Controlled Polymerization of Amino acid Derivatives

by

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Abstract

This dissertation can be broken into two parts comprising different strategies to synthesise novel poly-amino acid based polymers.

The use of recently developed nickel(0) and cobalt(0) metal catalysts for the living polymerization of *α-amino acid*-N-*carboxyanhydrides* (NCAs) to synthesise novel poly-amino acid polymers, comprising a polar, hydrophilic block and a neutral hydrophobic block, were investigated in the first part of this study. The hydrophilic block was made up of a random sequence of arginine (Arg, R), glycine (Gly, G) and aspartic acid (Asp, D) - poly-RGD. This was followed by a polyleucine (Leu, L) hydrophobic block. Success was limited with this system due to polymer precipitation during the polymerization reaction. Because of this precipitation, the amino acid composition of the hydrophilic block was changed to a random sequence of glutamic acid (Glu, E), cystein (Cys, C) and aspartic acid – poly-ECD. Here also, the success was limited because of polymer precipitation.

A novel approach to the synthesis of hybrid poly-amino acid – synthetic polymer materials constitutes the second part of this study. The final polymeric structure can be described as a *carboxylic acid functionalized polyethylene glycol (PEG) sheathed polylysine polymer*. The technology involves the synthesis of a lysine NCA functionalized at the ε-amino group with an α,ω-bis(carboxymethyl) ether PEG. The distal carboxylic acid group was protected as a benzyl ester during synthesis and subsequent polymerization of the PEG-lysine-NCA macro-monomer. The polymerization was successfully initiated using *n*-butyl amine to form short homopolymer strands. Copolymerization with lysine-NCA was also achieved as well as the successful initiation using a generation 1.0 dendritic amine initiator, *N,N,N',N'*-tetrakis(3-aminopropyl)-1,4-butanediamine (DAB-Am-4). These polymers were characterized by ^{1}H NMR.

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Uittreksel

Die proefskrif kan in twee opgedeel word wat verskillende strategië beskryf om nuwe polimere, gebasseer op amino sure, daar te stel.

Die gebruik van onlangs ontwikkelde nikel(0) en kobalt(0) katalisatore vir die lewendige polimerasasie van α-*amino suur*-N-*karboksianhidriede* (NCAs) vir die sintese van nuwe poli-aminosuur-gebasseerde polimere wat bestaan uit ‘n polêre, hidrofiliese blok en ‘n neutrale hidrofobiese blok was die eerste gedeelte van die studie. Die hidrofiliese blok het bestaan uit ‘n lukraak volgorde van argenien (Arg, R), glisien (Gly, G) en aspartiensuur (Asp, D) - poli-RGD. Dit was gevolg deur ‘n polileusien (Leu, L) hidrofobiese blok. Sukses is beperk in dié geval a.g.v. polimeer presipitasie tydens die polimerisasie reaksies. Weens die presipitasie is die aminosuur samestelling van die hidrofiliese blok verander na ‘n lukraak volgorde van glutamiensuur (Glu, E), sisteïen (Cys, C) en aspartiensuur – poli-ECD. Hier ook is die sukses beperk deur polimeer presipitasie.

‘n Nuwe beandering tot die sintese van poli-aminosuur – sintetiese polimeer materiale word in die tweede gedeelte ondersoek. Die finale polimeerstruktuur kan beskryf word as ‘n *karboksielsuur gefunksionaliseerde poliëtileenglikol (PEG) skede rondom ‘n polilisien polimeer*. Die tegnologie sluit in die sintese van ‘n lisien NCA, waarvan die ε-aminogroep gefunksionaliseer is met ‘n α,ω-bis(karboksimetiel)eter PEG. Die vêrste karboksielsuurgroep is beskerm deur ‘n bensielester tydens die sintese en die gevolglike polimerisasie van die PEG-lisien-makromonomeer. Die polimerisasie is suksesvol afgeset deur *n*-butiel amien om kort homopolimere te vorm. Kopolimerisasie met lisien-NCA is suksesvol asook die effektiewe polimerisasie afgesetting deur die gebruik van ‘n generasie 1.0 dendritiese amienafsetter, *N*,*N*,*N’*,*N’*-tetrakis(3-aminopropiel)-1,4-butaandiamien (DAB-Am-4). Die polimere is hoofsaaklik gekarakteriseer deur ^{1}H KMR.

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To:

My wife, my mother, my parents in law and all of my great friends for their enormous support over the years.

Thank you!

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List of Abbreviations

ADAM	A Disintegrin And Metalloproteinase domain
AMM	Active Monomer Mechanism
Bipy	2,2'-Bipyridyl
Bzl	Benzyl-
C	L-Cystein (Cys)
COD	Cyclooctadiene
D	L-Aspartic acid (Asp)
DAB-Am-4	*N*,*N*,*N'*,*N'*-Tetrakis(3-aminopropyl)-1,4-butanediamine
DCC	*N*,*N'*-Dicyclohexyl carbodiimide
DCM	Dichloromethane
DCMME	Dichloromethyl methyl ether
DCU	*N*,*N'*-Dicyclohexyl urea
DIPAM-resin	Diisopropyl amino methyl functionalized polystyrene resin
DIPEA	Diisopropyl ethyl amine
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethylsulfoxide
E	L-Glutamic acid (Glu)
ECM	ExtraCellular Matrix
EI-MS	Electron Impact ionisation Mass Spectroscopy
ES-MS	Electron Spray ionisation Mass Spectroscopy
F	L-Phenylalanine (Phe)
Fmoc	Fluorenylmethoxycarbonyl-
FT-IR	Fourier Transform Infrared Spectroscopy
G	L-Glycine (Gly)
HOBt	*N*-Hydroxybenzotriazole
HOSu	*N*-Hydroxysuccinimide
L	L-Leucine (Leu)
MDC	*N*-terminal Metalloproteinase domain, a Disintegrin domain and Cystein rich *C*-terminus
mPEG	Methyl ether terminated poly(ethylene glycol)

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NCA	α-Amino acid-*N*-Carboxy Anhydrides
NMM	*N*-Methylmorpholine
NMR	Nuclear Magnetic Resonance
PEG	Poly(ethylene glycol)
PhEt	2-Phenylethyl-
PhEt-OH	2-Phenylethanol
PyBOP	Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
R	L-Arginine (Arg)
SPS	Solid Phase Synthesis
Su	Succinimide
tBoc	tert.Butyloxycarbonyl-
TEA	Triethylamine
TFA	Trifluoro acetic acid
THF	Tetrahydrofuran
Tos	Tosyl-
Trt	Trityl-
Z	Benzyloxycarbonyl-

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Chapter 1

Introduction

Polyamino acids and their syntheses can be grouped into two categories. The first is the synthesis of biologically active peptides or short proteins. Different amino acids, natural and unnatural, are used to synthesize these peptides. The most common method for this peptide synthesis is through the use of solid phase synthesis techniques and protocols for the step-by-step coupling of the amino acids to the growing polypeptide[1].

The second category can be described as amino acid polymers, rather than peptides or proteins. These polymers would usually consist out of one type of amino acid, making up homo-polymers or homo-polymer blocks in more complex copolymers. These polymers are obtained by the ring opening polymerization (ROP) of appropriate amino acid derivatives.

Recently, we became interested in the second of the two categories mentioned, to find application in the synthesis of amino acid based bio-materials and more specifically in the formation of hydrogels. Two approaches for achieving this goal were investigated. The first is the synthesis of *block-copoly(amino acids)* and the second was through the synthesis of *hybrid poly(amino acid)–synthetic polymer* materials.

Polymerization of *α-amino acid*-N-*carboxyanhydrides* (NCAs)[2] takes place through ring opening polymerization (ROP). The different strategies for the synthesis of NCAs as well as possible complications because of the formation of byproducts are discussed in chapter 2. Also discussed are ways to circumvent this byproduct formation and finally three generally applied synthetic procedures for the syntheses of NCAs are described.

Traditionally NCA polymerizations are initiated through the use of 1º, 2º or 3º amines and alkoxides. Usually this method of initiation lacks absolute control over the

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polymerization because of unwanted side and termination reactions[2]. Hadjichristidis et al. proved that by using high vacuum techniques living polymerization of NCAs through 1^{o} amine initiation is indeed possible[3, 4]. Deming et al. introduced the controlled living polymerization of NCAs by means of zero-valent nickel or cobalt catalysts[5]. This technology found application in the synthesis of narrow polydispersed *block-copoly(amino acids)*[6]. Jhurry et al. applied aluminium Schiff's base complexes to initiate NCA polymerization for the synthesis of homo, random and block copolypeptides[7, 8]. The polymerization mechanisms and synthesis of these initiators and catalysts are described in Chapter 3.

We had a keen interest in the nickel(0) and cobalt(0) NCA polymerization catalyst systems as an immerging technology. These catalyst systems were employed to synthesize *block-copoly(amino acids)* consisting of a hydrophilic, charged poly-electrolyte block followed by a neutral, hydrophobic block. The charged block was either polylysine (+) or polyglutamic acid (-) and the hydrophobic block consisted of polyleucine. These *block-copoly(amino acids)* form hydrogels at very low concentrations[6]. The gel formation is influenced mainly by the length and conformation of the hydrophobic blocks which self assemble in an aqueous environment. The hydrophilic blocks play a lesser role in gel formation, through interstrand charge repulsion, compared to the hydrophobic interaction contributed by the hydrophobic blocks[9].

It was the intention to expand this technology by an application-driven study into the synthesis of new block-copoly(amino acids). It has already been proven that the conformation and length of the hydrophobic block is the crucial component in self-assembly and gel-formation of these polymers – this leaves the hydrophilic block open to further investigation. Chapter 4 describes the study and synthesis of three-component, random poly(amino acid) copolymers to make up the hydrophilic block. Random copolymers of arginine (R), glycine (G) and aspartic acid (D) (poly-RGD) and also glutamic acid (E), cystein (C) and aspartic acid (D) (poly-ECD) were investigated as suitable and functional alternatives to make up the hydrophilic block. The utility lies in the known cell-binding abilities of the RGD and ECD tri-peptide sequences. The aim was thus to synthesize poly(amino acid) polymers that would self

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assemble in an aqueous medium to form hydrogels with built-in cell-binding capabilities[10].

A novel approach to the synthesis of *hybrid poly(amino acid) – synthetic polymer* materials is described in chapter 5. The final polymeric structure can be described as a *carboxylic acid functionalized polyethylene glycol (PEG) sheathed polylysine polymer*. The technology entails the synthesis of a lysine NCA functionalized at the ε-amino group with a α,ω-bis(carboxymethyl) ether PEG. The distal carboxylic acid group is protected as an ester during synthesis and subsequent polymerization of the PEG-lysine-NCA macro-monomer. After polymerization the protecting ester will be removed to avail the carboxylic acid group at the distal end of the PEG. This acid group could then act as a synthetic handle for the attachment of bio-molecules and peptides or be used for either ionic or covalent cross-linking. Also, because of the PEG 'spacer' group the polymer should be water soluble without the distal carboxylic acid groups being neutralized. With this being the case it is hypothesised that cooperative hydrogen bonding between the distal acid groups may result in the self-assembly of the polymer strands. Together with the PEG spacer group's potential water trapping ability may lead to gel-formation in an aqueous medium.

