive component (such as in hydrolysis experiments), its presence is effectively seen as homogeneous and undifferentiated. As one can see when comparing such a medium to that involved in biological cells, such homogeneity cannot be taken for granted across the scale of the cell itself. This means that laboratory prebiotic experiments most frequently simulate what might be considered local bulk-water situations rather than scenarios in which the water itself exists in different phases.

Alternatives to lowering water activity through the process of freezing have been explored, most notably ice and salt-ice phases (e.g., (71)). As water freezes, the remaining solution becomes progressively more saline as pure water crystallises preferentially in the ice phase resulting in a freezing salt solution becoming more concentrated. This can have the effect of altering the rates of chemical reactions of interest to prebiotic chemists, such as the formation of organonitrogen nucleobases, polymers based on ribonucleic acids and those on the peptide bond (71). This in turn has led to much geological interest in salt-containing ice-water phases such as brinicles which are effectively examples of non-equilibrium chemical garden (72) and hence bear some similarities to the hydrothermal vent systems, albeit existing in colder rather than hotter environments (71).

1.4 Cytosol, gels and Cytosol's possible links to the origins of life

Membranes, one of the key materials of both cells themselves and of cellular compartments or vacuoles, are boundaries separating such domains from the environment. Such boundary conditions must exist in a cell to prevent leakage of solutes. This, however, comes from the presupposition that cells are an aqueous suspension of constrained solutes. Pollack (69) has made the observation that membranes do not act as a container, keeping the cell's

interior within, but that it is the cytoplasm which performs this function. Similar conclusions have been made by others. Pollack noted that the cytoplasmic environment within a biological cell has the properties of a hydrogel-like phase, and it is in no small part due to those soft-matter physicochemical properties that the cell's internal composition is maintained. Gels are loosely defined as soft, solid or solid-like materials which consist of two or more components of which one is a liquid (73). If the liquid in question is water, then the gel is called hydrogel. Examples of gels that allow water to adsorb to itself include PEG, Ficoll and Dextran, and inorganic materials such as silica and inorganic clays. It is, arguably, the latter classes of gel materials that may be of particular relevance to prebiotic chemistry in the first instance (e.g., silica gels which are important phases in rock formation).

The biological cytosol is a molecularly crowded environment, i.e., tightly constrained with many proteins and bio-macromolecules (70, 74). For example, Ostrowska et al. (75) comment that biological cells contain between 200 and 400 g/L of macromolecules which include proteins, nucleic acids, ribosomes, and lipids. As a result of this heterogeneity, along with different phases of water contained within such an environment, molecular diffusivity of ions and molecules is strongly affected (76).

In a series of publications inspired by Pollack's work (69), Trevors' has introduced an OoL model based on this idea that prebiotic chemical processes might behave very differently in a hydrogel environment compared to purely aqueous or aqueous salt systems (76-79). Pollack and Trevors have argued that the natural state of the gel phase is conducive to a molecularly crowded environment. Moreover, Trevors (78) provided a list of plausible features of the composition and organisation of the first prebiotic cytoplasm, i.e., a proto-cytoplasm that led to the first living bacterial cells. These features include: 1) providing a necessarily stable micro-environment for organisation to chemical evolution; 2) being an environment which permitted transfer or diffusion of essential ions (e.g., Na^+, K^+, Mg^{2+}, etc) without the need for specific molecular pumps, and 3) capable to undergo partition, akin to cell division, due to physical forces that will indicate "growth" and "division"

However, like most prebiotic chemistry expositions, this idea comes with open questions that require scrutiny. In the first instance, it is not so straight-forward to envision what such a proto-cytoplasm may have been made of and how it would have formed in a prebiotic environment. One might assume that primitive proteinaceous materials or some other organic macromolecule

might have been capable of forming the first gel-based environments. However, if that were the case, then we are back to the origin and composition of the first, unstructured cytoplasm (78) with the same problems, i.e., difficulty in making them, unless other chemicals made such architecture. Some work to accommodate the crowded cytoplasm, pores of mica (80), mineral surface (81), gel (78), and also hydrothermal (HT) vents (82, 83) are regarded as potential geological candidates where gel-phases may exist. Thus, interest has been shown in silica gels which have been shown to provide porous, high surface area environments for chemical reactions to take place, which include phosphorus-transfer processes (84).

Specifically, it was found that mixtures of inorganic H-phosphonate (H_2PO_3-) and H-phosphate (H_2PO^{4-}) undergo coupling to (ultimately) pyrophosphate ($H_2P_2O^{7-}$) under aerobic conditions within a silica hydrogel environment in the presence of ferrous ions. The same couple reactions do not proceed under the same conditions in an aqueous phase (84) (Figure 1.7).

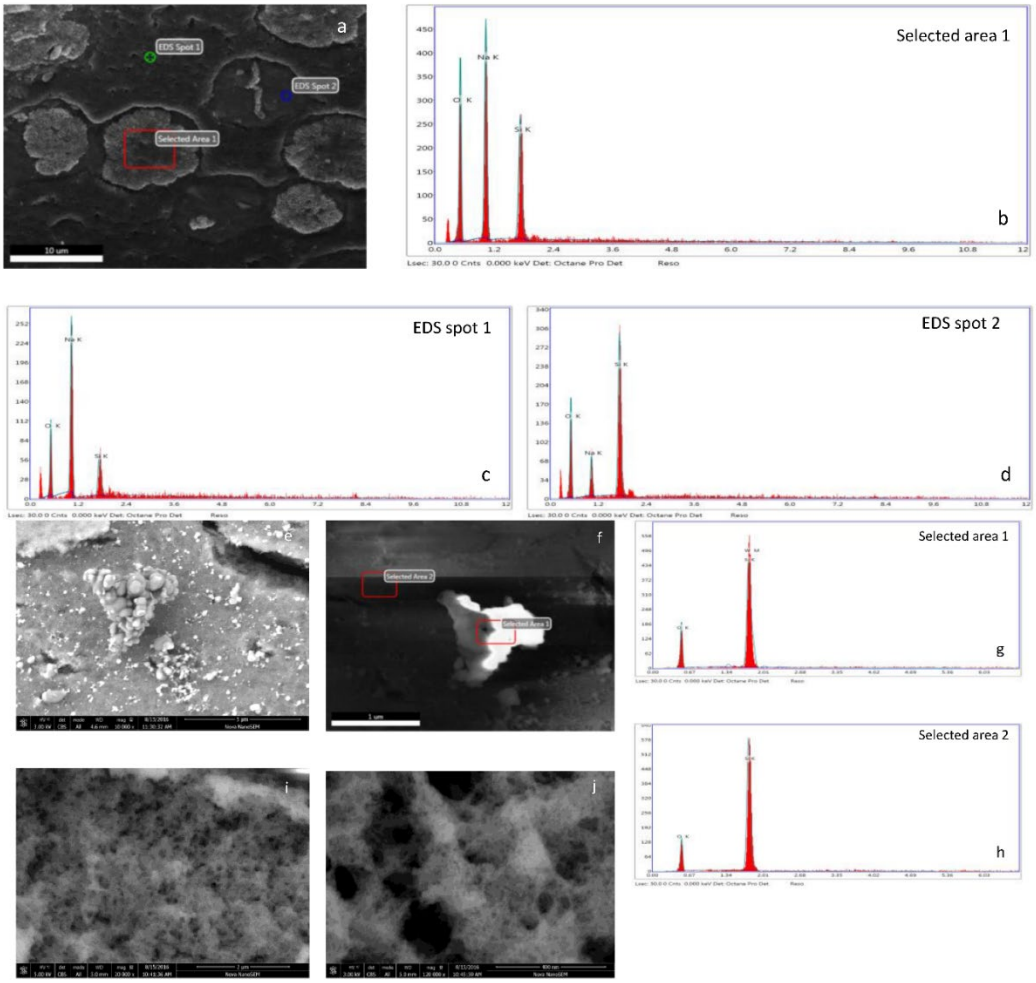

Figure 1.7 Scanning electron microscope images (secondary electron) of the solid mineral matrix of silica hydrogel (SHG) preparations, post-critical point drying; matrix resulting from 1.0 M SHG. (A–D) reveals the presence of sodium salts in addition to silica; (E–H) illustrate spherical silica formation and (i, j) reveal the more open alveoli structure of the silica matrix.

In addition to natural geological mineral gels, synthetic minerals, or alumino-silicate clays, have been demonstrated to form gels very easily in water. The smectite clays, laponite, are one such family of synthetic minerals which have been examined as potential reaction media, proto-cytoplasms, for transcription and translation reactions of RNA's which were found to be consistently enhanced over analogous processes in the aqueous phase alone (85). The point has been made that the gel phase could provide a protective environment for fragile molecular systems (76-79). Indeed, some gels such as etherified polyvinyl alcohol (86) have been shown to be resistant to UV radiation, a potentially valuable property of a prebiotic organismal container. This however follows the multifaceted need for a prebiotic gel to be robust to a given prebiotic environment and at the same time have the ability for proto-biological functions such as visco-elastic behaviour and morphological changes. It is not especially obvious what such prebiotically "valuable functions" may have been but alongside the more recognisable functions of permitting material to flow both in and out and providing a sufficiently stable environment for chemical networks to develop one might also expect more sophisticated functions to emerge such as the transduction of periodic chemical behaviour such as might be found within a nascent metabolism, to bulk phase changes, motions or divisions of a gel phase as a medium. Focusing on functional interconnections between chemistry and phase-change physics is an aspect of prebiotic chemistry that is still rather on the fringes of the field. For example, how might primitive, proto-metabolic chemistry be linked to the rheology of primitive cells? Questions such as these have clear relevance for cellular functions such as growth and division. In this regard, while much is known about contemporary cellular cytoskeletons and rheology, relatively little attention is given to this science in a prebiotic context, despite the obvious functional relevance. It is therefore worth noting that mineral hydrogels offer primitive "organisms" the twin advantage of a hydrogel cytoplasm and a structural cytoskeleton. While the debate between metabolism-first vs information-first scenarios for the OoL is still ongoing, there is also growing recognition that the answer may lie less in which system came first but how what appear at first glance to be

disparate systems grew together contiguously (87). Given that both these camps are closer to the top-down part of the OoL in terms of framing of questions, the need to understand complex, interconnected prebiotic chemistry (a systems chemistry approach) that involves “messy” chemical environments will be important too given that that was the condition on the early earth.

Also, given that at present it may be argued that a knowledge gulf still exists between what we know of abiotic chemistry and how these might have evolved into primitive biochemistry, the functionality of present biology is unlikely to be similar during prebiotic times. Input from synthetic biology and biomimicry fields may fill these gaps. For example, recently, a chromosome-removed cell, collectively known as Simcell (or simple cell) (88) was created from Escherichia coli, Pseudomonas putida, and Ralstonia eutropha. The chromosomes were replaced with synthetic genetic circuits (selected DNA plasmids) in amounts just sufficient to operate a functioning metabolism (e.g., glycolysis) and programmes for minimal living. This work, while not directly related to the OoL, may provide a valuable insight into building a synthetic cell. In other words, creating a new kind of life. By new kind of life, we do not mean to imply a tinkering with the genome of contemporary biology in order to produce an organism which is biochemically indistinct from what we know already (or by creating life in silico (e.g., Artificial Life). No, we mean here: a new life based on non-biochemical materials which may or may not evolve into, converge to, a contemporary biochemistry but nevertheless is, in and of itself, chemical and mechanistically (if not functionally) distinct from life that we currently know.

1.5 Summary

In this piece, we have laid out some critical problems that are pertinent in prebiotic chemistry, focusing on biomolecules, and we have highlighted new research approaches that might circumvent these problems. We have also included the view that origins of life studies may benefit from being integrated with soft-matter science and that such integration warrants further effort. There are good reasons to believe that understanding the chemical emergence of biology, the origins of life, is more than just an amalgamation of biomolecules that could copy themselves or transduce new molecular entities. All biological organisms as we know them are capable of linking (transducing) the behaviours of chemical systems to bulk, phase-changes of

a gel-based (cellular) environment. One of the significant problems in getting an effective handle on just which chemical systems might have been most likely to have been prominent in such primitive organisms lies in the “messiness” of prebiotic chemical spaces, and the seamlessly easier route to make higher-ordered structures utilising non-biomolecules compared to biomolecules, that may give prebiotic synthetic preferences to non-biomolecules (31, 89). Equally importantly, even if the specific origin of biological organisms as we know them was paved by a relatively smaller set of biomolecules compared to non-biomolecules that existed in prebiotic chemical spaces, then why is it that we haven’t come to a coherent working model of primitive life thus far? Perhaps we could benefit from taking a small step to one side, removing the focus from specific “brands” of biomolecules, and taking up new approaches, whereby one looks at how chemical behaviours could link to phase-change behaviours of chemical system ensembles. This may then allow us to look at other groups of molecular chemistries, which perhaps play little or no role in contemporary biology.

Investigations have been done into how chemistry might be different when performed in a purely aqueous environment and then again in other environments where water activity can be modified (including wet-dry cycling, in compartments, proto-cells or membranes and in ices). More is now being done in environments which try to better model contemporary biological cells, using hydrogel proto-cytoplasmic media and exploring how chemistry can interface with cellular rheology. By so doing, we may learn more about how links between chemistry and protocellular physics emerged and ultimately how functional biochemistry came to be central to life on earth (17).

1.6 Aims and Objectives

Lactic acid (LA) is the simplest α-hydroxy acid and has been demonstrated to exist on primitive planetary and pre-planetary bodies. The polymerisation of such α-hydroxy acids leads to polymers connected via ester bonds, not unlike the peptide linkages which form the basis for amino acid polymers. Generally, though, polyesters are found to be more readily prepared (47, 56) than, and more hydrolytically stable than, their peptide cousins. Polymerising LA might play a key role in forming potential prebiotic compartments and providing plausible places to protect, exchange and encapsulate promotive components on the prebiotic earth. These LA oligomers were randomly and

reversibly forming and persisted for long-term periods. Long wet-dry cycles contributed to increasing mean polymer length and expanding oligomer libraries under the prebiotic environment, which had been evidenced as able to lower the barrier to formation of polyacids (97), esters (47, 98), oligopeptides (99, 100), nucleosides (101), and polynucleotides (102, 103).

In this project, I report my studies on the: (i) wet-dry LA oligomerisation and (ii) hydrolysis of PLA in aqueous saline solution through simulating the early earth environment.

Within the confines of these two complementary approaches, I have been specifically concerned to examine:

(i) How the molecular weight range of LA oligomers changes as a function of the numbers of wet-dry cycles

(ii) How the PLA degradation is influenced by salts and time in aqueous solution.

In order to address problem (i), nuclear magnetic resonance spectroscopy and mass spectrometry have been used to analyse LA oligomer formation as a function of cycle numbers. In addressing problem (ii), liquid chromatography-Mass spectrometry (LC-MS) could identify and quantify the type of oligomers formed through PLA hydrolysis and indeed, in LA oligomerisation processes.

In addition, scanning electron microscope (SEM) and energy dispersive X-ray (EDX) spectroscopy have been used to examine the structural detail of partially digested PLA.

Chapter 2 Hydrolysis studies of Poly lactic acid (PLA)

2.1 PLA hydrolysis

Poly lactic acids (PLA) provide one example of a material that could have been available within an early earth environment where highly energetic processes generate organic polymeric materials (e.g., meteorite kerogen), which could have formed the basis for a primitive organismal matrix. PLA hydrolysis as the top-down method provides a pathway to enrich the LA library in prebiotic environment. One key feature of such a polymer's ability to serve in such a capacity would be its stability under hydrolysis. The work outlined in this Chapter aims to address a part of that question.

Due to PLA being the condensed polymer, chain scission is highly dependent on water, which could diffuse into the polymer, cleave ester bonds, and form LA oligomers with carboxyl and hydroxyl end groups (Figure 2.1). Hydrolysed LA and LA oligomers further diffuse through the PLA and dissolve in water when PLA is placed in the aqueous environment (90). The cleaved carboxyl end groups are able to catalyse the further hydrolysis of PLA to obtain shorter oligomers. PLA hydrolysis is a random process mainly depending on temperature, time, presence of salts, pH etc. Here, we investigated PLA hydrolysis at 60°C or 80°C for 24, 240 and 480 hours in different solutions.

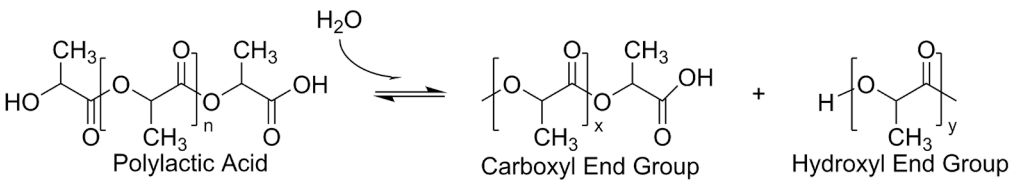

Figure 2.1 The mechanism of Polylactic acid (PLA) hydrolysis (modify based on ref.90)

2.2 PLA hydrolysis in water

The PLA had good solubility with dimethylformamide (DMF), and its molecular weight was measured *via* Advanced Polymer Chromatography (APC) which used DMF as the mobile phase. The molecular weight of the

PLA that we used was measured at *ca*. 45000 Da, while the molecular weight of hydrolytic PLA placed in water at 60°C for 24 hours fell to *ca*. 42000 DA under the same test conditions, indicating that slight depolymerisation had occurred. However, APC with the minimum molecular weight threshold of about *ca*.5000 Da could not detect any signal of PLA with increasing hydrolysis time or temperature which indicates that PLA was completely transferred to the smaller molecule.

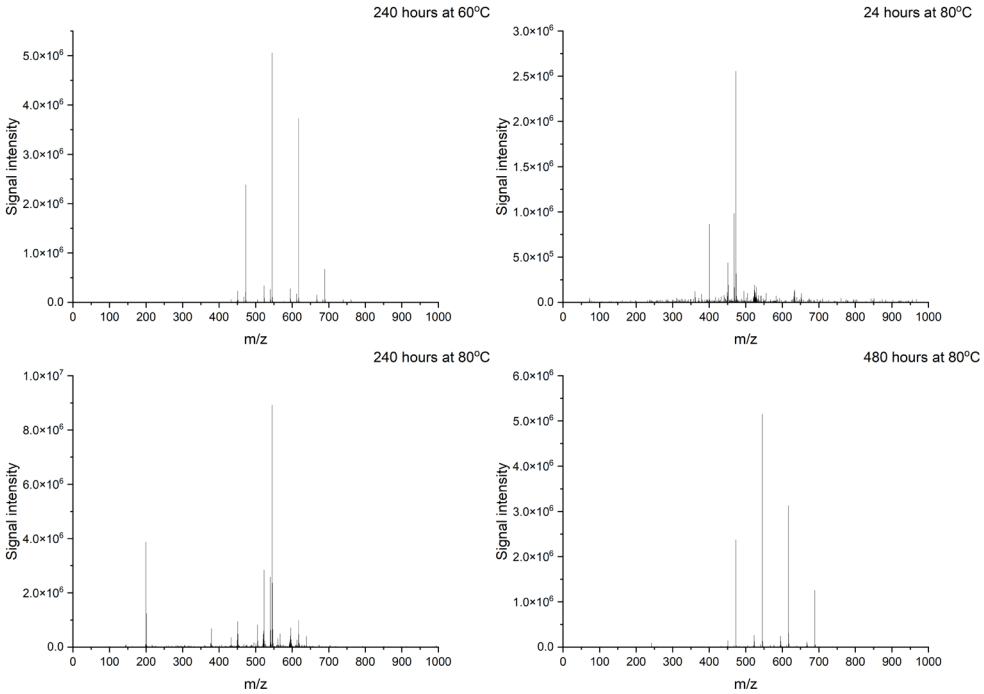

Figure 2.2 Liquid Chromatography–Mass Spectrometry (LC-MS) data for PLA hydrolysis in water.

To further investigate PLA hydrolysis in water, the LC-MS method was used to detect hydrolysed LA oligomers. Many types of ionised adduct species could be identified in positive LC-MS mode, including MH^+, MNa^+ and MNH_4^+. In detected LA oligomers, the evidence of a mass difference of 72.02 Da allowed us to identify LA oligomers and presented LC-MS data and range from 100-800 mass-to-charge ratio (Figure 2.2), due to the increasing repeating ester units -(OCH(CH_3)CO)-. The maximum mass-to-charge ratio of 761.21 indicated the presence of a MNa^+ 10-mer LA oligomer. All samples used the same volume of solvent when testing the LC-MS. Thus, in the following LC-MS figures, the abscissa of LC-MS figures was set to the

number of LA oligomers instead of the mass-to-charge ratio.

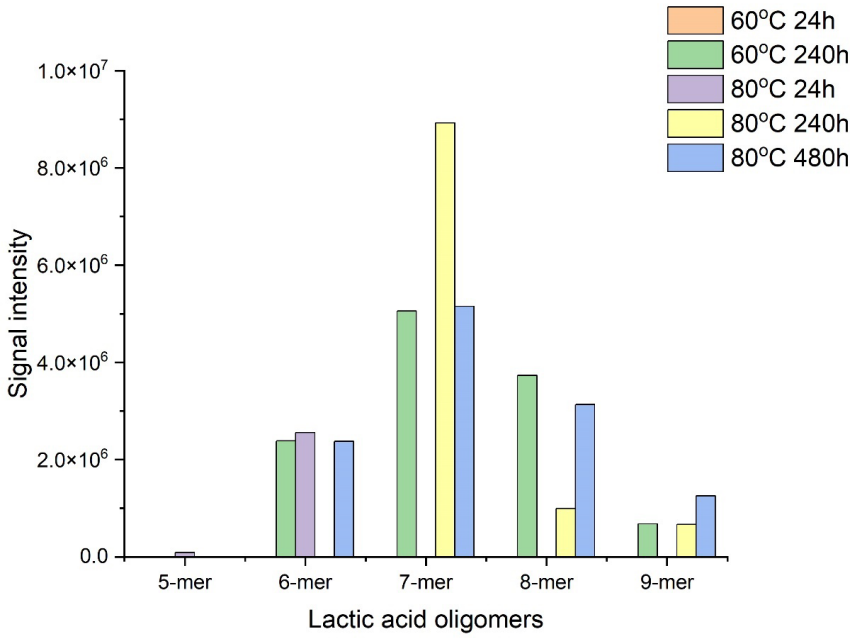

Figure 2.3 PLA hydrolysis in water.

Figures 2.2 and 2.3 present a summary of my results detailing the hydrolysis stability of PLA in water. Over the course of the first 24 hours, immersion in distilled water led to PLA weight loss due to LA and LA oligomers being released from and diffusing through PLA (91). We can evaluate the mechanism of hydrolysis degradation (erosion) via three basic mechanisms: (i) surface erosion, (ii) bulk erosion and (iii) autocatalytic bulk erosion, depending on the diffusion rate and the hydrolysis rate (91-93), which was not discussed in this thesis. It did not prove possible to observe the 24-hour hydrolytic PLA at 60°C using LC-MS, presumably because the amount of hydrolysate might be too low or the hydrolysed polymeric product might be too long to identify. However, when hydrolysis time was increased to 240 hours, the PLA hydrolysis products only could be detected by LC-MS instead of APC. The 7-mer was the leading hydrolysate while 6-mer, 8-mer and 9-mer also presented. The increasing temperature could contribute to PLA hydrolysis, corresponding to the presence of 5-mer and 6-mer from 24-hour 80°C hydrolytic PLA. As the hydrolysis time increased, the relatively weak 5-mer and 6-mer signals disappeared, and the signal of 7-mer became the most dominant component in the system with signals due to the 6-mer, 8-mer and 9-mer growing. The longer oligomers generated could be attributed to PLA bulk further hydrolysis rather than the oligomerisation of LA oligomer

where no shorter oligomers were detected. An abnormal phenomenon observed in PLA hydrolysis for 480 hours at 80°C where the hydrolysis products were similar with the sample hydrolysed for 240 hours at 60°C, not 80°C, might be caused by different kinetics of PLA hydrolysis.

