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Synthesis of Components for Amphiphilic Tri-Arm Star Copolymer for Gene Delivery

A Thesis Presented

by

Chai Kit Ngai

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Master of Science

in

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Stony Brook University

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Abstract of the Thesis

Synthesis of Components for Amphiphilic Tri-Arm Star Copolymer for Gene Delivery

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Amphiphilic block copolymers are polymers that consist of hydrophobic and hydrophilic blocks. These polymers have been of interest because of their potential use in medical applications such as gene delivery. The gene delivery vector that our group is currently synthesizing is an amphiphilic tri-arm star copolymer. The tri-arm star copolymer consists of a hydrophillic polyethylene glycol (PEG), a hydrophobic poly (lactic acid) (PLA) block, and a short cleavable oligolysine chain that will complex with DNA. In this document, model studies of lactic acid (LA) polymerization from 3-hydroxy-N-(2-methoxyethyl)-2-[(phenylmethoxy)carbonyl] propanamide (compound 1), and the synthesis of the components of the third arm; which are the cleavable disulfide compound *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) and the short oligolysine peptides chain.

Model studies of LA polymerization were conducted using tin (II) octanoate as catalyst and compound (1) as the initiator for ring opening polymerization. The ring opening polymerizations were conducted in both bulk and solution; with target molecular weights ranging from 2,000 to 10,000 g/mol.

The components necessary to make the third arm of the tri-arm star block copolymer are SPDP and oligolysine peptides. The synthesis of SPDP was accomplished in a two-step reaction, done according to literature. Oligolysine-rich peptides KKC and KKKC were prepared by solution phase peptide synthesis using orthogonal protecting group

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List of Abbreviations

^{1}H NMR- Proton nuclear magnetic resonance

GPC- Gel Permeation Chromatography

PEO- Poly(ethylene oxide)

PLA- Poly(lactic acid)

SPDP- N-Succinimidyl 3-(2-Pyridyldithio) Propionate

K or Lys- Lysine

C or Cys- Cysteine

Boc- t-Butyloxycarbonyl

Fmoc- 9-Fluorenylmethoxycarbonyl

Cbz- Carboxybenzyl

Trityl or Trt- Triphenylmethyl

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I would like to thank Professor Grubbs for allowing me to work in his lab for the past two and a half years. I have learned so much during this time. I want to thank Dr. Yu Cai for training and helping me throughout my research. I could not have asked for a better mentor. I would also like to thank my group members (past and present) Tianyuan, Bingyin, Laura, Dan, Zhe, and Dave for all the help and support. Thanks to Jim Marecek for his training and help with the NMR instrumentation.

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Introduction

Gene Therapy

Gene therapy is the incorporation of genetic material into cells for the treatment of illnesses. [1] The concept of gene therapy has been exploited by nature for as long as we can remember. Viruses have been designed by nature to deliver genetic material into host cells. In doing so the viruses are able to commandeer the cell to reproduce their own genetic information. Researchers want to be able to utilize the ability to incorporate foreign genetic information into a cell, i.e., gene delivery. Gene delivery can even be a potential treatment method for genetic diseases because these diseases are caused by a defect in specific genes. Therefore, if a normal version of the gene can be incorporated into the cell and expressed, this in theory should treat the disease.

The current gene delivery methods are divided into two categories: those that use viral vectors and those that use non-viral vectors. Modified viral vectors have been explored for gene delivery because they show good transfections efficiency both *in vitro* and *in vivo*. The concerns with viral vectors are often ones of safety, such as cell toxicity and immunogenic effects due to the viral vector. [2] There are inherent risks associated with using viral vectors for medical applications because it is difficult to completely control something designed by nature. For example, a clinical gene therapy trial using retroviral vectors was stopped due to serious adverse events in two patients that had developed leukemia from the treatment. [3]

Non-viral vectors have therefore been a promising alternative method for gene delivery because they overcome some of the concerns that arise with viral vectors. Issues such as immunogenic response are virtually eliminated because the components used to construct these synthetic vectors are biocompatible and biodegradable. In an ideal world immunogenic effect

would be the only hurdle that would need to be overcome in using non-viral vectors for gene delivery, but that is not the case. Synthetic vectors also have their limitations and problems, such as having low transfection rates, and high cytotoxicity. Figure 1 shows some of the most studied gene delivery polymers such as polylysine, polyethylenimine (PEI) and polyamidoamine (PAMAM). The reason these particular polymers have been studied is because of their ability to complex with DNA (deoxyribonucleic acid). The amines of these polymers are positively charged and DNA is negatively charged, so this allows them to complex together by electrostatic interactions.[4] The positive charges that these cationic polymers have can be cytotoxic to cells when not complexed to DNA. This is due to the free cationic polymer's ability to interact with the cell membranes and extracellular matrix proteins which can result in apoptosis. [5,6] The goal is then to limit the number of cationic charges on the polymers without compromising electrostatic interactions needed for the polymer to complex with DNA.

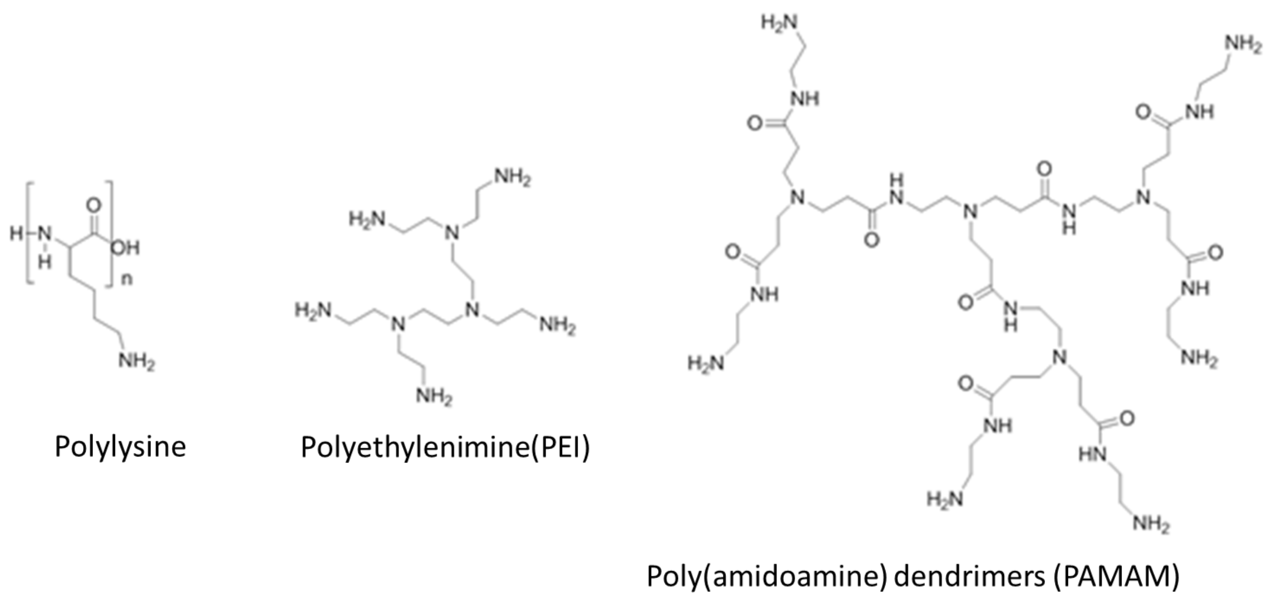


Figure 1: Three polymers commonly studied for gene delivery: polylysine, polyethylenimine (PEI), and polyamidoamine dendrimer (PAMAM) (generation 3). [4]

We are interest in synthesizing amphiphilic tri-arm star copolymer for gene delivery (Figure 2), where one of the arms is a cleavable cationic segment which is made up of a short oligolysine chain. The hope is by making the cationic segment shorter it should minimize cytotoxicity. [7] This alone would not be enough to make an efficient gene delivery vector, which is why the cationic segment is attached to an amphiphilic copolymer in hopes to improve the transfection rate. The amphiphilic copolymer should be able to protect the gene from the external environment and aid passing through the membrane of the cell.

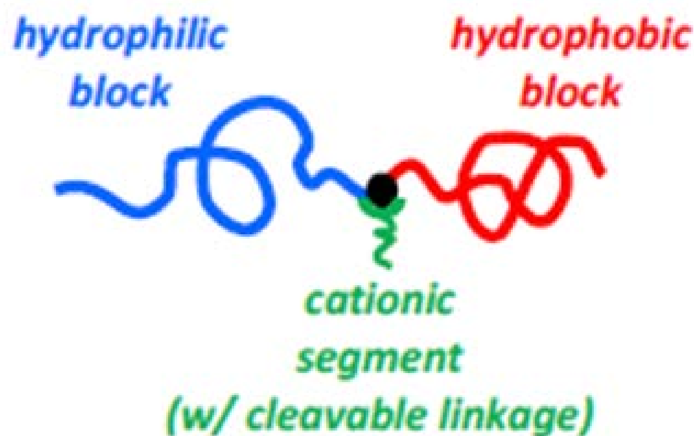


Figure 2: Amphiphilic tri-arm star copolymer with cleavable cationic segment

Amphiphilic Tri-Arm Star Copolymer

Amphiphilic block copolymers are polymers consisting of hydrophilic and hydrophobic blocks. They have the ability to self-assemble into different shapes and sizes depending on the ratios of the polymer blocks.[8,9] When these polymers are exposed to an aqueous environment

they can self-assemble into micellar structures. This can be especially useful when it comes to biological applications such as gene or drug delivery because the polymer can protect the gene or drug from an external environment, and even improve solubility. [10, 11] The amphiphilic tri-arm star copolymer that our group is making for gene delivery is comprised of a hydrophilic block of polyethylene glycol (PEG), a hydrophobic block made of poly (lactic acid) (PLA), and lastly a cleavable cationic segment that can complex with DNA; this is shown in Figure 3.

PEG is a good candidate to be the hydrophilic arm for the amphiphilic tri-arm star copolymer because it is biocompatible and has already been studied in making other types of amphiphilic copolymers. These copolymers include thermoresponsive di and triblock copolymers, [12, 13] ABC triblock for peptide delivery,[14] and even multiblock copolymers as a non-viral gene delivery method.[15] Furthermore, PEG is not only used to make copolymers but it has already been widely studied and used for medical applications, such as a laxative or even a potential treatment for certain types of cancers. [16, 17] PEG is also commercially available at various molecular weights, so it is not necessary to synthesize it.

The second arm is the PLA arm which is the hydrophobic block of the polymer. PLA homopolymer is too fragile and it is hydrophobic which limits its practical applications. That is why researchers have incorporated PEG to PLA making AB diblock copolymers, $(AB)_n$ multiblock copolymer, ABA triblock copolymers, and other types of varies block copolymers. By combining homopolymers together this can changes the properties of the new polymers made. Amphiphilic AB diblock copolymers have been of interest because of the potential medical applications it could be used for. PLA has been shown to be biocompatible and biodegradable. [18] PLA is a polyester that can be hydrolyzed into lactic acid which is the method that PLA decomposes. [19] One of the methods that PLA is commonly synthesized by is ring

opening polymerization of lactide using tin(II) octanoate ($Sn(Oct)_2$) as a catalyst and a hydroxyl group as an initiator.[20] Figure 3 shows the synthetic scheme of PEG coupling and PLA polymerization to make the amphiphilic copolymer and finally the coupling of olgiolysine to a disulfide bond, makes the tri-arm copolymer.