All of the following chapters have their own introduction. Chapters 2 and 3 serves as a concise yet detailed background into the synthesis, possible complications and relevant reaction mechanisms of the technologies applied as described in Chapters 4 and 5.

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References

1. Kates, A. S.; Albericio, F., *Solid-phase synthesis - A practical guide*. Marcel Dekker, New York, Basel, **2000**

2. Kricheldorf, H. R., *α-Amino acids-N-carboxy-anhydrides and related heterocycles: Syntheses, properties, peptide synthesis, polymerization*. Springer-Verlag, Berlin Heidelberg, **1987**.

3. Aliferis, T.; Iatrou, H.; Hadjichristidis, N., Living polypeptides. *Biomacromolecules* **2004,** 5, 1653-1656.

4. Hadjichristidis, N.; Iatrou, H.; Pispas, S.; Pitsikalis, M., Anionic polymerization: High vacuum techniques. *J. Polym. Sci., Polym. Chem.* **2000,** 38, 3211-3234.

5. Deming, T. J., Facile synthesis of block copolypeptides of defined architecture. *Nature* **1997,** 390, 386-389.

6. Nowak, A. P.; Breedveld, V.; Pakstis, L.; Ozbas, B.; Pine, D. J.; Pochan, D. J.; Deming, T. J., Rapidly recovering hydrogel scaffolds from self-assembling diblock copolypeptide amphiphiles. *Nature* **2002,** 417, 424-428.

7. Bhaw-Luximon, A.; Jhurry, D.; Belleney, J.; Goury, V., Polymerization of γ-methylglutamate *N*-carboxyanhydride using Al-Schiff's base complexes as initiators. *Macromolecules* **2003,** 36, 977-982.

8. Goury, V.; Jhurry, D.; Bhaw-Luximon, A.; Novak, B. M.; Belleney, J., Synthesis and characterization of random and block copolypeptides derived from γ-methylglutamate and leucine *N*-carboxyanhydrides. *Biomacromolecules* **2005,** 6, 1987-1991.

9. Deming, T. J., Polypeptide hydrogels via a unique assembly mechanism. *Soft Matter* **2005,** 1, 28-35.

10. Evans, J. P., Fertilin β and other ADAMs as integrin ligands: insights into cell adhesion and fertilization. *BioEssays* **2001,** 23, 628-639.

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Chapter 2

Synthesis of α-Amino acid-*N*-Carboxy Anhydrides (NCAs)

2.1 Introduction

Most aspects of NCA synthesis, characterization and polymerization can be found in the book of Kricheldorf, H.R., "α-Amino acid-*N*-Carboxy-Anhydrides and Related Heterocycles – Synthesis, Properties, Peptide Synthesis, Polymerization"[1]. A condensed version can be found as a section in the book "Models of Biopolymers by Ring-Opening Polymerization" edited by Penczek, S[2]. Unless referenced differently it can be assumed that the stated facts can be referenced to these two publications.

α-Amino acid-N-*carboxy anhydrides* (NCAs) synthesis can be classified into two groups depending on the nature of the amino acid substrate. The first is the *Leuchs* method and is based on the cyclization of *N*-alkoxycarbonyl amino acid halides to form the α-amino acid-*N*-carboxy anhydrides (oxazolidine-2,5-dione). The second, *Fuchs-Farthing* method, involves the direct phosgenation of unprotected α-amino acids.

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2.2 Leuchs method for NCA synthesis

This method was first discovered by Herman Leuchs in 1906 when he used N-alkoxycarbonyl amino acid chlorides for the purpose of stepwise peptide synthesis. Upon heating (50 – 70 °C) cyclization occurred together with the formation of alkyl chlorides.

Leuchs used thionyl chloride as chlorinating agent, which has the advantage of having gaseous byproducts. Phosphorous pentachloride is more reactive and the formation of NCAs can be achieved at lower temperatures, which reduces the risk of high temperature decomposition of certain NCAs. The phosphoric oxide trichloride byproduct may, on the other hand, interfere with the crystallization of the NCA product.

It is postulated that during NCA formation the acid halogenide group attacks the carbonyl oxygen of the carbamate protecting group forming a cyclic dioxacarbenium ion intermediate. The rate determining step is the nucleophilic attack by the halogenide on the alkyl group resulting in the formation of the NCA and the alkylhalide byproduct (Fig. 1).

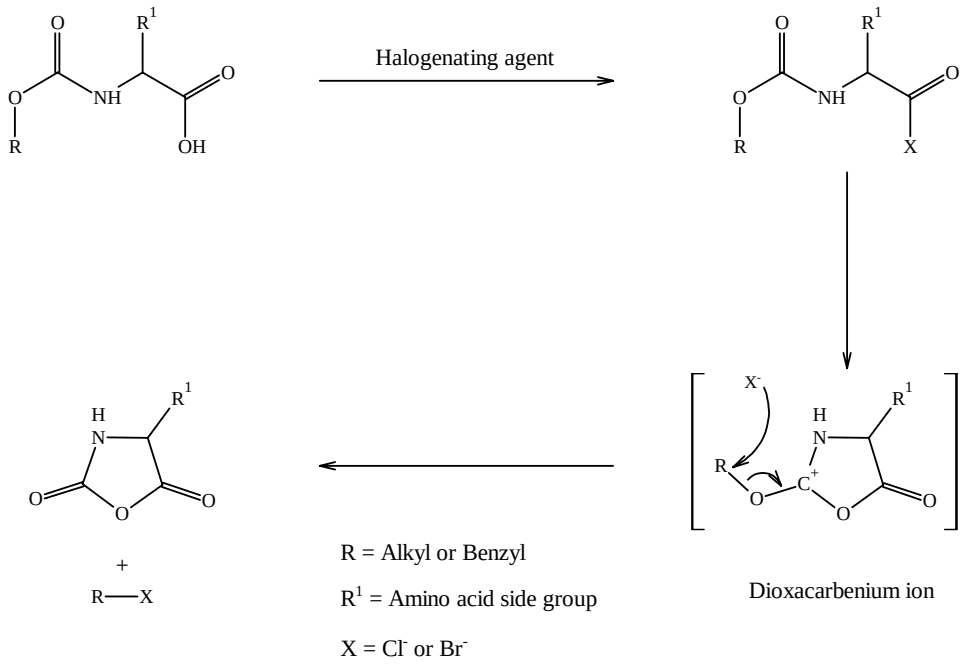

Figure 1: Leuchs method for the synthesis of NCAs.

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Not only does the halogenating agent play a role in the ease of the NCA formation, but the type of halogen is also a factor – a bromide ion is a better nucleophile in the rate determining step than the chloride ion. This makes phosphorus tribromide a preferred halogenating agent. The nature of the *N*-alkoxycarbonyl groups also influences the rate of cyclization. The rate of cyclization increases with an increase in the electrophilic nature of the alkyl group as illustrated in the following:

Ethyl- < Methyl- < Allyl- < Benzyloxycarbonyl

For the above reasons, some benzyloxycarbonyl amino acid bromides can cyclize below room temperature.

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2.3 Fuchs-Farthing method for NCA synthesis

This method is probably the most widely used method for the synthesis of NCAs. It involves the direct phosgenation of the α-*N*-unprotected amino acids. Cyclization proceeds through the formation of *N*-chloroformyl amino acid intermediates and the loss of a second HCl molecule completes the NCA formation (Fig. 2).

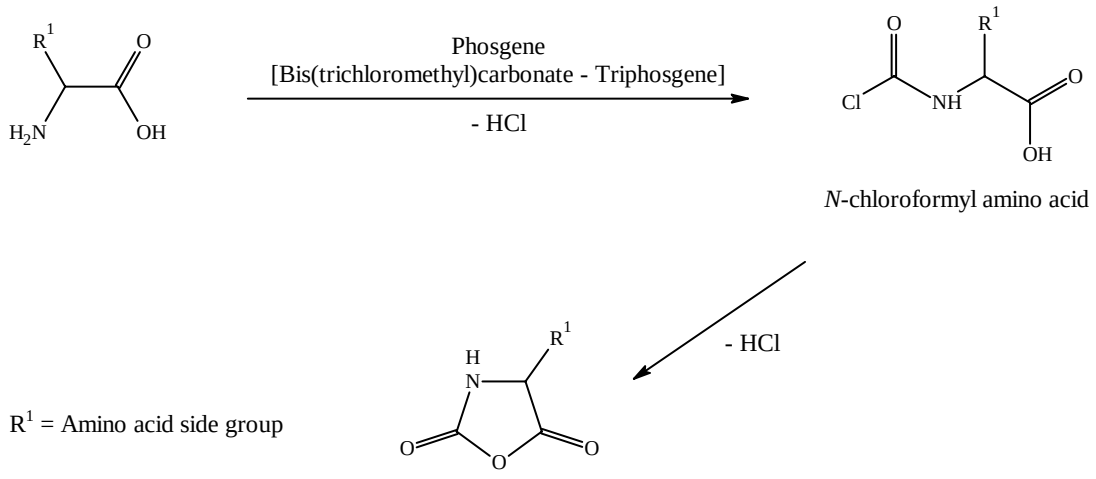

Figure 2: Fuchs-Farthing method for NCA synthesis.

THF, dioxane and ethyl acetate are appropriate solvents to use due to their inertness towards phosgene. In larger scale synthesis (> 0.5 mol) the use of a 1:1 boiling mixture of THF/dioxane and dichloromethane (DCM) are recommended – the HCl byproduct is less soluble under these conditions than in pure ethers. This limits the formation of byproduct contamination due to a high concentration of HCl. The contaminants are amino acid chloride hydrochlorides formed by the HCl cleavage of the NCA ring. These amino acid chloride hydrochlorides can be phosgenated in a second step to form α-isocyanato acid chlorides (Fig. 3).

Phosgene gas can be bubbled into the reaction mixture, but lacks stoichiometric control and results in excessive use and the formation of the contaminants mentioned above. Trichloromethyl chloroformate (diphosgene – 'phosgene dimer') which supplies 2 molar equivalents of phosgene to the reaction is available in liquid form and has improved the stoichiometric control[3]. A crystalline equivalent, bis(trichloromethyl) carbonate (triphosgene – 'phosgene trimer') is also available to

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deliver 3 molar equivalents of phosgene. When these agents are used it is done so at elevated temperatures in order to break up the dimers and trimers to release the reactive phosgene molecules. Reactions are typically carried out at 50°C when triphosgene is used[4].