2.3 PLA hydrolysis in seawater

The same experiment was carried out in seawater to investigate the mixed ion effect in PLA hydrolysis. The LC-MS signal intensity of hydrolysate shown in Figure 2.4 was much higher than in water, indicating that PLA hydrolysis was more violated in seawater. The PLA hydrolysis for 24 hours at 60°C was characterised by APC first and showed *ca.* 43000 Da, higher than PLA hydrolysis in water but lower than PLA. The early hydrolysis process seemed to be blocked by seawater. The same situation occurred in the first 24 hours at 80°C hydrolysis that LC-MS only could detect weaker signal intensity of 6-mer. However, as hydrolysis time increased, the yield of PLA hydrolysis grew. At 60°C, the distribution of 240-hour hydrolysate ranged from 5-mer to 10-mer of lactic acid, while the 5-mer and 10-mer were not major components when the reaction medium was water.

For 80°C hydrolysis, in the first 24 hours, only 6-mer was detected, while from 5-mer to 10-mer were identified at 240- and 480-hour hydrolysis. Interestingly, the yield of shorter chain oligomers such as 5-mer, 6-mer and 7-mer doubled and 8-mer and 9-mer surged, while 10-mer kept stable in the second 240 hours. The leading hydrolysis product in seawater was changed from 7-mer to 8-mer in 480-hour 80°C hydrolysis compared with water.

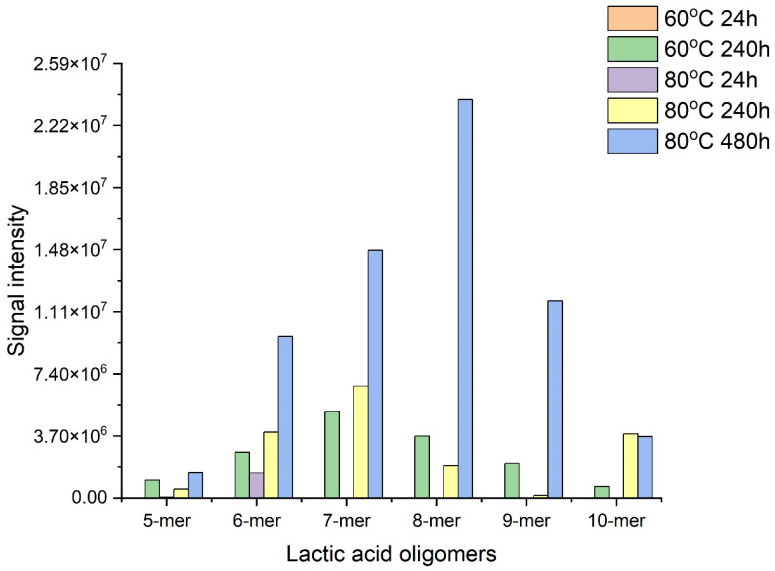

Figure 2.4 PLA hydrolysis in seawater. For raw LC-MS data, see Figure 6.1.

2.4 PLA hydrolysis in controlled salt-water mixtures

Five salt waters were prepared to distinguish each effect of the selected salt solution from seawater. NaCl, $NaNO_3$, $NaHCO_3$, Na_2SiO_3 and Mg_2Cl were chosen as the main components of seawater, and their solution concentration was based on seawater (94).

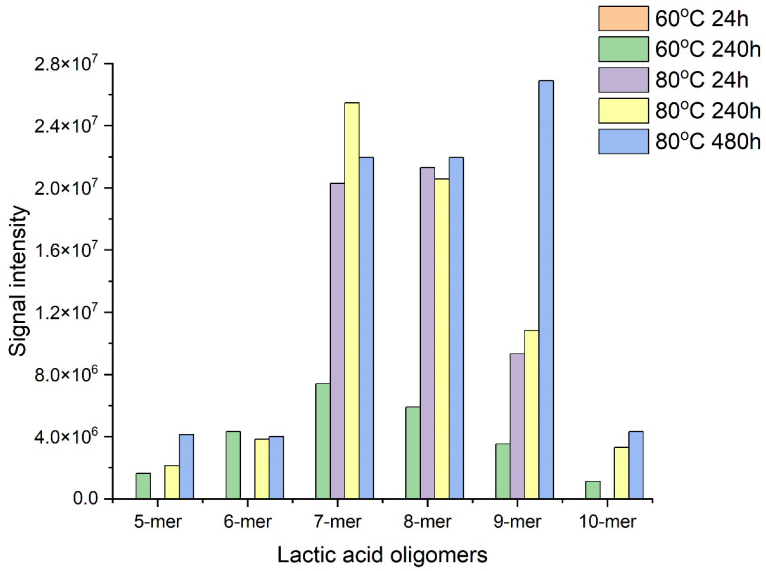

Figure 2.5 PLA hydrolysis in NaCl solution. For raw LC-MS data, see Figure 6.2.

Starting with 24-hour PLA hydrolysis at 60°C in NaCl solution, the APC result showed the molecular mass of PLA hydrolysate was *ca*. 45000 Da but no oligomer signal could be detected in LC-MS (Figure 2.5), indicating short oligomers might not cleave from PLA at this stage. With time increased to 240 hours, many short oligomers formed from PLA from 5-mer to 10-mer. PLA hydrolysis in NaCl solution also benefitted from rising temperature. Higher temperature environment brought 7-mer, 8-mer, and 9-mer at the first 24-hour stage, and 5-mer, 6-mer, 7-mer, 8-mer 9-mer and 10-mer at the both 240-hour and 480-hour stages. The same length oligomers were found at two 240-hour and one 480-hour hydrolysis, but the content and ratio were different. 7-mer and 9-mer dominated hydrolysate in the first 240 hour at both temperatures, until after 480-hour hydrolysis the production of 9-mer surged.

The APC identified PLA hydrolysis in $NaNO_3$ solution for 24 hours at 60°C was *ca.* 44000 Da; meanwhile, the LC-MS detected weak signals of 5-mer, 6-mer and 7-mer as shown in Figure 2.6. Increasing hydrolysis time to 240 hours led to more oligomers including 5-mer, 6-mer, 7-mer, 8-mer, 9-mer and 10-mer cleaved from PLA, and 7-mer was the has the highest yield. Increasing temperature brough 6-mer and 7-mer in the first 24 hours, 6-mer, 7-mer and 8-mer over 240 hours, and 4-mer, 5-mer, 6-mer 7-mer and 8-mer over 480 hours. The 6-mer dominated hydrolysate at 80°C hydrolysis. It was noted that the 9-mer and 10-mer only formed at 60°C for 240 hours, whereas the 4-mer only formed at 80°C for 480 hours.

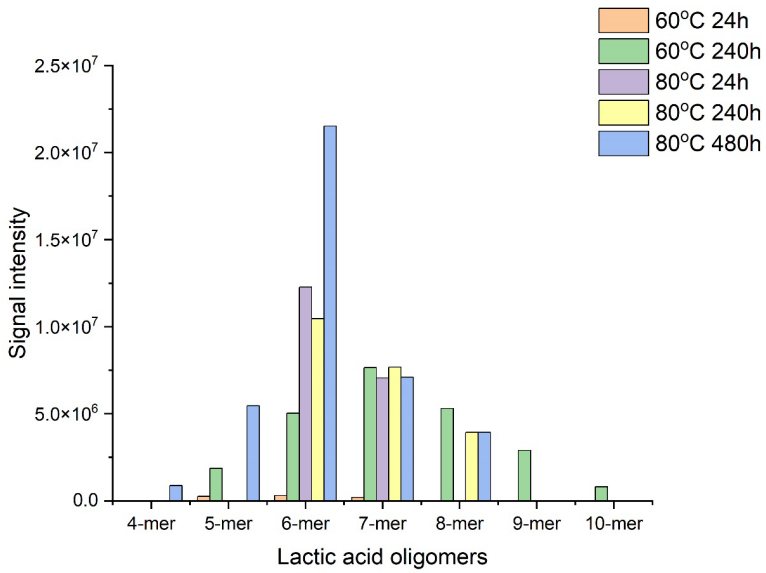

Figure 2.6 PLA hydrolysis in $NaNO_3$ solution. For raw LC-MS data, see Figure 6.3.

The 24-hour PLA hydrolysis at 60°C in $NaHCO_3$ solution was carried to APC and revealed ca. 44000Da, without any signal detected in LC-MS as shown in Figure 2.7. More hydrolysis time led to oligomers from 5-mer to 10-mer found, while higher temperature caused 5-mer and 6-mer formed in the first 24 hours, from 5-mer to 10-mer over 240 hours, and from 5-mer to 10-mer over 480 hours. Higher temperature and hydrolysis time have a signature impact on PLA hydrolysis in $NaHCO_3$ solution than in water. The signal of hydrolysed LA almost doubled in each period.

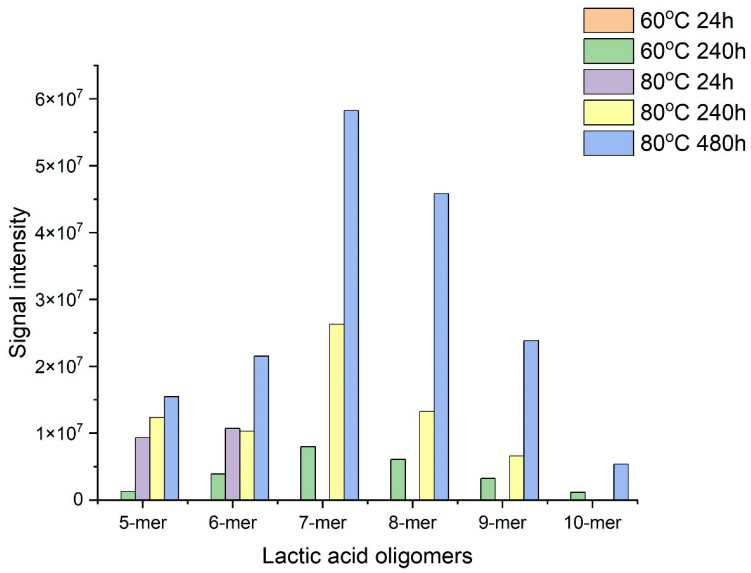

Figure 2.7 PLA hydrolysis in $NaHCO_3$ solution For raw LC-MS data, see Figure 6.4.

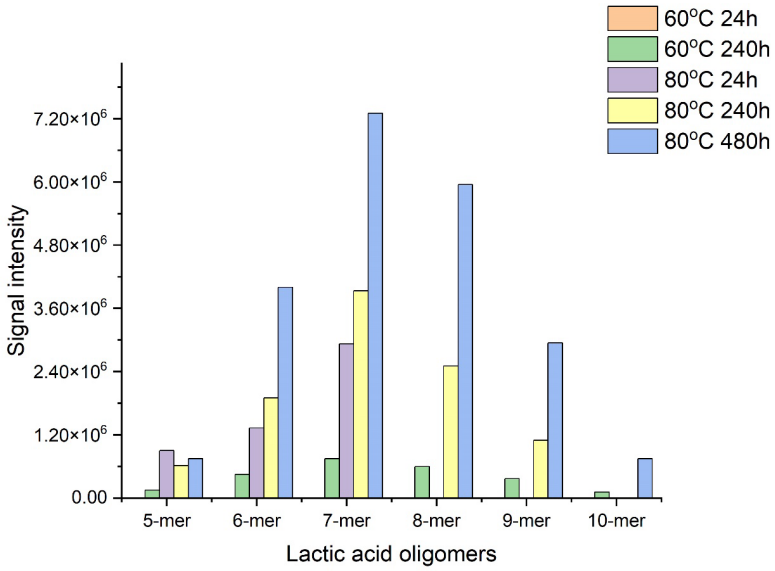

Figure 2.8 PLA hydrolysis in Na_2SiO_3 solution. For raw LC-MS data, see Figure 6.5.

Sodium silicate as the nutrient material in contemporary seawater plays a vital role for all phytoplankton, but it may make a difference in the prebiotic sea environment. The molecular mass of 24-hour PLA hydrolysis at 60°C in Na_2SiO_3 solutions was *ca.* 44000 Da was measured by APC. There was no signal in the first 24 hours, but 5-mer, 6-mer, 7-mer, 8-mer, 9-mer and 10-mer signals in extended hydrolysis time (Figure 2.8). At 80°C hydrolysis, 5-mer, 6-mer and 7-mer were formed first, then extra 8-mer and 9-mer formed found in 240 hours with much higher signal than 60°C. 9-mer was finally cleaved at 80°C. In Na_2SiO_3 solution, the 7-mer always dominated hydrolysate. During the whole hydrolysis period, Na_2SiO_3 solutions slightly inhibited PLA hydrolysis in each stage according to lower signal intensity than in waters.

The PLA hydrolysis in $MgCl_2$ solution at 60°C for 24 hours performed *ca.* 44000 DA in APC. Figure 2.9 showed no oligomer detected, while only 5-mer and 6-mer captured after 240 hours. Higher temperature facilitated 5-mer to first 24 hours, 5-mer, 6-mer and 7-mer to 240 hours and extra 8-mer, 9-mer and 10-mer to 480 hours. The weak intensity also showed $MgCl_2$ solution had inhibition effect on PLA hydrolysis.

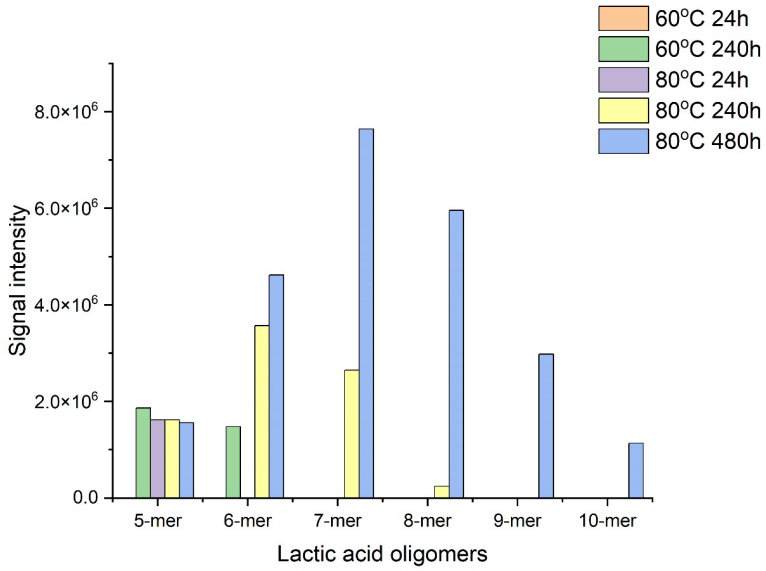

Figure 2.9 PLA hydrolysis in $MgCl_2$ solution. For raw LC-MS data, see Figure 6.6.

2.5 Structure of PLA post-hydrolysis

The microstructure and element distribution of hydrolytic PLA was analysed by a combination of scanning electron microscopy (SEM) and energy dispersive X-ray (EDX). The microscopic characterisation aimed to provide a morphological map of how the PLA polymer that was used in this study, changed during the process of slat-effected hydrolysis. Here we chose three

types of hydrolytic PLA to conduct structural characterisation according to the different degree of hydrolysis prevalent.

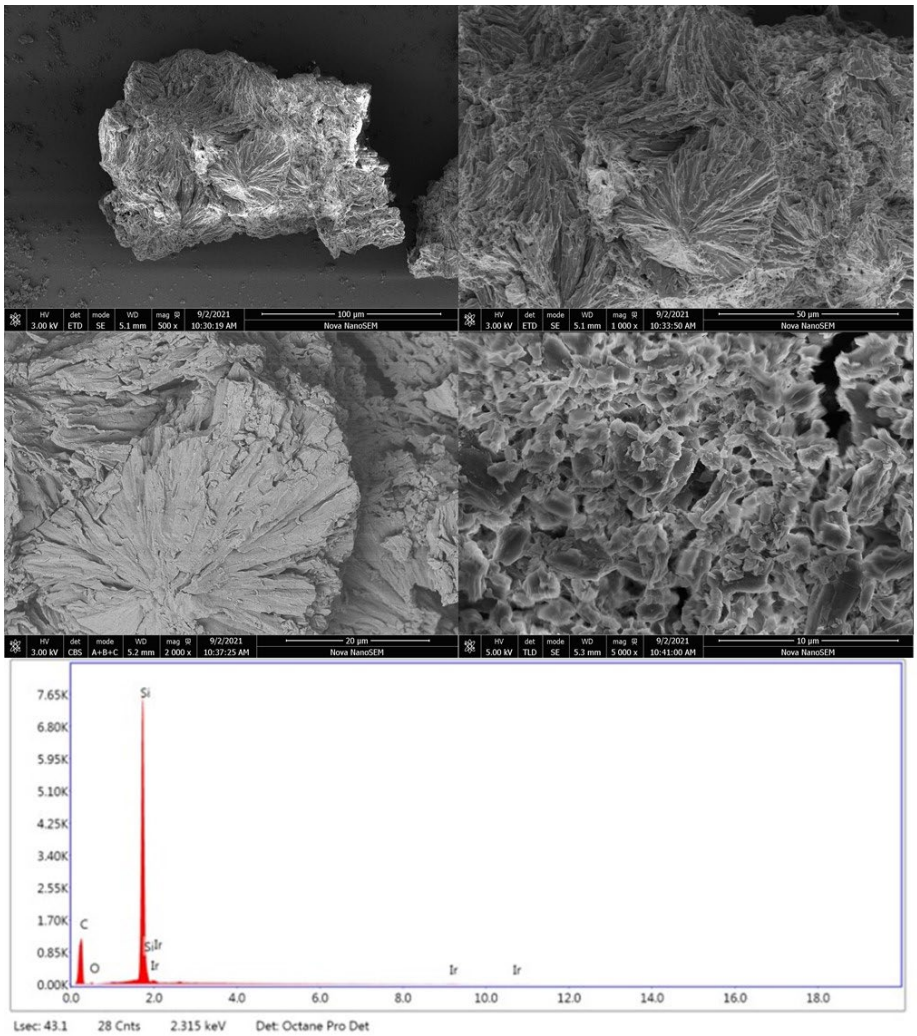

Figure 2.10 SEM micrographs of PLA hydrolysis in water at 80°C for 480 hours.

The PLA hydrolysis after 480 hours at 80°C in water was first studied by SEM Figure 2.10. The hydrolytic PLA size was about 150 µm, and no apparent pores or tunnels could be observed at the surface. As can be observed from the images in Figure 2.10, the material appeared fibrous with very few if any obvious pores. EDX identified C, O, Si and Ir in the sample where C and O were the main elements and Si was from sample preparation. Ir did not exist in any preparation stages but in EDX conductive coatings, which could be ignored. Then the Brunauer–Emmett–Teller (BET) surface area measurements were attempted to measure the sample surface area and number and distribution of potential pores. However, the decomposition temperature of PLA around 85°C was too low to be stable in

the 120°C BET test, which caused the correlation coefficient to be lower than necessary threshold of 0.999.

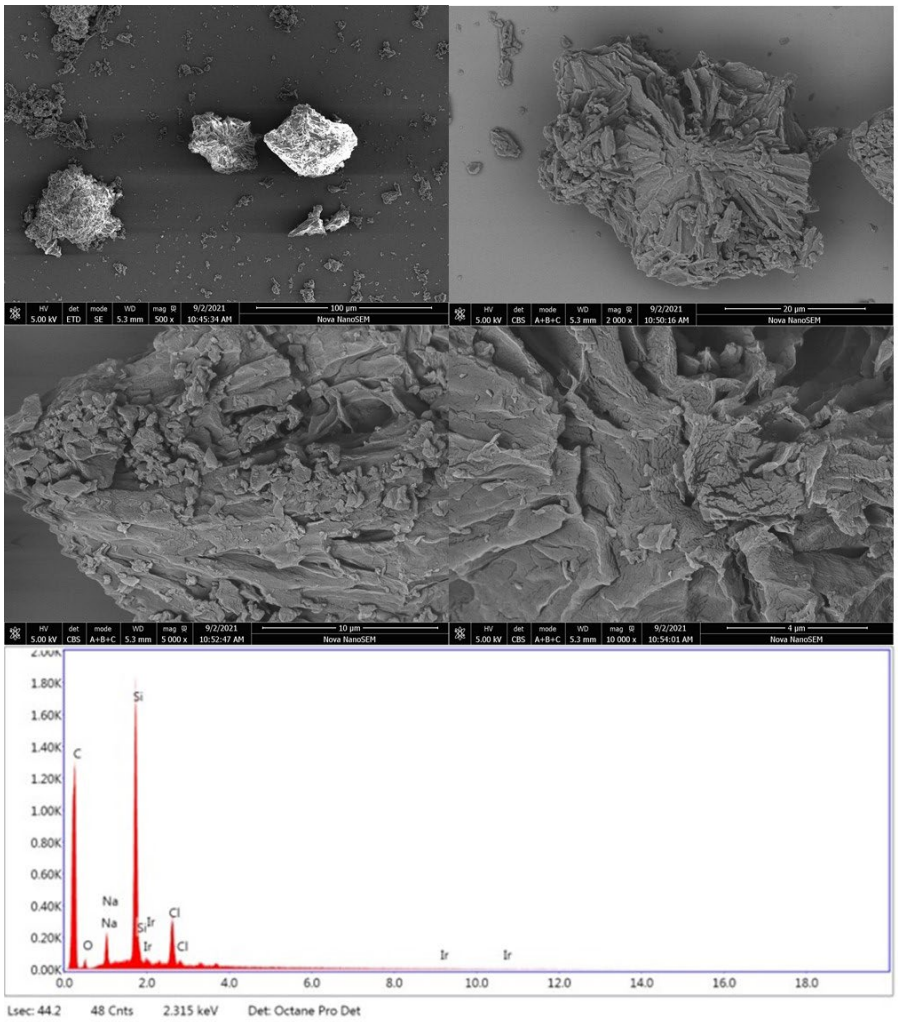

Figure 2.11 SEM micrographs of PLA hydrolysis in seawater at 80°C for 480 hours.

The PLA hydrolysis after 480 hours at 80°C in seawater and Na_2SiO_3 solution were also characterised by SEM and EDX as shown in Figures 2.11 and 2.12. For hydrolysis in seawater sample, the PLA was hydrolysed into smaller particles ca. 50μm than in water, and EDX charactered C, O, Na, Cl, Si, and Ir in PLA hydrolysis in seawater while C, O, Na, Si, and Ir in PLA hydrolysis in Na_2SiO_3 solution. Na and Cl were the most abundant elements in seawater, existed on the surface and inside the sample, while the C and O were the main PLA elements. Si was present in seawater, Na_2SiO_3 solution and preparation stage, and Ir was necessary element for EDX conductive coating. The smaller particle was connected to more oligomers formed in

seawater and more completed hydrolysis. Interestingly, the different structural changes occurred in PLA hydrolysis Na_2SiO_3 solution where the particle was cleaved with a 'layer-like' structure, corresponding to a different erosion pathway.

