L-Amino Acid and Sugar-based Biodegradable Poly(ester-urethane) for Drug Delivery Applications



MS-Thesis

BY

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20171138

UNDER THE SUPERVISION OF

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Certificate

This is to certify that this dissertation entitled "**L-Amino Acid and Sugar-based Biodegradable Poly(ester-urethane) for Drug Delivery Applications"** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Aditi Ashok Gujare at Indian Institute of Science Education and Research, Pune, under the supervision of Prof. M. Jayakannan, Professor, Department of Chemistry during the academic year 2021-2022.

Date: 9th April 2022

Place: Pune



Signature of the Student

Date: 9th April 2022

Place: Pune



Signature of the Supervisor

Declaration

I hereby declare that the matter embodied in the report entitled "**L-Amino Acid and Sugar-based Biodegradable Poly(ester-urethane) for Drug Delivery Applications"** are the results of the work carried out by me at the Department of Chemistry, Indian Institute of Science Education and Research, Pune, under the supervision of Prof. M. Jayakannan and the same has not been submitted elsewhere for any other degree.

Date: 9th April 2022

Place: Pune



Signature of the Student

Acknowledgement

I would like to thank my supervisor ***Prof. M. Jayakannan*** for his constant support and guidance throughout my MS Thesis, which helped me a lot to grow as an independent researcher and learn new skills. His enthusiasm towards research encouraged me to work hard in any situation and think positively.

I'm thankful to ***Dr. S. Sandanaraj Britto***, my Thesis Advisory Committee (TAC) member, for his guidance and helpful discussion during the mid-year presentation that helped me rise as a confident masters student.

I thank ***Prof. Jayant B. Udgaonka****r*, Director of IISER Pune, for providing the opportunity to conduct research. I also thank all the faculty members for providing a fruitful learning experience.

I would also like to thank my lab members Devesh, Keerthana, Utreshwar, Dheeraj, Khuddus, Ruma, Mishika, Rahul, Shahid, Pranav, Parshu, Mehak and Kajal for their immense support and for making this learning experience memorable. I wholeheartedly thank my friends Utkarsh, Devesh, Riya, Yashi, Meenu, Prajakta, Prachi and Vibhor for supporting me all along and making my experience at IISER Pune fun-filled and memorable.

I'm deeply indebted to my parents and especially to my brother Aryan for always being there and making my life cheerful and filled with joy.

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Abbreviations

L-DOPA	l-3,4-dihydroxyphenylalanine
DOX	Doxorubicin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NMR	Nuclear Magnetic Resonance
DBTO	Dibutyltin(IV)oxide
PTSA	p-Toluene sulphonic acid
THF	Tetrahydrofuran
TBDMSCl	tert-Butyldimethylsilyl chloride
HF	Hydrogen fluoride
DMF	Dimethylformamide
TEA	Triethylamine
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
TGA	Thermogravimetric analysis
GPC	Gel Permeation Chromatography
M_n	Number Average Molecular Weight
M_w	Weight Average Molecular Weight
PDI	Polydispersity Index
X_n	Degree of Polymerisation
p	Extent of Reaction
RALS	Right Angle Light Scattering
DSC	Differential Scanning Calorimetry
DLC	Drug Loading Content
DLS	Dynamic Light Scattering
C8	L-DOPA monomer and 1,8-octane diol based polymer
C10	L-DOPA monomer and 1,10-decane diol based polymer
C12	L-DOPA monomer and 1,12-dodecane diol based polymer
DAPI	4′,6-diamidino-2-phenylindole

Abstract:

Melt-condensation approach is well known for the transesterification to synthesise polyesters and was developed for synthesising polyurethanes and poly(ester-urethane) from our group by using amino-acid, a bioresource for the synthesis of monomer. Though amino acid makes the polymer biocompatible, the diols used in this condensation chemistry are petroleum-based, which doesn't make the polymer completely biodegradable. By synthesising a sugar-based diol, the polymer is made completely biodegradable. The polymer self-assembles to form a nanocarrier capable of carrying drugs and would be easily degraded by the lysosomal enzymes. This makes the nanocarrier suitable for cancer treatment. The amino acid chosen is L-DOPA(l-3,4-dihydroxyphenylalanine); its carboxylic group is converted into ester, the amine group into urethane and the catechol group is silyl protected. The catechol unit allows for further functionalisation of the polymer side chain, which allows for a change in hydrophilicity of the polymeric side chain. The sugar diol is synthesised from D-mannitol by the acetal protection of secondary alcohol groups. The melt-condensation was done at 150℃, and the polymer was compared with other polymers synthesised using commercially available diols. They were characterised, and their thermal properties were studied. After deprotection of the silyl and acetal groups of the amino acid and sugar diol-based, fully biodegradable polymer, the dye and the drug loading were checked, and cell viability was studied for the nascent, the dye loaded and the drug-loaded polymer. The deprotected polymer was then loaded with the Nile Red dye and Doxorubicin(DOX) to check the drug and dye loading separately. The absorbance was measured, and using MTT assay, the cytotoxicity of the dye and drug were checked at various concentrations. Cellular uptake studies were also carried out. Poly(ester-urethane)s from our group synthesised by the melt-condensation approach are known to degrade by lysosomal enzymes. The complete biodegradability of the polymer would open a new method for developing polymeric nanomedicine, by the solvent-free melt-condensation approach, for cancer treatment.

Introduction:

The development of drug delivery systems for the treatment of various diseases is a well-known approach in biomedical research. Various nanomedicines are being developed for specifically targeting the diseases. Metallic, organic, inorganic and polymeric nanostructures have been developed for biomedical applications[1]. Enzyme, pH, and even some biomolecule responsive nanoparticles have been designed for drug delivery applications[2, 3]. Drug encapsulation and transportation are some of the important parameters to consider, where the size and geometry of the nanocarrier is an important factor [4]. Further, the biocompatibility of nanomedicine is crucial in drug delivery applications. Large numbers of polymer nano-carriers are made based on the non-biodegradable platform; thus, the degradation of polymer chains at the cellular level and their excretion from the biological system under physiological conditions are not clearly understood. Design and development of biodegradable polymer nano-assemblies from bio-resources is an important area of research for delivering anticancer drugs to improve the efficacies in cancer treatment. For this, we develop a solvent-free melt-condensation approach that involves using naturally available resources and using an environment-friendly process of synthesising the nanomedicine.

The solvent-free melt-condensation approach is an environmentally friendly process for synthesising polyesters, polyurethanes and poly(ester-urethane)s. It was first developed by *Whinfield* for polyester synthesis[5] by transesterification. From our group, *Deepa et al.* developed a melt-condensation approach for polyurethane synthesis for replacing the hazardous isocyanate pathway[6]. Later, *Anantharaj* from our group combined these two chemical pathways and developed this approach for synthesising poly(ester-urethane)[7] by taking amino acid as a bioresource (*Figure 1.1*). Amino acid-based polymers are an important class of biomaterials. Polymers also have applications in developing a nanocarrier for drug delivery applications. Considering these aspects, we aim to use amino acids as a bioresource for developing nanocarriers for drug delivery applications for cancer treatment.

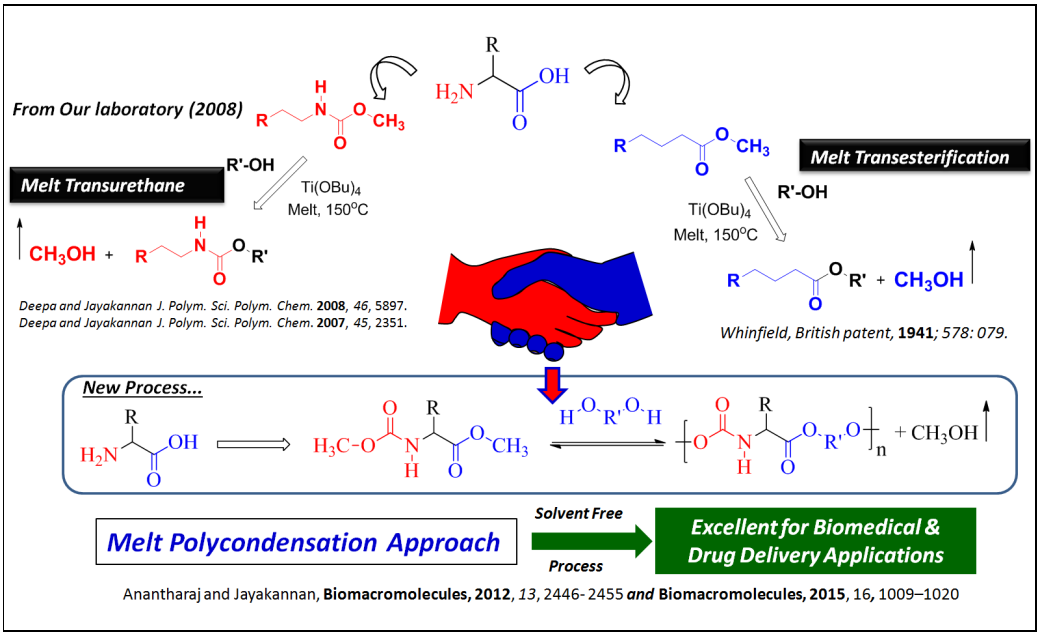


Figure 1.1 *Melt-Condensation Approach*

Synthesising the polymer, observing their self-assembly, drug loading, drug delivery, and polymer degradation are the steps involved in developing such a nanomedicine for cancer treatment. One of the aspects to consider for developing such a polymer is its biodegradability. Some commercially available polyesters and polyurethanes are synthesised using commercially available petroleum-based diols. Using bioresources for polymer syntheses, such as amino acids and sugar, makes the polymers biocompatible and enzymatically biodegradable.

L-DOPA(l-3,4-dihydroxyphenylalanine), an amino acid having an amine and a carboxylic group, along with a catechol group that allows further functionalization of the amino acid, is an excellent bioresource for poly(ester-urethane) synthesis. Converting the carboxylic group into ester and the amine group into urethane and polymerizing it with a diol is our strategy to develop biodegradable polymers.

L-DOPA is involved in the biosynthetic pathway for catecholamines[8]. Noradrenaline, adrenaline and dopamine are the naturally occurring catecholamines. They are found in animal and vegetable tissues but are well known for their role in the nervous tissue of animals[9]. L-DOPA is known for treating Parkinson's disease in humans as it can easily cross the hematoencephalic barrier and enter nerve cells and later decarboxylate into dopamine to reduce its deficiency, which is the major cause of the disease. L-DOPA is also involved in the formation

of melanin in cells. In some plants, such as *Mucuna pruriens*, L-DOPA is known to have a strong allelopathic activity which contributes to increasing the yield of the plant[10]. L-DOPA is commercially produced by asymmetric hydrogenation but has a poor conversion rate, low enantioselectivity and is expensive. Other production methods under consideration include microbial fermentation, enzymatic conversion by hydroxylation of L- tyrosine using tyrosinase and electroenzymatic synthesis. These are some of the processes for L-DOPA synthesis that are being researched for its commercial synthesis[11].

The amine part of the L-DOPA is converted into urethane, the carboxylic group is converted into ester, and the catechol group allows further functionalization of the amino acid to form a hydrophobic[12] or hydrophilic side chain[13], as was previously done in our group (*Figure 1.2*), for developing nanocarriers for cancer treatment. It is, therefore, an excellent bioresource for poly(ester-urethane) synthesis. Hence we choose this as a bioresource for polymer synthesis.

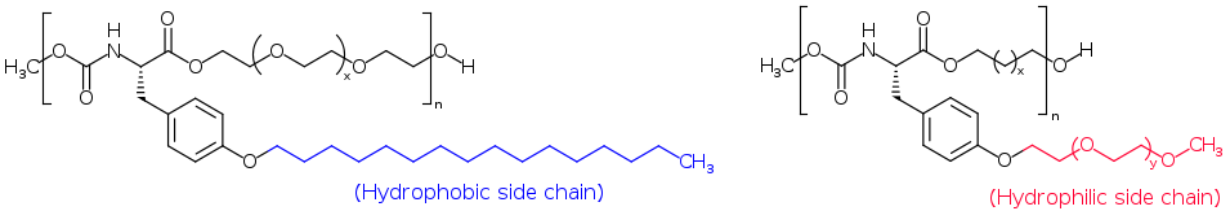


Figure 1.2 *L-tyrosine based Amphiphilic Poly(ester-urethane)s with hydrophobic and hydrophilic functionalized side chains*[16,17].

In our group, a solvent-free melt-condensation approach has been developed by taking amino acids as the bioresource for synthesising polyesters, polyurethanes and poly(ester-urethane)s for drug delivery applications as previously mentioned[5-7, 13, 21-27]. These polymers were partially bio-based as they were synthesised using amino acid and a commercially available petroleum-based diol (*Figure 1.3*). Thus, making the polymer non-biocompatible. To replace these commercially available diols, D-mannitol based diol is synthesised.

D-mannitol is a naturally occurring sugar found in olive trees, the bark of *Fraxinus ornus L.*, and fruits and vegetables like strawberries, pumpkin and onion. Certain bacteria and fungi are

also capable of producing D-mannitol[14]. Currently, it is commercially produced from fructose. But only about 50% of it is converted into mannitol; the rest is converted into sorbitol by this method. Other approaches for the production of D-mannitol include enzymatic hydrogenation and production by microbes, which are being researched. D-mannitol is extensively used in pharmaceutical industries; it is used to decrease cellular edema. It has a lower calorific value compared to glucose. It is also metabolised independent of insulin and is thus used in diabetic food products[15]. D-mannitol is known in the literature involving the synthesis of polyurethanes with tunable hydrophilicity[16], AABB-type polyamides[17], Aliphatic Polyesters[18], PET copolyesters[19] and some hyperbranched polymers[20].