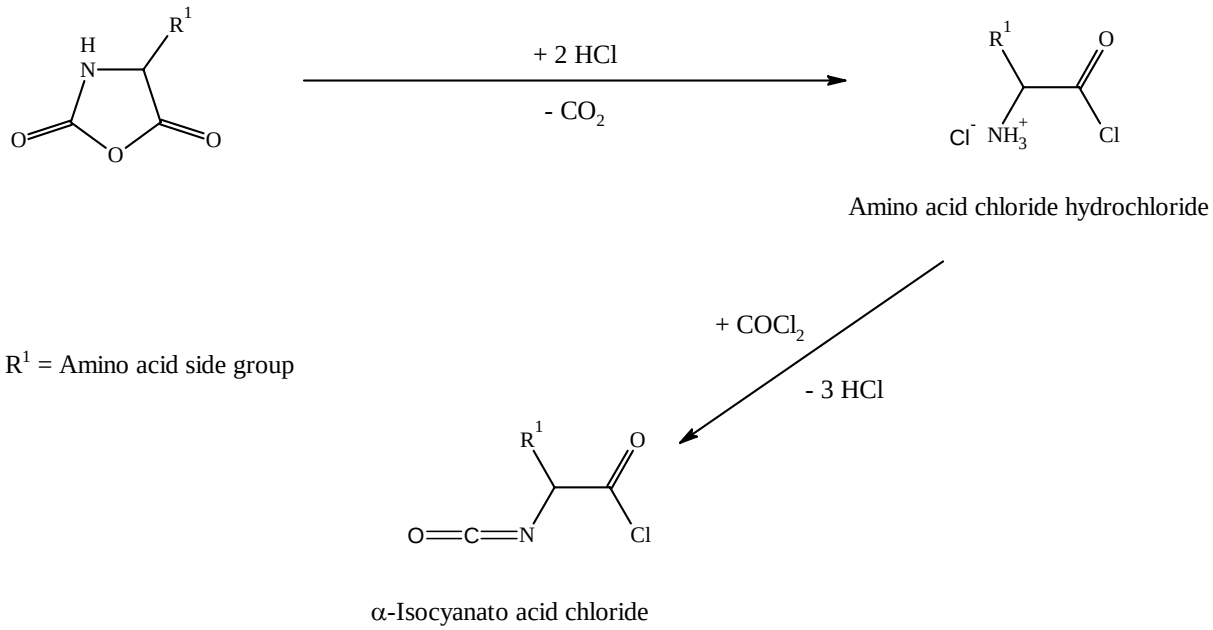

Figure 3: Contaminant formation due to an excess of phosgene and HCl.

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2.4 Solving the HCl problem

The problem with HCl, as a byproduct, has led to the investigation of alternative or modified procedures for the HCl free synthesis of NCAs. To follow are a couple of examples of these alternative processes.

Wilder et al. used triphosgene to react with *tert.*-butyloxycarbonyl (tBoc-) α-NH protected amino acids in the presence of triethylamine as a mild, room temperature method to synthesize NCAs (Fig. 4)[5].

Kricheldorf showed that the phosgenation of silylated amino acids are an effective means to eliminate the formation of HCl in the reaction mixture. This can be illustrated by the phosgenation of per-silylated serine in a Fuchs-Farthing type method for NCA synthesis (Fig. 5)[1].

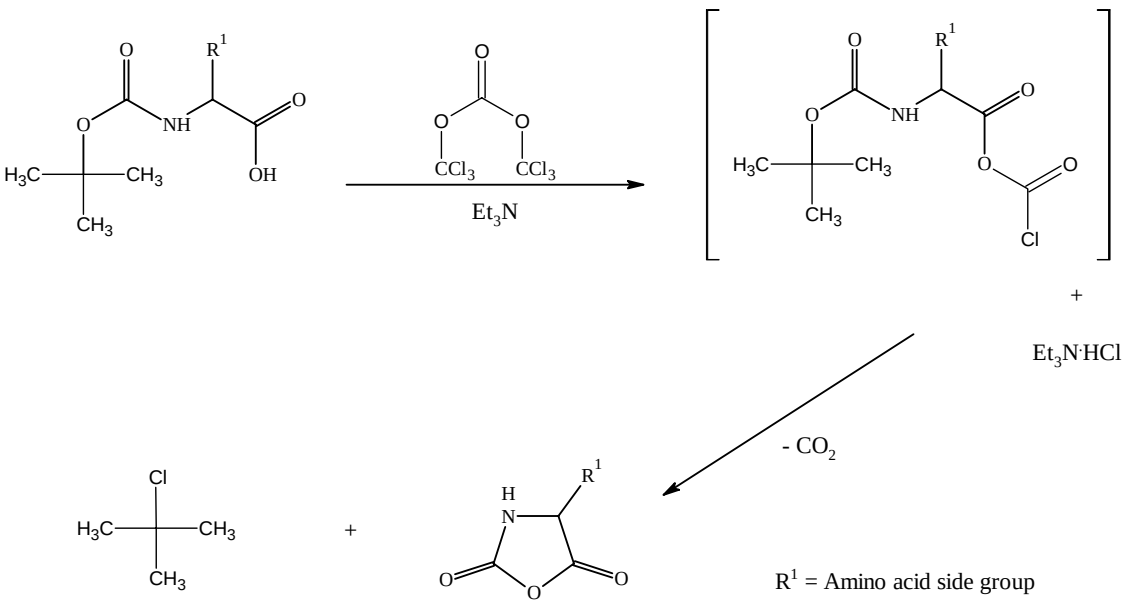

Figure 4: NCA synthesis through the use of triphosgene and triethylamine.

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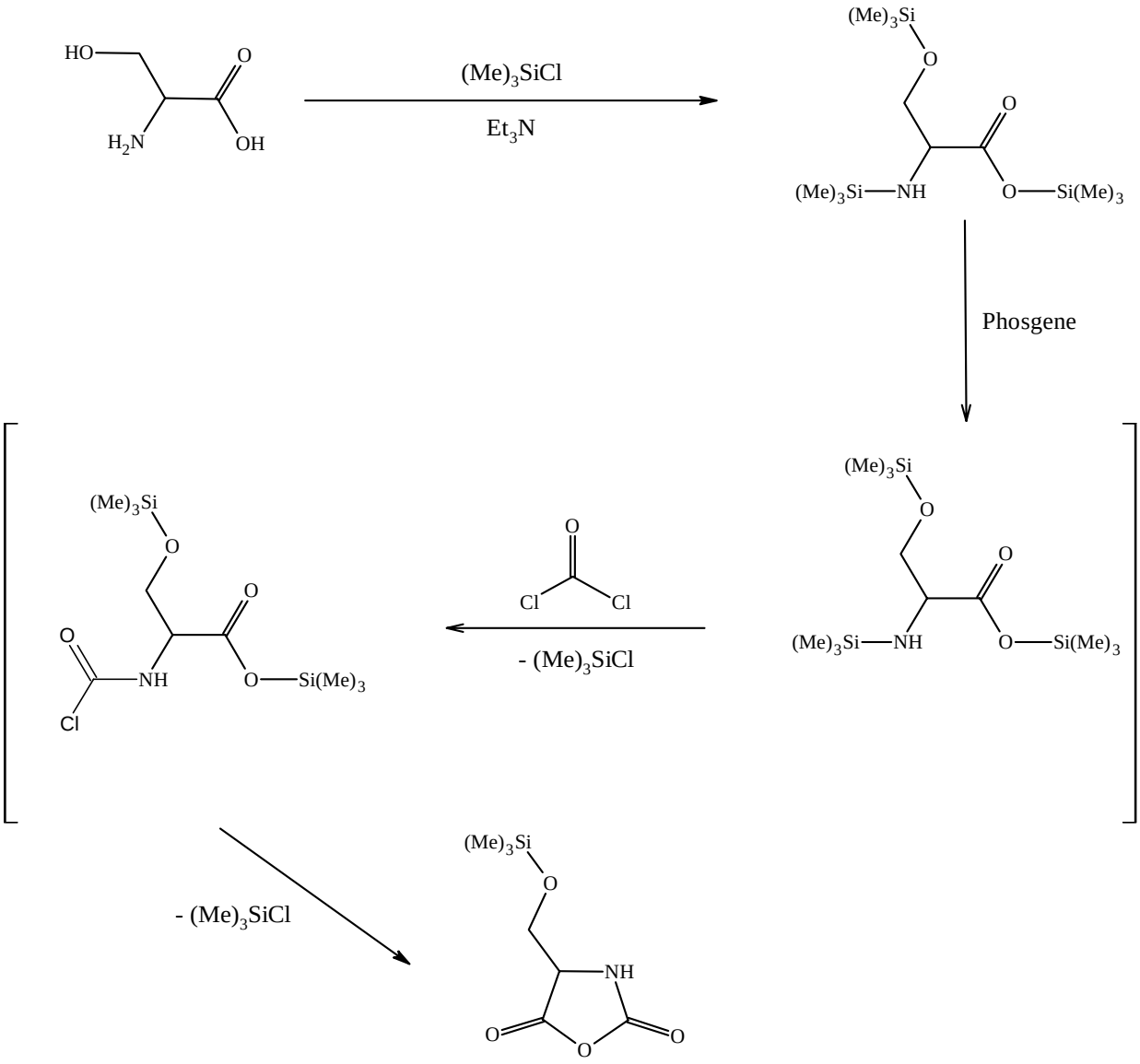

Figure 5: The per-silylation of serine and phosgenation to prepare Ser(SiMe$_3$)-NCA without the formation of HCl.

Johnston et al. also made use of silylated amino acid intermediates, in a modified Leuchs-type NCA synthesis, as another alternative to circumvent the problem of HCl formation during the synthesis of NCAs. As the first step, tBoc-α-*N* amino acids were silylated with *tert*-butyldimethylsilyl chloride in the presence of triethylamine to form the silyl ester. This was subsequently reacted with oxalyl chloride, with a few drops of DMF as activator, to form the NCA under very mild conditions free of HCl (Fig.6)[6].

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A practical problem with the use of organic bases in the alternative syntheses is the quantitative removal of ammonium chloride salts from the product. Repeated product specific recrystallizations are necessary to achieve complete removal of the salts. This might not always be possible, as some NCAs are not able to crystallize.

The use of proton scavengers like (+)-limonene proved effective in preventing byproduct formation, due to the high HCl content, in the large scale synthesis of L-leucine-NCA[7, 8].

