

Interactions of Amphiphilic Polymers with Proteins.

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ABSTRACT

A range of polymeric glucosides were synthesised and characterised for the use as biodegradable drug delivery implants with the added ability to stabilise proteins during freeze drying. Freeze drying is a process which pharmaceutical products commonly have to undergo during formulation.

Poly(D,L-lactide) (PDLL) and poly(ϵ -caprolactone) (PCL) are recognised as suitable polymers for the controlled release of protein based drugs. These two types of polymers were synthesised at various low molecular weights by using a bifunctional initiator and tin(II) octoate as the catalyst. Low molecular weights were achieved by accurately controlling the monomer to initiator ratios. The polymers were synthesised with a low degree of polydispersity and also with hydroxyl end groups which were necessary for the next stage of the synthetic route. Poly(ethylene glycol) (PEG) which is not recognised for its controlled release properties, but has been noted to have the ability to stabilise proteins at low temperatures, was also investigated in this research. The three types of polymer were characterised using NMR, GPC and the relatively new technique, MALDI-TOF mass spectrometry. The results obtained from each technique were used to see how well the techniques compared.

Due to the ability of sugars to stabilise proteins during freeze drying, the PEG, PCL and PDLL were functionalised by end capping the polymers with D-glucose *via* the Koenigs-Knorr reaction, which yielded the respective glucosides. The glucosides were isolated and characterised.

The ability of the polymeric glucosides to stabilise proteins during freeze drying was evaluated by using the enzyme, β -galactosidase. All the polymeric glucosides exhibited the ability to stabilise β -galactosidase.

DECLARATION

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THE AUTHOR

The author of this thesis graduated from the University of Manchester in July 1996 with a BSc. (Hons.) in Chemistry with Medicinal Chemistry. Between October 1996 and October 1999 she has been engaged in full-time research at the University of Manchester, which forms the basis of this thesis.

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LIST OF ABBREVIATIONS

CL	ϵ -caprolactone
CMC	critical micelle concentration
GPC	gel permeation chromatography
DCM	dichloromethane
LDH	lactate dehydrogenase
MALDI	matrix assisted laser desorption ionisation
M_n	number-average molecular weight
M_r	relative molecular mass
MTAG	methyl tetra- <i>o</i> -acetyl- β -D-glucopyranoside
M_w	weight-average molecular weight
MWD	molecular weight distribution
NMR	nuclear magnetic resonance
ONPG	<i>o</i> -nitro- β -D-galactopyranoside
PCL	poly(ϵ -caprolactone)
PCLG	poly(ϵ -caprolactone) glucoside
PDLL	poly(D,L-lactide)
PDLLG	poly(D,L-lactide) glucoside
PEG	polyethylene glycol
PFK	phosphofructokinase
ppm	parts per million
Prep GPC	preparative gel permeation chromatography
TAG	tetra- <i>o</i> -acetyl- α -D-glucose
TAGB	tetra- <i>o</i> -acetyl- α -D-glucopyranosyl bromide

TAGC	tetra- <i>o</i> -acetyl- α -D-glucopyranosyl chloride
T _m	melting temperature
TRIS	tris(hydroxymethyl) methylamine

1. Introduction

1.1 PROTEINS AND POLYPEPTIDES

Proteins are a unique class of macromolecules in being able to specifically recognise and interact with highly diverse molecules.¹

Amino acids are the basic structural units of proteins and consist of an amino group, a carboxyl group, a hydrogen atom and a distinctive side chain known as the R group. Amino acids are linked by peptide bonds to form polypeptides. The formation of a peptide bond is shown in Figure 1-1.



Figure 1-1 Formation of a peptide bond

Polypeptides are chains of many amino acid residues and their relative molecular mass (M_r) can extend up to several thousands. Proteins are polypeptide agglomerates which have a biological function, with an M_r between several thousands and several million.

The critical determinant of a biological function of a protein is its conformation which is the three dimensional arrangement of the atoms in the molecule. In describing this three dimensional structure of proteins it is usual to refer to four separate levels of organisation. The primary structure is the number and sequence of amino acids in the polypeptide chain. The secondary structure refers to the spatial arrangements of the amino acids that are near one another in the linear chain and the tertiary structure refers to the spatial arrangement of amino acids that are far apart in the linear sequence. Proteins containing more than one polypeptide chain exhibit an additional level of

structural organisation. Each polypeptide chain in such a protein is called a subunit and the quaternary structure refers to the spatial arrangement of such subunits and the nature of their contacts.

1.2 THERAPEUTIC PROTEINS AND PEPTIDES

In recent years there have been great advances in the field of biotechnology leading to the development of therapeutic proteins and peptides.² This progress is expected to continue and escalate in the near future. Therapeutic peptides and proteins are expected to alleviate suffering in the future, for example as anticancer agents, hormones, growth factors, analgesics, anti-hypertensives and thrombolytics (anti-blood coagulation agents).³

The administration and storage of these large molecular weight polypeptides can not be performed in conventional ways as they are susceptible to proteolysis, chemical modification and denaturation. A great deal of research has been performed to investigate formulations which stabilise proteins significantly over long periods of time and the main problems that have arisen are protein aggregation, proteolytic degradation and chemical modification.

Delivery of proteins orally is not a viable option as proteins are denatured at gastric pH levels and can be degraded by proteolytic enzymes in the small intestine.⁴ Parenteral delivery of proteins and peptides is one remedy to the problem. Parenteral administration involves delivering drugs by routes other than the alimentary tract, i.e. into the body tissue at some level under the surface of the skin. This means the proteins do not have to pass any biological barriers, pharmacological levels of circulating protein

over a short period of time can be achieved and it offers a relatively facile way of administering the drug. This will be discussed in greater detail in Section 1.4.

1.3 STABILITY OF THERAPEUTIC PEPTIDES AND PROTEINS

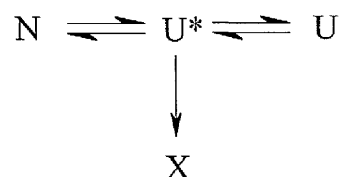
A major challenge to the formulation of peptides and proteins is to ensure the desired activity is still present even after the rigours of formulation and storage prior to administration. The instability of proteins can be classified as physical or chemical instability. Physical instability refers to a change in the secondary, tertiary or quaternary structure of the protein and includes denaturation, aggregation, precipitation and/or adsorption to surfaces. Chemical instability is the result of chemical modifications such as hydrolysis, deamidation, oxidation, disulfide exchange, β -elimination and racemization.⁵ All the above reactions can occur for a given peptide, but not all are sufficiently rapid to compromise the shelf life of a peptide. The reactions which have the most significant effect on protein stability are discussed in Section 1.3.1.

1.3.1 Physical Instability

1.3.1.1 Denaturation.

Denaturation of a protein involves the disruption of the higher ordered structure, such as the secondary or tertiary structure, which typically involves the unfolding of the protein. Denaturation can be reversible or irreversible and can be caused by many factors such as thermal stress, extremes of pH or denaturing chemicals. The unfolding of the protein can involve a sharp transition in structure from native to an unfolded state at the melting temperature. The melting temperature, T_m , is defined as the temperature where 50% of the molecules are completely unfolded. Even though unfolding involves a sharp

transition from native to unfolded state, an intermediate state can exist in some systems. The representation shown below gives a general mechanism for reversible and irreversible thermal denaturation of proteins.



where N and U are the native and unfolded states, U* is an unfolding intermediate, and X is a collection of inactive molecules, such as aggregated protein, that are kinetically and/or thermodynamically blocked from changing to N or U.⁶ If the protein cannot revert back to its native state by refolding then denaturation is considered irreversible.

1.3.1.2 Aggregation.

Protein molecules often self-associate by physical or chemical forces to form dimers, trimers, tetramers or higher oligomers. This self-association or aggregation is a common problem during formulation development and pharmaceutical processing. Although aggregation is a physical instability, it can lead to a loss of biological activity. Research into the occurrence of aggregation is crucial to developing a successful peptide or protein formulation.

1.4 FORMULATION OF THERAPEUTIC PEPTIDES AND PROTEINS.

Formulation of peptide and protein drugs is very different from the formulation of traditional drugs. As discussed in previous sections, physical and chemical degradation can occur making their formulation very complicated and difficult to generalise.

Advances in biotechnology can provide us with highly purified proteins, but these are not in their natural environment which normally contributes to their stability. This natural environment may include other proteins, carbohydrates, lipids and salts which can help to stabilise the structure.⁷ Some of the factors which are important considerations for formulation development are choice of appropriate buffer systems, the pH of the protein formulation, protein solubility, the selection of the solvent system and the use of preservatives.

There are three types of formulation used in the delivery of therapeutic proteins. Depos are small cylindrical implants which are inserted under the skin by using a wide gauge injection. They are formed by extrusion of the polymer-protein composition at temperature above the T_g . Microspheres or nanospheres are another class of formulation and are administered by injection in an aqueous diluent through a narrow gauge needle. Hydrogels are also used to deliver protein drugs and are administered *via* an injection.

Once a formulation has been identified, the process can be scaled up to meet commercial demands. It is during such a scale up and subsequent manufacture that the peptide or protein is exposed to several types of stress. These include the generation of extensive air-water interfaces due to turbulence in mixing tanks, foaming, adsorption to filters or tubing, freezing and drying stress during lyophilisation and other situations such as exposure to light, organic solvents or heavy metals.

1.4.1 Freeze Drying As A Pharmaceutical Process.

Very few proteins have sufficient stability to be marketed as solutions with a shelf life of a year or more, so the proteins are best distributed in the dry form. Since proteins are heat sensitive, such drying must be done without using elevated temperatures to prevent degradation. Freeze drying, also known as lyophilisation, is the most popular method used to accomplish this. In addition to preventing degradation, freeze drying also results in a product with a very high specific surface area which results in good dissolution during product reconstitution. Another advantage of freeze drying is that the process can be carried out aseptically resulting in a sterile product. This allows the formulation of injectible products which is the most common method of administration.

Freeze drying is a technique which involves freezing a solution of the drug, and removing the ice crystals that form by converting them straight into water vapour under vacuum, a process known as sublimation.⁸ A typical freeze-dryer consists of a vacuum pump, a condenser, a refrigeration system and a site for placing the sample such as a chamber or receptacles for flasks.

A typical freeze drying process comprises of three stages, (i) freezing the sample, followed by (ii) primary drying and finally (iii) secondary drying. During the first stage, the water and solutes are completely frozen, usually by submersion of the flask in liquid nitrogen. After freezing, the pressure in the sample chamber is reduced and the temperature of the drying chamber is kept at room temperature or slightly elevated to provide energy for primary drying. Primary drying removes water under vacuum through sublimation. The driving force for sublimation is the difference in vapour

pressure of water within the system. Once the bulk of the water has been removed, the remaining unfrozen water actually bound to the product is removed through secondary drying.

1.4.2 Protein Denaturation During Lyophilisation.

On the face of it freeze drying would seem to be the ideal solution to problems with formulating proteins as the proteins can be rendered dry without heat. Unfortunately, freeze drying causes conformational instability making the protein susceptible to degradation in the solid state. This is because once the protective coat of water is removed from the protein, it exposes the charged groups on its surface which can react chemically with other species. Two different forms of stress are subjected to the protein during freeze drying. The mechanism of protein denaturation is different for both forms of stress, the first stress is due to the freezing and the second is due to the drying process.⁹

Since the mechanisms of protein denaturation during freezing and drying are different, it follows that excipients (additives) may stabilise the protein during freezing while others will stabilise during drying. The excipients that give stability to proteins during freezing are called cryoprotectants and those that help against drying are called lyoprotectants. The excipients display these different methods of protection as their function depends on whether the protein molecules are in the dried, solution or frozen state.

1.4.2.1 Cryoprotectants

Stabilisation of proteins in the frozen state by cryoprotective excipients is based on a mechanism known as preferential exclusion or preferential hydration.¹⁰ This can be described as the cryoprotectant stabilising the protein by being preferentially excluded from the surface of the proteins. This depletion of excipient in the domain of the protein results in enhancement of hydration of the protein.

This can be explained in greater detail if we discuss the two state equilibrium between the native (N) and denatured (X) states of the protein, as shown in Figure 1.2.

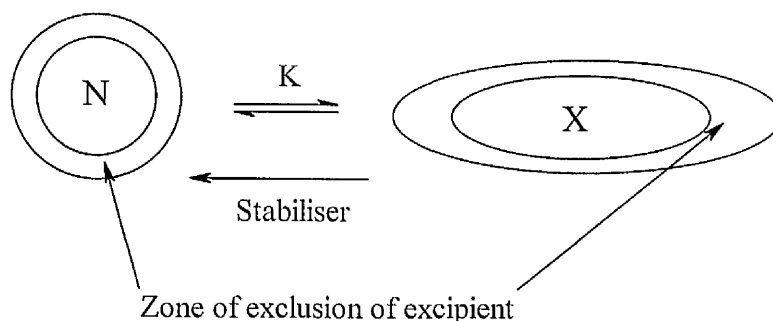


Figure 1-2 Two states of protein conformation

Adding the stabilising excipients moves the equilibrium to the left, making the equilibrium constant dependent on the concentration of excipient. The reason for this is that the added excipient increases the chemical potential of the protein, leading to an increase in the free energy of the system.^{11,12} This creates a thermodynamically unfavourable situation which means the protein will not unfold since that would increase the contact surface area between protein and excipient. The native structure of the protein is favoured, resulting in stabilisation.

In 1961, Shikama and Yamazaki¹³ gave the first quantification of denaturation of an isolated enzyme, catalase, during freeze-thawing. In their study they demonstrated that a range of stabilising solutes added to catalase solutions could increase, to varying degrees, the recovery of activity after thawing. The types of compound they found to exhibit cryoprotective properties were sugars, polyols, other proteins and certain salts. In subsequent years there has been a great deal of research into the effectiveness of stabilising excipients, usually using sugars and polyols as cryoprotectants.

Carpenter and Crowe¹⁴ tested the ability of 28 different compounds, including sugars, polyols, amino acids, methylamines and lyotropic salts, that were added to lactate dehydrogenase (LDH) to protect it from damage during freeze-thawing. All of the compounds tested, except NaCl, preserved the enzyme activity to varying degrees. The common characteristic of the additive was that it was preferentially excluded from the protein surface in aqueous solution. The sugars that they tested included glucose and sucrose and when LDH was frozen in their presence, greater than 60 and 80%, respectively, of the initial activity was recovered. As well as testing polyols, polyethylene glycol (PEG) with a molecular weight of 600 was investigated for cryoprotective properties and was found to stabilise LDH with almost no loss of activity.

Carpenter and colleagues^{15,16} have shown that combinations of certain divalent cations (e.g. Zn^{2+}) and organic solutes (e.g. sugars, polyhydric alcohols and amino acids) can provide synergistic protection for labile enzymes. They demonstrated that the full activity of phosphofructokinase (PFK) could be recovered when frozen in the presence of 0.6 mM zinc sulphate and 5 mM trehalose. With either additive alone, they claimed that no activity was measurable at the respective concentrations after freeze-thawing.

This phenomenon could have practical advantages in protein formulation since reduced amounts of stabiliser could be used.¹⁷

To investigate the structure of proteins in ice and the nature of their loss of activity, Strambini and Gabellieri employed the phosphorescence emission properties of the amino acid, tryptophan.¹⁸ This technique was used as an intrinsic probe to disclose conformational changes in the protein in response to variation in its environment. It was concluded from this work that freezing alters the native fold of the protein due to adsorption onto the surface of the ice. The consequence of adding cryoprotectants was also observed and their stabilisation effects were proposed to be due to decreasing the adsorption of the protein to the ice, as well as preferential hydration and the lowering of the freezing temperature.

Polyethylene glycol is believed to stabilise the proteins by steric exclusion from the protein molecules.^{19,20} This speculation is based on the observation that the degree of hydration increases with the increase in PEG's molecular weight. Only the water can penetrate the protein structure thus hydrating and stabilising the protein.

1.4.2.2 Lyoprotectants

Lyoprotectants stabilise proteins by binding to the dried protein and behave as a "water substitute" when the hydration shell of the protein is removed. Binding to the protein is usually achieved through hydrogen bonding. For this to occur effectively the lyoprotectant must be in an amorphous form. Amorphous excipients stabilise a protein in the solid state by altering the glass-transition temperature which affects the reactant mobility and protein flexibility. Amorphous excipients also reduce the effective

concentration of the protein in the solid which reduces the rate of intermolecular decomposition pathways in the presence of moisture. Also, the amorphous excipients are often hygroscopic and may act to “buffer” any water that is absorbed in the solid state.

Sugars have gained particular interest as lyoprotectants over the past few years and the inspiration for this comes from nature. A cryptobiont is an organism which displays the ability to survive in a suspended state of animation, under extreme conditions, such as desert or arctic climates.²¹ During drought some cryptobiotic desert plants and organisms can lose up to 99 per cent of their water and still manage to keep their cells intact and this is due to a disaccharide called trehalose. This forms a glassy matrix when the plant dries out, preserving internal structures until the next rainfall when the plant miraculously returns to life. The sugar molecules protect the biologically active molecules by supporting the active structure preventing it from denaturing when the water molecules are removed. Unlike pure water, a sugar solution stays liquid well below its expected freezing point. The water in the solution then begins to form ice crystals, increasing the proportion of sugar to water left in the liquid. Further cooling creates a thick viscous liquid in which molecular motion is slowed down. Eventually the syrup solidifies (vitrifies), but without ever crystallising.²² This under-cooled liquid state is called a glass (the molecular motion is zero) which keeps the biologically active molecule in a state of suspended animation.

To gain better understanding of how trehalose and other sugars protect proteins in the solid state various studies have been performed. Carpenter and Crowe used Fourier

transform infrared spectroscopy to characterise the interaction of stabilising carbohydrates with dried proteins.²³ Their work showed that replacement of water lost during lyophilisation by hydrogen bonding of the carbohydrate's hydroxyl groups to the protein's polar groups was responsible for preventing dehydration-induced inactivation.

Hellman *et al.*²⁴ discovered that polymers such as polyvinylpyrrolidone, which are sterically hindered from hydrogen bonding to dried proteins, can protect multimeric enzymes during freeze-drying by an alternative mechanism. Anchordoquy and Carpenter²⁵ investigated this further to suggest that the polymer stabilised the protein by firstly maintaining the quaternary structure in the frozen state during primary drying and by preventing the formation of an acidic environment through salt precipitation.

1.4.2.3 Stabilisation using a Combination of Cryoprotectants and Lyoprotectants.

Now that it is well established that proteins need protecting against both freezing and dehydration stresses during lyophilisation, a system that provides both cryoprotective and lyoprotective properties is needed. Carpenter *et al.*^{26,27} provided the first example of the stabilisation of proteins during lyophilisation through separate and specific treatments of the fundamentally different stresses of freezing and dehydration. They found that almost the full enzyme activity of phosphofructokinase and lactate dehydrogenase was recovered after freeze-drying and rehydration in the presence of both a sugar (such as glucose or trehalose) and PEG. PEG stabilises the proteins during freezing due to preferential exclusion from the protein surface. The sugar protects the

protein during dehydration by hydrogen bonding to the dried protein. In effect it behaves as a water substitute.

1.5 BIODEGRADABLE POLYMERS FOR THE DELIVERY OF PROTEINS AND PEPTIDES.

The use of biodegradable polymer systems for controlled release of therapeutic proteins has created great interest over the past few decades.^{28,29} This involves implanting into the patient a reservoir of the protein drug embedded into a biodegradable polymeric carrier material. The polymer is degraded in the body by hydrolysis or enzymatic digestion resulting in the controlled release of the protein.³⁰

Biodegradable polymeric controlled release drug delivery systems have several advantages compared to conventional drug therapies. Improved patient compliance is observed due to there being one or very few administrations to give months of medication as opposed to maybe daily or more regular dispensations. Injections can cause discomfort, instil fear in some people and require trained people to administer them, so for the patient to have only one administration of the drug is advantageous.³¹ The peaks and troughs of drug plasma levels associated with conventional injections is avoided and a constant level is achieved. The localised delivery of the drug to a particular body compartment or cell type is much more advantageous as this lowers the systemic drug level. The implants give better protection of the drugs that are rapidly degraded in the body and this gives improved drug efficacy. As the polymers utilised are biodegradable it means surgical removal of the implant once the drug supply is depleted is avoided.

A polymer which is to be used in a biodegradable drug delivery system must be tailor made to meet a number of specific requirements.³² The polymer should be soft and pliable which means little or no crystallinity, as this minimises tissue irritation. Tissue irritation can also be reduced if the polymer surface is smooth. The glass transition temperature should not be higher than body temperature as the polymer needs to be in the rubbery state to give higher permeability. It is important that the polymer is compatible and unreactive towards the drug and the polymer itself and its degradation products are biocompatible. Biocompatible has been defined as 'the ability of a material to perform with an appropriate host response in a specific application'.³³ The concept of biocompatibility might be extended to consider the material's direct interaction with the immunological system of the host.³⁴ By summarising these two definitions, for a material to be biocompatible, it has to be able to perform the desired requirement without causing any ill effects to the host. The polymer is preferably obtained from inexpensive, commercially available starting materials by a simple polymerisation process. Most importantly, the permeability to the drug and the rate of biodegradation should be compatible with the application in mind.

Each type of delivery system needs to be tested independently with each protein in order to evaluate the specific interactions. It is these interactions along with the degradation of the polymer which will determine the protein release rate. Once an ideal protein-polymer system has been developed, there are still problems to face with regards to maintaining the integrity and activity of the incorporated proteins. During the processing of the implants the proteins may be exposed to extreme stress, heat, shear forces, pH extremes, organic solvents, freezing and drying. When the biodegradable polymer drug delivery systems are administered, the incorporated proteins may become

hydrated at relatively high concentrations for prolonged periods of time. Proteins in this environment are susceptible to denaturation and aggregation.³⁵ Also when a polymer begins to degrade following administration, a highly concentrated microenvironment is created from the released protein and polymer breakdown products in and around the implants. Proteins may be susceptible to hydrolytic degradation and chemical modification in such an environment. The drug delivery systems also have to be able to withstand prolonged storage prior to administration. The proteins may undergo reversible or irreversible adsorption to polymeric materials used which can affect the drug delivery rate.

There are several ways in which polymeric delivery systems can be modified to stabilise proteins and reduce denaturation. As discussed already in Section 1.4.2, the addition of stabilising agents prevents protein aggregation or adsorption to polymer surfaces. Excipients can be incorporated to increase hydration of the system and enhance protein diffusion and polymer degradation. The modification of the protein or polymer with water soluble polymers can also be performed to prevent protein aggregation and adsorption.

1.6 DEGRADATION MECHANISMS OF BIODEGRADABLE POLYMERS.

Biodegradable polymers as vehicles for drug delivery are very attractive because of the lack of surgery to remove the implant after depletion of the drug supply.³⁶ Polymers commonly used for this purpose can be natural such as polysaccharides or polypeptides, or synthetic such as polyesters, polyamides, poly(ortho esters) or polyanhydrides. The

drug may be dissolved or dispersed uniformly throughout the polymer matrix; incorporated within an erodable reservoir or the drug may be an integral part of the polymeric chain. So the drug may be released either by degradation of the polymer matrix or by hydrolytic cleavage of a polymer-drug bond.

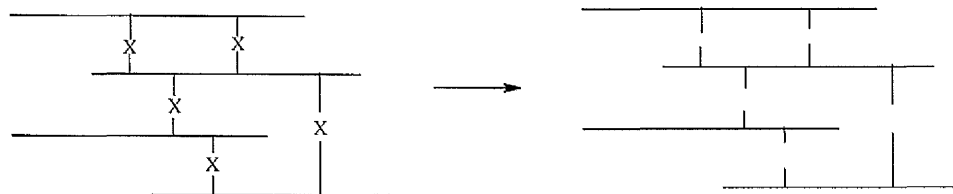
Many degradable systems such as polyesters have labile bonds that are hydrolysed and the polymeric chain breaks down to oligomers that are easily removed by the body. Degradation can also occur by incorporation of bonds that can be cleaved by proteolytic enzymes at the site of delivery or by inclusion of polymeric chains with specific side groups that undergo chemical modification which subsequently cause dissolution of the initially insoluble polymer.

The degradation mechanisms can be described both physically and chemically. Chemical degradation can be divided into three types of mechanism as has been described and quoted in several publications by Heller,³⁷ and the physical degradation has two sub-divisions.

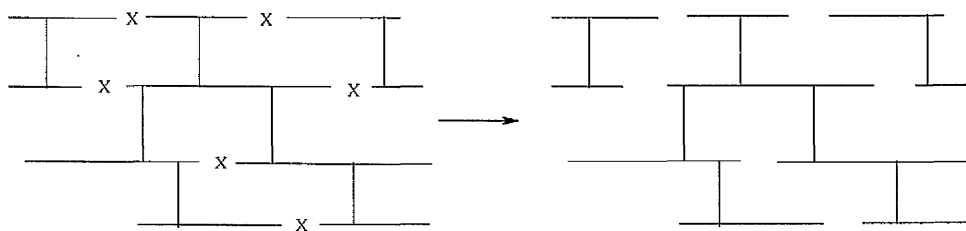
1.6.1 Chemical Erosion.

Mechanism I

Mechanism I demonstrates the mechanism of degradation of water-soluble macromolecules that are cross-linked to form a three-dimensional network and it is these cross links that cause the matrix to be insoluble. Degradation of these systems can occur at the cross links to form soluble backbone polymeric chains as shown in Mechanism IA below.

Mechanism IA

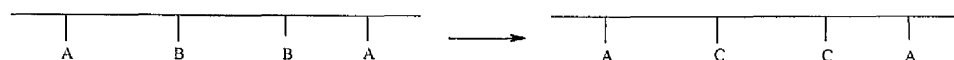
Degradation can also occur by cleavage of the main chain to form water-soluble fragments as shown in mechanism IB.

Mechanism IB

Hydrogels are an example of controlled release system which degrade *via* Mechanism 1. The degree of cross-linking controls the extent to which the polymer can swell and so hence controls the rate of release.

Mechanism II

Mechanism II describes the dissolution of water-insoluble polymers with side groups that are converted to water-soluble polymers as a result of ionisation, protonation or hydrolysis of the groups. The polymer remains intact during the release of the drug and there is little change to molecular weight. The following diagram describes this process.

Mechanism II

A represents a hydrophobic substituent

B \rightarrow C represents hydrolysis, ionisation or protonation.

Examples of polymers which display this type of degradation are cellulose acetate derivatives and maleic anhydride.

Mechanism III

This mechanism demonstrates the degradation of insoluble polymers with labile bonds. Hydrolysis of these labile bonds causes scission of the polymer backbone which forms low molecular weight, water soluble polymers.

Mechanism III



Polymers which undergo this type of degradation include, polyesters such as polylactides, polyglycolides and poly(ϵ -caprolactone), polyamides and polyanhydrides.

1.6.2 Physical Degradation

Physical degradation can be classed as either heterogeneous or homogeneous. Heterogeneous degradation which is also called surface erosion, involves erosion of the

polymer at its surface whilst maintaining its physical integrity. For these systems, the drug release kinetics are predictable and zero-order release kinetics are obtainable. This desirable form of degradation is the less dominant with most biodegradable polymers, most polymers undergo homogeneous degradation. This mechanism involves hydrolysis throughout the whole matrix at a constant rate and the polymers exhibiting this degradation tend to be more hydrophilic than those exhibiting surface erosion.

Both heterogeneous and homogeneous degradation are the extreme cases, and most biodegradable drug delivery systems are a combination of both processes. Other factors apart from the hydrophobicity or hydrophilicity that can govern the rate of degradation are polymer morphology, structural sequences and degree of crystallinity. The addition of the drug also affects these mentioned properties, so each polymer/drug system must be studied individually, so it can be seen that all factors contribute to the type and rate of degradation.

1.7 POLYESTERS AS BIODEGRADABLE POLYMERS

Polyesters such as polylactides, polyglycolides and their copolymers, and poly(ϵ -caprolactone) are among the most extensively studied and documented biodegradable polymers. The degradation mechanism of the polyesters seems to be a homogenous process. The relative rate of degradation and hence rate of release of the drug depends on the bond energy of the ester and the ratio of monomers if it is a copolymer.

The research undertaken for this thesis has mainly involved poly(ϵ -caprolactone) (PCL) and poly(D,L-lactide) (PDLL) and these two polymers are described in more detail in the next two sections.

1.7.1 Properties and Characteristics of Polylactides

The most widely investigated polymers for the purpose of biodegradable polymer drug delivery vehicles are the aliphatic polyesters based on lactic and glycolic acids. Figure 1-3 shows the structures of these two polymers and the monomers they are synthesised from *via* a ring-opening polymerisation.

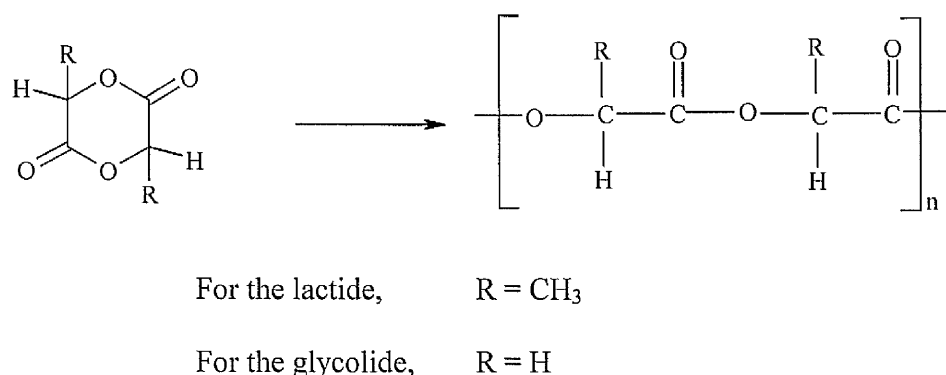


Figure 1-3 Structures of Polylactide and Polyglycolide and their respective monomers

The homo- and copolymers derived from these two monomers have received considerable attention since 1973 as materials for drug delivery. The properties of these polymers which make them very attractive as drug delivery excipients are biocompatibility, predictability of biodegradation kinetics, ease of fabrication and regulatory approval in commercial suture application. For the wide applications desired by controlled drug delivery, it is necessary that a range of rate and durations of drug release be achievable. A broad array of performance characteristics with the polylactides may be obtained by careful manipulation of four main variables:- monomer

stereochemistry, comonomer ratio, polymer chain linearity and polymer molecular weight.³⁰

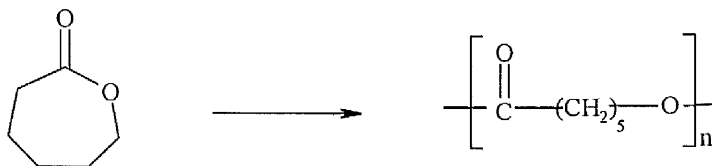
The racemic poly(D,L-lactide) has a lower degree of crystallinity and has a lower melting point than the two stereoregular polymers, poly(D-lactide) and poly(L-lactide). The glass transition temperature of poly(D,L-lactide) is 57-59 °C, which is not ideal as it is higher than body temperature, but due to its low degree of crystallinity the release of the drug is not hindered. An interesting feature of the copolymers of the lactide and glycolide is that they are less crystalline than either of the two homopolymers. The polylactide is found to be more hydrophobic than the polyglycolide due the presence of the methyl group.

Poly(D,L-lactide) degrades to the naturally occurring lactic acid which is found in the body as a product of glycolysis. The first stage of biodegradation is a decrease in the molecular weight due to random hydrolytic cleavage of the ester linkages. The second stage is the onset of weight loss and a change in the rate of chain scission. Matrix structure is one of the factors that determines the capacity of hydration of these hydrophobic polymers and hence will influence both the diffusional and erosional release.³⁸ Poly(D,L-lactide) requires about 12-16 months to completely biodegrade whereas poly(L-lactide) can take up to 2 years. This may be due to it being more crystalline and less hydrophilic. In clinical studies, polymers of D,L-lactide have been more successful than with the homopolymers, and this may be due to the poly(D,L-lactide) being less crystalline and more permeable to the drugs. However, their degradation rate is rather low for most applications,³⁹ and efforts to increase degradation

rates have been performed by copolymerising with monomers such as malic acid and glyceric acid which are present in the body as metabolites.^{40,41}

1.7.2 Properties and Characteristics of Poly(ϵ -caprolactone).

The success achieved through the use of polymers of lactic acid and glycolic acid as biodegradable drug delivery systems led naturally to the evaluation of other aliphatic polyesters, and hence to the discovery of the degradability of poly(ϵ -caprolactone) (PCL) *in vivo*. PCL is usually produced by the ring opening of ϵ -caprolactone monomer.



PCL is a semi-crystalline polymer melting in the range of 59-64 °C, depending on crystallite size. The crystallinity of PCL varies with its molecular weight and for molecular weights in excess of 100,000, the degree of crystallinity is about 40%, rising to 80% as the molecular weight decreases to about 5000.^{4,42} Crystallinity is known to be an important property when determining both permeability and biodegradability.⁴² The permeability is affected because the bulk crystalline phase is inaccessible to water and other pigments. An increase in crystallinity reduces the permeability by reducing the solute solubility. The biodegradation rate is reduced by the decrease in accessible ester bonds.

Poly(ϵ -caprolactone) hydrolyses to 6-hydroxyhexanoic acid and its biodegradation rate is governed by the accessibility of the ester groups which undergo hydrolysis. Ester

groups in the crystalline region are inaccessible to undergo hydrolysis, so the rate of chain cleavage increases with decreasing crystallinity.³⁷

As mentioned in Section 1.5, the glass transition temperature of a polymer which is to be employed as drug delivery implant, should not be higher than body temperature as the polymer needs to be in the rubbery state to give higher permeability. The glass transition temperature of PCL is -60 °C, which means it is in the rubbery state at body temperature (37 °C). This is ideal as the permeability is higher above the T_g due to an increase in chain mobility and free volume. PCL is known to have a high permeability to therapeutically active macromolecules and along with its ideal mechanical properties, tissue compatibility and ease of processing render it a suitable excipient for use in long term delivery of proteins and peptides.⁴³

However, the hydrophobic and crystalline nature of PCL give rise to poor entrapment and diffusion of high molecular weight proteins. The diffusion process occurs primarily through the fluid filled pores, i.e. the non-crystalline phase, so an increase in matrix water content may contribute favourably to the volume available for solute diffusion.⁴⁴ Attempts to enhance the water permeability and hydrophilic nature of PCL have included statistical and block copolymerisation^{45,46,47} and blending⁴⁸ with a second monomer such as lactides, glycolides, δ -valerolactone and ethylene oxide. This induced increase in hydrophilic character by copolymerisation is usually however accompanied by diminished mechanical strength. Positive results have been achieved using ethylene oxide and aliphatic polyethers (e.g. Synperonic L61) in enhancement of the water content and permeability of the hydrophobic PCL matrix.⁴⁴

1.8 MODIFICATION OF POLYMERS USING SUGARS

The aim of this research was to synthesise new materials for use as drug delivery implants for the controlled release of therapeutic proteins or peptides. These new materials were based on the polymers poly(ϵ -caprolactone), poly(D, L-lactide) and polyethylene glycol. By end capping these polymers with a sugar, such as glucose, it was hoped to have dual benefits. Firstly, as glucose is an extremely hydrophilic moiety due to the numerous hydroxyl groups, it was predicted to increase the hydrophilicity of the hydrophobic polymers PCL and PDLL which would improve their water permeability and hence give better drug diffusion. Secondly, it has been shown that glucose has good cryoprotective and lyoprotective properties, so its presence in polymers to be formulated as drug delivery implants is very attractive.

As well as being highly hydrophilic, sugars are inexpensive and biocompatible. One drawback of using sugars is that they tend to be “overfunctionalised” for many purposes, i.e. have many reactive functional groups, but this can be overcome using protecting groups. So the idea of end capping polymers with sugars may seem like complicated chemistry, but molecules of this kind are found widely distributed in nature. These are classed as glycosides and can be generally considered as a sugar in which the hydrogen atom of the anomeric carbon atom has been replaced by an alkyl, aralkyl or aryl group. The term glycoside is used in the generic sense and specific glycosides are named by replacing the ending “ose” of the parent sugar by “oside” and by adding the name of the side chain group and the symbol α or β to designate the configuration of the glycosidic or anomeric carbon atom.

Alkyl glucosides or alkyl polyglycosides as the industrially manufactured products are of great interest due to their surfactant properties.⁴⁹ These molecules consist of a glucose moiety with a fatty acid or alkyl group attached at the anomeric carbon of the sugar as shown in Figure 1-4. The first patent application describing the use of alkyl glucosides in detergents was filed in Germany in 1934.⁵⁰

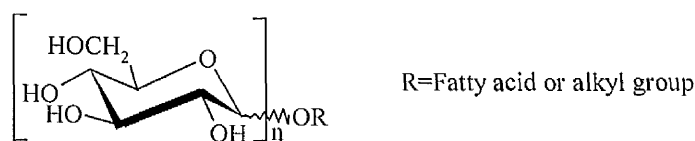


Figure 1-4 Molecular structure of a glycoside

1.9 SURFACTANTS

A surface active agent or surfactant is a compound which will adsorb at an air-water or oil-water interface.⁵¹ The ability of surfactants to change the interfacial free energy and surface charge results from their adsorption and this enables compounds to act as emulsifying or suspending agents.

Typically a surfactant molecule is characterised by having both a polar and non-polar component. For ionic surfactants the polar or hydrophilic region of the molecule may carry a positive or negative charge giving rise to cationic and anionic surfactants. For nonionic surfactants, the hydrophilic component is commonly a polyoxyethylene or polyoxypropylene derivative but polymers such as poly(vinyl alcohol) are also used. The non-polar or hydrophobic portion of the molecule is most commonly a flexible chain hydrocarbon. The hydrophilic part is often referred to as the head and the hydrophobic portion as the tail and represented by the following diagram in Figure 1-5.