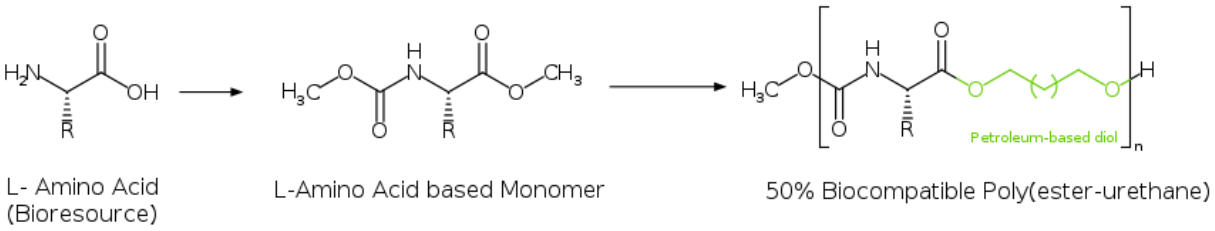


Figure 1.3 *Polymer synthesised from Amino Acid and Petroleum-based Diol*

Using these two bioresources, L-DOPA and D-mannitol, we aim to synthesise a fully biodegradable poly(ester-urethane) that would self-assemble into a nanocarrier for cancer drugs. We observe its drug loading and drug delivery ability to cancer cells.

Experimental Methods

Materials: L-DOPA, D-mannitol, 1,8-Octanediol, 1,10-Decanediol, 1,12-Dodecanediol, 1,4-Benzenedimethanol, Titaniumtetrabutoxide($Ti(O^tBu)_4$), Dibutyltin(IV)oxide(DBTO), Benzoyl chloride, dry pyridine, 2,2-dimethoxypropane, dry acetone, p-Toluene sulphonic acid (PTSA), toluene, sodium methoxide, dry methanol, dry chloroform, Thionyl chloride, Methyl chloroformate, Tetrahydrofuran(THF), Water, Sodium bicarbonate, tert-Butyldimethylsilyl chloride(TBDMS-Cl), Imidazole, 4-Dimethylaminopyridine, hydrogen fluoride(HF), Dimethylformamide(DMF), triethylamine(TEA), Dichloromethane(DCM), ethyl acetate, methanol, diethyl ether, Dimethyl sulfoxide(DMSO), Doxorubicin (DOX),

General Procedures: For the ^1H NMR and the ^{13}C NMR, 400MHz BRUKER and JEOL NMR spectrophotometer were used and $CDCl_3$ and DMSO-D6 were used for sample preparation. The thermogravimetric analysis(TGA) was performed to check the thermal stability of the monomers and polymers using Perkin Elmer STA 6000 Stimulus Thermal Analyzer. Further, TA Q-20 Differential Scanning Calorimeter was also used to observe the thermal properties of the polymers, with a heating and cooling rate of 10℃/min and a continuous nitrogen purge. The number average and the mass average molecular weight of the polymers were determined by gel permeation chromatography(GPC) using polystyrene as a standard at 25℃, Viscotech RALS detector was used and the samples were prepared in chloroform.

Synthetic Procedures for Monomers and Polymers:

Synthesis of methyl 3‐(3,4‐dihydroxy phenyl)‐2‐[(methoxycarbonyl) amino] propanoate (1): L-DOPA(10g, 50.7mmol) was weighed in a 250ml 2-neck round-bottomed flask and dry methanol(100ml) was added to it. Thionyl chloride(4.05ml, 55.8mmol) was added dropwise under ice-cold(0℃) and nitrogen conditions and the reaction mixture was stirred for 12 hours at room temperature. After 12 hours, the reaction was dried by vacuum distillation. At room temperature, a solution of sodium bicarbonate(4.7g, 55.8mmol) and water(40ml) was added to the reaction mixture. THF(50ml) and the solution of methyl chloroformate(4.4ml, 55.8mmol) and THF(10ml) were added to the reaction mixture. The reaction was stirred for 12 hours at

room temperature. The product was extracted by DCM and dried by rotary evaporation. Yield = 7.8g (62.1%). ^{1}H NMR (400MHz, $CDCl_3$) δppm: 6.74(d, 1H), 6.64(s, 1H), 6.51(d, 1H), 5.30(d, 1H), 4.58(d, 1H), 3.73(s, 3H), 3.66(s, 3H), 2.98(m, 2H).

Synthesis of methyl 3 -{3,4 -bis [(tert-butyldimethylsilyl) oxy]phenyl} -2 -[(methoxycarbonyl) amino] propanoate (2): Compound 1(7.8g, 29.0mmol) was taken in a 2-neck round-bottomed flask. Imidazole(7.9g, 11.6mmol) and DMAP(0.1mol%) were added at room temperature. Add dry DMF(70ml) and then a solution of dry DMF(10ml) and TBDMS-Cl was added to it under nitrogen conditions at room temperature. Stir the reaction at room temperature for 12 hours. The product was extracted by DCM and dried using a rotary evaporator. The product was then purified by column chromatography at 30% ethyl acetate in pet ether concentration. Yield = 11.2g (77.3%). ^{1}H NMR (400MHz, $CDCl_3$) δppm: 6.73(d, 1H), 6.59(s, 1H), 6.54(d, 1H), 5.09(d, 1H), 4.57(q, 1H), 3.71(s, 3H), 3.66(s, 3H), 2.98(d, 2H), 0.98(d, 18H), 0.18(d, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δppm: 172.25, 122.30, 121.22, 54.86, 52.43, 37.58, 26.06, 18.56.

Synthesis of C8 Polymer: Compound-2 (0.76g, 1.5mmol) and 1,8-Octanediol (0.22, 1.5mmol) were weighed in a polymer tube. This was allowed to melt and then 0.1 mol% $Ti(O^tBu)_4$ catalyst was added. The polymerization was done using the melt-condensation approach, at 150℃ with continuous stirring, first four hours under nitrogen purge and the next two hours under vacuum(10^{-2}mbar). The polymer was precipitated in methanol. ^{1}H NMR (400MHz, $CDCl_3$) δppm: 6.72(d, 1H), 6.60(s, 1H), 6.54(d, 1H), 5.09(d, 1H), 4.55(q, 1H), 4.02(m, 4H), 2.98(d, 2H), 1.30(s, 12H), 0.98(d, 18H), 0.17(s, 12H).

Synthesis of C10 Polymer: Compound-2 (0.500g, 1.01mmol) and 1,10-Decanediol (0.18g, 1.06mmol) were weighed in a polymer tube. This was allowed to melt and then 0.1 mol% $Ti(O^tBu)_4$ catalyst(0.1mol%) was added. The polymerization was done using the melt-condensation approach, at 150℃ with continuous stirring, first four hours under nitrogen purge and the next two hours under vacuum(10^{-2}mbar). ^{1}H NMR (400MHz, $CDCl_3$) δppm: 6.72(d, 1H), 6.60(s, 1H), 6.54(d, 1H), 5.09(d, 1H), 4.55(q, 1H), 4.02(m, 4H), 2.97(d, 2H), 1.58(m, 4H), 1.28(s, 12H), 0.97(d, 18H), 0.18(s, 12H).

Synthesis of C12 Polymer: Compound-2 (0.6g, 1.20mmol) and 1,12-Dodecanediol (0.26g, 1.23mmol) were weighed in a polymer tube. This was allowed to melt, and then 0.1 mol% $Ti(O^tBu)_4$ catalyst was added. The polymerization was done using the melt-condensation approach, at 150℃ with continuous stirring, first four hours under nitrogen purge and the next two hours under vacuum(10^{-2}mbar). ^{1}H NMR (400MHz, $CDCl_3$) δppm: 6.72(d, 1H), 6.60(s, 1H), 6.54(d, 1H), 5.09(d, 1H), 4.55(q, 1H), 4.03(m, 4H), 2.97(d, 2H), 1.58(m, 4H), 1.26(s, 16H), 0.98(d, 18H), 0.18(s, 12H).

Synthesis of 6-(benzoyloxy)-2,3,4,5-tetrahydroxyhexyl benzoate (3): D-mannitol (55g, 301.9mmol) was weighed in a 2-neck round-bottomed flask, and dry pyridine(500ml) was added. Benzoyl chloride was added under ice-cold conditions(0°C) with continuous stirring. The reaction was stirred at room temperature for five hours under nitrogen conditions. The reaction was then precipitated in ice-cold water and filtered. The reaction was given chloroform wash and recrystallized in ethanol. It was then dried using rotary evaporation. Yield = 45.9g (52%). ^{1}H NMR (400MHz, DMSO-d6) δppm: 8.04(d, 4H), 7.66(t, 2H), 7.53(t, 4H), 4.54(d, 2H), 4.28(q, 2H), 3.86(m, 2H), 3.76(d, 2H).

Synthesis of {8-[(benzoyloxy)methyl]-2,2,6,6-tetramethyl-hexahydro-[1,3] dioxino [5,4-d] [1,3]dioxin-4-yl} methyl benzoate (4): Compound 3(25g, 64.1mmol) was weighed in a 2-neck round-bottomed flask and dry acetone(175ml) was added to it. PTSA(0.1mol%) and 2,2-dimethoxy propane were added, and the reaction was stirred at room temperature for 12 hours under nitrogen conditions. The product was purified by column chromatography at 40% ethyl acetate in pet ether. Yield = 18g (59.4%). ^{1}H NMR (400MHz, DMSO-d6) δppm: 7.97(dd, 4H), 7.67(m, 2H), 7.54(m, 4H), 4.37(m, 5H), 4.03(m, 3H), 1.31(m, 12H).

Synthesis of [8-(hydroxymethyl)-2,2,6,6-tetramethyl-hexahydro-[1,3] dioxino [5,4-d] [1,3] dioxin-4-yl] methanol (5): Compound 4(18g, 38.3mmol) was weighed in a 2-neck round-bottomed flask and sodium methoxide(4.2g, 76.6mmol) was added to it. Dry methanol(20ml) and dry chloroform(60ml) was added. The reaction was stirred at room temperature under nitrogen conditions for 12 hours. The product was washed with chloroform and purified by column chromatography at 50% ethyl acetate in pet ether. Yield = 4.3g (42.8%).

^{1}H NMR (400MHz, DMSO-d6) δppm: 4.71(s, 2H), 3.72(d, 2H), 3.42(m, 6H) 1.26(d, 12H). ^{13}C NMR (100 MHz,DMSO-d6) δppm:

Synthesis of L-DOPA Silyl and Sugar-based Diol Polymer: Compound-2 (0.50g, 1.01mmol) and Compound-5 (0.28g, 1.06mmol) were weighed in a polymer tube. This was allowed to melt and then 0.1 mol% DBTO catalyst was added. The polymerization was done using the melt-condensation approach, at 150℃ with continuous stirring, first four hours under nitrogen purge and the next two hours under vacuum(10^{-2}mbar). ^{1}H NMR (400MHz, $CDCl_3$) δppm: 6.70(m, 3H), 5.14(d, 1H), 4.60(m, 1H), 4.24(m, 4H), 3.78(m, 4H), 2.98(m, 2H), 1.32(d, 12H), 0.97(m, 18H), 0.18(s, 12H).

Deprotection of L-DOPA Silyl and Sugar-based Diol Polymer: 200mg Polymer was weighed in a round-bottomed flask and dissolved in 2ml Pyridine. 2ml 70%HF in pyridine was added to it dropwise under ice-cold conditions and stirred for 6 hours. It was dialysed for 24 hours and then lyophilised for 24 hours.

Drug and Dye Encapsulation, Cell Viability Assay and Cellular Uptake Studies:

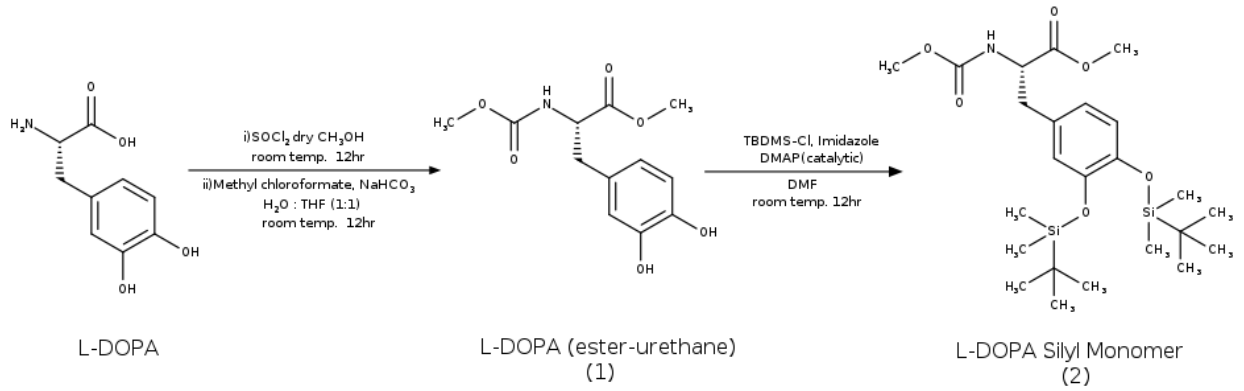
5mg deprotected polymer and 0.5mg drug(DOX)/dye(Nile Red) were dissolved in 2ml DMSO. 3ml water was added dropwise under continuous stirring at room temperature. It was then transferred to a 3.5kD dialysis membrane and dialysed for 24 hours. After 24 hours, absorbance and emission were recorded. The cytotoxicity of the nascent polymer, as well as the drug-loaded polymer, was checked using MTT Assay. Sterile 96 well plate was seeded with a cell density of 1000 cells per well in 100 μL DMEM (with 10% FBS) and incubated at 37℃ for 16 hr. After which different concentrations of the polymer were added and again incubated at 37 0℃ for 72 hr and after adding MTT, they were again incubated for 4 h at 37℃. Cellular uptake and confocal imaging, for the drug-loaded polymer, were then performed after confirming its cytotoxicity toward cancer cells.

Results and Discussion

Synthesis and Characterization of Monomers:

L-DOPA Silyl Monomer:

Amino acid L-DOPA has an amine group and a carboxylic group. The carboxylic group was converted into ester and the amine group into urethane. The hydroxyl groups of the catechol unit were silyl protected (*Scheme 1*). The monomer was characterised by ^{1}H NMR and ^{13}C NMR.