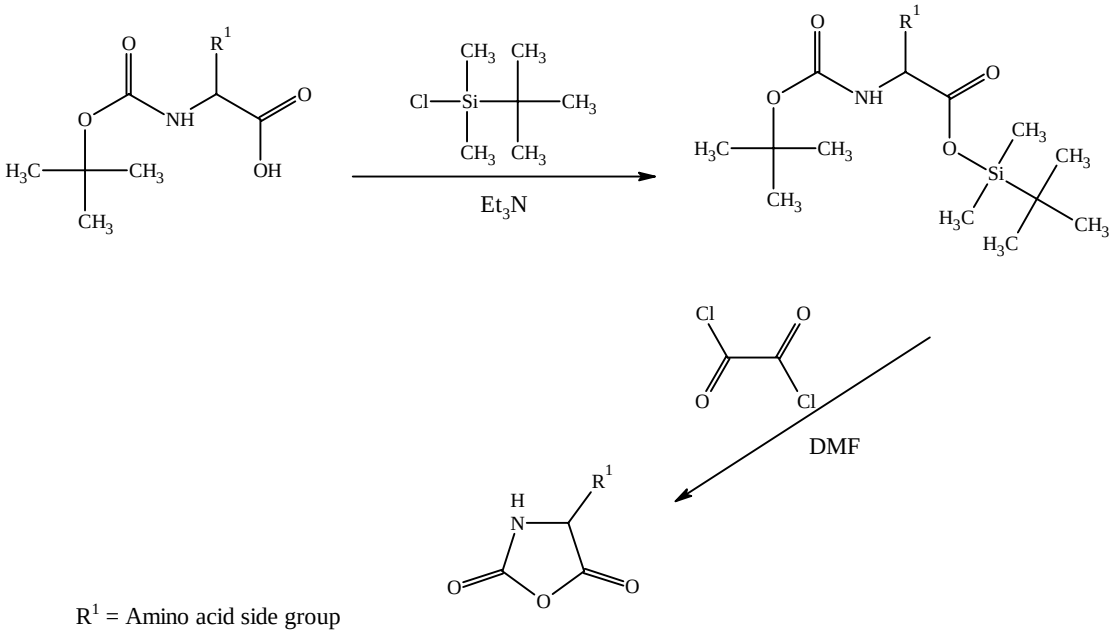

Figure 6: The use of silyl intermediates in the synthesis of NCAs

Poché et al. used cold, weak solutions of sodium bicarbonate to wash their NCAs, dissolved in organic solvents, to remove the HCl contamination. This was suggested for oily NCAs which cannot be purified by crystallization[9].

These synthetic techniques and especially the alternative procedures proved vital in the approach to some of the synthetic challenges in this research work.

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2.5 General procedures for the synthesis of NCAs

2.5.1 NCA synthesis from α-amino unprotected amino acids

This procedure is applicable for amino acids which are unprotected at the α amino- and carboxylic acid groups. Side-group protection should not be acid labile [eg. *tert.*-butyloxycarbonyl (tBoc)], since HCl is a byproduct of this reaction.

The amino acid was dissolved or suspended in THF, in a three-neck round-bottom flask fitted with a condenser, dropping funnel and argon atmosphere (for difficult to dissolve amino-acids, it might help to pulverise the amino acid with a mortar and pestle – in some cases the amino acid is drawn into solution during the reaction). The THF solution/suspension was heated to between 40 and 50°C. The bis(trichloromethyl)carbonate (triphosgene) was added (between ½ and $^1/_3$ eq., dissolved in THF), drop-wise to the amino acid solution, and the reaction stirred for 3 – 4h at 40 – 50°C. The reaction mixture was then filtered through celite to remove any unreacted amino acid, and the filtrate concentrated and layered with hexane and left to precipitate/crystallize in the freezer. The precipitate was filtered and recrystallised from THF and hexane (repeat 3 times), and dried under vacuum. This procedure is effective for the following amino acids: alanine, valine, leucine, phenylalanine, γ-benzyl-glutamate, β-benzyl-aspartate, ω-*N*-benzyloxycarbonyl-lysine and *O*-benzyl-serine. See the synthesis of Cys(Trt)-NCA (Chapter 4) as a practical example.

2.5.2 NCA synthesis from tBoc-α–amino protected amino acids

This procedure is suitable where solubility of the amino acid is a problem and the product can easily be crystallized. tBoc-α-amino protected amino acids are generally readily soluble in ethyl acetate or THF.

The tBoc-amino acid was dissolved in ethyl acetate in a round-bottom flask, fitted with a dropping funnel and argon atmosphere. Triethylamine (1 eq.) was added to the solution followed by the drop-wise addition of triphosgene ($^1/_3$ eq.). At this point a white precipitate, triethylammoniun chloride ($Et_3N.HCl$) formed. The argon inlet was

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removed and an oil bubbler attached to monitor the evolution of CO_2. The reaction mixture was stirred for at least 3h until evolution of CO_2 ceased. This sometimes required overnight stirring. Once the reaction is complete, the mixture was cooled down to 0^oC. This was to maximise the precipitation of the $Et_3N.HCl$. The precipitate was filtered and the filtrate concentrated. More $Et_3N.HCl$ may form upon evaporation of the solvent. The process was repeated until all the $Et_3N.HCl$ was removed. The final product was then isolated by precipitation and recrystallisation from appropriate solvents. THF/diethyl ether, THF/hexane, ethyl acetate/diethyl ether or hexane usually worked very well.

The use of ethyl acetate as reaction solvent is very important, since the $Et_3N.HCl$ was insoluble in this solvent, whereas it may be partly or completely soluble in other organic solvents. The final product often needs to be recrystallised once or twice more to remove the last traces of $Et_3N.HCl$. THF may be used as an alternate solvent when the reagents are insoluble in ethyl acetate, then *N*-methylmorpholine is a better base because the HCl salt of this base is less soluble than $Et_3N.HCl$ in THF.

2.5.3 NCA synthesis from tBoc- and Z-α–amino protected amino acids

This is a very convenient method to synthesize NCAs since most commercially available amino acids are protected with either of these protecting groups. These protecting groups enhance dissolution of the amino acids in organic solvents. This procedure is practically free of contaminants

For a 0,5g-scale reaction, the amino acid was dissolved in dichloromethyl methyl ether (DCMME) (3 – 5ml) in a small schlenk-tube. The tube was evacuated and the cap properly secured before heating the reaction mixture to 60°C for 30 min. The reaction was then removed from the heat and stirred at room temperature for 2h[10]. The DCMME was removed under vacuum to yield the crude NCA. The product may then be finally purified by precipitation/recrystallisation from the appropriate solvents.

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2.6 Conclusion

This chapter covered the chemistry involved in the synthesis of *α-amino acid*-N-*carboxy anhydrides* (NCAs) from different types of amino acid substrates. Also shown were the possible side-reactions and byproducts to be mindful of during the preparation of these NCAs. Special attention was given to procedures employed to circumvent the potential synthetic dangers posed by HCl generated in NCA syntheses.

Three general procedures for NCA synthesis were discussed – these procedures will again be encountered in chapters 4 and 5 and detailed synthetic methods of the relevant NCAs will be given there.

Refer to Appendix 1 for a table of important ^{1}H and ^{13}C NMR chemical shifts in the synthesis of NCAs.

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References

1. Kricheldorf, H. R., *α-Amino acids*-N-*carboxy-anhydrides and related heterocycles: Syntheses, properties, peptide synthesis, polymerization*. Springer-Verlag, Berlin Heidelberg, **1987**.

2. Penczek, S.; Kricheldorf, H. R.; Le Borgne, A.; Spassky, N.; Uryu, T.; Klosinki, P., *Models of biopolymers by ring-opening polymerization*. CRC Press, Inc.: Boca Ranton, **1990**.

3. Mijs, W. J., *New methods for polymer synthesis*. Plenum Press, New York and London, **1998**.

4. Daly, W. H.; Poché, D., The preparation of *N*-carboxyanhydrides of α-amino acids using bis(trichloromethyl)carbonate. *Tetrahedron. Lett.* **1988,** 29, 5859-5862.

5. Wilder, R.; Mobashery, S., The use of triphosgene in the preparation of N-carboxy-α-amino acid anhydrides. *J. Org. Chem.* **1992,** 57, 2755-2756.

6. Mobashery, S.; Johnston, M., A new approach to the preparation of *N*-carboxy α-amino acid anhydrides. *J. Org. Chem.* **1985,** 50, 2200-2202.

7. Smeets, N. M. B.; Van der Weide, P. L. J.; Meuldijk, J.; Vekemans, J. A. J. M.; Hulshof, L. A., A scalable synthesis of L-leucine-*N*-carboxyanhydride. *Org. Process. Res. Dev.* **2005,** 9, 757-763.

8. Copp, J. D.; Tharp, G. A., Optimization of the penicillin ring expansion reaction through the use of an alkene as an HCl scavenger. *Org. Process Res. Dev.* **1997,** 1, 92-94.

9. Poché, D. S.; Moore, M. J.; Bowles, J. L., An unconventional method for purifying the *N*-carboxyanhydride derivatives of γ-alkyl-L-glutamates. *Synthetic Comm.* **1999,** 29, 843-854.

10. Lange, M.; Fischer, P. M., Efficient synthesis of differentially protected (*S,S*)-2,7-diaminooctanedioic acid, dicarba analogue of cystine. *Helv. Chim. Acta* **1998,** 81, 2053-2061.

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Chapter 3

NCA Polymerization Initiators and Catalysts

3.1 Introduction

This chapter will focus on the different initiators and catalysts used in this study and their relevant mechanisms for the polymerization of the NCAs.

The discussion will be broken up into three sections focussing on the specific types of initiators or catalysts in NCA polymerization. Traditionally NCAs have been polymerized by either initiation through primary-, secondary-, tertiary amines or alkoxides. The first section will focus on the use of these organic bases for initiating the polymerization of NCAs. More recently controlled polymerization of NCAs was achieved through the use of zero-valent metal complexes and aluminium alkoxides. This is the focus of the second and the third sections of this discussion, respectively.

In this section each of the above will be discussed in detail, highlighting their applications, reaction mechanism and their synthesis. Polymerization using amines will be discussed first as it will allow a fundamental background to NCA polymerization and will put the zero valent metal and aluminium catalysts/initiators into context.

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3.2 NCA polymerization using organic bases

Comprehensive information on NCA polymerization through the use of organic bases, like for the synthesis and characterization of NCAs (Chapter 2), can be found in the book of Kricheldorf, H. R., "α-Amino acids-*N*-Carboxy-Anhydrides and Related Heterocycles – Synthesis, Properties, Peptide Synthesis, Polymerization"[1]. A condensed version can be found as a section in the book "Models of Biopolymers by Ring-Opening Polymerization" edited by Penczek, S[2]. The facts in this section can be referenced to these two works.

When looking at the NCA heterocycle, four reactive sites can be identified. These include two electrophilic centres (C–2 carbamoyl- and the C–5 carbonyl group) and two nucleophilic sites (NH and α-CH) which after deprotonation yield highly reactive amide anions and carbanions, respectively.