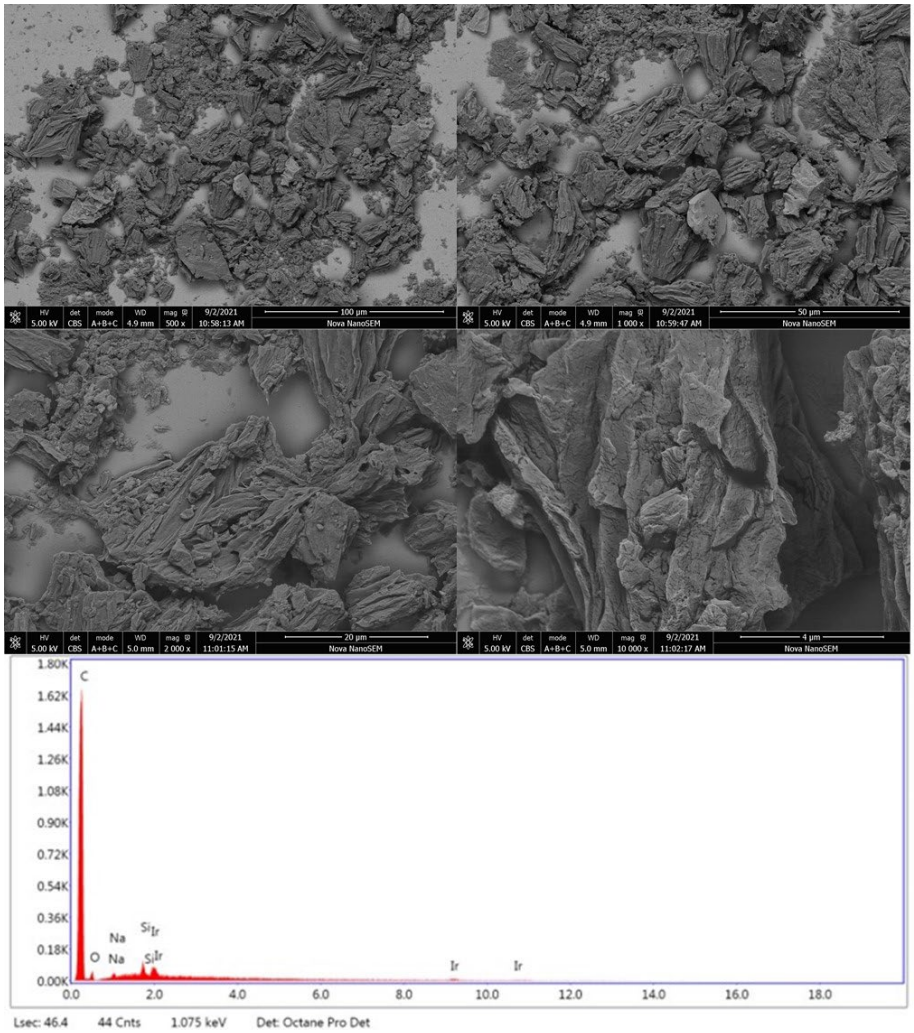

Figure 2.12 SEM micrographs of PLA hydrolysis in Na_2SiO_3 solution at 80°C for 480 hours.

2.6 Conclusion

The chain's scission during PLA hydrolysis releases H^+ acid groups, which the acid is retained in the material or the aqueous medium. As pKa of PLA's carboxylic acid end group and its oligomers is lower than most carboxyl groups leading to a faster hydrolysis rate and a more acidic environment. As $MgCl_2$ is acidic in solution, NaCl and $NaNO_3$ are neutral, and seawater, $NaHCO_3$ and Na_2SiO_3 are alkaline, they may have different effects on PLA

hydrolysis in the early stage. However, due to the relatively low salt concentration, PH did not dominate PLA hydrolysis. For all hydrolysis experiments at 480 hours, the cumulative LA oligomer intensity from all aqueous saline solutions was higher than in water. In the short-term 24-hour or 240-hour experiments, the acidic $MgCl_2$ solution did not facilitate LA oligomer production, while the basic Na_2SiO_3 did not reduce the production.

In this chapter, the main conclusions may be summarised as:

(1) High molecular weight PLA has been found to hydrolyse in water at 60°C and 80°C and this could be identified through combined APC and LC-MS.

(2) Higher temperature and longer hydrolysis time led to more extensive hydrolysis and more oligomer production.

(3) Up to 10-mer and down to 4-mer are found to exist in the selected solution.

(4) Dissolved salts affect PLA hydrolysis through changing PLA hydrolysis dynamic balance and pathway.

Thus, different salts solutions had different impacts on PLA hydrolysis. Compared with water, seawater, $NaNO_3$, NaCl and $NaHCO_3$ solutions boosted PLA hydrolysis while the $Na2SiO_3$ and $MgCl_2$ had an inhibitory effect in each stage of hydrolysis.

Chapter 3 Wet-Dry Cycling of Lactic acid (LA)

3.1 Lactic acid (LA) oligomer formation driven by wet-dry cycles

Lactic acid (LA) is the simplest α-hydroxy acid and has been demonstrated to exist on the prebiotic earth and to have the potential to play a key role in oligomers formation. One pivotal feature is a simple acid's ability to enrich the peptide library in primordial soup. This chapter aims to demonstrate a potential pathway to oligomer formation.

Peptide (amide) bond formation has been considered to be unfavourable thermodynamically. While this is now well established, there is still debate over how important a consideration this issue is for abiogenesis in aqueous solutions (95). The forming and breaking of bonds are reversible, with the result that oligomer chain length is especially sensitive to environmental drivers in prebiotic chemical environments. One possible pathway of formation of compartments in prebiotic environments might be facilitated through longer polymer chain molecules created through wet (cold/night)-dry (warm/day) cycles (31, 47). In the dry stage, evaporated solvent promotes bonds formation and longer polymers. In the wet stage, bonds are expected to hydrolyse to shorter chains. The wet-dry cycle had been proven to be a value in the formation of polyacids (96), esters (47, 97), oligopeptides (98, 99), nucleosides (100), and polynucleotides (101, 102) in prebiotic environments without the need for modern catalysts. It seems reasonable to 'create' the first far-from-equilibrium polymers based on the prebiotic chemistry environment (Figure 3.1) (97). Here, we have studied the possible prebiotic oligomerisation of lactic acid in a simulated aqueous seawater environment and explained how ions affected the distribution of oligomers formed.

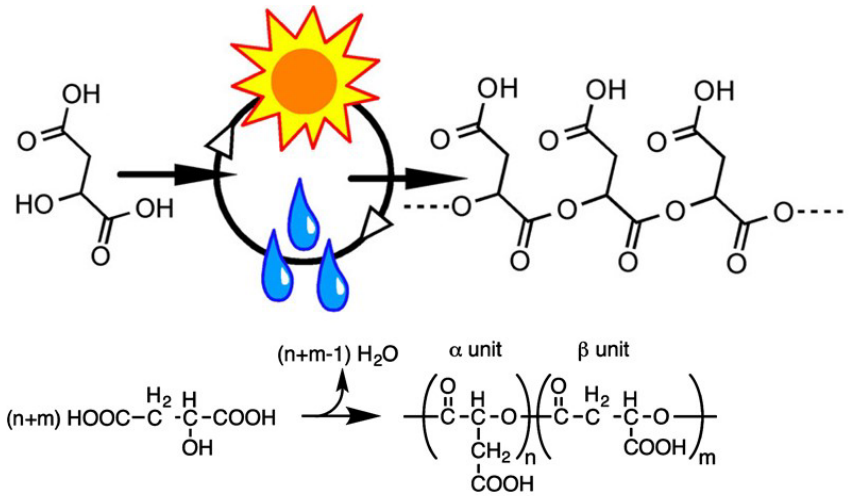

Figure 3.1 example image of polymers emerged from the wet-dry cycle. L-malic acid monomers form metastable oligomers under dry-night cycles (97).

We used LC-MS and nuclear magnetic resonance (NMR) spectrometry to observe the oligomerisation behaviour of LA based on wet-dry cycles, starting with one cycle including 6-hour wet-night and 18-hour dry-day sections in oven to simulate the day-night cycles with humidity change on the prebiotic earth (Figure 3.2). Since the LA oligomers could form through self-esterification (47), the humidity, temperature and ions would have an impact on oligomers formation. Several cycles number from 1, 2, 5, 10, 20 and 30 in different solutions to investigate the various ionic effect in prebiotic environment.

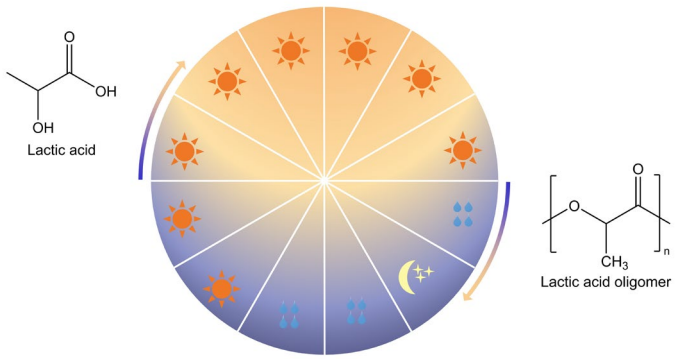

Figure 3.2 Schematic of wet-dry cycles of LA in oven.

3.2 Wet-dry cycles of LA in water

NMR spectroscopy was used to identify LA oligomers which were formed as a result of 1, 2, 5, 10, 20 and 30 wet-dry cycles using water as a medium as shown in Figure 3.3 and 6.14. The peak at 4.80 ppm logically corresponds to residual hydrogen in deuterium oxide solvent and remains unchanged across in all samples NMR spectra, while differences are observed mainly at low field. The signals resulting from the single wet-dry cycle signals in low field regions were assigned to CH- group (4.03 ppm) and CH_3- group (1.23 ppm) from the LA monomer, whereas the signals with weak intensity were attributed to the LA oligomer with two different H signals from CH- (4.92 ppm and 4.19 ppm) and CH_3- (1.38 ppm and 1.27 ppm) (103). When various chemical shift regions of 1.2-1.6 ppm and 4.0-4.4 ppm were observed (Figure 3.3), peaks showed a slight shift to the higher field as the wet-dry cycle increased. Relatively higher chemical shift indicated the stronger electron with-drawing groups formed around the terminal CH_3- where they de-shielded the nucleus and resulted in a relatively larger chemical shift. It was evidenced by stronger electron-withdrawing groups that the longer LA oligomers were forming.

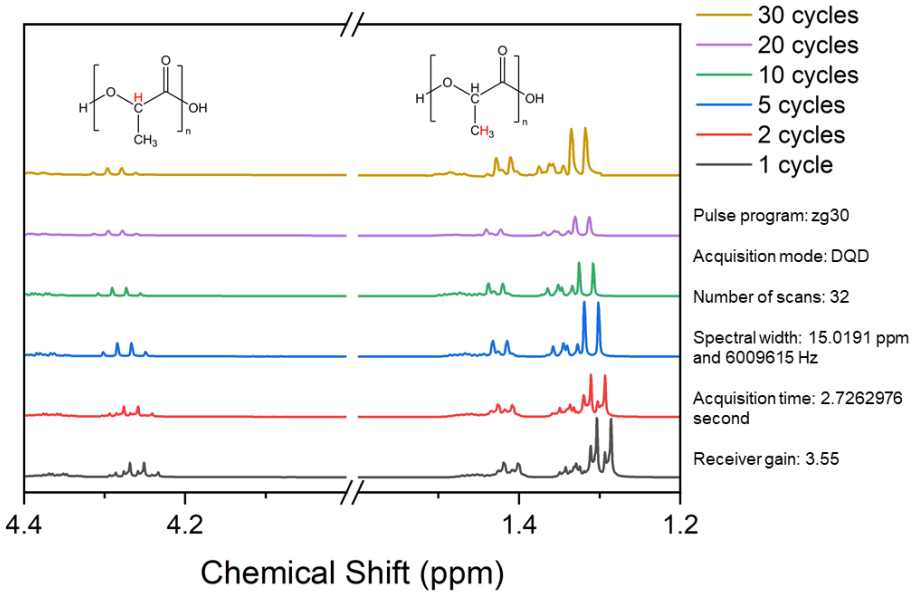

Figure 3.3 Nuclear magnetic resonance (NMR) spectra in H_2O/D_2O (1:1) for processed LA oligomer undergoing 1, 2, 5, 10, 20 and 30 cycles in water with a specific zoom region with 1.2-1.6 ppm and 4.0-4.4 ppm. Full NMR full spectra, see Figure 6.7.

To further investigate LA oligomerisation in wet-dry cycles, LC-MS was used to recognise oligomers. The repeated -(OCH(CH_3)CO)- group with Δ 72.02 Da mass difference is evidenced to distinguish various LA oligomers as shown in Figures 3.4 and 6.8. In oligomerisation of LA in water, up to 9-mer could be identified, corresponding to 689.19 Da as the MNa^+ linear oligomer.

Here we also simplified the mass-to-charge ratio into the number of LA oligomers. Figure 3.3 shows 2-mer, 3-mer, 4-mer, 5-mer and 6-mer were characterised in LC-MS over the course of the first cycle, 3-mer, 4-mer, 5-mer, 6-mer and 7-mer formed in the second cycle. As the cycle goes by, 4-mer, 5-mer, 6-mer and 7-mer were found in the fifth cycle, whereas only 6-mer and 7-mer existed in the tenth cycle. Longer oligomers were formed over more cycles. In the twentieth cycle, there were 6-mer, 7-mer and 8-mer, while 7-mer, 8-mer and 9-mer in the thirtieth cycle. It was observed that the longer LA oligomer formed with wet-dry cycles increasing, and repeated cycling led to a significantly longer length distribution with short oligomers disappearing. In the whole oligomerisation process in water, eight types of oligomers were detected, and the reason for the disappearance of short oligomers might be completely transferring to longer LA oligomers.

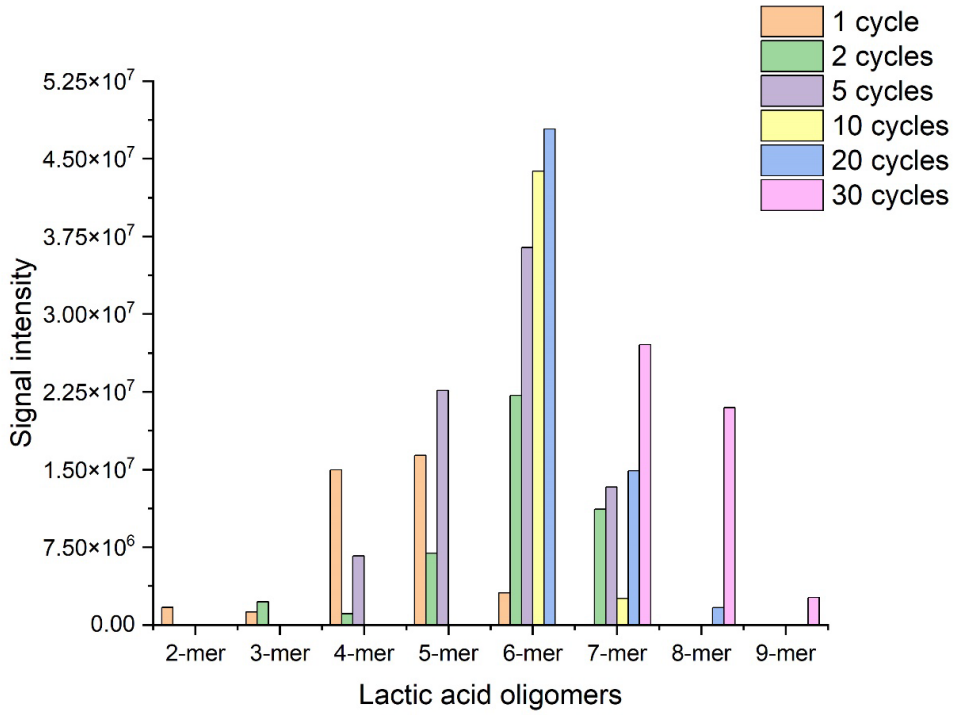

Figure 3.4 Oligomerisation of LA in water. For raw LC-MS data, see Figure 6.8.

3.3 Wet dry cycles of LA in seawater

Multiple ion effects on LA oligomerisation were also studied with seawater as the solvent medium. As cycles increased, a similar chemical shift was recorded at 1.2-1.6 ppm and 4.0-4.4 ppm regions to the higher field as shown in Figure 6.9, indicating that LA oligomers had been formed in seawater. LC-MS spectra (Figure 3.5 and 6.9) revealed that a different speciation or collection of oligomers are produced across different timelines of wet-dry cycling. During the first and second cycles, only 2-mer and 3-mer LA oligomers were formed, while longer 5-mer and 6-mer LA oligomers began to appear in the product mixture across the fifth and tenth cycles. The 5-mer disappeared in the twentieth cycles where only 6-mer and 7-mer existed but 5-mer re-formed in thirtieth cycle. In all cycles, five types of oligomers excluding 4-mer were found. This might imply that multiple ion effects impede 4-mer formation. Compared with LA oligomerisation in water, seawater showed an inhibition effect in the early stage of oligomerisation regarding to lower LC-MS signal intensity. The Multiple ion effects also reflected on the length of the oligomers that only 7-mer was recorded shorter than 9-mer in water.

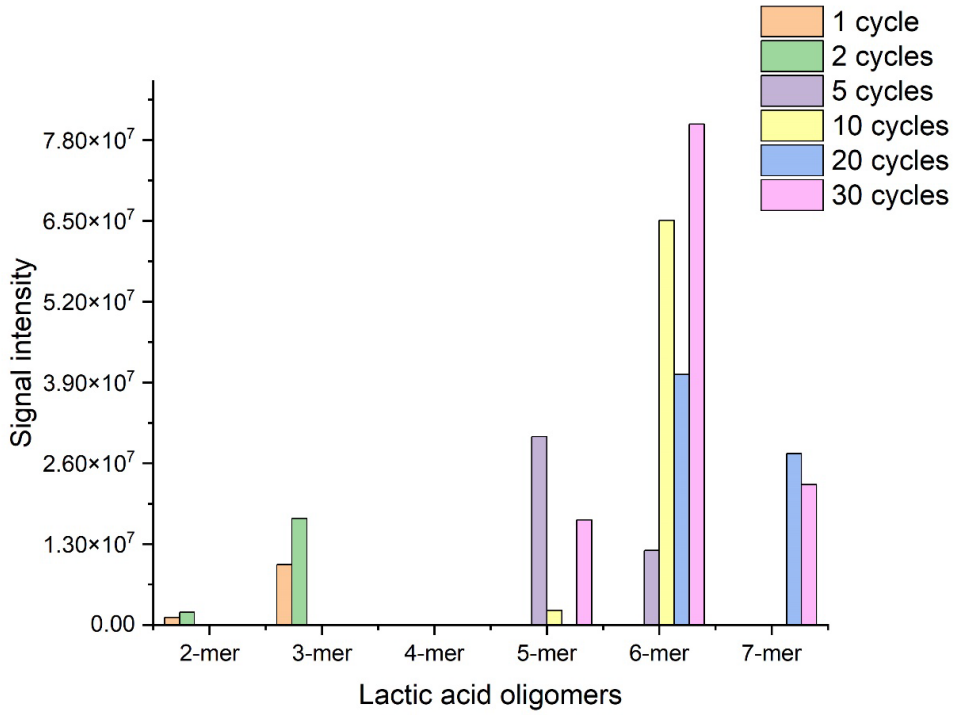

Figure 3.5 Oligomerisation of LA in seawater. For raw LC-MS data, see Figure 6.10.

3.4 Wet dry cycles of LA in controlled salt-waters mixtures

Each single ion effect was investigated through NaCl, $NaNO_3$, $NaHCO_3$, Na_2SiO_3 and $MgCl_2$ solutions. The wet-dry cycling in controlled salt-waters in NaCl, $NaNO_3$, $NaHCO_3$ and Na_2SiO_3 solutions were carried first to NMR. Figure 6.11-6.14 indicated similarly slight chemical shift at regions of 1.2-1.6ppm and 4.0-4.4ppm to higher field, representing the existence of longer oligomers. The oligomers formed with ester bonds resulted in de-shielding of the H_α resonance and new signals of CH- and CH_3- were assigned for *ca.* 4.92ppm and 1.38ppm (103). However, Mg^{2+} ion will activate a strong magnetic field effect in NMR system, which blocked NMR locking magnetic field from recording. The LA oligomerisation in $MgCl_2$ solutions was not recorded by NMR.

The LC-MS spectra Figure 3.6 (detailed data as shown in Figure 6.15) revealed much stronger oligomer signal intensity in NaCl solution than in water or seawater over each cycle. The first cycles in NaCl solutions drove LA to oligomerise to 2-mer, 3-mer and 4-mer, while 6-mer formed in the second cycle. From five wet-dry cycles, the yield of 6-mer and 7-mer were increased significantly, regarding much higher signal intensity. Longer LA oligomers 9-mer and 10-mer generated separately in the twentieth and thirtieth cycle. Seven types of LA oligomer were formed, but they were not continuous due to lack of 5-mer and 8-mer.

LA oligomerisation in $NaNO_3$ solution was slightly stronger than in water, because the higher signal intensity from LC-MS spectra shown in Figures 3.7 and 6.16, and six types of oligomers were captured. Starting with very slow and weak oligomerisation, a few 1-cycle products of 3-mer and 4-mer could be detected and 2-mer 3-mer, 4-mer, 5-mer and 6-mer formed in next cycle. More cycles contributed products distributed in wider lengths from 2-mer to 7-mer, while the shorter oligomers decreased over cycling. Unlike in water and seawater, an unusual oligomerisation occurred in that shorter average oligomers were generated at the twentieth cycle, where the length distribution was not increased compared to the tenth cycle.

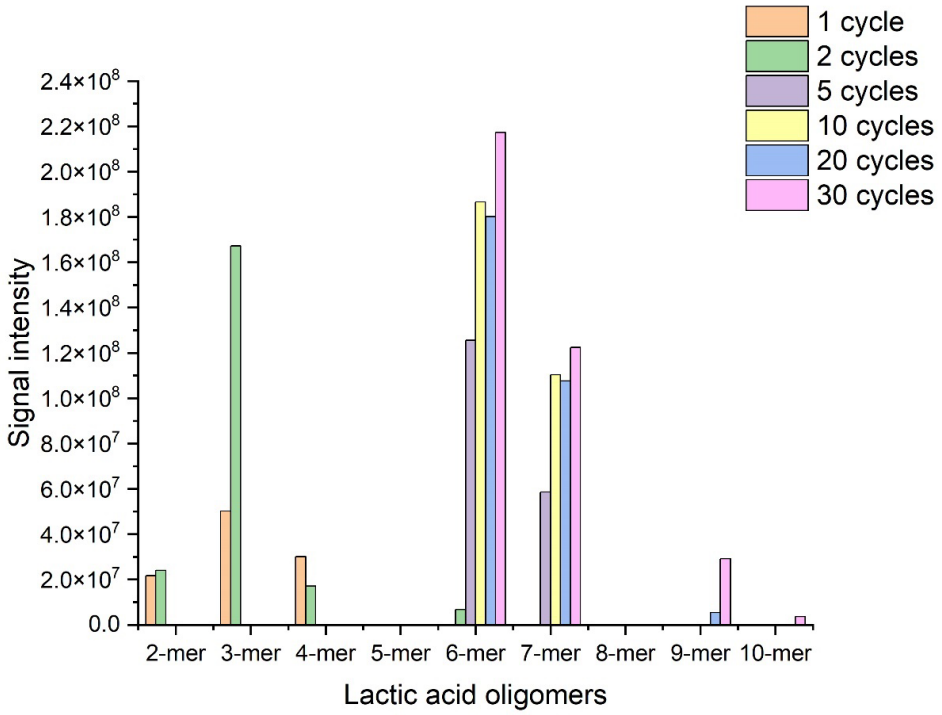

Figure 3.6 Oligomerisation of LA in NaCl solution. For raw LC-MS data, see Figure 6.15.