***Scheme 1**: Synthesis of L-DOPA Silyl Monomer (Compound 2)*

The ^{1}H NMR for compound 1 showed aromatic peaks at 6.74(H_b), 6.64(H_a) and 6.51(H_c)ppm. The peaks at 5.30ppm and 4.58ppm correspond to the N-H(H_f) and the proton attached to chiral carbon(H_e) respectively. The peaks at 3.73ppm and 3.66ppm correspond to the methoxy protons of ester(H_h) and urethane(H_g) respectively. The peak at 2.98 corresponds to the -CH_2(H_d) protons. In the ^{1}H NMR for compound 2, we see the appearance of silyl peaks at 0.98ppm and 0.18ppm that correspond to (H_j) and (H_i) respectively after silyl protection, rest of the peaks remain the same.

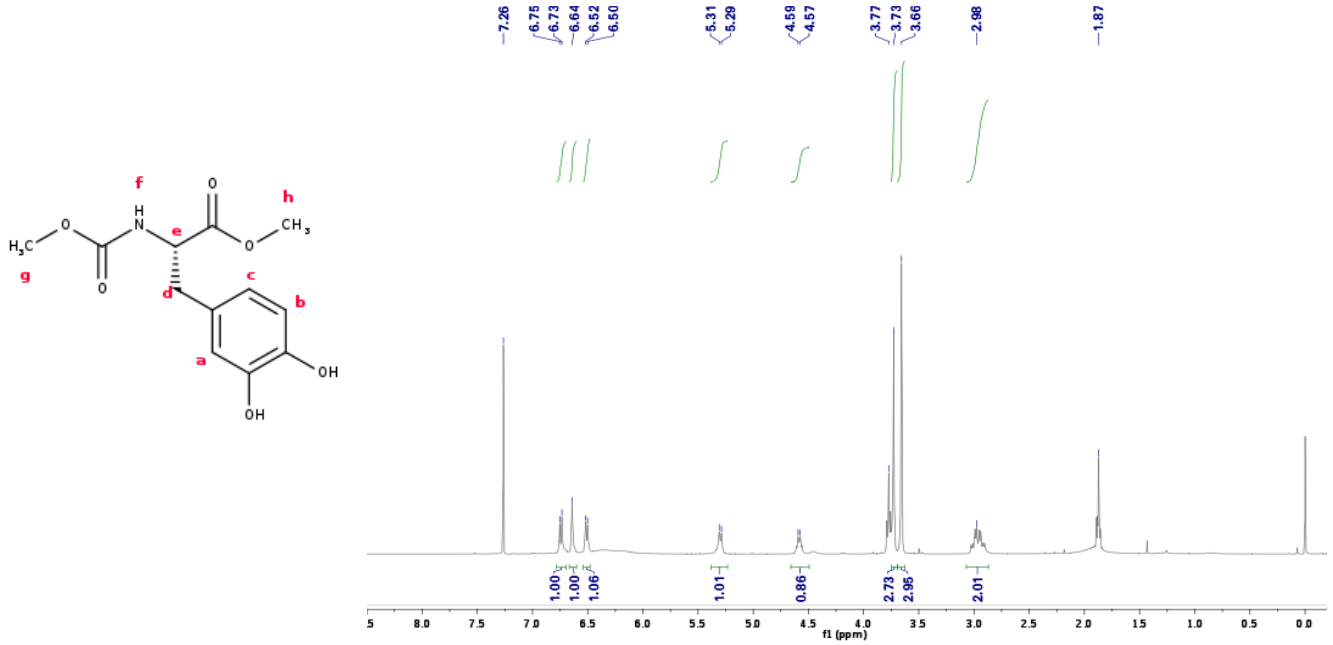


Figure 3.1 *^{1}H NMR of Compound 1*

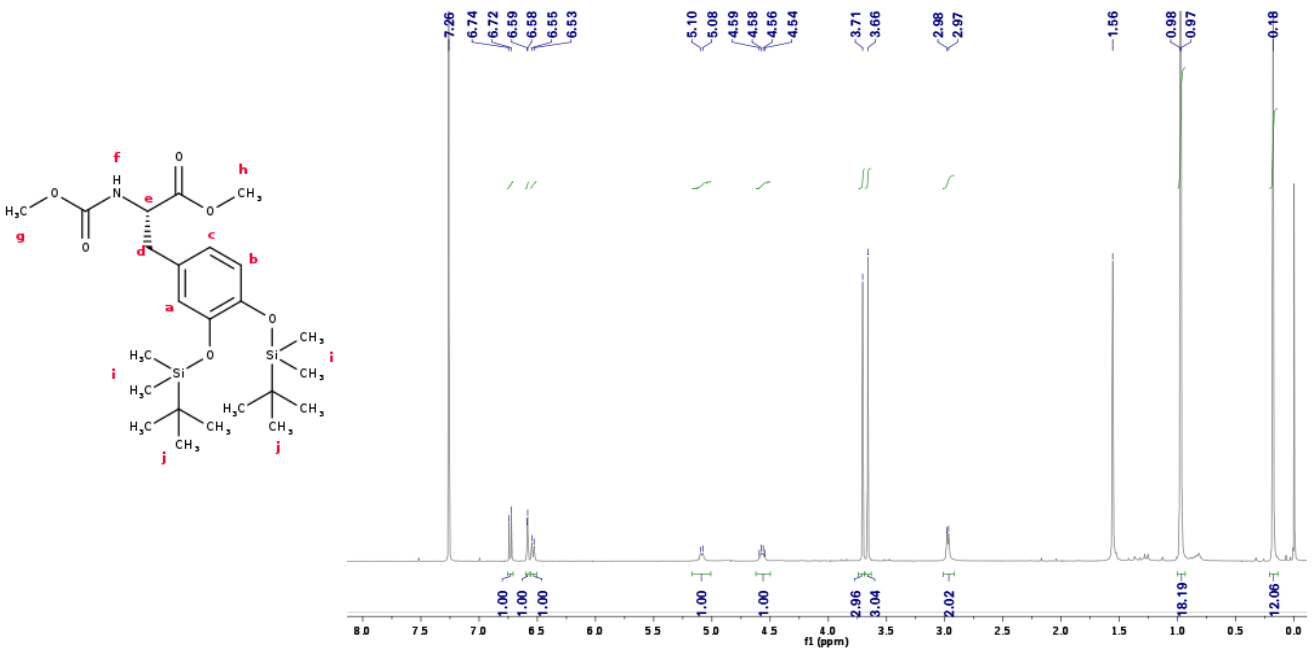


Figure 3.2 *^{1}H NMR of Compound 2*

Compound 2 was further confirmed by ^{13}C NMR where we see a peak corresponding to the carbonyl carbon at 172.25ppm, aromatic peaks at 122.30ppm, 121.22ppm, methoxy peaks at 54.86ppm(H_g) and 52.43ppm(H_h), -CH_2 (H_d) at 37.58ppm, and the silyl peaks at 26.06ppm(H_j) and 18.56ppm(H_i).

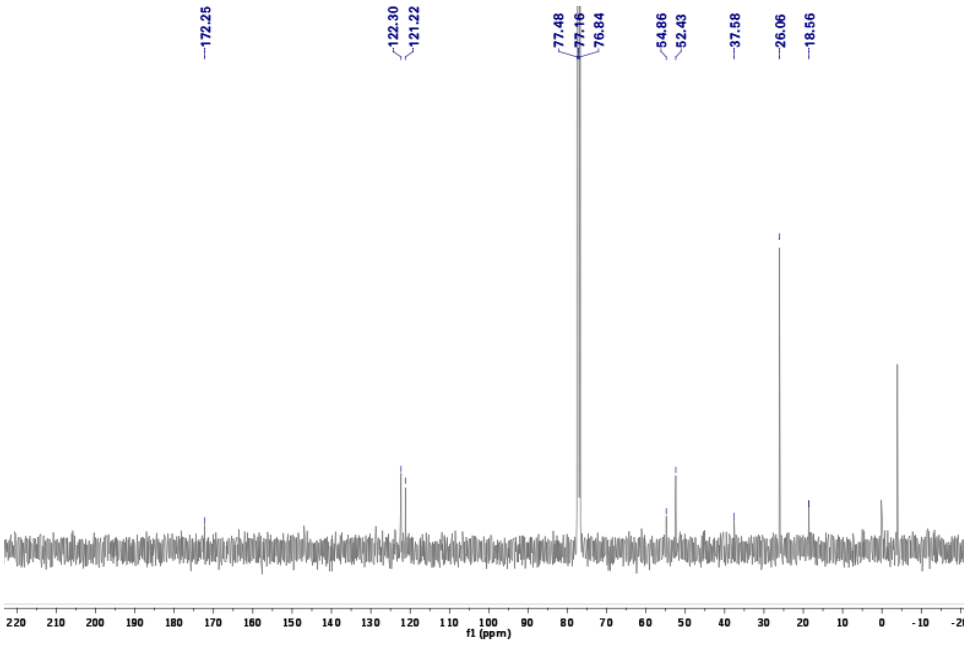


Figure 3.3 *^{13}C NMR of Compound 2*

After characterization by ^{1}H NMR and ^{13}C NMR, the L-DOPA Silyl Monomer was further confirmed by Infrared(IR) Spectroscopy and the thermal properties were checked using thermogravimetric analysis(TGA).

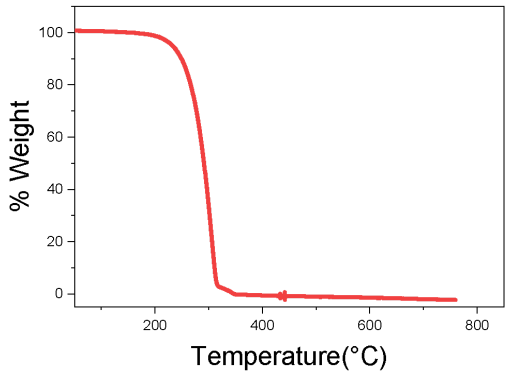


Figure 3.4 *TGA plot of Compound 2*

The monomer was stable up to 190℃ and then as the temperature is increased it starts decomposing as can be seen from the TGA plot. At 190℃, we observe that the %weight starts decreasing and goes to zero. This makes it evident that the monomer is melt-stable and can be used for polymerization at 150℃ by the melt-condensation approach. In the IR spectrum, we see **N-H** stretching frequency at around 3000 cm^{-1}, carbonyl at 1750 cm^{-1}, the aromatic hydrocarbon stretching frequency occurs at 1500 cm^{-1}, RCO**O-CH$_3$** (ester/ urethane) at 1250-1050 cm^{-1} and the **O-Si** in the silyl groups at 900-700 cm^{-1}.

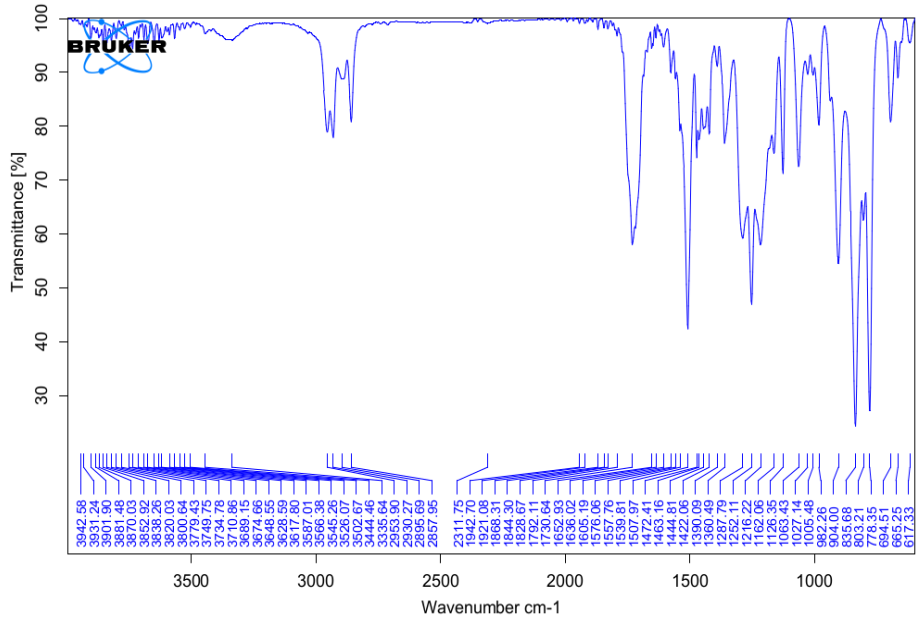
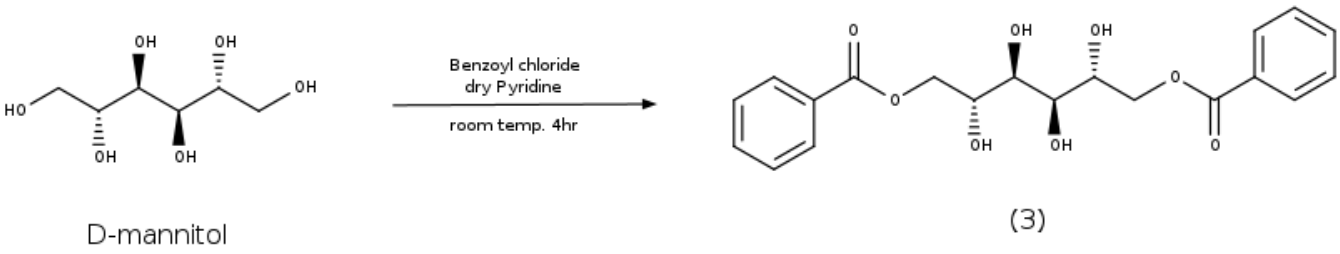


Figure 3.5 *IR Spectrum of Compound 2*

D-mannitol diol:

The first step in the synthesis of D-mannitol diol is the esterification of primary hydroxyl groups (*Scheme 2*). The ^{1}H NMR for compound 3 showed aromatic peaks at 8.04ppm(H_a), 7.66ppm(H_c) and at 7.53ppm(H_b). The peaks appearing from 4.29ppm to 3.34ppm correspond to the -CH_2 and -CH protons.