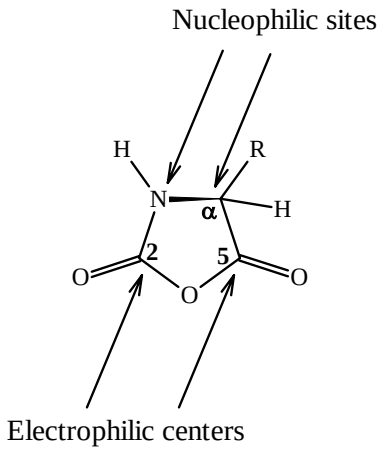

Figure 1: The NCA heterocycle – indicated are the four potential sites through which organic base initiated polymerization can take place.

The multiplicity of these reactive sites is one of the factors that contribute to the complexity in assigning potential mechanisms for polymerization. Other factors that play a role include: the low solubility of oligo- and polypeptides in organic solvents which affects the reactivity of the endgroups and conformational changes that influence the rate of propagation.

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Reaction pathways depend on the nature of the initiator. Radicals, cations and acids do not initiate polymerization of NCAs; whereas common initiators include protic- and aprotic nucleophiles, aprotic bases and certain organometallic compounds.

Initiators can be classified in terms of a ratio between the basic (thermodynamic) and the nucleophilic (kinetic) attributes of the initiator. Other classifications can be made in terms of protic- or aprotic initiators and if the initiator is able to form a stable endgroup.

Depending on the nature of the organic base, one of two initiation routes is possible. In the case where the nucleophilic character exceeds the basic strength of the base - nucleophilic attack on the C_5=O takes place. Thereafter, depending on the basic nature of the initiator, one of two intermediates is possible. The first scenario is where the initiator is not basic enough to stabilize a carbamic acid intermediate through deprotonization, thereby in decarboxylation. This yields an amino acid α-amine terminus that is maintained through the polymerization and is known as the *amine mechanism* or *normal* propagation (Fig. 2). The second scenario is where unreacted base (used as initiator) is basic enough to stabilize the carbamic acid through deprotonation. The carbamate intermediate can also be stabilized by amino endgroups of the growing polypeptide in much the same way as the initiating base. This stabilized carbamate can act as a nucleophile to attack an NCA through the *carbamate mechanism* (Fig. 2).

If the initiator is more basic than nucleophilic in character, the NCA N-H is deprotonated. The NCA anion reacts with a second NCA forming a dimer with a highly electrophilic *N*-acyl NCA endgroup and a nucleophilic carbamate group. Chain propagation can take place either through the carbamate mechanism or further nucleophilic attack by the next NCA anion on the NCA endgroup of the growing polymer. This is referred to as the *activated monomer mechanism* (AMM) (Fig. 3).

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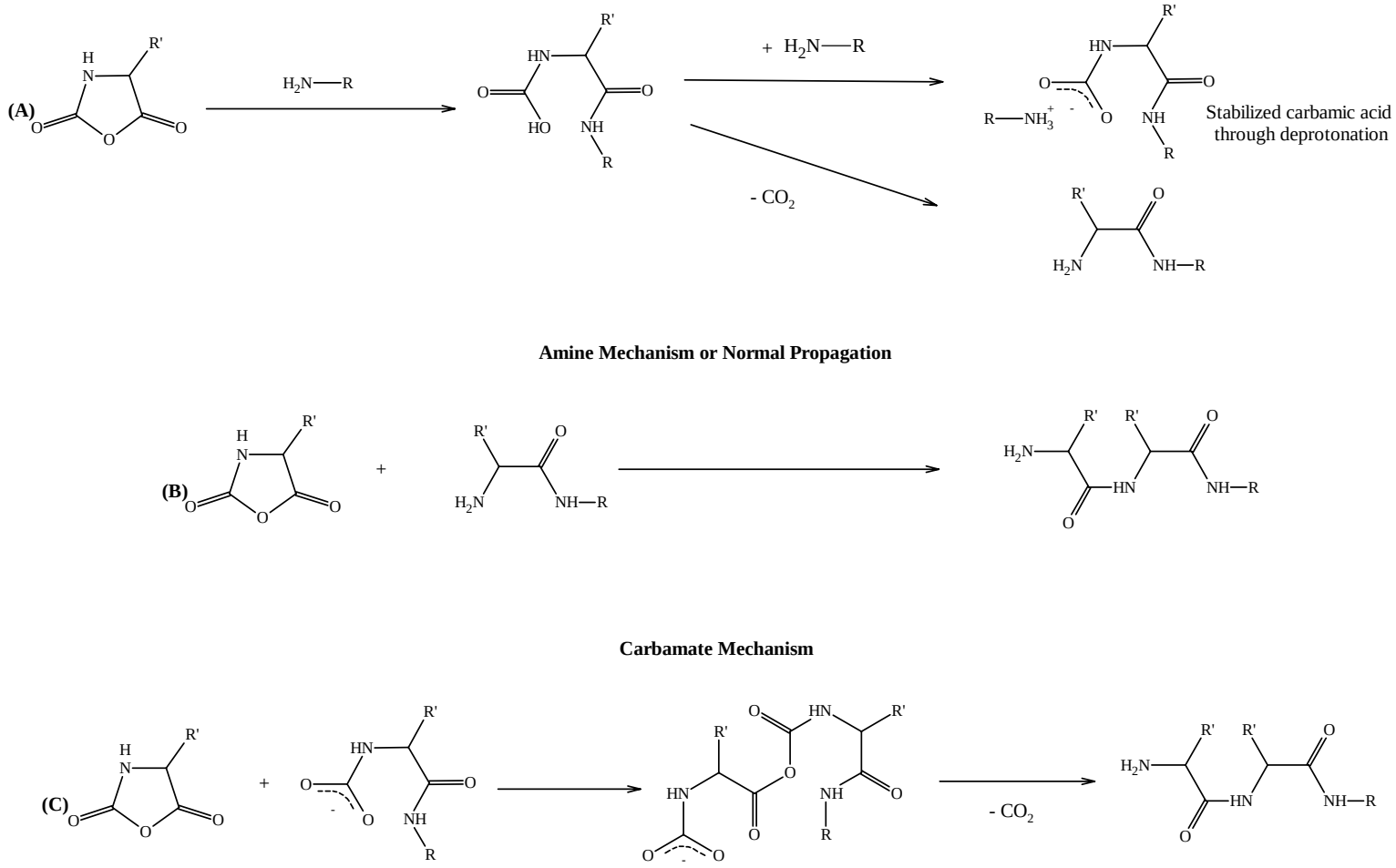

Figure 2: Chain growth pathways after amine initiation (A), highlighting the amine- (B) and the carbamate- (C) mechanisms.

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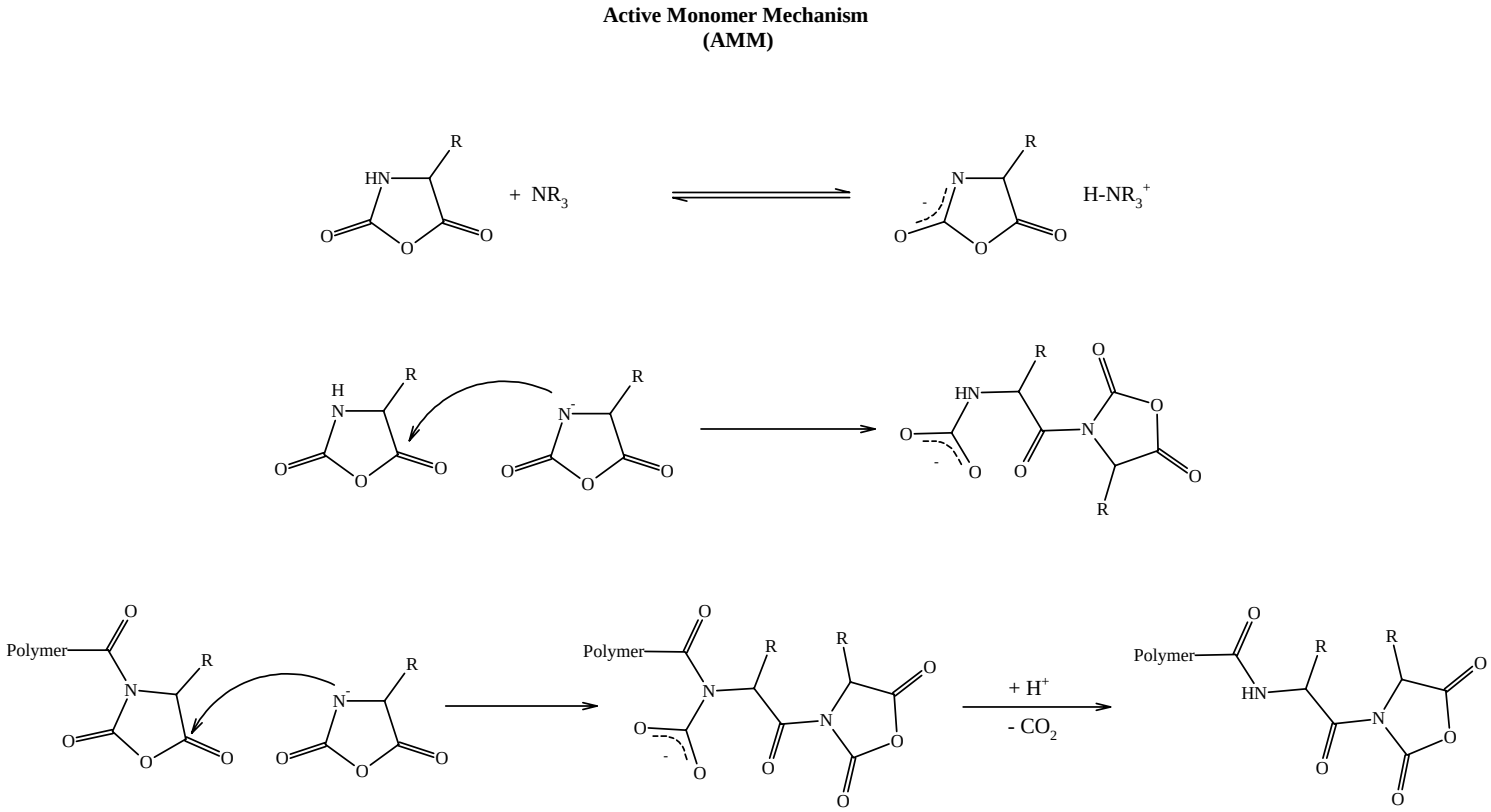

Figure 3: Polymerization through the activated monomer mechanism (AMM).

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3.2.1 Primary amines

Primary amines can be divided into three groups with respect to the way they react with NCAs.