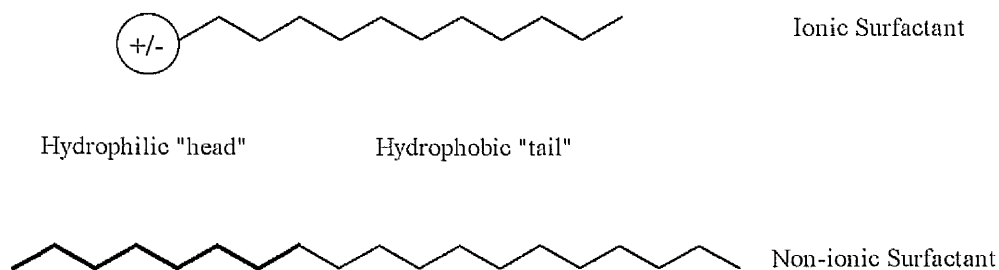


Figure 1-5 Diagrammatic representation of surfactant molecules

A molecule which incorporates two moieties, one having an affinity for water and the other being repelled is termed amphiphilic. This dual nature is responsible for the phenomenon of surface activity and of micellisation.

1.9.1 Surface Activity in Aqueous Solution

Two processes have an important influence on the surface activity of surfactants in aqueous solution. One concerns the effect a surfactant has on the structure of water and the other concerns the freedom of motion of the hydrophobic groups.

The solution of an amphiphilic molecule in pure water leads to a disruption or distortion in the highly structured hydrogen bonding of water as the surfactant is accommodated. In the case of the hydrophobic region there is no possibility of hydrogen bonding with the water molecules.

Experimental evidence suggests that the water molecules in the immediate vicinity of the hydrophobic chain restructure into an even more ordered arrangement than in pure water.⁵² This phenomenon has been termed 'hydrophobic hydration'. The overall effect

is an entropy decrease, making the dissolution of the hydrocarbon chain an unfavourable process.

The hydrocarbon chain of the amphiphile is brought into solution by means of its attachment to a hydrophilic group. The ability of the hydrophilic group more than compensates for the initial disruption process. The amphiphile in solution may be thought of as being surrounded by a cage of highly structured water molecules. A consequence of this situation is that the internal torsional vibrations of the hydrocarbon chains are restricted in solution. It has been suggested that this is the reason for the entropy decrease on the dissolution of hydrocarbon rather than hydrophobic hydration.

It is clear that the removal of the hydrophobic portion of the molecule from its aqueous environment is an entropically favourable process leading to the disruption of the highly organised water structure and the removal of the mobility constraints on the hydrocarbon chains. For these reasons the amphiphile will tend to accumulate on the water-air or water-oil interface in such an orientation that its hydrophobic portion is extended into the gaseous or oil phase. This is illustrated in Figure 1-6.

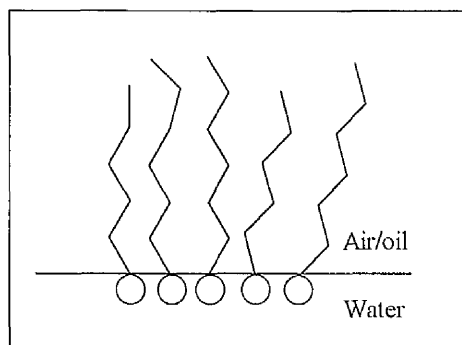


Figure 1-6 The assembly of surfactants at a water-oil/air interface

The molecule is anchored at the interface by the hydrophilic head group which remains in contact with the water. A consequence of the intrusion of the amphiphilic molecules into the surface or interfacial layer is that some of the water molecules are effectively replaced by the hydrocarbon or other non-polar groups. Since the forces of intermolecular attraction between water molecules and non-polar groups are less than those existing between just water molecules, the contracting power of the surface is reduced and so is the surface tension.

1.9.2 Effect of Surfactant Structure on Surface Activity.

The surface activity of a particular surfactant depends on the balance between its hydrophilic and hydrophobic properties. For the simplest case of a homologous series of surfactants, an increase in the length of the hydrocarbon chain results in increased surface activity. The relationship between hydrocarbon chain length and surface activity is expressed in Traube's rule which states that in dilute aqueous solutions of surfactants belonging to any one homologous series, the molar concentrations required to produce equal lowering of the surface tension of water decrease three fold for each addition of a methylene group in the hydrocarbon chain of the solute.

1.9.3 Micellisation

The characteristic property of amphiphiles in accumulating at air-water or oil-water interfaces can be discussed in terms of the entropy gain caused by the disruption of organised water structure and the removal of the mobility constraints as the hydrophobic region was removed from an aqueous environment.

An alternative to the crowding of the interface as the surfactant concentration is increased is provided by the formation of small aggregates or micelles in the bulk of the solution. The hydrophobic moieties compose the core of the micelle, being shielded from the surrounding solvent by the shell of hydrophilic heads. There is much experimental evidence in support of the observation that the mobility of hydrocarbon chains in the micellar interior resembles that in a liquid hydrocarbon. Hence the process of micellisation does indeed represent a method by which hydrocarbon chains can regain their mobility.

The concentration at which micelles first appear is the critical micelle concentration (CMC). This is determined experimentally from the inflection point of some physical property such as surface tension, conductivity, light scattering intensity or osmotic pressure.

1.10 AIMS OF RESEARCH

The aims of the research were based on synthesising and characterising novel products for use as biodegradable drug delivery implants with the added ability to stabilise proteins during freeze drying. To achieve these aims, the research was broken down into the following objectives.

(I) Poly(D,L-lactide) (PDLL) is well established as a polymer for use in the controlled release of drugs. The potential of poly(ϵ -caprolactone) (PCL) is also well recognised and so these polymers were investigated. These polymers were to be end capped via the Koenigs-Knorr reaction which requires the presence of hydroxyl end groups. Studying the success of end capping a polymer is simplified if a polymer of low molecular weight is used. So the first objective was to synthesise PDLL and PCL with low molecular weight and low polydispersity with hydroxyl end groups and to characterise these fully.

(II) Polyethylene glycol (PEG) has been noted as a good cryoprotectant and was investigated alongside PDLL and PCL. PEG is frequently used to standardise characterisation techniques and for this research it was used to compare the relatively new characterisation technique Matrix Assisted Laser Desorption Ionisation Mass Spectrometry (MALDI-MS) with more traditional methods such as Gel Permeation Chromatography (GPC) and Nuclear Magnetic Resonance (NMR).

(III) The next objective was to functionalise the PEG, PDLL and PCL by end capping the polymers with D-glucose *via* the Koenigs-Knorr reaction to produce the

corresponding glucosides. The glucosides were then isolated, purified and characterised.

(IV) The final objective was to study how the polymeric glucosides interacted and stabilised proteins. An assay was required to establish the effectiveness of the polymeric glucosides in stabilising proteins during freeze drying.

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2. Synthesis and characterisation of Poly(ϵ -caprolactone), Poly(D,L-lactide) and Polyethylene Glycol

2.1 INTRODUCTION TO CHAPTER 2

As explained in section 1.10., low molecular weight poly(ϵ -caprolactone) and poly(D,L-lactide) with hydroxyl end-group functionality are required to allow end-capping with glucose *via* the Koenigs-Knorr reaction. This chapter offers a review of the commonly used methods that are available to prepare the homopolymers. The reasoning behind the particular chosen method is given followed by the details of experimental procedures used to make the polymers. The chapter ends with the characterisation of the synthesised polymers and a comparison of the results obtained from three different characterisation techniques.

2.2 METHODS FOR THE SYNTHESIS

POLY(ϵ -CAPROLACTONE)

The preparation of PCL can be carried out by using five different mechanisms categorised as anionic, cationic, co-ordination, radical and the more recent enzymatic. Each method has unique qualities, providing different degrees of control of molecular weight, molecular weight distribution and end-group functionality.¹

2.2.1 Anionic Polymerisation

Anionic polymerisation is a useful method for the synthesis of low molecular weight hydroxy-terminated oligomers and polymers that are to be further processed. The mechanism of this polymerisation is believed to involve the uncatalysed cleavage of the acyl-oxygen bond during initiation with propagation through an alkoxide ion,² as shown in Figure 2-1.

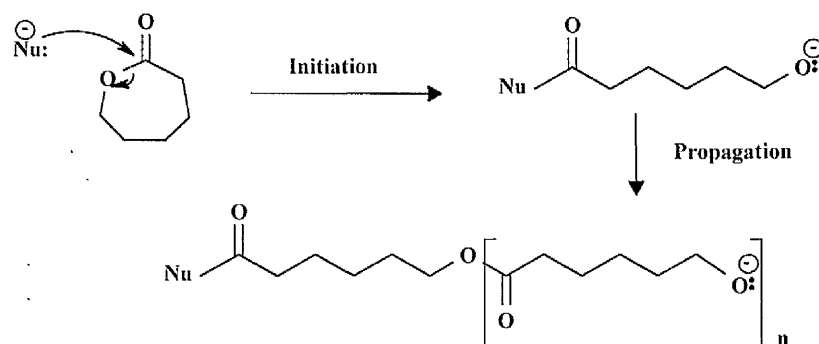


Figure 2-1 Anionic Polymerisation of ϵ -caprolactone

In the anionic polymerisation of lactones, initiators such as alkali metals, organometallic compounds, tertiary amines, carboxylates and alcoholates are extensively used. An example of anionic polymerisation is ethylene glycol-initiated polymerisation of ϵ -caprolactone which requires 35 h at 190 °C for quantitative conversion.³ Rates are substantially increased by using the more nucleophilic alkali alcoholates, the sodium salt of diethylene glycol reduces the conversion time to 17 h at 120 °C.⁴ The use of anionic initiators containing sodium or potassium ions for the synthesis of polymers to be used in drug delivery systems is favoured in some cases.⁵ The reasoning for this is that alkali metal ions are found to participate in the metabolism of living cells and so do not have to be removed from the polymer prior to its use. The more frequently used heavy metal catalysts are toxic to living organisms and so require careful removal before use. These simple alkoxide initiators containing sodium or potassium counterions initiate “living” anionic polymerisation. Problems encountered with anionic polymerisation are that propagation is accompanied by inter- and intramolecular transesterification reactions which are responsible for broadening of the molecular weight distribution and formation of cyclic oligomers. An example is the polymerisation of ϵ -caprolactone with potassium *t*-butoxide in THF.⁶ The predominant product is the cyclic dimer, formed by backbiting degradation of the initially formed linear polymers.

2.2.2 Cationic Polymerisation

The main advantage of cationic polymerisation is the ability to obtain otherwise inaccessible copolymers. Various classes of cationic initiators have been used to polymerise lactones such as protic acids, Lewis acids, acylating agents and alkylating agents.

Alkyl sulfonates are very effective cationic initiators of ϵ -caprolactone. The mechanism of polymerisation in the presence of these initiators is believed to involve alkylation of the exocyclic carbonyl oxygen, followed by partial ring opening of the activated lactone by the counteranion.⁷ Figure 2-2 shows the mechanism for this reaction.

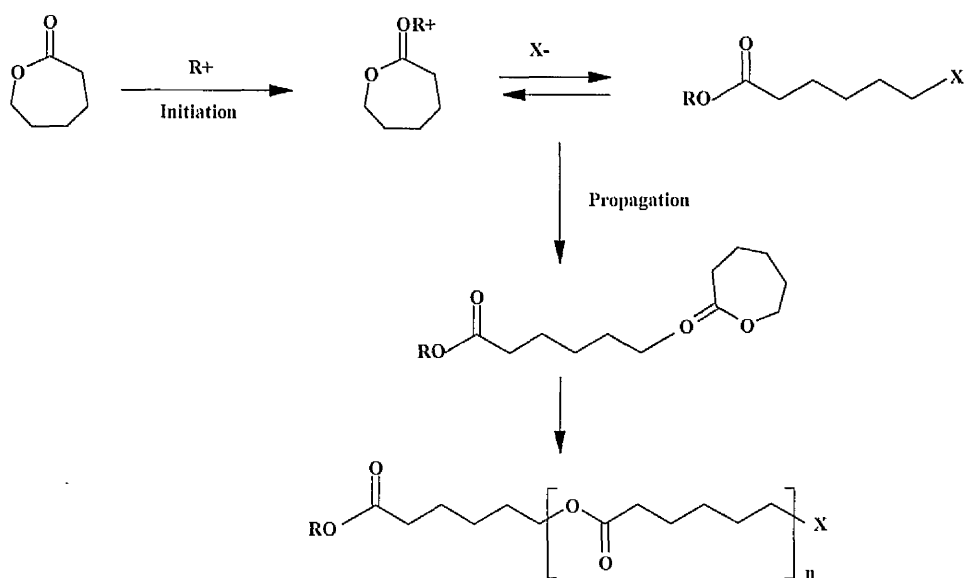


Figure 2-2 Cationic Polymerisation of ϵ -caprolactone

The use of triethyloxonium hexafluorophosphate has been studied as a strong alkylating agent for the initiation of cationic polymerisation of ϵ -caprolactone. This provided low molecular weight polymer *via* an activated monomer mechanism.⁸

2.2.3 Co-ordination Polymerisation

Co-ordination polymerisation has become the most popular method for preparing PCL and its copolymers, affording high molecular weights and conversions and excellent control over molecular weight and end group functionality. Again, this method is a living chain polymerisation. The number of available initiators for the synthesis of PCL using this polymerisation method is increasing rapidly, each new initiator being discovered offering different efficiency, control and selectivity. Initiators used in the polymerisation of CL include aluminium compounds, such as aluminium alkoxides^{9,10,11} and triisobutylaluminium,¹² zinc compounds such as dibutylzinc,^{12,13} rare earth complexes such as yttrium alkoxides,¹⁴ lanthanide phenolates,¹⁵ and rare earth halides,¹⁶ and tin compounds such as tin (II) octoate.^{17,18} The general mechanism of co-ordination polymerisation of ϵ -caprolactone is shown in Figure 2-3.

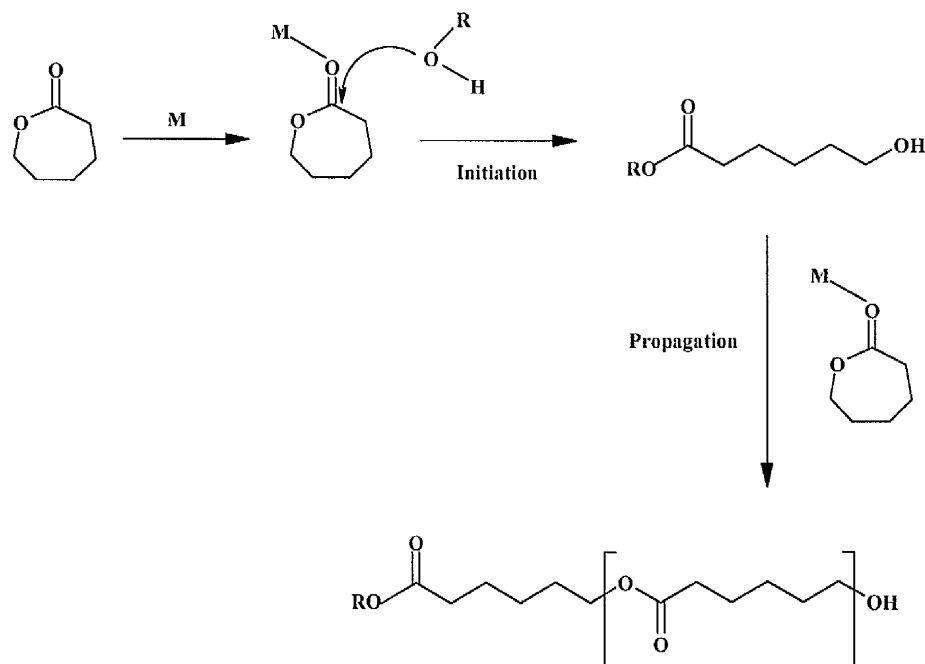


Figure 2-3 Co-ordination Polymerisation of ϵ -caprolactone

Tin(II) octoate is one of the most commonly utilised initiators for synthesising PCL and its mechanism is discussed in greater detail in section 2.3.3.1.

2.2.4 Radical Polymerisation

There is an indirect method of making PCL by free radical polymerisation. This involves ring opening polymerisation of 2-methylene-1,3-dioxepane with AIBN as the initiator.¹⁹ The mechanism is shown below in Figure 2-4.

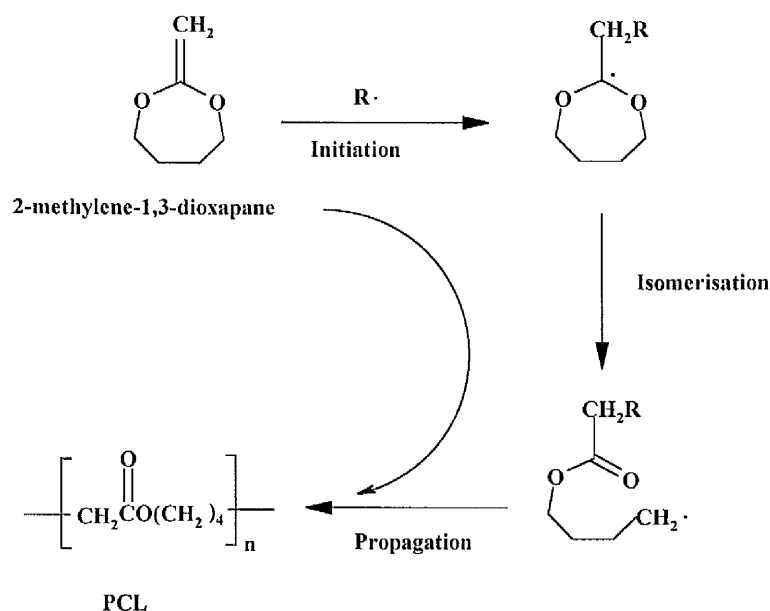


Figure 2-4 The synthesis of poly(ε-caprolactone) *via* radical polymerisation

2.2.5 Enzymatic Polymerisation

The enzyme catalysed polymerisation of ε-caprolactone was first reported in 1993 using lipases from *P. Fluorescens*, *Candida cylindracea* and porcine pancreatic lipase.²⁰ Bacterial proteases have also been investigated as potential catalysts with encouraging results.²¹ Studies undertaken by Henderson *et al.*²² on lactone ring opening polymerisation reactions using porcine pancreatic lipase as the catalyst and

ϵ -caprolactone as the monomer, supplemented with butanol or butylamine produced the reaction scheme shown in Figure 2-5.

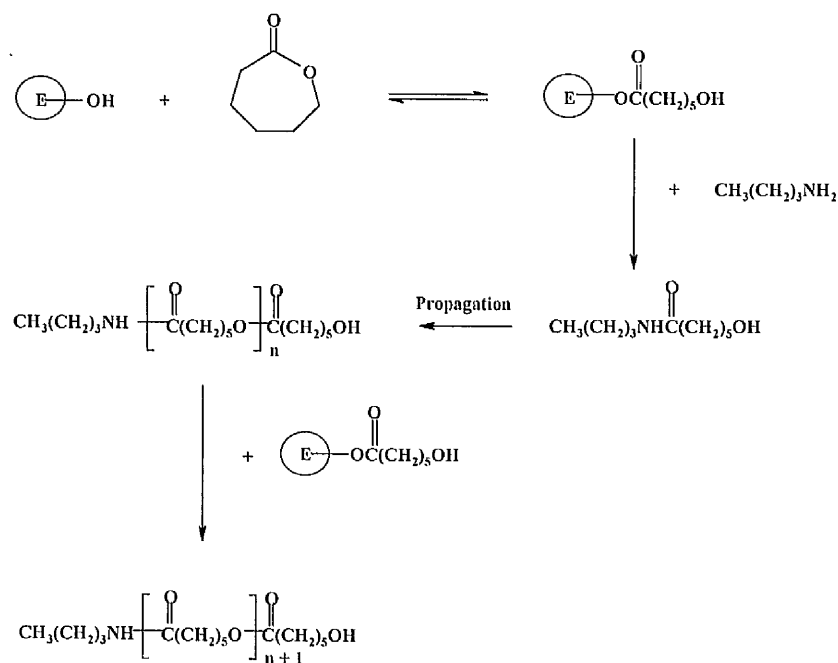


Figure 2-5 Enzymatic Polymerisation of ϵ -caprolactone

In general, enzyme catalysed polymerisations have been found to require long reaction times for complete monomer conversion and have resulted in low number-average molecular weight polyesters. But there is a great deal of interest in this area and active research is being undertaken to make this method of polymerisation an efficient and viable process.

2.3 METHODS FOR THE SYNTHESIS OF POLY(D,L-LACTIDE)

Poly lactides can be synthesised by two quite different mechanisms, by polycondensation of lactic acid or by ring-opening polymerisation of the cyclic dimer, the lactide,²³ as shown in Figure 2-6

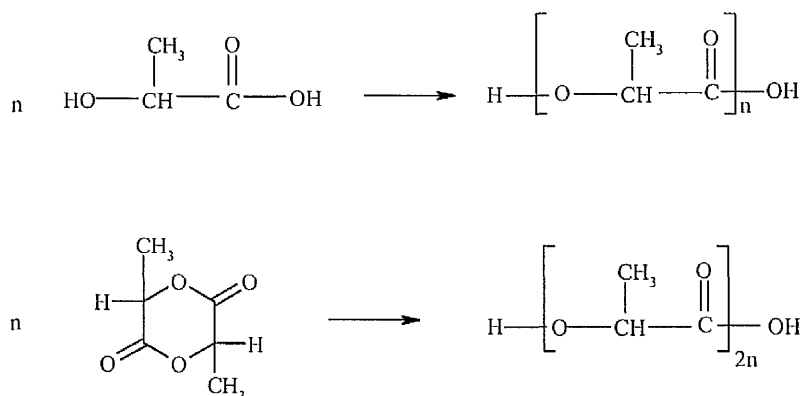


Figure 2-6 Polymerisation of Lactic acid and Lactide.

The polycondensation method has many disadvantages which include difficulty in obtaining high molecular weights, lack of control of molecular weight and end-group variation and problems with preparing copolyesters. However, it is a very inexpensive process and appears to be an attractive method for the synthesis of polylactides to be used as drug delivery implants^{24,25} as a catalyst or initiator is not necessarily required meaning clean, uncontaminated products can be obtained.

The ring-opening polymerisation of the lactide, of which there are four stereoisomers, is the most extensively used approach for the synthesis of polylactides. There are three different types of ring-opening polymerisation, cationic, anionic and co-ordination-insertion and each method is evaluated in the following sections.

2.3.1 Cationic Polymerisation

Cationic polymerisation involves the alkylation of the exocyclic oxygen and cleavage of the alkyl-oxygen bond of the lactide.²⁶ Trifluoromethanesulfonic acid and methyl triflate are useful initiators and the mechanism for this polymerisation utilising methyl triflate is shown in Figure 2-7.

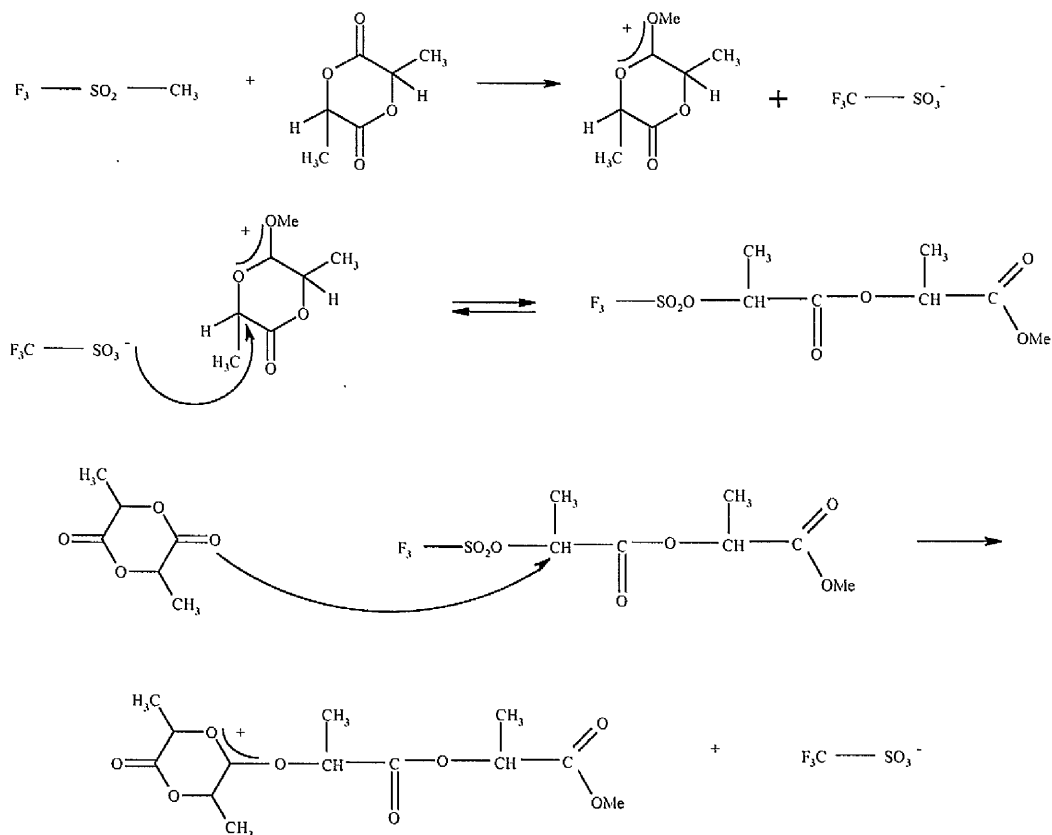


Figure 2-7 Cationic Polymerisation of Lactide

Each propagation step is a substitution at the chiral centre so this mechanism generates a high risk of racemisation and this effect is increased with increasing temperatures. Other downfalls of the mechanism are that it is difficult to obtain high molecular weights and only a few initiators are reactive enough to promote cationic polymerisation.²⁷ Also, this method does not yield random copolymers of lactide and glycolide. Therefore it can be seen that cationic polymerisation is not a very attractive approach to the synthesis of polylactides.²⁸

2.3.2 Anionic Polymerisation.

Various initiators can be used for the anionic polymerisation of lactides such as butyllithium,²⁹ alkali alkoxides,^{29,30} and potassium benzoate.²⁹ Anionic polymerisation is more rapid than cationic. However, there are a number of complications. The chain

growth involves the nucleophilic attack of the alkoxide ion at the carbonyl carbon atom with acyl-oxygen bond scission. The reaction mechanism below in Figure 2-8 demonstrates anionic polymerisation of the lactide initiated with potassium methoxide.

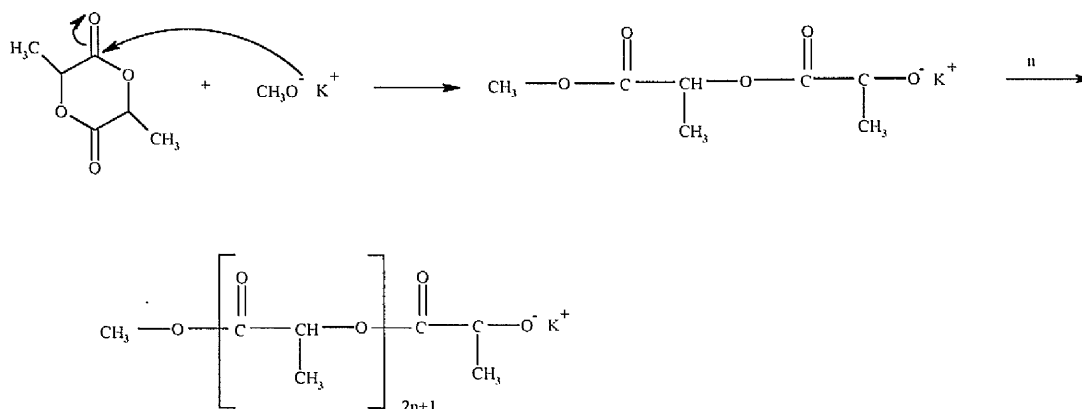


Figure 2-8 Anionic Polymerisation of Lactide

Chain growth allows deprotonation of the monomer by the initiator or by the active chain end which causes partial racemisation. The deprotonation of the monomer by the active chain end is a chain transfer to the monomer and this strongly limits the molecular weights. High molecular weights can only be achieved by using a combination of butyllithium and crownethers, but with a view to use these polymers as drug delivery implants this method is not viable as lithium is a poisonous metal. When poly(D,L-lactides) of lower molecular weights are required, sodium or potassium salts are useful as initiators as they are biocompatible.

2.3.3 Co-ordination-Insertion Polymerisation.

Co-ordination-insertion polymerisation is the most commonly used method for the synthesis of polylactides as this mechanism affords polymers of high molecular weight with a good control of stereochemistry. Other advantages of this method are that the molecular weights can be easily controlled by variation of the monomer/initiator ratio, a broad range of end-groups can easily be obtained and numerous copolyesters can be

synthesised. The most commonly used initiators used for the coordination-insertion mechanism are tin and aluminium compounds such as tin tetrachloride,³¹ stannous octoate,^{32,33} tetraphenyl tin,³⁴ tributyl tin methoxide,³⁵ aluminium bromide,³² and triisobutylaluminium. Zinc alkoxide³⁶ and zirconium chloride³² have also been reported as effective initiators. The common feature of these initiators is that they have covalent oxygen bonds and energetically favourable unoccupied p- and d-orbitals. These initiators induce the co-ordination-insertion mechanism through two steps, firstly association of the monomer and co-initiator followed by cleavage of the acyl-oxygen bond which is shown in Figure 2-9, with tin(II) octoate as the catalyst.

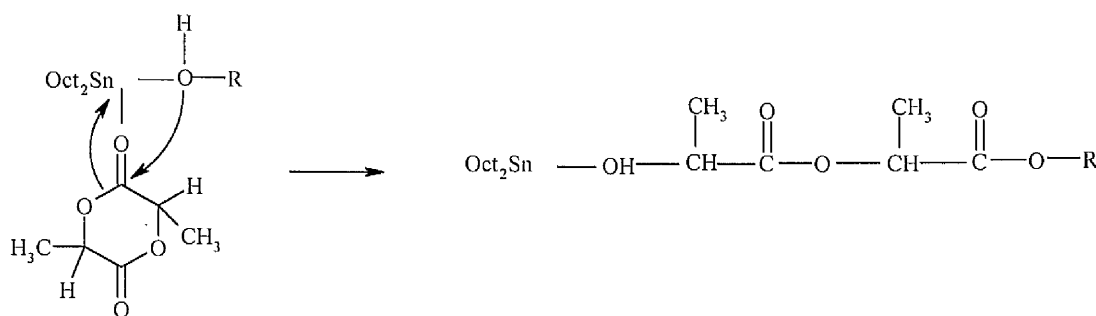


Figure 2-9 Co-ordination-Insertion Polymerisation of Lactide

Tin(II) 2-ethylhexanoate or tin(II) octoate is one of the most widely used catalysts for the synthesis of polylactides as it is highly efficient, allowing complete conversion of monomer with high optical purity and has low toxicity which is an important consideration for pharmaceutical applications. Tin(II) octoate is a strong Lewis acid which forms complexes with alcohols including the hydroxyl end-groups of a growing polylactide chain.³⁷ A great deal of research has been conducted into the precise polymerisation mechanism of tin(II) octoate, and this is discussed in section 2.3.3.1.

2.3.3.1 Tin (II) octoate Catalysed Polymerisation.

With tin(II) octoate or tin(II) bis (2-ethylhexanoate) catalysed polymerisations, the metal species is believed to function as the catalyst and an alcohol serves as an initiator.

Figure 2-10 shows the structure of tin (II) octoate.

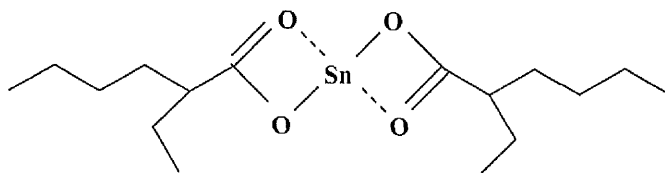


Figure 2-10 Structure of Tin(II) Octoate

With respect to the tin(II) octoate-catalysed polymerisation of lactones, the first step in the role of the catalyst is co-ordination of the lactone carbonyl to free p or d orbitals of the tin(II) octoate.³³ This produces the following resonance structures, as shown in Figure 2-11.

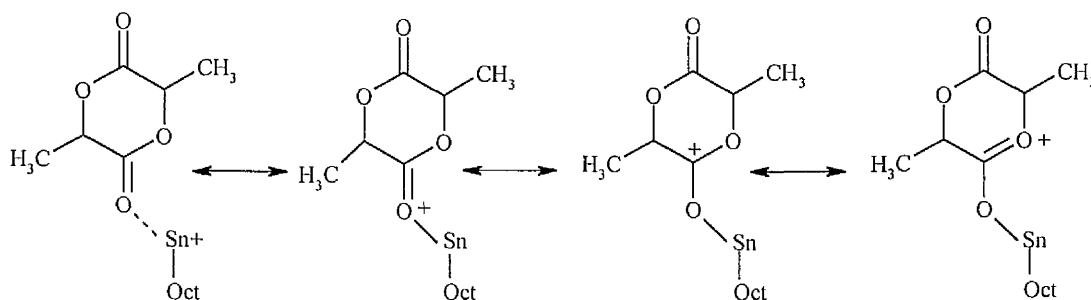


Figure 2-11 Resonance structures of a lactide during Initiation

As a result of the resonance, electron density at the carbon is decreased, making it susceptible to nucleophilic attack by hydroxyl-containing compounds which are actually the true initiators of the polymerisation. Figure 2-12 shows the mechanism for the polymerisation as determined by Nijenhuis *et al.*³³

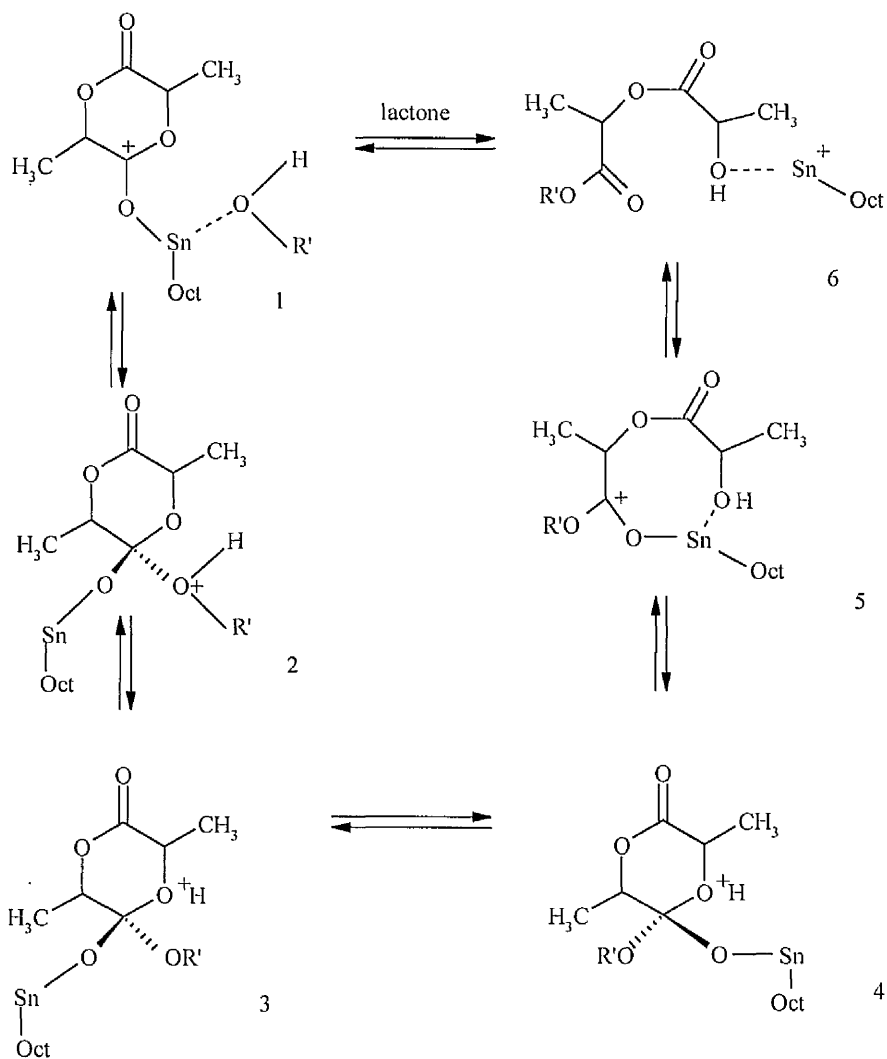


Figure 2-12 Mechanism of Polymerisation of a lactide with Tin (II) octoate as the catalyst

The polymerisation starts when the hydroxyl-containing compound $R'OH$ reacts with the lactone-tin(II) octoate complex through a nucleophilic attack at the carbon (structures 1 and 2). Once co-ordination complex 6 has been formed, the co-ordination with a new lactone will generate species 1 again, where R' is the growing polymer chain. It is thought that the catalyst is not chemically bound to the growing chain end. This means the catalyst can switch from one chain end to another and that the number of polymer chains able to polymerise can be larger than the number of catalyst molecules.

2.4 INTRODUCTION TO SYNTHESIS OF POLY(ϵ -CAPROLACTONE)

The synthesis of the PCL homopolymers was carried out by using the co-ordination-insertion method. A bifunctional initiator, 1,5-pentanediol was used with tin(II) octoate as the catalyst. This system was chosen because of the narrow molecular weight distributions afforded by the catalyst and the hydroxyl end group functionalisation as described in Section 2.2.3. The choice of initiator, 1,5-pentanediol, was governed by the fact that PCL is composed of five carbon units and will therefore give continuation in the structure of the polymer. As low molecular weight polymers were being used, the composition of the initiator can have a considerable effect on the overall polymer, so these effects are reduced using 1,5-pentanediol. From the work done by Schindler *et al.*¹⁷ and Morris³⁸ on the conditions of ϵ -caprolactone polymerisations initiated with difunctional alcohols, it was possible to choose the temperature and length of time required for the polymerisation. It was found that the molecular weight distribution (MWD) of the polymers was narrow at temperatures of 80 and 100°C when terminated at low conversions. On increasing the polymerisation temperature to 140-180°C and conversion (>95%), the MWD is mainly governed by ester interchange. Because 1,5-pentanediol is dihydric, it generates two statistically independent chain segments, each possessing a most probable MWD due to ester interchange. As a narrow MWD was required, a temperature of 85°C for 48hrs was chosen and the desired molecular weight was achieved by controlling the monomer to initiator ratio.

2.4.1 Preparation of Poly(ϵ -caprolactone)

All reagents and glassware were rigorously dried as any water present can act as a site for initiation which would in turn effect the molecular weight and polydispersity. CL (Aldrich) was dried over calcium hydride for 24 hours then distilled taking the middle cut at reduced pressure and stored over molecular sieve (4A) under dry nitrogen. 1,5-Pentanediol (Aldrich) was dried for 24 hours over molecular sieve and then distilled at reduced pressure. Methanol (AnalaR) and tin(II) octoate (Sigma-Aldrich, 98%) were used as supplied.