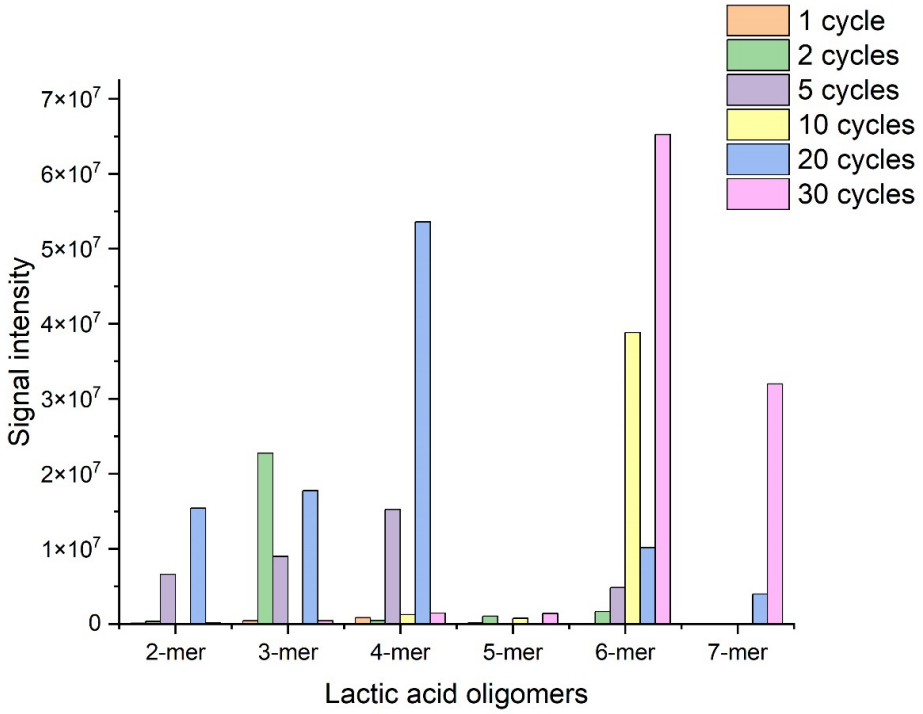

Figure 3.7 Oligomerisation of LA in $NaNO_3$ solution. For raw LC-MS data, see Figure 6.16.

Oligomerisation of LA in $NaHCO_3$ solution was revealed through LC-MS (Figure 3.8 and detail data as shown in Figure 6.17). 3-mer and 6-mer were recorded in the first two cycles. Then 3-mer, 4-mer and 5-mer formed in the following three cycles. Only 6-mer was found in the tenth cycle, and 7-mer existed after the twentieth cycle. In total, five types of LA oligomers presented in $NaHCO_3$ solution.

In Na_2SiO_3 and Mg_2Cl solutions, only four kinds of oligomers existed during all cycles. 2-mer, 3-mer, 4-mer and 5-mer could be detected in all cycles in Na_2SiO_3 solution, except that 5-mer did not form over 1 cycle (Figure 3.9 and detailed data as shown in Figure 6.18). Oligomers with lower yield than in Mg_2Cl solution were 2-mer, 3-mer and 4-mer in all cycles as shown in Figure 3.10 (detailed data as showed in Figure 6.19). In addition, 5-mer presented after 20 cycles.

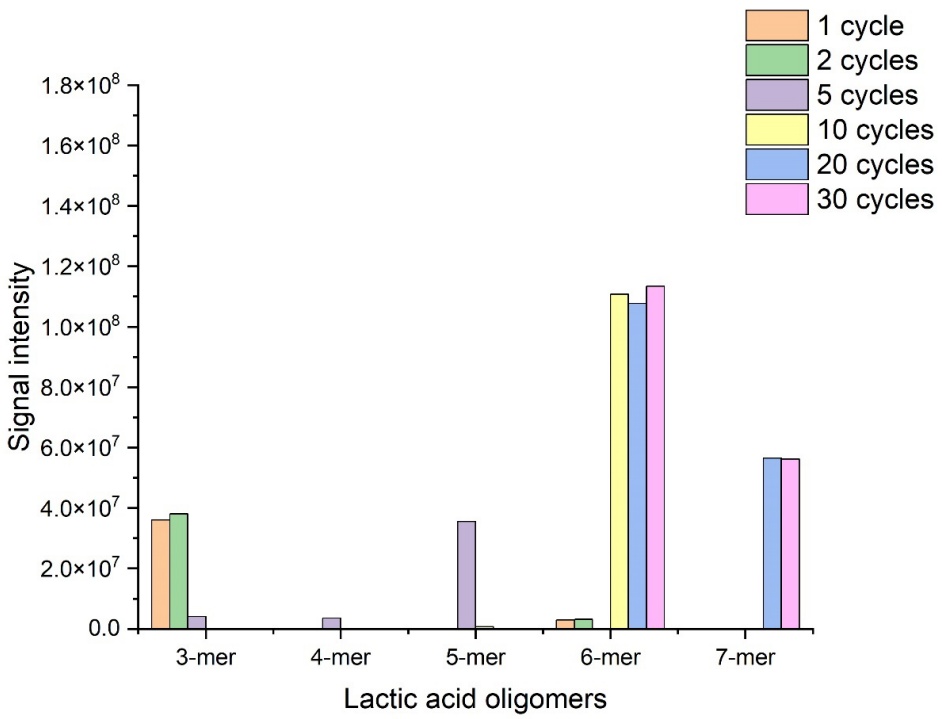

Figure 3.8 Oligomerisation of LA in $NaHCO_3$ solution. For raw LC-MS data, see Figure 6.17.

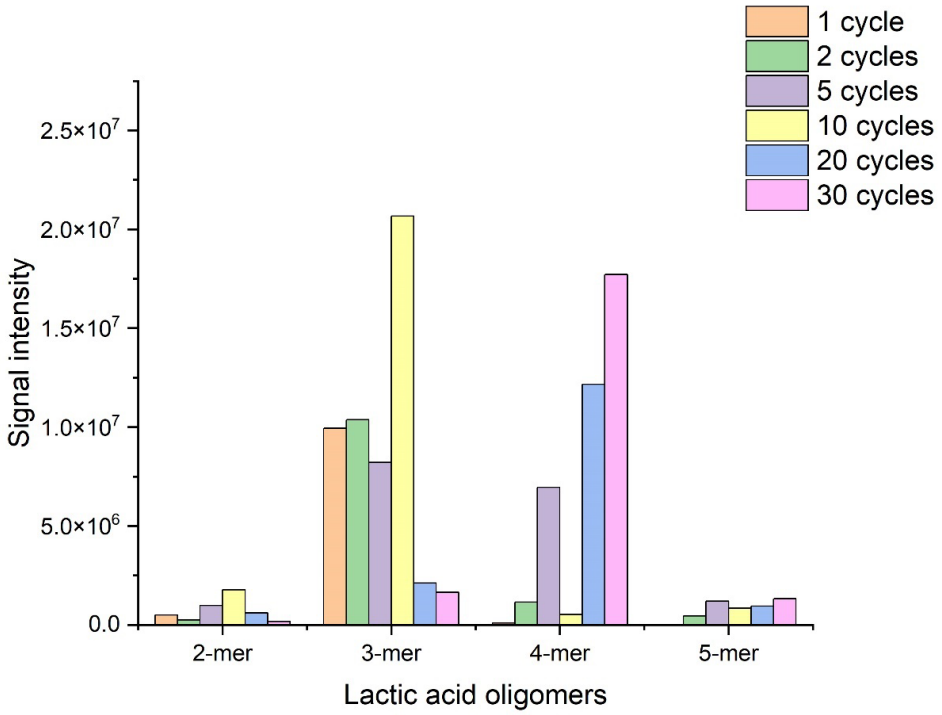

Figure 3.9 Oligomerisation of LA in Na_2SiO_3 solution. For raw LC-MS data, see Figure 6.18.

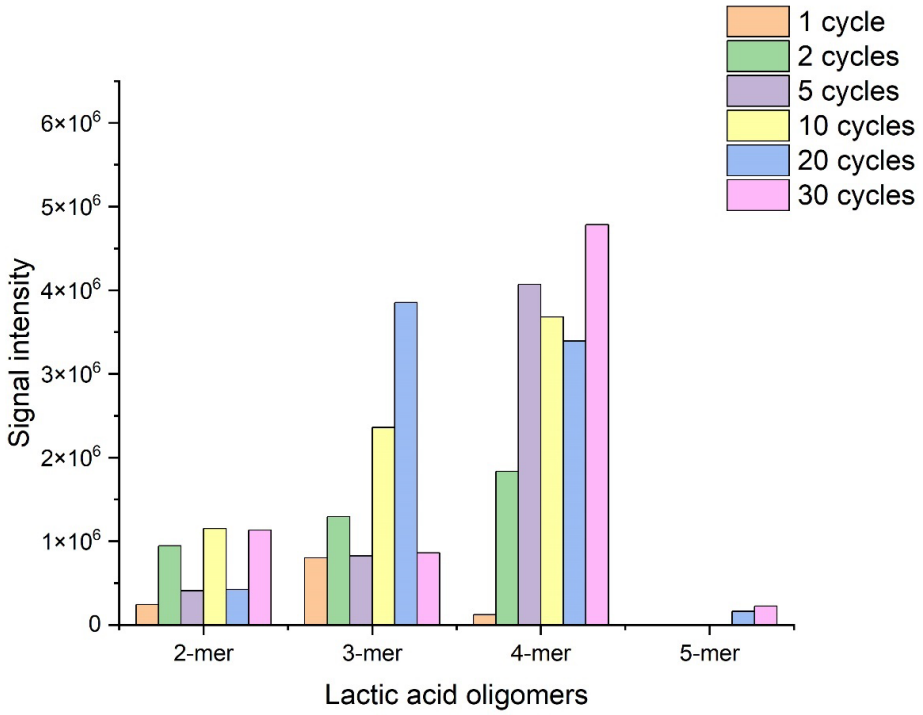

Figure 3.10 Oligomerisation of LA in $MgCl_2$ solution. For raw LC-MS data, see Figure 6.19.

3.5 Conclusion

Overall, the content of this section could be summarised as:

(1) Lactic acid (LA) would polymerise under wet-dry cycles and could be detected through combined NMR and LC-MS.

(2) More cycles tend to lead to the observation of longer oligomers.

(3) Single salt-solutions appear to have different impact on LA oligomerisation yield. Compared with in water alone, the presence of aqueous $NaNO_3$, NaCl and $NaHCO_3$ salts seem to facilitate more LA oligomerisation, whilst aqueous Na_2SiO_3 and $MgCl_2$ solutions appear to show less of an effect.

(4) Another single ion effect reflects in the length of the LA oligomer chains. The 5-mer is detected in Na_2SiO_3 and $MgCl_2$; 7-mer is generated in seawater, $NaNO_3$, and $NaHCO_3$ solutions, 9-mer is formed in water and up to 10-mer is found in NaCl solution.

Chapter 4 Analysis and discussion based on PLA hydrolysis and LA oligomerisation

4.1 Analysis of experiments in water and seawater

To investigate the ion effect on PLA hydrolysis and LA oligomerisation and the connection between these processes in different solutions, all LC-MS spectra have been combined into one figure according to each ion. Before characterising under LC-MS, the samples were separately prepared to the same concentration according to hydrolysis and oligomerisation experiments. A blank was washed after each LC-MS injection to prevent any sample-to-sample contamination. By doing this, the LC-MS signal intensity can be compared in the PLA hydrolysis or LA oligomerisation group to reflect the relative concentrations of oligomers. Because the solvents of PLA hydrolysis and LA oligomerisation were different for LC-MS, it is difficult to define an absolute reference unit. We therefore chose the highest 7-mer signal intensity of PLA hydrolysis in $NaHCO_3$ solution at 80°C for 480 hours as a relative intensity unit in hydrolysis experiments, and 6-mer signal intensity of LA oligomerisation in NaCl solution after 30 wet-dry cycles as a relative intensity unit in LA oligomerisation experiments separately, which reflected in a simple way the yield of each product relative to the highest product in both processes. Accordingly, the title of the abscissa would be simplified to a combination of hydrolysis time and temperature for PLA hydrolysis, or ‘C’ plus cycle number for LA oligomerisation. For example, PLA hydrolysis for 24 hours at 80°C would be simplified to ‘24_60’ and LA oligomerisation after 1 cycle would be marked as ‘C1’. The formation of LA oligomers resulting from top-down PLA hydrolysis could be considered as a reverse reaction from bottom-up LA oligomerisation. Thus, comparing both pathways of LA oligomer formation could provide more valuable and complementary information on the stability of both LA polymers and monomer sand short-length oligomers.

In water, the process of LA oligomerisation was only dependant on thermodynamics without salt ion effect, which was a blank reference and represented an original LA formation pathway. At 80°C hydrolysis, the stacked intensity increases over time (Figure 4.1), indicating that PLA continued hydrolysing. The 6-mer existed in 24 and 480 hours but was absent in 240 hours, indicating that in the first 240 hours all 6-mer increased

to longer length chain; while more 6-mer cleaved from PLA and the rate of generation was greater than the consumption. It also provided evidence that the PLA hydrolysis was not a single process but was accompanied by oligomerisation. An increase in hydrolysis time in terms of PLA break-down led to shorter oligomers while employing more wet-dry cycles resulted in longer oligomers of LA.

In the wet phase, ester bonds hydrolysis is most likely to occur, whereas diffusion and reformation of new ester bonds with the release of water is likely to be preferred in subsequent dry phase cycles with increasing oligomer length. Over the course of time, the longer products generated and accumulated. The yield of 6-mer kept increasing gradually and was found to be a dominant product during the first 20 wet-dry cycles. Until the thirtieth cycle, 6-mer fully transformed into longer oligomers including 7-mer, 8-mer and 9-mer. This might indicate that 6-mer was less favoured in long-term water environment. A higher mean length distribution was favoured in both hydrolysis and oligomerisation in water over time. Up-to 9-mer formed in both processes may approach the limit under current experimental conditions. The 7-mer, 8-mer and 9-mer existed stably long-term in water, evidenced by no further strong hydrolysis in the second 240 hours and thirtieth wet-dry cycle. It was summarised that long-time reaction would also benefit prebiotic libraries with more kinds of oligomers with relatively longer chains, corresponding with the theoretical model (104).

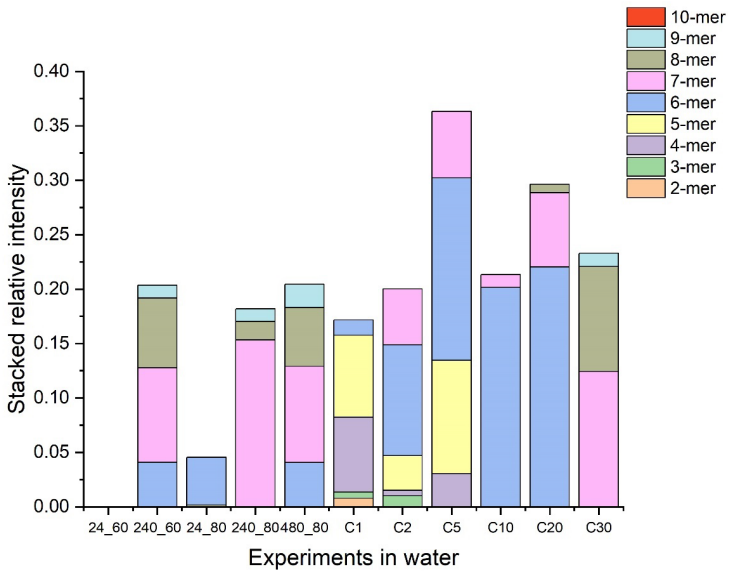

Figure 4.1 Stacked relative intensity of LA oligomers from PLA hydrolysis (left five bars) and LA oligomerisation (right five bars) in water.

Seawater led to the different equilibrium length distribution to those observed in aqueous, salt-free, solution as illustrated in Figure 4.2. During PLA hydrolysis in seawater, stacked signal intensity from LA oligomerisation was higher than in water, indicating a stronger hydrolysis process and more LA involved oligomerisation. Five types of LA oligomers and up to 10-mer formed from PLA hydrolysis; while only 3 types were recorded in the thirtieth cycle. The seawater environment drove 5-mer and 10-mer formation in 240_80 and 480_80 experiments, which were not present in water. Compared with 7-mer and 8-mer dominating 30 wet-dry cycles in water, 6-mer became the biggest part in seawater. From the twentieth to the thirtieth cycle, the 7-mer slightly decreased without any longer oligomer forming, implying that seawater may block generation of the longer oligomers. The equilibrium length distribution changed in seawater, and two kinds of change could be observed. In PLA hydrolysis process, seawater benefitted longer and shorter oligomers formation at the same time; however, in LA oligomerisation process, it reduced the mean length distribution.

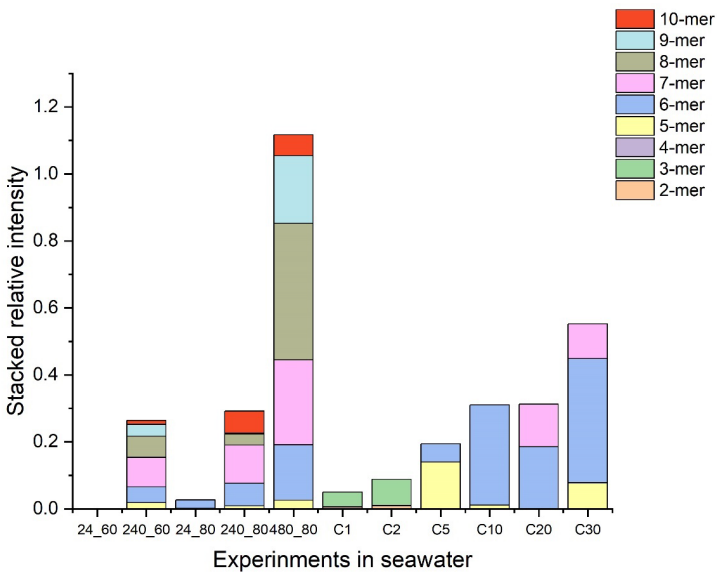

Figure 4.2 Stacked relative intensity of LA oligomers from PLA hydrolysis (left five bars) and LA oligomerisation (right five bars) in seawater.

4.2 Analysis of experiments in selected salt-water

Comparing water and seawater, PLA hydrolysis and LA oligomerisation were both found to be favoured in the presence of NaCl compared with both salt-

free water and mixed-salt seawater. Figure 4.2 indicates much higher stacked relative intensity, representing higher oligomer yield in both hydrolysis and oligomerisation processes at each stage. 6-mer, 7-mer 9-mer and 10-mer existed stably in long-term PLA hydrolysis and La oligomerisation, which indicates these oligomers may be sustainable as a result of reaction equilibrium in certain prebiotic environment. Compared with salt-free water, the mean length increases and more types of oligomers in NaCl solution.

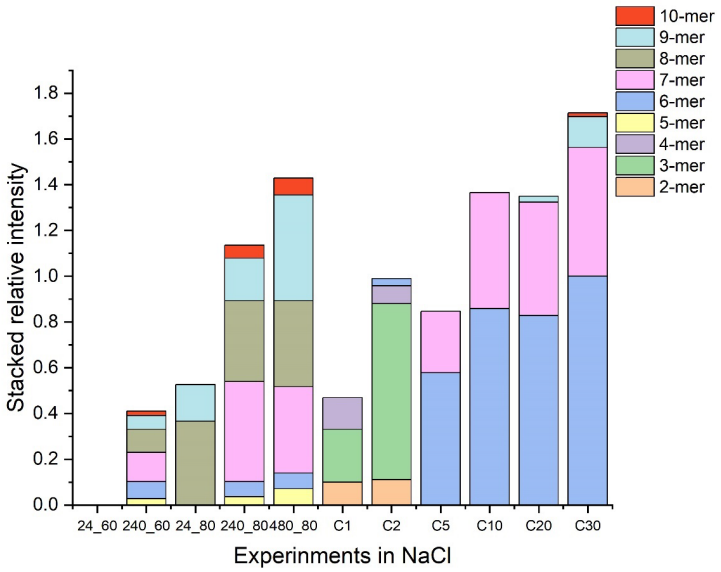

Figure 4.3 Stacked relative intensity of LA oligomers from PLA hydrolysis (left five bars) and LA oligomerisation (right five bars) in NaCl solution.

$NaNO_3$ solution brought the shorter oligomers after 480-hour hydrolysis and 30 cycles as shown in Figure 4.4. 4-mer and 5-mer were formed in the second 240-hour PLA hydrolysis at 80°C, while the 2-mer and 3-mer fully disappeared and 4-mer and 5-mer were formed after 20 cycles in LA oligomerisation. The detected 4-mer in 480_80 experiment was the shortest oligomer among all PLA hydrolysis experiments. It may indicate that the 4-mer and 5-mer were favoured in both hydrolysis and oligomerisation in $NaNO_3$ solution, which was different in water and seawater.

$NaHCO_3$ solution also facilitated the PLA hydrolysis and LA oligomerisation which results in higher yields of oligomers at higher relative intensity as shown in Figure 4.5. The yield of PLA hydrolysate almost doubled after the second 240-hour interval at 80°C and was 10 times higher than in water. $NaHCO_3$ solution also contributed to wider length distribution from 5-mer to

10-mer over 480 hours in PLA hydrolysis process. Although the $NaHCO_3$ solution single increased the yield of oligomer in LA oligomerisation, the mean length and the longest oligomer were decreased compared with a non-salt water environment. And oligomerisation process seemed to stop after the twentieth cycle, as there was only slight growing intensity without longer oligomers being detected.

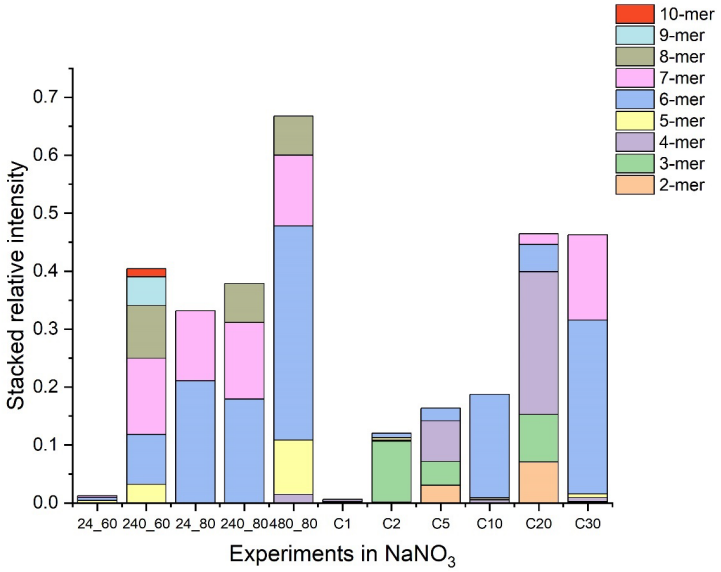

Figure 4.4 Stacked relative intensity of LA oligomers from PLA hydrolysis (left five bars) and LA oligomerisation (right five bars) in $NaNO_3$ solution.