Group 1: Aromatic amines, hydrazines and hydroxylamines

Group 2: α-Amino acid derivatives

Group 3: Aliphatic amines

Group 1: These amines are characterized by lower basicity and nucleophilicity and the reaction with NCAs, even in stoichiometric amounts, will lead to oligo- and polypeptides. The amine terminus of the growing oligopeptide is more nucleophilic than the initiator and reacts faster with the remaining monomers resulting in a propagation step that is faster than the initiation step and living polymerization kinetics cannot be applied. Therefore DP_n cannot be calculated from:

$$\text{Degree of Polymerization}: \overline{DP}_n = \frac{M}{I} \bullet \frac{\%\,\text{Conversion}}{100}$$

Equation 1: Degree of polymerization as calculated through the monomer (M) to initiator (I) ratios.

Chain-growth also though the carbamate chain end is probably less likely because of the inability of the weakly basic amines to stabilise the carbamic acid through deprotonation (Fig. 2).

Group 2: Carboxylic acid derivatives of α-amino acids would have similar characteristics as the terminal amine on the growing polymer. These ester derivatives are rarely used as initiators because of their instability during storage. Amino acid derivatives have found application in stepwise synthesis of well defined amino acid sequences.

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Group 3: Primary amines, especially *n*-butylamine and *n*-hexylamine, are probably the most widely used protic nucleophiles used in NCA homopolymerization. Their nucleophilicity is higher than that of the growing end of the polymer – the initiation step is faster than the propagation step and complies with equation 1. M/I ratios of up to 150 – 200 are possible with nearly quantitative conversion of the monomer. However, primary aliphatic amines do not furnish narrow molecular weight distributions. Primary aliphatic amines are also basic enough to stabilise the carbamic acid through deprotonation. Because of this polymerization can follow one of two routes – either through the amine mechanism (normal propagation) or through the carbamate mechanism (Fig. 2). Protonation of carbamate ions and decarboxylation of carbamic acids are both reversible reactions and is dependant on reaction conditions like temperature, solvent and CO_2 pressure and proton concentration, this will determine to which extent the reaction follows the amine or carbamate mechanism. The high reactivity of aliphatic primary amines to NCAs results in a too low concentration of free base to stabilize the carbamate ion and because of this indications are that the carbamate route only plays a minor role and has no significance in the preparative application of primary amine initiated polymerization.

Factors that cause the molecular weight distribution (MWD) to deviate from the theoretical is the termination reactions that can take place. The following is possible:

- Intramolecular chain termination through the nucleophilic attack of the amino chain end on a side group carbonyl.
- β-Sheet forming polypeptides might be responsible for termination in their chain growth.
- The formation of terminal hydantoic acid units through the reaction of the chain-ends with α-isocyanatocarboxylate ions which leads to inactive carboxylic acid terminated polymers (Fig. 4).

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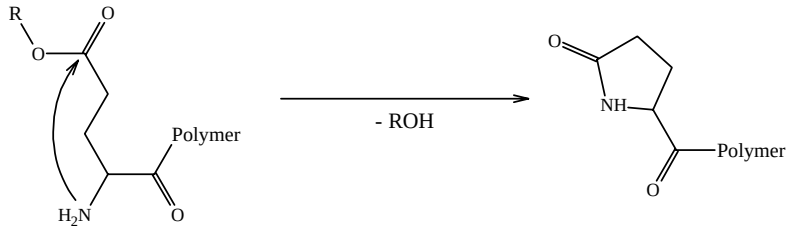
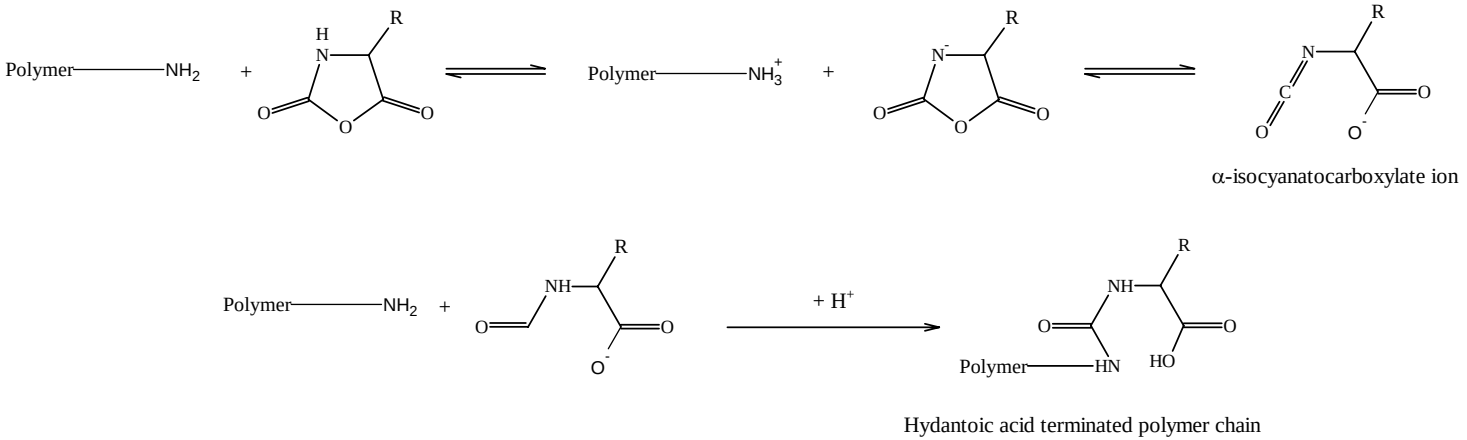


(A) Chain termination through terminal monomer cyclization



(B) Chain termination through reaction with α-isocyanatocarboxylate ion to form inactive hydantoic acid terminated polymer ends

Figure 4: Chain growth termination through (A) terminal monomer cyclization and (B) the formation of hydantoic acid terminated polymer ends[1, 2].

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3.2.2 Secondary amines

Secondary amines can also react with NCAs either as nucleophiles through the amine mechanism or as bases following the active monomer mechanism (AMM) route.

The polymerization route followed will depend on the nucleophilic *vs.* the basic nature of the secondary amine used. For the purpose of predicting the possible route followed by the initiation reaction, amines can be loosely classified into two classes – those with a *more basic character* and those with a *more nucleophilic character*.

Class 1: *More basic character* - Secondary amines with alkyl substituents bulkier than ethyl groups like: di-*n*-propylamine, dicyclohexylamine, *N*-methyl-α-methylbenzylamine, *N*-methylalanine amides.

Class 2: *More nucleophilic character* - Dimethylamine, diethylamine, morpholine, and pipiridine, *N*-methylaniline, *N*-methyl-ω-amino acid amides and aromatic secondary amines also belong here because of their low basicity.

3.2.3 Tertiary amines

Two groups of compounds fall into this category – namely the trialkylamines and the pyridines. When comparing their nucleophilic and basic characters the trialkylamines have higher basicities (pKa's ~ 11) than the pyridines (pKa's ~ 4 – 7) while pyridines have a higher nucleophilic character than the trialkylamines. The trialkylamines are sterically hindered which makes them weak nucleophiles, while the positive inductive effect of the alkyl groups makes them stronger bases. Pyridines on the other hand are less sterically hindered while the polarizability of the π-electrons favours nucleophilic attack.

Trialkylamines thus initiate polymerization through deprotonation of the NCA N-H, following the AMM.

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Due to the nucleophilic nature of pyridines it can be suggested that polymerization should be through nucleophilic attack on the -C_5(O)- carbonyl, analogous to the pyridine catalyzed hydrolysis of anhydrides through the formation of the intermediate acylpyridinium ion. However, it was shown that the rate of initiation depended on the basicity of the pyridine, as determined by the substituents and their bulkiness on the 2 and 6 positions, and that it can indeed also follow the AMM route.

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3.3 Zero valent metal catalysts for NCA polymerization

This section describes the use of zero-valent nickel(0) and cobalt(0) catalysts, for the controlled living polymerization of NCAs. This method was introduced in 1997, where Deming described the polymerization of γ-benzyl-L-glutamate *N*-carboxyanhydride [Glu(Bzl)-NCA] and ε-benzyloxycarbonyl-L-lysine *N*-carboxyanhydride [Lys(Z)-NCA] through the use of a zero valent nickel catalyst. The activation and polymerization through oxidative ring opening is very similar to the oxidative addition of nickel(0) to cyclic anhydrides to yield divalent nickel metallacycles. This oxidative metallic insertion takes place exclusively across the -$C(O)_5$-O- bond in the NCA heterocycle[3, 4].

Other transition metal complexes were also investigated for this catalytic activity. Palladium(0) and platinum(0) complexes, the same group as nickel, oxidatively added across the NCA N-H bond and not through the –C_5(O)-O- bond. Polymerization was initiated, albeit very poorly, through a mechanism similar to that of the active monomer mechanism (AMM) as seen with amine initiated polymerization[5].

Requirements for an effective metal catalyst were determined to be (i) a low valent metal capable of undergoing a 2-electron oxidative addition, (ii) the metal should be coordinated to strong electron donating ligands to promote the oxidative addition and (iii) the metal should be stable towards other functional groups in the monomer, like esters, amides and thioethers. Suitable candidates were found in cobalt(0)- and iron(0) tetrakis(trimethylphosphine) complexes. The iron reacted very fast when mixed with the NCA, but proved to be ineffective yielding only low molecular weight products. The tetrakis(trimethylphophine)cobalt(0) on the other hand reacted rapidly and effectively polymerized the NCAs[6].

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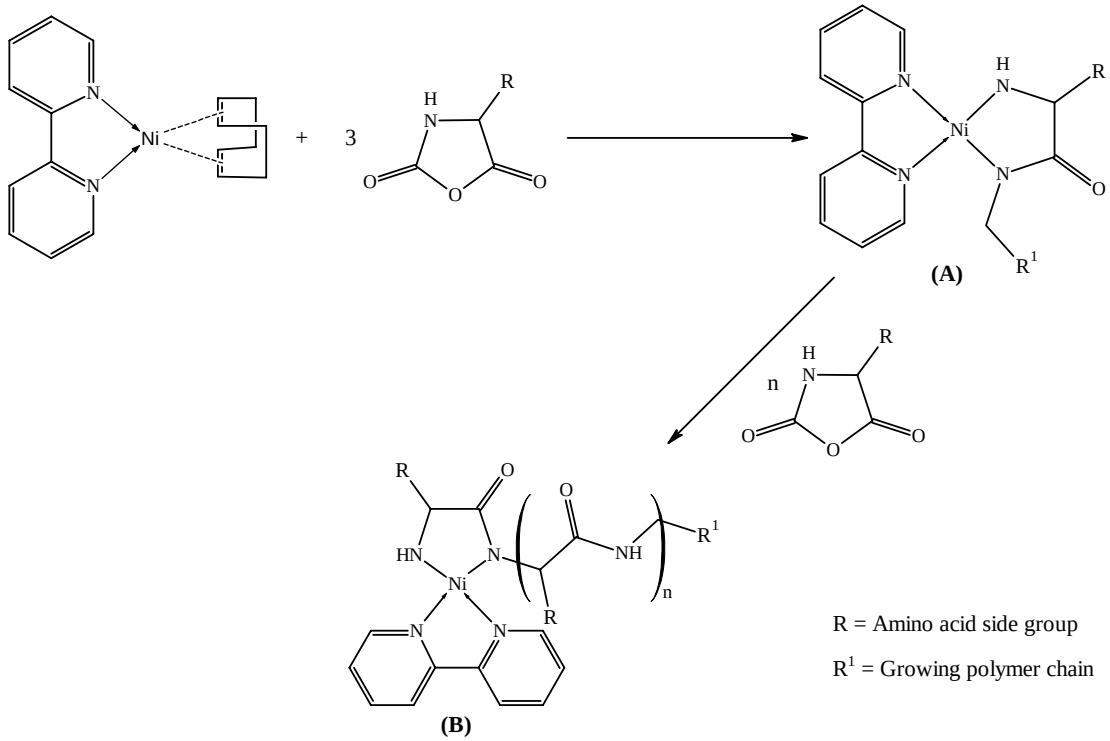

Figure 5: BipyNiCOD catalysed NCA polymerization. This would also apply to $Co(PMe_3)_4$ catalyzed polymerizations. Three NCA monomers are consumed to yield the active amido-amidate propagating intermediate (A) after which further NCA additions will contribute directly to the growing polymer with the catalyst coordinated at the living end (B) of the polymer.