All polymerisations were carried out in bulk using tin(II) octoate as the catalyst. Table 2-1 lists the amounts of initiator and monomer used. The polymerisations were conducted in ampoules of 150 cm³ capacity fitted with a B24 ground glass joint and Youngs Tap. The inner walls of the ampoules were treated with trimethylsilyl chloride to remove hydroxyl groups present, producing a monolayer organic film.

The following experimental procedure can be regarded as a general method for the polymerisations. The appropriate amount of monomer, initiator and tin(II) octoate were added to the ampoule under dry nitrogen. The ampoule was then sealed and degassed on the vacuum line, followed by placing the ampoule in the thermostatically controlled water bath at 85 °C for 48 h. After this time the reaction was quenched by reducing the temperature to ambient temperature. Quenching the reaction meant that transesterification was less likely to occur, hence keeping the molecular weight distribution narrow. After extraction and precipitation at 7°C with methanol, the polymer was filtered and washed and dried for 24 hours in a vacuum oven. Degree of

conversion was calculated from the mass of polymer recovered and is reported in Table 2-1.

Table 2-1 Reactants for synthesis of PCL

Polymerisation Designation	Initiator/mol	Monomer/mol	[I]:[M] Mole Ratio	% Conversion (Mass)
PCL-1	7.61×10^{-2}	0.229	1:3	98
PCL-2	3.80×10^{-2}	0.229	1:6	73
PCL-3	1.27×10^{-2}	0.178	1:14	74

2.5 INTRODUCTION TO SYNTHESIS OF POLY(D,L-LACTIDE)

Initial attempts to synthesise poly(D,L-lactic acid) were made using the polycondensation method.³⁹ This method was chosen initially as no catalyst or initiator was required. This method would yield uncontaminated polymers which is desirable for polymers to be used in biomedical applications.

2.5.1 Attempted Preparation of Poly(D,L-lactic acid) PDLA

100 ml of a 85-90 % D,L-lactic acid aqueous solution (Sigma) was concentrated by removal of the water by rotary evaporation under reduced pressure. The D,L-lactic acid was dried on a vacuum line under reduced pressure. The vessel, still be kept under vacuum, was then placed in a sand bath and heated to 140 °C for 3 days. After 3 days, a solid mass should have been present, but the resulting product was still a liquid. The

resulting product was then dissolved in acetone and should have precipitated in water. The product was found to be soluble in water and the product could not be recovered.

2.5.2 Preparation of Poly(D,L-lactic acid) (PDLLA)

The second attempt to synthesise poly(D,L-lactic acid) utilised the lactic acid monomer again as well as an initiator and a catalyst. The method was based on the work by Hiltunen *et al.* and involved using 1,4-butanediol and tin(II) octoate.⁴⁰ The following procedure was used to synthesis poly(D,L-lactic acid).

10.5 ml of a 85-90 % D,L-lactic acid aqueous solution (Sigma) was concentrated by removal of the water by rotary evaporation under reduced pressure. The D,L-lactic acid was dried on a vacuum line under reduced pressure. 1.0 ml of 1,5 pentanediol and 2 drops of tin (II) octoate was then added. A small magnetic stirrer bar was also added and the vessel was then degassed and sealed under vacuum. The vessel was then placed in a sand bath and heated to 160 °C 24 hours. The resulting product was a viscous liquid. The product was analysed using GPC and the results are shown in Section 2.6.2.2

It was decided that the polycondensation reaction was not proving to be successful as the molecular weight obtained using this method was 340 and this proved difficult to successfully reproduce. It was also proving difficult to recover the D,L-lactic acid from solution prior to the polymerisation. Any water present acts like the pentanediol as an initiator for the polymerisation and this means that it is difficult to control the molecular weight of the polymer.

The next attempt at synthesising the polymer used a ring opening polymerisation of the D,L lactide monomer rather than the polycondensation of the D,L-lactic acid. The method decided upon for the synthesis of the PDLL homopolymers was co-ordination-insertion, using tin(II) octoate as the catalyst and ethylene glycol as the initiator. As mentioned before in Section 2.4, the initiator was chosen to give as much continuation as possible to the polymer structure. From the work carried out by Nijenhuis *et al.*³³ and Kricheldorf *et al.*³⁵, the following procedure was composed, performing the polymerisation in bulk as a melt.

2.5.3 Preparation of Poly(D,L-lactide)

It was essential that all reagents and glassware were extremely clean and dry. D,L-lactide (Lancaster) was used as received. Ethylene glycol (Lancaster) was dried over molecular sieve for 24 h then distilled under reduced pressure taking the middle cut and stored over molecular sieve and under nitrogen. Tin(II) octoate, dichloromethane and heptane were used as received.

All the polymerisations were carried out as a melt using tin(II) octoate. Table 2-2 lists the amounts of initiator and monomer used. The polymerisations were carried out in a round bottomed flask of 100 cm³ capacity fitted with a B24 ground glass joint and Youngs Tap containing a small stirrer bar. The inner walls of the ampoule were treated with trimethylsilyl chloride to remove hydroxyl groups present.

Table 2-2 Reactants for synthesis of PDLL

Polymer Designation	Initiator/mol	Monomer/mol	[I]:[M] Mole Ratio	% Conversion (Mass)
PDLL-1	7.63×10^{-2}	0.134	1:1.8	80
PDLL-2	3.59×10^{-2}	0.134	1:3.7	71
PDLL-3	1.80×10^{-2}	0.134	1:7.5	97

The following experimental procedure can be regarded as a general method for all the D,L-lactide polymerisations. The appropriate amount of monomer, initiator and tin(II) octoate were added to the ampoule which was then degassed on the vacuum line. It was then sealed and placed in a thermostatically controlled sand bath at 120°C for 48 h. It was crucial that none of the ampoule was exposed by the sand causing cool regions on the surface of the ampoule as D,L lactide sublimes. The melt was agitated by the magnetic stirrer bar to give good mixing of reaction mixture by using a stirrer hotplate. After 48h the reaction was quenched by reducing the temperature to ambient temperature. Finally, the reaction mixture was diluted in dichloromethane and then filtered to remove contaminants. Heptane was then added with stirring until the solution became turbid. This mixture was then left at 6°C overnight to induce phase separation. The polymer, precipitated as a concentrated solution was isolated and any remaining solvent removed by rotary-evaporation. The resulting polymer was an amorphous, transparent material. The degree of conversion was calculated from the mass of polymer recovered and is shown in Table 2-2.

2.6 CHARACTERISATION OF POLY(ϵ -CAPROLACTONE), POLY(D,L-LACTIDE) AND POLYETHYLENE GLYCOL

The following section describes the techniques which were used to characterise the polymers, PCL and PDLL that were synthesised in Sections 2.4.1 and 2.5.1. In addition, three polyethylene glycols (M_n 300, 1000 and 2000, Hoechst) were characterised. The techniques included NMR, GPC and MALDI and the results obtained are compared and discussed.

2.6.1 Nuclear Magnetic Resonance (NMR)

High-resolution solution state NMR is an effective and versatile technique which is widely used in all areas of polymer chemistry. It can be used to great effect in obtaining accurate qualitative and quantitative information of the chemical structure of a polymeric material. Both ^1H and ^{13}C NMR are widely used for routine purposes such as polymer identification, conformation of chain structure, calculation of molecular weight *via* end group analysis and chain stereochemistry classification. For copolymers, NMR is a useful tool for average copolymer composition and statistics of sequence structure evaluation.

NMR spectroscopy is based on the fact that many atoms possess nuclei that have an associated magnetic moment which can be affected by a magnetic field. When such nuclei are placed in a strong magnetic field, B_0 , they align themselves in a certain definite orientation with respect to the field. The most important nuclei in NMR (^1H , ^{13}C , ^{15}N , ^{19}F , ^{29}Si , ^{31}P) all have a spin state of $\frac{1}{2}$ and can take one of two orientations, a low energy orientation aligned with the applied field and a high energy orientation opposed to the applied field. The magnetic field causes the moments to resonate in the direction of B_0 . The resonance frequency ν_0 is given by,

$$\nu_0 = \gamma B_0 / 2\pi \quad (\text{eq.1})$$

where γ is the magnetogyric ratio of the nucleus.

To obtain an NMR spectrum, the nuclei are subjected to a second small magnetic field, B_1 , which is orientated perpendicular to B_0 . In Fourier Transform (FT)-NMR, the field B_1 is applied in the form of a short intense pulse of the order of microseconds. An FT-NMR spectrometer monitors the superimposed absorption of all the resonances with time after the pulse as the signal decays due to various relaxation processes (free induction decay, FID). The spectrum obtained is the Fourier transformation of the FID.

The resonance frequency of a nucleus depends on its chemical environment and so is affected by several structural factors such as bond hybridisation, the nature of neighbouring atoms, delocalisation, stereochemistry and hydrogen bonding. The frequency depends on the chemical environment because the surrounding electrons, in response to the applied field, B_0 , generate a small induced field opposed to B_0 , thus shielding the nucleus from B_0 . This effect can be quantified as a shielding constant σ , according to,

$$B' = B_0(1-\sigma)$$

where B' is the reduced field at the nucleus. From equation 1, the resonance frequency becomes

$$\nu_0 = \gamma B_0 (1-\sigma) / 2\pi$$

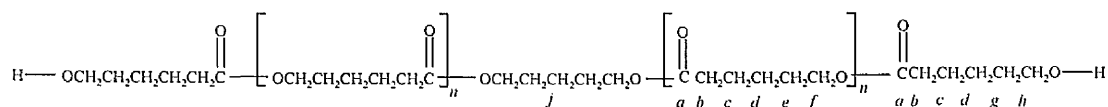
The variation of ν_0 with structure is known as the chemical shift. From the examination of the spectra of known structure, correlations between chemical shift and structure have been developed.

In this section NMR was used to determine the molecular weight using end group analysis and confirm the stereochemistry of PDLL.

2.6.1.1 NMR of Poly(ϵ -caprolactone)

The spectra were obtained in this work using deuteriochloroform (CDCl_3) solution ($\sim 100 \text{ mg cm}^{-3}$) and performed on a Varian Unity 500 spectrometer operating at 125 MHz.

The spectra for PCL-1, PCL-2 and PCL-3 were obtained and Figure 2-13 shows the spectrum for PCL-2. The peak assignments are shown in Table 2-3 and these can be applied to all the PCL homopolymers.

Table 2-3 ^{13}C Chemical Shifts for PCL

Carbon Atom	^{13}C Chemical Shift (ppm)
a	173.6-173.8
b	34.1-34.2
c	25.2-25.5
d	24.5-24.6
e	28.2-28.3
f	64.1-64.3
g	32.2
h	62.4
j	22.4

These results were concurrent with the end groups analysis of PCL by R. F. Storey and A E Taylor⁴¹. Within the main-chain ϵ -caprolactone repeat unit, the carbon resonances were assigned as follows; a, 173.6-173, b, 24.1-34.2, c, 25.2-25.5, d, 24.5-24.6, e, 28.2-

28.3. The splitting observed in the carbonyl region and the main-chain ϵ -caprolactone repeat unit region was due to the terminal repeat unit and the initiator unit. Their work also shows that for diol-initiated systems, the resulting polymer carries two hydroxyl end groups, the resonance of the carbon bonded directly to the hydroxyl end group occurring at 62.1 ppm.

The number of repeat units and hence the molecular weight (M_n) of the polymers PCL-1, PCL-2 and PCL-3 were calculated using the integrals from peaks due to a carbon atom in the repeat unit and the corresponding carbon atom from an end group. The following example shows how the calculation was performed for PCL-2 from the integrals shown in Figure 2-13 and a summary of the results obtained from NMR spectroscopy are shown in Table 2-4.

$$n = \frac{2 \times I(\text{Repeat} - \underline{\text{CH}_2\text{O}})}{I(\text{End group} - \underline{\text{CH}_2\text{O}})}$$

(the factor of 2 takes into consideration that the integral for the end group represents 2 carbon atoms).

$$n = \frac{(2 \times 151.27)}{43.51} = 6.95$$

(the value of n does not include the end groups so an extra 2 monomer units have to be added as well as the initiator, 1,5-pentane diol, to obtain the value of M_n)

$$M_n = (6.95 \times 114.15) + (2 \times 114.15) + 104.15$$

$$M_n = 1130 \pm 113$$

This value for M_n is quoted to the nearest factor of 10 and an estimated uncertainty error of 10% is associated with this value. The values of M_n obtained from NMR for the other polymers were treated in the same way.

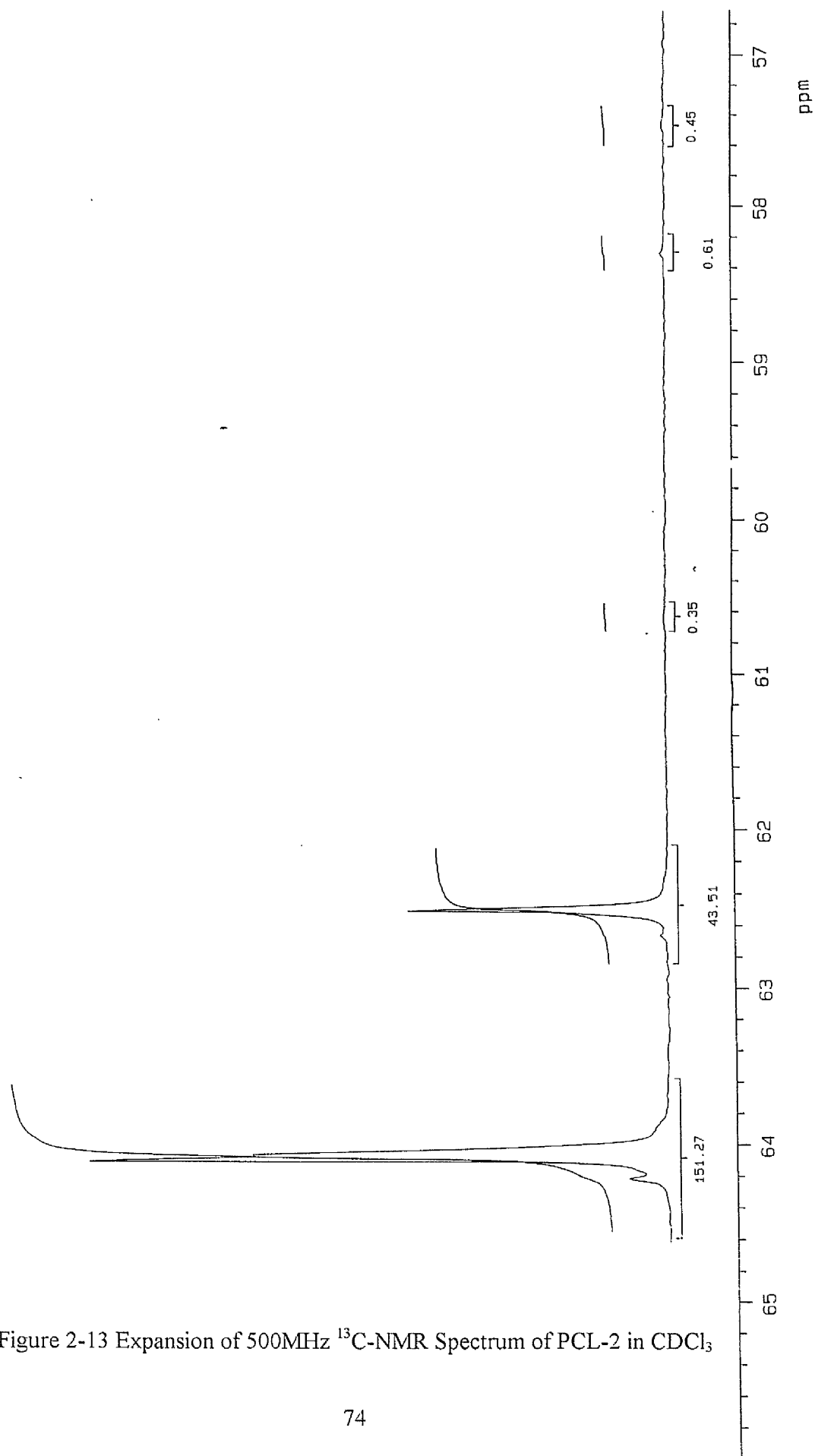


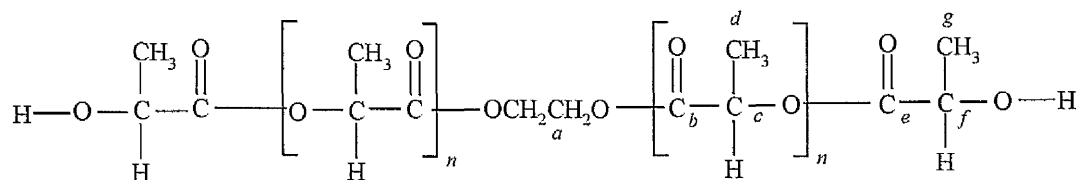
Figure 2-13 Expansion of 500MHz ^{13}C -NMR Spectrum of PCL-2 in CDCl_3

Table 2-4 Results obtained from NMR of PCL

Polymer Designation	[I]:[M] Mole Ratio	<i>n</i>	<i>M_n</i>
PCL-1	1:3	2.95	670 ± 67
PCL-2	1:6	6.95	1130 ± 113
PCL-3	1:14	16.67	2240 ± 224

2.6.1.2 NMR of Poly(D,L-lactide)

The structure of PDLL-1, PDLL-2 and PDLL-3 were confirmed using ^{13}C -NMR and the spectra for PDLL-2 is shown in Figure 2-14. The peak assignments are shown in Table 2-5.

Table 2-5 ^{13}C Chemical Shifts for PDLL

Carbon Atom	^{13}C Chemical Shift (ppm)
a	62.7
b	169.1-169.88
c	68.9-69.1
d	16.6-16.7
e	174.9-175.0
f	66.6-66.7
g	20.11-20.38

The following calculation using PDLL-2 as an example, demonstrates how the molecular weight of the PDLL's were calculated using end group analysis. The expansion of the

NMR in the CH_3 region is shown in Figure 2-15. The results obtained for PDLL-1, PDLL-2 and PDLL-3 are shown in Table 2-6.

$$n = \frac{2 \times I(\text{Repeat} - \text{CH}_3)}{I(\text{End group} - \text{CH}_3)}$$

$$n = \frac{(2 \times 117.43)}{(23.28 + 23.01)} = 5.07$$

Two integral values are observed in the spectrum for the end group due to the D- and L-stereochemistry.

$$M_n = (5.07 \times 72.06) + (2 \times 72.06) + 62.49$$

$$M_n = 570 \pm 57$$

Table 2-6 Results obtained from NMR of PDLL

Polymer Designation	[I]:[M] Mole Ratio	n	M_n
PDLL-1	1:1.8	1.87	340 ± 34
PDLL-2	1:3.7	5.07	570 ± 57
PDLL-3	1:7.5	11.00	1000 ± 100

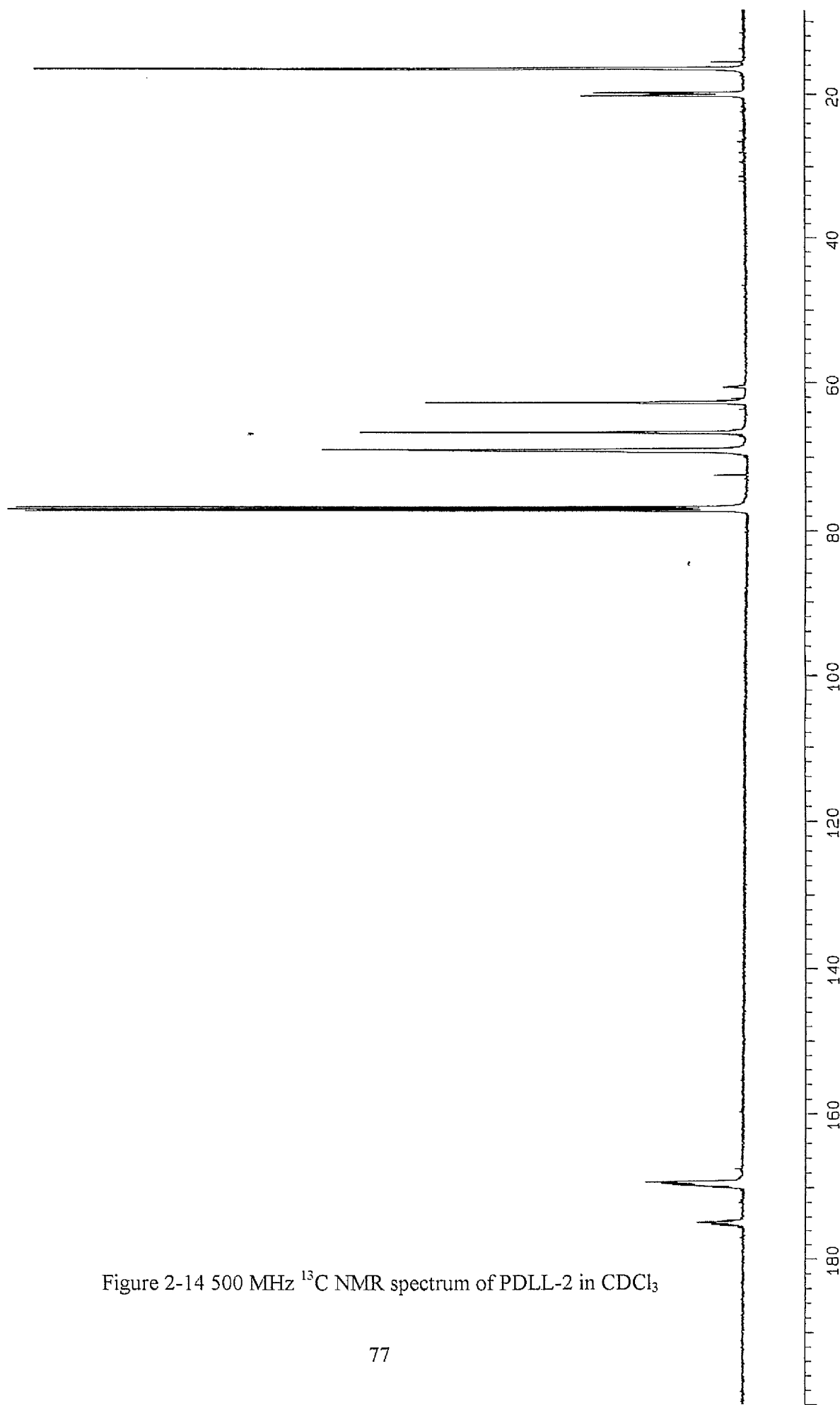


Figure 2-14 500 MHz ^{13}C NMR spectrum of PDLL-2 in CDCl_3

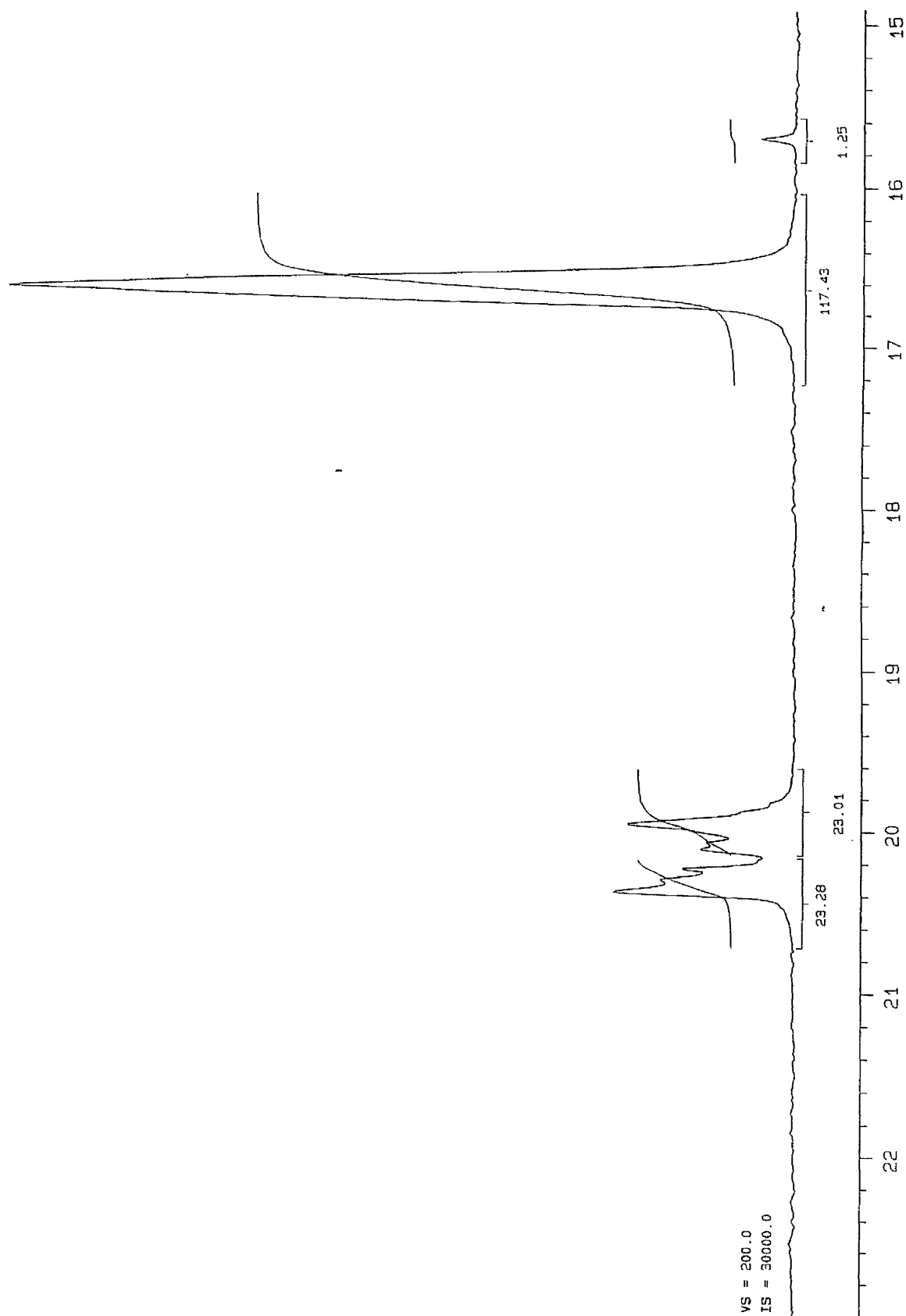


Figure 2-15 Expansion of 500 MHz ^{13}C spectrum of PDLL-2 in CDCl_3

2.6.1.3 NMR of Poly(ethylene glycol)

The structure of PEG-1, PEG-2 and PEG-3 were confirmed using NMR and the chemical shifts observed are shown in Table 2-7. The molecular weight of each polymer was calculated using end group analysis and the value of M_n was then compared with the manufacturer's value of M_n . All PEGs were purchased from Hoechst.

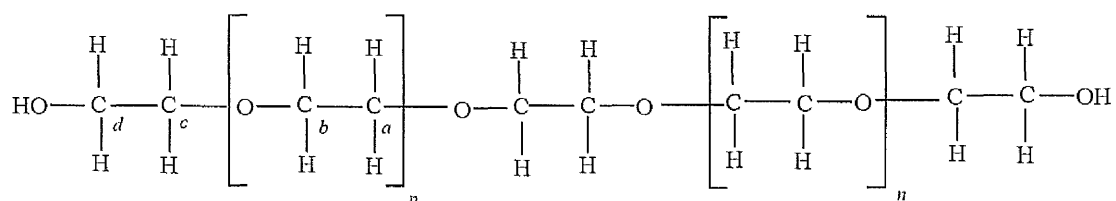


Table 2-7 ^{13}C Chemical shifts of PEG

Carbon Atom	^{13}C Chemical Shift (ppm)
a, b	70.1-70.4
c	72.4-72.7
d	61.3-61.4

The following calculation using PEG-2 as an example, demonstrates how the M_n was calculated for each polymer. Figure 2-16 shows the expanded NMR spectrum for PEG-2.

$$n = \frac{I(\text{Repeat} - \underline{\text{CH}_2} -)}{I(\text{End group} - \underline{\text{CH}_2} -)}$$

For this calculation the integrals of the end groups and repeat units can be compared without the multiplication of a factor of 2 as the repeat unit integral represents both carbon atoms of the repeat unit.

$$n = \frac{1716.2}{75.68} = 22.67$$

$$M_n = (22.67 \times 44.05) + (2 \times 44.05) + 18$$

$$M_n = 1110 \pm 111$$

Table 2-8 shows the results obtained from NMR spectroscopy for PEG-1, PEG-2 and PEG-3.

Table 2-8 Results obtained from NMR of PEG

Polymer Designation	Manufacturers value for M_n	n	M_n
PEG-1	300	4.26	290 \pm 29
PEG-2	1000	22.65	1110 \pm 111
PEG-3	2000	34.28	1620 \pm 162

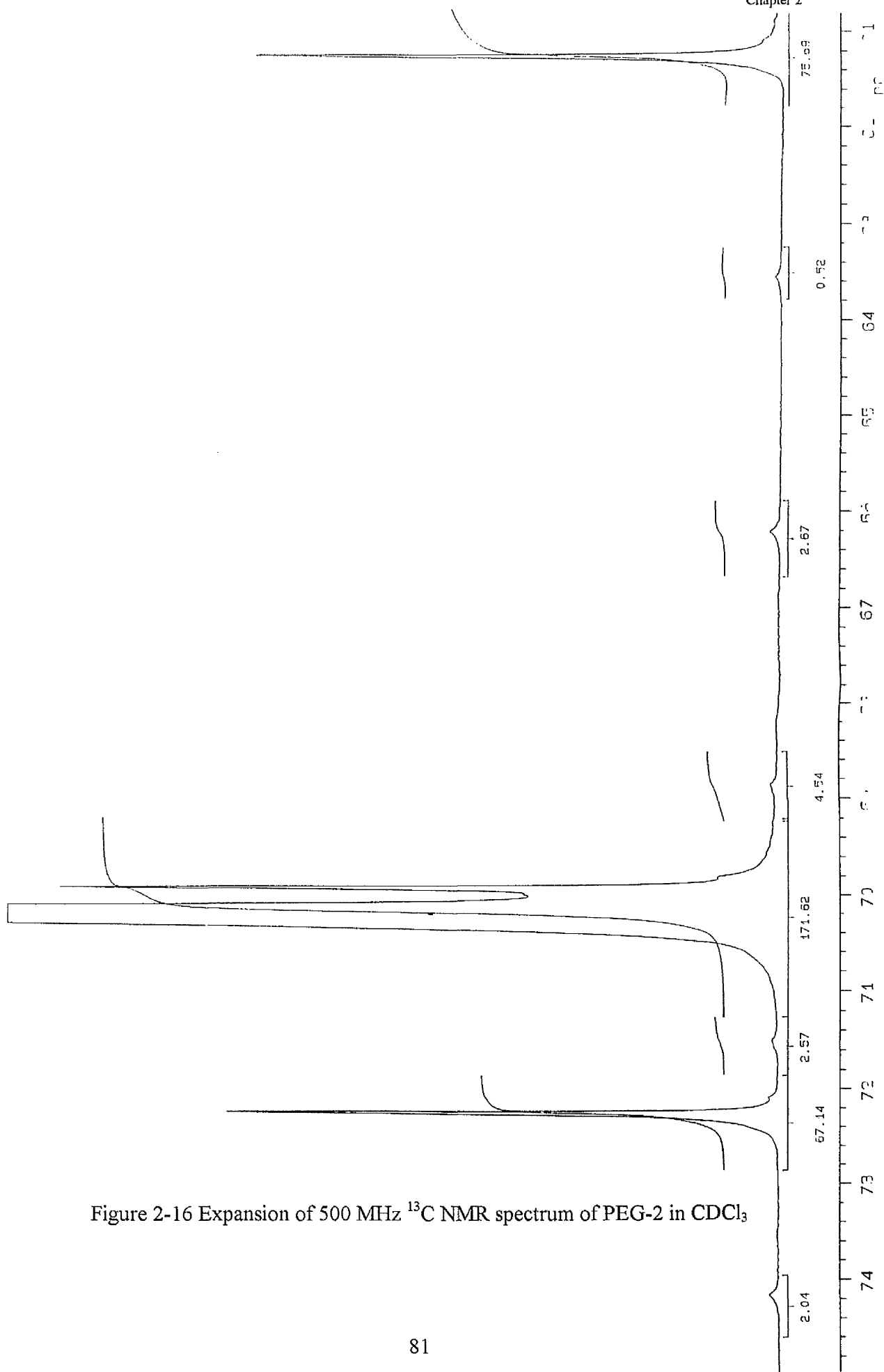


Figure 2-16 Expansion of 500 MHz ^{13}C NMR spectrum of PEG-2 in CDCl_3

2.6.2 Gel Permeation Chromatography (GPC)

Gel permeation chromatography or size exclusion chromatography is a technique in which polymer molecules are separated according to hydrodynamic volume.⁴² With calibration, one can determine a molar mass distribution. The separation is achieved on a column packed with a highly porous material that separates the polymer molecules according to size. Small molecules are able to diffuse into the pores of the column more efficiently, and hence they travel through the column more slowly and higher molecular weight fractions are eluted first.

Calibration is required for an individual GPC column for a particular polymer. Standard samples, with different average molar masses and narrow molar mass distributions, may be used for calibration. Polystyrene standards having polydispersity indexes close to unity are available over a wide range of molecular weights and these are often used. If a polymer other than polystyrene is being studied, the molecular weights obtained are only approximate and can not be relied on as being accurate. This problem is overcome using universal calibration.

The universal calibration is based on the observation that the product of intrinsic viscosity and molecular weight is independent of polymer type. This product, $[\eta]M$, is called the universal calibration parameter. A plot of $\log ([\eta]M)$ versus elution volume in a solvent such as tetrahydrofuran, yields a single curve, approximately linear, for a widely diverse group of polymers. So $\log ([\eta]M)$ may be considered a constant for all polymers for a given column, temperature and elution volume. If we assume that the reference polymer (e.g. polystyrene) is polymer 1 and the polymer to be separated is polymer 2, it follows that,

$$[\eta]_1 M_1 = [\eta]_2 M_2$$

From the Mark-Houwink-Sakurada relationship,

$$[\eta]_1 = K_1 M_1^{a_1}$$

$$[\eta]_2 = K_2 M_2^{a_2}$$

Combining these equations and solving for $\log M_2$, we obtain

$$\log M_2 = \left[\frac{1}{1 + a_2} \right] \log \left[\frac{K_1}{K_2} \right] + \left[\frac{1 + a_1}{1 + a_2} \right] \log M_1$$

To determine the molecular weight of polymer 2(M_2) at a given retention volume, the column must first be calibrated with the standard polystyrene fractions. Constants K and a are normally obtainable, and substituting these and the value of M_1 for a particular retention volume from the calibration plot, M_2 can be readily calculated. So GPC provides an efficient and convenient method of obtaining molecular weight distribution once the appropriate calibrations have been performed.

The GPC system used consisted of 4 Styragel columns (Water Associates) with tetrahydrofuran as eluent at a flow rate of $1 \text{ cm}^3 \text{ min}^{-1}$. Samples were dissolved in THF at a concentration of 0.2% w/v and one drop of an internal marker (dodecane) was added to each solution. All experiments were carried out at room temperature. The eluent was detected using a differential refractometer (Water Associates Model 410), and the detector response was plotted against retention time.

The on-line microcomputer and GPC 6000 software (Jones Chromatography Ltd.) enabled calibration of the results using universal calibration. The Mark-Houwink coefficients used for polystyrene were $K = 0.93 \times 10^{-4} \text{ dL g}^{-1}$ and $a = 0.74$, and for

poly(ϵ -caprolactone) were $K = 1.4 \times 10^{-4} \text{ dL g}^{-1}$ and $a = 0.79$.⁴³ For poly(D,L-lactide), $K = 5.49 \times 10^{-4} \text{ dL g}^{-1}$ and $a = 0.64$.⁴⁴

2.6.2.1 GPC Results for Poly(ϵ -caprolactone)

The results obtained from GPC of PCL-1, PCL-2 and PCL-3 are shown in Table 2-9. The values for M_n and M_w are quoted to the nearest factor of 10 and an estimated uncertainty of 10% is associated with these values. The polydispersity (M_w/M_n) is quoted to two decimal places. The values of M_n , M_w and M_w/M_n obtained from GPC for the other polymers were treated in the same way. The GPC traces obtained are shown in Figure 2-17 and it can be seen from the traces of PCL-1 and PCL-2 the presence of peaks for the low molecular weight oligomers.

Table 2-9 Results obtained from GPC for PCL

Polymer Designation	[I]:[M] Mole Ratio	M_n	M_w	M_w/M_n
PCL-1	1:3	770 \pm 77	940 \pm 94	1.22
PCL-2	1:6	1060 \pm 106	1520 \pm 152	1.44
PCL-3	1:14	3170 \pm 317	3570 \pm 357	1.13

2.6.2.2 GPC Results for Poly(D,L-lactide) and Poly(D,L-lactic acid)

Table 2-10 shows the results obtained from GPC for PDLL-1, PDLL-2 and PDLL-3 and the GPC traces for the three polymers is shown in Figure 2-19. The results obtained for poly(D,L-lactic acid) are also shown in Table 2-10 and the GPC trace for this polymer is also shown in Figure 2-17. For PDLL-1 and PDLLA, the presence of peaks for the low molecular weight oligomers is noted.

Table 2-10 Results obtained from GPC for PDLL and PDLLA

Polymer Designation	[I]:[M] Mole Ratio	M_n	M_w	M_w/M_n
PDLL-1	1:1.8	400 ± 40	490 ± 49	1.22
PDLL-2	1:3.7	740 ± 74	930 ± 93	1.25
PDLL-3	1:7.5	1150 ± 115	1630 ± 163	1.41
PDLLA	1:10	320 ± 32	420 ± 42	1.31

2.6.2.3 GPC Results for Poly(ethylene glycol)

The results obtained from GPC of PEG-1, PEG-2 and PEG-3 are shown below in Table 2-11 and the traces are shown in Figure 2-18.