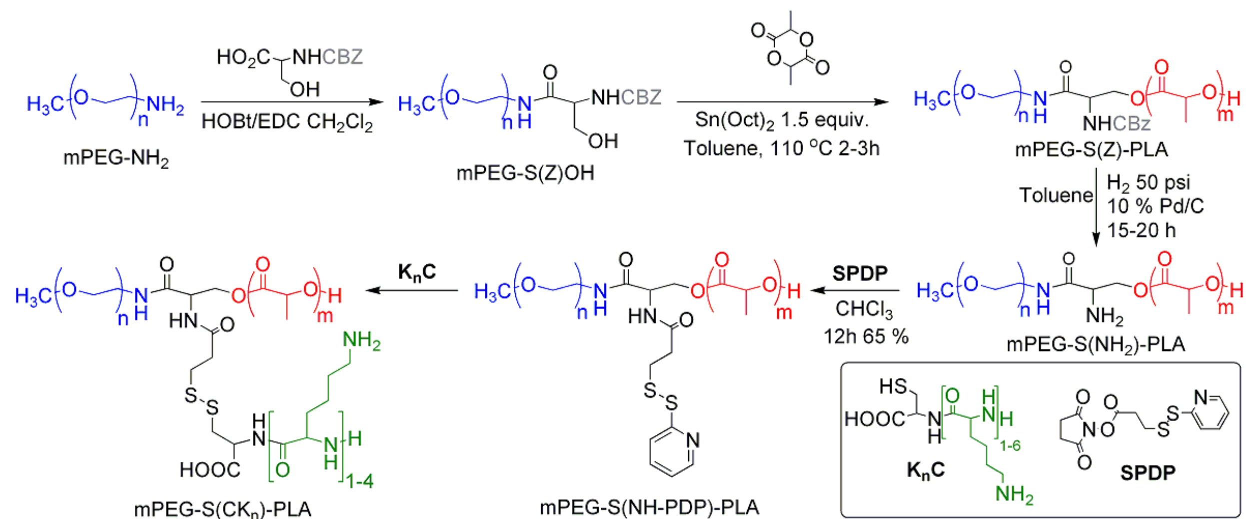
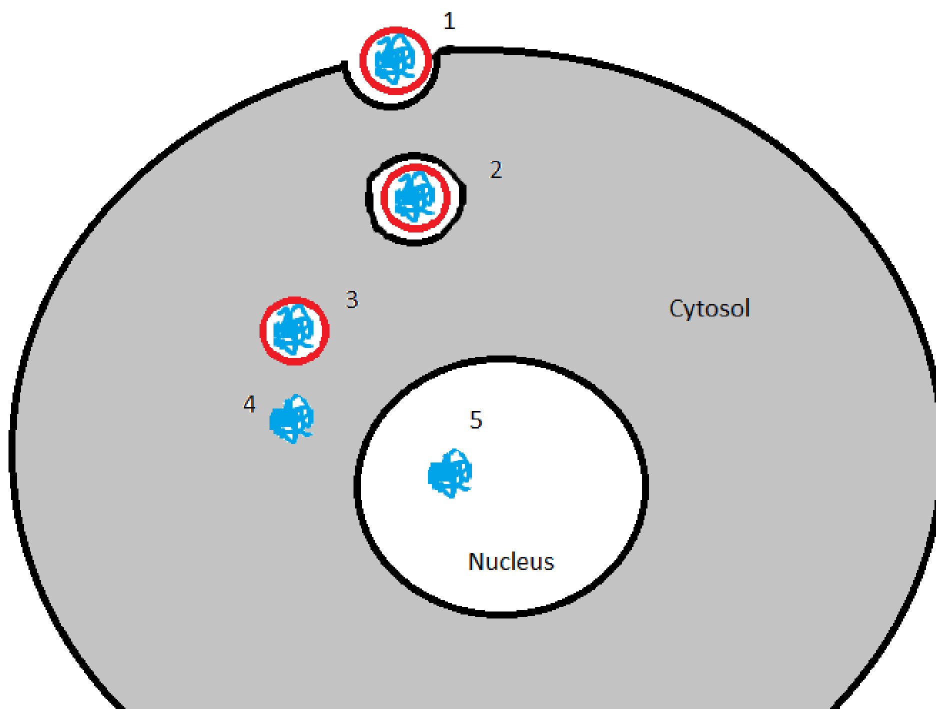


Figure 3: Synthesis of amphiphilic tri-arm star copolymer for DNA delivery

In order to make the oligolysine chain cleavable a disulfide bond has been incorporated between the oligolysine chain and the rest of the copolymer. The cleavable disulfide linkage should facilitate the release of the DNA in the cell and could improve the transfection efficiency. Figure 4 shows an idealized representation of how the DNA should be delivered into the nucleus of the cell. First, the tri-arm star copolymer will complex to the desired gene to be delivered. The polymer will encapsulate the DNA and depending on the external environment either the hydrophilic or hydrophobic blocks will be exposed. The polymer will be transported into the cell via endocytosis. When the polymer is inside the cell the disulfide bond will be cleaved due the high concentration of glutathione inside the cell. [21, 22] By cleaving the disulfide bond, the

oligolysine/DNA complex will separate from the polymer vector. The DNA will then enter the nucleus in order to be transcribed by the cell and produce the proteins that the DNA was encoded for.



1.DNA Encapsulated inside polymer, 2. Endocytosis of polymer, 3. Escape from endosome, 4. DNA is cleaved from polymer, 5. DNA enters nucleus to be transcribed

Figure 4: Pathway for polymer and DNA delivery into cell [23]

The goal of this research had three parts; one was to conduct a model study of the PLA polymerization, using compound (1) to optimize the PLA polymerization without having to account for PEG interaction during polymerization. The second was the synthesis of *N*-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP), this is the disulfide molecule that will be

bonded to the polymer and the oligolysine. Finally, the last part of this research is the synthesis of the short oligolysine peptide chain.

The full synthetic pathway for the oligolysine (compound 6 and 10) are shown in Figure 5. The peptide synthesis was conducted in solution phase, in order to produce the oligolysine peptide on a multigram scale. Compound (6) and (10) were synthesized using base and acid labile protecting groups. The acid labile protecting groups that were used during the synthesis were triphenylmethyl (Trt or Trityl) and *t*-butyloxycarbonyl (Boc). Compound (2) was protected with Trt for the thiol group of the cysteine. Two Boc protecting groups were used to protect the amines of compound (C), one on the side chains of the lysine and the other amine terminus. Compound (A) was protected with one Boc to protect the amine of the lysine side chain and one 9-fluorenylmethoxycarbonyl (Fmoc) used to protect the N terminus of the amino acid. The reason Trt, Boc and Fmoc groups were used for the peptide synthesis was because Trt and Boc are acid labile protecting groups whereas Fmoc groups can be removed under basic conditions, typically by treatment with piperidine. By deprotection of the Fmoc it will expose the N terminus which can be further coupled to another amino acid, growing the oligolysine chain. Trt and Boc are not affected by basic conditions. Trt can be removed under mild acidic conditions, whereas Boc deprotection requires a stronger acid, such as trifluoroacetic acid (TFA); therefore TFA can remove both Trt and Boc protecting group. Trt and Boc protecting groups are therefore orthogonal to the Fmoc. [24]

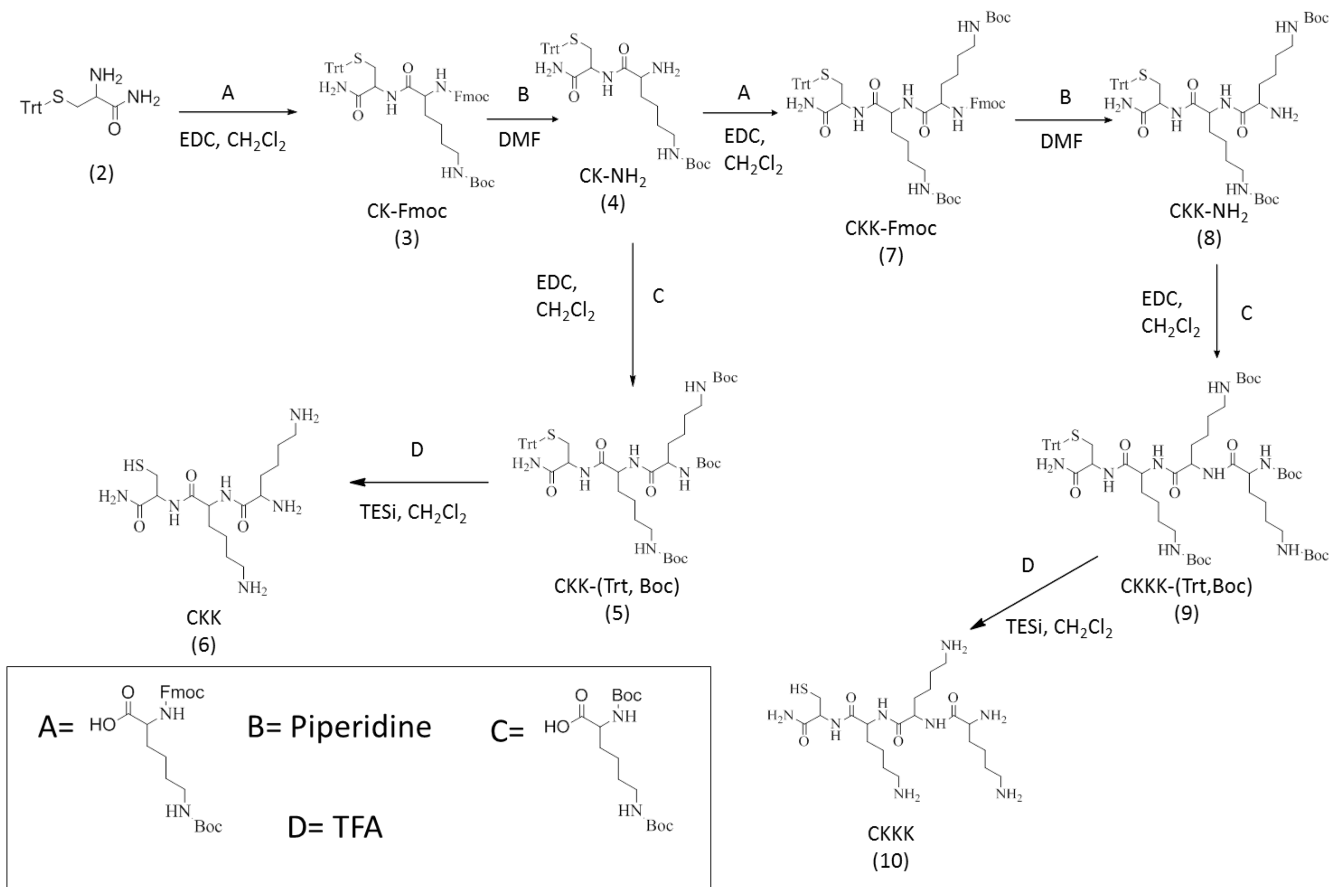


Figure 5: Synthetic pathways for oligolysines (KKC and KKKC)

Experimental

Materials

H-Cys(Trt)-NH2, Fmoc-Lys(Boc)-OH, Boc-Lys(Boc)-OH (all from Aapptec) were used as received. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (98+%), N,N'-dicycohexyl-carbodiimide (DCC)(99%), and triethylsilane (TESi) (99%) (all from Acros Organics). Hydroxybenzotriazole hydride (HOBt)(Advance ChemTech), 2-Methoxyethylamine (98%, Alfa, Aesar), trifluoroacetic acid (TFA) (99.5+% biochemical grade, Alfa Aesar), 3-mercaptopropanoic acid (99%, Alfa Aesar), 2,2-dipyridyl disulfide (98%, Alfa Aesar; and TCI), *N*-hydroxysuccinimide (NHS) (98+%, Alfa Aesar), dichloromethane (99.8%, HPLC grade, EMD), dimethylformamide (99.9%, EMD), ethyl acetate (HPLC grade, EMD) hexane(98.5%, HPLC grade, EMD), magnesium sulfate (anhydrous, J.T. Baker and general, BDH) anhydrous 200 proof ethyl alcohol (ACS/USP grade, Pharmco-Aaper), acetic acid (glacial, J.T. Baker), aluminum oxide, powder(J.T. Baker), chloroform (99%, J.T. Baker), hydrochloric acid (36.5-38.0%, J.T. Baker), methanol (99.8%, J.T. Baker), piperidine (99%, Sigma Aldrich), silica gel (40-63µm, Silicycle), *N*-CBZ-DL-serine(TCI), and sodium chloride (99%, VWR). Unless specified, all reagents were used as received.

Molecular sieves 4Å 1-2mm beads (Alfa Aesar) were activated by heating oven prior to using. Triethylamine (100%, J.T. Baker) was passed through alumina oxide column before coupling reactions. Toluene (99.5%, VWR) was purified by distillation over lithium aluminum hydride and stored under N2. Tin(II) 2-ethylhexanoate (tin(II) octanoate) (96%, Alfa Aesar) was dissolved with dry toluene to make catalyst solution which ranged from 9.1-9.4mg/mL, prepared inside glove box. Ninhydrin stain was prepared by dissolving 0.1g of ninhydrin (99%, Alfa

Aesar) with ethanol (95 mL) and 2, 4, 6-collidine(99%, Alfa Aesar) (5 mL). 3,6- Dimethyl-1,4-dioxane-2,5-dione[D,L lactide] (Sigma Aldrich) was recrystallized from toluene and sublimed under vacuum; and stored in glove box before use.

Instrumentation

^{1}H NMR spectroscopy was conducted on a 300 MHz Varian Gemini 2300 spectrometer using $CDCl_3$, DMSO, CD_3OD, and CD_2Cl_2 (all from Cambridge Isotope Laboratories, Inc) as solvents. GPC was performed at room temperature using THF (HPLC grade, J.T. Baker) with a flow rate of 1.0mL/minute. The GPC consisted of a Viscotek VE2001 GPC solvent/ sample module (Viscotek), VE3210 UV/Vis detector (Viscotek), VE 3580 RI detector (Viscotek), ViscoGEL columns (300 X 7.8mm,I-MBHMW-3078). Acquired data was analyzed against narrow polydispersity polystyrene standards in the molecular weight range of 580-400,000 g/mol (EasiCal PS-2, Polymer Laboratories).