Cobalt reacted faster in the oxidative addition step than the nickel catalyst, especially in less polar solvents such as THF, making it an effective catalyst for the synthesis of short polymers or block-copolymers[7], since all the chain ends are activated well before the monomers are consumed. Higher molecular weights were observed when the nickel catalyst was used in less polar solvents like THF, dioxane, ethyl acetate and toluene. This is because of the loss of active catalyst through CO trapping by the metal, ineffective ring contraction of the amido-alkyl intermediates (see discussion on the mechanism to follow) and the aggregation/dimerization of the propagating species. In a polar solvent like DMF the BipyNiCOD catalyst was very effective in the controlled polymerization of the NCAs yielding narrow molecular weight distributions and molecular weights almost equal to that predicted by the initiator-to-monomer ratios[8].

The following reaction mechanism was proposed for the nickel(0) and cobalt(0) catalysed NCA polymerization (Figs. 6 and 7)[8].

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In the first step regioselective oxidative-addition of the metal takes places across the -C_5(O)-O- bond in the NCA heterocycle. This is followed by a decarbonylation to form a *five membered carbamate metallacycle*. (Some of the released CO may be trapped by the metal complexes quenching a small fraction of the catalyst through the formation of inert carbonyl complexes.) This five membered carbamate metallacycle reacts with a second NCA monomer and through the release of CO_2 the ring contracts to a *six-membered amido-alkyl metallacycle*. The addition of another NCA monomer to the amido-alkyl metallacycle, the liberation of another CO_2 molecule and the subsequent proton migration from the tethered amide to the metal-carbon bond yields the active *amido-amidate propagating intermediate* (Fig. 6).

Chain growth takes place through the nucleophilic attack by the ammido group, in the ammido-amidate propagating intermediate, on the $C(O)_5$ of the next NCA monomer. Proton migration from the new amidate to the tethered amidate releases the end of the growing polymer chain. Elimination of CO_2 contracts the ring back to the propagating intermediate (Fig. 7).

3.3.1 Synthesis of the zero valent metal catalysts

3.3.1.1 Synthesis of BipyNiCOD

BipyNiCOD is synthesized in situ by dissolving equimolar amounts of $Ni(COD)_2$ and 2,2'-bipyridyl in DMF. $Ni(COD)_2$ was not commercially available at the time and it was necessary to synthesize $Ni(COD)_2$ in-house. Two fundamental procedures were found in the literature. The first uses triethylaluminium ($AlEt_3$) as reducing agent[9] and the second makes use of diisobutylaluminium hydride (DIBAH) to reduce the Ni^{2+} to Ni(0)[10].

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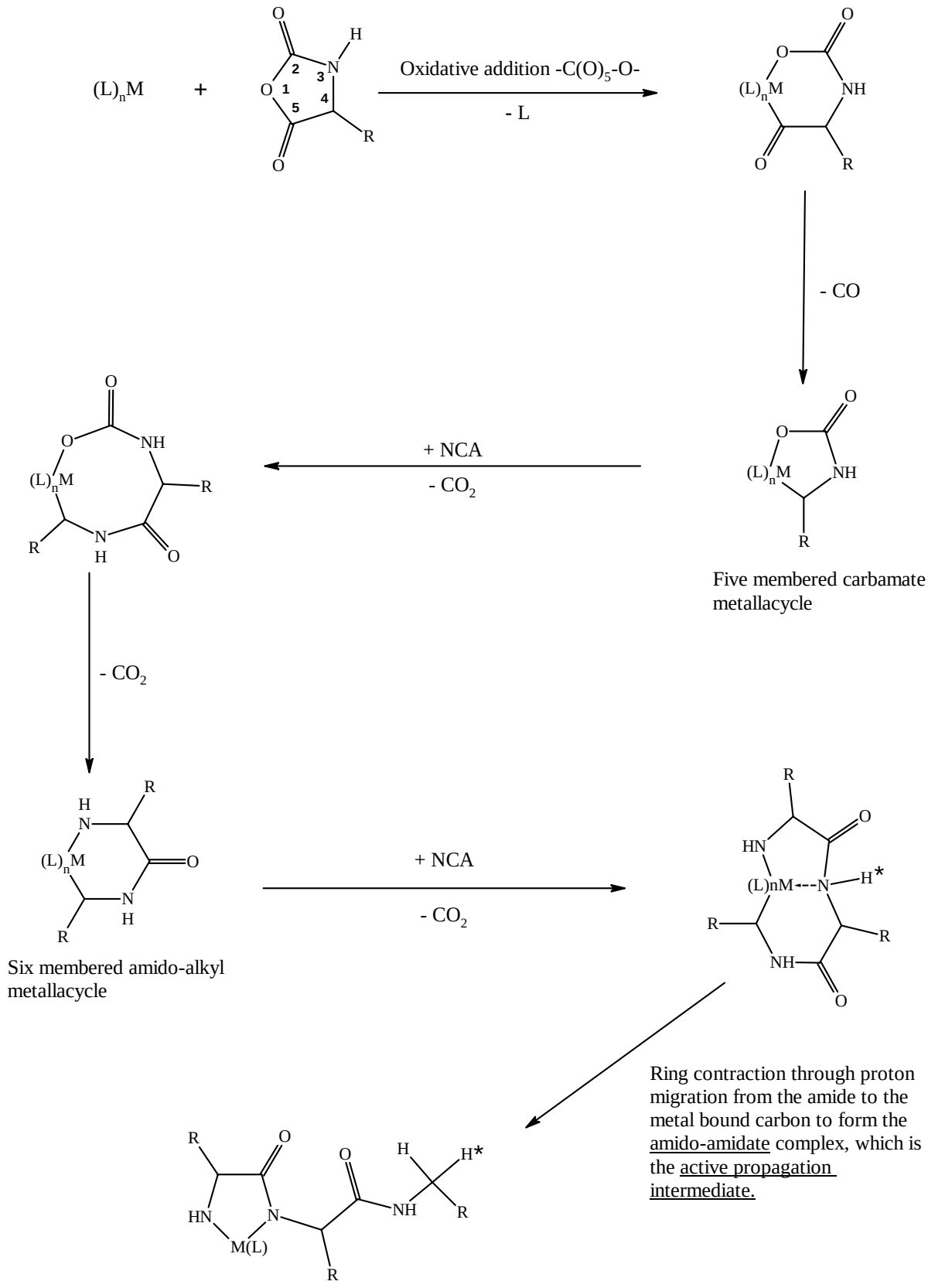

Figure 6: Formation of the active polymerization intermediate in NCA polymerization by Ni(0) and Co(0) catalysts[8].

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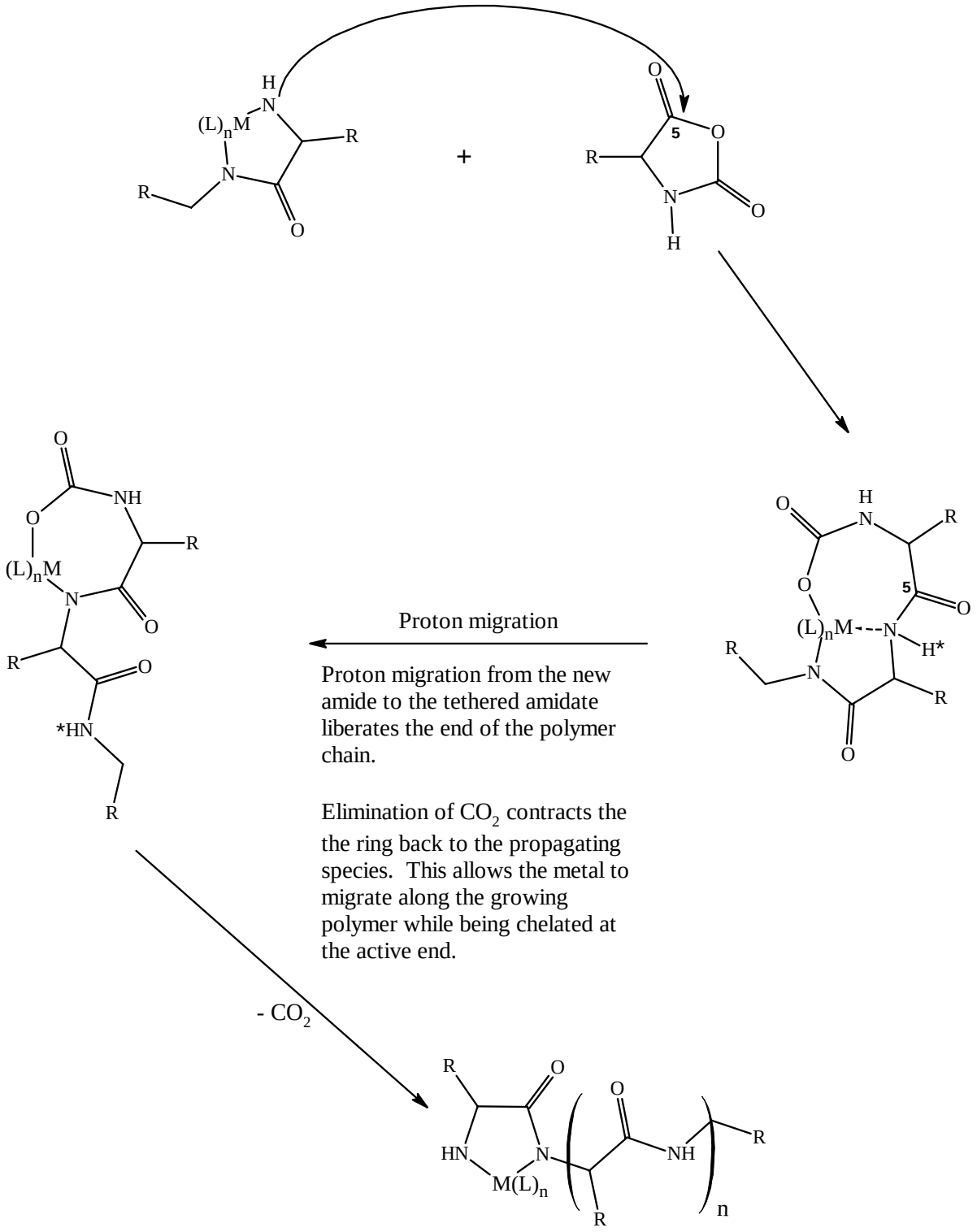

Figure 7: Living chain growth once the active intermediate has been formed[8].