***Scheme 2**: Protection of Primary Alcohol*

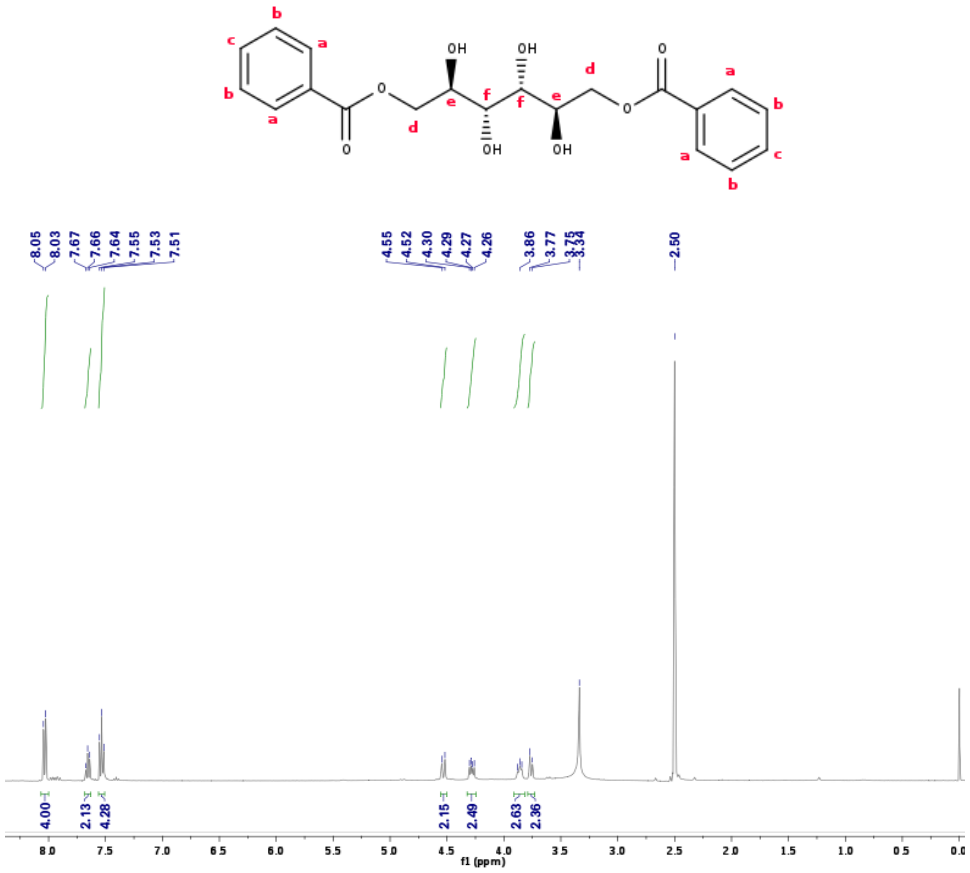
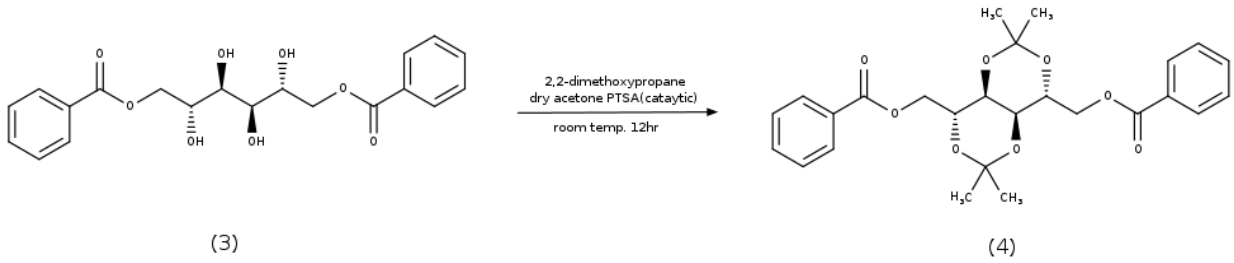


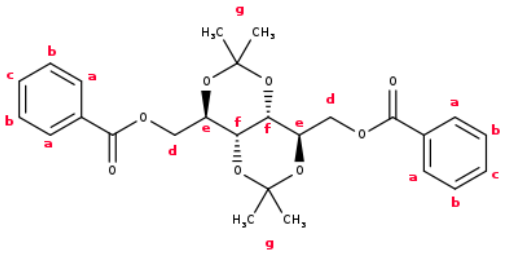
***Figure 3.6** [1]H NMR of Compound 3*

In the second step (*Scheme 3*), secondary hydroxyl groups are protected by the acetal groups. New peaks corresponding to the methyl groups of the acetal appear at 1.42, 1.28ppm(five-membered ring isomer) and 1.33, 1.26ppm(six-membered ring isomer).



(3) (4)

***Scheme 3**: Protection of Secondary Alcohol*



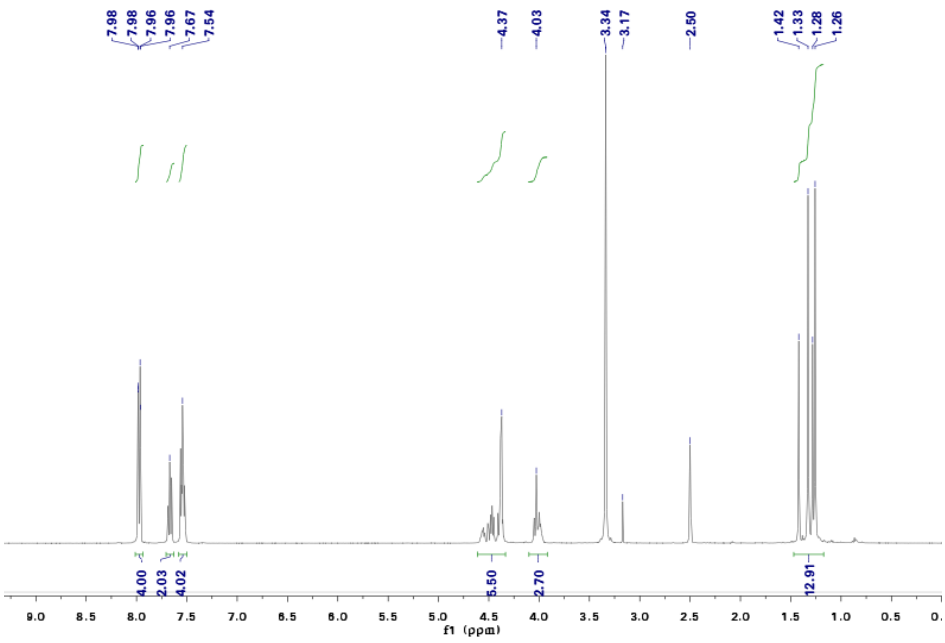
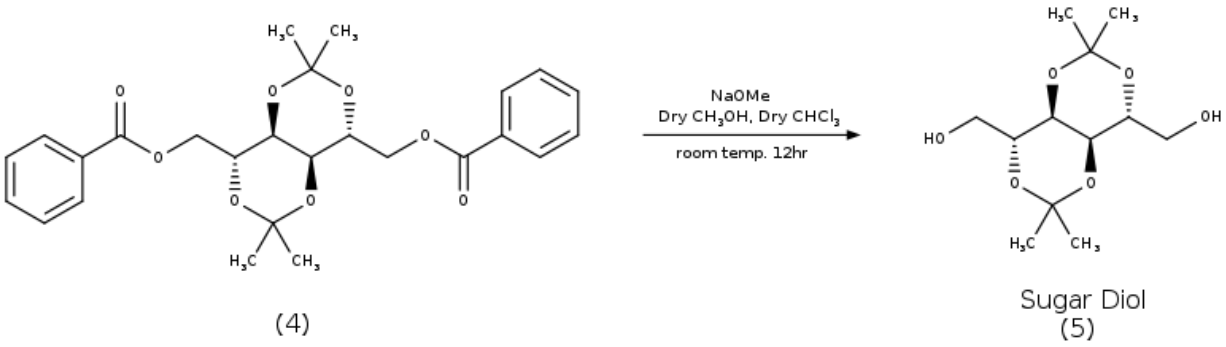


***Figure 3.7** ^{1}H NMR of Compound 4*

In the next step (*Scheme 4*), the primary alcohol is deprotected to form sugar diol(compound 5). The six-membered ring isomer is purified by column chromatography and is characterised by ^{1}H NMR, ^{13}C NMR, IR and TGA. In the ^{1}H NMR spectrum, we see that the aromatic peaks have disappeared and the -OH peaks have appeared. We see the -OH peaks appearing at 4.71ppm and no peaks corresponding to the five-membered ring isomer. In the ^{13}C NMR we see the peak corresponding to quaternary carbon at 101.06ppm, -C-H_e at 70.30ppm, -C-H_f at 67.81ppm, -C-H_d at 63.11ppm and the peaks corresponding to the methyl group of the acetal at 24.93ppm and 23.92ppm.



***Scheme 4**: Deprotection of Primary Alcohol*

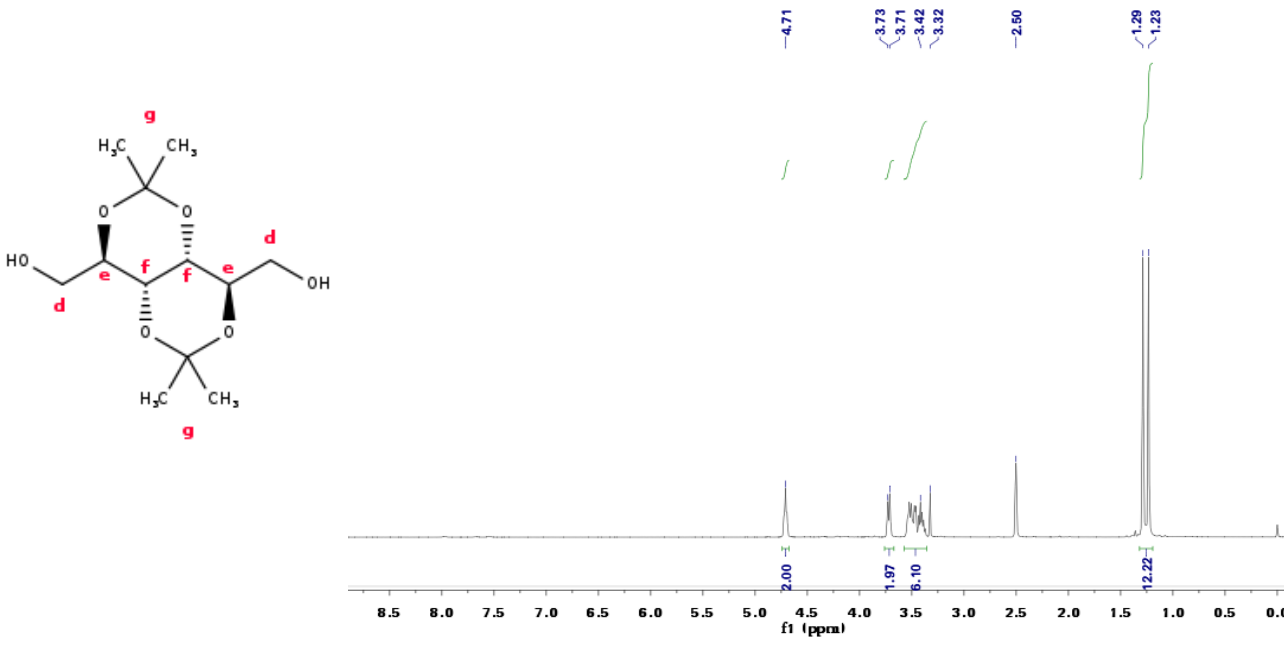


***Figure 3.8** ^{1}H NMR of Compound 5*

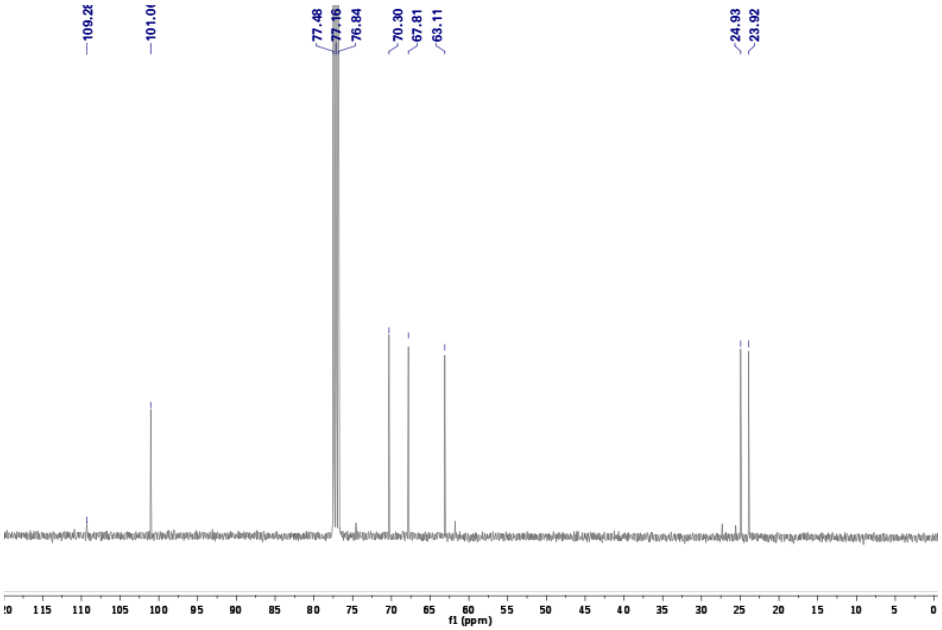


Figure 3.9 *^{13}C NMR of Compound 5*

In the IR spectrum we can see a broad peak for **O-H** stretching frequency at 3250 cm^{-1}, C-H stretch at around 3000 cm^{-1}, the C-H bending and two rocking and the C-O stretching frequency, all occur between 1700-700 cm^{-1}.