Synthesis of 3-hydroxy-*N*-(2-methoxyethyl)-2-[(phenylmethoxy)carbonyl]propanamide (compound 1) for PLA polymerization

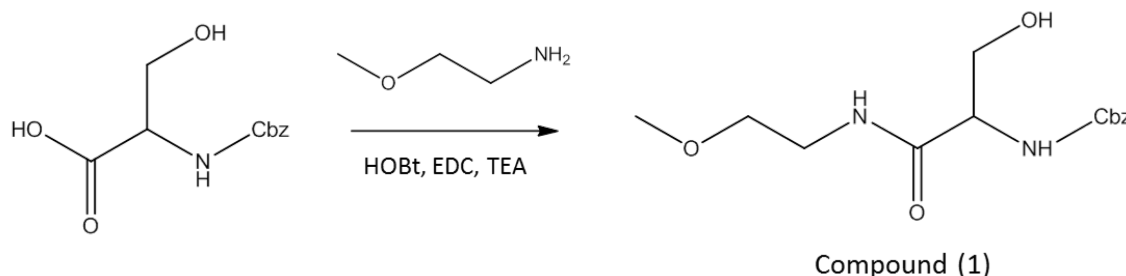


Figure 6: Synthesis of 3-hydroxy-*N*-(2-methoxyethyl)-2-[(phenylmethoxy)carbonyl]propanamide (compound 1) by EDC coupling

N-CBZ-DL-serine (500 mg, 2.09 mmol), HOBt (320 mg 2.09 mmol), and EDC (400 mg, 2.09 mmol) were dissolved in CH_2Cl_2 (~10mL) by stirring with a magnetic stir bar in a 100 mL round bottom flask. 2-methoxyethylamine (204 mg, 2.72 mmol) and triethylamine (211.4 mg, 2.09 mmol) were both dissolved separately in dichloromethane (~2 mL each) and added to mixture solution. The round bottom flask was then placed in an ice bath, and allowed to warm back to room temperature, and was stirred for a total of 24 hours. Mixture was washed with about 30 mL of deionized water and about 30 mL brine solution. The organic layer of dichloromethane was dried with $MgSO_4$ for about 20 mins, filtered, and concentrated by rotary evaporation to afford a viscous yellow oil, which was further purified by flash chromatography ($SiO_{2,}$ 30:1 chloroform/methanol) in a 25mm column with 50 times SiO_2 to crude compound (1). Single spot fractions (with R_f of 0.33 in 10:1 chloroform/methanol) were pooled concentration by rotary evaporation and drying in vacuum oven the resulting product was a white solid (128.4 mg ~20%), confirmed by ^{1}H NMR (Appendix 1)

An alternative method of purification after washing was recrystallization, product was dissolved in 1 mL CH_2Cl_2 poured into 20 mL of ethyl acetate/ hexane (1:9 v/v), this yields compound (1) which was a solid white crystals after drying (332.4 mg, ~50%), confirmed by ^{1}H NMR (Appendix 2).

Typical Polymerization of Lactide(Bulk)[25]

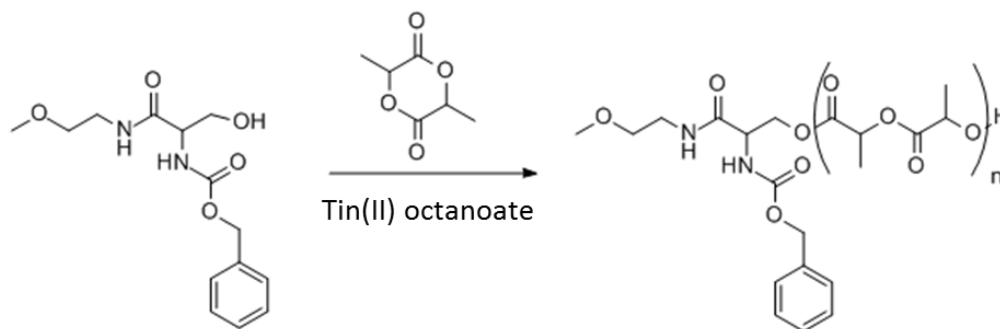


Figure 7: Ring opening polymerization of lactide from compound (1) with tin (II) octanoate

25 mL Schlenk tube (a) was filled with 4Å molecular sieves and sealed with a rubber septum. Tube (a) was evacuated and refilled with nitrogen for 4 cycles, and left under positive nitrogen flow. 6 mL of the tin octanoate solution (9.1 mg/mL) was transferred to tube (a) via syringe and left over sieves under positive nitrogen flow for one hour.

Compound (1) (30.3 mg, 0.1022 mmol) was dissolved in CH_2Cl_2(1 mL) and the resulting solution was transferred to 25 mL Schlenk tube (b), which was sealed and left under vacuum in order to remove dichloromethane. After removal of CH_2Cl_2, tube (b) was opened to air in order to add lactide (1.14 g, 7.88 mmol) and resealed with rubber septum. Tube (b) was then evacuated and refilled with nitrogen for 4 cycles and left under nitrogen. 2.3 mL (21 mg, 0.0511 mmol) of the catalyst solution was removed via syringe from tube (a) and transferred to tube (b). The solvent was then removed under vacuum over about 0.5h from tube (b). Tube (b) with the compound (1), monomers, and catalysis was then heated in an oil bath at 130°C. After 0.5h, the reaction mixture was removed from oil bath, cooled to room temperature then dissolved in CH_2Cl_2. Crude polymer solution was quenched with about ~1M of acidic methanol solution (1

mL). The solution was concentrated to about 0.5 mL by rotary evaporation and then precipitated into hexanes (17-19 mL). The resulting polymer was precipitated into hexanes twice. After drying the polymer was a brittle white solid (0.9784 g, 85%) Appendix 3 shows the ^{1}H NMR for crude product and appendix 4 shows the ^{1}H NMR for twice precipitated polymer.

Typical Polymerization of Lactide(solution)[12,20]

25 mL Schlenk tube (a) was prepared the same as for the bulk polymerization described above; the concentration of the tin octanoate solution was 9.5 mg/mL. The initiator (31.5 mg, 0.106 mmol) was added to 25 mL Schlenk tube (b) and then brought into the glove box. The lactide monomers (260 mg, 1.81 mmol), toluene (7mL), and a magnetic stir bar were added to tube (b) inside the glove box. Tube (b) was then sealed removed from the glove box, and the lactide solution was allowed to stir for about 0.5-1h until homogeneous at room temperature. The tin octanoate solution 2.3mL (21.9 mg, 0.0531 mmol) from tube (a) was then added via syringe to tube (b). The mixture solution was heated in an oil bath at 111°C for 4h. After 4h the reaction mixture was removed from oil bath, cooled to room temperature, and then quenched with ~1M of acidic methonal solution (~1 mL). The solution was concentrated to about 0.5 mL and then precipitated into hexanes (17-19 mL). The resulting polymer was precipitated into hexane twice. After drying the polymer was a brittle white solid (283 mg, 97%). ^{1}H NMR (Figure 18) appeared almost identical to bulk polymerization (appendix 4).

Glass stoppers were used in most reactions. Rubber septa were used for reactions in which samples were withdrawn via syringe during the polymerization. These samples were

quenched, precipitated into hexanes and vacuum dried before being analyzed by ^{1}H NMR and GPC.

<u>Synthesis of *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) [22, 26-27]</u>

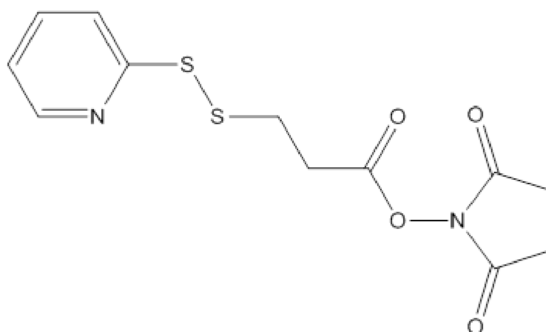


Figure 8: *N*-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP),

The synthesis of *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) was a two-step process. First, 3-mercaptopropionic acid was reacted with 2,2'-dipyridyl disulfide to yield 2-carboxyethyl 2-pyridyl disulfide (PDP). In the second step, PDP was reacted with *N*-hydroxysuccinimide by an esterification to produce SPDP

Preparation of 2-carboxyethyl 2-pyridyl disulfide (PDP)

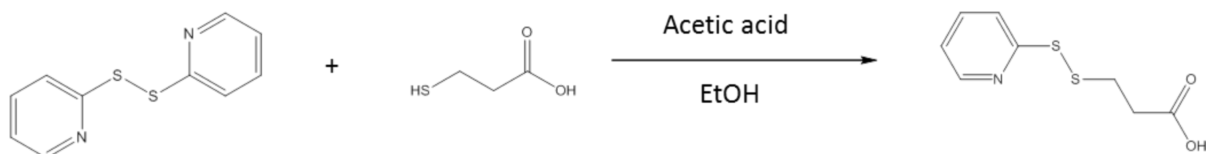


Figure 9: Synthesis of 3-(2-pyridyldithio)-propanoic acid (PDP)

To a clear solution of 2,2-dipyridyldisulfide(DPDS) (2.5 g, 11.3 mmol) in ethanol (20 mL), a solution of 3-mercaptopropanoic acid (0.6 g, 5.65 mmol) in acetic acid (0.7 mL) was slowly added over a period of 10-15 minutes. The resulting yellow solution was left to stir at room temperature for 2 h. The solution was concentrated with a rotary evaporator and dried under vacuum to afford a pale yellow oil. The crude oil was then dissolved in dichloromethane/ethanol (3:2 v/v) and eluted through an Al_2O_3 column (2cm x 24cm). The column was washed with 3:2 dichloromethane/ethanol until all of the yellow color (2,2-dipyridyldisulfide and 2-mercaptopyridine) had been removed. The PDP product was then eluted with CH_2Cl_2/EtOH/HOAc (60:40:4) and dried under vacuum, to afford a white solid. ^1H NMR (300MHz; -DMSO-d_6): δ 8.4(dt, 1H), 7.8(dt, 2H), 7.2(m, 1H), 2.9 (t, 2H, *J*=7Hz), 2.2 (t, 2H, *J*=7Hz)

Preparation of *N*-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP)

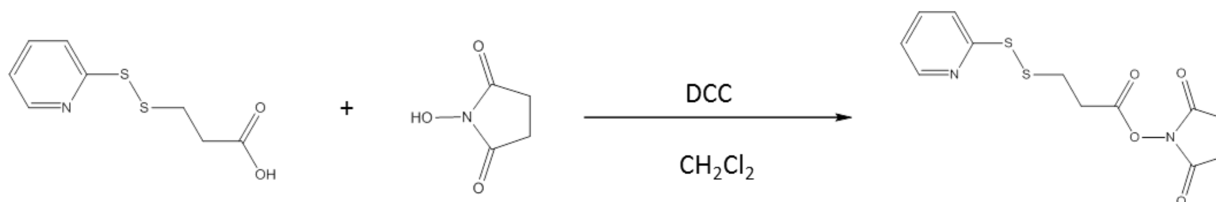


Figure 10: Synthesis of *N*-succinimidyl-3-(2-pyridyldithio) propionate by DCC coupling

After most of the acetic acid was removed, PDP (808.6mg) was dissolved in dichloromethane (10 mL). NHS (519.0 mg, 4.51 mmol) was added to the solution and the resulting mixture was stirred in an ice bath for 5 to 10 minutes. Separately DCC (838.1 mg, 4.06 mmol) was dissolved in dichloromethane (4 mL) and chilled in an ice bath. The DCC solution was added to the NHS/PDP solution. The resulting clear solution turned cloudy after a few minutes and was left to stir for 3.5h. After the reaction $MgSO_4$ was added and the resulting mixture was stirred at room temperature for a few minutes, cooled in an ice bath for a few minutes then filtered to remove the urea. The filtrate was concentrated and dried in a vacuum oven. To afford a yellowish oil, which was then dissolved in ethanol (~5-10 mL) (with some heating if needed, about 40°C) and left in the freezer(~ -20°C) for about a day until solid white precipitate (fluffy white balls) formed on the bottom of the vial. This process was completed twice. The solid was dried and then dissolved with dichloromethane (~1 mL) and poured into isopropanol (~19 mL) and left in the freezer for about a day until rod like crystals formed. This process was repeated twice, and afforded the final product (236 mg). ^{1}H NMR (300MHz, $CDCl_3$): δ 8.5(dt, 1H), 7.6 (dt, 2H), 7.1(m, 1H), 3.1(m, 4H), 2.8 (s, 4H).

Quantifying the amount of NHS acetate was accomplished by adding NHS acetate as internal standards for ^{1}H NMR. Figure 20 is^{1}H NMR SPDP (10 mg) and Figure 21 is the ^{1}H

NMR for SPDP (10mg) with NHS acetate (1 mg). This then allowed us to back calculate the remaining amount of NHS acetate which is ~2-3% of NHS acetate.

Peptide Synthesis [24, 28]

Dimer Synthesis (CK Fmoc) (3)

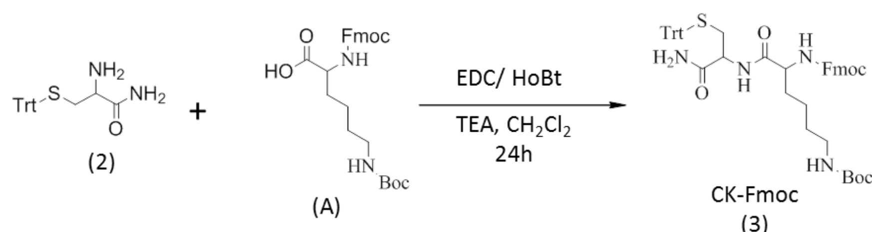


Figure 11: Dimer Synthesis, Compound (3)

Compound (2) (3.89 g, 10.7 mmol), HOBt (1.73 g, 11.3 mmol), and compound (A) (5.53 g, 11.8 mmol) was dissolved in dichloromethane (180 mL). The solution was stirred in an ice bath for 15-20mins. Triethylamine (1.09 g, 10.7 mmol) was then added to chilled solution. Lastly, chilled EDC (2.16 g, 11.3 mmol) in dichloromethane (~20 mL) was added. The mixture was allowed to slowly warm back to room temperature, and stirred for a total of 24h in air.