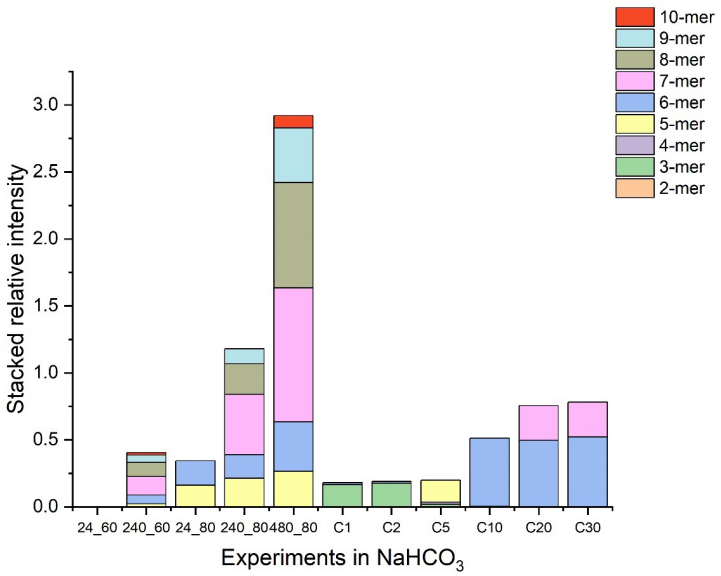

Figure 4.5 Stacked relative intensity of LA oligomers from PLA hydrolysis (left five bars) and LA oligomerisation (right five bars) in $NaHCO_3$ solution.

Na_2SiO_3 showed a slight inhibition effect on both PLA hydrolysis and LA oligomerisation such that the yield of oligomers at each stage was much lower than in water (Figure 4.6). Although the types of oligomers in hydrolysis process were more than in water, it was as the same as in seawater. The inhibition effect was reflected in the length of oligomer in that the mean length was much lower than in water where only 3-mer 4-mer and 5-mer were formed after the thirtieth cycle

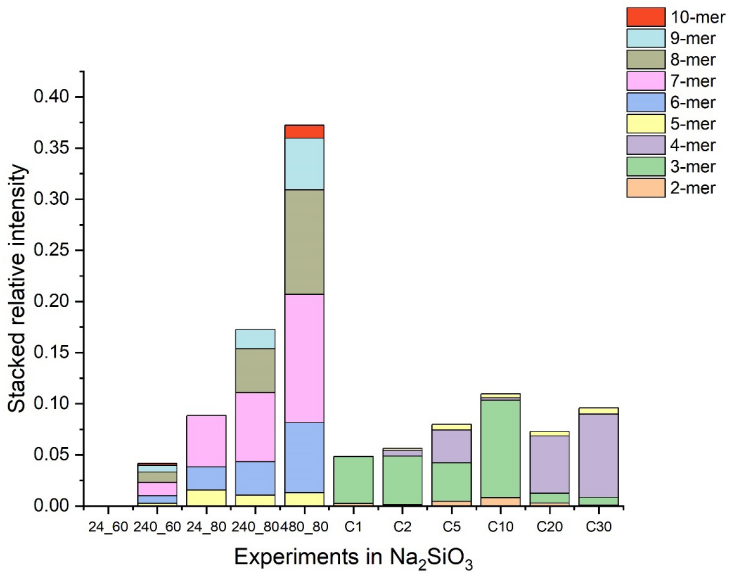

Figure 4.6 Stacked relative intensity of LA oligomers from PLA hydrolysis (left five bars) and LA oligomerisation (right five bars) Na_2SiO_3 solution.

Figure 4.7 shows in the similar ion effect on Mg_2Cl solution that it inhibited the first 240-hour oligomer yield. However over 480 hours, five types of oligomers from 5-mer to 10-mer were generated in PLA hydrolysis experiments and the stacked relative intensity was double that in water. The inhibition effect was stronger on both length and yield of oligomer in wet-dry cycling experiments. Only few 2-mer, 3-mer and 4-mer were detected in the oligomerisation process.

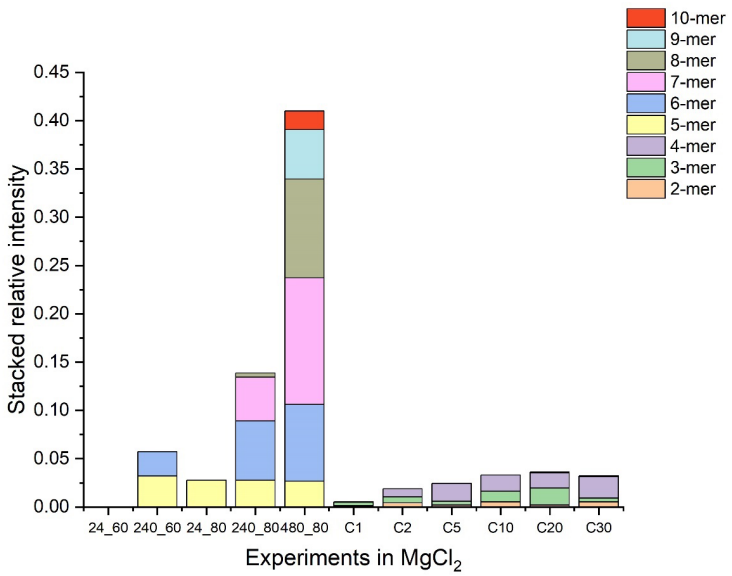

Figure 4.7 Stacked relative intensity of LA oligomers from PLA hydrolysis (left five bars) and LA oligomerisation (right five bars) in $MgCl_2$ solution.

4.3 Discussion of cross-comparison

The all oligomers with different length were be recorded in Table 4.1. Any more oligomers formed in salt solutions were highlight in red, or highlighted in black.

More connections are revealed in Table 4.1 that seawater, NaCl, $NaHCO_3$, $NaNO_3$, Na_2SiO_3, and $MgCl_2$ solutions facilitate more LA oligomers in PLA hydrolysis than water while they reduce in LA oligomerisation. Furthermore, except that NaCl facilitates longer oligomer in both processes, the other saline solutions only lead to longer chains in the PLA hydrolysis but shorter in the LA oligomerisation

Table 4.1 The type of LA oligomers formed

Salts		Types	Up to	Down to	Notes
Water	PLA hydrolysis	5	9	5	
	LA Oligomerisation	8	9	2	
Seawater	PLA hydrolysis	**6**	**10**	5	
	LA Oligomerisation	**5**	**7**	2	No 4-mer
NaCl	PLA hydrolysis	**6**	**10**	5	

	LA Oligomerisation	**7**	**10**	2	No 5-mer and 8-mer
$NaNO_3$	PLA hydrolysis	**7**	**10**	**4**	
	LA Oligomerisation	**6**	**7**	2	
$NaHCO_3$	PLA hydrolysis	**6**	**10**	5	
	LA Oligomerisation	**5**	**7**	**3**	
Na_2SiO_3	PLA hydrolysis	**6**	**10**	5	
	LA Oligomerisation	**4**	**5**	2	
$MgCl_2$	PLA hydrolysis	**6**	**10**	5	
	LA Oligomerisation	**4**	**5**	2	

Through accumulating the stacked relative intensity of LA oligomers in each experiment, Table 4.2 shows that seawater, NaCl, $NaNO_3$, and $NaHCO_3$ solutions facilitate high-yield LA oligomers, while Na_2SiO_3 and $MgCl_2$ block it compared with water.

Based on the prebiotic chemistry environment, the thermodynamic ester bonds formation (ΔG = ~ 0 kcal mol^{-1}) compared to amide bonds ΔG = ~ 3.5 kcal mol-1. The ester bonds seem to be preferred as the first prebiotic polymers. However, such ester linkages are less thermodynamically and kinetically robust than amide bonds. If they were capable of performing some of the essential functions of primitive cells, they might have potential in prebiotic terms. The research about the dynamics and thermodynamics of LA showed that they are both critical (50). If only thermodynamics change is applied for oligomerisation, LA will only be remained in the dry phase without cycling. Although the dry condition is suitable for polymerisation, it does not usually allow diffusion, or at least, diffusion is likely to be much slower than in solution. Monomers may only link together if they encounter others, so only monomers that are very close after the drying procedure are able to polymerise further.

Thus the benefit of the wet phases is that they permit the rearrangement of molecules and bring molecules together that may polymerise in the following dry phase. If the wet phase is relatively short, then the net effect of cycling may increase polymerisation relative to a single dry phase with no cycling. Now, the wet-dry cycle has been shown to lower the thermodynamic barrier in the formation of polyacids and esters.

Table 4.2 The yield of LA oligomers

	Water	Seawater	NaCl	$NaNO_3$	$NaHCO_3$	$Na2SiO_3$	$MgCl_2$
24 hours 60°c	0.000	0.000	0.000	0.013	0.000	0.000	0.000
240 hours 60°c	0.203	0.264	0.411	0.404	0.404	0.041	0.057
24 hours 80°c	0.045	0.027	0.526	0.332	0.344	0.088	0.028
240 hours 80°c	0.182	0.292	1.135	0.379	1.180	0.173	0.139
480 hours 80°c	0.205	1.117	1.429	0.667	2.921	0.372	0.410
1 cycle	0.172	0.050	0.469	0.006	0.179	0.048	0.005
2 cycles	0.200	0.088	0.989	0.120	0.190	0.056	0.019
5 cycles	0.363	0.194	0.847	0.164	0.199	0.080	0.024
10 cycles	0.213	0.310	1.366	0.188	0.513	0.110	0.033
20 cycles	0.296	0.312	1.350	0.464	0.755	0.073	0.036
30 cycles	0.233	0.552	1.714	0.463	0.780	0.096	0.032

4.4 Conclusion

The presence of some salts promotes the production of more oligomers from the oligomerisation reaction and limits the distribution of chain lengths. We believe that the importance of some salts is that they can provide relatively high concentrations of oligomers so that the high concentration of oligomers in the prebiotic chemical library may reach a critical value for the following reaction.

Different ions have different degrees of inhibition on the types of oligomers, but the production can be divided into promotion and inhibition. In this project, since it is not an open system, it cannot be determined whether the inhibition of length distribution is due to the effect of salt ions or the lack of the reactant lactic acid. However, from the PLA hydrolysis experiments, the aqueous saline solution produced longer oligomers than PLA hydrolysis in water which may indicate that the inhibitory effect of oligomerisation comes from converting oligomerisation without enough lactic acid.

Overall, the content of this Chapter could be concluded as:

(1) Based on different ion effects, the length and yield of oligomer would be affected due to different dynamic equilibrium. However, the common trend of hydrolysis is for the generation of longer and shorter oligomers over time. As the wet-dry cycle goes on, oligomers favour longer length.

(2) Compared with water, seawater, NaCl, $NaHCO_3$, $NaNO_3$, Na_2SiO_3 and $MgCl_2$ solutions widen the length distribution of LA oligomer in PLA hydrolysis. Up to 10-mer is found in seawater, NaCl, $NaHCO_3$, Na_2SiO_3 and $MgCl_2$ solutions, while down to 4-mer only is found in $NaNO_3$ solution.

(3) Based on water, only longer length oligomer 10-mer is detected in NaCl solution over long wet-dry cycling. The shorter oligomer 2-mer are identified in Na_2SiO_3 and $MgCl_2$ solutions, 4-mer in $NaNO_3$ solution, 5-mer in seawater and 6-mer in NaCl and $NaHCO_3$ solutions.

Chapter 5 Experimental details

5.1 Chemicals and instruments

Table 5.1 The list of chemicals used in this research

Name	CAS	Supplier	LOT	Notes
DL-lactic acid	50-21-5	Fluka	BCBG9428V	
Sodium Nitrate	7631-99-4	British Drug Houses LTD		
Sodium Decarbonate	144-55-8	British Drug Houses LTD		
Sodium Silicate	1344-09-8	Fisher Chemical	K47791221 644	
Sodium Chloride	7647-14-5	Fisher Chemical	1871051	
Magnesium Chloride	7791-18-6	VWR Chemicals BDH		
Potassium Chloride	7447-40-7	British Drug Houses LTD		
Sodium Sulphate	7757-82-6	Fisher Chemical	1861552	
Potassium Bromide	7758-02-3	Vickers Laboratories		
Boric Acid	10043-35-3	Aldrich		
Strontium Chloride	10024-70-4	Acros Organics	A0345328	
Sodium Fluoride	7681-49-4	Aldrich	83679	
Calcium Chloride	10035-04-8	Hopkin & Williams		
Polylactic Acid	4511-42-6			synthesis by another

				research group

All chemicals were directly used without further purification.

Table 5.2 The list of instruments

Technique Name	Supplier and model
Liquid chromatography-mass spectrometry	Bruker amaZon series
Nuclear magnetic resonance spectroscopy	Bruker AV3HD
EDX analysis with SEM	Hitachi SU8230
Advanced polymer chromatography	Water ACQUITY Advanced Polymer Chromatography System

5.2 Preparation of simulated seawater and salt solutions

The simulated seawater was prepared following the Table 5.3 (94). After adding all components in deionised water, the resulting solution was stirred magnetically for a period of at least 2 hours to ensure all chemicals were fully dissolved in solution. The NaCl, $NaHCO_3$, $NaNO_3$, and Mg_2Cl solutions were prepared separately following the concentration profiles illustrated in Table 5.3. The Na_2SiO_3 solution was prepared at the concentration of 0.01293 g/L.

Table 5.3 The list of seawater components and concentration

Salt	Mass g/L	Molar Mol/L
Sodium Chloride	23.9260	0.409
Sodium Sulphate	4.0080	0.028
Potassium Chloride	0.6770	0.009
Sodium Decarbonate	0.1960	0.002
Potassium Bromide	0.0980	0.0008
Boric Acid	0.0260	0.00042

Strontium Chloride	0.000054	0.00000034
Sodium Fluoride	0.003	0.07145
Sodium Nitrate	0.150	0.0018
Calcium Chloride	0.0078	0.000070
Magnesium Chloride	0.0250	0.0002626

5.3 PLA hydrolysis

Poly-lactic acid PLA (100 mg; Mw= 45000) was placed in a small glass bottle (5 ml) with water, seawater or salt solutions (1.8 mL) and DMF (0.2 mL) and the bottle was sealed. The hydrolysis was carried out in an oven maintained at a temperature of 60°C or 80°C. After being maintained in the sealed oven for 24, 240 or 480 h, the samples were removed and subsequently kept at room temperature until analysed.

5.4 LA oligomerisation

Reactions were started with 500 µL deionised water, seawater or salt solutions with 100 µL lactic acid. Each one completed wet-dry(day-night) cycle should be divided into two stages. In the dry-day-warm stage, the solution was deployed in an oven and allowed to dry at 85 ^{o}C for 18 hours. In the subsequent wet-night-cool stage, each dried sample was rehydrated with 500 µL deionised water and capped at 65°C for 6 hours which ensured the same ion concentration in every cycle. The longer-cycle samples were prepared by repeating these stages for the expected number of cycles.

5.5 Preparation for Advanced Polymer Chromatography (APC) analysis

Polymer molecular weights were measured *via* a Waters Acquity advanced polymer chromatography system with a bed of three 150 mm length Acquity columns (XT 125, XT 200 and XT 450). The columns have a 4.6 mm inner diameter and contain 2.5 µm diameter particles with different pore sizes depending on the column. Dimethylformamide (DMF) with 1g mol^{-1} lithium bromide was used as the mobile phase at a flow rate of 0.5 mL min^{-1} under 40°C column temperature. The APC instrument was calibrated against standard poly(methyl methacrylate) samples in DMF. To perform APC to

analyse the samples, the solvent change from DMF 1 g mol^{-1} LiBr to DMF is necessary to ensure correct mobile phase to purge columns at a flow rate of 0.3 mL min^{-1}, while setting column and refractive index detector temperature to 40°C. Once pressure and temperature are steady, increase the flow rate to 0.5 mL min^{-1}. The samples are ready to place in the APC when the refractive index detector detects a smooth curve. The hydrolysed PLA was extracted with 10 mL DMF to keep samples near to 10 mg/ml and filtered *via* Agilent PTFE syringe filter (0.2 μm, 25 mm). Between each sample, the DMF is run as the blank sample to remove any potential pollution in the APC system. After all samples are detected, the solvent changes to DMF to flush the APC system for an hour. While the data has been exported from APC, the retention time will be obtained from the APC chromatograms Figure 6.20-6.27. Through bringing retention time into the calibration formula, the M_w of each sample will be determined.

$$m_w = e^{15.00832 - t * 0.90761}$$

5.6 Preparation for nuclear magnetic resonance (NMR) analysis

^{1}H Nuclear Magnetic Resonance spectra were performed on a Bruker AV4 NEO 11.75 T 500 MHz spectrometer fitted with a 5 mm Bruker C/H cryoprobe. Chemical shifts were referenced to a tetramethylsilane standard which has a chemical shift of 0 ppm. The NMR tubes for NMR are made by NORELL and the model is XR-55-7. The dried LA oligomer samples were obtained after the dry stage and were rehydrated in 350 μL D_2O and 350 μL to record on NMR at 25°C choosing the ‘proton’ experiment and ‘D_2O’ solvent. The pulse program chosen was ‘zg30’, and acquisition mode was set as the ‘DQD’, which eliminates quad images and O1 spikes. Under this setting, the number of scans was set as 32; receiver gain as 3.55; spectral width as 15.0191 ppm and 6009.615 Hz and the acquisition time as 2.7262976 seconds.

5.7 Preparation for Liquid Chromatography–Mass Spectrometry (LC-MS) analysis

Liquid Chromatography–Mass Spectrometry (LC-MS) was recorded on Bruker amaZon system (LC from Thermo Dionex; MS from Bruker HCT/esquire) with an electrospray ionization source. The LC-MS system performs at a flow rate of 0.2 mL min^{-1} under the positive polarity mode. Analysis was performed using a Thermo Scientific Ultimate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA), interfaced with a mass spectrometer equipped with an electrosparay-ionization source operated in the positive mode (Bruker amaZon Speed, Bruker Daltonik GmbH, Billerica, MA, United States). Chromatographic separations were performed using a Kinetex C18 (2.1×50 mm i.d., 2.6 μm particle size; Phenomenex, Torrance, CA, USA) at a column temperature of 40℃. The mobile phases were (A) 0.1 % Formic acid in water and (B) 0.1 % Formic acid in acetonitrile. A gradient was used starting at 98 % of A and 2 % of B over 1.2 minutes, ending with 2 % A and 98 % B at a flow rate of 1.3 mL/min.

The ‘quickLC_posneg’ method was used for the sample characterised. The 5 mL acetonitrile was the solvent to ensure removing the water from PLA hydrolysis samples and the organic component dissolved. The dried samples obtained after the dry-day-warm stage were rehydrated and diluted in 500 μL deionised water for LC-MS deionised. For LC-MC, A water blank was injected between each sample to guard against bleed and pollution.

5.8 Preparation for Scanning Electron Microscopy (SEM) with Energy Dispersive X-Ray Analysis (EDX)

The PLA hydrolysis samples were left for one day without caps so that aqueous solutions could be vapoured. Freeze drying was performed to remove all aqueous solutions until the white uniform powder was left. Then another two days were freeze-drying under reduced pressure. An SEM-EDX instrument characterised the obtained white powder.

Chapter 6 Conclusion and further work

6.1 Conclusion

Under a prebiotic environment, one of the critical questions is abiotically forming long polymers to fold without the presence of enzymes. However, it is widely believed that the origin of life highly depends on the abiotically produced organics. The primitive planet's conditions make it is not easy to facilitate some polymers for contemporary biochemistry. Some chemistry compounds not used in contemporary biochemistry may play a vital role in the origin of life. Various simple or oligomers components were available in prebiotic chemistry from diverse sources, including atmospheric and geochemical synthesis and extraterrestrial input under hot springs, seawater or dried land. Because of such diversity of the prebiotic environment, the core of prebiotic life-like molecules might differ from modern biochemistry. The prebiotic existed α-hydroxy acids could be induced into polymers which have the potential to create a new phase that enables a closed chemical environment with higher molecule concentration. These prebiotic environment conditions are compatible with the primitive earth environment.

Here we show that LA oligomers spontaneously form from presumably abundant prebiotic lactic acid under conditions likely to be present on primitive planetary and pre-planetary bodies.

We discuss the ion effect of PLA hydrolysis on the dried environment and LA oligomerisation under wet-dry cycling through various characterisation methods to reveal different pathways to form LA oligomers. The experiments showed that LA oligomers were affected by specific seawater environments where each ion has a different effect. NaCl facilitates the formation of longer LA oligomers, whereas simulated seawater only leads to 7-mer formation. The Na_2SiO_3 and $MgCl_2$ had an inhibitory effect on long oligomer formation and oligomer yield. The experiments demonstrated that a mixture including LA could give rise to oligomers in a plausible prebiotic reaction. LA as a typical αH-hydroxy acid of abundant prebiotic monomers could be oligomerised to form long-lived oligomers and stable libraries. These libraries could be expanded through various reaction conditions, including temperature, time, concentration, and types of αH-hydroxy acids and salt ions. These results supported a hypothesis of OoL that wet-dry cycles might

drive the plausible prebiotic compartments based on combinatorial rearrangement from oligomer libraries in the early sea (47, 96, 97).

The further experiment may focus on the more prebiotically available component. Through mixtures of prebiotic α-hydroxy acids and α-amino acids, it will form the oligomers with both ester and amide linkages, resulting in reaction products being more complex even with the functional side. The high-sequence heterogeneity polymer library may scaffold the origin of life.

We also noticed that there is contentiousness that changed the pH, temperature and ionic strength of seawater as a function of time crossing earth's early evolution. Each new chemical environment on the prebiotic earth created conditions that allowed the new reaction under non-direction environment change, driven by the prebiotic environment. It is not easy to determine which stage of earth plays a decisive role in the origin of the earth, but we know geology always keeps changing. Understanding the potential prebiotic chemistry environment, including chemistry diversity, potentially contributes to the origin of life.

6.2 Future work

Based on the results of this project, the next steps to supplement that hypothesis can be divided into three levels:

1. Enrich the oligomer library by adding different reactants.

    (1) More types of αH-hydroxy acids should be tested through wet-dry cycling in different salt-solutions to identify various length distribution.

    (2) Using prebiotic α-amino acids to perform wet-dry cycling to reveal their different equilibrium length distribution.

    (3) Combined with αH-hydroxy acids and α-amino acids, mixed reactant added into wet-dry cycles in different solutions. The reported work showed the peptides might be formed from wet-dry cycles with combined acids (47). The ions existing in prebiotic ‘wet and dry phases’ would lead into more complicated libraries of oligomers and peptides and formation a variety of oligomers with both ester and amide linkages.