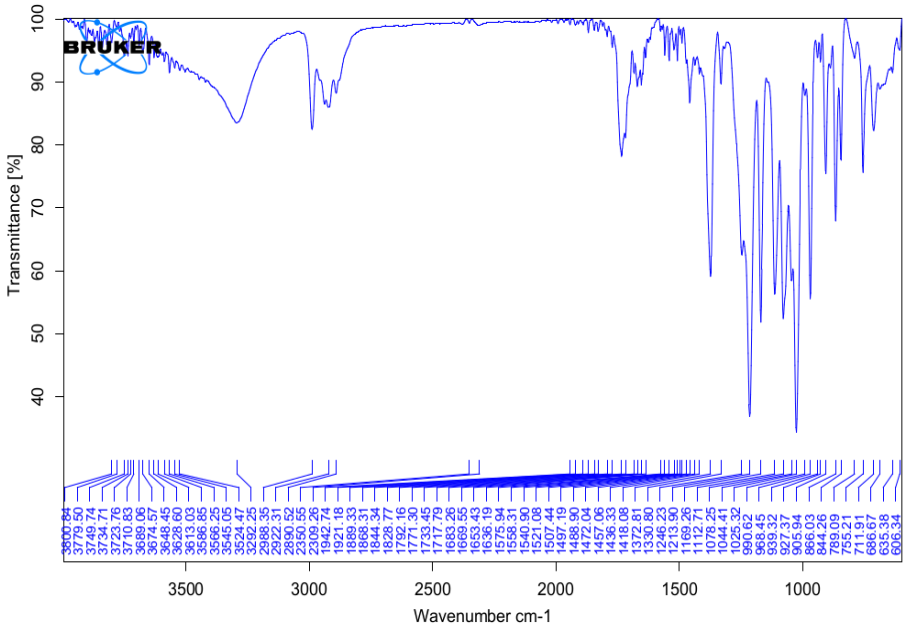
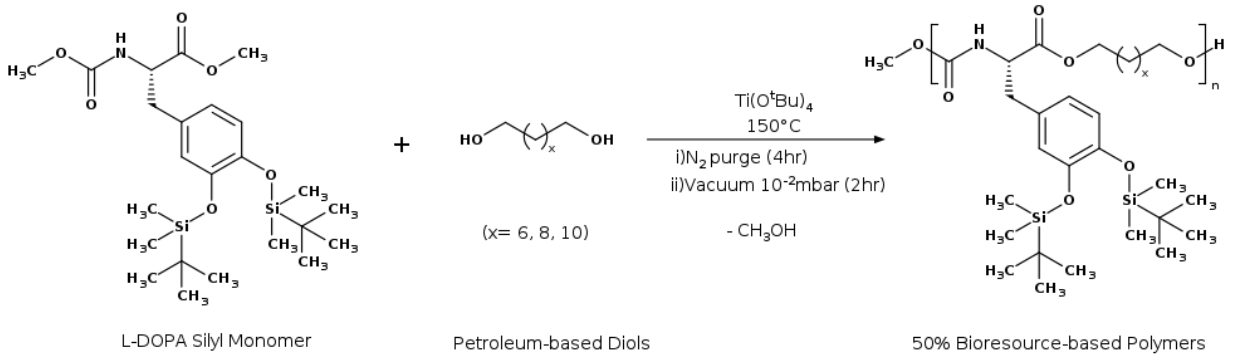


Figure 3.10 *IR Spectrum of Compound 5*

Poly(ester-urethane) from L-DOPA Silyl Monomer and Petroleum-based Diols:

Three polymers (C8, C10 and C12) from the L-DOPA silyl monomer and different chain lengths of diols were synthesised (*Scheme 5*).



Scheme 5*: Poly(ester-urethane) from Amino acid and Petroleum-based diol*

1,8-octane diol, 1,10-decane diol and 1,12-dodecane diol were used, respectively, for poly(ester-urethane) synthesis. These polymers were characterised by ^{1}H NMR, ^{13}C NMR, GPC, TGA, DSC and IR. The molecular weights of the polymer were determined from ^{1}H NMR and GPC. Their thermal properties were studied from TGA and DSC.

In the ^{1}H NMR spectrum of the C8 polymer, we see the methoxy peaks disappearing and the new peaks corresponding to the diol appearing at 4.02ppm and 1.30ppm. The polymer was also characterised by ^{13}C NMR where we see methoxy peaks disappearing which were in the range 50-55ppm and the new peak corresponding to the diol appearing at 28.64ppm.

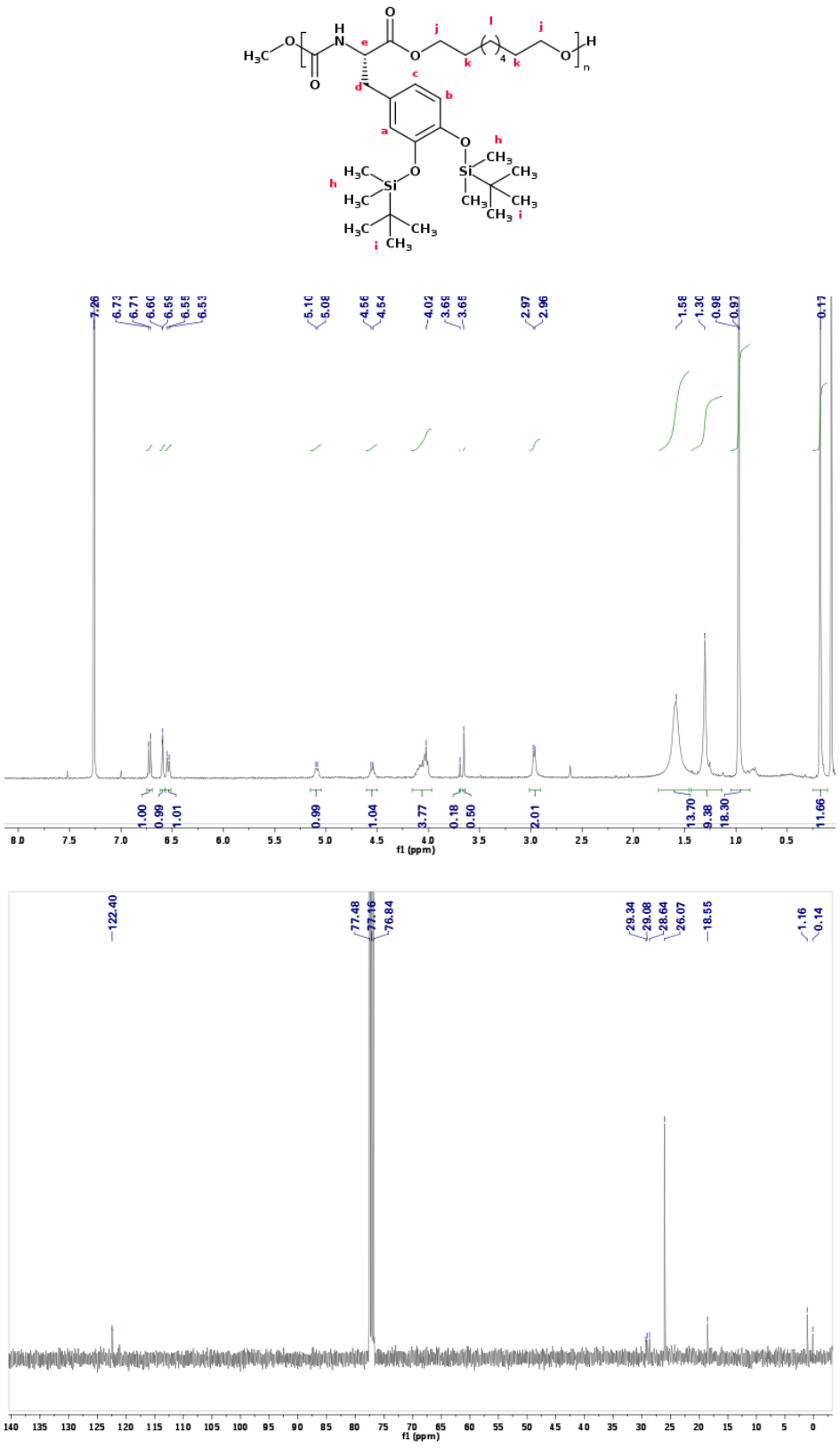


Figure 3.11 *^{1}H and ^{13}C NMR of C8 Polymer*

The number average molecular weight was determined from the ^{1}H NMR spectrum as follows:

$$Number\ of\ Repeating\ Units\ = \frac{New\ Peak\ Intensity/4}{End\ group\ Peak\ Intensity/6}$$

$$Number\ of\ Repeating\ Units\ = \frac{3.77/4}{0.68/6}\ = 8$$

$$Number\ Average\ Molecular\ Weight(Mn)\ = Repeating\ Unit\ Mass\ \times\ Number\ of\ Repeating\ Units$$

$$= 580\ \times\ 8$$

$$= 4640\ g/mol$$

Similarly in the ^{1}H NMR spectrum of the C10 polymer, we see the methoxy peaks disappearing and the new peaks corresponding to the diol appearing at 4.02ppm and 1.28ppm. The polymer was also characterised by ^{13}C NMR where we see the peaks corresponding to ester and urethane have disappeared and a new peak has appeared at 29.09ppm that corresponds to the diol.

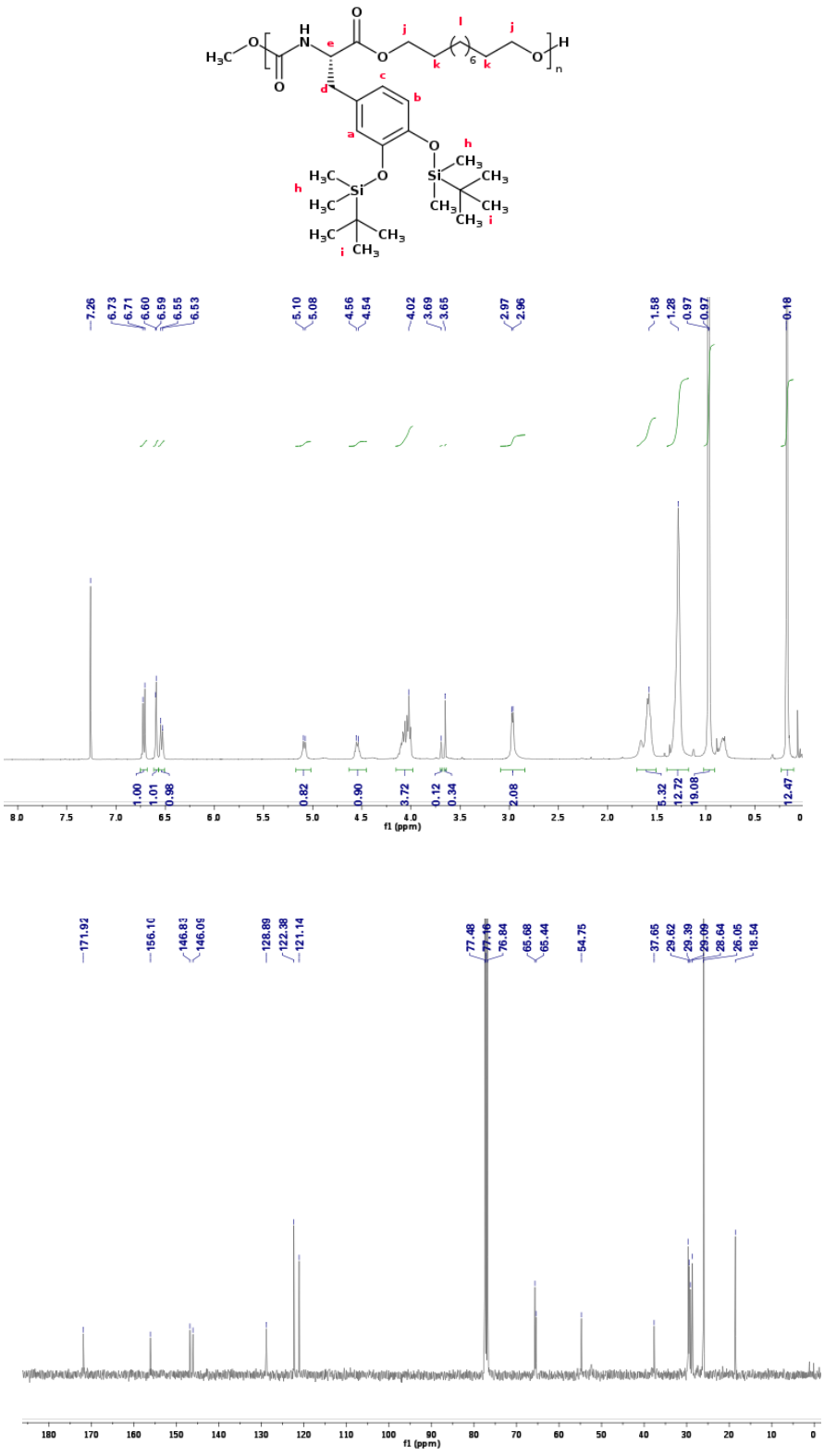


Figure 3.12 *^{1}H and ^{13}C NMR of C10 Polymer*

The number average molecular weight was determined from the ^{1}H NMR spectrum as follows:

$$Number\ of\ Repeating\ Units = \frac{New\ Peak\ Intensity/4}{End\ group\ Peak\ Intensity/6}$$

$$Number\ of\ Repeating\ Units = \frac{3.72/4}{0.46/6} = 12$$

$$Number\ Average\ Molecular\ Weight(Mn) = Repeating\ Unit\ Mass \times Number\ of\ Repeating\ Units$$

$$= 608 \times 12$$

$$= 7296\ g/mol$$

Similarly in the ^{1}H NMR spectrum of the C12 polymer, we see the methoxy peaks disappearing and the new peaks corresponding to the diol appearing at 4.03ppm and 1.26ppm. The polymer was also characterised by ^{13}C NMR where we see the peaks corresponding to ester and urethane have disappeared and a new peak has appeared at 28.64ppm that corresponds to the diol.