The dark orange mixture was washed with deionized water (~150 mL) and brine (~150 mL). The organic layer was dried with $MgSO_4$ and filtered. The solution was concentrated by rotary evaporation to afford, an orange solid. That was purified by flash chromatography (SiO_2, 25:1 $CHCl_3$/MeOH). The product was concentrated and dried under vacuum to afford a light orange solid (7.692 g ~86%). Product was tracked via TLC aided by UV and ninhydrin stain.

General scheme for Fmoc deprotection (compound 3 to 4 and 7 to 8)

Fmoc-protected oligolysine (compound 3 or 7) was dissolved in 25% piperidine in DMF and the resulting solution was stirred for about 3 hours under air. After completion of the reaction, the reaction mixture was evaporated to dryness. The crude solid was dissolved with a $CHCl_3$/MeOH mixture and then applied to silica column, the oligiolysine product remained on column until adding 0.5 mL triethylamine was added to the eluent (chloroform: methanol).

Deprotection of Fmoc (3 to 4)

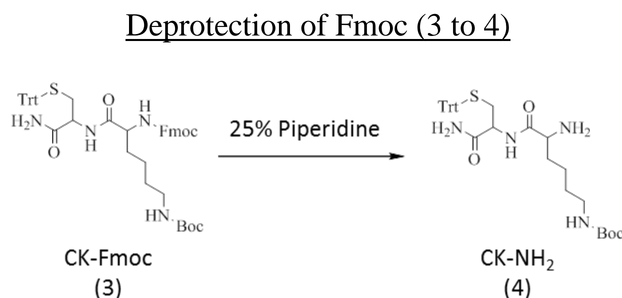


Figure 12: Deprotection of Fmoc, compound (3) to (4)

A pale yellow solution of compound (3) (4.12g, 5.07mmol) in DMF (30 mL) and piperidine (10 mL) was stirred for 3 hours at room temperature in air. The reaction mixture was evaporated to dryness and then crude compound (4) was dissolved with CH_2Cl_2 and precipitated into hexane twice (50 mL of hexane per 1mL of CH_2Cl_2). The precipitated product was dried and purified by flash chromatography (SiO_2, 20:1 $CHCl_3$/MeOH) until all byproduct had been eluted off (3 spots with R_f greater than 0.40, and was not stainable with ninhydrin with 10:1 $CHCl_3$/MeOH). Then, compound (4) was washed out by $CHCl_3$/MeOH/Et_3N (20:1:0.5). Compound (4) was tracked on TLC (R_f of 0.18 with 10:1 $CHCl_3$/MeOH) with ninhydrin staining; it appeared light brown on TLC. The solution was concentrated by rotary evaporation and dried under vacuum to afford a white solid compound (4) 2.47g (82%).

Deprotection of Fmoc (7 to 8)

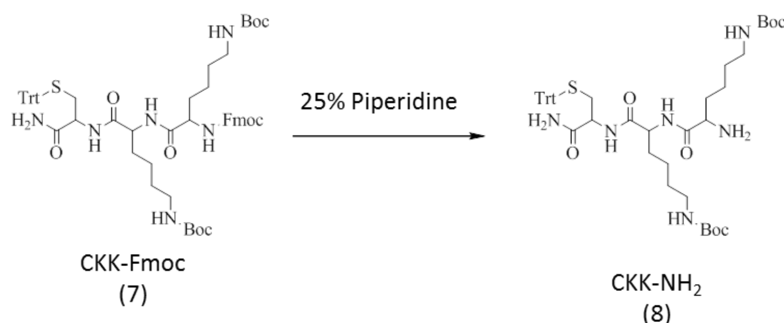


Figure 13: Deprotection of Fmoc, compound (7) to (8)

A pale yellow solution of compound (7) (0.500 g, 0.48mmol) was dissolved with DMF (30 mL) and piperidine (10 mL) was stirred for 3 hours at room temperature in air. The reaction mixture was evaporated to dryness and then crude compound (8) was dissolved with CH_2Cl_2 and precipitated into hexane twice (50 mL of hexane per 1mL of CH_2Cl_2). The precipitated product was dried and purified by flash chromatography (SiO_2, 20:1 $CHCl_3$/MeOH) until all byproduct had been eluted off (3 spots with R_f greater than 0.28 was not stainable with ninhydrin with 20:1 $CHCl_3$/MeOH). Compound (8) remained on column until $CHCl_3$/MeOH/Et_3N (20:1:0.5) was added to column. Compound (8) (R_f 0.06 in 20:1 $CHCl_3$/MeOH) was tracked on TLC with ninhydrin staining. The solution was concentrated by rotary evaporation and dried under vacuum to afford a white solid yielding compound (8) 0.301 g (~77%).

Compound (4) coupling to (C) forming (5)

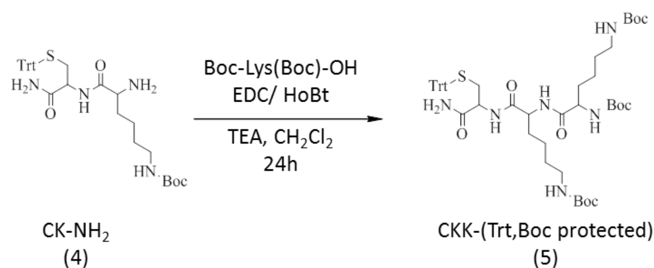


Figure 14: Coupling of compound (4) with (C) forming compound (5)

Compound (4) (1.445 g, 1.933 mmol), HOBt (0.3109 g, 2.0297 mmol), and compound (C) (0.7735 g, 2.126 mmol) were dissolved in CH_2Cl_2 (40 mL). The solution was stirred in an ice bath for 15-20mins. Triethylamine (0.1956 g, 1.933 mmol) was then added to chilled solution. Lastly, chilled EDC (0.3892 g, 2.0297 mmol) in CH_2Cl_2 (20 mL) was added. The mixture was allowed to slowly warm back to room temperature, and stirred for a total of 24h in air.

Mixture was washed with deionized water (~50 mL) and brine (~50 mL). The organic layer of dichloromethane was dried with $MgSO_4$ and filtered. The solution was concentrated by rotary evaporation and dried under vacuum to afford a slight yellow/white solid (1.238 g, ~59%). Product was tracked via TLC aided by UV and ninhydrin stain. Product was not applied to column due to lack of solubility in CH_2Cl_2.

Compound (8) coupling to (C) forming compound (9)

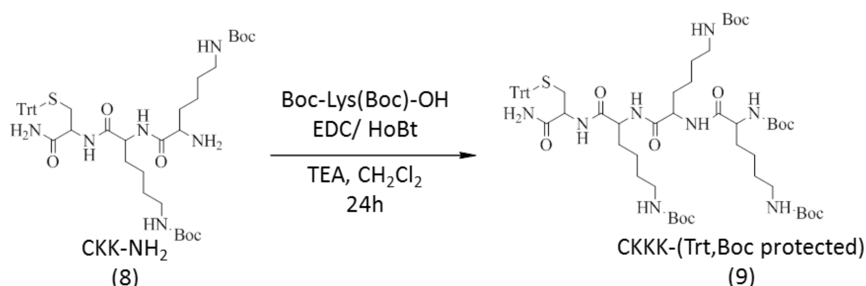


Figure 15: Coupling of compound (8) to (C) forming compound (9)

Compound (8) (269.5 mg, 0.3285mmol), HOBt (52.82 mg, 0.3449 mmol), and compound (C) (125.2mg, 0.3613mmol) were dissolved in CH_2Cl_2 (20 mL). The solution was stirred in an ice bath for 15-20mins. Triethylamine (33.3 mg, 0.3285mmol) was then added to chilled solution. Lastly, chilled EDC (66.1 mg, 0.3449mmol) in CH_2Cl_2 (10 mL) was added. The mixture was allowed to slowly warm back to room temperature, and stirred for a total of 24h in air. The solution after reaction was cloudy white.

Mixture was washed with deionized water (~30 mL) and brine (~30 mL). The organic layer of dichloromethane was dried with $MgSO_4$ and filtered. The cloudy white solution was concentrated by rotary evaporation and dried under vacuum to afford a slight yellow/white solid (351 mg). Product was tracked via TLC aided by UV and ninhydrin stain. Product was not applied to column due to lack of solubility in CH_2Cl_2.

General scheme for Trt and Boc deprotection (compound 5 to 6, 9 to 10)

Trt and all Boc protected oligolysine (compound 5 or 9) were dissolved with trifluoroacetic acid (TFA)/CH_2Cl_2 (1:1). The bright yellow mixture solution turned into a clear colorless solution after the addition of triethylsilane. The mixture solution was stirred for 1 hour at room temperature in air. Mixture solution was concentrated by rotary evaporation and left under vacuum. Crude product was then dissolved with methanol and precipitated into chloroform. The solution did not mix immediately; it was a cloudy white interphase between the two layers. Solution was vortexed to remove the cloudy white interphase from precipitation; this was left in freezer overnight. Precipitation yielded a clear solid that was spread around vial. Products was confirmed by ^{1}H NMR (300MHz, CD_3OD)

TFA deprotection of compound (5) to (6)

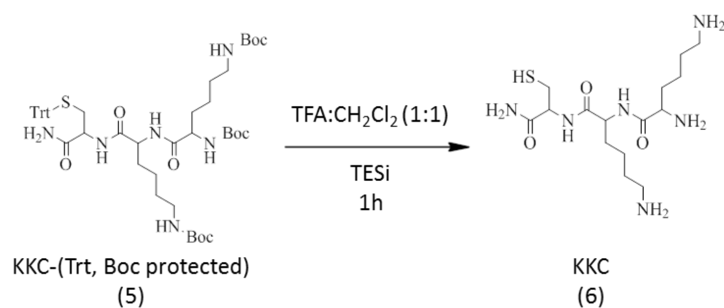


Figure 16: TFA deprotection of compound (5) to (6)

Compound (5) (253.2 mg, 0.2712 mmol) was dissolved in CH_2Cl_2 (20 mL) and TFA (25 mL). The bright yellow mixture solution became clear and colorless after the addition of CH_2Cl_2 (5 mL) triethylsilane (TESi) (159.3 mg, 1.356 mmol) solution. The mixture solution was stirred

for 1h at room temperature in air. Mixture solution was concentrated by rotary evaporation and dried under vacuum. Crude compound (6) was dissolved with methanol (~1 mL) and precipitated into chloroform (~18 mL). Precipitated product formed a cloudy white interphase; mixture was then vortexed until homogeneous. This mixture was then left in the freezer for one day. Chloroform was decanted off and product was dried under vacuum which afforded a clear solid (98.6 mg, 96%). Product was confirmed by ^{1}H NMR (300MHz, CD_3OD): 4.48(q, 1H, *J*=6Hz), 4.39(q, 1H, *J*=6Hz), 3.95 (t, 1H, *J*=6Hz), 2.80(m, 6H, 2H per amino acid side chain), 1.97-1.35 (m, 12H, 6H per Lys side chain) shown in Figure 22.

TFA deprotection of compound (9) to (10)

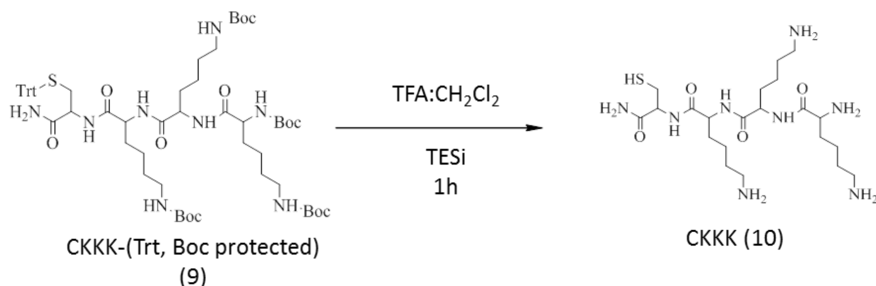


Figure 17: TFA deprotection of compound (9) to (10)

Compound (9) (319.5 mg, 0.2781 mmol) was dissolved CH_2Cl_2 (20 mL) and TFA (25 mL). The bright yellow mixture solution became clear and colorless after the addition of CH_2Cl_2 (5 mL) triethylsilane (TESi) (159.3 mg, 1.390 mmol) solution. The mixture solution was stirred for 1 hour at room temperature in air. Mixture solution was concentrated by rotary evaporation and dried under vacuum. Crude compound (10) was dissolved with methanol (1 mL) and precipitated into chloroform (18 mL). Precipitated product formed a cloudy white interphase;

mixture was vortexed until homogeneous. This mixture was then left in the freezer for one day. Chloroform was decanted off and product was dried under vacuum which afforded a clear solid (95.2 mg, 68%). Product was confirmed by ^{1}H NMR (300MHz, CD_3OD): 4.48(q, 1H, *J*=6Hz), 4.39(m, 2H), 3.95 (t, 1H, *J*=6Hz), 2.80(m, 8H, 2H per amino acid side chain), 1.35-1.97 (m, 18H, 6H per Lys side chain) shown Figure 23.

Results and Discussion

Compound (1)

The successful synthesis of compound (1) was confirmed by ^{1}H NMR spectroscopy. The spectrum for compound (1) from column and precipitation (Appendix 1 and 2) appear to be identical. Both methods of purification were efficient in isolating compound (1) but precipitation afforded the product in larger yields.

Solubility was conducted in order to find proper precipitation conditions for isolating compound (1). Pure compound (1) was isolated by column and tested against 2 methoxyethylamine (excess reactant for coupling reaction). Under ethyl acetate/ hexane (1:9 v:v) the 2 methoxyethylamine was soluble and compound (1) was not.