Table 2-11 Results obtained from GPC for PEG

Polymer Designation	Manufacturer's value for M_n	M_n	M_w	M_w/M_n
PEG-1	300	340 ± 34	380 ± 38	1.09
PEG-2	1000	1030 ± 103	1060 ± 106	1.03
PEG-3	2000	1730 ± 173	1790 ± 179	1.04

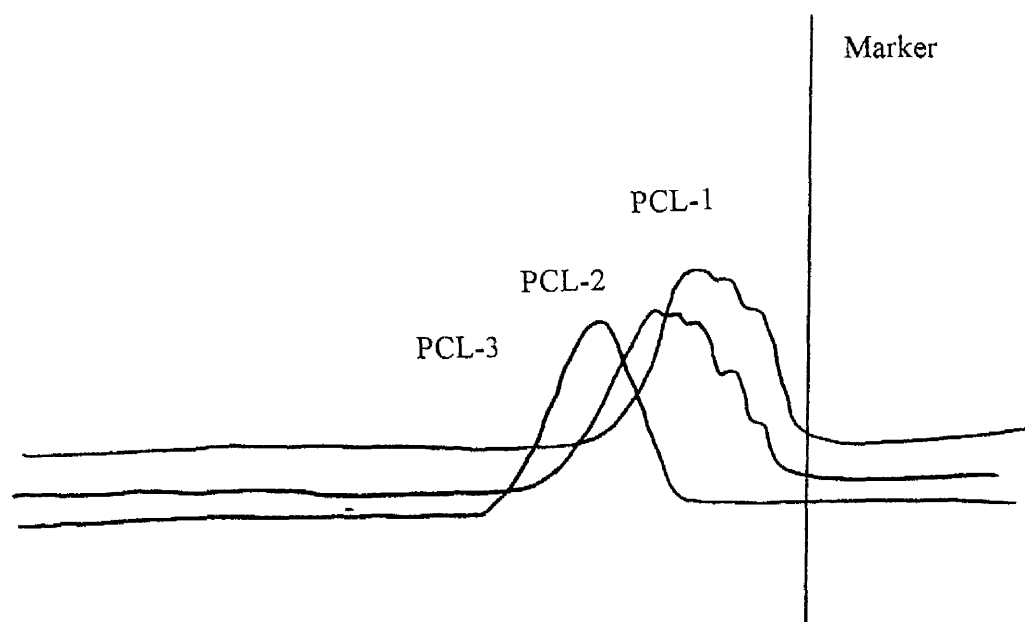


Figure 2-17 GPC traces for PCL-1, PCL-2 and PCL-3

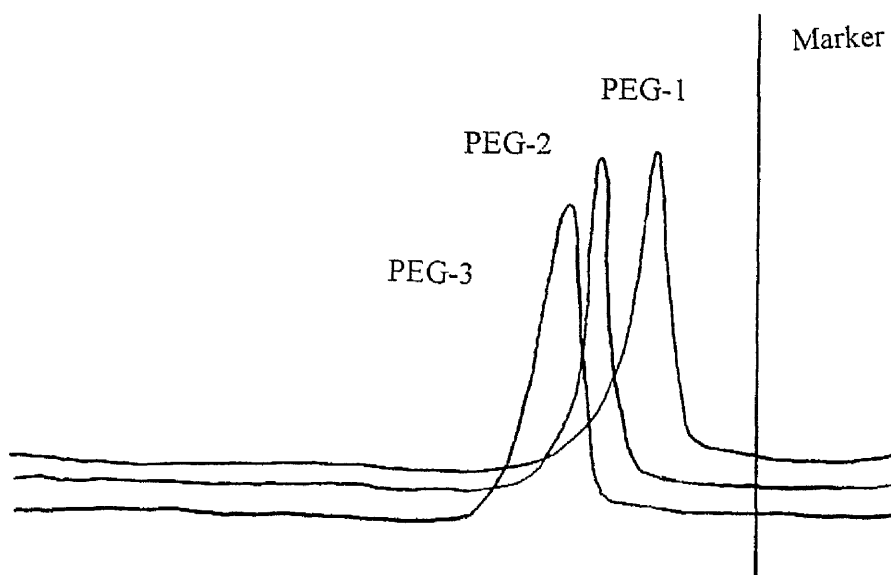


Figure 2-18 GPC traces for PEG-1, PEG-2 and PEG-3

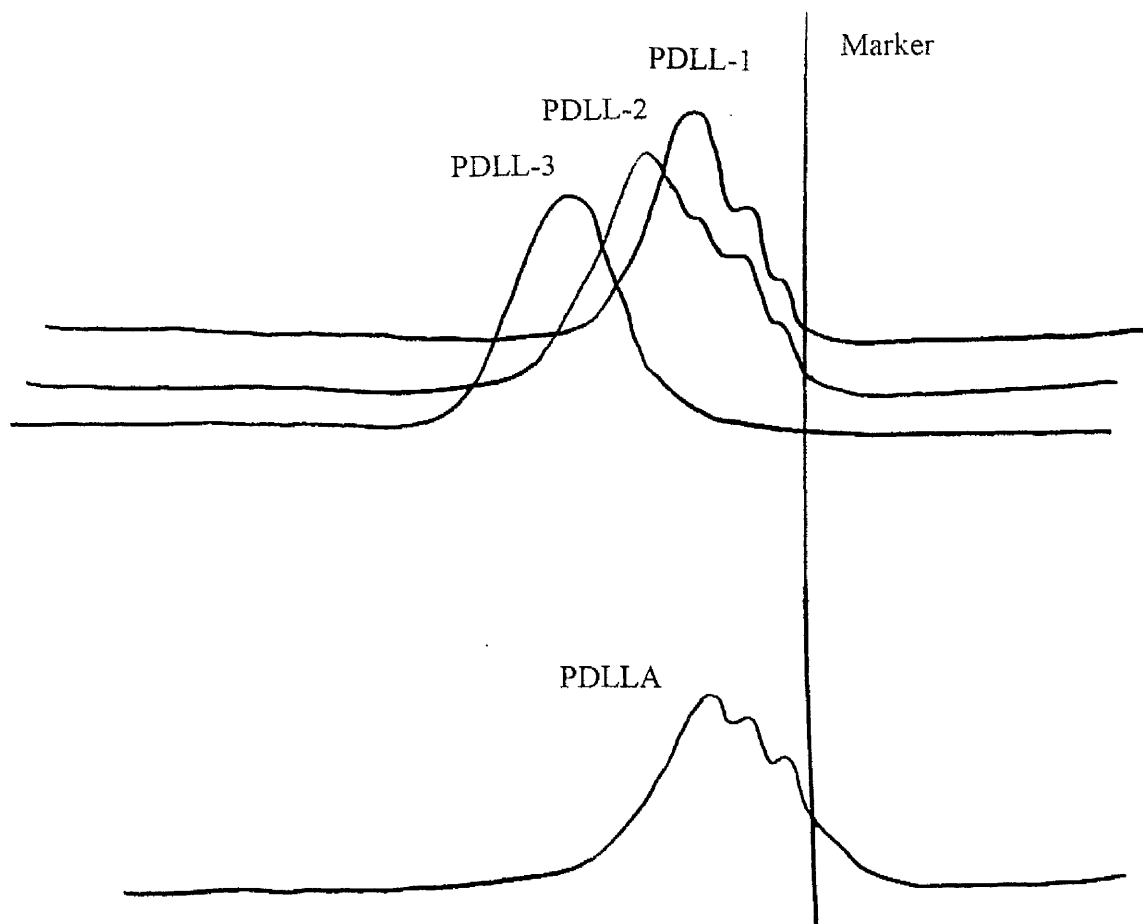


Figure 2-19 GPC traces for PDLL-1, PDLL-2 PDLL-3 and PDLLA

2.6.3 Matrix Assisted Laser Desorption and Ionisation Mass Spectrometry (MALDI-MS)

MALDI-MS has recently become an effective analytical tool for the analysis of polymers. MALDI-MS is a soft ionisation process and has a great advantage over other mass spectroscopy methods as it offers the identification of polymeric materials with minimum fragmentation. Previous analysis of polymers by mass spectrometry has not been effective due to the difficulty in forming stable ions of the polymers in the gas phase. This is overcome in MALDI-MS by using a low molar mass organic matrix to assist the ionisation process. The polymer is incorporated into the matrix and then desorbed by irradiation with an ultraviolet laser and molecular ions are formed indirectly as a result of matrix-polymer interactions. The energy from the laser is adsorbed by the matrix molecules, resulting in an instantaneous phase transition from the solid phase to the gas phase. This gas phase expands into a vacuum and both positive and negative ions are created. The type of ion detected depends on the polarity of the extraction field. Since it is the matrix and not the polymer that adsorbs the laser energy then no fragmentation of the polymer ions should occur prior to detection.

A crucial part of the overall process of using MALDI-MS is sample preparation. The choice of matrix is very important as well as preparation. For this work, samples were prepared by uniformly mixing the matrix and the polymer in a ratio of 10:1 matrix : polymer in a suitable solvent such as acetone or dichloromethane. A few microlitres of this mixture is deposited onto a target plate, dried and placed in to the mass spectrometer. This technique of sample preparation is referred to as the 'dried droplet' method.

In MALDI-MS spectra the occurrence of peaks due to cation attachment has been noticed. In this research, it was found that this gave complicated spectra so this was controlled by enhancing cationisation by adding a salt solution such as sodium chloride solution or potassium iodide solution prior to analysis.

All MALDI-TOF spectra were obtained by using a Micromass TofSpec mass spectrometer equipped with a nitrogen laser operating at 337 nm with 4 ns pulse width. Ions were accelerated by 20 kV to the TOF analyser, which was used in the reflection mode to detect positive or negative ions.

All of the polymer samples were prepared by uniformly mixing the matrix (10mg/ml) and the analyte, at a ratio of 10:1 matrix/analyte in a suitable solvent such as DCM. The matrix employed in all of the analysis was dithranol. One microlitre of the resultant solution was deposited onto a stainless steel probe and allowed to cocrystallise in air ('dried droplet' method). The probe was then introduced into the vacuum of the instrument for analysis.

2.6.3.1 MALDI-TOF MS Results of Poly(ϵ -caprolactone)

The results obtained from MALDI-TOF MS for the PCL polymers are displayed in Table 2-12 and the spectrum obtained for PCL-2 is shown in Figure 2-20. The values for M_n and M_w are quoted to the nearest factor of 10 and an estimated uncertainty of 10% is associated with these values. The polydispersity (M_w/M_n) is quoted to two decimal places. The values of M_n , M_w and M_w/M_n obtained from MALDI-TOF MS for the other polymers were treated in the same way.

Table 2-12 Results obtained from MALDI-MS for PCL

Polymer Designation	[I]:[M] Mole Ratio	M_n	M_w	M_w/M_n
PCL-1	1:3	790 ± 79	970 ± 97	1.23
PCL-2	1:6	1420 ± 142	1770 ± 177	1.25
PCL-3	1:14	2090 ± 209	2400 ± 240	1.13

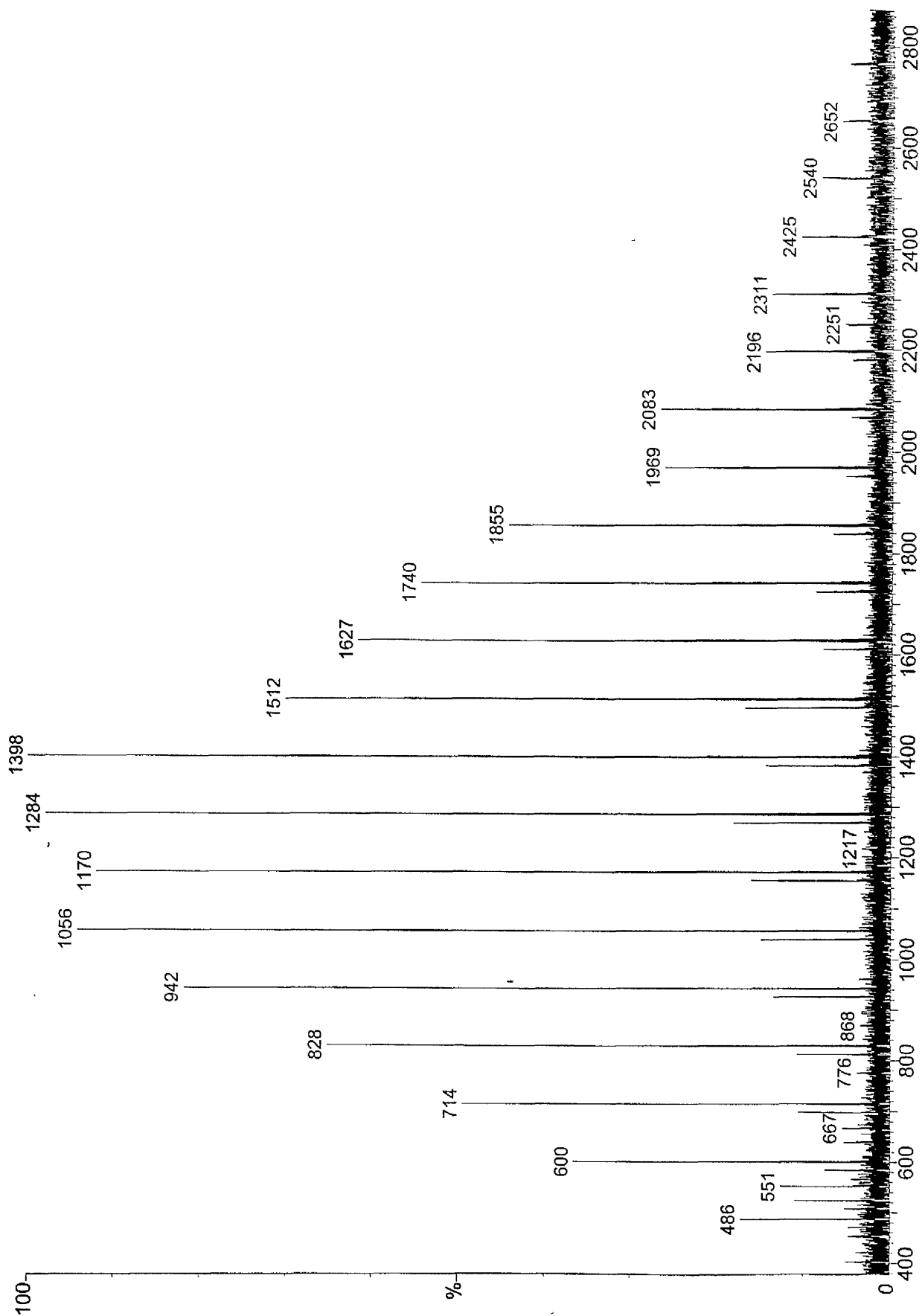


Figure 2-20 MALDI-TOF mass spectrum of PCL-2

2.6.3.2 MALDI-TOF MS Results of Poly(D,L-lactide)

Table 2-13 displays the results obtained from analysing PDLL-1, PDLL-2 and PDLL-3 using MALDI-TOF MS and Figure 2-21 shows the spectra obtained for PDLL-2.

Table 2-13 Results obtained from MALDI-TOF MS for PDLL

Polymer Designation	[I]:[M] Mole Ratio	M_n	M_w	M_w/M_n
PDLL-1	1:1.8	560 ± 56	660 ± 66	1.17
PDLL-2	1:3.7	1050 ± 105	1220 ± 122	1.16
PDLL-3	1:7.5	1760 ± 176	2210 ± 221	1.25

2.6.3.3 MALDI-TOF MS Results of Poly(ethylene Glycol)

The results are displayed in Table 2-14 that were obtained from MALDI-TOF MS of the PEG polymers and Figure 2-22 shows the spectra obtained for PEG-2.

Table 2-14 Results obtained from MALDI-TOF MS for PEG

Polymer Designation	Expected value of M_n	M_n	M_w	M_w/M_n
PEG-1	300	400 ± 40	420 ± 42	1.06
PEG-2	1000	1050 ± 105	1110 ± 111	1.05
PEG-3	2000	1970 ± 197	2020 ± 202	1.02

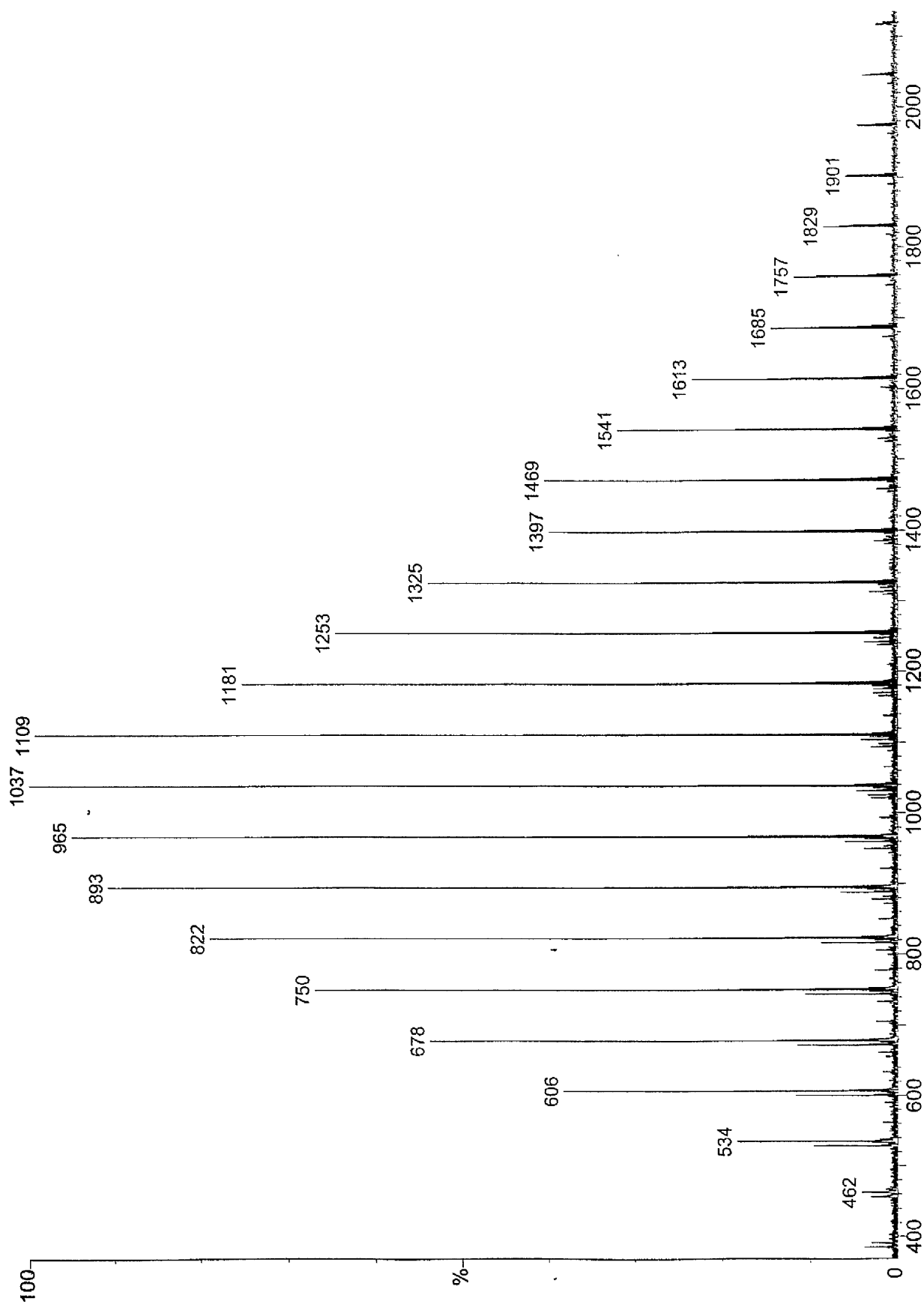


Figure 2-21 MALDI-TOF MS spectrum of PDLL-2

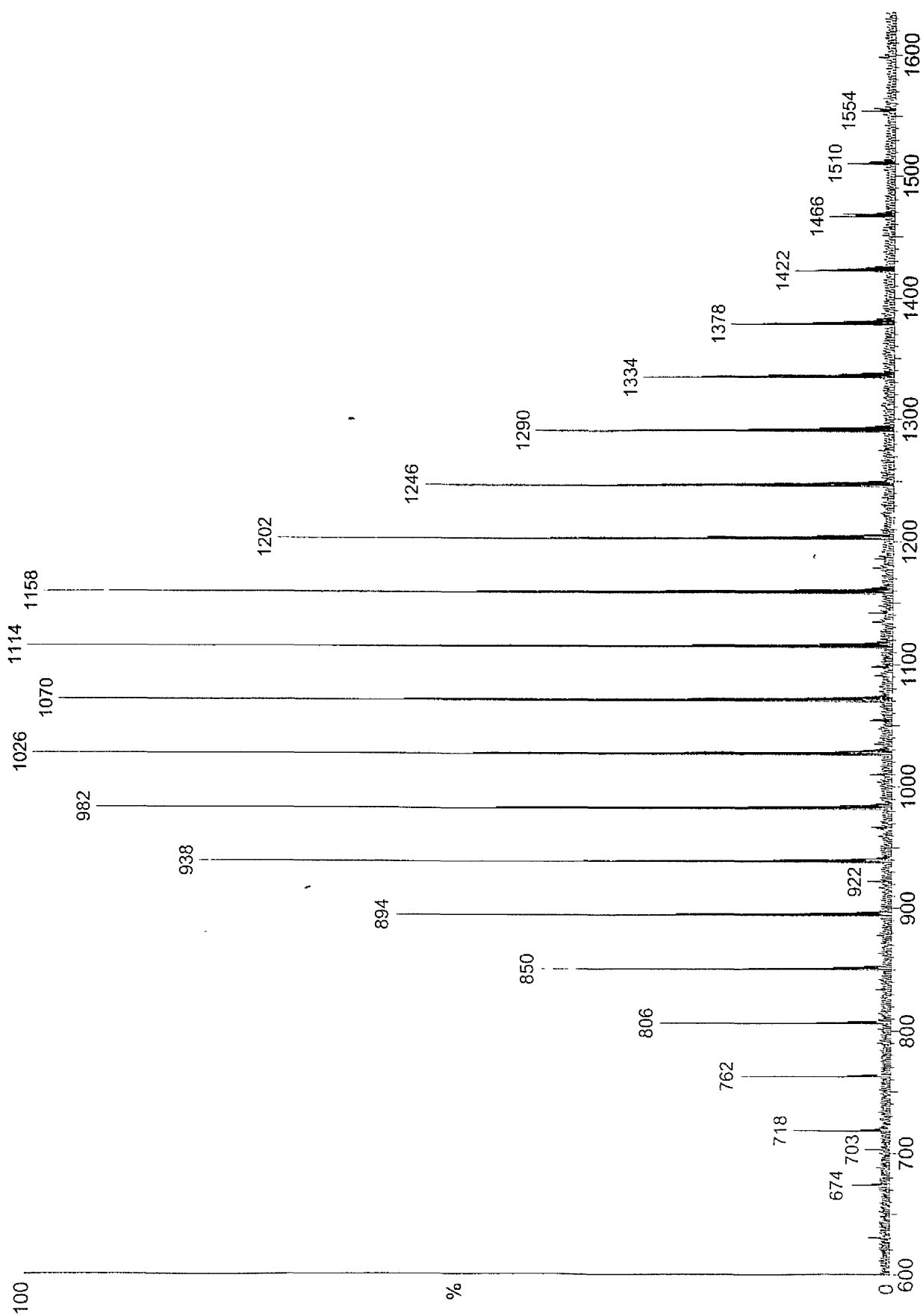


Figure 2-22 MALDI-TOF MS spectrum of PEG-2

2.7 COMPARISON OF RESULTS FROM CHARACTERISATION TECHNIQUES

This section will compare the results obtained from NMR, GPC and MALDI-TOF MS and discuss how well the values for M_n relate.

2.7.1 Comparison of Characterisation Results for PCL

The results obtained from the characterisation of poly(ϵ -caprolactone) by NMR, GPC and MALDI-TOF MS are shown in Table 2-15.

Table 2-15 Values of M_n obtained from NMR, GPC and MALDI-TOF MS for PCL

Polymer Designation	M_n NMR	M_n GPC	M_n MALDI-TOF MS
PCL-1	670 ± 67	770 ± 77	790 ± 79
PCL-2	1130 ± 113	1060 ± 106	1420 ± 142
PCL-3	2240 ± 224	3170 ± 317	2090 ± 209

The values of M_n obtained from the three techniques for PCL-1 compare well taking into consideration the errors associated with the values. For PCL-2, the values of M_n obtained from NMR and GPC show good correlation but the value obtained from MALDI-TOF MS was higher. The values of M_n obtained for PCL-3 from NMR and MALDI-TOF correlate well taking into consideration the errors associated with the results. The value of M_n from GPC for PCL-3 was higher and this may be due to errors associated with calibration of the GPC equipment by the universal calibration procedure.

2.7.2 Comparison of Characterisation Techniques for PDLL

The results obtained from the characterisation of poly(D,L-lactide) by NMR, GPC and MALDI-TOF MS are shown in Table 2-16.

Table 2-16 Values of M_n obtained from NMR, GPC and MALDI-TOF MS for PDLL

Polymer Designation	M_n NMR	M_n GPC	M_n MALDI-TOF MS
PDLL-1	340 ± 34	400 ± 40	560 ± 56
PDLL-2	570 ± 57	740 ± 74	1050 ± 105
PDLL-3	1000 ± 100	1150 ± 115	1760 ± 176

For PDLL-1, the values of M_n obtained from NMR and GPC compare well when considering the errors associated with the values. The value of M_n obtained from MALDI-TOF MS was found to be higher than the values from NMR and GPC. Similarly for PDLL-2, the values of M_n from GPC and NMR are similar but the value from MALDI-TOF MS is again higher. The same scenario was observed with the value from MALDI-TOF MS being higher than the other two techniques.

The results obtained from NMR and GPC compare well for all the PDLL polymers but the M_n values from MALDI-TOF MS are almost double those obtained from NMR. In reality, the difference in M_n is approximately 500 and this would be negligible at an M_n of many thousands. MALDI-TOF MS is a relatively new characterisation technique and its weaknesses are still to be identified for certain systems.

2.7.3 Comparison of Characterisation Techniques for PEG

The results obtained from the characterisation of PEG by NMR, GPC and MALDI-TOF MS are shown in Table 2-17.

Table 2-17 Values of M_n obtained from NMR, GPC and MALDI-TOF MS for PEG

Polymer Designation	M_n NMR	M_n GPC	M_n MALDI-TOF MS
PEG-1	290 ± 29	340 ± 34	400 ± 40
PEG-2	1110 ± 111	1030 ± 103	1050 ± 105
PEG-3	1620 ± 162	1790 ± 179	1970 ± 197

For PEG-1, there is a good correlation between the values of M_n from all the techniques but the value from NMR is slightly lower. For PEG-2 and PEG-3, the values of M_n from all the techniques compare very well taking into consideration the estimated errors associated with the values.

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3. Synthesis and Characterisation of Polymeric Glucosides

3.1 INTRODUCTION TO CHAPTER THREE

This chapter will firstly outline the various methods which can be used to synthesise glycosides and then discuss previous attempts to synthesise polymeric glucosides.

The research undertaken will then be discussed. The glycosylation of poly(ethylene glycol) was investigated using PEG-2 and its characterisation was discussed in Chapter 2. The glycosylation of poly(ϵ -caprolactone) will then be demonstrated and this was performed using PCL-1. The synthesis and characterisation of this polymer was discussed in Chapter 2. Finally, the glycosylation of poly(D,L-lactide) will be demonstrated using PDLL-2. The synthesis and characterisation of PDLL-2 was discussed in Chapter 2.

3.2 INTRODUCTION TO METHODS FOR SYNTHESIS OF GLYCOSIDES

There is currently much interest in glycosides since their chemistry is important in nature. In the synthesis of glycosides, a polyfunctional sugar component is combined with a nucleophile such as an alcohol, carbohydrate or protein. The polyfunctionality of the sugar due to the many hydroxyl groups means that for a selective reaction at one of these hydroxyl groups, all other potential sites of reaction have to be protected in a first reaction step. In principal, enzymatic or microbial procedures, due to their natural selectivity, can replace the complicated chemical protection and deprotection steps where regioselective formation of the glycosides is required. However, the controlled use of enzymes for glycoside synthesis is still in its infancy so the chemical procedures are still preferred at present. Although many methods for the synthesis of glycosides

example below in Figure 3-2 shows the preparation of methyl tetra-*O*-acetyl- β -D-glucopyranoside (MTAG) by treatment of tetra-*O*-acetyl- α -D-glucopyranosyl bromide (TAGB) with methanol and silver carbonate.

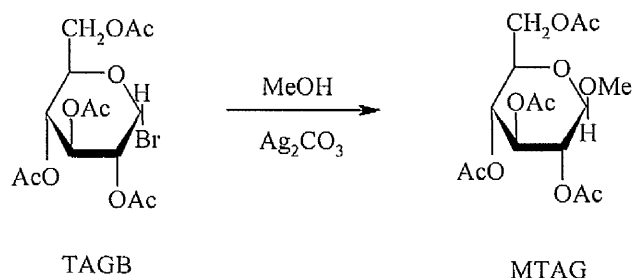


Figure 3-2 Synthesis of MTAG *via* Koenig-Knorr Method

3.2.2 Fischer Method

The Fischer synthesis is a fundamentally different approach to the synthesis of alkyl glucosides. This method is applicable with alcohols but not with phenols and is particularly suited to the preparation of glycosides with lower aliphatic alcohols.⁴ The reaction involves heating a solution of the monosaccharide in the alcohol in the presence of a few percent of hydrogen chloride as catalyst. The formation and hydrolysis of the glycoside is a reversible reaction, but the reaction can be forced in one direction by use of a large excess of alcohol or water.

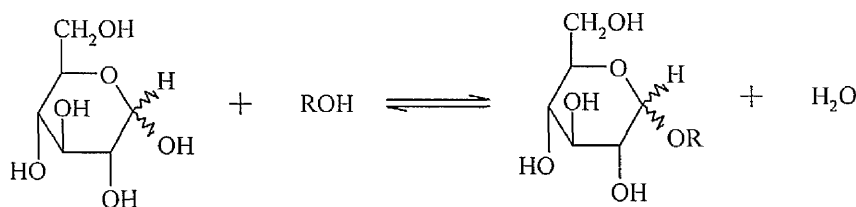


Figure 3-3 Formation and Hydrolysis of a Glycoside

3.2.3 Glycosides from Acetals and Thioacetals

This involves a ring closure reaction of an acetal or thioacetal. Capon and Thacker investigated the hydrolyses of the acyclic dimethyl acetals of D-glucose and D-galactose in dilute aqueous hydrochloric acid.⁵ This reaction produced the methyl furanosides by a concurrent ring closure. The following scheme in Figure 3-4 is the proposed mechanism for this reaction.

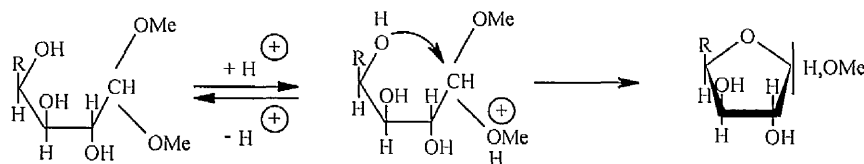
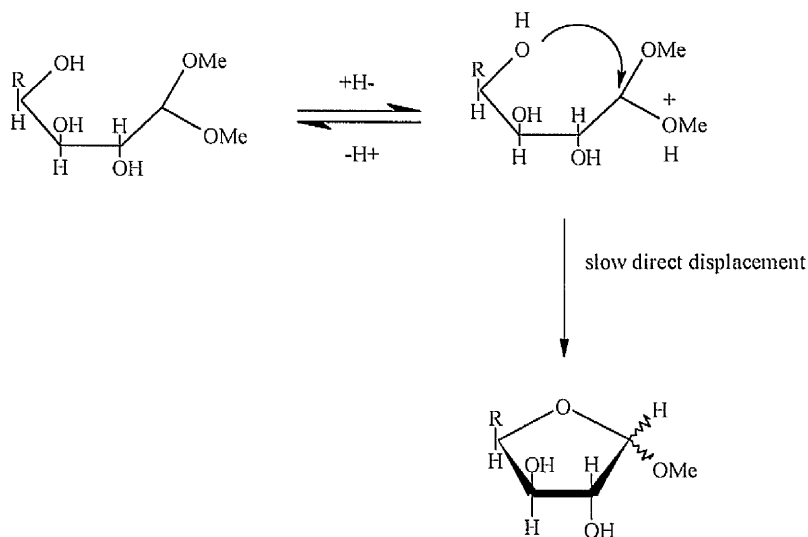


Figure 3-4 The synthesis of a glycoside from an acetal

3.2.4 Helferich Method⁶

6.2.1

This reaction involves heating the acetylated sugar with a phenol in the presence of an acid catalyst, usually zinc chloride or *p*-toluenesulfonic acid. This reaction is useful for the synthesis of aromatic glycosides, *via* replacement of the labile acetoxy group at C-1 by a phenoxy group. A shortcoming of this reaction is that both isomers are formed. The mechanism for this reaction is shown in Figure 3-5.

Figure 3-5 Synthesis of Glycoside *via* Helferich method

3.2.5 Glycosides from Glycosyl Fluorides

Glycosyl fluorides do not react under the conditions of the Koenigs-Knorr reaction, but are easily converted into the glycosides by the action of the corresponding alkoxides in boiling alcohol. This reaction may be used to obtain some of the more inaccessible alkyl α -D- glycosides.

3.2.6 Glycosides from Ortho Esters

Under the slightly basic conditions of the Koenigs-Knorr reaction an alkoxide anion can attack the “closed-ion” that results from participation of an acetoxy group at C-2 in replacement reactions with acetyl glycosylhalides. This results in a stable ortho ester, as shown in Figure 3-6.

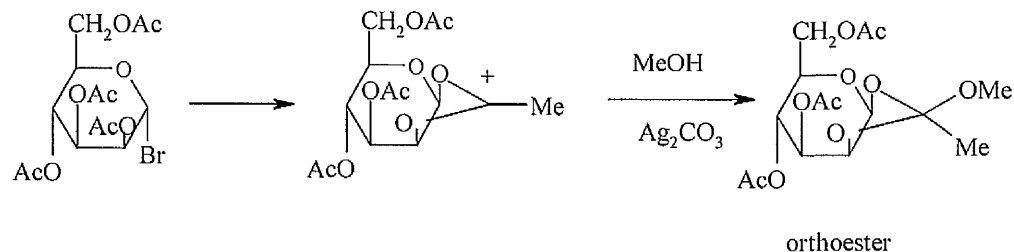


Figure 3-6 Synthesis of glycosides from orthoesters

3.2.7 Enzymatic Glycosylation⁷

Chemical synthesis of alkylglucosides gives a mixture of α - and β -anomers, although conditions can strongly prefer one anomer over the other. Enzymatic synthesis gives stereoselective control. There may be two ways of enzymatically synthesising the alkyl glucosides, through condensation and transfer reactions. Because water is the by-product in enzymatic synthesis by condensation, recovery of the product is easy.

3.2.8 Koenigs-Knorr Reaction

The method chosen for the addition of glucose to the polymers was the Koenigs-Knorr method. As mentioned previously, this reaction involves the treatment of *o*-acetylglucosyl halide with the alcohol or phenol in the presence of a heavy metal salt such as a silver salt, which acts as an acid acceptor. Shinda *et al.* reported using silver oxide in the synthesis of β -D-octyl glucoside.⁸ Numerous variations and improvements have been made to the original method. The use of Drierite (calcium sulphate) is often beneficial, and a preliminary stirring of the tetraacetate, silver salt and solvent is

recommended.¹ The presence of iodine in the reaction mixture may improve the yield,⁹ and the use of mercuric and ferric salts has also been investigated.

The stereochemistry at the anomeric carbon of the resulting glycoside is dependent on the solvolysis of the acylated glycosyl halides. The mechanism of solvolysis can be divided into two main classes, (i) those that involve an “open—ion” intermediate (I) with no participation by a neighbouring group, and (ii) those having a “closed-ion” intermediate (II) resulting from participation by the neighbouring acyloxy group. These ions are shown in Figure 3-7.



Figure 3-7 The possible intermediate structures of the acylated glycosyl halide during the Koenigs-Knorr Reaction

Halides having an acyloxy group at C-2 *cis* to the halogen at C-1 normally react with inversion, whereas the corresponding *trans* halides react with predominant retention of configuration.

3.2.9 Previous work

Attempts at the addition of glucose to polymers *via* the Koenigs-Knorr reaction have been made by Ricardo,¹⁰ using the following general scheme, shown in Figure 3-8.

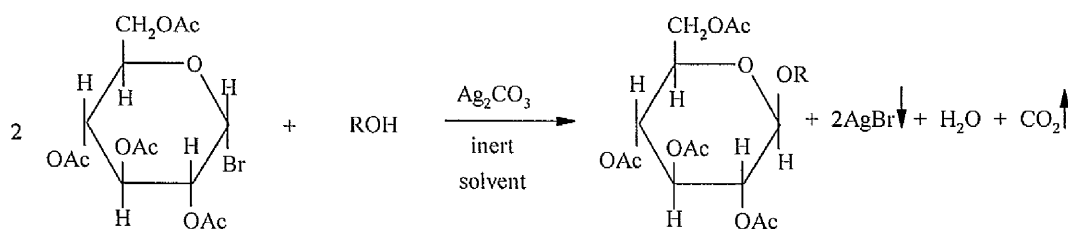


Figure 3-8 Reaction Scheme for Koenigs-Knorr Reaction

In the work done by Ricardo, 2,3,4,6-tetra-*o*-acetyl- α -D glucopyranosyl (TAGB) was reacted with a series of polymers including poly(ϵ -caprolactone) diol, decanol and dodecanol in the presence of silver carbonate as the condensing agent. A desiccant, CaSO_4 was present in the system to reduce the hydrolysis of the TAGB. The alcohol was added very slowly to the TAGB in order to minimise the amount of liberated water, which would compete with the alcohol in reacting with the TAGB. Deacetylation of the condensation product was carried out using a methanol/triethylamine/water mixture (2:1:1 v/v), and the reaction was followed using thin layer chromatography.

The results obtained suggested that the glycosides had been produced, but also suggested that for the PCL glycoside, there had been cleavage at the ester linkages to the initiator unit during deacetylation. This would be expected as deacetylation involves the removal of the protecting groups by breaking an ester bond, and as PCL is a polyester, cleavage of the polymer is expected.