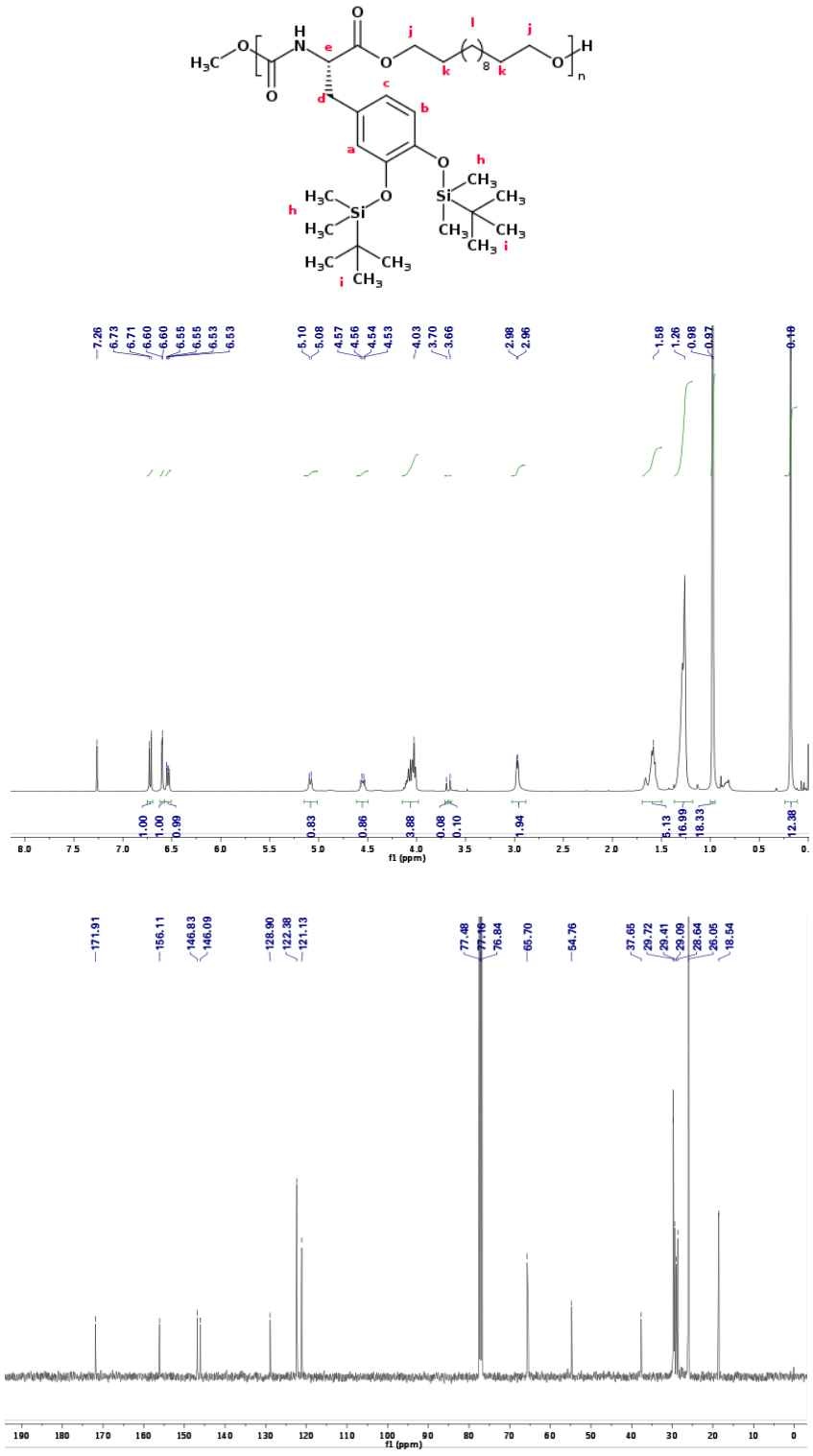


Figure 3.13 *^{1}H and ^{13}C NMR of C12 Polymer*

The number average molecular weight was determined from the ^{1}H NMR spectrum as follows:

$$Number\ of\ Repeating\ Units\ =\ \frac{New\ Peak\ Intensity/4}{End\ group\ Peak\ Intensity/6}$$

$$Number\ of\ Repeating\ Units\ =\ \frac{3.88/4}{0.18/6}\ =\ 32$$

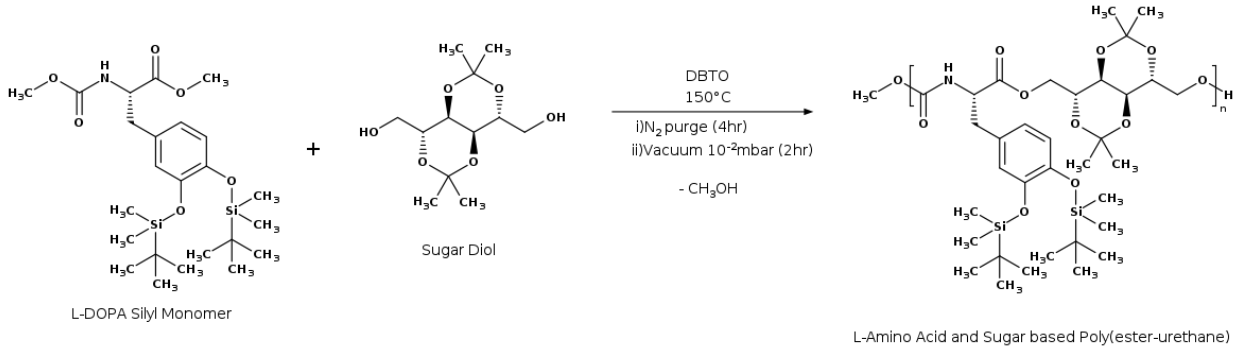
$$Number\ Average\ Molecular\ Weight(Mn)\ =\ Repeating\ Unit\ Mass\ \times\ Number\ of\ Repeating\ Units$$

$$=\ 636\ \times\ 32$$

$$=\ 20,350\ g/mol$$

Poly(ester-urethane) from L-DOPA Silyl Monomer and Sugar-based Diol:

To synthesise a fully biodegradable poly(ester-urethane), L-DOPA Silyl monomer and D-mannitol diol were synthesised, and the polymer was synthesised by melt-condensation approach (*Scheme 6*).



Scheme 6*: Poly(ester-urethane) from Amino acid and Sugar-based diol*

The polymer was characterised by ^{1}H NMR, ^{13}C NMR, GPC, TGA, DSC and IR. The ^{1}H NMR spectrum showed new peaks between 4.50ppm and 3.50ppm and reduced intensity of methoxy peaks of the L-DOPA Silyl monomer. Other new peaks at 1.36ppm and 1.28ppm correspond to the acetal groups. The polymer was also characterised by ^{13}C NMR, where we see the disappearance of methoxy peaks of ester and urethane of the monomer and the appearance of new peaks(**C**-H_g) at 25.76ppm. The peaks corresponding to the sugar diol are at 101.20ppm, 68.36ppm, 68.31ppm and 24.69ppm, as observed previously in its ^{13}C NMR.

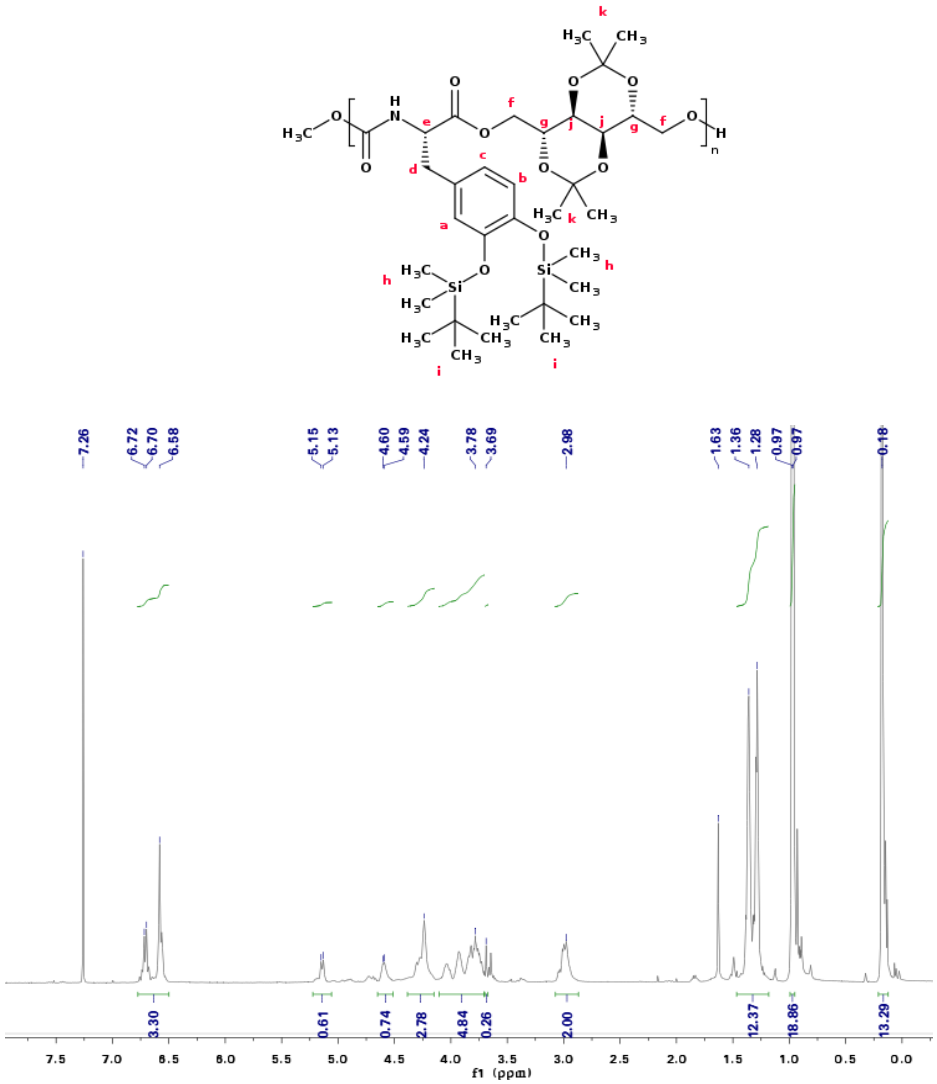


Figure 3.14 *[1]H NMR of Sugar diol-based Polymer*

The number average molecular weight of this sugar diol based polymer was determined from the [1]H NMR spectrum as follows:

$$Number\ of\ Repeating\ Units\ =\ \frac{New\ Peak\ Intensity/8}{End\ group\ Peak\ Intensity/6}$$

$$Number\ of\ Repeating\ Units\ =\ \frac{7.6/8}{0.25/6}\ =\ 23$$

$$Number\ Average\ Molecular\ Weight(Mn)\ =\ Repeating\ Unit\ Mass\ \times\ Number\ of\ Repeating\ Units$$

$$=\ 698\ \times\ 23$$

$$=\ 16,045\ g/mol$$

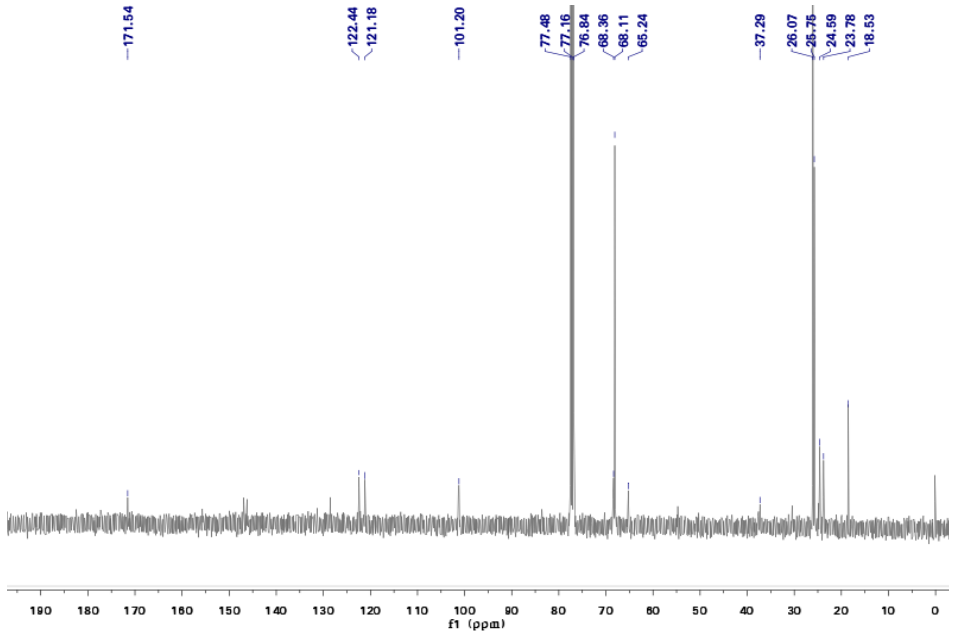


Figure 3.15 *^{13}C NMR of Sugar diol-based Polymer*

The extent of polymerization is determined by:

$Degree\ of\ Polymerization(Xn) = \frac{1}{1-p}$; where p is the extent of reaction

$Number\ Average\ Molecular\ Weight(Mn) = Degree\ of\ Polymerization(Xn) \times Repeating\ Unit\ Mass$

Polymer	X_n	Molecular Mass of Repeating Unit Unit (g/mol)	M_n (g/mol)	p
C8	8	580	4,600	87%
C10	12	608	7,300	92%
C12	32	636	20,400	97%
Sugar-based	23	698	16,000	96%

Table 1*: Number Average Molecular Weight(M_n) and Extent of Reaction(p) calculated from NMR*

The TGA plots for the C8, C10 and C12 were compared (*Figure 3.16)*. From this plot, we observe that the higher chain length diol is stable up to higher temperatures than the lower chain

length diol. The order of stability with respect to temperature is C8 < C10 < C12. The sugar diol-based polymer showed a comparable decomposition temperature as that of the petroleum-based diols, which is 195℃.

a) b)