Poly(D,L-lactide)(PLA) homopolymers

The goal of this project was to try to successfully synthesize PLA homopolymer with narrow molecular weight distribution at a target molecular weight. The target molecular weight varied from 2,000 to 10,000 g/mol. The reaction times for the series of reactions ranged from 25 minutes to 4 hours. The successful synthesis of the PLA homopolymer was confirmed by ^{1}H

NMR spectroscopy. The typical spectrum (Figure 18) of the PLA homopolymer is consistent with what would expect from PLA polymerization, broad peaks at 5.10-5.25 (methine hydrogens) and 1.46-1.60 (methyl hydrogens) ppm. Samples with very broad molecular weight distribution could not be determined by ^{1}H NMR alone; GPC was used to determine the molecular weight distribution (against polystyrene standards).

A series of trials were conducted to synthesize the PLA homopolymer (Table 1). The earliest reactions (1 and 2) were conducted in bulk with a targeted molecular weight of 10,000 g/mol. The reaction times were varied from 1 hour to 0.5 hour in order to see if narrower molecular weight distribution could be obtained by the decrease in reaction time, this would give an approximation of reaction rate in bulk. The results polymerization in bulk occurred very quickly with little change in polydispersity. This would be very difficult to control for lower molecular weight polymers.

For solution phase polymerization, the temperature was decreased from 130°C to 111°C because the lactide monomers only needed slight heating to completely dissolve. Reaction time was initially prolonged to 4 hours in order to gauge the extent of the polymerization in solution. Aliquots were taken for reactions (5 and 7) in order to examine molecular weight by ^{1}H NMR and GPC at different time intervals of the reactions. A typical spectrum for the PLA homopolymer is shown in Figure 18.

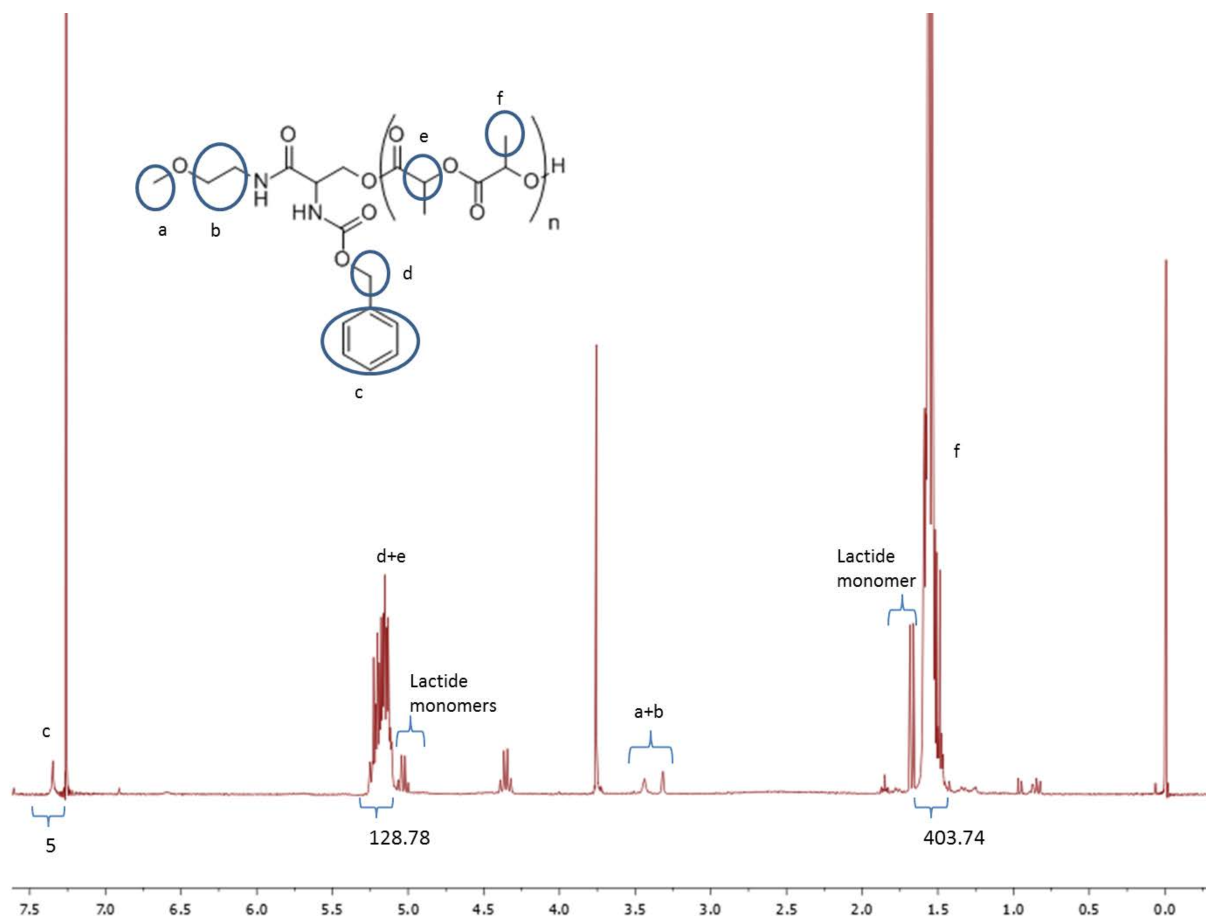


Figure 18: Typical ^{1}H NMR solution polymerization (reaction 6 after three precipitations into hexanes)

The M_n calculation for the ^{1}H NMR was performed by setting the integration for the CBZ to 5. The CBZ peak is at 7.35 ppm and was set as the reference in which all calculations were estimated from. The peak at 5.10-5.25 ppm corresponds to the two methine hydrogens per polylactic acid repeating unit and the methylene of the initiator. The peaks at 1.46-1.60 ppm correspond to the six hydrogens of the two methyls on the PLA repeat units. This method was only used as a rough estimation of molecular weight, and was then compared to gel permeation chromatography (GPC). The reaction conditions and estimated molecular weight by ^{1}H NMR and GPC results are shown in Table 1.

In Figure 18, the peaks at 5.0 and 1.7 ppm are the lactide monomer which were not completely removed by precipitation. The peaks at 0.8 to 0.9 ppm are most likely due to hexane which was not completely removed under vacuum. Lastly, singlet peak at ~3.75 ppm and the quartet at ~4.4 ppm we speculate that these peaks are methyl lactate that forms from acidic methanol quenching. When these results were compared to fellow group members, who also conducted ring opening polymerization of lactide using macroinitiator, they also saw this peak at 3.75 ppm which they were able to remove by hexane precipitation. The reason this peak is prominent in these samples is likely due the crude solution being over concentrated and viscous, therefore precipitation into hexane was not as effective.

Table 1: Results for poly (D, L-lactide) (PLA) polymer synthesized by ring opening polymerization

Reaction	Compound (1)/monomer Ratio	Reaction time(h)	Target MW(g/mol)	Amount of toluene solution (temp)	Estimated M_n(g/mol) by H-NMR (methine/methyl) Phenyl ring ref.	M_n(g/mol) (from GPC)	PDI (M_w/M_n)
1	1:69	1h	10,000	Bulk(130°C)	11,100/11,800	13,500	1.62
2	1:69	0.5h	10,000	Bulk(130°C)	10,200/10,800	8,900	1.62
3	1:14	4h	2,000	7mL(111°C)	3,000/3,200	3,300	1.59
4	1:14	2.5h	2,000	7mL(111°C)	2,000/2,100	2,300	1.46
5	1:14	0.5h 1h	2,000	6mL(111°C)	1,300/1,500 1,500/1,700	1,400 1,800	1.24 1.27
6	1:69	1h	10,000	7mL(111°C)	9,100/9,700	8,100	1.22
7	1:55	0.25h 0.5h 0.75h 1h	8,000	8mL(111°C)	3,800/5,200 5,600/6,800 5,800/6,500 7,200/7,900	2,800 4,700 5,500 6,000	1.18 1.17 1.20 1.21

*Catalysis amount (with respect to initiator) = 0.5eq for all reactions

The series of ring opening polymerization reactions were all conducted with 0.5 equivalents of catalysis amount with respect to initiator. For the majority of the reactions, the estimated M_n by ^{1}H NMR seems fairly close to the GPC M_n, with exceptions to the bulk reactions. Although the GPC uses polystyrene standards to measure M_n; polystyrene standards have been used before to characterize PLA homopolymers molecular weight .[29] The broad molecular weight distribution from bulk polymerization is most likely due to polymerization occurring faster in bulk, and likelihood of transesterification, which leads to a broader polydispersity for those reactions.

3-(2-Pyridyldithio)-propanoic acid (PDP)

Synthesis of 3-(2-pyridyldithio)-propanoic acid was confirmed by ^{1}H NMR spectroscopy. The chromatographic purification procedure used for PDP was different from the original paper by Carlsson. Carlsson and coworkers reported using basic aluminum oxide column in order to purify the PDP product, but instead neutral aluminum oxide was used. The spectrum for the PDP product matched those that had been reported, with the exception of the peak that corresponds to acetic acid. Figure 19 shows the spectrum for PDP, the 4 aromatic hydrogens (a) ranged from 8.4ppm to 7.2 having a 1:2:1 ratio. The remaining 4 aliphatic hydrogens (b) are at 2.9 (t, 2H, J=7Hz), 2.2 (t, 2H, J=7Hz).

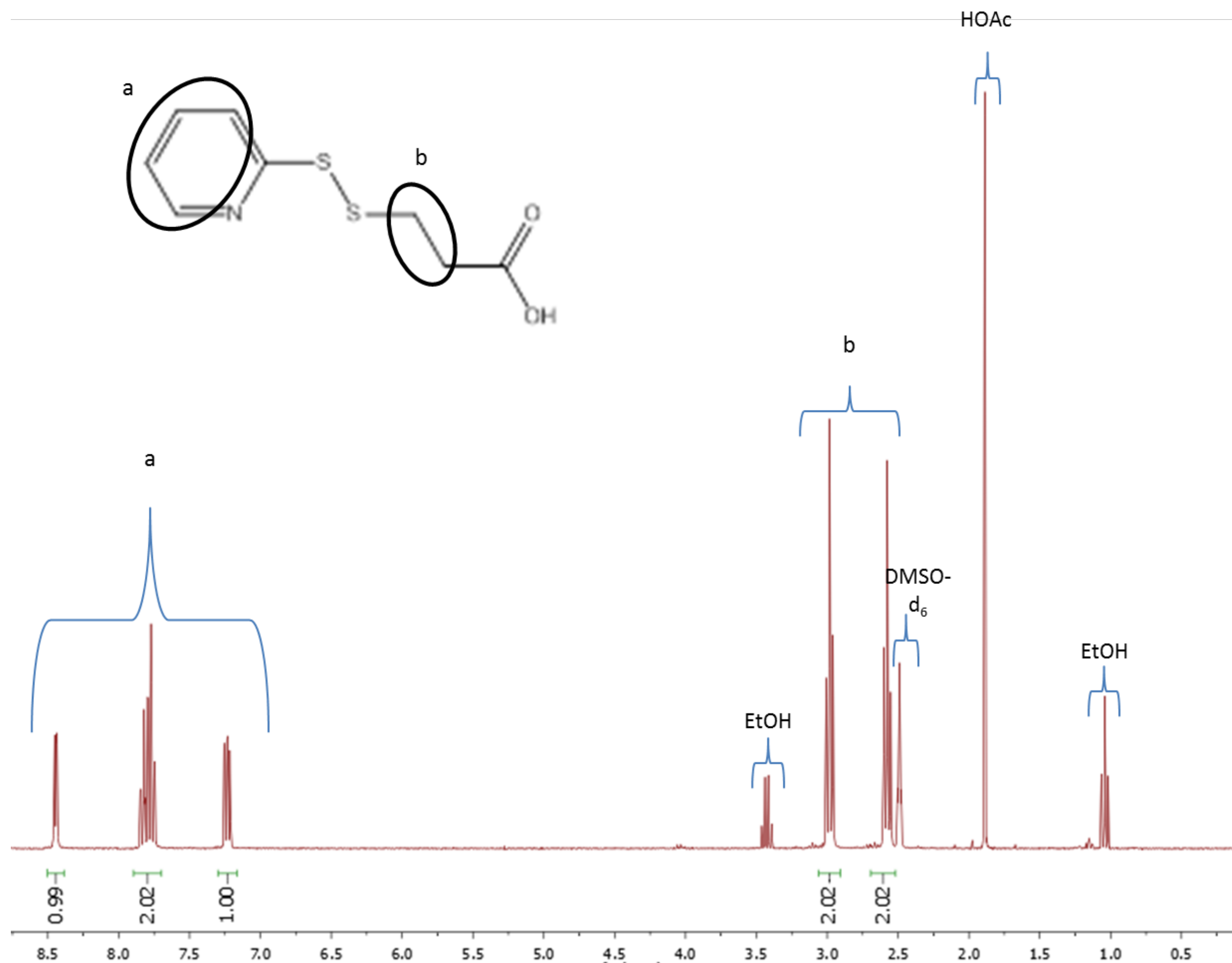


Figure 19: Typical ^{1}H NMR spectrum of PDP (DMSO)

The procedure for the aluminum oxide column was different from the original paper. The authors used a basic aluminum oxide column and ours were neutral. The removal of the excess nonpolar starting material 2,2- dipyridyl disulphide and the byproduct pyridine-2-thione could be visually seen by the removal of the yellow band on column. This was confirmed by ^{1}H NMR were only PDP and acetic acid are seen.