The starting materials used by N. Ricardo was another issue to be considered. The poly(ϵ -caprolactone) diol (Aldrich) was of very high polydispersity due to the presence of numerous low molecular weight oligomers, so an alternative source would be

appropriate. The use of TAGB also purchased from Aldrich was found to be a problem as this compound proved to be thermally and hydrolytically unstable. TAGB cannot be stored for more than a few days, even under a dry inert atmosphere, before degradation to glucose occurs. A synthesis needed to be found to be able to use freshly prepared TAGB.

Before attempting to synthesise the glucosides of poly(ϵ -caprolactone) and poly(D,L-lactide), which have the complication of chain scission during deacetylation, the glucoside of polyethylene glycol was attempted which was considered to be robust to the reaction conditions.

3.3 INTRODUCTION TO SYNTHESIS OF POLYETHYLENE GLYCOL GLUCOSIDE.

The Koenigs-Knorr synthesis was used to prepare polyethylene glycol glucoside. The procedure was based on the work done by Ricardo, but with several modifications.

After identifying the instability of the TAGB, a method for making the TAGB was found so the reactant could be prepared fresh just prior to the Koenig-Knorr reaction. The procedure used by Ricardo involved using an excess of the polymer compared to the TAGB. Due to the instability of the sugar, this was reversed for this synthesis and the TAGB was reacted in excess of the polymer. A molecular sieve was used in Ricardo's synthesis to remove any water present in the system, but this was replaced by Drierite (calcium sulphate) here. Chloroform was also replaced with dichloromethane (DCM) as no difference was found in their function yet DCM is less toxic. An alternative procedure for deacetylation was decided upon, rather than using a mixture of

methanol, triethylamine and water in an overnight reaction, the Zemplén procedure was used.¹¹ This involved using a catalytic amount of sodium methoxide in methanolic solution for one hour.

3.3.1 Preparation of 2,3,4,6-tetra-*o*-acetyl- α -D-glucopyranosyl bromide.¹²

All reagents were used as purchased except 30/40 petroleum ether which was dried over calcium hydride and diethyl ether which was dried over magnesium sulphate. The magnesium sulphate was dessicated in an oven at 200 °C for 3-4 hours.

A 250 ml three necked flask equipped with a magnetic stirrer bar, a dropping funnel and a thermometer was set up. The flask was immersed in an ice-salt bath on a labjack. Acetic anhydride (50 ml, 0.53 mol) was added to the flask and cooled to 4°C. 60% perchloric acid (0.3 ml) was added with stirring and then the cooling bath removed. Once the reaction mixture had warmed to room temperature, dry powdered α -D-glucose (12.5 g, 0.07 mol) was added in small portions with stirring whilst maintaining the temperature of the reaction mixture at between 30 and 40 °C. The reaction mixture was then cooled to 20 °C and red phosphorus (3.88 g, 0.125 mol) was added, followed by bromine (7.5 ml, 1.13 mol) drop wise at such a rate that the temperature did not exceed 20 °C. Distilled water (4.5 ml) was then added over a period of about 15 minutes, whilst continuing to stir and maintaining the temperature below 20 °C. The reaction mixture was then allowed to stand for 2 hours at room temperature.

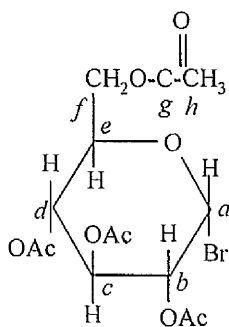
After this period, the reaction mixture was diluted with dichloromethane (40 ml), and filtered though a large glass funnel with a glass wool plug. The reaction flask and

funnel were rinsed with small portions of dichloromethane and then the filtrate and washings were transferred to a separating funnel and washed by rapidly shaking vigorously with two 100 ml portions of iced water (*caution*). The lower organic layer from the second washing was run into a stirred saturated solution of aqueous sodium hydrogen carbonate (65 ml) to which some crushed ice had been added. The mixture was then transferred to a separating funnel and the dichloromethane layer run into a large flask containing anhydrous magnesium sulphate (1.25g). After 10 minutes, the magnesium sulphate was removed by filtration, and the solvent removed under reduced pressure using a rotary-evaporator on a water bath maintained at 60 °C. Towards the end of the solvent removal, the syrup mass crystallises as a thick layer around the inside of the flask. At this stage, the flask was removed and the crystalline mass broken away from the insides of the flask. The remaining solvent was then removed under pressure without further heating. Portions of the solid were then transferred to a mortar and ground with a 2:1 mixture of dry petroleum ether and dry ether. The combined slurry was then filtered and the filtrate washed with the petroleum ether-ether solvent mixture and then with previously chilled dry ether (7 ml). The crude product was recrystallised from the petroleum ether-ether solvent mixture. The resulting purified glucosyl halide (20.24g, 71%) was stored in a flask under nitrogen placed in a desiccator containing indicator silica gel.

3.3.1.1 Characterisation of 2,3,4,6-tetra-O acetyl- α -D glucopyranosyl bromide (TAGB) using ^{13}C NMR.

The structure of TAGB, which is shown below, was confirmed using ^{13}C -NMR. The chemical shifts were compared with work done by K. Bock and C. Pedersen,¹³ showing

that this synthesis was a viable preparation of the starting material. The chemical shifts obtained are shown in Table 3-1 and the spectrum of TAGB is shown in Figure 3-9.



TAGB

Table 3-1 ^{13}C Chemical Shifts for TAGB

Carbon Atom	^{13}C -Chemical Shift
a	86.5
b	70.6
c	72.1
d	67.1
e	70.1
f	60.9
g	169.4-170.5
h	20.5-20.6

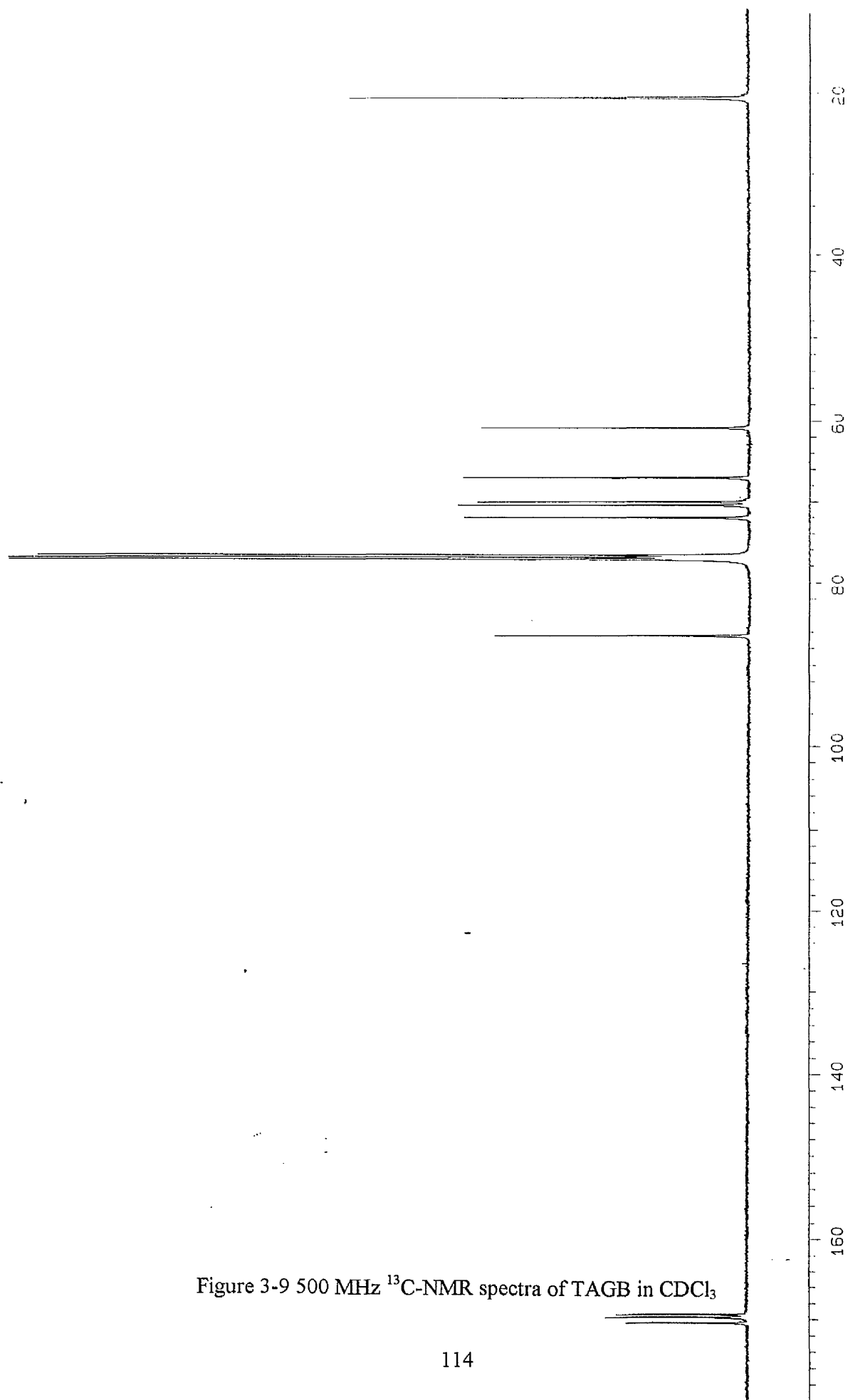


Figure 3-9 500 MHz ^{13}C -NMR spectra of TAGB in CDCl_3

3.3.2 Preparation of Silver Carbonate.

A solution of sodium carbonate (1.02g, 9.623×10^{-3} mol in 11.5 ml of water) was added to a solution of silver nitrate (3.06g, 0.018 mol in 35.6 ml of water), very slowly with vigorous stirring. The silver carbonate was filtered, washed with a little acetone and then air dried. All operations were carried out in dim light in covered vessels.

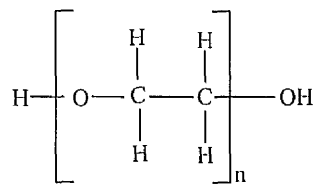
3.3.3 Preparation of Polyethylene Glycol Glucoside.

2,3,4,6-tetra-*O*-acetyl- α -D-pyranosyl bromide (TAGB) (16.0 g, 0.039 mol), silver carbonate (5.73 g, 0.0195 mol), calcium sulfate (32 g) and dry dichloromethane (DCM) (60 ml) were placed in a three necked flask equipped with a dropping funnel. The flask was wrapped in tin foil and all operations were carried out in dim light. The contents of the flask was stirred for 30 min to ensure the complete absence of water. Polyethylene glycol $M_n = 1000$ (20.0 g, 0.02 mol) dissolved in dichloromethane (40 ml) was then charged to the dropping funnel and added dropwise over the course of one hour. The reaction mixture was then further stirred for a period of 48 hours. The DCM was then removed from the reaction mixture by rotary evaporation and remaining solid washed several times with methanol to remove the silver bromide produced and calcium sulphate. The methanol was then removed leaving a viscous yellow solution comprising the unreacted PEG, tetra-*o*-acetyl-glucose (TAG) and the acetylated PEG glucoside. TAGB that had not undergone glycosidation was hydrolysed to TAG during the Koenigs-Knorr reaction by any water present or during washing of the reaction mixture with methanol. The TAG was removed from the reaction mixture by washing with water and filtering off the TAG which is insoluble. The resulting mixture of PEG and acetylated PEG glucoside could not be separated through solvation and precipitation

methods due to the similar solubilities of the two species. Preparative Gel Permeation Chromatography (Prep GPC) was used for isolation of polyethylene glycol glucoside and this is discussed in Section 3.2.4.

The acetylated PEG glucoside and PEG mixture was characterised at this stage using MALDI-TOF MS and the resulting spectrum is shown in Figure 3-11. This was compared with the spectrum for the homopolymer PEG shown in Figure 3-10. Figure 3-11 shows three overlapping groups of peaks corresponding to the homopolymer PEG, PEG end capped with one acetylated sugar, and the final peak corresponds to PEG end capped at both ends with the sugar groups. The spectrum shown in Figure 3-11 was obtained without doping and used as it gave the clearest representation of the three different products. The molecular masses were obtained from a sample doped with potassium salt.

Using the molecular structure of the PEG shown below, the molecular mass can be calculated by multiplying the number of repeat units (n) by the molecular mass of the repeat unit (44.05) plus the addition of the molecular mass of the end groups (17.01) and the molecular mass of potassium (39.10) which it is doped with. (Note that the molecular mass of the end groups takes into consideration of the loss of H^+ during the ionisation process). Table 3-2 shows the calculated predicted molecular weights and the actual observed weight from the spectrum, and there is very good agreement.



$$\text{Molecular mass of PEG} + \text{K}^+ = (44.05 \times n) + 17.01 + 39.10$$

where n = number of repeat units

Table 3-2 Results of MALDI-TOF MS of PEG

n	Predicted molecular mass	Recorded molecular mass
16	760.91	761
17	804.96	805
18	849.01	849
19	893.06	893
20	937.11	937
21	981.16	981
22	1025.21	1025
23	1069.26	1069
24	1113.31	1113
25	1157.36	1157

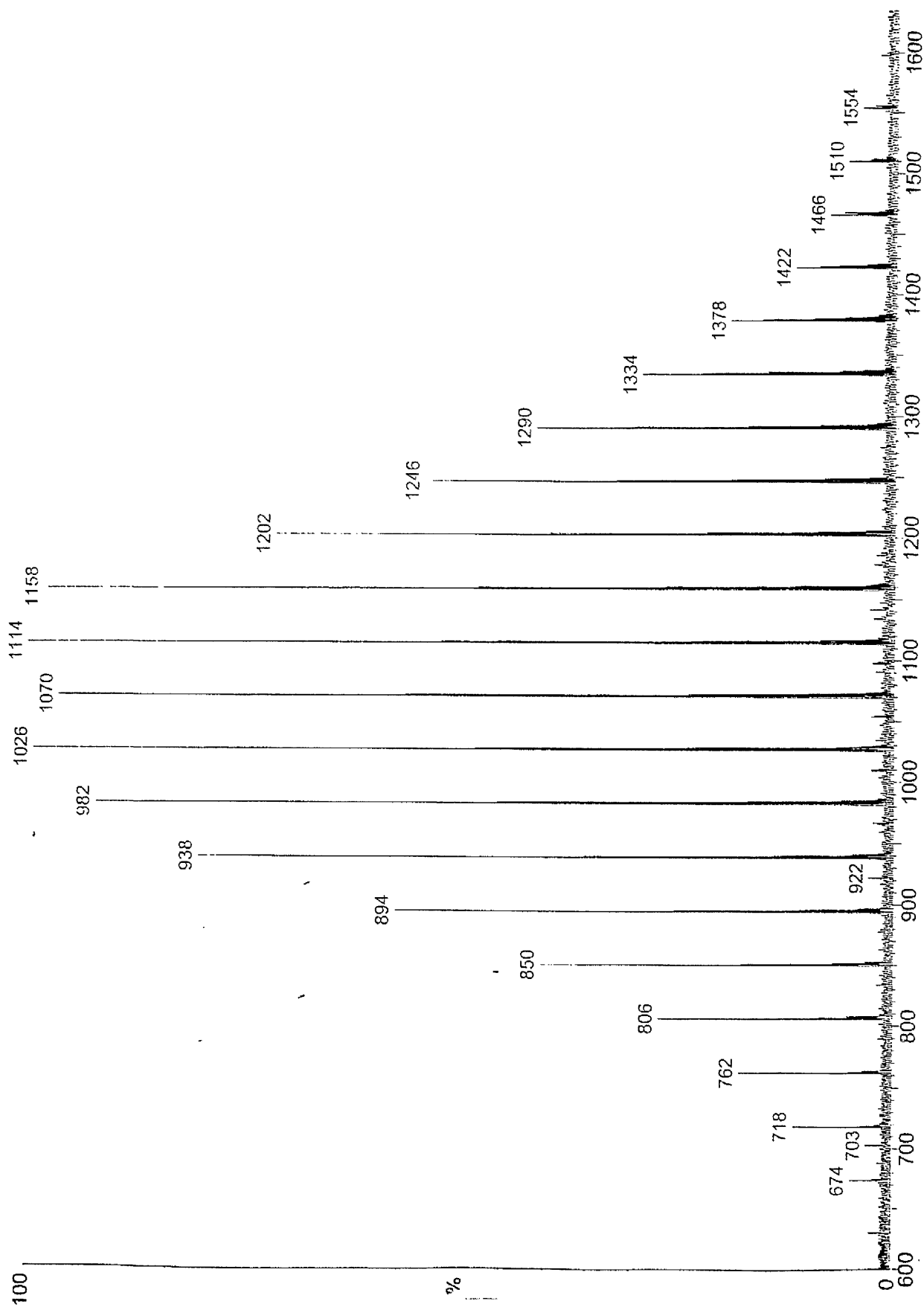


Figure 3-10 MALDI-TOF MS spectrum of PEG

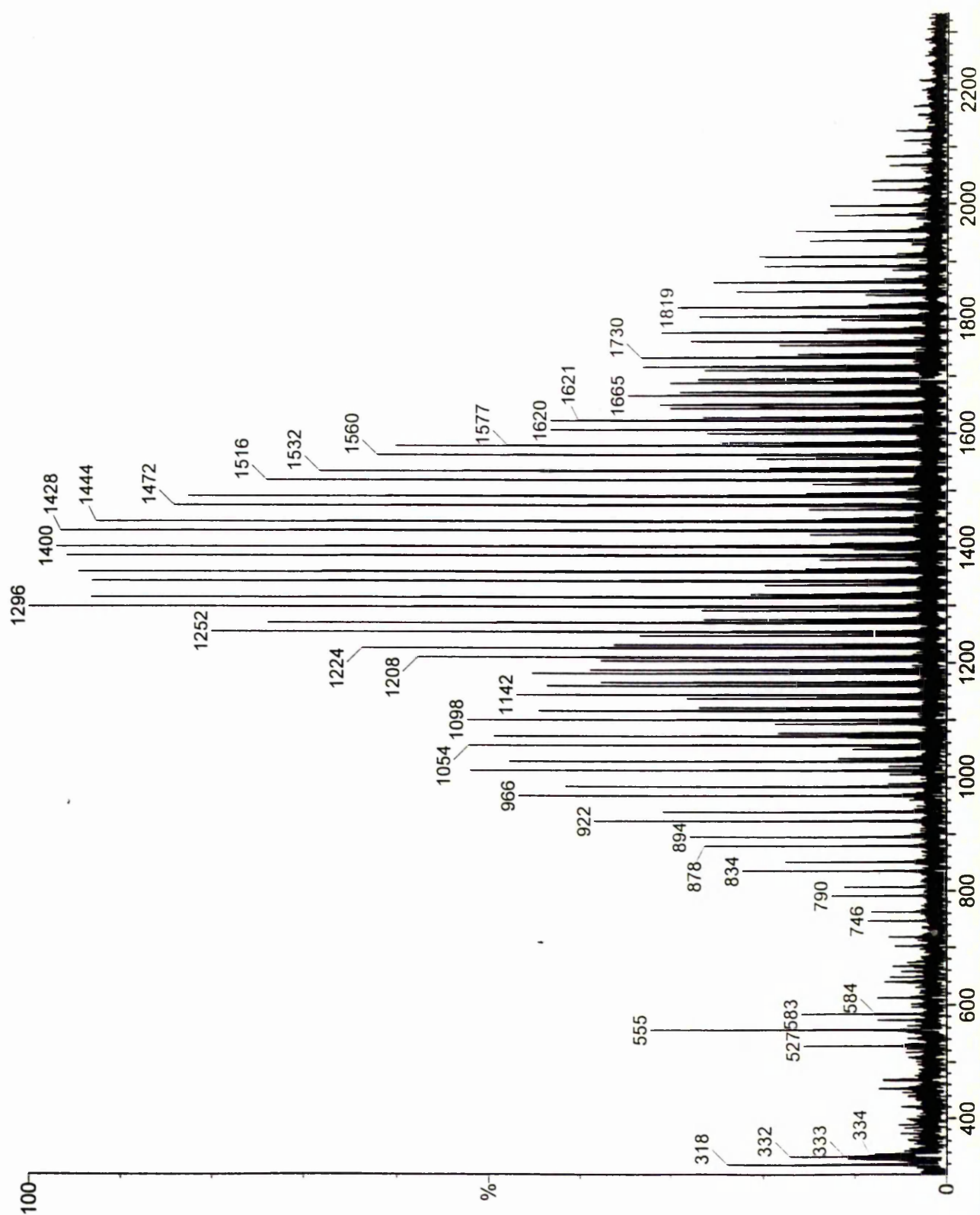
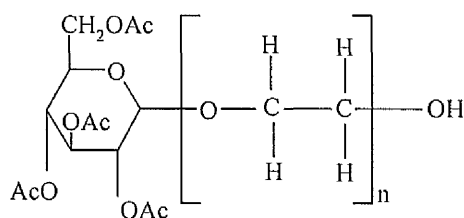


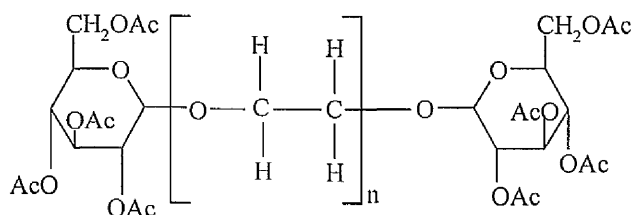
Figure 3-11 MALDI-TOF MS spectrum of PEG and acetylated PEG glucoside

The same method of calculating the molecular mass of the acetylated PEG glucosides can be used by using the molecular masses from the PEG homopolymer above and adding the molecular mass of one or two acetylated sugar end groups. The results are shown in Table 3-3.



Molecular mass of monofunctional Acetylated PEG Glucoside + K^+ (1-A-PEG-G) =

$$\text{MW of PEG} + 331.30$$



Molecular Mass of Difunctional Acetylated PEG Glucoside + K^+ (2-A-PEG-G) =

$$\text{MW of PEG} + (2 \times 331.30)$$

Table 3-3 Results from MALDI-TOF MS of Acetylated PEG glucosides

Predicted MW of PEG	Predicted MW of 1-A-PEG-G	Recorded MW of 1-A-PEG-G	Predicted MW of 2-A-PEG-G	Recorded MW of 2-A-PEG-G
760.91	1091.22	1092	1421.53	1422
804.96	1135.27	1136	1465.58	1466
849.01	1179.32	1180	1509.63	1510
893.06	1223.37	1224	1553.68	1554
937.11	1267.42	1268	1597.73	1598
981.16	1311.47	1312	1641.78	1642
1025.21	1355.52	1356	1685.83	1686
1069.26	1399.57	1400	1729.88	1730
1113.31	1443.62	1444	1773.93	1774
1157.36	1487.67	1488	1817.98	1818

3.3.4 Isolation of Acetylated Polyethylene Glycol Glucoside using Preparative GPC.

Preparative GPC (Prep GPC) is based on the same principle as analytical GPC described in Chapter 2 which involves a solute being passed through a chromatographic column. The solutes movement depends upon the bulk flow of the mobile phase and upon the diffusion of the solute molecules into and out of the stationary phase. The large molecules which rarely enter the stationary phase, move through the chromatographic bed fastest while the smaller molecules which enter the gel pores move slowly through the column, since they spend a proportion of their time in the stationary phase. Molecules are therefore eluted in order of decreasing molecular size. Larger columns tend to be used in Prep GPC than in analytical GPC so that typically grams of solute can be separated rather than milligrams. The different fractions are then collected as they are eluted from the column.

From the spectrum in Figure 3-11, it can be seen that the PEG, monofunctional acetylated PEG glucoside and difunctional acetylated PEG glucoside do not exist as discrete ^{entities} peaks as each peak is only separated by a molecular weight of 330. Prep GPC will not be able to give fractions just containing each individual component, but it would give the opportunity to separate the homopolymer PEG from the glucosides. Each fraction would also give a progressively lower content of glucose which would enable the study of the effect the glucose has on the cryoprotective properties.

The Prep GPC system used consisted of a Sephadex LH-20 column (Pharmacia Biotech) with ethanol as the eluent at a flow rate of $0.5 \text{ cm}^3 \text{ min}^{-1}$. Samples were dissolved in ethanol at a concentration of 0.25% w/v and all experiments were carried out at room

temperature. The eluent was detected using a differential refractometer (Water Associates) and fractions of 6 cm³ were collected at intervals of 12 min. The ethanol from each fraction was removed by rotary evaporation. The remaining product was then analysed using MALDI-TOF MS and ¹³C NMR.

3.3.4.1 Results of Isolation of Acetylated PEG Glucosides using Preparative GPC.

The results of the MALDI-TOF MS from the Prep GPC of the PEG glucosides are shown in Figure 3-12. Fraction 1 predominantly consisted of the difunctional PEG glucoside. Fraction 2 was mainly the monofunctional PEG glucoside. Fractions 3-7 showed a mixture of monofunctional PEG glucoside and PEG with an increasing proportion of PEG in the later eluted fractions. Figure 3-13 shows fraction 3 and this spectra clearly shows the 2 peaks corresponding to PEG and monofunctionalised PEG.

The percentage of hydroxyl groups which underwent glycosylation was 75%.

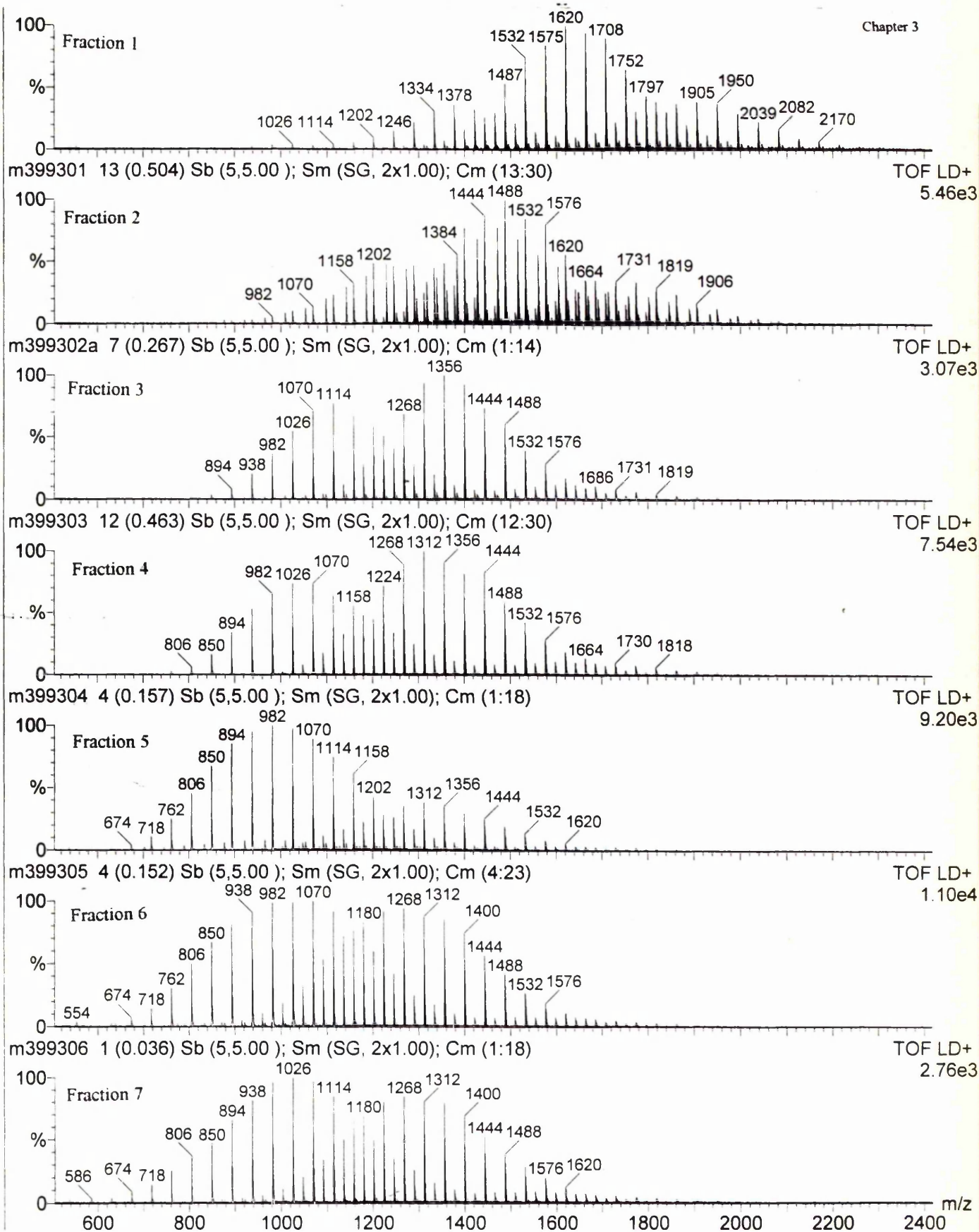


Figure 3-12 MALDI-TOF mass spectra of fractions obtained from Prep GPC of

Acetylated PEG Glucosides

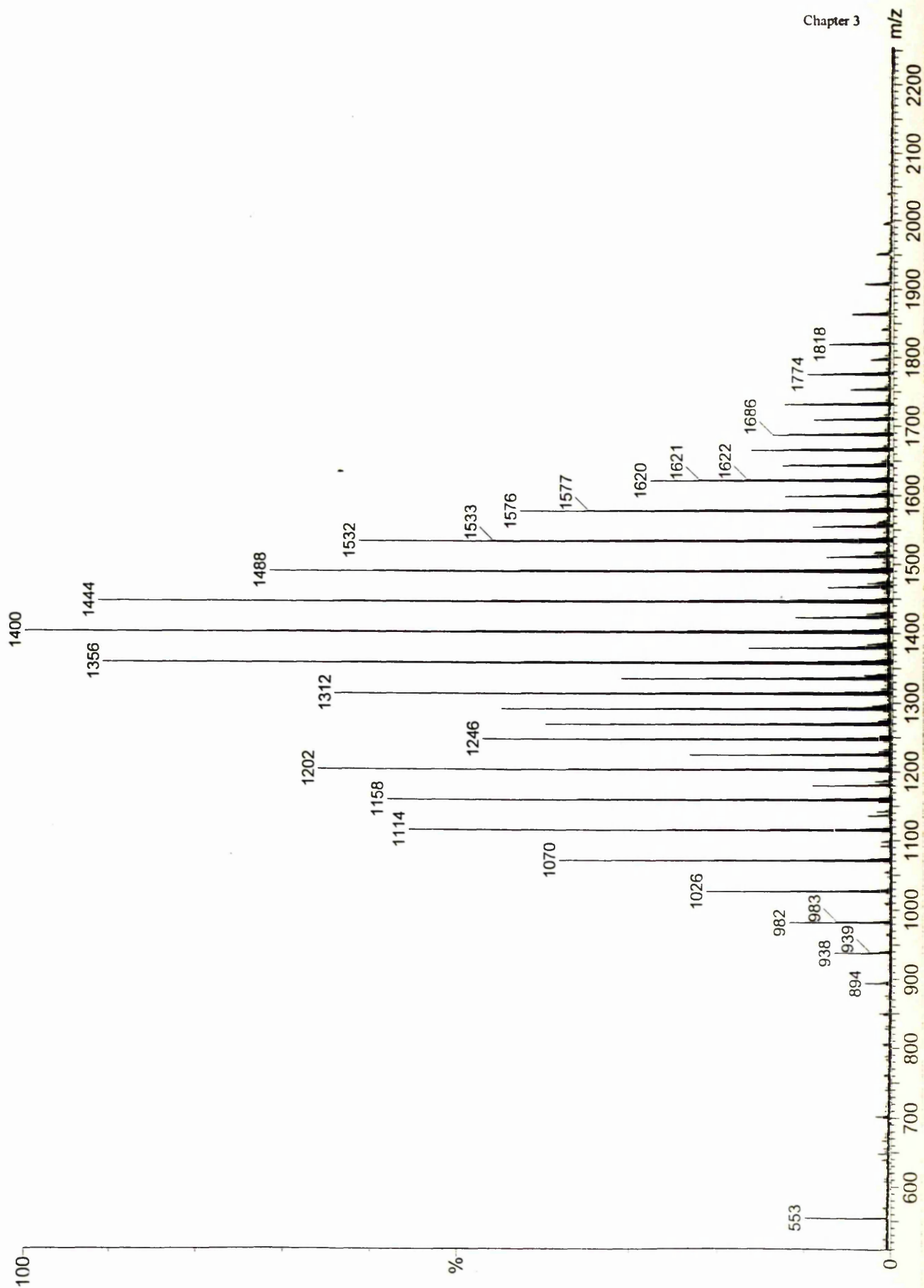


Figure 3-13 MALDI-TOF mass spectrum of fraction 3 from Prep GPC

3.3.4.2 Characterisation of Acetylated PEG Glucosides using ^{13}C -NMR

The polymer from each recovered fraction was analysed using ^{13}C NMR in order to confirm the structure of the acetylated PEG glucoside. Figure 3-14 shows the ^{13}C NMR spectrum of the polymer product obtained from fraction 3. The chemical shifts obtained are displayed in Table 3-4.

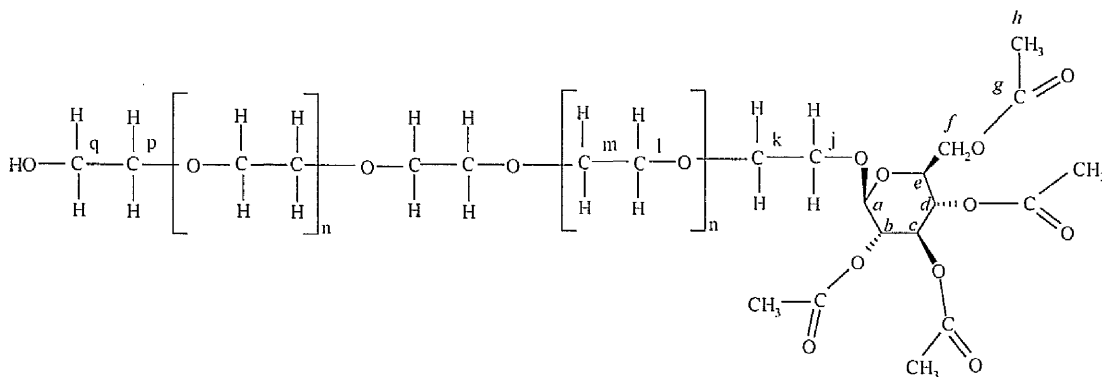


Table 3-4 Chemical Shifts obtained for Acetylated PEG Glucoside

Carbon Atom	Chemical Shift (ppm)
<i>a</i>	100.6
<i>b</i>	71.6
<i>c</i>	72.7
<i>d</i>	68.3
<i>e</i>	71.05
<i>f</i>	61.7
<i>g</i>	169.2
<i>h</i>	20.3-20.5
<i>j</i>	68.9
<i>k</i>	*
<i>l,m</i>	70.0-70.4
<i>p</i>	61.45
<i>q</i>	72.3

* Peak not observed due to being hidden by broad peak at 70.0-70.4

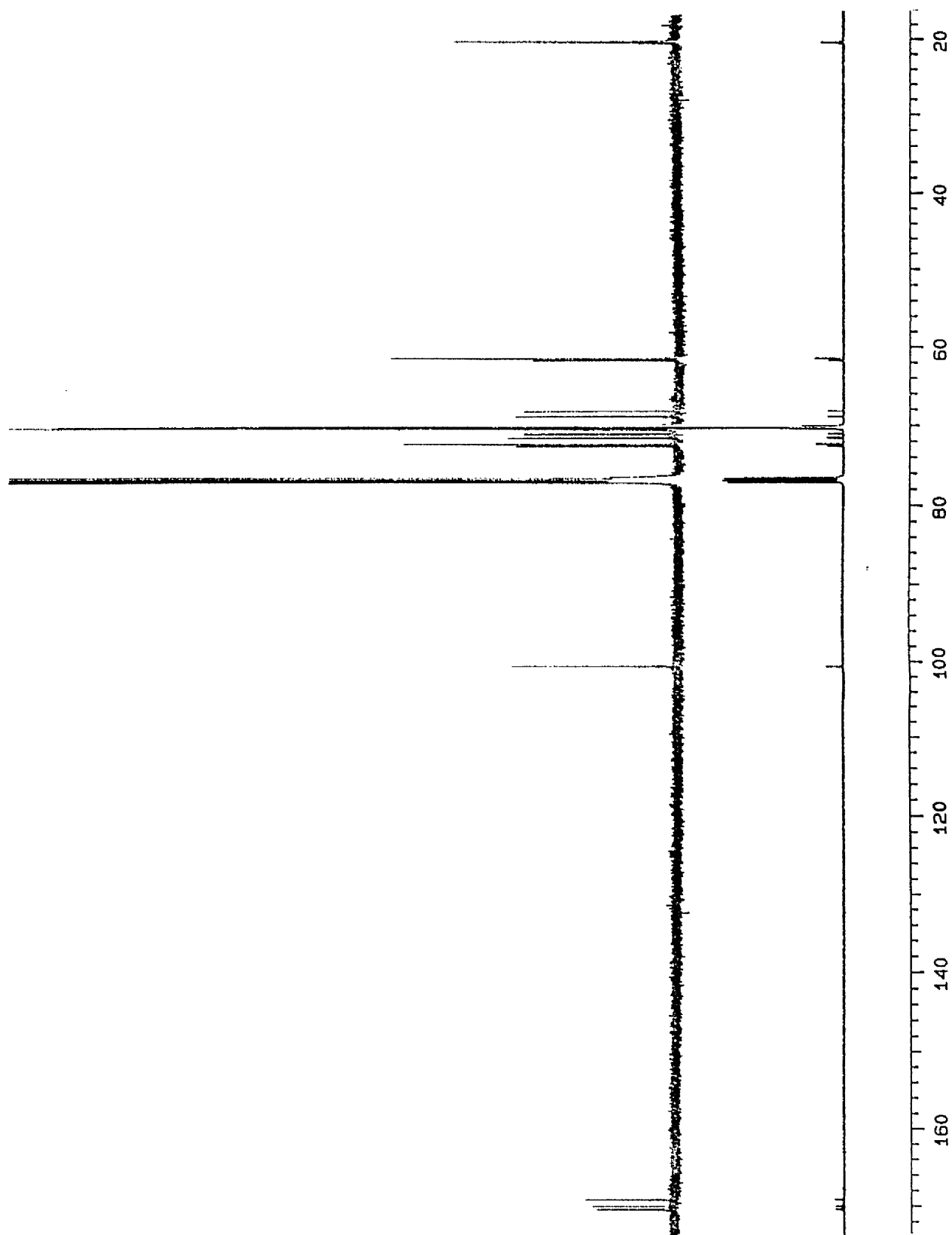


Figure 3-14 500 MHz ^{13}C NMR of fraction 3 from Prep GPC of Acetylated PEG glucoside in CDCl_3

3.3.5 Deacetylation of Acetylated PEG Glucoside

The Zemplén procedure was used to remove the protecting groups from the glucose end groups *via* a deacetylation reaction. The reaction employs sodium methoxide in catalytic amounts in methanolic solution and this is shown in Figure 3-15.