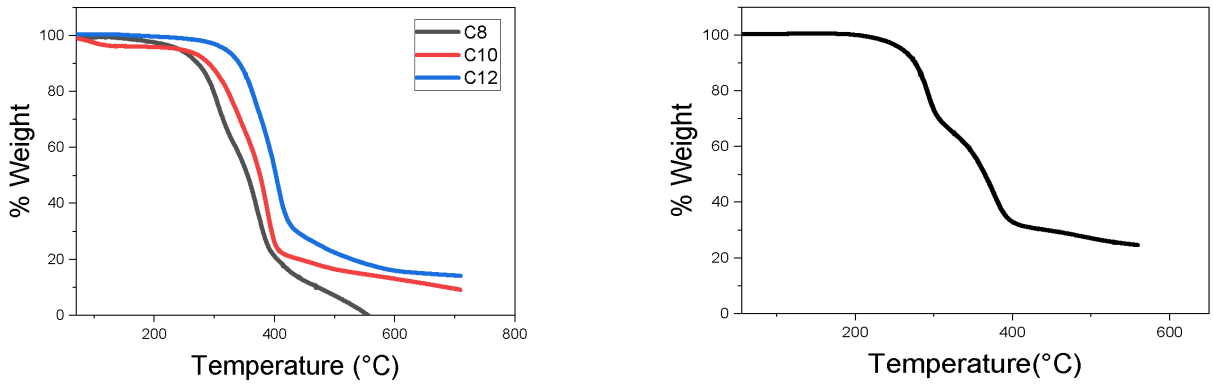


Figure 3.16 *a) TGA plot of petroleum-based diol polymers(C8, C10 and C12) b)TGA plot of sugar-based diol polymer*

Using Gel Permeation Chromatography(GPC), the number average molecular weight(M_n) and the weight average molecular weight(M_w) were determined for all four polymers. The data plotted is as follows:

a) b)

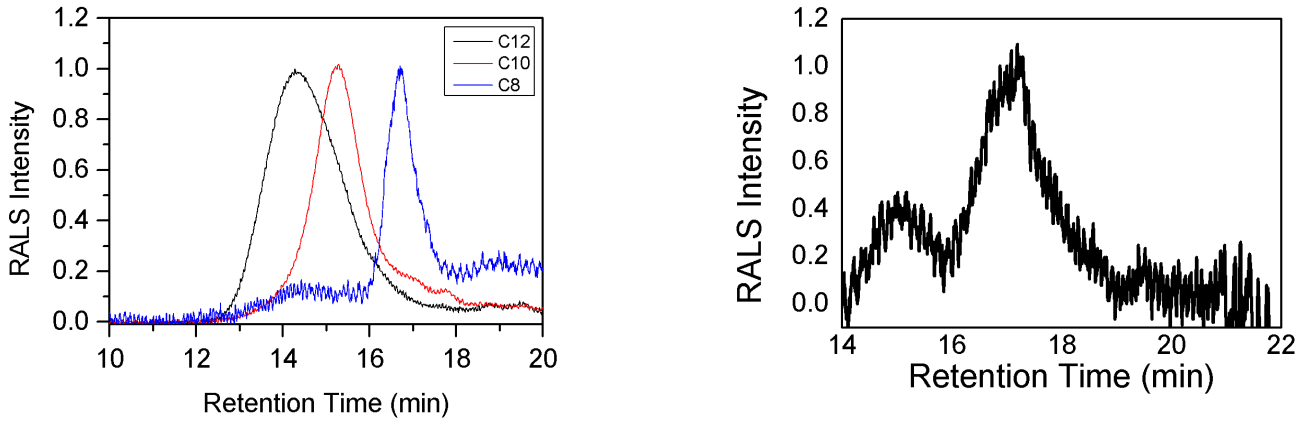


Figure 3.17 *a) GPC plot of petroleum-based diol polymers(C8, C10 and C12) b)GPC plot of sugar-based diol polymer*

From the GPC plots of C8, C10 and C12 we observe that the retention time for C12 is lower than C10, and C10 is lower than C8. This shows that C12 has a longer chain length, which is also observed from the number of repeating units calculated from the ^{1}H NMR. Also, high molecular weight chains are observed as the diol chain length increases which also leads to the same conclusion that the higher chain lengths are in the order C12 > C10 > C8. The M_n for the sugar-based diol polymer, is highly underestimated by GPC, which means that polystyrene is not a good standard for estimating molecular weights from GPC for this particular polymer (*Table 2*). The average molecular weights and the polydispersity index(PDI) determined from NMR and GPC are as follows:

Polymer	**M_n (NMR) (g/mol)**	**M_n (GPC) (g/mol)**	**M_w (g/mol)**	**PDI**
C8	4,600	8,400	10,800	1.3
C10	7,300	6,800	10,200	1.5
C12	20,400	8,800	63,500	7.2
Sugar-based	16,000	2,100	6,800	3.2

***Table 2**: Average Molecular Weights of the Polymers determined from NMR and GPC*

a) b)

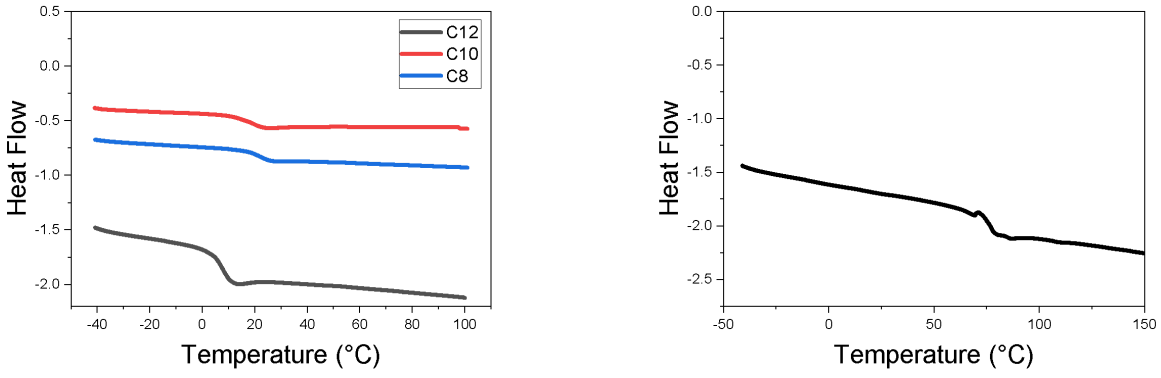


***Figure 3.18** a) DSC Thermograms of petroleum-based diol polymers (C8, C10 and C12) b)DSC Thermogram of sugar-based diol polymer*

We determine the glass transition temperature from DSC thermograms. There's a significant difference between the glass transition temperature of the petroleum-based diol polymers, which comes in the range of 0℃ - 25℃ and that of the sugar-based diol polymer, which comes to be 77℃. IR Spectrum corresponding to C8 (*Figure 3.19*), C10 (*Figure 3.20*), C12 (*Figure 3.21*) and Sugar-based diol polymer (*Figure 3.22*) we see **N-H** stretching frequency at around 3000 cm^{-1}, carbonyl at 1750 cm^{-1}, the aromatic hydrocarbon stretching frequency occurs at 1500 cm^{-1}, RCO**O-CH$_3$** (ester/ urethane) at 1250-1050 cm^{-1} and the **O-Si** in the silyl groups at 900-700 cm^{-1}.

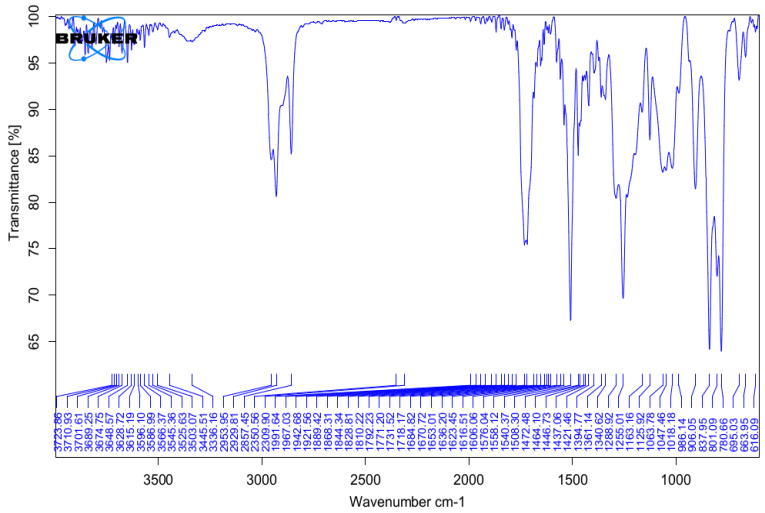


Figure 3.19 *IR spectrum of C8 Polymer*

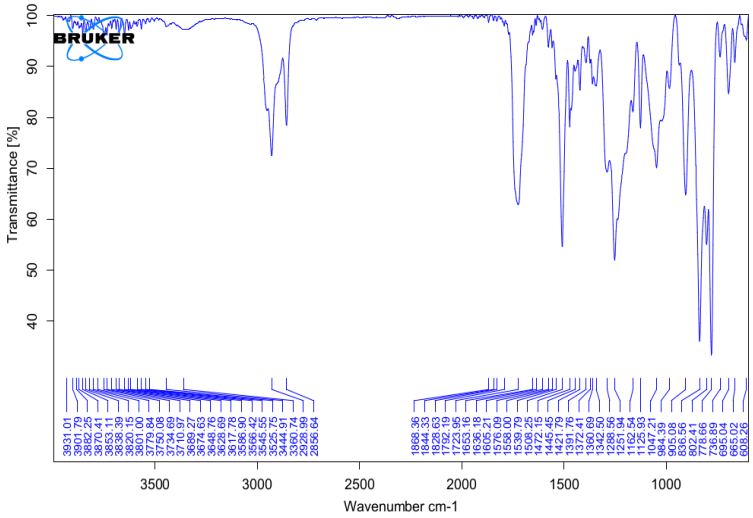


Figure 3.20 *IR spectrum of C10 Polymer*

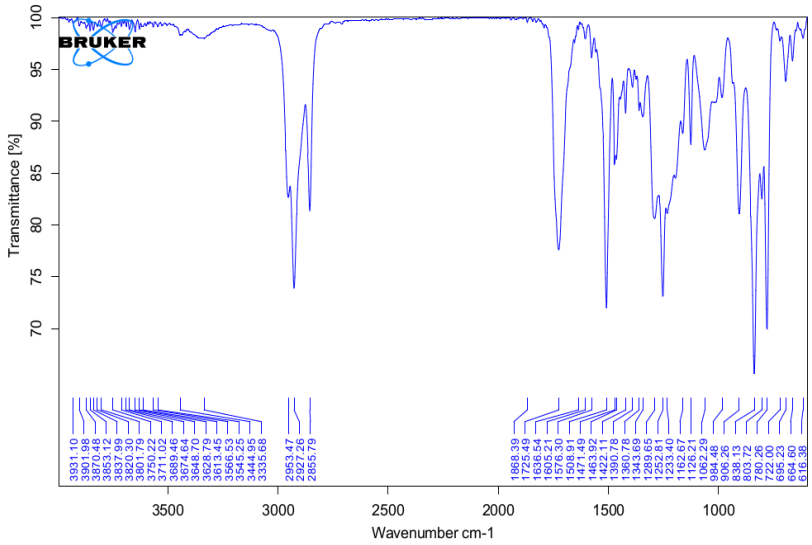


Figure 3.21 *IR spectrum of C12 Polymer*

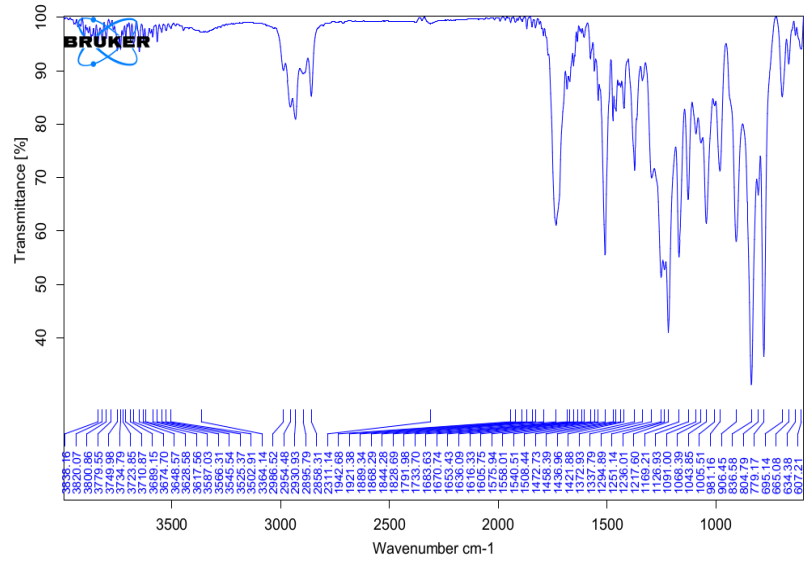
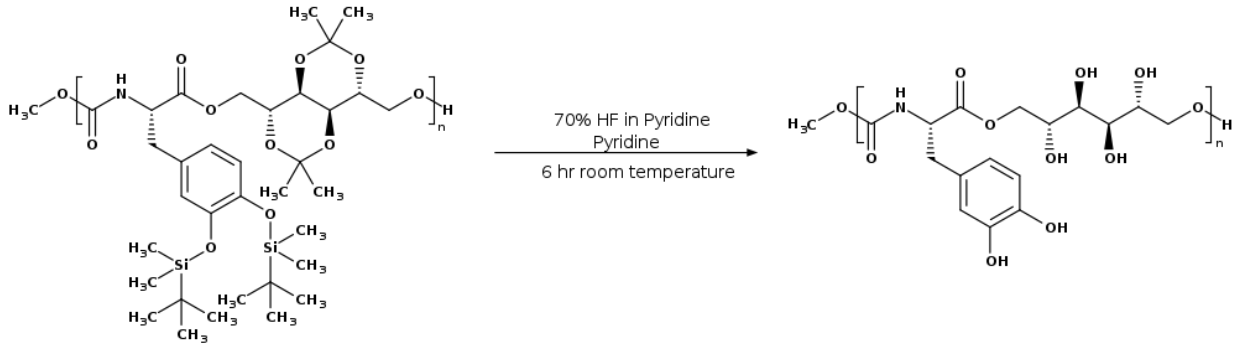


Figure 3.22 *IR spectrum of Sugar diol-based Polymer*

Deprotection of L-DOPA Silyl and Sugar Diol-based Poly(ester-urethane):

The polymer was deprotected using HF (*Scheme 7*). The ^{1}H NMR showed the disappearance of the silyl and the acetal peaks(*Figure 3.23*). The acetal peaks appear at 1.36 and 1.28 as a doublet, which has disappeared, and the silyl peaks appear at 0.97ppm and 0.18ppm, which have also disappeared, as seen in the ^{1}H NMR. The polymer self-assembles to form a nanoparticle. Its size was confirmed by the Dynamic Light Scattering (DLS) technique, which was 4.36 nm while the drug loaded polymer was 12.18 nm (*Figure 3.25*), both at a concentration of 0.3mg/ml.