The singlet at 1.9ppm is the peak for acetic acid; attempts were made to remove acetic acid at this stage. Our hope was that by removing acetic acid after column there would be no need to remove the byproduct formed by the reaction of acetic acid and NHS. The issue at this

stage was that acetic acid was still very difficult to remove. Precipitation into heptane was explored because the crude PDP did not appear soluble and acetic acid was miscible. However, the precipitate had low solubility in DMSO after precipitation and acetic acid concentration appeared to be similar to unprecipitated PDP.

Concentrated PDP product after aluminum oxide column was redissolved with ethanol and dried under vacuum on Schlenk line. This method still was not able to completely remove acetic acid. The remaining acetic acid not only produced byproduct which had to be removed but it also consumed the NHS that is needed in order to fully convert the PDP to SPDP.

N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP)

The *N*-succinimidyl 3-(2-pyridyldithio) propionate that was made was synthesized from PDP with residual acetic acid. The SPDP synthesized was confirmed by ^{1}H NMR spectroscopy (Figure 20). The two triplets at 2.9 and 2.2ppm of PDP shift to 3.10 ppm after coupling and becomes a multiplet with an integration of four for hydrogen (b). The singlet at 2.83ppm is from the protons of the NHS (c). The peak at 2.33 is the NHS acetate that forms from the coupling of residual acetic acid and NHS. The NHS peak of NHS acetate overlaps with the SPDP's NHS at 2.83. A relatively purified SPDP spectrum is show in Figure 20.

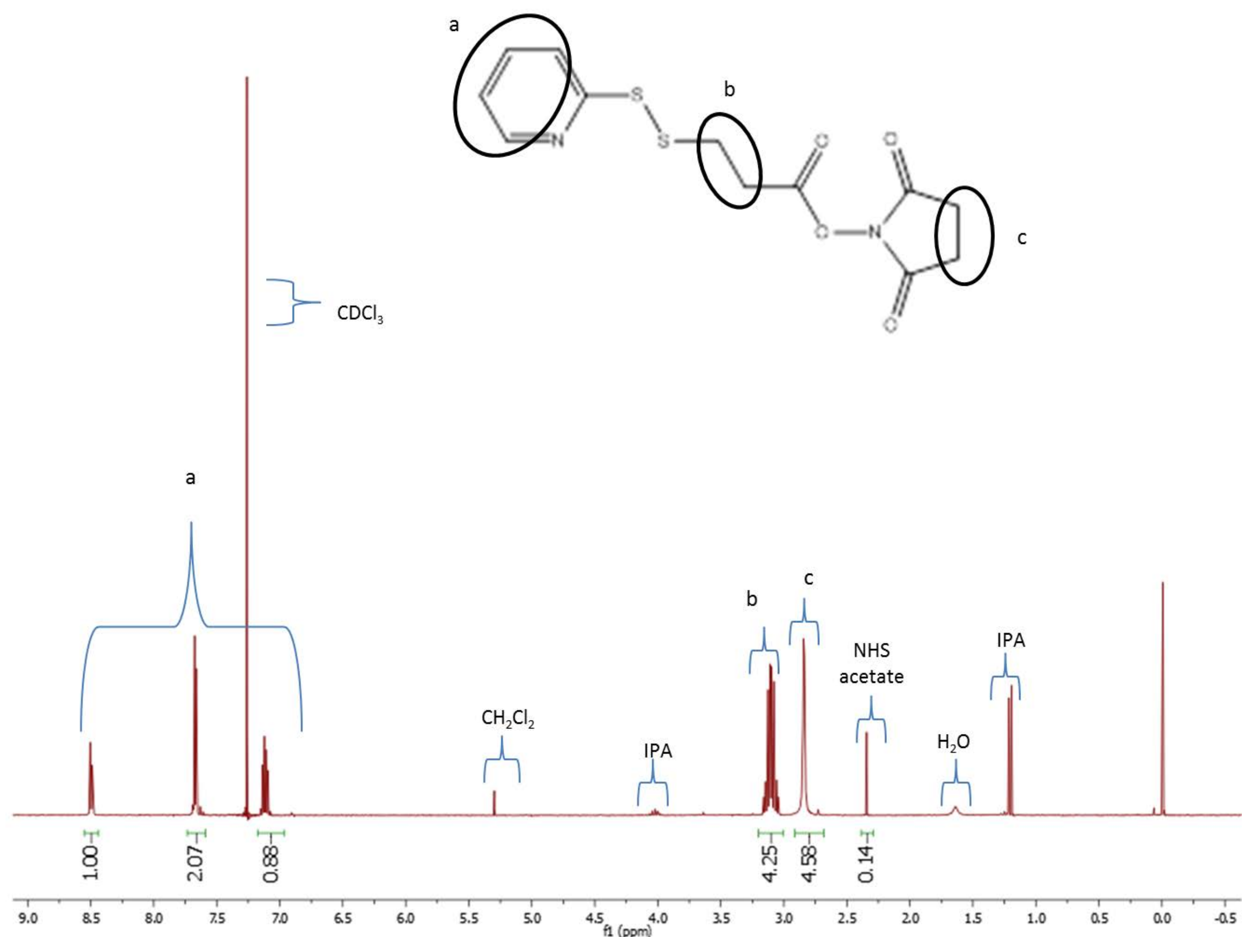


Figure 20: ^{1}H NMR spectrum of SPDP (10mg)

After filtration to remove $MgSO_4$ and urea byproduct, the concentrated product afforded a yellow oil with trace amounts of urea byproduct. According to the literature, recrystallizing the product from ethanol should have yielded pure SPDP, but the purified material still contained NHS acetate (appendix 7, p 47). Solubility tests showed that pure NHS acetate was soluble in isopropyl alcohol and the recrystallized SPDP did not appear to be very soluble. The SPDP was then dissolved with CH_2Cl_2 and poured into isopropyl alcohol and left in a freezer (~-20°C) for about a day before rod-like crystals formed, which lead us to believe that SPDP had a low solubility in isopropyl alcohol. After the rounds of purification, the majority of the NHS acetate

was removed. Furthermore, the isopropyl alcohol supernatant still contained SPDP, was able to be recovered after leaving the supernatant in the freezer for about 3-4 weeks for the SPDP to recrystallize forming small white fluffy balls. The yield of SPDP was not reported because SPDP from the supernatant was not accounted for; furthermore acetic acid was not quantified at PDP stage. The byproduct (NHS acetate) from SPDP synthesis was only quantified after purification.

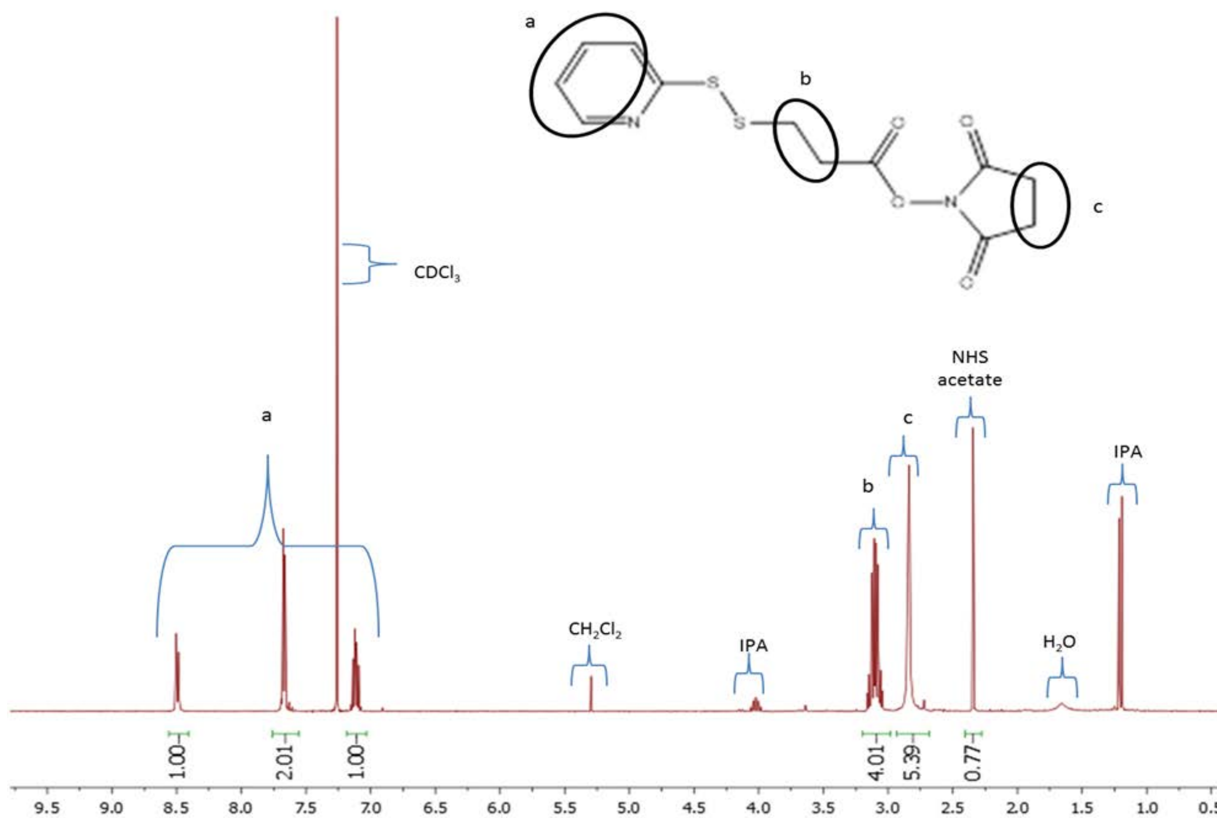


Figure 21: ^{1}H NMR spectrum of SPDP (10 mg) with NHS acetate (1 mg)

Compound (6) and (10)

The peptides that were synthesized were KKC (compound 6) and KKKC (compound 10). The synthetic scheme used to make these compounds consisted of EDC coupling of protected lysine (Compound A or C), deprotection of Fmoc(compound A) by piperidine, and deprotection of Trt and Boc by TFA. The synthesis of oligolysines KKC (compound 6, Figure 22) and KKKC (compound 10, Figure 23) were confirmed by ^{1}H NMR spectroscopy. Intermediate compounds (2-5, 7-9) were too complicated to be examine by ^{1}H NMR because it was difficult to find a reference peak for the compounds.

Compound (6) has three methine peaks (labeled (a) in Figure 22) that appear around 3.9 to 4.5 ppm. There are six hydrogens at the methylene peaks (b in Figure 22) at 2.8 ppm. Lastly, the aliphatic hydrogen of the lysine chain appears at 1.40 to 1.97 ppm which has 12 hydrogens. The integration for methylene and aliphatic hydrogens(c in Figure 22) is not exactly six and 12; this error is most likely due to instrumental error.

Compound (10) has four methine hydrogens (labeled (a) in Figure 23) and the integration from 3.9 to 4.5 ppm is 4. The two middle lysines peaks overlapped at ~4.35 ppm giving an integration of 2. There are eight methylene hydrogens (b in Figure 23) at 2.8 ppm. Lastly, the aliphatic hydrogens (c in Figure 23) of the lysine chains appear at 1.40 to 1.97 ppm which has 18 hydrogens. The error in integration again is most likely due to instrumental error.

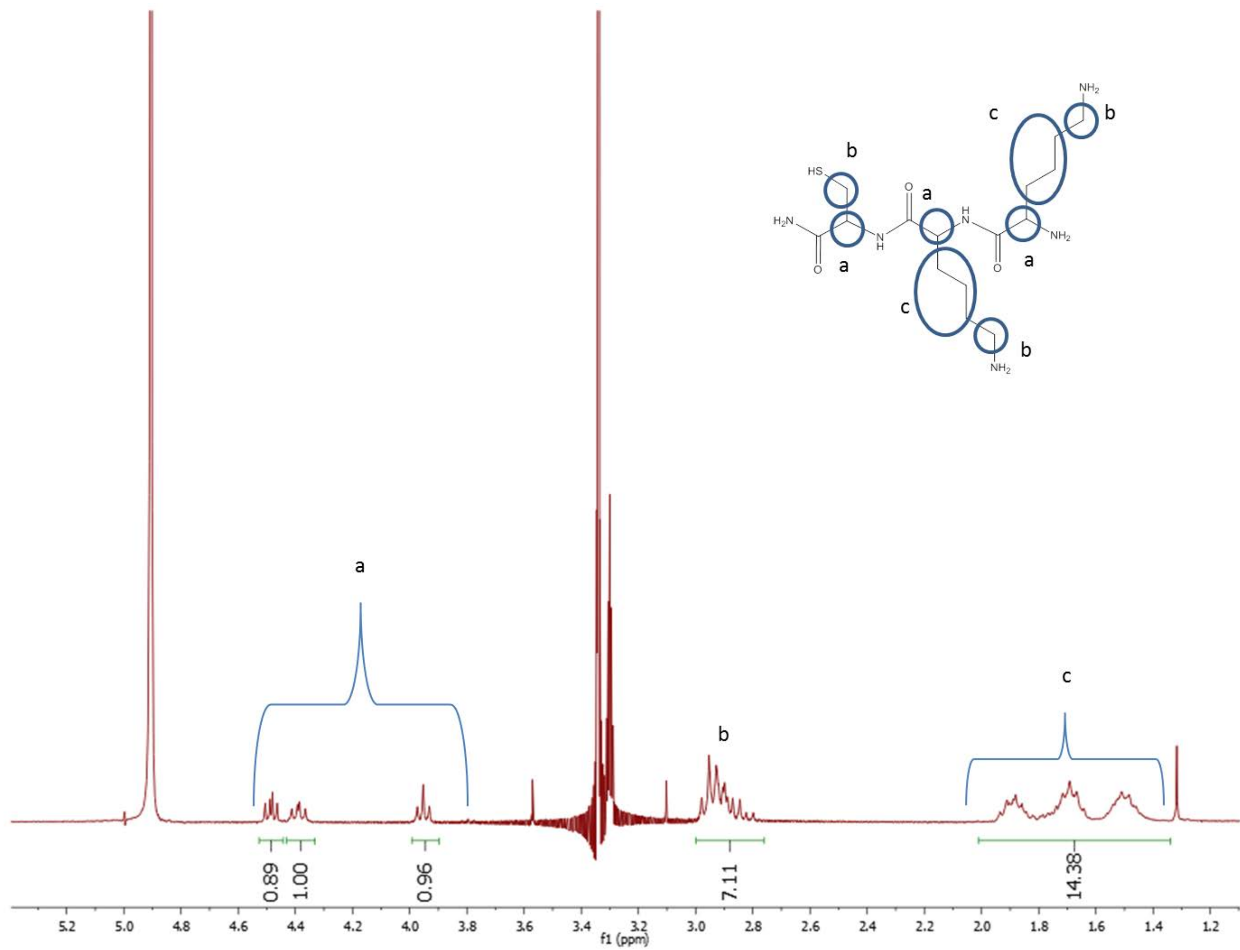


Figure 22: ^{1}H NMR spectrum of KKC (compound 6)