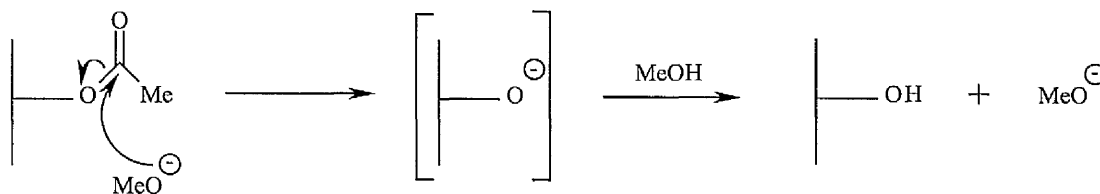


Figure 3-15 Zemplén Reaction

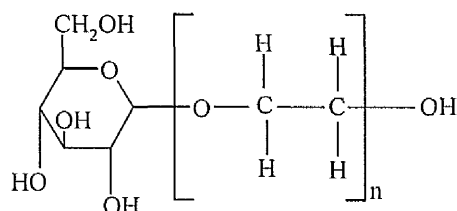
The reaction was terminated by the addition of a lump of solid carbon dioxide to the reaction mixture.

3.3.5.1 Experimental Details of Deacetylation of Acetylated PEG Glucoside.

Firstly, a solution of sodium methoxide in methanol was prepared by the cautious addition of sodium (0.1 g) to methanol (20 ml). A known amount of glucoside was dissolved in dry methanol and 0.66 ml of the methoxide solution was added per mmol of acetylated glucose. The solution was allowed to stand for an hour and after this time a lump of solid carbon dioxide was added to quench the reaction. The solvent was removed under reduced pressure to yield the glucoside product. This was performed for each fraction and the products characterised by MALDI-TOF MS and ^{13}C -NMR.

3.3.6 Characterisation of PEG Glucoside

The structure of the PEG glucoside was confirmed using MALDI-TOF MS and the spectrum for fraction 3 is shown in Figure 3-16. As demonstrated in previous sections, we can confirm the structure of the glucoside by predicting its mass and then seeing if there is any correlation between the predicted and observed values. As a sodium salt was used during the deacetylation, sodium was used to dope the matrix sample rather than potassium. Using the structure below of the PEG glucoside we can predict the mass using the following calculation. The results are shown in Table 3-5.



Molecular Mass of monofunctional PEG Glucoside + Na⁺ =

$$(44.05 \times n) + 17.01 + 162.14 + 22.99$$

Table 3-5 Results obtained from MALDI-TOF MS of PEG glucoside

n	Predicted molecular mass	Recorded molecular mass
19	1040.1	1040
20	1084.15	1084
21	1128.20	1128
22	1172.25	1172
23	1216.30	1216
24	1260.35	1260
25	1304.40	1304

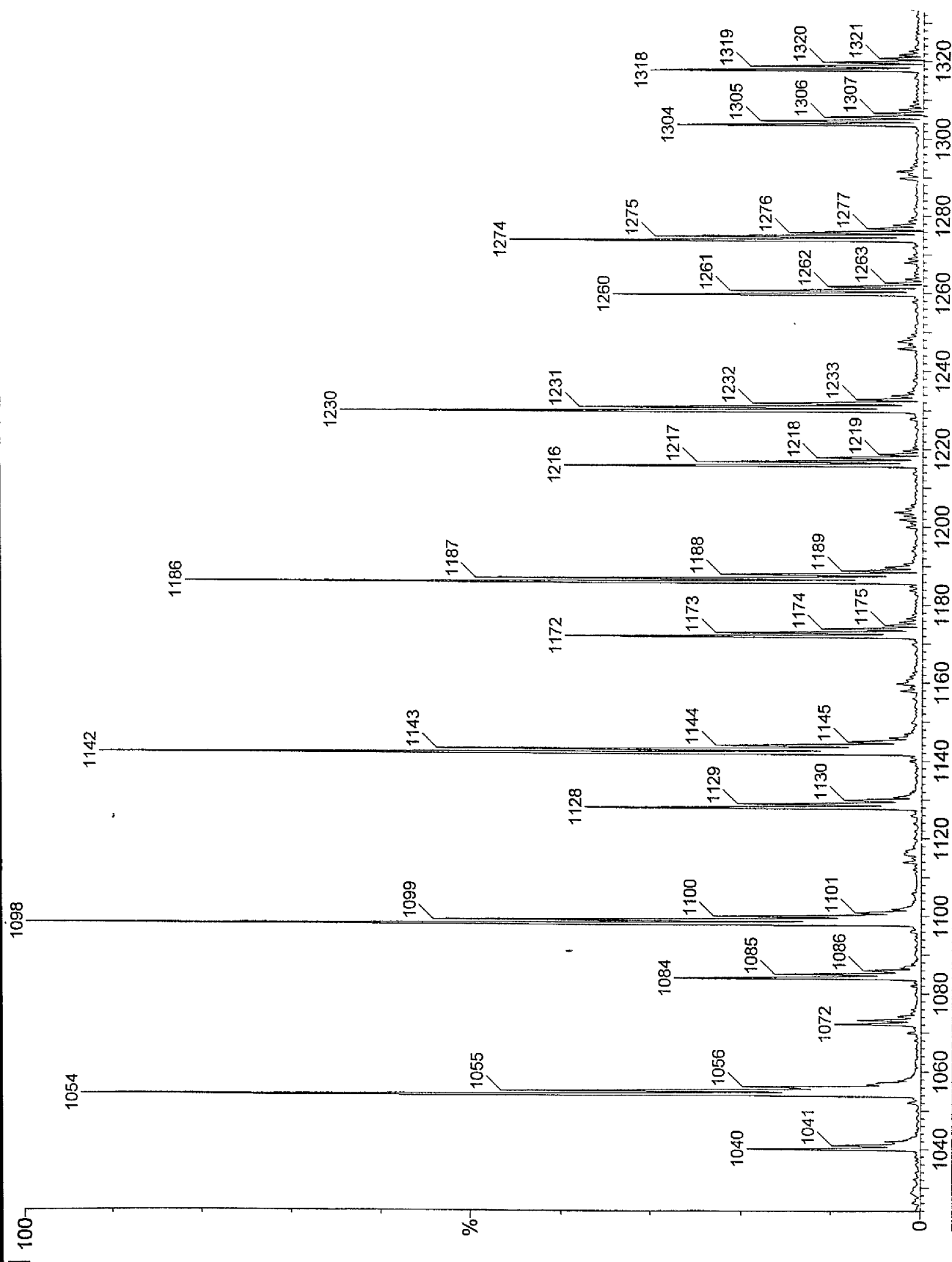


Figure 3-16 MALDI-TOF MS spectrum for PEG glucoside

The structure of PEG glucoside was also confirmed using ^{13}C NMR and Figure 3-17 shows the spectra obtained for fraction 3 and the chemical shifts are displayed in Table 3-6.

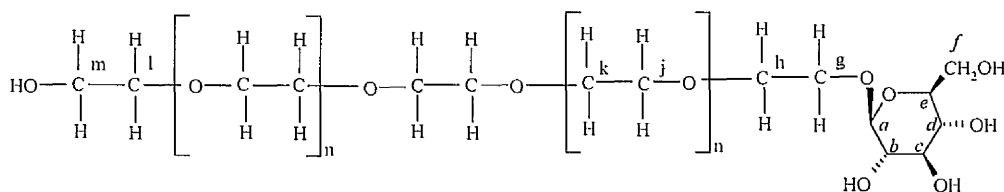


Table 3-6 Chemical Shifts for PEG Glucoside

Carbon Atom	^{13}C Chemical Shift (ppm)
<i>a</i>	104.2
<i>b</i>	74.8
<i>c</i>	78.0
<i>d</i>	71.7
<i>e</i>	77.5
<i>f</i>	63.0
<i>g</i>	69.1
<i>h</i>	*
<i>j, k</i>	71.2-71.3
<i>l</i>	62.0
<i>m</i>	73.5

*Peak hidden by broad peak at 71.2-71.3

The results compared well with previous NMR studies of glucosides.^{14,15}

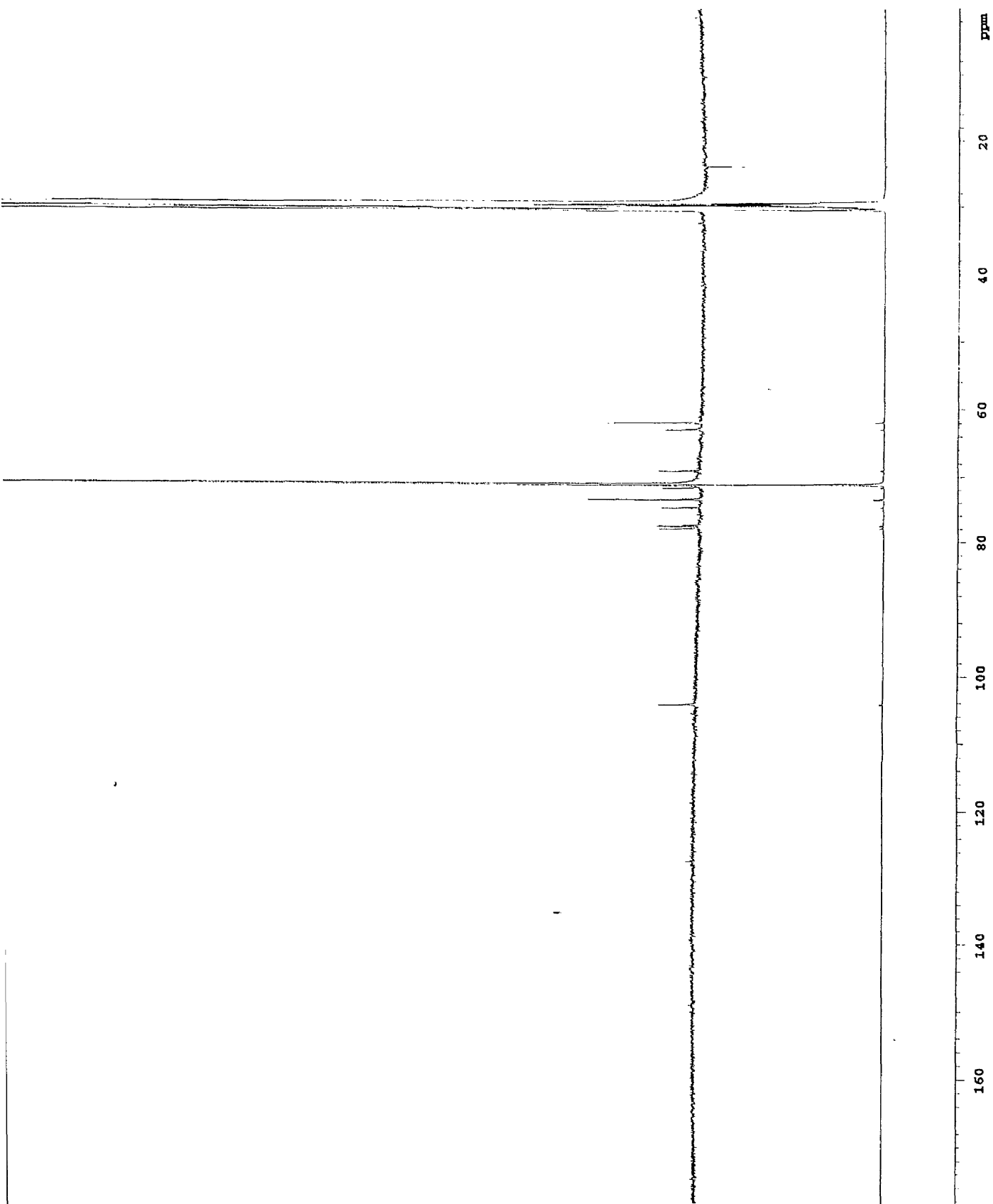


Figure 3-17 500 MHz ^{13}C NMR spectra of PEG Glucoside in CDCl_3

3.4 INTRODUCTION TO THE SYNTHESIS OF POLY(ϵ -CAPROLACTONE) GLUCOSIDE

The synthesis of the PEG glucoside proved successful and so the same general procedure was followed for the synthesis of poly(ϵ -caprolactone) glucoside (PCLG), but with a few modifications.

The first difference was how the deacetylation of the acetylated glucoside was performed. As mentioned in section 3.1.9 with regard to the research performed by Ricardo, deacetylation of the glucosides causes chain scission of the polyester component. To reduce this effect, deacetylation *via* the Zemplen method was performed at 0 °C.

The main distinction from the PEG glucoside synthesis was the purification of the product. Isolation of the PEG glucoside proved to be very difficult due to the fact that the addition of the glucose end-group to the PEG had little effect on its solubility and polarity as it was already a very hydrophilic species. Preparative GPC was performed to separate the PEG from the glucoside product, but this is a long and laborious process and is best avoided. This could be avoided for the separation of the PCL glucoside from the unreacted PCL as the addition of the glucose end groups greatly enhanced the hydrophilicity of the PCL producing a surfactant. This meant that the glucoside product could be isolated by extraction from two immiscible liquids, i.e. water and chloroform.

3.4.1 Preparation of Acetylated Poly(ϵ -caprolactone) glucoside.

2,3,4,6-tetra-*O*-acetyl- α -D-pyranosyl bromide (TAGB) (30.8 g, 0.075 mol), silver carbonate (10.35 g, 0.0375 mol), calcium sulphate (32 g) and dry dichloromethane (75 ml) were placed in a three necked flask equipped with a dropping funnel. The flask was wrapped in tin foil and all operations were carried out in dim light. The contents of the flask was stirred for about 30 min to ensure the complete absence of water. Poly(ϵ -caprolactone) (PCL-1) (15 g, 0.02 mol) dissolved in dichloromethane (40 ml) was charged to the dropping funnel and then added dropwise over the course of about one hour. The stirring was then continued over a period of 48 hours. The DCM was then removed from the reaction mixture by rotary evaporation and remaining solid washed several times with methanol to remove the silver bromide produced and calcium sulphate. The methanol was then removed leaving a viscous yellow solution composing of unreacted PCL, tetra-*o*-acetyl-glucose (TAG) and the acetylated PCL glucoside. TAGB that does not undergo glycosidation is hydrolysed to TAG during the Koenigs-Knorr reaction by any water present or during washing the reaction mixture with methanol. The crude reaction mixture was analysed at this stage using ^{13}C NMR.

3.4.1.1 Characterisation of Acetylated Poly(ϵ -caprolactone) Glucoside

Attempts were made to characterise the acetylated poly(ϵ -caprolactone) glucoside using MALDI-TOF MS but a spectrum was not obtained. The intermediate product was successfully analysed using ^{13}C -NMR and the spectrum obtained is shown in Figure 3-18. The presence of the intermediate compound was confirmed and the chemical shifts obtained are shown in Table 3-7.

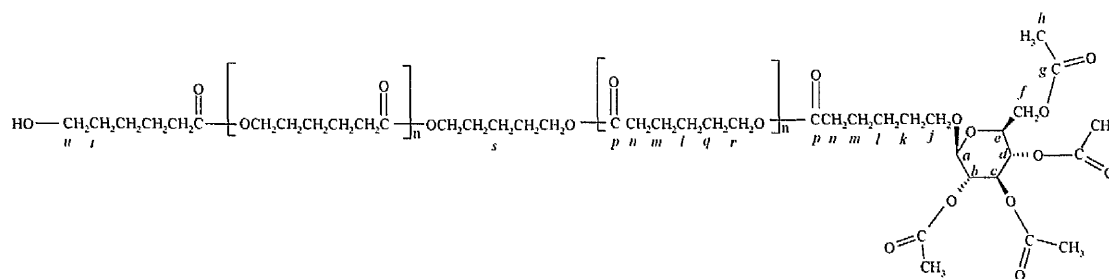


Table 3-7 Chemical Shifts obtained for Acetylated PCL Glucoside

Carbon Atom	^{13}C Chemical Shift (ppm)
<i>a</i>	104.8
<i>b</i>	81.1
<i>c</i>	85.2
<i>d</i>	72.4
<i>e</i>	73.7
<i>f</i>	66.4
<i>g</i>	169.8
<i>h</i>	19.7
<i>j</i>	60.9
<i>k</i>	32.1
<i>l</i>	24.1
<i>m</i>	25.0
<i>n</i>	33.3
<i>p</i>	172.4-172.9
<i>q</i>	27.9
<i>r</i>	63.3
<i>s</i>	21.8
<i>t</i>	32.1
<i>u</i>	60.9

The results compared well with those obtained for the acetylated PEG glucoside, but relatively low yields were obtained, 20% of the hydroxyl groups had undergone glycosylation. This problem is discussed in Section 3.4.2.1, as the same low yields are observed with the synthesis of the PDLG glucoside.

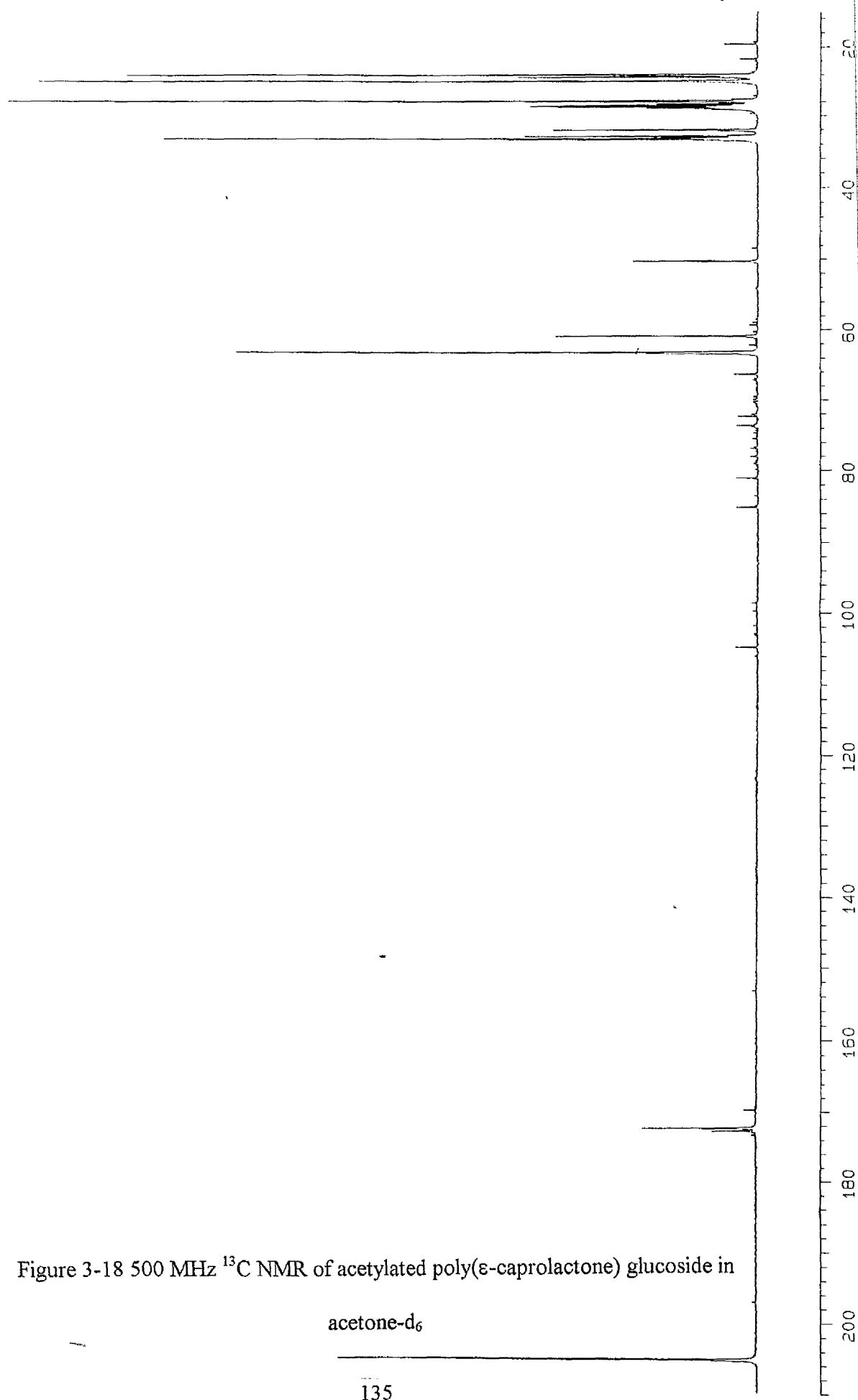


Figure 3-18 500 MHz ^{13}C NMR of acetylated poly(ϵ -caprolactone) glucoside in acetone- d_6

3.4.2 Deacetylation and Isolation of Acetylated Poly(ϵ -caprolactone)

Glucoside

10.0 g of the intermediate mixture consisting of acetylated PCLG, PCL and TAG was then dissolved in methanol (60 ml). The reaction mixture was then placed in an ice bath whilst being stirred to avoid any precipitation of the polymeric products. When the temperature had reached 1 °C, 12 ml of a solution of sodium methoxide in methanol (as described in section 3.2.5.1) was added. The deacetylation reaction was then left for 15 min and after this time the reaction was quenched by adding a lump of solid CO₂.

The intermediate reaction mixture consisted of PCLG, PCL and D-glucose. The insolubility of D-glucose in chloroform meant it was possible to remove it from the mixture by filtration. The solution of PCLG and PCL in chloroform was then washed with water in a separating funnel. An emulsion was formed, probably due to the surfactant-like glycoside molecules emulsifying the chloroform in the water. The water and chloroform layers did eventually separate after a few hours. The water layer contained the PCLG. The chloroform layer was washed two more times with water. The washings were then collected together and the water was removed by rotary-evaporation yielding the product.

3.4.2.1 Characterisation of Poly(ϵ -caprolactone) Glucoside.

Attempts to characterisation of poly(ϵ -caprolactone) glucoside using MALDI-TOF MS were made but this technique did not yield a spectrum. The structure of PCLG was confirmed using ¹³C-NMR and the spectrum obtained is shown in Figure 3-20. The chemical shifts for this molecule are listed in Table 3-8.

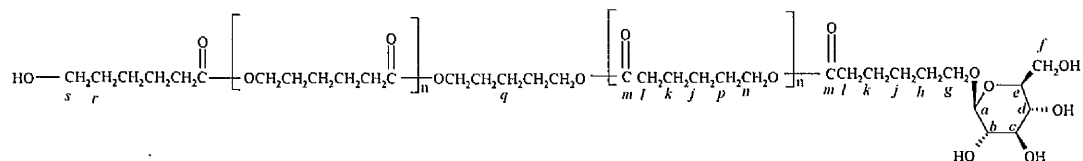


Table 3-8 Chemical shifts for PCLG

Carbon Atom	^{13}C Chemical Shift (ppm)
<i>a</i>	103.2
<i>b</i>	73.5
<i>c</i>	76.2
<i>d</i>	69.9
<i>e</i>	75.9
<i>f</i>	63.8
<i>g</i>	60.8
<i>h</i>	31.2
<i>j</i>	24.2
<i>k</i>	24.9
<i>l</i>	34.0
<i>m</i>	174.4
<i>n</i>	27.7
<i>p</i>	61.1
<i>q</i>	20.75
<i>r</i>	31.2
<i>s</i>	60.8

The isolation of the product was confirmed, but it was noted that chain scission of the poly(ϵ -caprolactone) component had occurred. This was evident from the comparison of the number of repeat units (*n*) of the starting polymer and the glycosylated polymer.

Using the values obtained from NMR for PCL-1,

$$n = 2.95 \text{ and } M_n = 670$$

For PCLG,

$$n = 1.82 \text{ and } M_n = 430$$

It can be seen there has been a significant decrease in the PCL moiety of PCLG. ^{Content} This is also observed with the GPC traces in Figure 3-19. It must be noted that the following results were for different experimental conditions but demonstrate the scission effect very clearly. PCL was left in the presence of the sodium methoxide in methanol as described in Section 3.3.2 for 1 hour rather than 15 minutes.

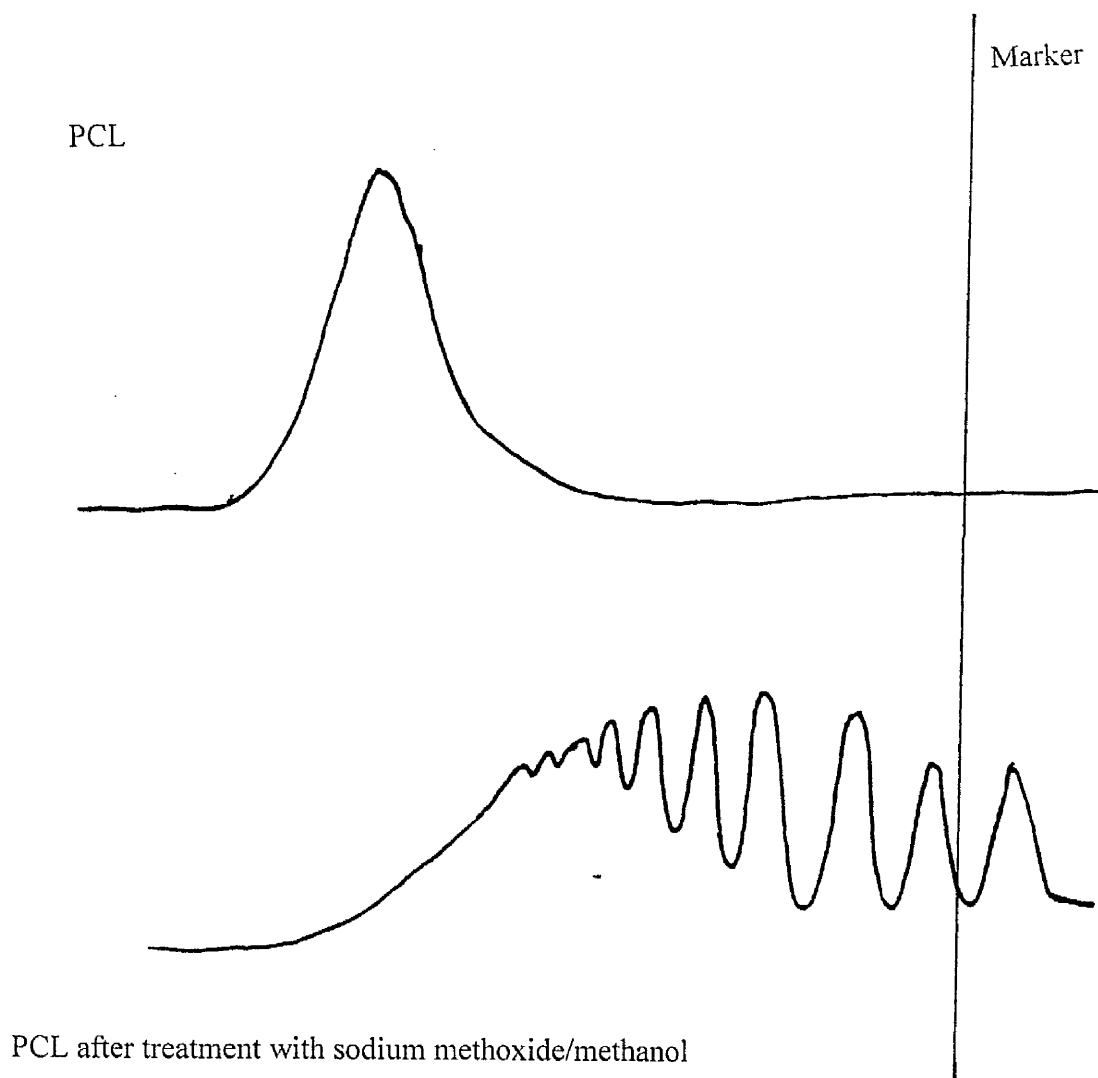


Figure 3-19 GPC traces showing chain scission of PCL

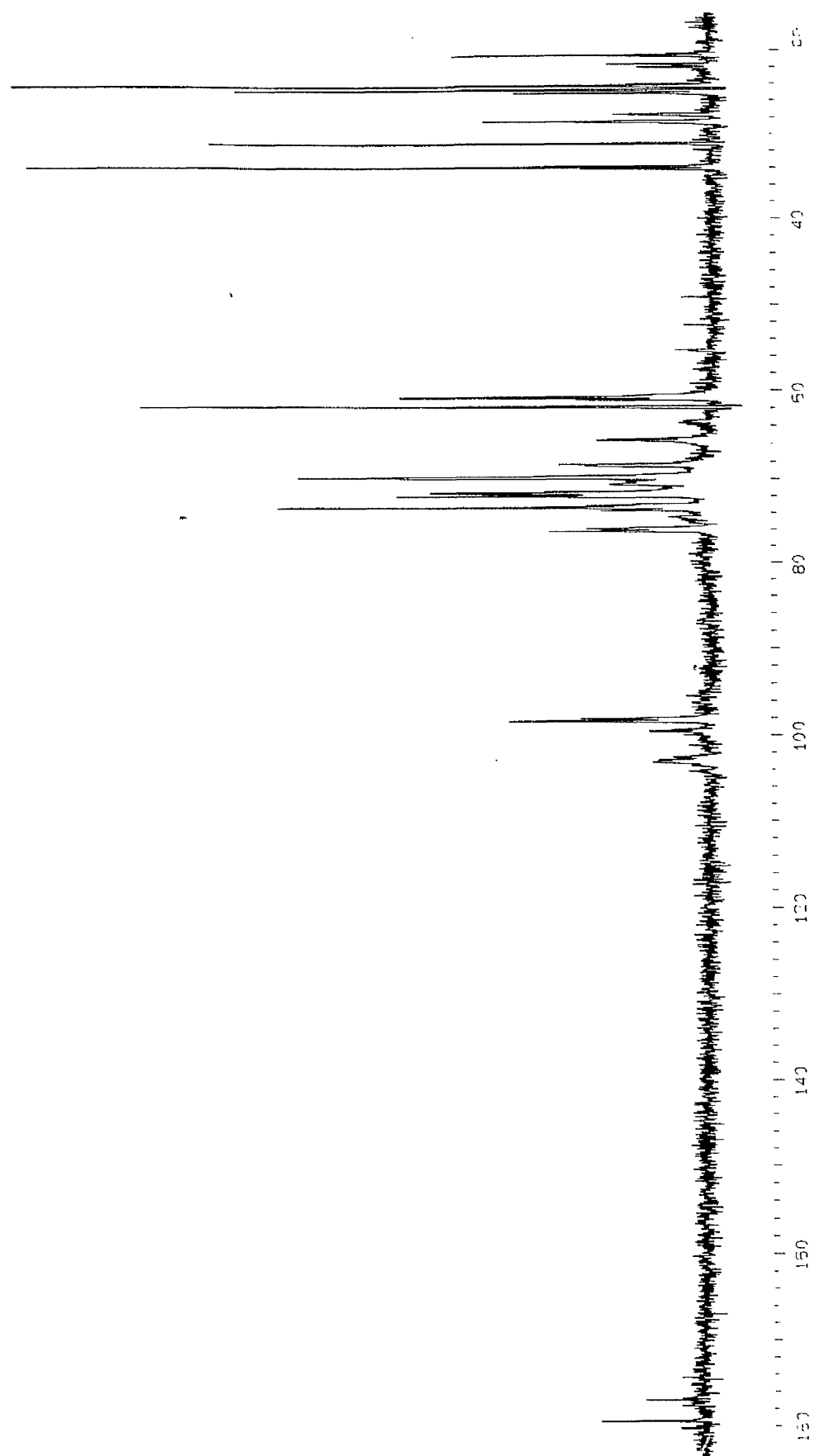


Figure 3-20 500 MHz ^{13}C NMR spectra of poly(ϵ -caprolactone) glucoside in D_2O

3.5 INTRODUCTION TO THE SYNTHESIS OF POLY(D,L-LACTIDE) GLUCOSIDE

The synthesis of poly(D,L-lactide) glucoside was carried out in the same way as for synthesis of poly(ϵ -caprolactone) glucoside.

3.5.1 Preparation of Acetylated Poly(D,L-lactide) glucoside.

2,3,4,6-tetra-*O*-acetyl- α -D-pyranosyl bromide (TAGB) (31.5 g, 0.075 mol), silver carbonate (10.35 g, 0.0375 mol), calcium sulphate (32 g) and dry dichloromethane (75 ml) were placed in a three necked flask equipped with a dropping funnel. The flask was wrapped in tin foil and all operations were carried out in dim light. The contents of the flask were stirred for about 30 min to ensure the complete absence of water. Poly(D,L-lactide) (PDLL-2) (15 g, 0.034 mol) dissolved in dichloromethane (40 ml) was then added dropwise over the course of about one hour. The stirring was then continued over a period of 48 hours. The DCM was then removed from the reaction mixture by rotary evaporation and the remaining solid washed several times with methanol to remove the silver bromide produced and calcium sulphate. The methanol was then removed leaving a viscous yellow solution composed of unreacted PDLL, tetra-*o*-acetyl-glucose (TAG) and the acetylated PDLL glucoside. TAGB that does not undergo glycosidation is hydrolysed to TAG during the Koenigs-Knorr reaction by any water present or during washing the reaction mixture with methanol. The crude reaction intermediate was then characterised by MALDI-TOF MS and ^{13}C NMR.

3.5.1.1 Characterisation of Acetylated Poly(D,L-Lactide) Glucoside.

MALDI-TOF MS was used to characterise acetylated poly(D,L-lactide) glucoside and the mass spectrum obtained is shown in Figure 3-22, with an expansion of the spectrum shown in Figure 3-23. The molecular mass was calculated and compared to the values obtained from the mass spectrum, and this confirmed the presence of acetylated poly(D,L-lactide) glucoside. The results obtained are shown in Table 3-9.

$$\text{Molecular mass of PDLL} + \text{K}^+ = (72.06 \times n) + 17.01 + 39.10$$

$$\text{Molecular mass of PDLLG} + \text{K}^+ = \text{Molecular mass of PDLL} + 331.30$$

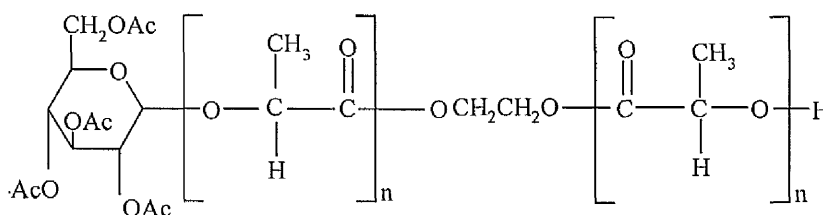


Table 3-9 Results from MALDI-TOF MS of Acetylated PDLL

n	Predicted molecular mass	Recorded molecular mass
2	574.58	575
3	646.64	647
4	718.70	720
5	790.77	791
6	862.83	863
7	936.47	935
8	1007.53	1008
9	1079.59	1080
10	1151.66	1152
11	1223.72	1224
12	1295.78	1296
13	1367.84	1368
14	1439.91	1440

It can be seen from these results that the Koenigs-Knorr synthesis has yielded the desired product, but the yield of 20% was considerably lower than the corresponding

PEG. It can also be seen that addition of the glucose to PDL of a molecular mass of 1300 or higher did not occur. This could be due to a solubility effect or the reactivity of the glycosyl acceptor, i.e the alcohol or polymer with hydroxyl end groups in this case.. All classes of alcohols can be used in the Koenigs-Knorr reaction but the efficiency decreases with increasing complexity of the alcohol.¹⁶ This is demonstrated in Figure 3-21 by the silver carbonate promoted glycosylation of methanol, ethanol, *n*-propanol and *t*-butanol.¹⁷

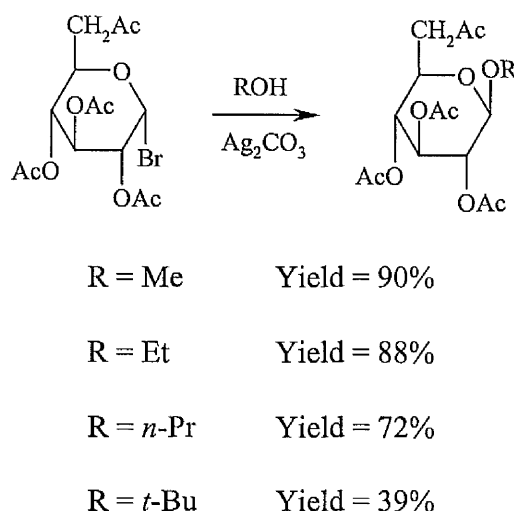


Figure 3-21 Silver carbonate promoted glycosylation of various alcohols.

These results show that with increasing molecular weight of the alcohol, a decrease in yield is observed. This concept could be translated to the use of polymers instead of alcohols. With increasing molecular weight of a polymer, a decrease in the yield of polymeric glucoside may be observed. The reduction in yield from the Koenigs-Knorr reaction with increasing molecular weight of alcohol could explain why the addition of glucose to PDL of a molecular mass of 1300 or higher did not occur.