    2. Change a single variable such as temperature, time, PH, concentration, or cycle number to study the oligomer library in the presence of only one reactant.

3. Prebiotic environment is an open system, and acids could be re-introduced/removed at any time. It necessary to consider adding/removing basic αH-hydroxy acids or α-amino acids in wet-dry cycle system.

6.3 Appendix

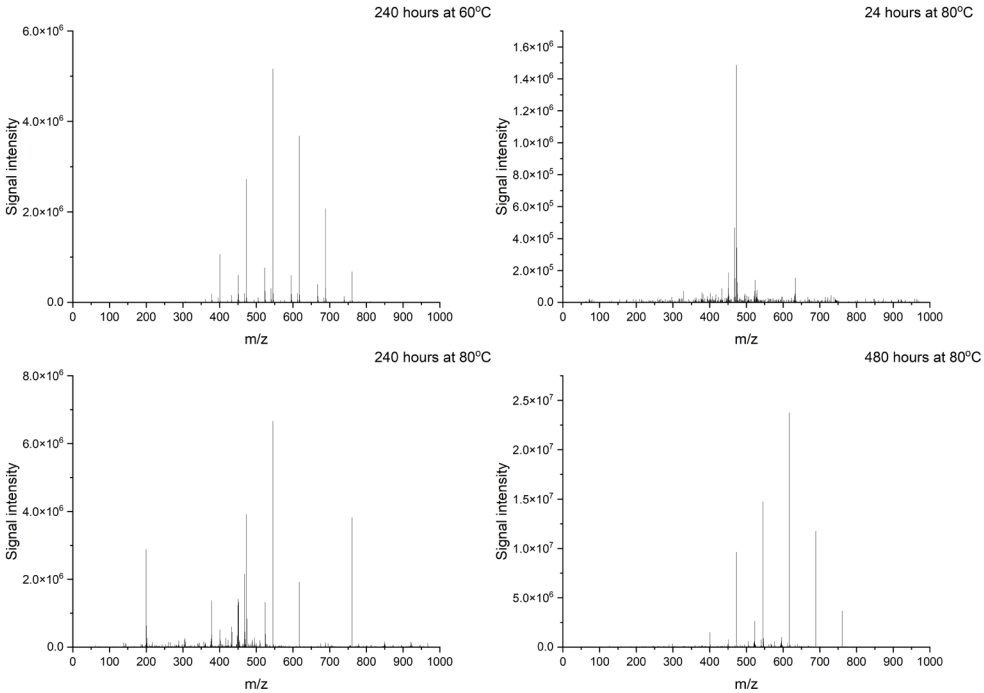

Figure 6.1 LC-MS data for PLA hydrolysis in seawater.

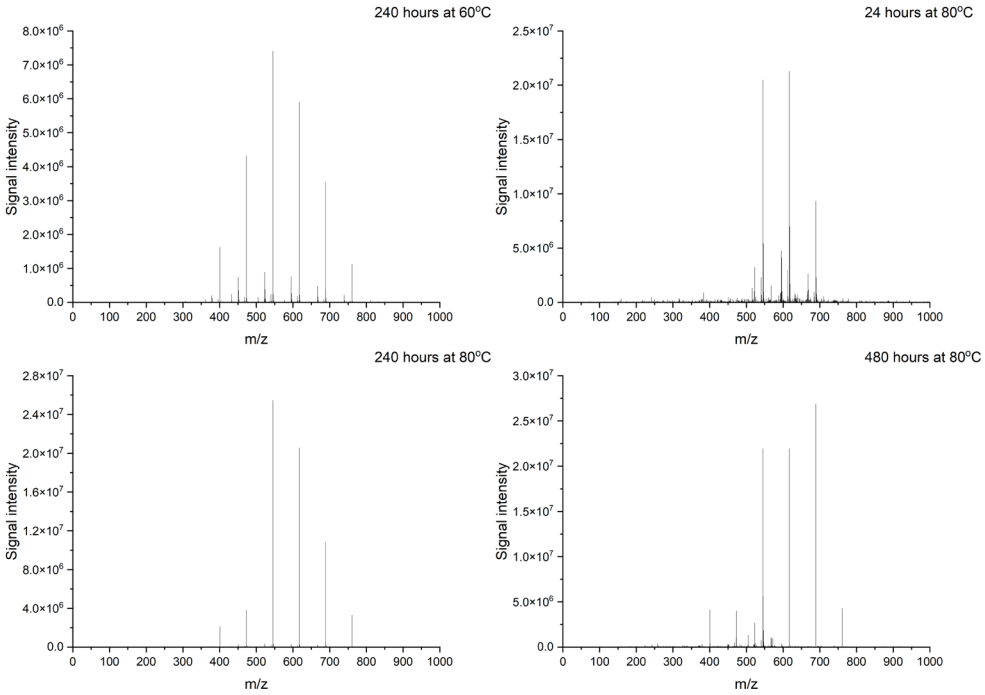

Figure 6.2 LC-MS data for PLA hydrolysis in NaCl solution.

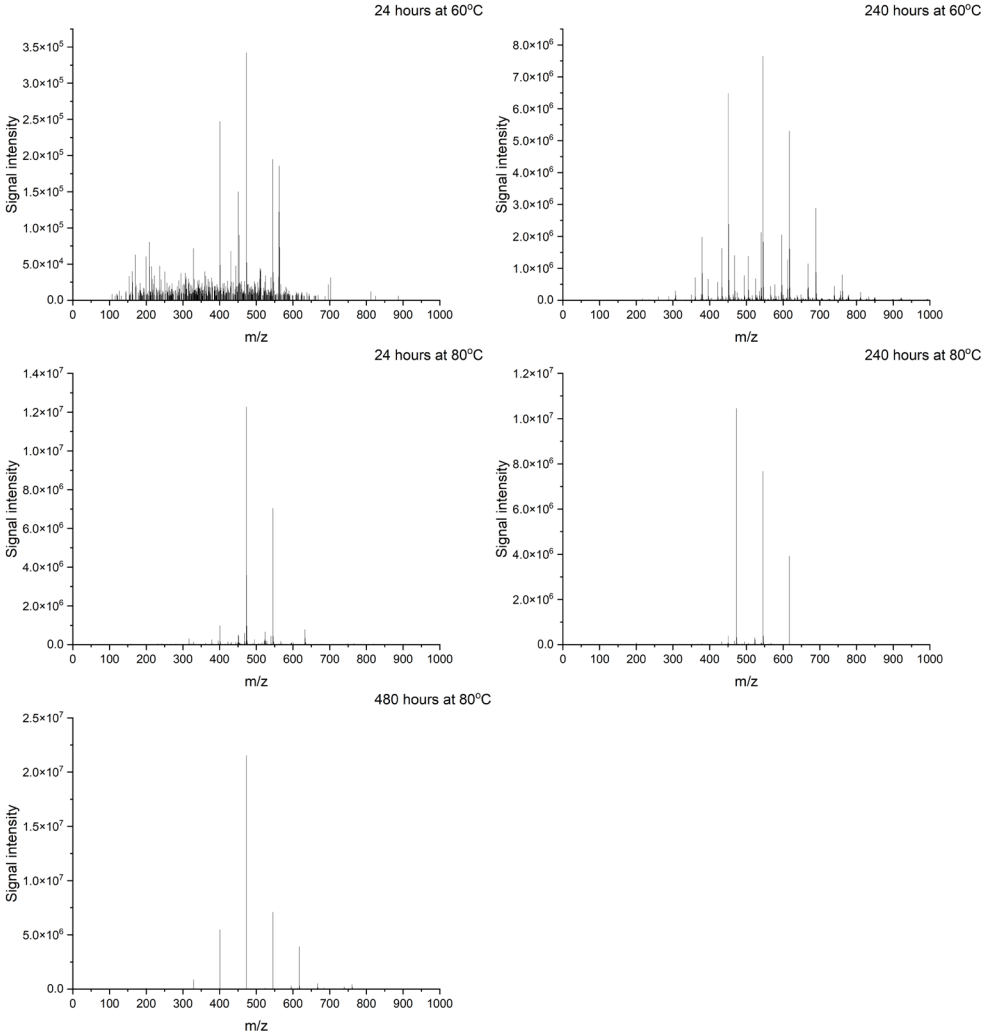

Figure 6.3 LC-MS data for PLA hydrolysis in $NaNO_3$ solution.

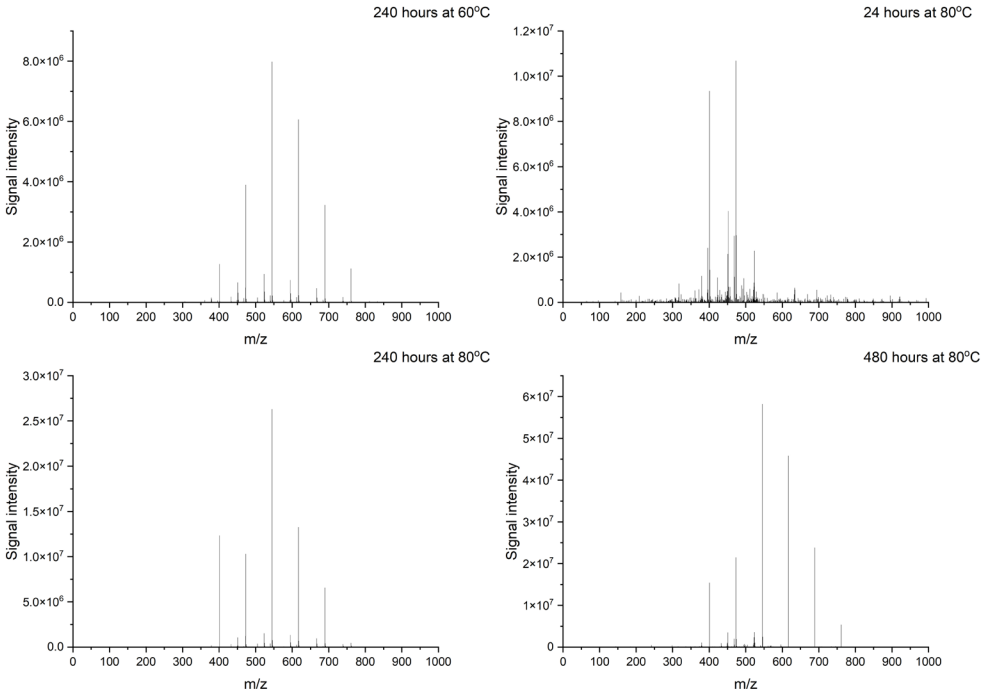

Figure 6.4 LC-MS data for PLA hydrolysis in $NaHCO_3$ solution.

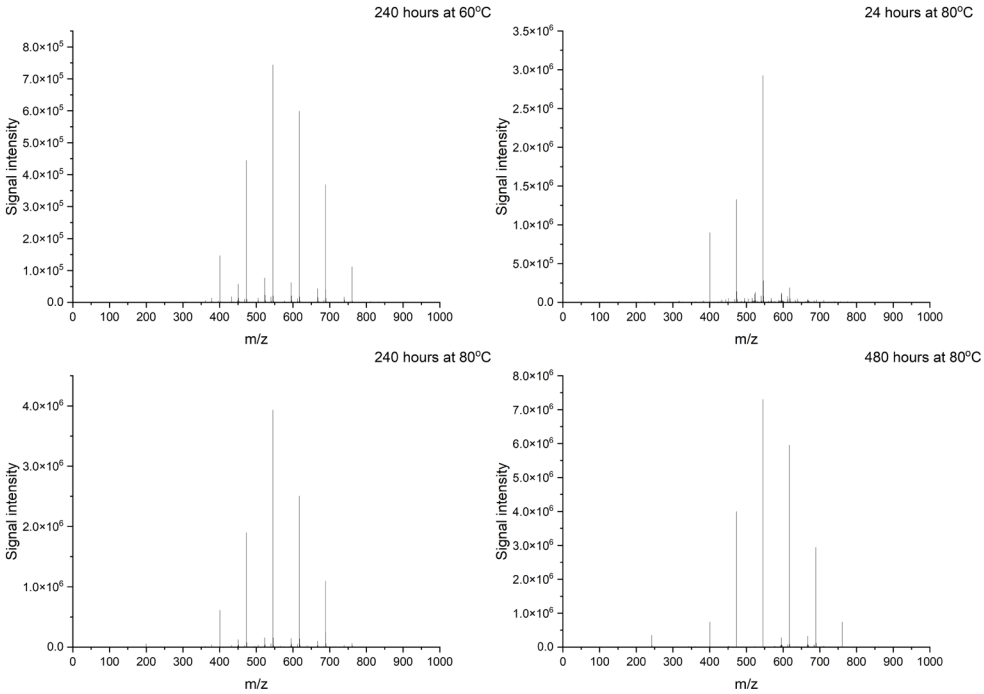

Figure 6.5 LC-MS data for PLA hydrolysis in Na_2SiO_3 solution.

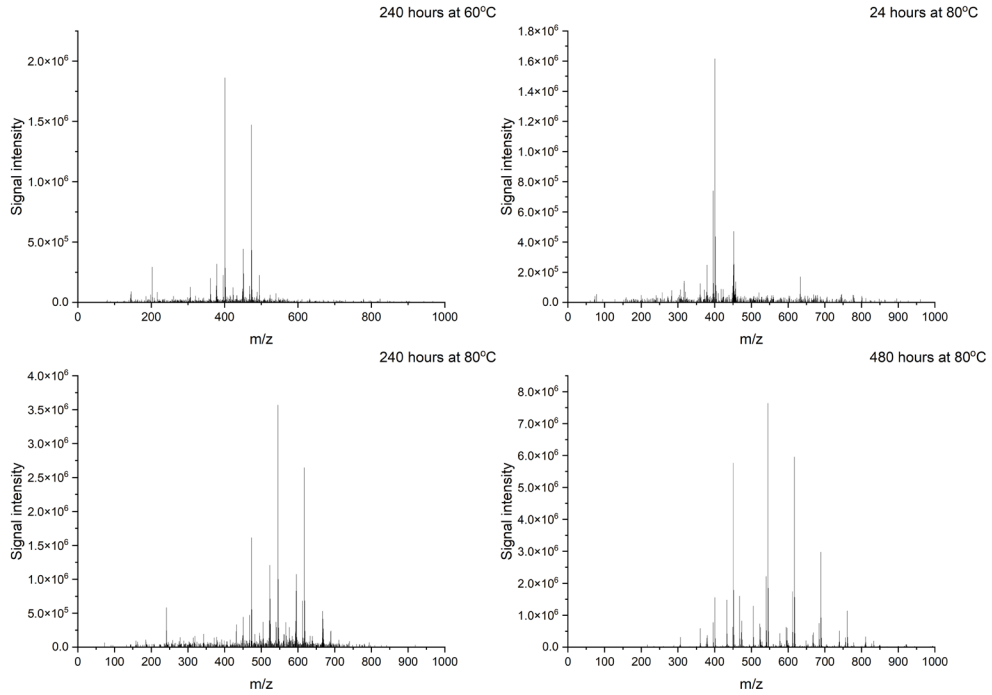

Figure 6.6 LC-MS data for PLA hydrolysis in $MgCl_2$ solution.

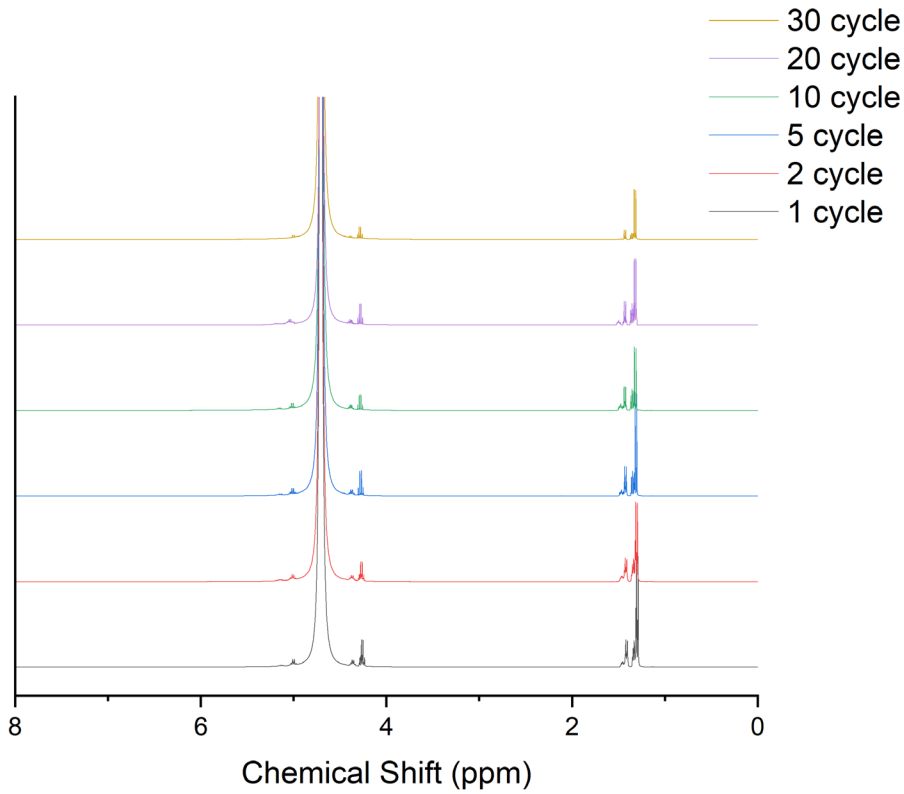

Figure 6.7 ^{1}H-NMR spectra in H_2O/D_2O (1:1) for processed LA oligomer undergoing 1, 2, 5, 10, 20 and 30 cycles in water.

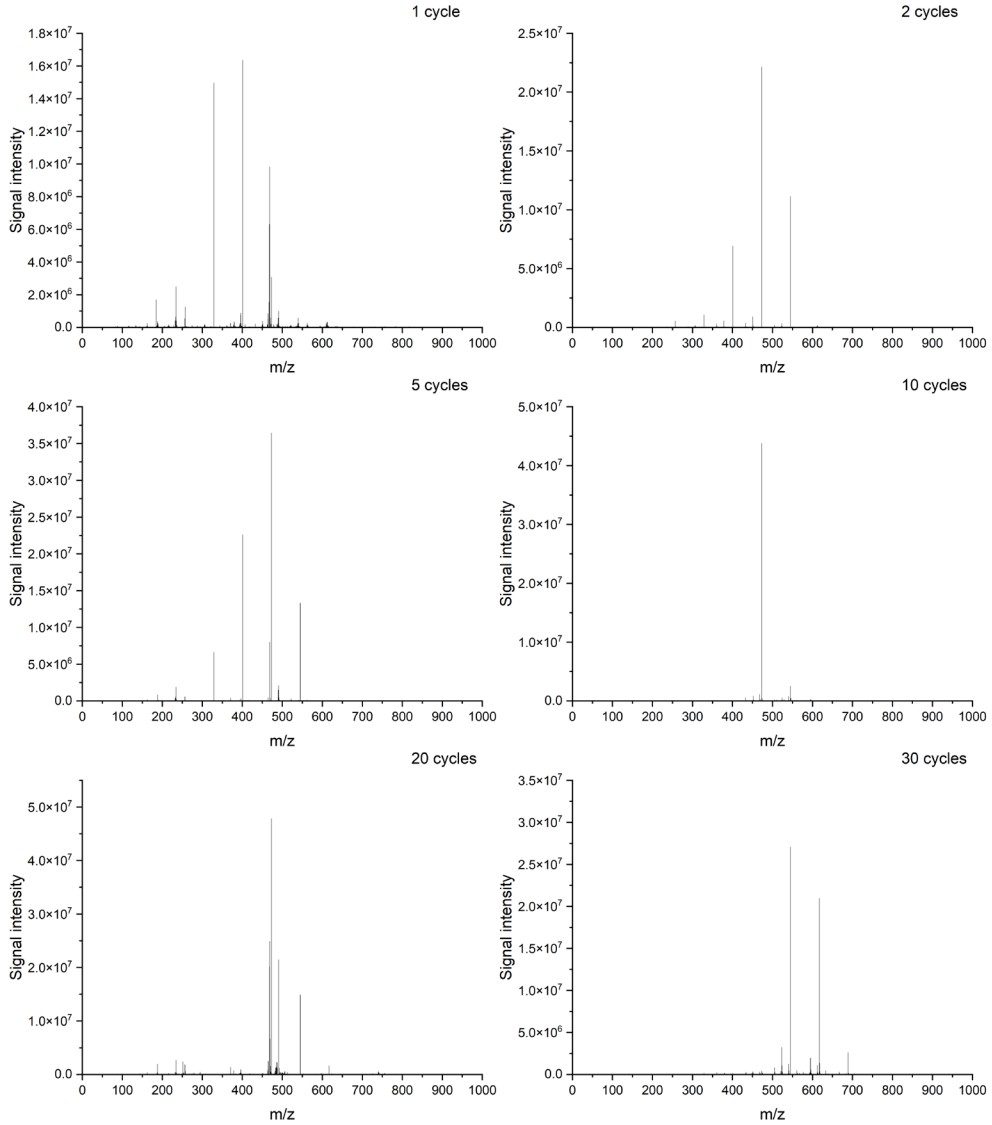

Figure 6.8 LC-MS data for LA oligomerisation in water.

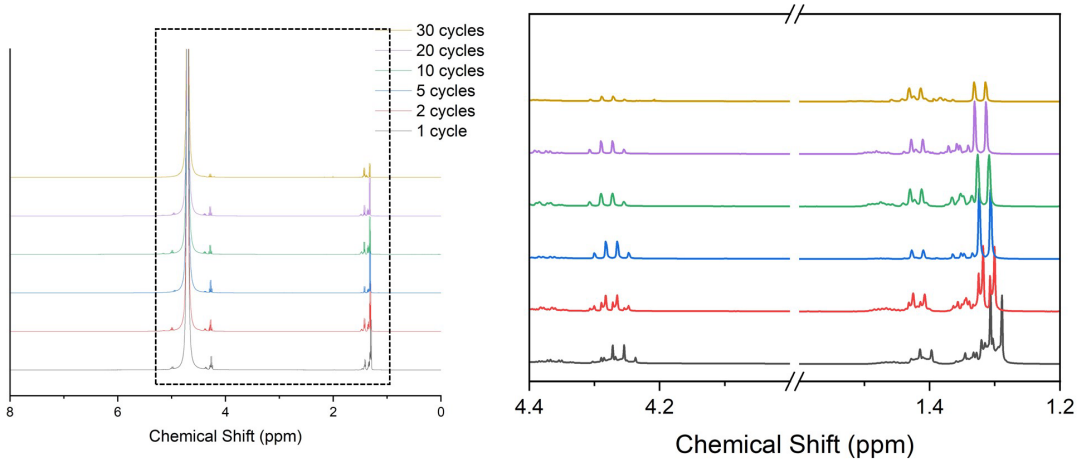

Figure 6.9 ^{1}H-NMR spectra in H_2O/D_2O (1:1) for processed LA oligomer undergoing 1, 2, 5, 10, 20 and 30 cycles in seawater and a specific zoom region with 1.2-1.6 ppm and 4.0-4.4 ppm.