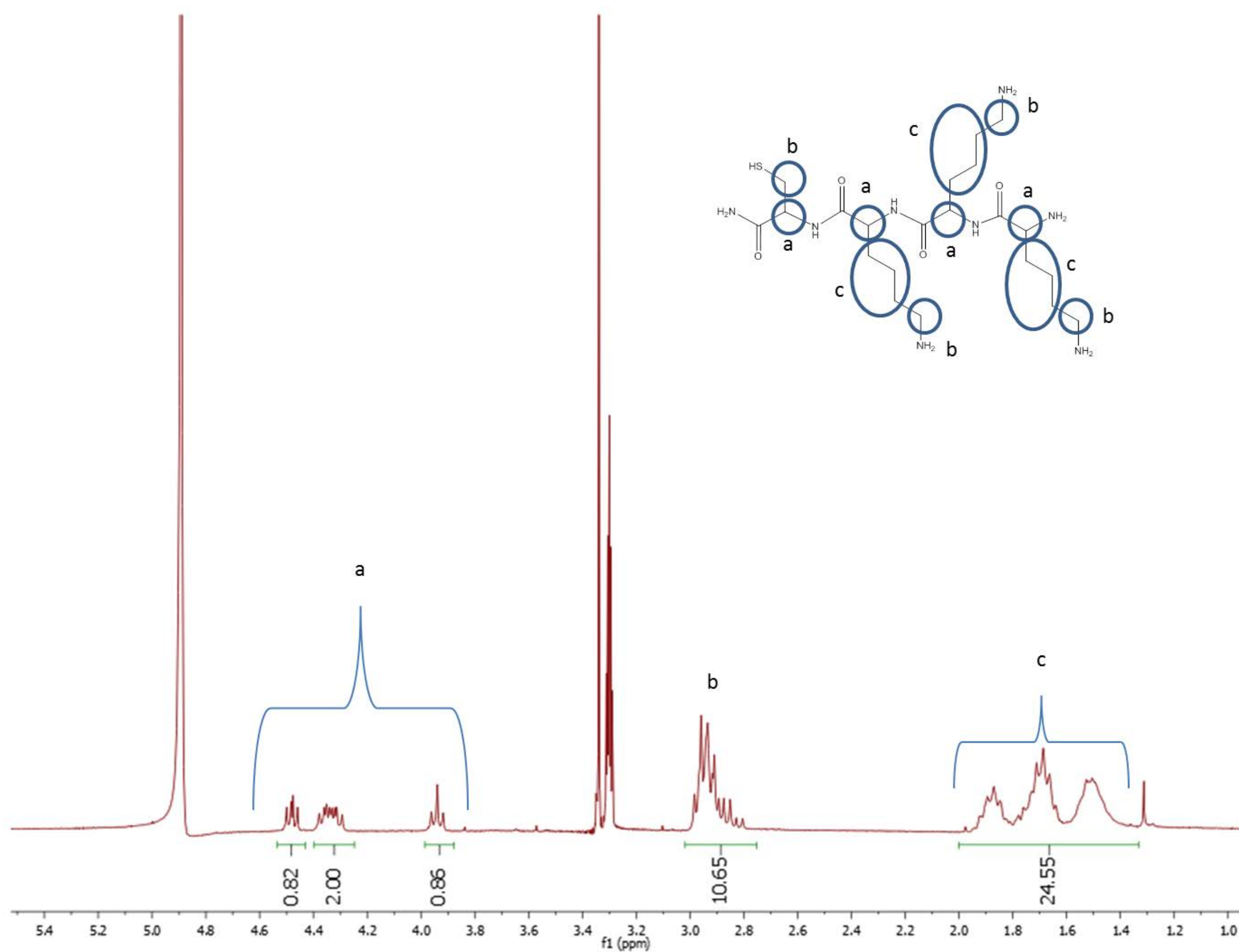


Figure 23: ^{1}H NMR spectrum of KKKC (compound 10)

Synthesis of the intermediate products (Figure 24) was tracked by TLC with UV and ninhydrin stain. Although this method was not ideal it was effective in tracking the products after reactions. For example, the coupling reactants to synthesize compound (7), compound (4) and (A) are both very polar, but after coupling compound (7) becomes nonpolar. This was detectable by UV and ninhydrin staining therefore the completion of each reaction was tracked by TLC. An alternative method of tracking the reaction at each stage could be mass spectroscopy because the increase and decrease in molecular weight at each stage.

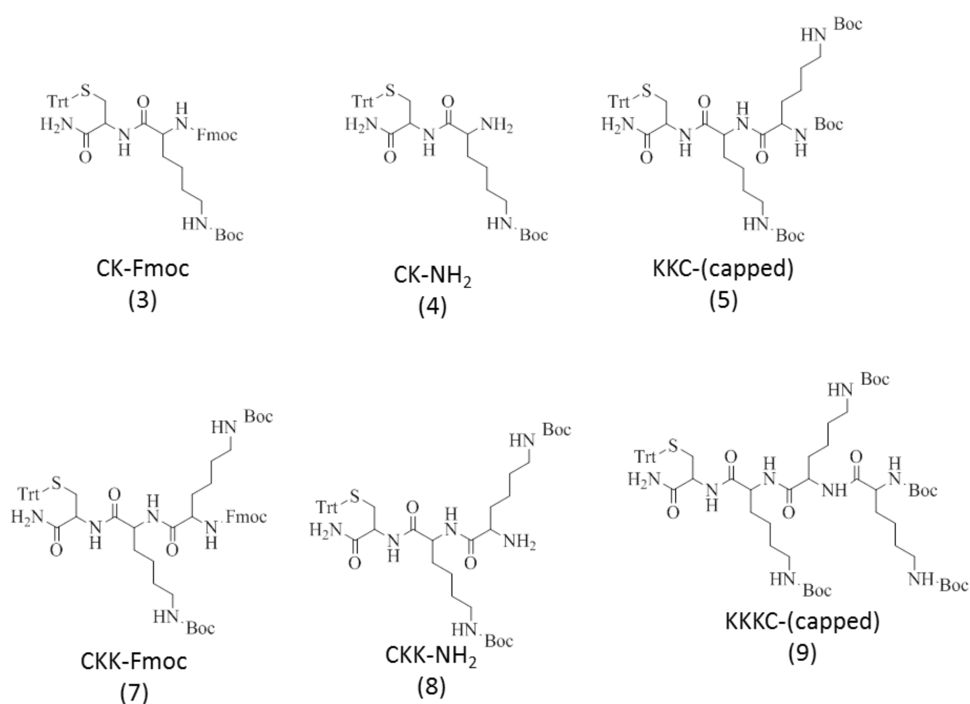


Figure 24: Intermediate products of oligolysine synthesis

Compounds (5 and 9) were deprotected with TFA and the chloroform precipitation purification was able to remove byproducts from the reaction and the reactants from the previous step; that is why compound (5) and (9) did not have to be purified by column. It appears that as the oligolysine chain increases the solubility in CH_2Cl_2 and chloroform decreases, which creates a problem with purification after each coupling. The only solvent that was found able dissolve the longer oligolysine chain was DMF. DMF would not be a practical solvent to use for flash chromatography due to high boiling temperature and polarity. Therefore synthesizing longer oligolysine peptides with 5 or more lysine units could pose a potential problem in solution. An alternative method of synthesizing longer oligolysine peptides could be solid-phase peptide synthesis.

Conclusion

PLA homopolymer were successfully synthesized and characterized by ^{1}H NMR and GPC. Results were compared to fellow group members who synthesized copolymers, their reactions time for PLA polymerization with 0.5 equivalents of catalyst was approximately 6 hours with a narrow PDI of about 1.1-1.2. Compound (1) was a small molecule so it is likely that it was able to react faster and therefore needed less reaction time. The macroinitiator for the copolymer had a molecular weight of about 5,000 g/mol, so it is likely the reaction rate was slower due to the size of the initiator.

SPDP was synthesized but still needs to be optimized in order to maximize yield and purity. The compound will most likely have to be purified at the PDP stage, therefore eliminating the undesirable side product of the NHS acetate. The synthesis of the deprotected KKC (compound 6) and KKKC (compound 10) oligolysine was successfully completed and was

confirmed by ^{1}H NMR. Mass spectra were also taken for a few of the intermediate products in order to further confirm that the oligolysines growing in molecular weight after each coupling. These results were not reported in this thesis because not all samples were examined. Solution phase peptide synthesis would have to be reevaluated for synthesizing longer oligolysine chains, due to solubility issues and concern of purity. There are alternative methods of synthesizing longer oligolysine chains. One could be solid phase peptide synthesis using a polymer resin. The concerns with this could be the amount of oligolysine that can be made with this method due to the capacity of the resins. The second method could be making poly(L-lysine) by ring opening polymerization but this method could have potential problems such as the oligolysine chains would not be perfectly identical. One oligolysine chain could have one or more lysine per chain as compared to another oligolysine chain from the same reaction. Future work would include coupling SPDP and oligolysine chains to the amphiphilic copolymer; and then conducting biological studies on cells.

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Appendix:

1) ^{1}H NMR spectrum of compound 1 from column……………………………………………………42

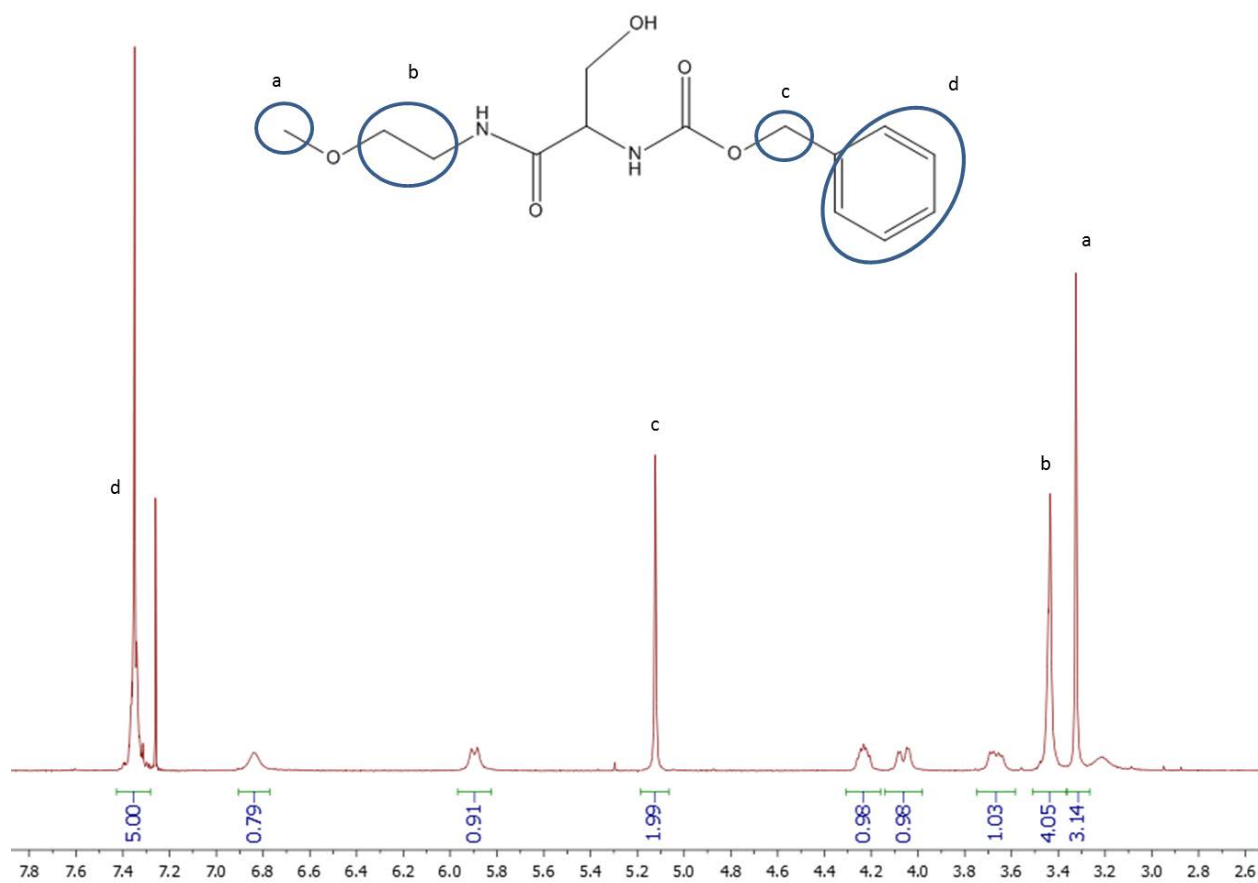
2) ^{1}H NMR spectrum of compound 1 from precipitation……………………………………….…...43