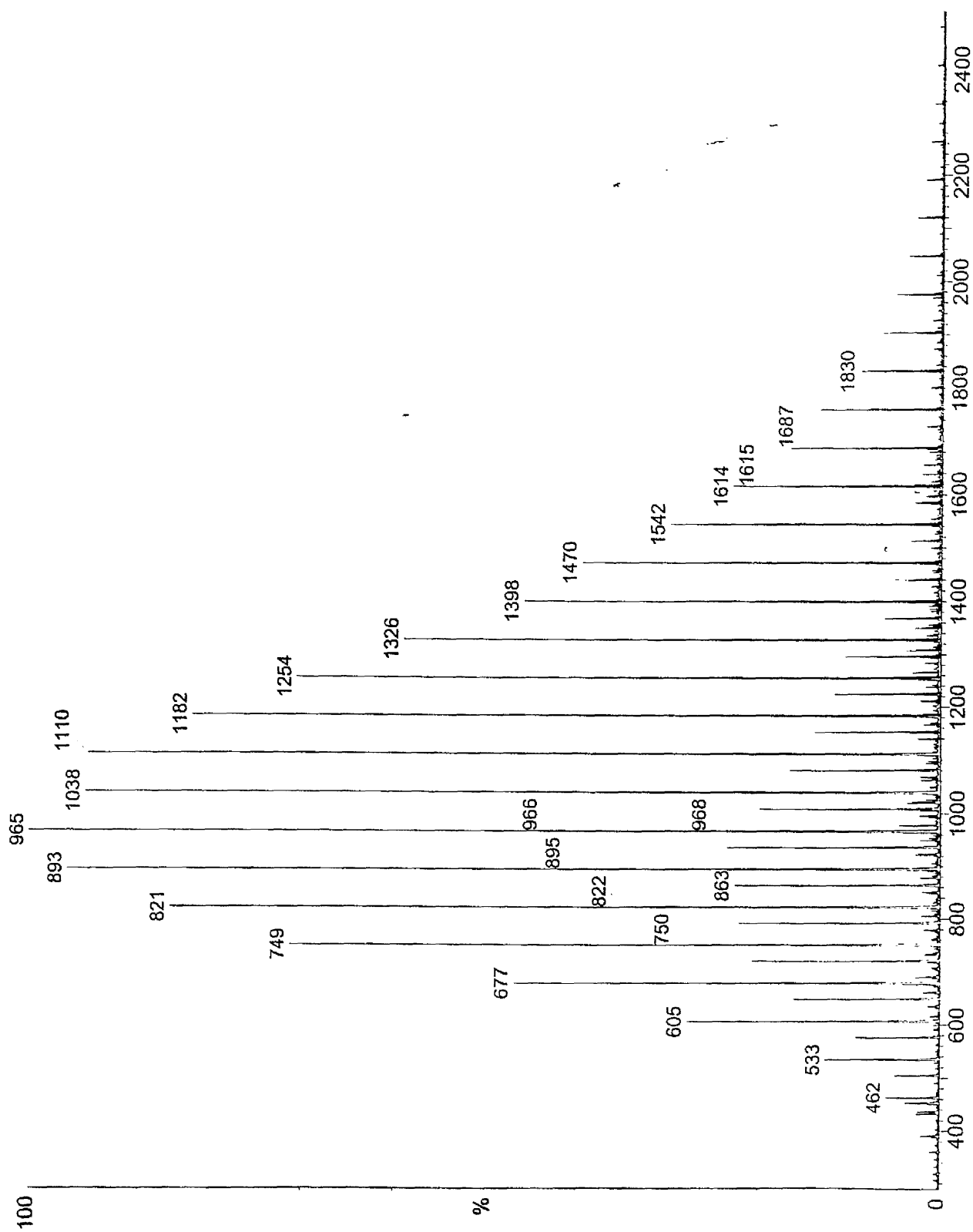


Figure 3-22 MALDI-TOF mass spectra of acetylated poly(D,L-lactide glucoside) and poly(D,L-lactide)

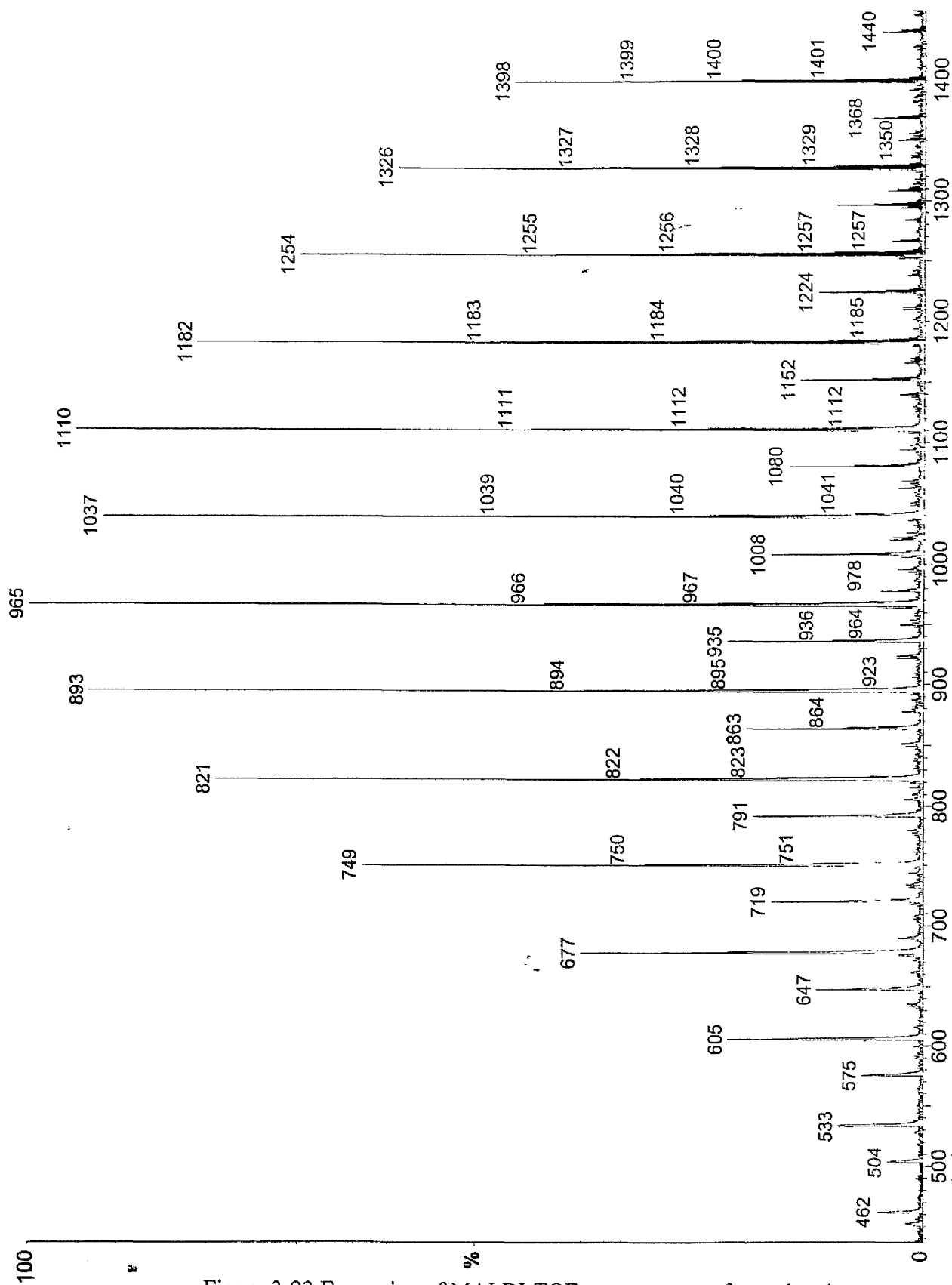


Figure 3-23 Expansion of MALDI-TOF mass spectra of acetylated poly(D,L-lactide glucoside) and poly(D,L-lactide)

3.5.2 Deacetylation and Isolation of Acetylated Poly(D,L-Lactide)

Glucoside

10.0 g of the intermediate mixture consisting of acetylated PDLLG, PDLL and TAG was then dissolved in 60 ml of methanol. The reaction mixture was then placed in an ice bath whilst being stirred to avoid any precipitation of the polymeric products. When the temperature had reached about 1 °C, 12 ml of a solution of sodium methoxide in methanol (as described in section 3.2.5.1) was added. The deacetylation reaction was then left for 15 min and after this time the reaction was quenched by adding a lump of solid CO₂.

The reaction mixture now consisted of PDLLG, PDLL and D-glucose. The insolubility of D-glucose in chloroform meant it could be easily removed from the mixture by filtration. The solution of PDLLG and PDLL in chloroform was then washed with water in a separating funnel. An emulsion was formed, probably due to the surfactant molecules emulsifying the chloroform in the water. The water and chloroform layers did separate after being left for a few hours, allowing the isolation of the water layer containing the surfactant. The chloroform layer was washed two more times with water. The washings were then collected together and the water was removed by rotary-evaporation yielding the product.

3.5.2.1 Characterisation of Poly(D,L-Lactide) Glucoside

Attempts were made to obtain a MALDI-TOF mass spectrum of poly(D,L-lactide) glucoside but this technique did not yield any results. The ^{13}C NMR spectra obtained is shown in Figure 3-25 and the chemical shifts displayed in Table 3-10

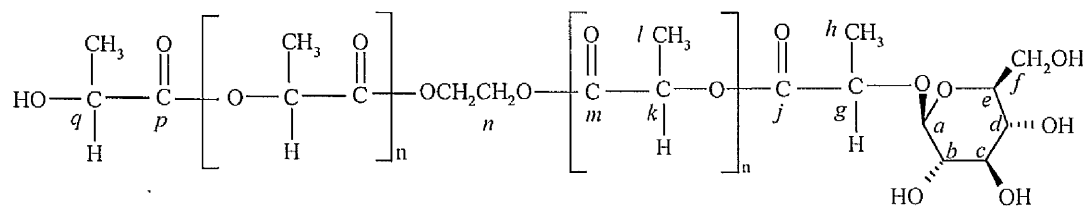


Table 3-10 Chemical Shifts obtained for PDLL Glucoside

Carbon Atom	^{13}C Chemical Shift (ppm)
<i>a</i>	103.9, 100.4
<i>b</i>	74.5
<i>c</i>	77.8
<i>d</i>	71.7
<i>e</i>	77.2
<i>f</i>	62.7
<i>g</i>	66.4-66.6
<i>h</i>	20.1-20.4
<i>j</i>	174.6-174.8
<i>k</i>	68.4-68.7
<i>l</i>	16.6-16.7
<i>m</i>	169.0-169.7
<i>n</i>	62.7
<i>p</i>	174.9-175.0
<i>q</i>	66.6-66.7

The above results confirm the presence of poly(D,L-lactide) glucoside, but it is observed that there has been chain scission and hence a reduction in the number of repeat units of the poly(D,L-lactide) component. The degree of chain scission could be quantified by comparing the NMR spectra of the starting polymer (PDLL-2) and the polymer component of PDLLG.

Using the values obtained from NMR for PDLL-2,

$$n = 5.07 \text{ and } M_n = 570$$

For PDLLG,

$$n = 1.54 \text{ and } M_n = 320$$

It can also be observed that deacetylation has not gone to completion, but the spectrum shown in Figure 3-26 shows the result of complete deacetylation. This spectrum shows the result of allowing the deacetylation reaction to proceed for 1 hour and it indicates that there has been complete chain scission, as there is no peak for the repeat unit of PDLL. The complete deprotection of the glucose end groups by the Zémplen procedure is at the expense of intactness of the PDLL moiety.

Another interesting observation from these NMR spectra is the presence of two peaks in the anomeric carbon atom region. This is probably due to the production of the α and β anomers, which are designated according to the location of the hydroxyl group, or in this case the substituted hydroxyl group. The α has the hydroxyl group *down* and the β has the hydroxyl group *up*. Nilsson and Söderman studied the α and β anomeric structures of n-octyl- α -D-glycoside and found that for the α -linkage, the hydrocarbon chain was attached to the glucose molecule equatorially, where as for the β -linkage, it was attached axially.¹⁸ They produced the representation of the anomeric forms shown in Figure 3-24.

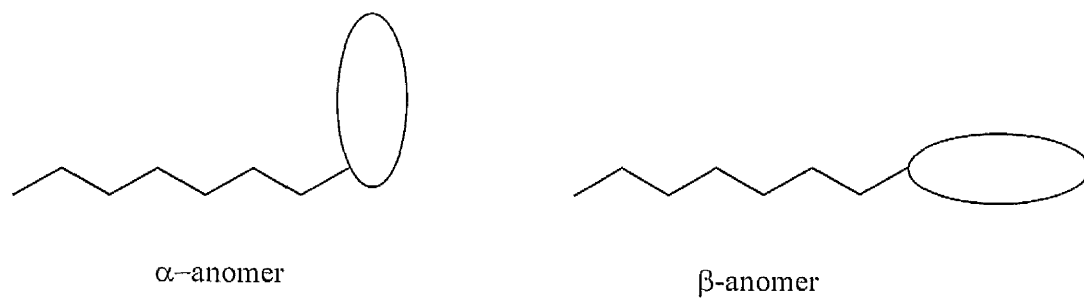


Figure 3-24 Representations of α - and β -anomeric configuration of n-octyl β -D-Glucoside

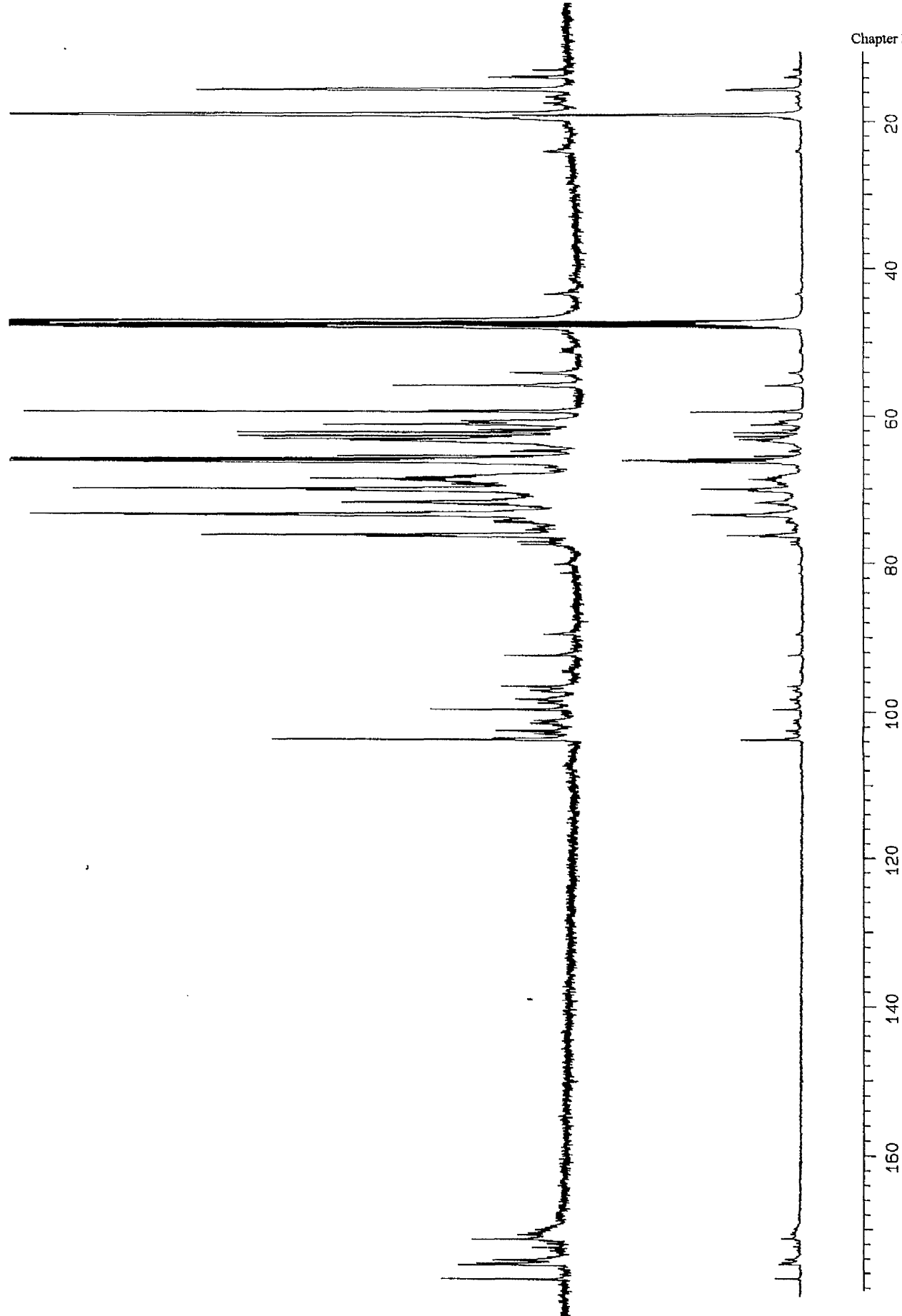


Figure 3-25 500 MHz ^{13}C NMR of Poly(D,L-lactide) Glucoside in CD_3OD

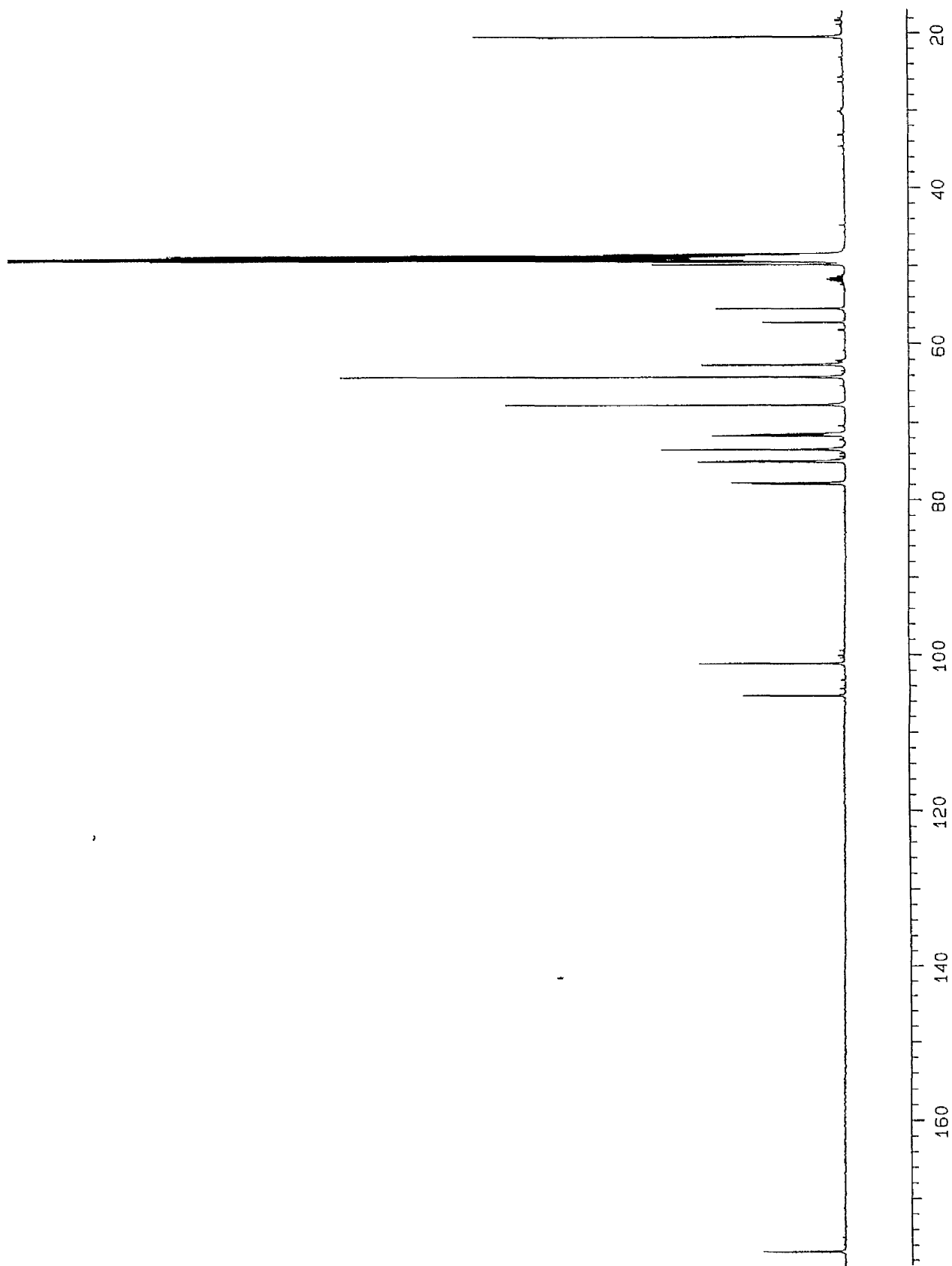


Figure 3-26 500 MHz ^{13}C NMR showing complete chain scission of Poly(D,L-lactide)

Glucoside in CD_3OD

3.6 CONCLUSIONS FROM CHAPTER THREE

The Koenigs-Knorr reaction was successfully used to synthesise poly(ethylene glycol) glucoside. The reaction yielded PEG end capped with two acetylated glucose groups, PEG end capped with one acetylated glucose group and PEG that had undergone no reaction. The use of MALDI-TOF mass spectrometry in conjunction with Preparative GPC greatly facilitated the isolation and characterisation of the products.

The reaction was repeated for poly(ϵ -caprolactone) and poly(D,L-lactide). It was hoped that the glucoside product would have surfactant-like properties and could be easily isolated from the more hydrophobic homopolymer. The difference in solubility was hoped to give a good method to isolate the glucoside products, rather than resorting to the more laborious Prep GPC. This did work to some extent, but the hydrophobic nature of the homopolymer seemed to have an effect on the yield after the Koenigs-Knorr reaction. It was also noted that only the lower molecular weight oligomers reacted during the reaction. It is known that the more complex the alcohol used in the Koenigs-Knorr reaction, the less efficient the reaction is. However the higher molecular weight poly(ethylene glycol) was observed to react during the Koenigs-Knorr reaction. The reason for the low yields observed for the glycosidation of PCL and PDLL may be due to the reactivity of the hydroxyl end group of the polymer. The reactivity of the hydroxyl end groups of different types of polymers in the Koenigs-Knorr reaction needs to be investigated.

The deacetylation of PCL and PDLL glucosides proved to have detrimental effects on the polymer component of the compound. This was expected but it was hoped that performing the deacetylation at low temperatures, would reduce the scission of the

polymer. This synthetic route is more ideal for polymers such as PEG which can withstand the harsh deacetylation conditions. Alternative synthetic routes for the production of PCL and PDLL glucosides are discussed in Chapter 5.

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4. Studies of Stabilisation Effects of Polymeric Glucosides on β -Galactosidase

4.1 INTRODUCTION

In this chapter, the ability of the polymeric glucosides discussed in Chapter 3 to stabilise the protein β -galactosidase during freeze drying will be discussed. The reasons for using this enzyme assay and its effectiveness will be addressed. Details of experimental procedures using the assay and the results obtained are also discussed in this chapter.

4.1.1 Introduction to Enzymes

Enzymes are a class of proteins which act as catalysts of biological systems. Enzymes are highly effective at catalysing chemical reactions due to their ability to specifically bind to substrates. The enzymes bring together the substrates in an optimal orientation and in essence stabilise transition states, the highest energy species in a reaction pathway. Enzymes are highly specific and determine which one of several potential chemical reactions actually occurs. Their specificity originates from the region of the enzyme where the substrate binds, which is called the active site. The active site is a three dimensional cleft or crevice where the substrate is bound by multiple weak attractions. The specificity of binding depends on the precisely defined arrangement of atoms in an active site. To fit into the site, a substrate must have a matching shape, as expressed in Emil Fischer's metaphor of the lock and key model, as shown in Figure 4-1

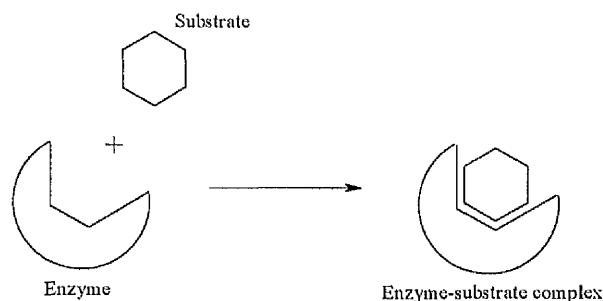


Figure 4-1 Lock and Key Model of the interaction of substrates and enzymes

As discussed in section 1.3.1.1, when a protein is denatured, there is disruption to the ordered structure, usually involving disarrangement of the secondary or tertiary structure, leading to the unfolding of the protein. For an enzyme, denaturation leads to loss of structure of the active site, rendering the enzyme inactive. It is this feature of enzymes that can be utilised to establish whether an additive can provide protection and stabilisation during freeze drying. The activity of an enzyme can be measured before and after freeze drying, and any differences in activity can be recorded.

4.2 β -GALACTOSIDASE ENZYME ASSAY

The effectiveness of the polymeric glycosides as protein stabilisers during freeze drying was assessed using a β -galactosidase enzyme assay. The activity of the enzyme was determined by its reaction with the substrate *o*-nitrophenyl- β -D-galactopyranoside (ONPG). This reaction liberates *o*-nitrophenol which is yellow in colour and so can be quantified spectrophotometrically. Figure 4-2 shows the mechanism for the enzyme assay.

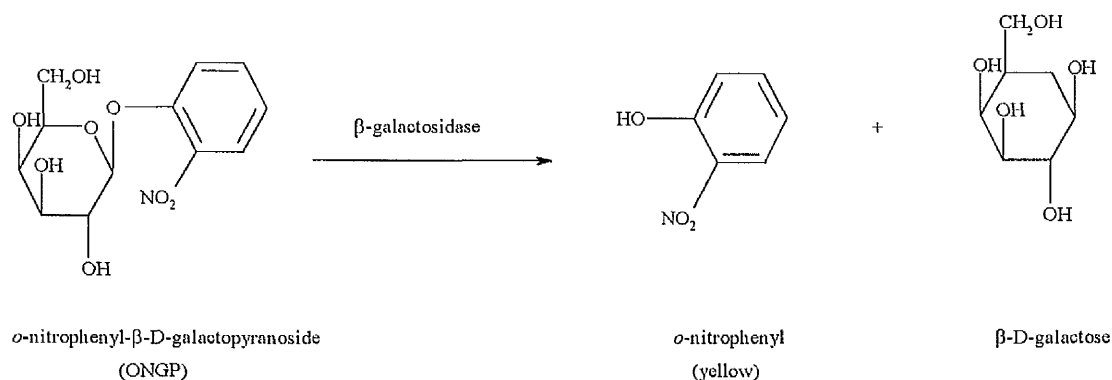


Figure 4-2 β -Galactosidase Assay

4.2.1 Experimental Procedure for β -galactosidase Assay

A 0.05 M buffer solution of tris(hydroxymethyl)methylamine (TRIS) (BDH) was corrected to pH 7.3 at 37 °C by the addition of dilute HCl. β -Galactosidase from *E. Coli* (Sigma) was accurately diluted with buffer solution to give 40 enzyme units ml⁻¹. This was separated into aliquots of 1 ml and placed in eppendorf tubes and flash frozen by submersion in liquid nitrogen. The frozen solutions were then stored at -10 °C prior to use. A solution of *O*-nitrophenyl- β -D-galactopyranoside (ONPG) (Lancaster) (5 mg in 10 ml of buffer) was prepared. A solution of NaCl (Aldrich) (1M, 0.584 g in 10 ml of buffer) was also prepared.

NaCl solution (0.25 ml), ONPG solution (1.0 ml) and buffer solution (1.75 ml) were placed in a U.V. cell (d = 1 cm) which was maintained at 37 °C by a thermostatically controlled U.V. cell holder. The reaction was started by the addition of the enzyme solution which had also been warmed to 37 °C prior to addition. The absorbance was recorded between the wavelengths of 250-500 nm every 15 seconds for 15 minutes and the activity was measured by the increase in the absorbance at 405 nm. This was performed on a Varian Cary 1 UV/VIS spectrometer, which is a double beam spectrometer; TRIS buffer solution was used as a blank.

The UV/VIS spectrum obtained from the above enzyme assay is shown in Figure 4-3 and from this the absorbance at 405 nm was recorded and plotted against time in Figure 4-4.

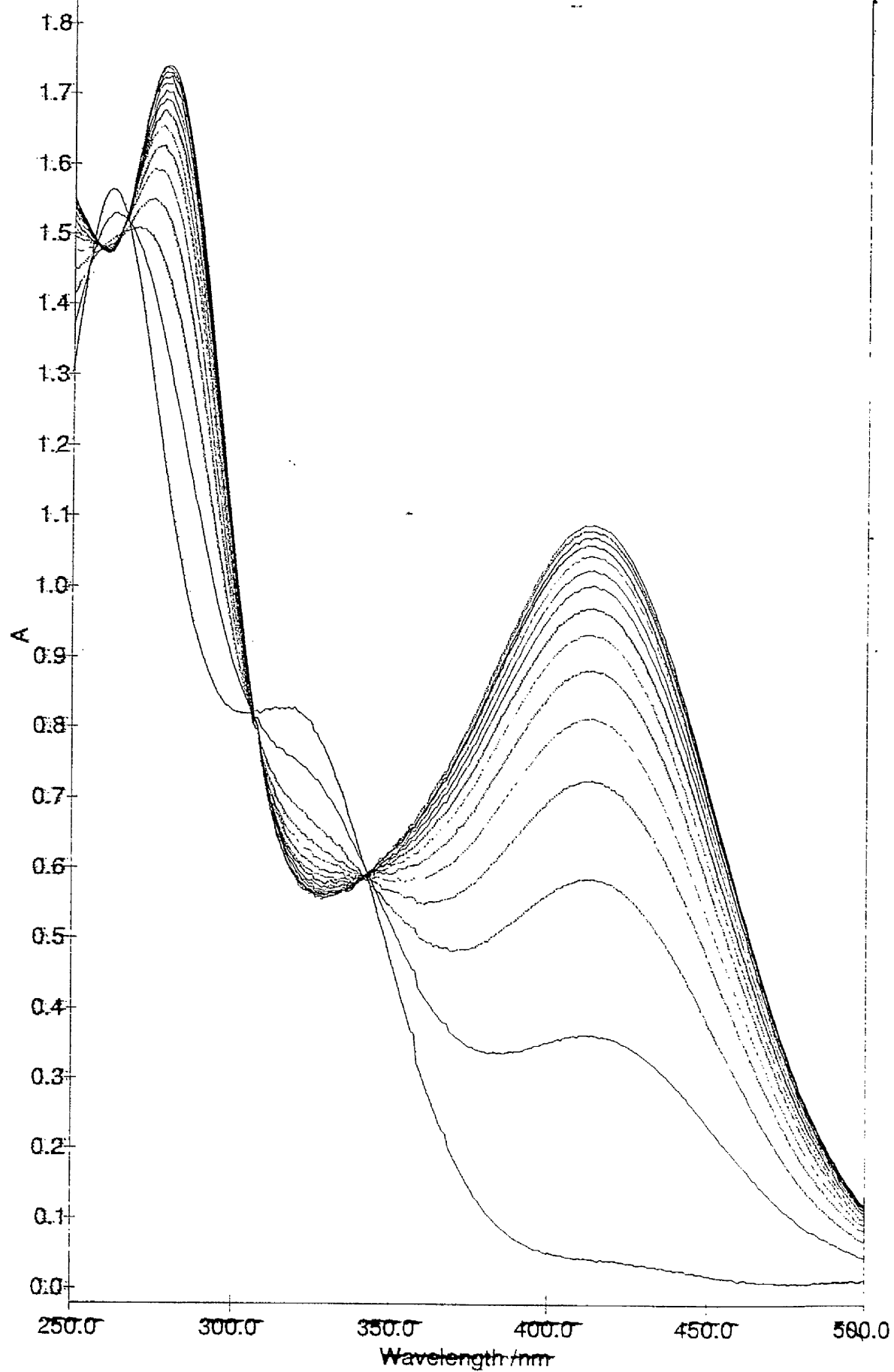


Figure 4-3 UV/VIS spectrum obtained from β -galactosidase enzyme assay

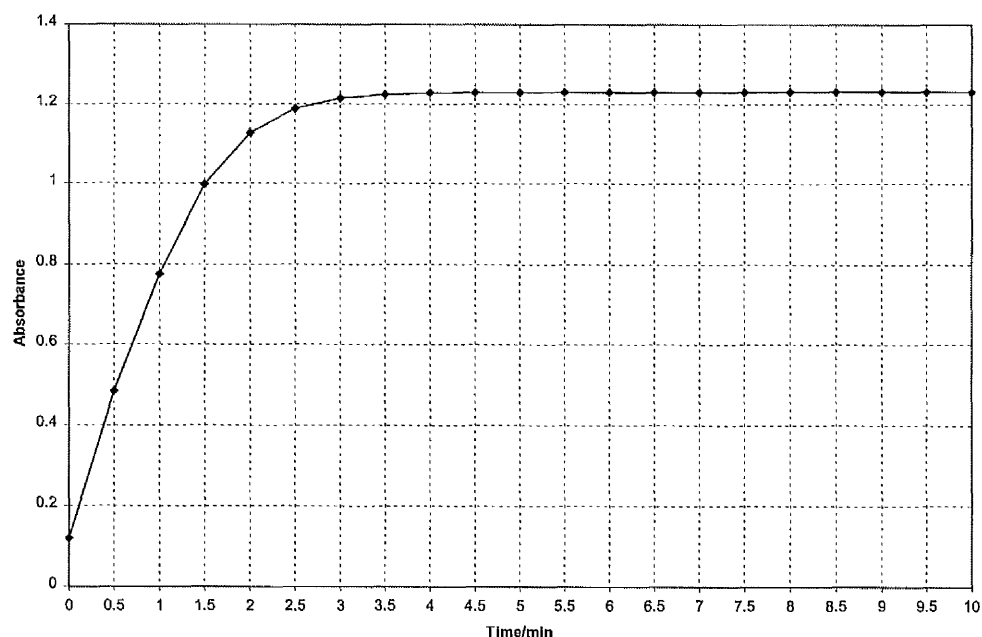


Figure 4-4 Graph of Absorbance vs time for β -galactosidase assay

The graph obtained in Figure 4-3 is a typical plot for an enzyme catalysed reaction. At a constant concentration of enzyme, the reaction will proceed until it reaches a maximal activity. At this stage, the activity remains constant due to all the active sites of the enzyme being filled with substrate. For the β -galactosidase assay, the activity reached a plateau after 5 min and reached a maximum absorbance of 1.23.

4.2.2 Activity of β -galactosidase after Freeze Drying

1 ml of β -galactosidase solution (40 enzyme units in 1ml TRIS buffer) was placed in a clean 25 ml round bottomed flask. The flask was attached a freeze drier (Edward) and the solution was left to freeze dry for 48 h. After this time, the enzyme was dissolved in distilled water (1 ml) and left for 1 hour for complete dissolution.

The same experimental procedure was followed as in Section 4.2.1, but using the solution of enzyme which had undergone freeze drying.

Figure 4-5 shows the UV/VIS spectrum obtained from the assay using β -galactosidase after being freeze dried. The absorbance at 405 nm was recorded and plotted against time in Figure 4-6. The plot for β -galactosidase which has not undergone freeze drying is also shown in Figure 4-6, so that a comparison of activities is observed.

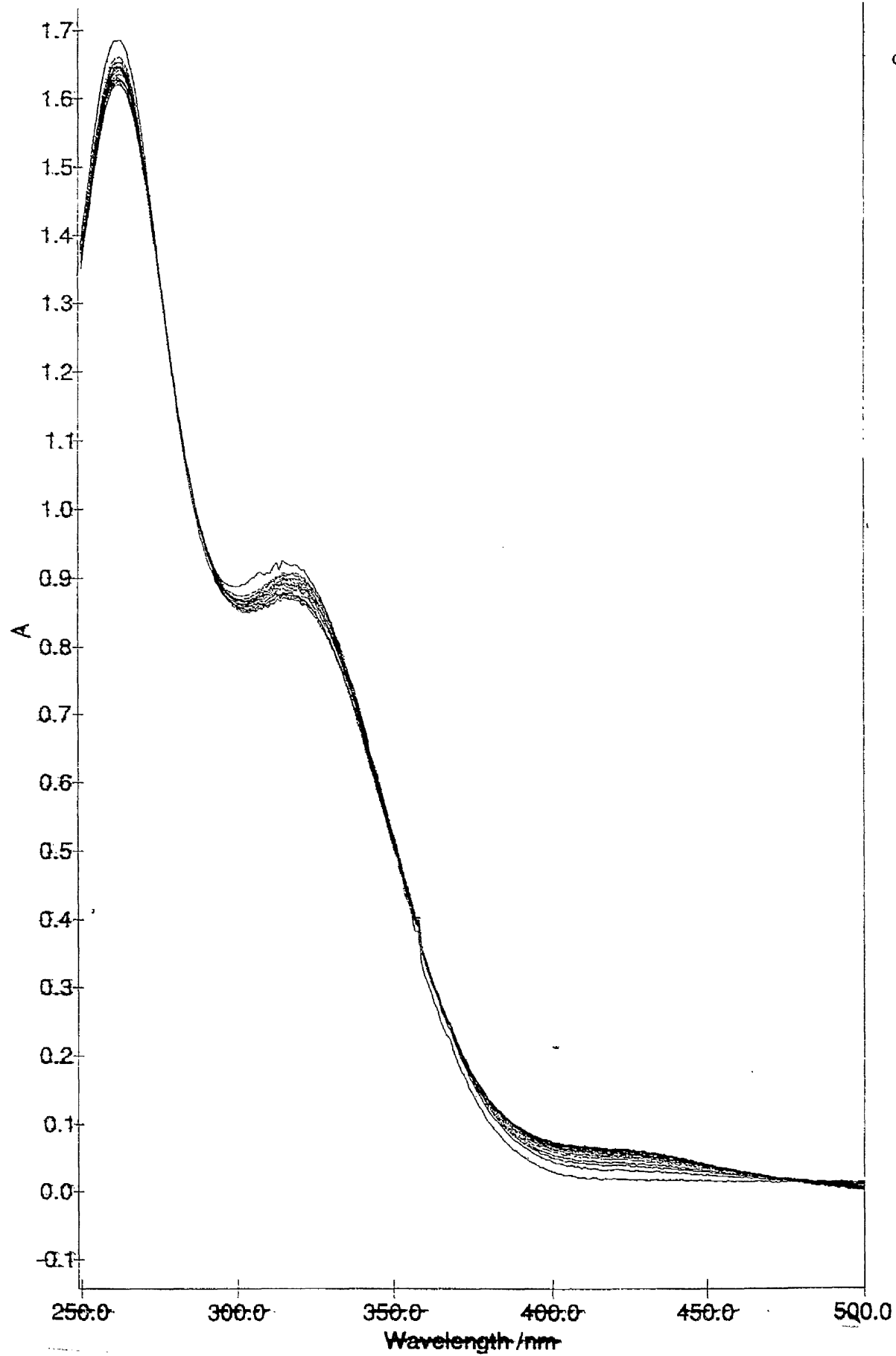
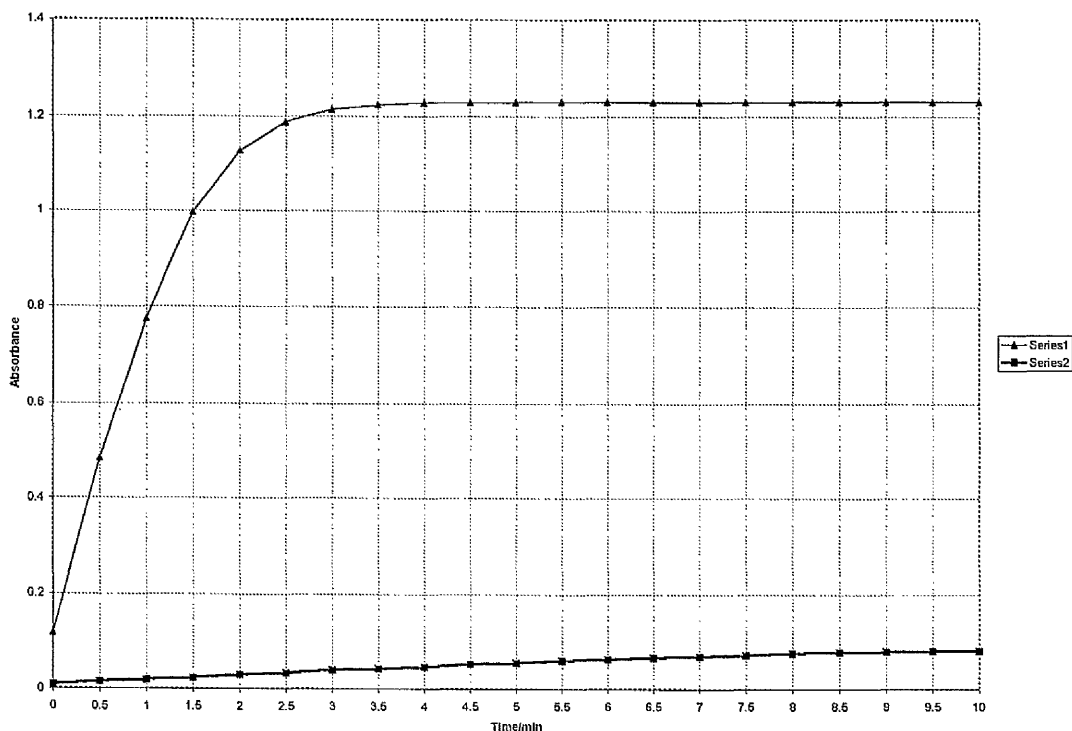


Figure 4-5 UV/VIS spectrum of β -galactosidase after freeze drying.