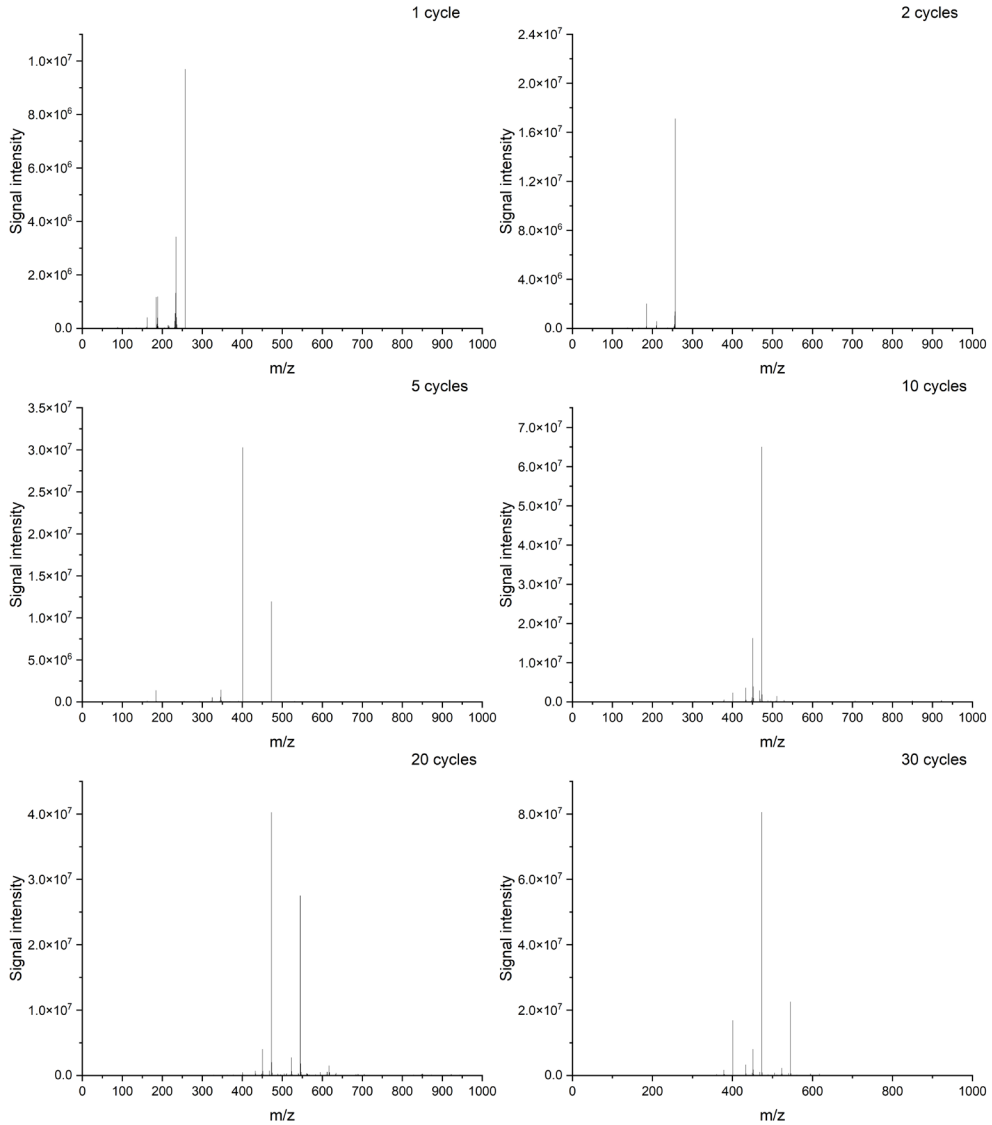

Figure 6.10 LC-MS data for LA oligomerisation in seawater.

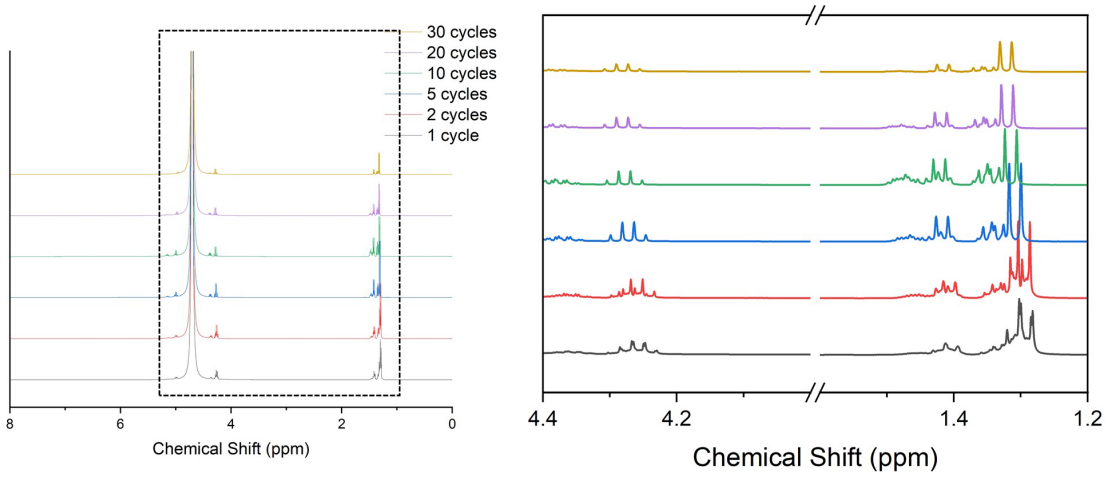

Figure 6.11 ^{1}H-NMR spectra in H_2O/D_2O (1:1) for processed LA oligomer undergoing 1, 2, 5, 10, 20 and 30 cycles in NaCl and a specific zoom region with 1.2-1.6 ppm and 4.0-4.4 ppm.

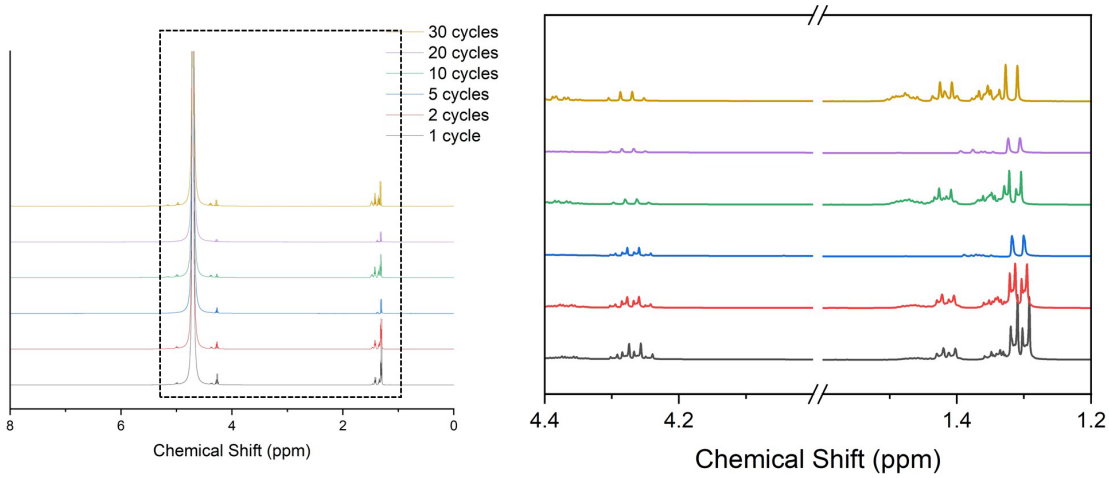

Figure 6.12 ^{1}H-NMR spectra in H_2O/D_2O (1:1) for processed LA oligomer undergoing 1, 2, 5, 10, 20 and 30 cycles in $NaNO_3$ and a specific zoom region with 1.2-1.6 ppm and 4.0-4.4 ppm.

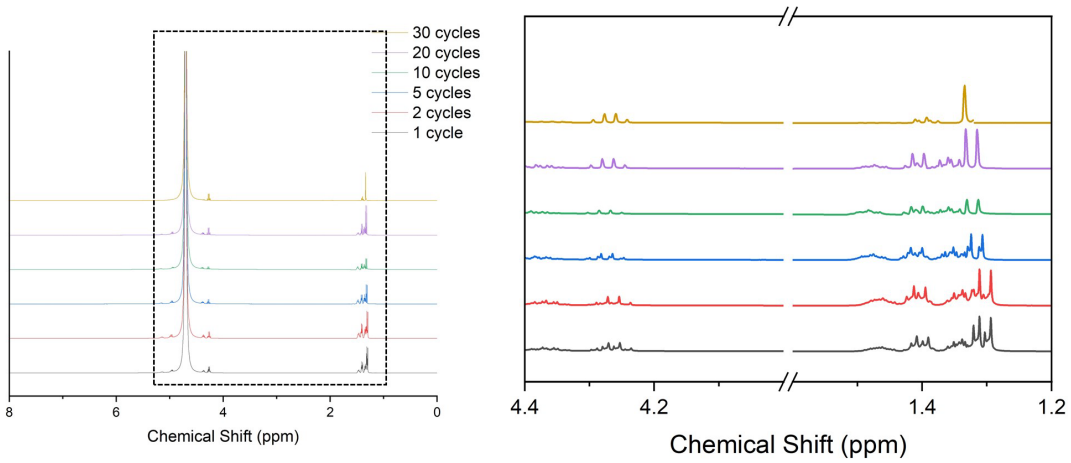

Figure 6.13 ^{1}H-NMR spectra in H_2O/D_2O (1:1) for processed LA oligomer undergoing 1, 2, 5, 10, 20 and 30 cycles in $NaHCO_3$ and a specific zoom region with 1.2-1.6 ppm and 4.0-4.4 ppm.

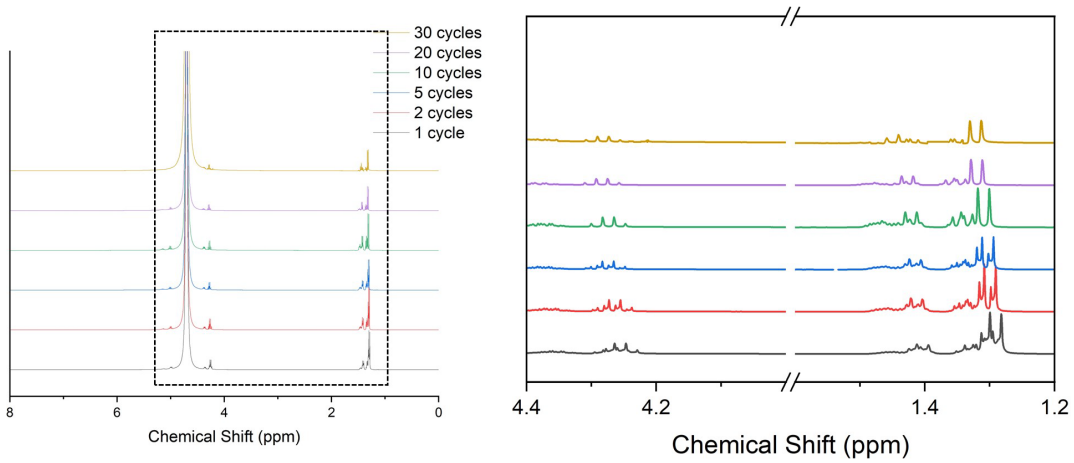

Figure 6.14 ^{1}H-NMR spectra in H_2O/D_2O (1:1) for processed LA oligomer undergoing 1, 2, 5, 10, 20 and 30 cycles in Na_2SiO_3 and a specific zoom region with 1.2-1.6 ppm and 4.0-4.4 ppm.

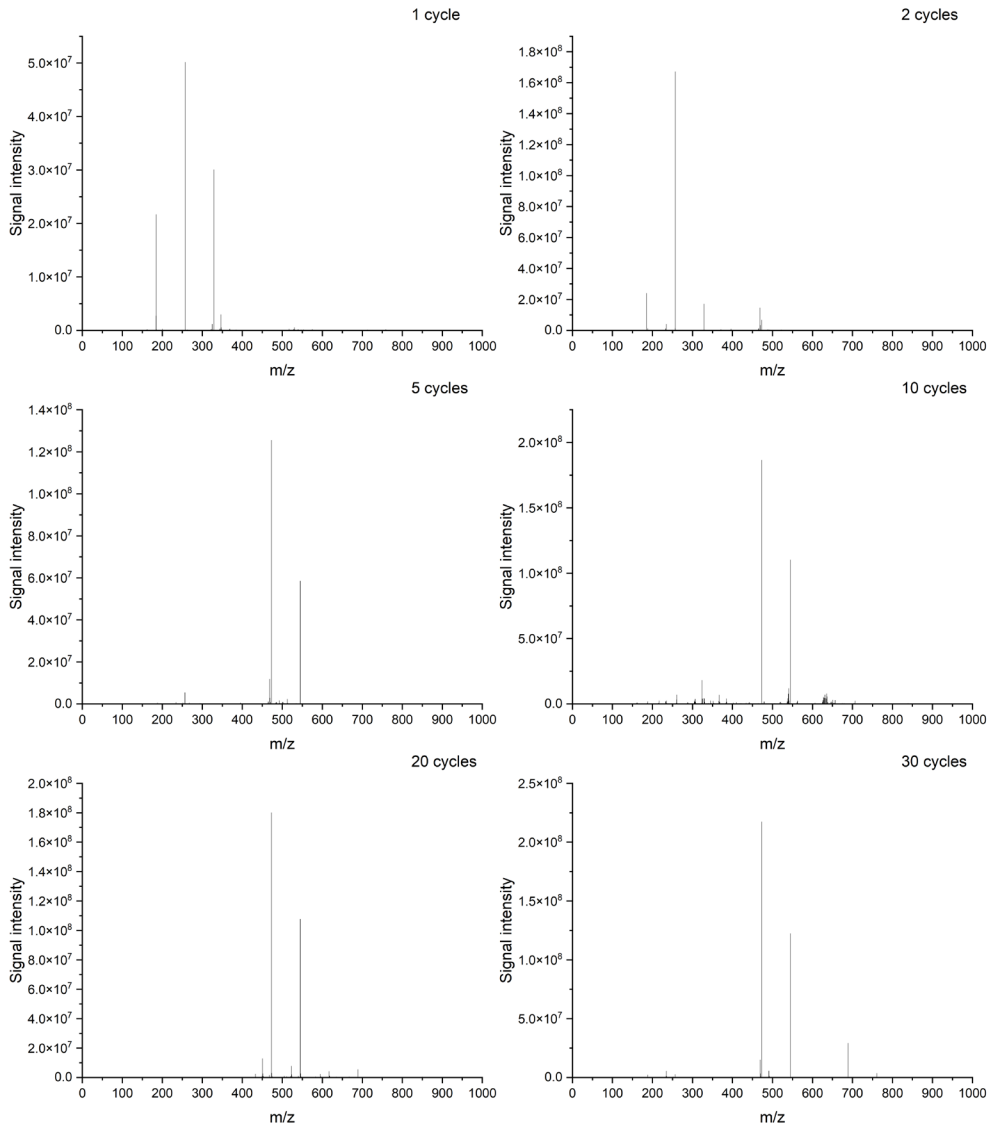

Figure 6.15 LC-MS data for LA oligomerisation in NaCl solution.

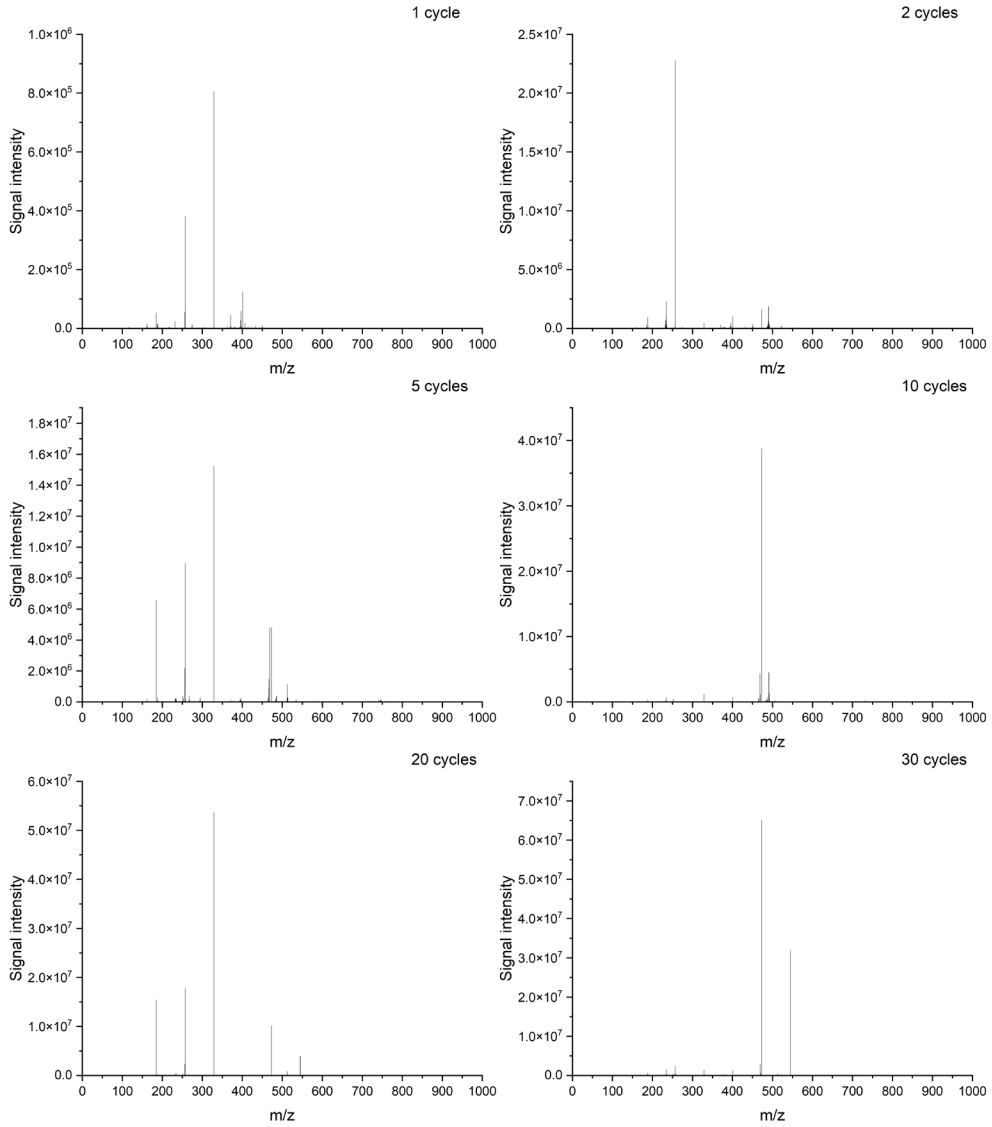

Figure 6.16 LC-MS data for LA oligomerisation in $NaNO_3$ solution.

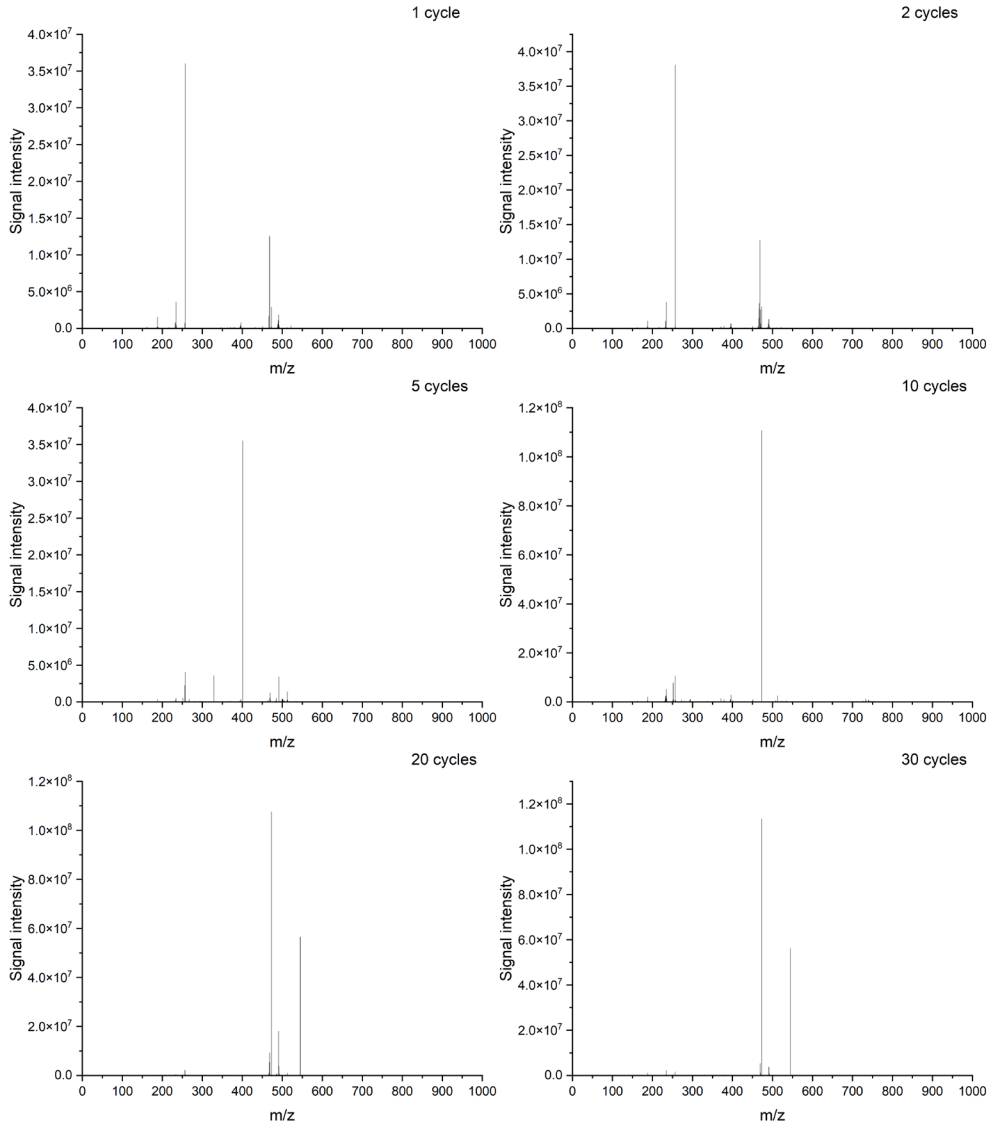

Figure 6.17 LC-MS data for LA oligomerisation in $NaHCO_3$ solution.