3) 1H NMR spectrum of Crude PLA 10K reaction (1)…………………...……………………..44

4) ^{1}H NMR spectrum of PLA 10K after 2 time precipiation reaction (1)………...……………..45

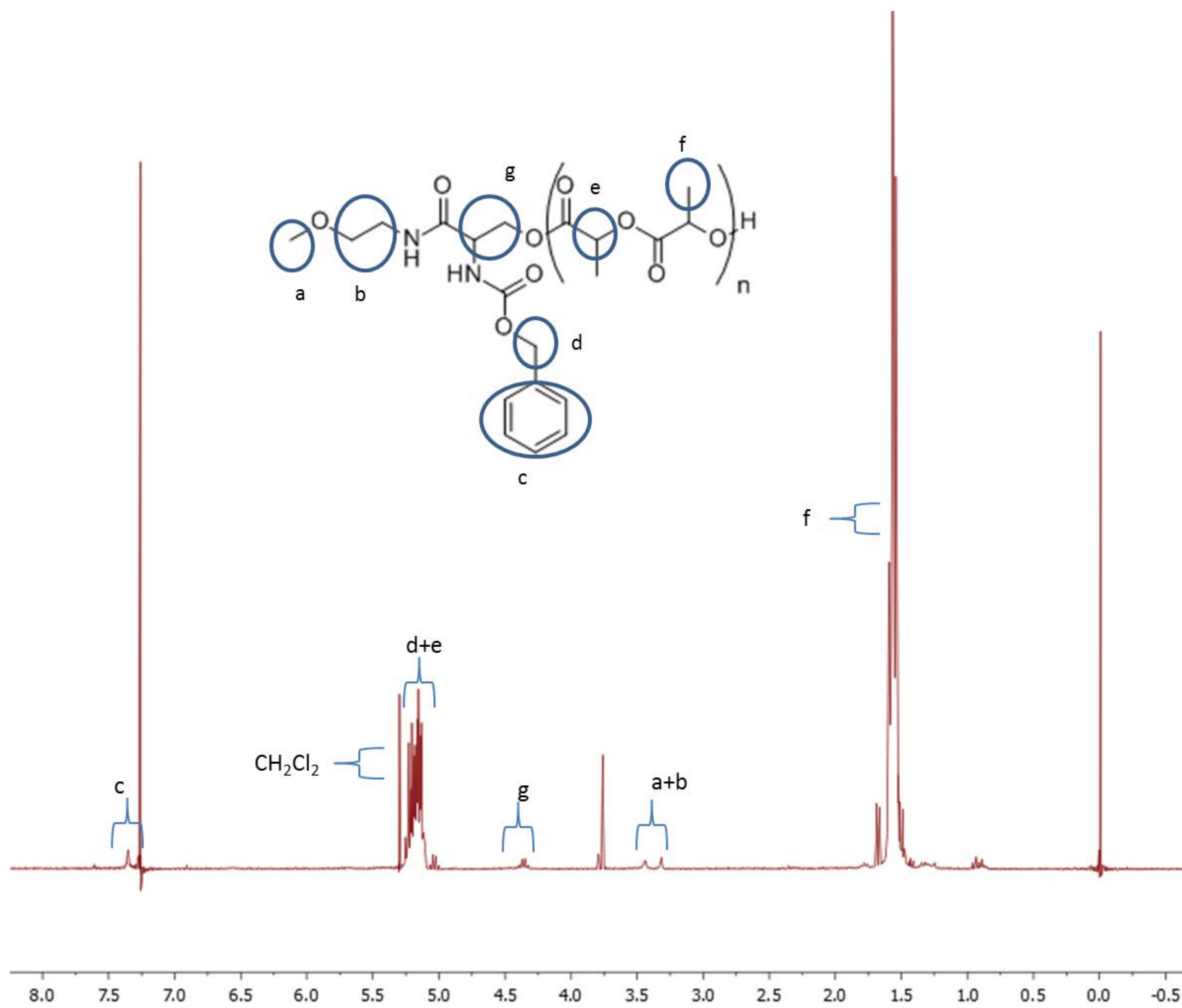
5) Calculated molecular weight for compounds 3-10……….……………………………………..46

6) ^{1}H NMR spectrum of NHS acetate……………………………………………………………47

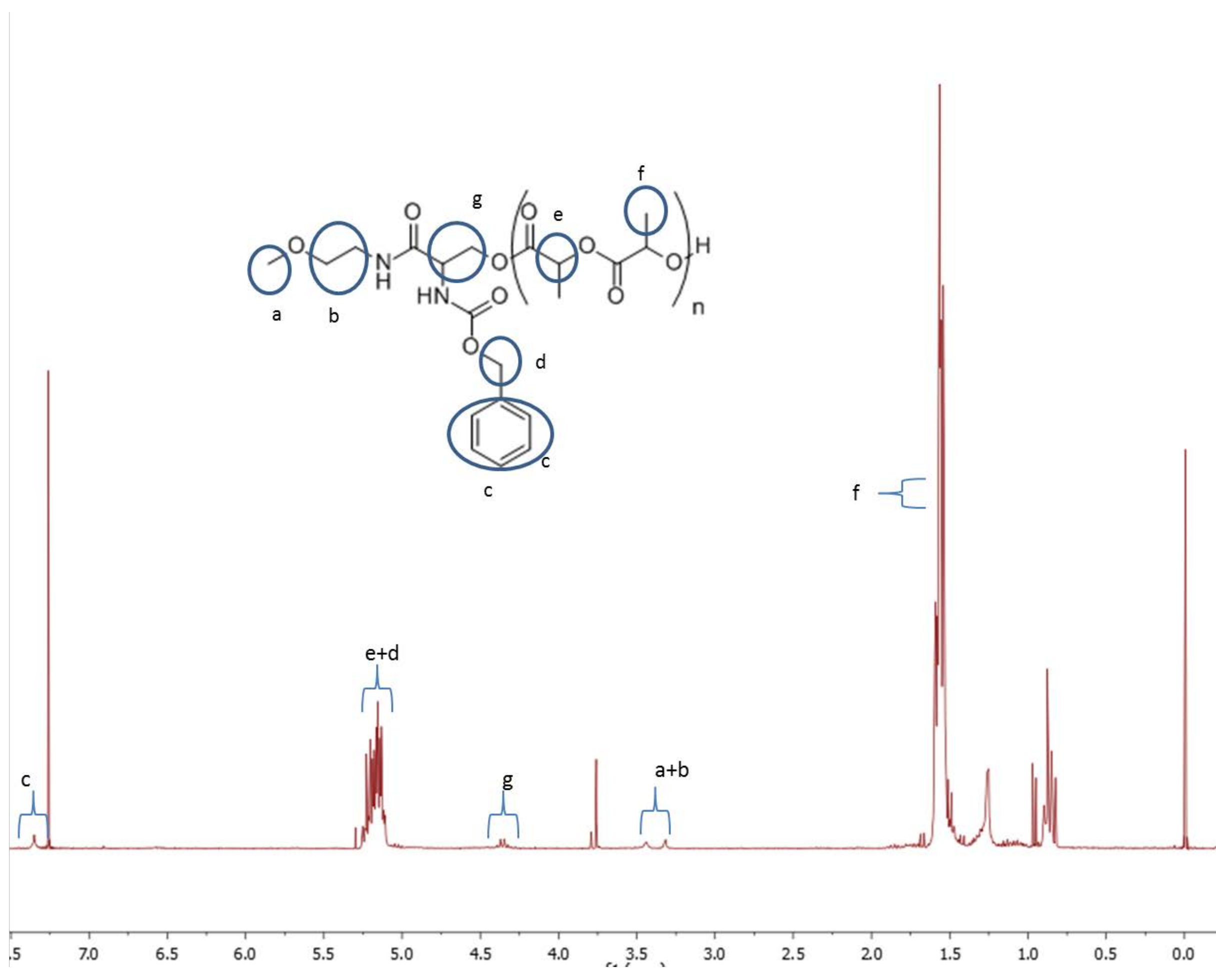


Appendix 1: ^{1}H NMR spectrum of compound (1) from column

Appendix 2: ^{1}H NMR spectrum of compound (1) from precipitation



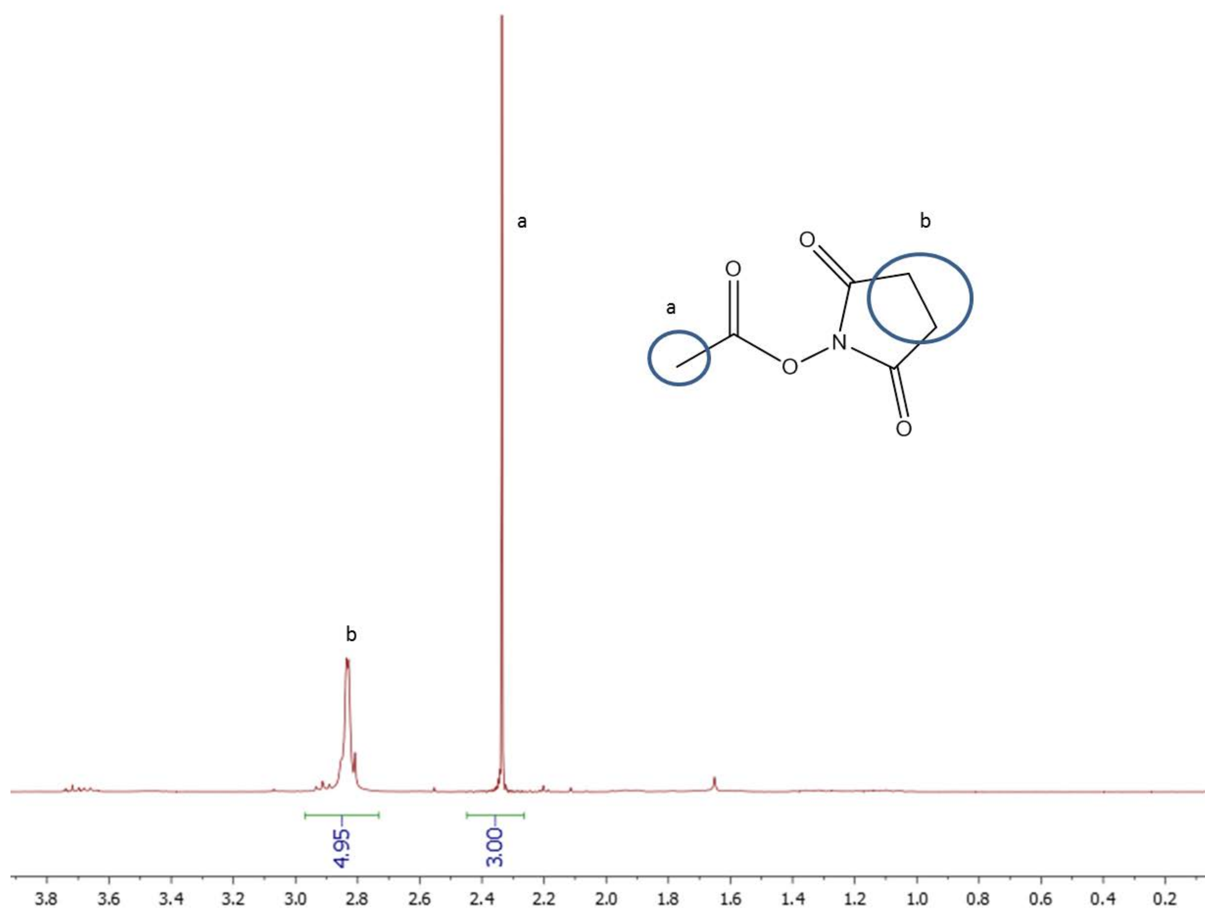
Appendix 3: ^{1}H NMR spectrum of Crude PLA 10K reaction (1)



Appendix 4: ^{1}H NMR spectrum of PLA 10K after 2 time precipiation reaction (1)

Compounds		Molecular Weight (g/mol)
3	CK-Fmoc	813.01
4	$CK\text{-}NH_2$	590.78
5	KKC(Trt,Boc)	919.18
7	CKK-Fmoc	1041.30
8	$CKK\text{-}NH_2$	819.06
9	KKKC(Trt,Boc)	1147.47
6	KKC	376.52
10	KKKC	504.69

Appendix 5: Calculated molecular weight for compounds 3-10



Appendix 6: ^{1}H NMR spectrum of NHS acetate