***Scheme 7**: Deprotection of the Silyl and Acetal Groups of the L-DOPA and Sugar Diol-based Polymer*

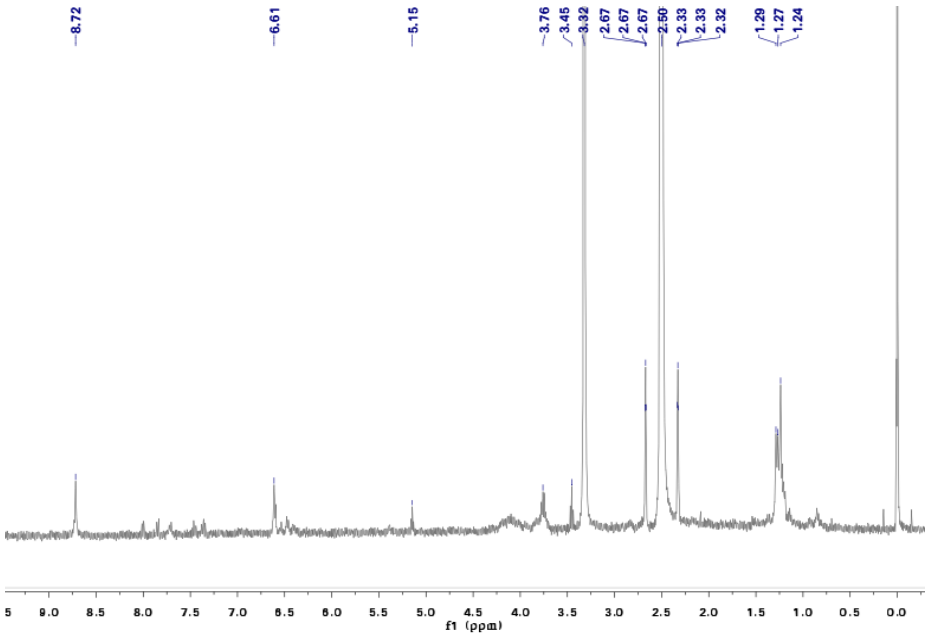


***Figure 3.23** ^{1}H NMR of Deprotected L-DOPA Silyl and Sugar Diol-based Polymer*

Drug and Dye Encapsulation, Cell Viability Assay and Cellular Uptake Studies:

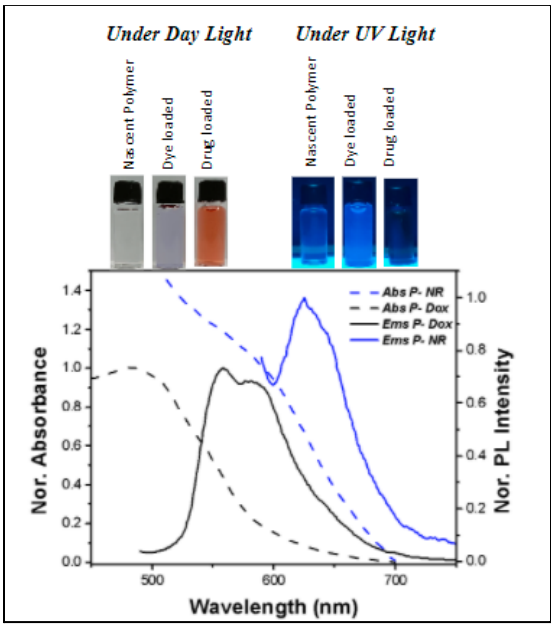


Figure 3.24 *Absorbance and Emission Plot*

The deprotected L-DOPA Silyl and Sugar Diol-based polymer was dialysed for 24 hours(the nascent polymer). For dye and drug encapsulation, 5mg polymer and 0.5mg drug/ dye were dissolved in DMSO and dialysed for 24 hours. The absorbance and emission were recorded and were plotted (*Figure 3.24*) for the dye loaded and drug loaded polymer where we can see that the Nile Red loaded polymer absorbs at 580 nm and emits at 640 nm and the Doxorubicin loaded polymer shows absorption at 480 nm and emission at 560 nm. This depicts that the dye and the drug have been successfully loaded in the polymer.

To calculate the drug loading content, concentration of the drug loaded polymer was calculated from the absorbance data, by using Beer-Lambert's Law. The drug loading capacity(DLC) was calculated to be 6% and the dye loading capacity was 1%.

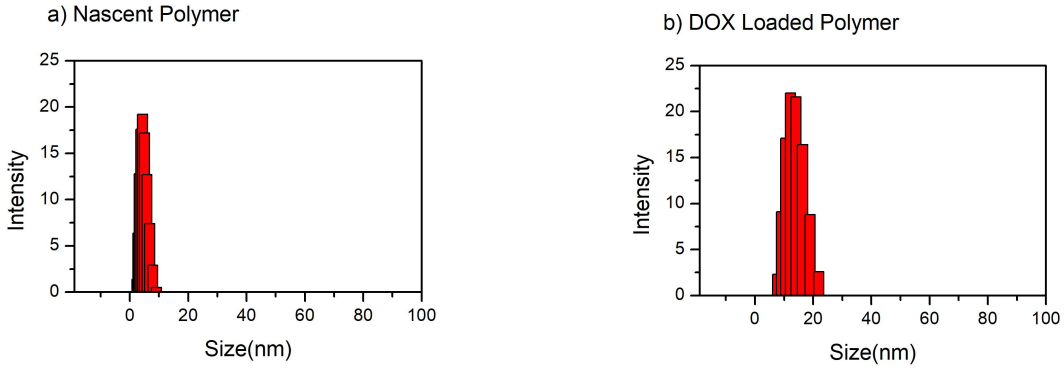


Figure 3.25 *(a) Size of Nascent Polymer (b) Size of DOX Loaded Polymer*

The cytotoxicity of the nanoparticle was studied using MTT assay in HeLa cell line. The cell killing was checked by taking the polymer alone and by taking the dox-loaded polymer. For this, the concentration of the nascent polymer was varied from 5.0 µg/mL to 80.0 µg/mL and the concentration of dox-loaded polymer was varied from 0.1 µg/mL to 0.8 µg/mL. The amino acid and sugar-based poly(ester-urethane) was non-toxic to cells in all these concentrations i.e. upto 80.0 µg/mL whereas the DOX-loaded polymer showed IC_{50} value as 0.4 µg/mL(*Figure 3.26*). The free DOX showed IC_{50} value as 0.4 µg/mL, as known in the literature. This shows that the amino acid and sugar-based poly(ester-urethane) is suitable for delivering DOX to the cervical cancer cells.

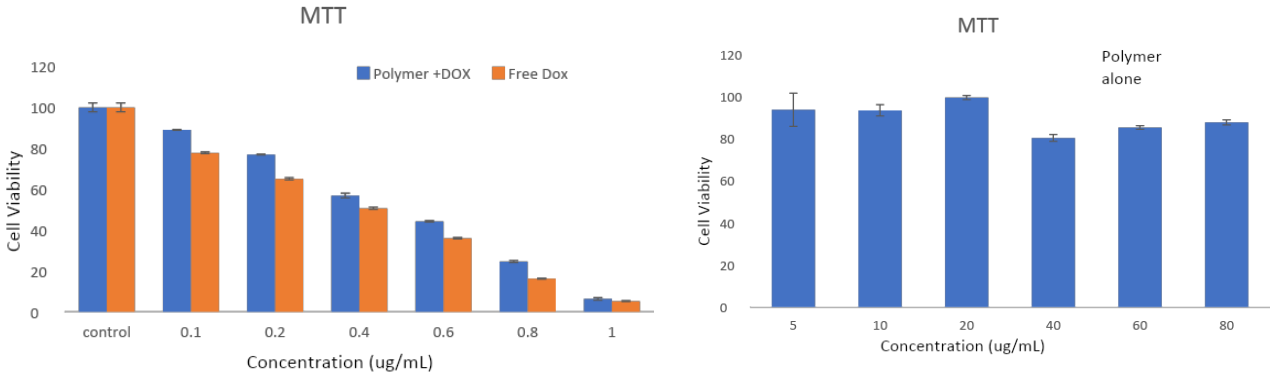


Figure 3.26 *Cytotoxicity of nascent polymer and DOX loaded nanoparticle and free DOX in HeLa cell lines.*

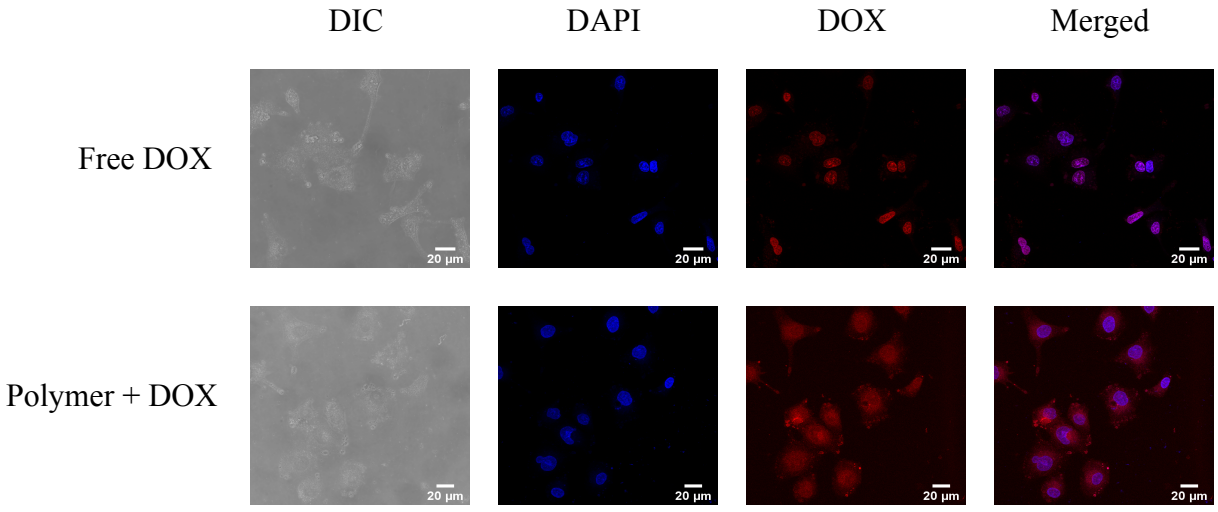


Figure 3.27 *Cellular Uptake Studies*

Further, cellular uptake studies were performed. 4',6-diamidino-2-phenylindole (DAPI) is a fluorescent stain that binds to the adenine and thymine rich regions of the cell; it, therefore, stains the nucleus of a cell. The cancer cells (MCF-7) were then administered with doxorubicin (DOX), and from the merged image, it was confirmed that DOX had entered inside the nucleus of the cells. A similar result was seen in the case of DOX loaded polymer. Thus from the cellular uptake studies, it is confirmed that the DOX loaded polymer has successfully entered the nucleus of the MCF-7 cancer cells, as is confirmed from the merged image shown above (*Figure 3.27*).

Hence the melt polymerisation of the D-mannitol based sugar diol and L-DOPA ester urethane monomers produced high molecular weight aliphatic poly(ester-urethane), which was stable under melt conditions as was confirmed by TGA. This polymer showed quite a higher glass transition temperature(77℃) than the petroleum-diol based polymers, whose glass transition temperature ranges between 5℃ and 25℃. The acetal and silyl groups were further deprotected to get an amphiphilic poly(ester-uretane). In this project, we emphasise using sugar and amino acids to design one of the first examples of completely renewable resource-based poly(ester-urethane).

Conclusion:

L-Amino Acid-based polymers with different types of diols were polymerized by the melt-condensation approach aiming to develop a nanocarrier for drug delivery to cancer cells. For this, three commercially available petroleum-based diols of varying chain length, 1,8-octane diol, 1,10-decane diol and 1,12-dodecanediol were used. Each repeating unit of the polymer had an amino acid and a petroleum-based diol, which makes the polymer only partially biodegradable. To make a fully biodegradable polymer, the petroleum-based diols were replaced by a sugar-based diol. The sugar-based diol was synthesised from D-mannitol, in which first the primary alcohol groups were protected, then the secondary alcohol groups were protected by acetal. The primary alcohol groups were then deprotected to give a sugar-based diol. The amino acid used was L-DOPA, in which the carboxylic group was converted into ester, the amine group was converted into urethane, and the catechol group was silyl protected. By using a sugar-based diol and an amino acid, by solvent-free melt-condensation, a fully biodegradable poly(ester-urethane) was synthesised. The polymer was deprotected, after which it self-assembled to form a nanocarrier of size 4.36nm. The dye Nile Red and the drug Doxorubicin were loaded separately, and their absorbance and emission were recorded. The size of the DOX-loaded nanoparticle was confirmed by DLS, which came out to be 12.18nm which is larger than the nascent polymer. From the absorbance, it was confirmed that the dye and the drug had loaded successfully in the nanoparticle separately. The drug showed 6% loading, whereas the dye showed 1% loading in the L-Amino acid and Sugar-based Poly(ester-urethane). The cytotoxicity studies were carried out in HeLa cell lines by MTT assay, where it was observed that the nanoparticle is capable of delivering DOX to the cervical cancer cells. From the cellular uptake studies, it was confirmed that the DOX loaded polymer has successfully entered the nucleus of the MCF-7 cancer cells. Hence, this fully bioresource based poly(ester-urethane) can be used for biomedical applications.

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