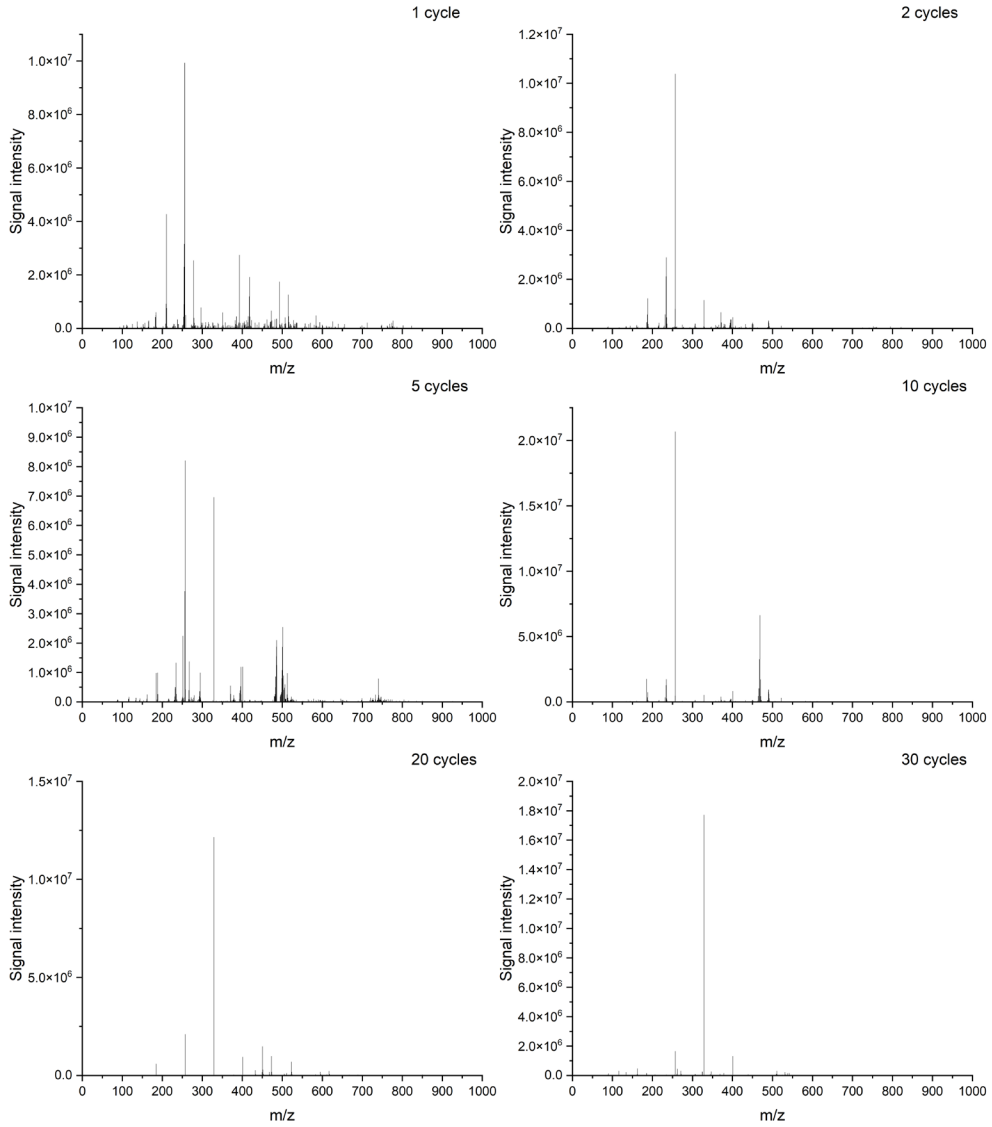

Figure 6.18 LC-MS data for LA oligomerisation in Na_2SiO_3 solution.

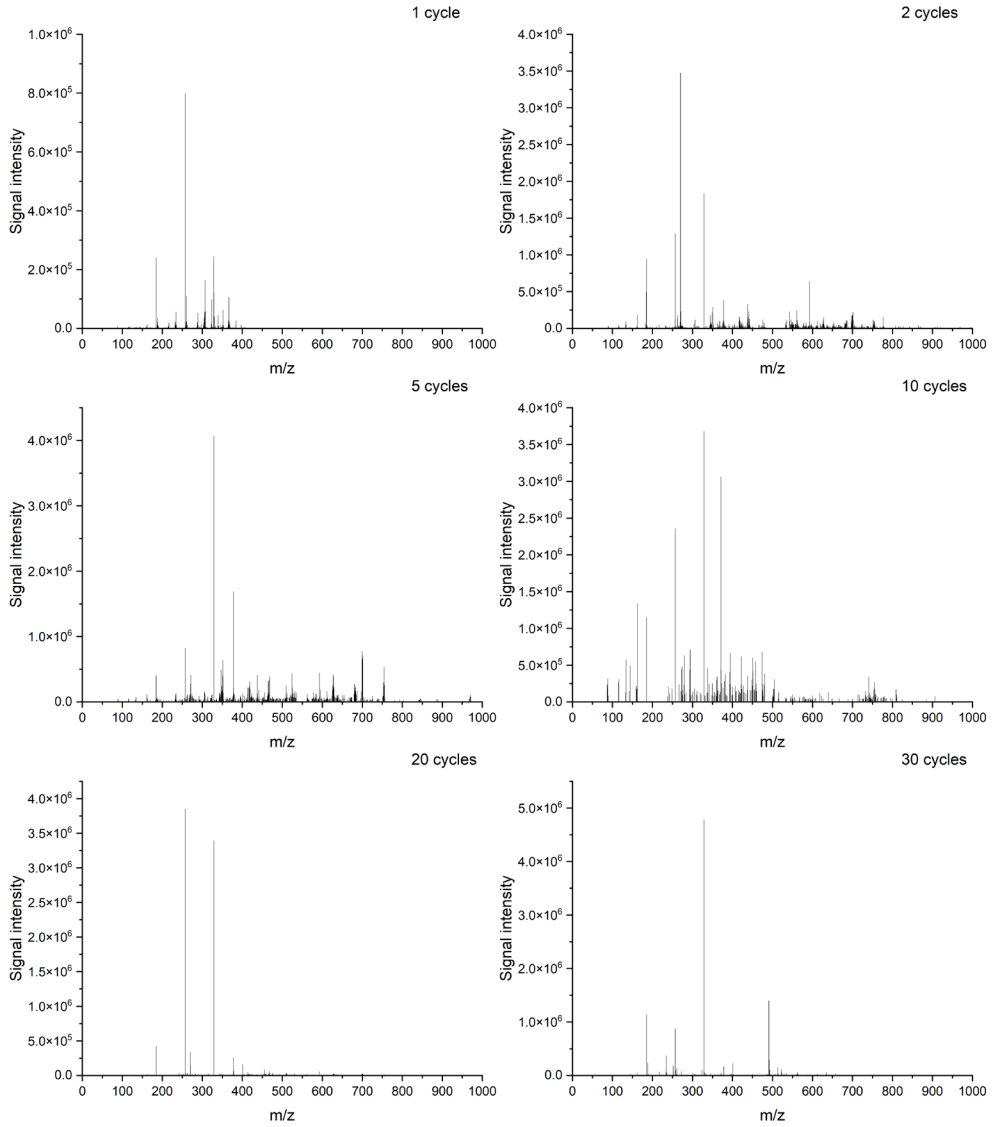

Figure 6.19 LC-MS data for LA oligomerisation in $MgCl_2$ solution.

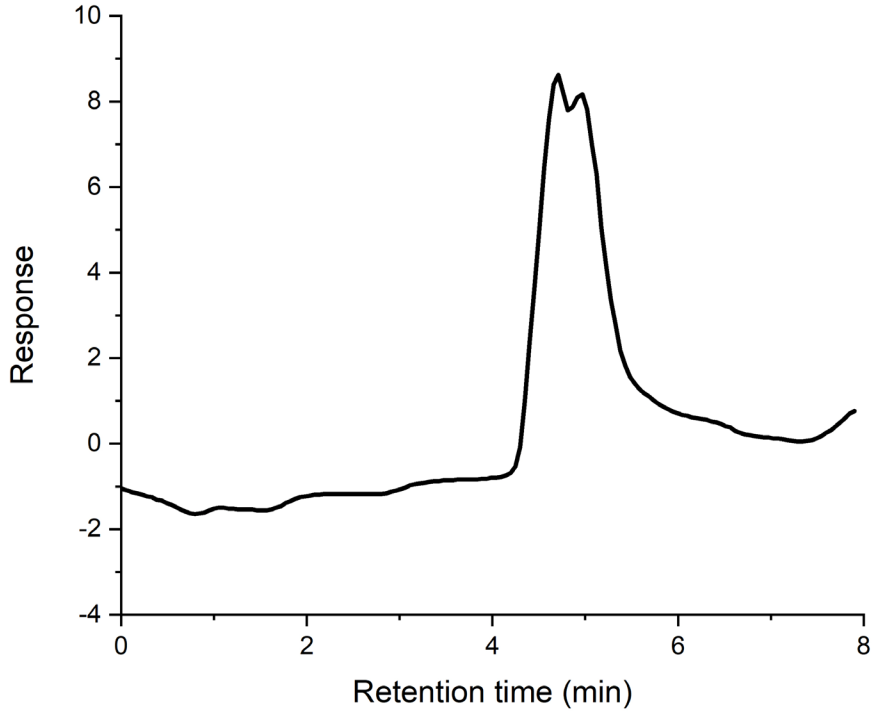

Figure 6.20 Advanced Polymer Chromatography (APC) chromatogram for PLA.

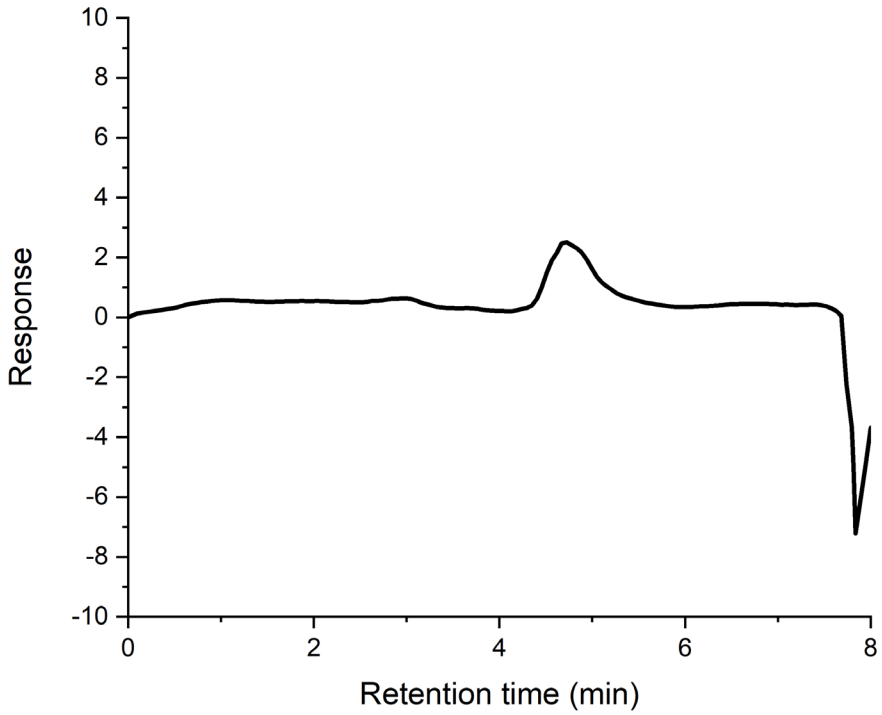

Figure 6.21 APC chromatogram for PLA hydrolysis in water after 24 hours.

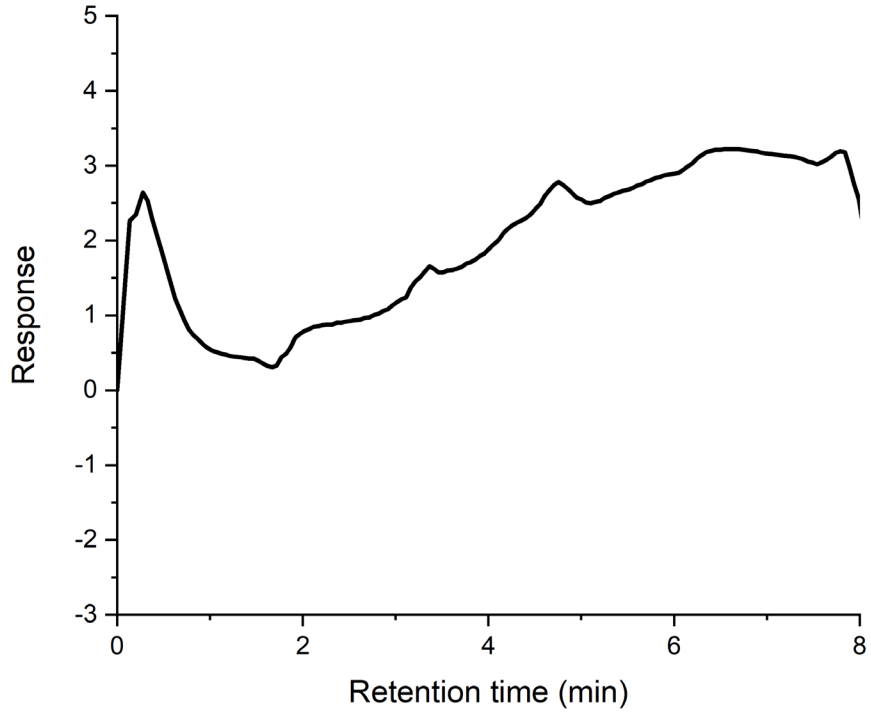

Figure 6.22 APC chromatogram for PLA hydrolysis in water after 24 hours.

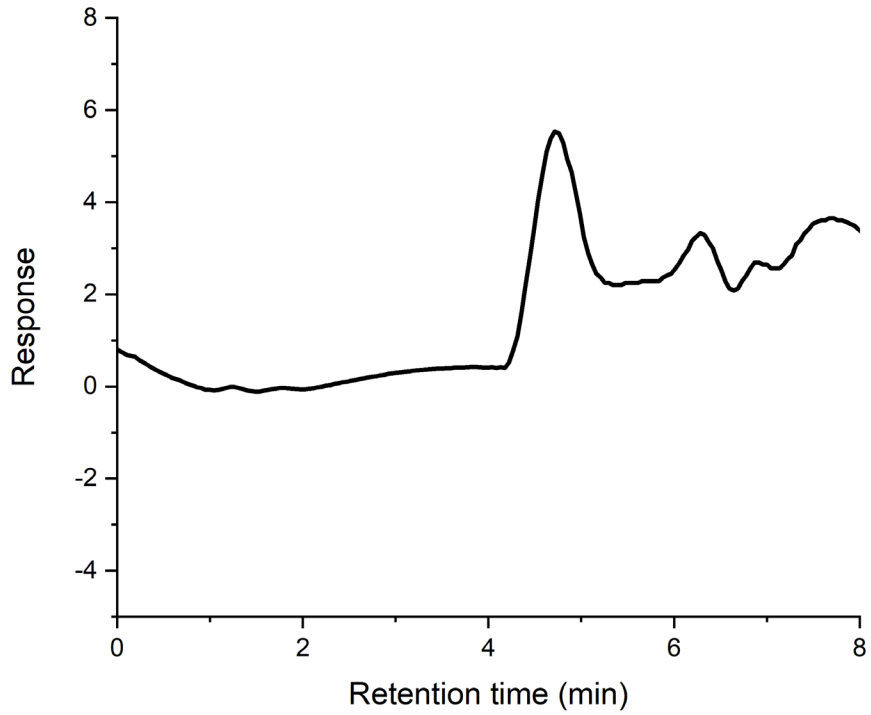

Figure 6.23 APC chromatogram for PLA hydrolysis in NaCl solution after 24 hours.

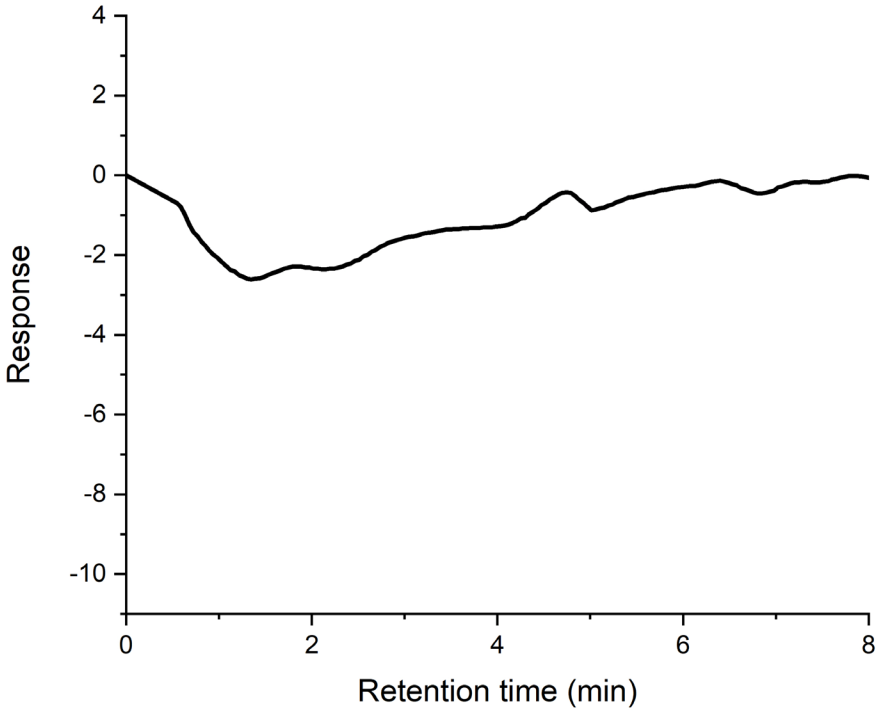

Figure 6.24 APC chromatogram for PLA hydrolysis in $NaNO_3$ solution after 24 hours.

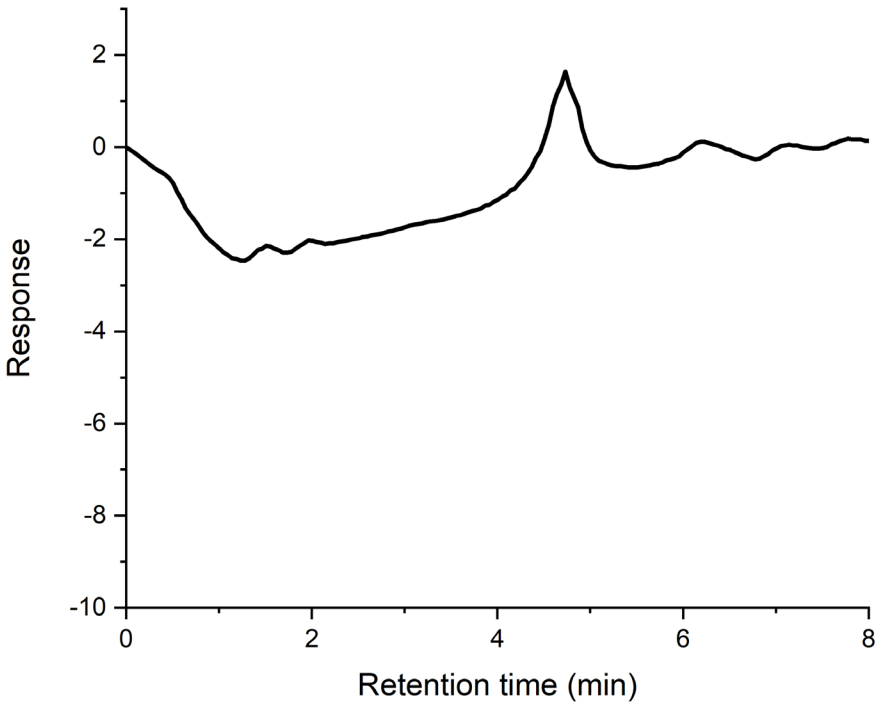

Figure 6.25 APC chromatogram for PLA hydrolysis in $NaHCO_3$ after 24 hours.

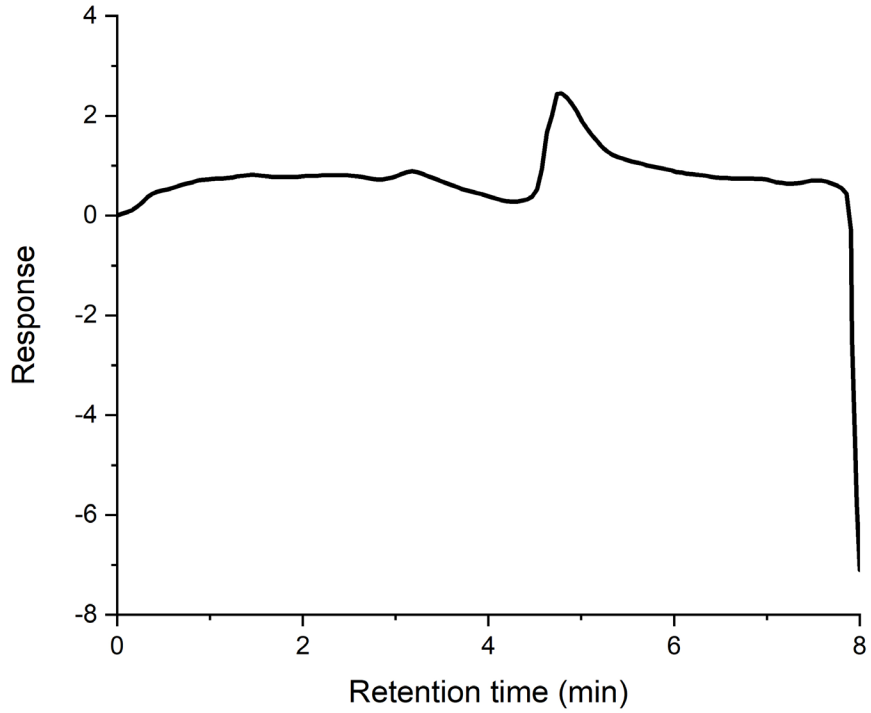

Figure 6.26 APC chromatogram for PLA hydrolysis in Na_2SiO_3 solution after 24 hours.

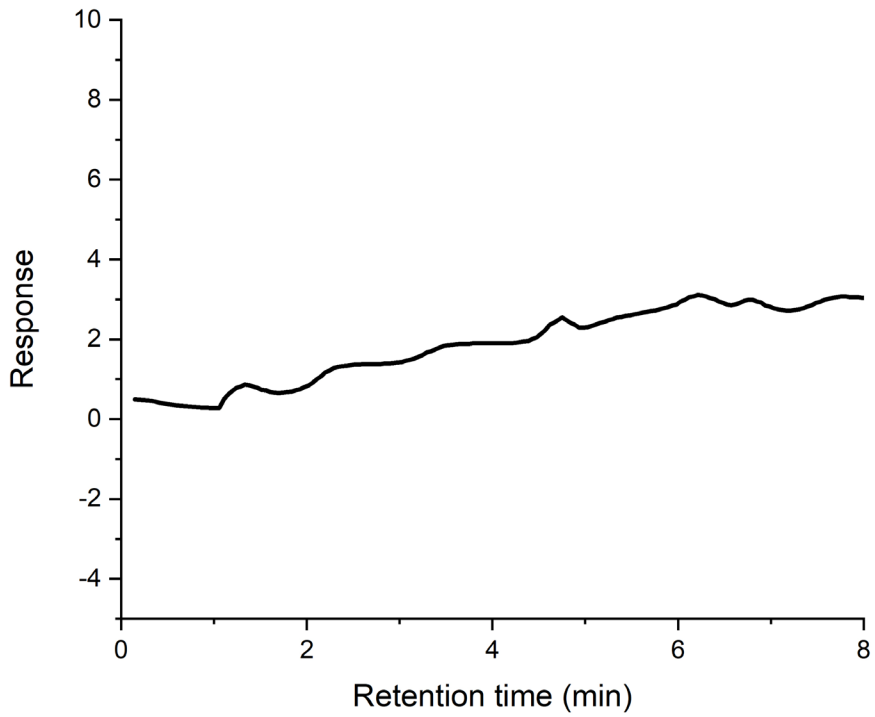

Figure 6.27 APC chromatogram for PLA hydrolysis in $MgCl_2$ solution after 24 hours.

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