Series 1 = Enzyme before freeze drying

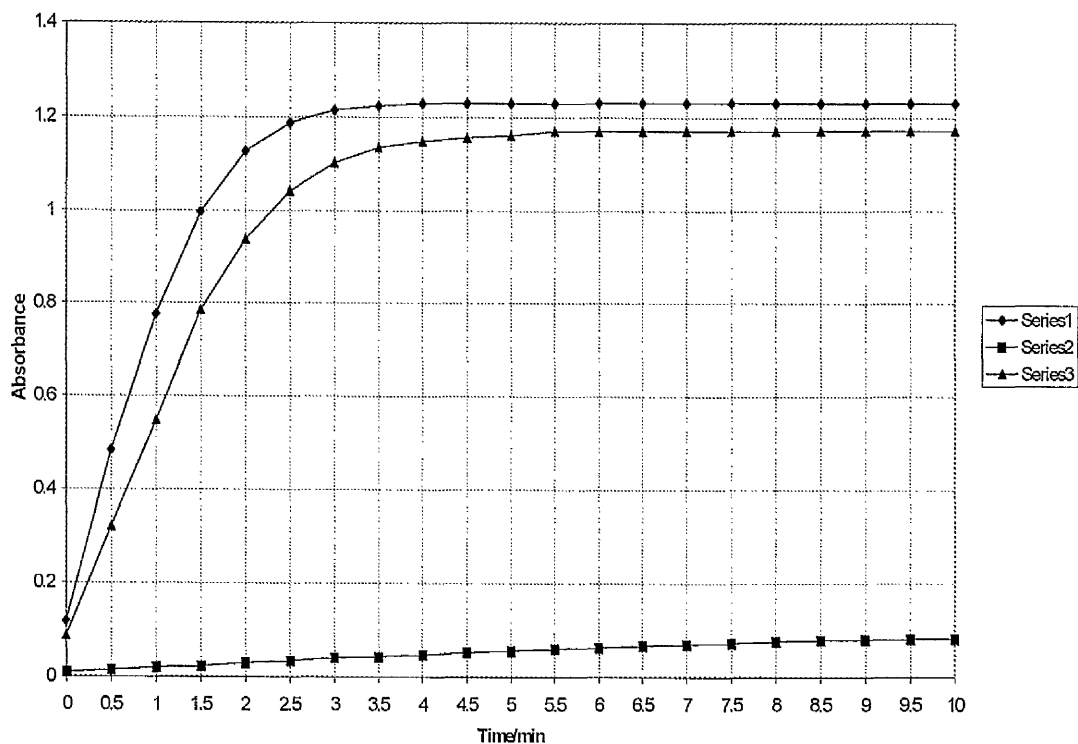
Series 2 = Enzyme after freeze drying

Figure 4-6 Graph comparing the activity of β -galactosidase before and after freeze drying

Figure 4-6 shows that freeze drying β -galactosidase considerably reduces its catalytic ability, and so it can be concluded that it has suffered denaturation. The significant difference in activity shown by this enzyme when it is subjected to freeze drying means it is an effective assay for studying protein stabilisation during freeze drying.

4.2.3 Freeze Drying β -galactosidase in the presence of D-Glucose

The same experimental procedure was followed as in section 4.2.2 but to the enzyme solution, D-glucose (9 mg) was added so that the stabilising additive was present at a weight ratio of 90:10. From the UV/VIS spectrum obtained from the assay using β -galactosidase after being freeze dried in the presence of D-glucose, the absorbance at 405 nm was recorded and plotted against time in Figure 4-7. The plot for β -galactosidase which has been freeze dried and also the one for the enzyme which has undergone no freeze drying are also shown in Figure 4-7, so that a comparison of activities is observed.



Series 1 = Enzyme with no freeze drying

Series 2 = Enzyme after freeze drying

Series 3 = Enzyme after freeze drying with D-glucose

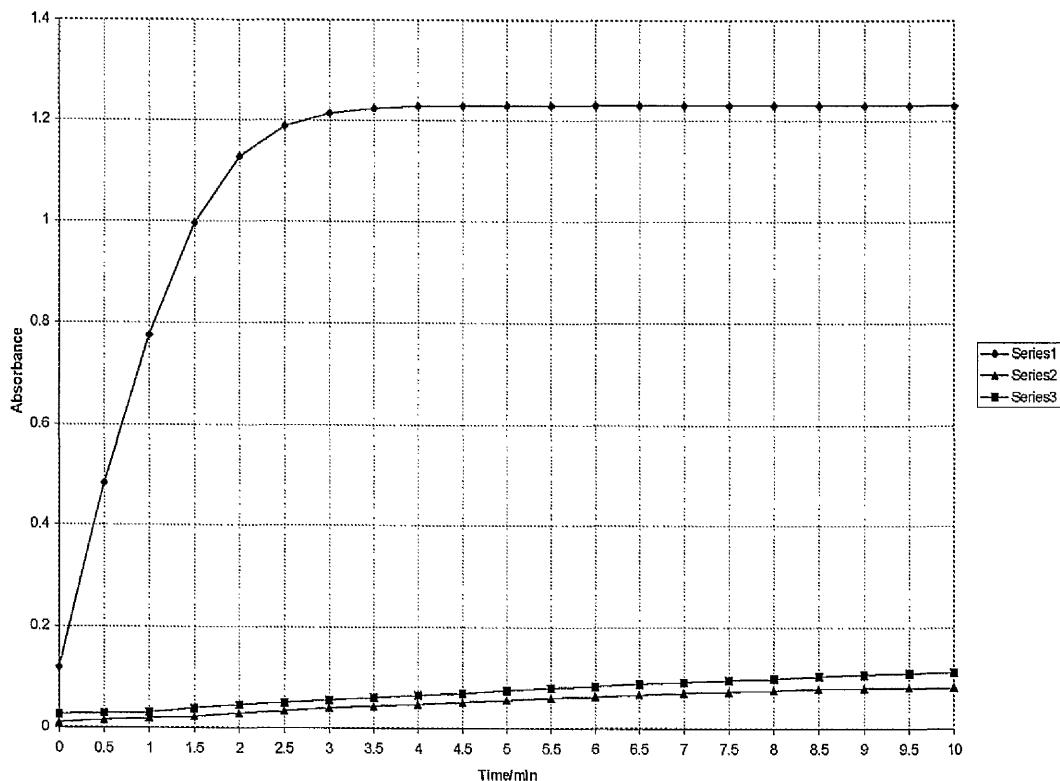
Figure 4-7 Graph comparing the activity of β -galactosidase after being freeze dried in the presence of D-glucose.

The results show that D-glucose has provided protection to the enzyme during freeze drying and it has retained 95% of its activity.

4.2.4 Freeze Drying β -galactosidase in the presence of PEG, PCL and PDLL

The same procedure was repeated as used in section 4.2.3. but using the polymers polyethylene glycol, (PEG-2) poly(ϵ -caprolactone) (PCL-1) and poly(D,L-lactide) (PDLL-2).

None of these polymers provided any protection to the enzyme from freeze drying. The β -galactosidase enzyme was rendered inactive in the presence of all the polymers. As mentioned in Section 1.4.2.1, polymers such as PEG are known to provide cryoprotection but not lyoprotection which has been evident from these results. Figure 4-8 shows the activity of β -galactosidase after being freeze dried in the presence of PEG-2.



Series 1 = Enzyme with no freeze drying

Series 2 = Enzyme after freeze drying

Series 3 = Enzyme after freeze drying in presence of PEG

Figure 4-8 Graph comparing the activity of β -galactosidase after being freeze dried in presence of PEG-2

4.2.5 Freeze Drying β -galactosidase in the presence of penta-acetate glucose

The theory that sugars act as “water substitutes” when the shell of hydration is removed from a protein during freeze drying has been well reported. The sugar is believed to form hydrogen bonds to the protein through the hydroxyl groups. It was postulated that this phenomenon could be investigated by comparing the activity of a protein that has

been freeze dried in the presence of a sugar with the activity of a protein freeze dried in the presence of the same sugar that has its hydroxyl groups protected.

Attempts to test this theory were performed by trying to observe the activity of β -galactosidase after freeze drying in the presence of D-glucose and penta-acetate glucose (PAG), i.e. D-glucose with the hydroxyl groups protected with acetyl groups. In practice, this was not simple due to the insolubility of PAG in the buffer solution. For an additive to have a stabilising effect on the protein, a co-solvent must be used.

Solvents other than water tend to act as denaturants for proteins.¹ Water miscible organic solvents cause the proteins to unfold by changing the dielectric constant of the aqueous medium. This change increases the solubility of the hydrophobic core and decreases the solubility of the charged surface causing the protein to unfold.^{2,3}

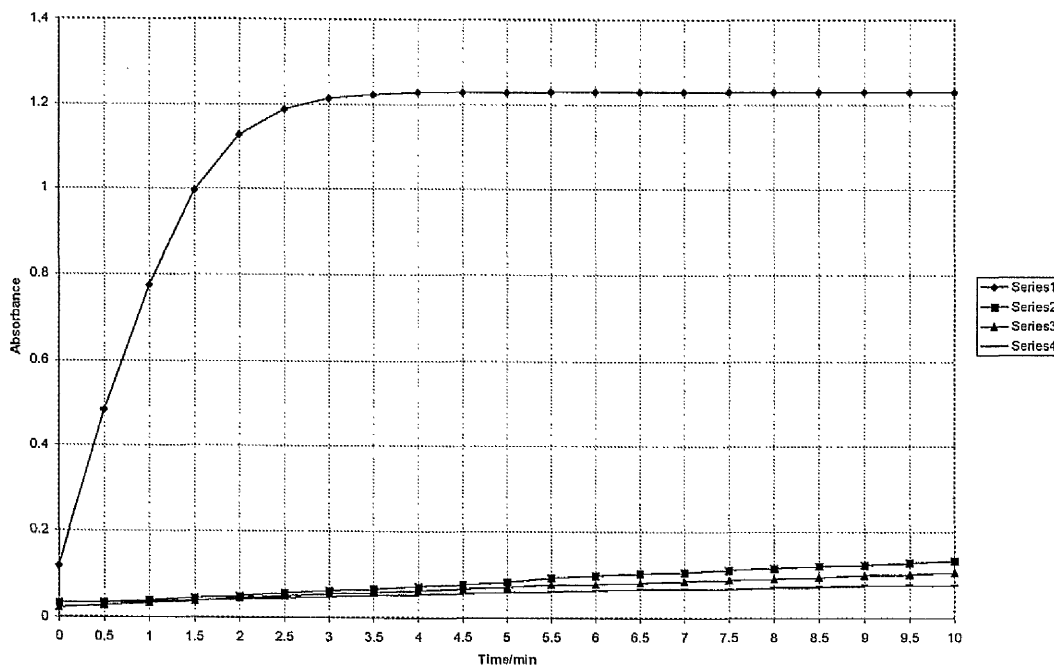
Franks has reported the use of cryosolvents to study the thermodynamic properties associated with the stability of proteins.⁴ Cryosolvents are organic water-miscible solvents that depress the freezing point of water and hence allow the behaviour of proteins below the freezing point of water to be observed. Solvents such as methanol, ethanol and dimethyl sulfoxide are typical solvents used. Douzou used cryosolvents to good effect in kinetics studies of enzyme-catalysed reactions.⁵ By the criteria chosen, high concentrations of organic solvents did not affect the enzyme kinetics. This work conflicts with the concept that organic solvents cause denaturation.

To investigate whether solvents such as ethanol and methanol could be used as a cosolvent for freeze drying enzymes in the presence of water insoluble additives, the

same experimental procedure as in Section 4.2.3 was used but with modifications. The enzyme (1 mg) and D-glucose (9 mg) dissolved in TRIS buffer (1 ml) was added to methanol or ethanol (1 ml). The solvents were then removed by freeze drying and the activity analysed as in previous experiments. This was then repeated using penta-acetate glucose instead of D-glucose.

Figure 4-9 shows the activity of β -galactosidase after freeze drying in the presence of D-glucose and PAG from 50:50 v/v solutions of TRIS:ethanol and TRIS:methanol. The enzyme showed very little activity after the use of either alcohol and the presence of D-glucose had no beneficial effect.

Conclusions about the ability of D-glucose to stabilise the enzyme through hydrogen bonding and this being suppressed when the hydroxyl groups are protected cannot therefore be drawn from this series of results owing to limitations posed by solubility issues.



Series 1 = Enzyme with no freeze drying

Series 2 = Enzyme after freeze drying with penta-acetate glucose in 50:50

TRIS:Methanol

Series 3 = Enzyme after freeze drying with D-glucose in 50:50 TRIS:Ethanol

Series 4 = Enzyme after freeze drying with D-glucose in 50:50 TRIS:Methanol

Figure 4-9 Graph comparing the activity of β -galactosidase after freeze drying in the presence of D-Glucose and PAG from ethanol and methanol

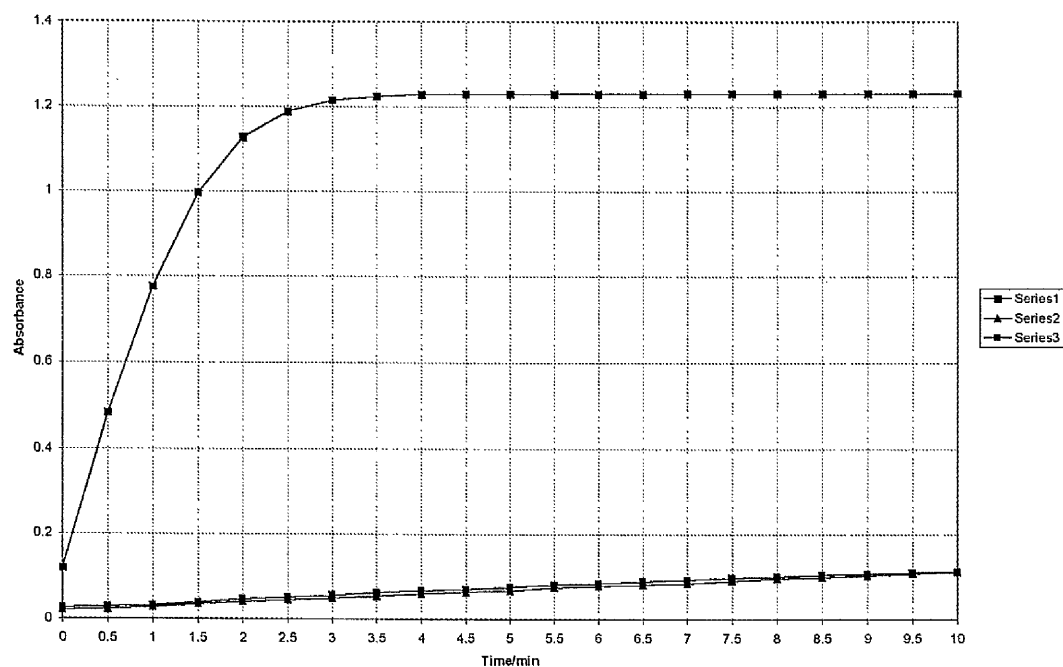
One point that can be concluded from these results is that the non-aqueous solvents used had a detrimental effect on the enzymes.

4.2.6 Freeze Drying β -galactosidase in the presence of Acetylated Polyethylene Glycol Glucoside

Efforts made in section 4.2.5 to establish the mechanism by which sugars stabilise proteins during freeze drying were unsuccessful. This was due to solubility problems as

discussed. One method of overcoming this problem is to attach the sugar, in this case penta-acetate glucose to a water soluble moiety. This was in effect achieved by the synthesis of acetylated polyethylene glycol as this product was soluble in water due to the PEG. Results from section 4.2.4 showed that PEG gave very little stabilisation during freeze drying and so any effects observed from freeze drying a protein in the presence of acetylated polyethylene glycol glucoside could be attributed to the acetylated sugar.

The same experimental procedure used in previous sections was carried out to freeze dry β -galactosidase in the presence of acetylated polyethylene glycol glucoside. The UV/VIS spectrum obtained from the assay was used to plot the absorbance at 405 nm against time. The graph obtained is shown in Figure 4-10.



Series 1 = Enzyme with no freeze drying

Series 2 = Enzyme freeze dried in the presence of Acetylated PEG Glucoside

Series 3 = Enzyme freeze dried in the presence of PEG-2

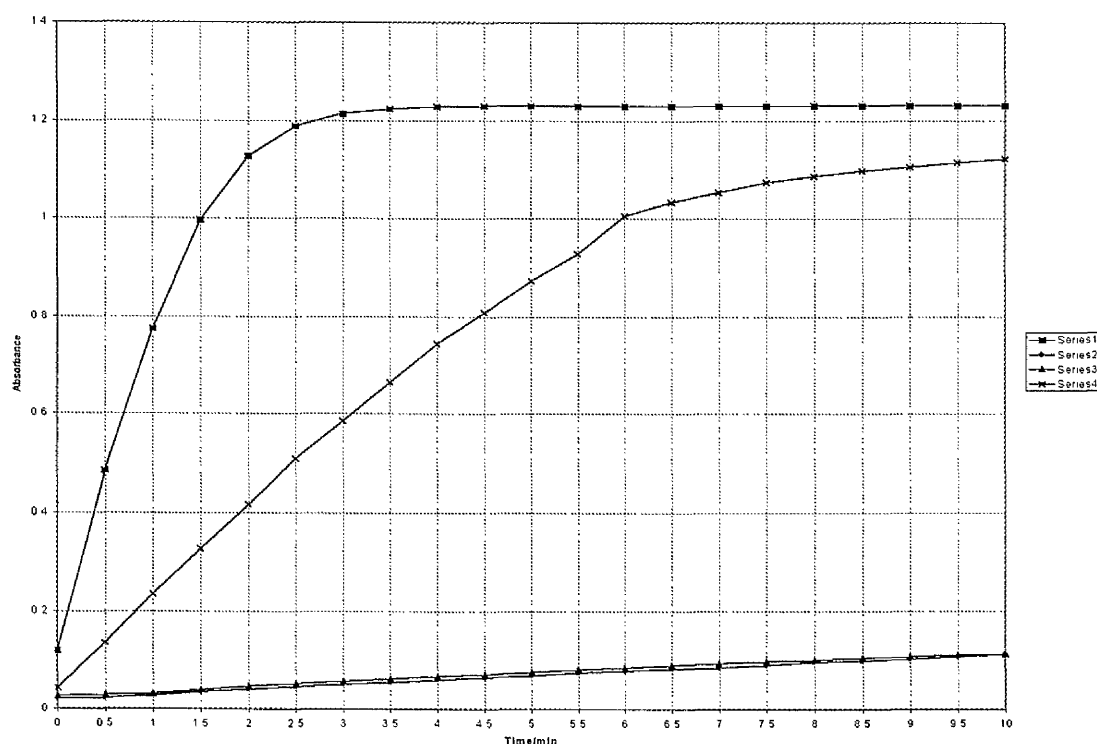
Figure 4-10 Graph comparing the activity of β -galactosidase after freeze drying in the presence of Acetylated PEG glucoside and PEG-2

It can be seen from these results that acetylated PEG glucoside gives no more protection to β -galactosidase during freeze drying than PEG. This indicates that the hydroxyl groups of D-glucose are required to give the enzyme stability, and this may ^{occur} through hydrogen bonding.

4.2.7 Freeze Drying β -galactosidase in the presence of Polyethylene

Glycol Glucoside

The same experimental procedure used in previous sections was carried out to freeze dry β -galactosidase in the presence of polyethylene glycol glucoside. The UV/VIS spectrum obtained from this assay is shown in Figure 4-12 and from this a graph of absorbance against time was plotted which is shown in Figure 4-11



Series 1 = Enzyme with no freeze drying

Series 2 = Enzyme freeze dried in the presence of Acetylated PEG Glucoside

Series 3 = Enzyme freeze dried in the presence of PEG

Series 4 = Enzyme freeze dried in the presence of PEG Glucoside

Figure 4-11 Graph comparing the activity of β -galactosidase after freeze drying in the presence of Acetylated PEG Glucoside and PEG Glucoside

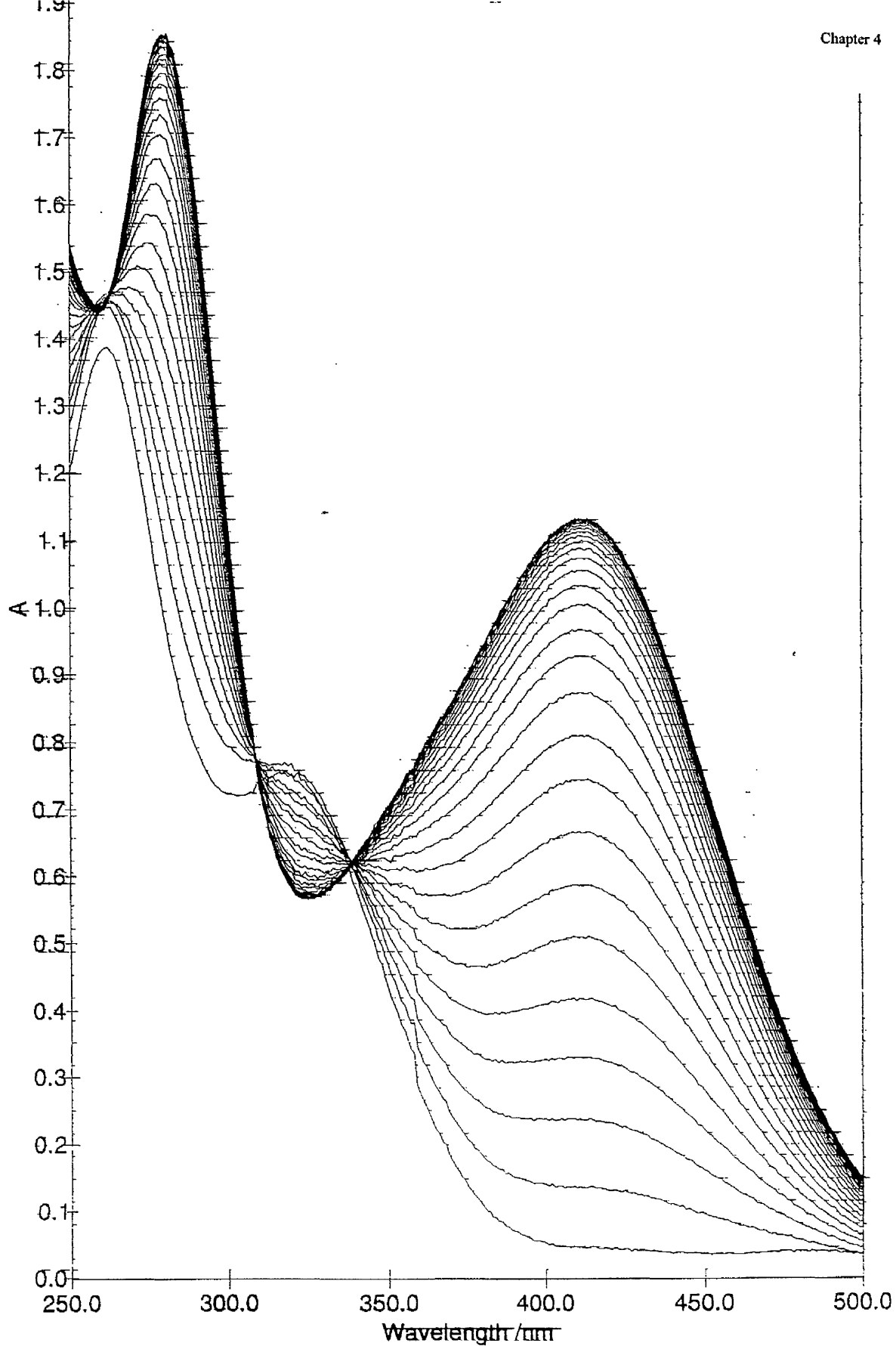


Figure 4-12 UV/VIS spectrum of β -galactosidase after freeze drying after freeze drying
in the presence of PEG glucoside

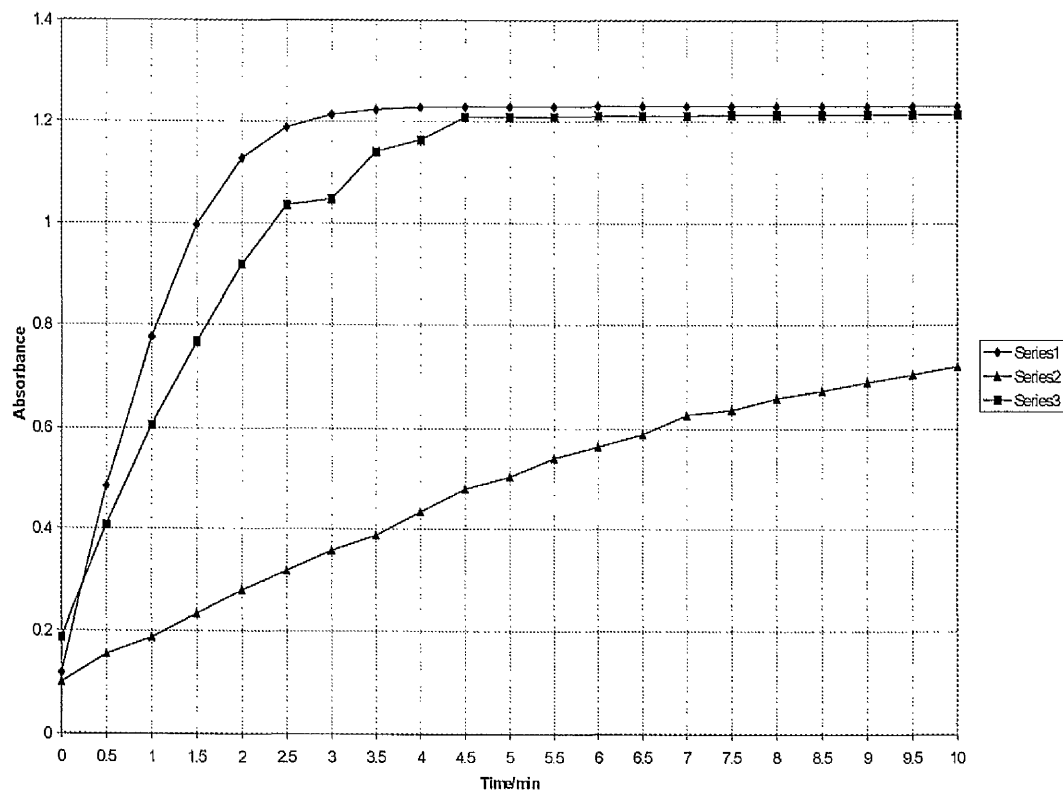
From these results, it can be seen that PEG glucoside can effectively stabilise proteins during freeze drying. When β -galactosidase is freeze dried in the presence of PEG glucoside, it retains 90% of its activity, but the rate at which the enzyme converts the substrate (ONPG) to *o*-nitrophenol, seems to have diminished.

4.2.8 Freeze Drying β -galactosidase in the presence of

Poly(ϵ -caprolactone) Glucoside and Poly(D,L-lactide) Glucoside

The same experimental procedure used in previous sections was carried out to freeze dry β -galactosidase in the presence of poly(ϵ -caprolactone) glucoside and poly(D,L-lactide) glucoside. The results from the assay are shown on Figure 4-13.

The results obtained show that the glucosides studied exhibited the ability to stabilise the enzyme.



Series 1 = Enzyme with no freeze drying

Series 2 = Enzyme after freeze drying in the presence of poly(D,L lactide) glucoside

Series 3 = Enzyme after freeze drying in the presence of poly(ϵ -caprolactone) glucoside

Figure 4-13 Graph comparing the activity of β -galactosidase after freeze drying in the presence of poly(D,L lactide) and poly(ϵ -caprolactone)

4.3 CONCLUSIONS

From the work presented in this chapter, it can be concluded that the β -galactosidase assay is an effective method with which to establish how polymeric glucosides interact and stabilise proteins.

It was established that D-glucose can successfully stabilise β -galactosidase during freeze drying and this may be due to hydrogen bonding through the hydroxyl groups. To establish whether it was the hydroxyl groups causing the stabilisation, the enzyme was freeze dried in the presence of penta-acetate glucose. This is essentially D-glucose with the hydroxyl groups protected by acetyl groups, meaning they cannot hydrogen bond to the enzyme. Unfortunately, penta-acetate glucose was insoluble in the aqueous system so no results were obtained using this method. To overcome this problem, the penta-acetate glucose was attached to a water soluble moiety, PEG and this overcame the solubility problems. PEG had been shown to give very little stabilisation during freeze drying and so any effects observed from freeze drying the protein in the presence of acetylated PEG glucoside could be attributed to the acetylated sugar. The results showed that the acetylated PEG glucoside gave no stabilisation to the enzyme and indicates that stabilisation during freeze drying occurs through hydrogen bonding with the hydroxyl groups.

It was observed that the polymers PEG, PCL and PDLL provide little protection when used alone to stabilise proteins, but it has been reported they stabilise proteins during the freezing process.

Results showed that β -galactosidase is denatured in the presence of methanol and ethanol and an aqueous environment was required when formulating the enzyme with the stabilising excipient.

The glucosides of PEG, PCL and PDLL all exhibited the ability to protect β -galactosidase during freeze drying. To fully evaluate the potential of these glucosides as protein stabilisers, a full array of enzymes and proteins needs to be studied in the future to ensure that the effect is not specific to this particular enzyme.

4.4 REFERENCES FOR CHAPTER FOUR

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5. Conclusions and Suggestions for Further Work

5.1 CONCLUSIONS

The aim of this research was to synthesise novel products for the use of biodegradable drug delivery implants with the ability to stabilise proteins during freeze drying.

One of the first steps to achieving this was to synthesise low molecular weight poly(ϵ -caprolactone) (PCL) and poly(D,L-lactide) (PDLL) with a low degree of polydispersity. It was also necessary for the polymers to have hydroxyl end groups for the next stage of the synthetic route. This was attained by using a bifunctional initiator and tin(II) octoate as the catalyst. Low molecular weights were achieved by accurately controlling the monomer to initiator ratios.

A range of low molecular weight PCLs and PDLLs were produced and along with a range of low molecular weight poly(ethylene glycol)s, they were characterised using NMR, MALDI-TOF NMR and GPC. The results obtained from each technique were compared for each set of polymers. By looking at the value for the number-average weight molecular (M_n) given by each technique, it was possible to see how well the techniques compared.

The end capping of PEG with D-glucose was achieved *via* the Koenigs-Knorr reaction. The reaction yielded PEG end capped with two acetylated glucose groups, PEG end capped with one acetylated glucose group and PEG which had not undergone any reaction. Due to the similar solubilities, separation of the three compounds proved difficult. Isolation and characterisation of the products was achieved by using MALDI-TOF mass spectrometry in conjunction with Preparative GPC.

The reaction was carried out with poly(ϵ -caprolactone) and poly(D,L-lactide). The reaction yielded the respective glucosides with surfactant properties. The difference in solubility of the glucoside and the starting homopolymer made separation and isolation simple. However, low yields were obtained for these two systems and this was probably due to the polymers being non polar and having an electron withdrawing effect on the hydroxyl end group. This reduces the reactivity of the hydroxyl group and decreases the ability to undergo glycosidation in the Koenigs-Knorr reaction.

The deacetylation of the PCL and PDLL glucosides proved difficult as the reaction had the expected detrimental effects on the polymer component of the product. Using reduced temperatures during the deacetylation reaction did not sufficiently reduce the cleavage of the polymer. Reducing the length of the reaction would not yield the fully deacetylated product and it proved difficult to obtain a good balance between the two opposing reactions.

To study the ability of the polymeric glucosides, the β -galactosidase enzyme assay was used. The enzyme showed a marked decrease in activity after being subjected to freeze drying, and so formed the ideal basis for assessing the ability of the glucosides to protect it.

D-glucose was used to stabilise the enzyme during freeze drying and was found to be very effective, the enzyme maintaining 95% of its original activity. Studies were carried out to show that the hydroxyl groups were necessary for the stabilisation process. Initial attempts were made by freeze drying the enzyme in the presence of penta-acetate glucose, which is glucose with the hydroxyl groups protected by acetyl groups. This

meant that the hydroxyl groups could not hydrogen bond to the enzyme as predicted. However, penta-acetate was insoluble in the aqueous system and no conclusive results could be obtained. To overcome this problem, the penta-acetate glucose was attached to PEG to give the desired water solubility. PEG had shown to give very poor protection to the enzyme during freeze drying and so any effects observed from freeze drying β -galactosidase in the presence of acetylated PEG glucoside could be attributed to the acetylated glucose. The results showed that acetylated PEG glucoside gave no stabilisation to β -galactosidase during freeze drying, indicating that the hydroxyl groups may hydrogen bond to the protein and act as a water substitute as reported by several publications mentioned in Chapter 1.

Results showed that β -galactosidase is denatured in the presence of methanol and ethanol and an aqueous environment was required when formulating the enzyme with the stabilising excipient. This shows that modifying hydrophobic PCL and PDLL polymers with the hydrophilic sugar groups was necessary to give water solubility and hence the capacity to formulate the enzyme in an aqueous environment.

The glucosides of PEG, PCL and PDLL all exhibited the ability to protect and stabilise β -galactosidase during freeze drying due to their cryoprotective and lyoprotective properties. To fully evaluate the potential of these compounds as excipients which can be used to stabilise therapeutic protein drugs, the glucosides must be tested with a full array of proteins and enzymes.

5.2 SUGGESTIONS FOR FURTHER WORK

The poor yields obtained from the synthesis of PCL and PDLL glucosides indicate that the Koenigs-Knorr reaction is not the best synthetic route. One potential route is to use a protected glucose with one hydroxyl group unprotected and use this as an initiator for the polymerisation of the PCL and PDLL. In this way, the polymer is 'grown' from the glucose rather than 'added' to the complete polymer. This is explained in Figure 5-1.

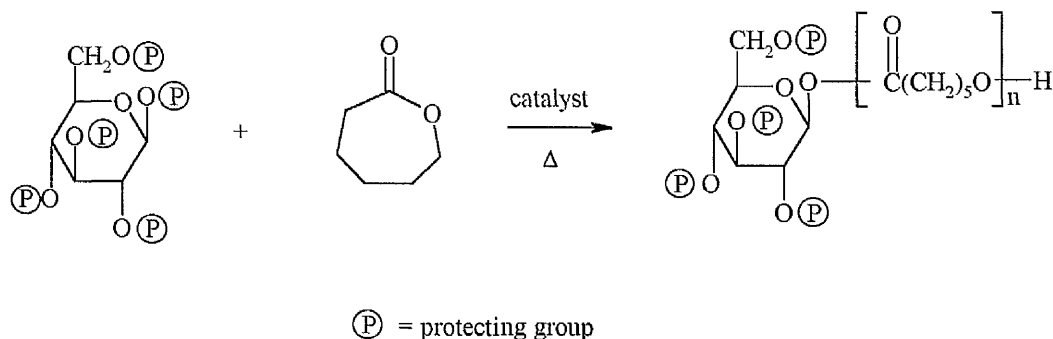


Figure 5-1 Alternative method for synthesis of PCL Glucoside

The use of acetyl protecting groups has proved not suitable for the synthesis of PCL and PDLL glucosides as the deprotection causes chain scission. Alternative protecting groups could be used such as chloroacetates and silyl groups.

For the PEG, PCL and PDLL glucosides to be used as drug delivery compounds, they would have to be used as an 'envelope' to encase the protein drug. A further coating of a non-water soluble polymer would then have to be added to encase the envelope, as the surfactant-like glucosides presented in this work would be solubilised too quickly *in vitro*, hence defeating the object of controlled release. Further studies would then have to be carried out to evaluate the ease with which the glucosides could be formulated as microcapsules or depots. Alternatively, the level of glucose content in the formulation

could be experimented with to give the desired release profile of the drugs, but still exhibiting protection.