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After various attempts using both methods, the first approach proved to be more effective. This was only achieved after considerable experimentation with the reaction conditions. The reaction conditions in the literature were for relatively large scale synthesis (> 100g NiAcAc as precursor)[9]. It was necessary to scale the reaction down to around 1g – this was because of the amount of final product required, cost and availability of some of the reagents (especially the 1,3-butadiene gas). This much smaller reaction scale and the resulting unavoidable higher dilution of the final product, amounted to toluene being unsuitable as solvent since the final product was unable to crystallize from the reaction mixture. Under these conditions THF proved to be more appropriate as solvent than toluene, resulting in a higher yield than the established literature procedure.

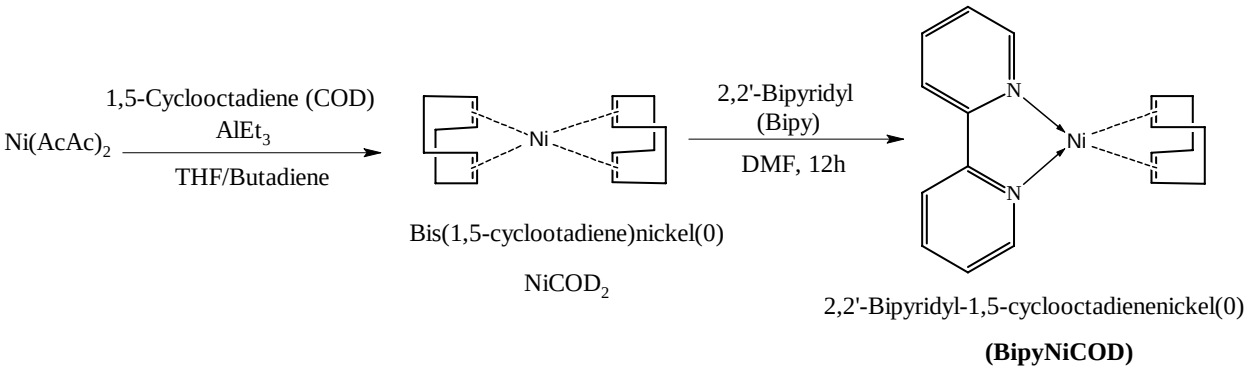

Figure 8: Synthesis of 2,2'-bipyridyl-1,5-cyclooctadiene nickel(0).

$Ni(COD)_2$ synthesis and reactions with 2,2'-bipyridyl had to be repeated very often because of the decomposition of the $Ni(COD)_2$. When even the smallest particle of $Ni(COD)_2$ decomposes the process of decomposition spreads very quickly to the rest of the crystals - this happens in solutions as well. Great care must be taken in keeping the solvents and glassware completely dry and deoxygenated. The solvents used have to contain copious amounts of 1,3-butadiene to prevent colloidal Ni(0) from forming. Use of diethyl ether and butadiene mixtures at 0^oC is better for washing the final product crystals than toluene. All contact of the $Ni(COD)_2$, in solution or crystalline, with glass joints and vacuum grease has to be avoided at all times. Once the $Ni(COD)_2$ is successfully synthesized it can be stored for extended times under Ar in the absence of light[11].

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Preparation of the Bis(acetylacetonate)nickel(II) [Ni(AcAc)$_2$]

Bis(acetylacetonate)nickel(II) Ni(AcAc)$_2$ (2g) in a 50ml round bottom flask was refluxed in dry toluene (30ml) for 2h. The toluene was then distilled off to near dryness. The residue was re-dissolved in dry toluene and filtered through celite. The toluene was removed under vacuum and the residue dried for 16h at 80^{o}C under vacuum.

Synthesis of bis(1,5-cyclooctadiene)nickel(0) [Ni(COD)2]

A schlenk tube containing *ca* 100ml of dry THF was cooled in an acetone/dry ice bath and saturated with 1,3-butadiene (the 1,3-butadiene supplied in a lecture bottle was bubbled into the THF using a Teflon cannula fitted with a 12 inch needle). In a separate schlenk tube dried NiAcAc (1.375g; 5.0mmol) was weighed and cooled in ice/salt bath to -15^{o}C, 30ml of the THF/butadiene was then added to create a suspension. Cyclooctadiene (3.1ml; 25mmol) was added to the suspension through a syringe. The NiAcAc suspension was allowed to warm up to 0^{o}C. A dropping funnel was fitted to the schlenk tube and loaded with THF (5ml) and triethyl aluminium (2.1ml; 15mmol). The triethyl aluminium solution was added drop-wise to the NiAcAc suspension, while stirring at 0^{o}C. The reaction mixture turned from green to a very dark brown with the evolution of ethane gas. Once all the triethyl aluminium was added the reaction mixture was allowed to warm to room temperature. The reaction was stirred for 30 min at this temperature before being cooled down to -78^{o}C in a dry ice/acetone bath. Crystallization rapidly occurred and was allowed to reach completion during 16h at -78^{o}C. The reaction was then once again warmed to room temperature and all the crystals dissolved. This mixture was filtered though a filter-column packed with 5cm Celite (washed with dry THF and then dry diethyl ether and dried under vacuum). Black colloidal nickel stayed behind on the filter while the filtrate was yellow-brown in colour. The Celite was washed with a cold THF/butadiene mixture (3x10ml). The filtrate was again cooled down to -78^{o}C and left to crystallize for 16h. The resulting bright yellow crystals were isolated and washed with cold (0^{o}C) diethyl ether (3x30ml) and dried under high vacuum. Yield bis(cyclooctadiene)nickel(0) (1.088g; 79%).

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3.3.1.2 Synthesis of tetrakis(trimethylphosphine)cobalt(0) [$Co(PMe_3)_4$]

The literature offers two methods for reducing $CoCl_2$ to Co(0) and the synthesis of tetrakis(trimethylphosphine)cobalt(0) [$Co(PMe_3)_4$] – the first is by means of sodium amalgam in diethyl ether[12, 13] and the second with the use of magnesium metal turnings in THF[13]. Because of toxicity considerations, the second method, using Mg, was preferred above the use of mercury, although it is not as reactive as the Na amalgam. The Mg sometimes needed activation by treating the turnings with a small amount of 1,2-dibromoethylene in diethyl ether for a few minutes, before washing the turnings with diethyl ether (3 times) and drying them under vacuum. This treatment was sometimes the difference between a speedy reduction and no reaction at all.

The author of the referenced publications stated that the $Co(PMe_3)_4$ can be sublimated at 80°C/1 torr. Three different sublimation apparatus were tested for sublimating the final product with little success. After prolonged periods of time (1-2 days) very little sublimated product could be recovered. The recovery of the amorphous/micro-crystalline product was also very difficult because it had to be done under 100% oxygen free conditions. It was later found that a pentane extraction of filtered residue is sufficient and that sublimation or crystallisation is not necessary to yield the pure product[14].

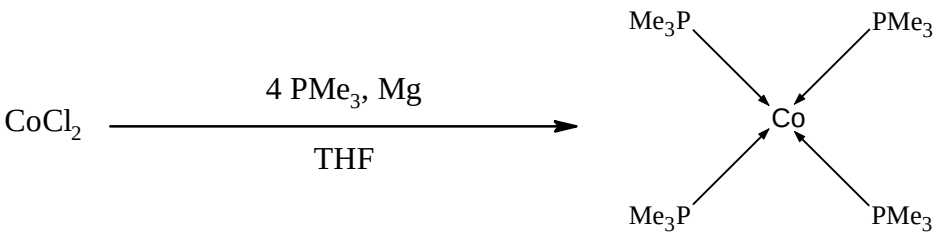

Figure 9: Synthesis of tetrakis(trimethylphosphine)cobalt(0).

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Synthesis of tetrakis(trimethylphosphine)cobalt(0): 0,7% Na/Hg reduction.

Sodium (0,143g; 6.2mmol) was added to mercury (20,357g) in a schlenk tube under argon to form 0.7% sodium amalgam. Cobalt chloride (0.375g; 3.0mmol) was dissolved in 1M/THF trimethylphosphine solution (15.0ml; 15.0mmol). This solution was then added to the amalgam under argon. The solution above the amalgam turned rapidly from a very dark blue to dark yellow-brown as the reduction of the cobalt commenced. The reaction was stirred for 4h at room temperature, and then filtered though Celite. The diethyl ether and excess trimethylphosphine was removed under vacuum. The crude residue was extracted with dry pentane and filtered through Celite (whenever Celite was used in any of the synthetic steps, it was washed three times with THF and then once with diethyl ether before being dried under vacuum). The pentane was evaporated under vacuum to yield tetrakis(trimethylphosphine)cobalt(0) (0.512g; 47%).

Synthesis of tetrakis(trimethylphosphine)cobalt(0): Mg reduction.

Magnesium turnings (81.0mg; 3.3mmol) was activated with 1,2-dibromoethane (30µl; 0.3mmol) in THF (15ml). The THF was decanted once gas evolution stopped. The magnesium was washed with diethyl ether (3x10ml) and dried under vacuum. Cobalt chloride (0.375g; 3.0mmol) was dissolved in 1M/THF trimethylphosphine solution (15.0ml; 15.0mmol). This solution was added to the activated magnesium under argon. The solution turned from a very dark blue to dark yellow-brown as the reduction of the cobalt commenced. The reaction was stirred for 16h at room temperature, and then filtered though Celite. The THF and excess trimethylphosphine was removed under vacuum. The crude residue was extracted with dry pentane and filtered through Celite. The pentane was evaporated under vacuum to yield tetrakis(trimethylphosphine)cobalt(0) (0.664g; 61%). (Yields usually vary from reaction to reaction depending on the amount of decomposition.)

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3.4 NCA polymerization via aluminium initiators

Recently Jhurry et al. introduced a new approach to initiate polymerization of NCAs through the use of aluminium-Schiff's base complexes[15]. This method was extrapolated from the use of aluminium complexes for the controlled polymerization of lactides[16, 17]. The complexes used were derived from aluminium methoxides or isopropyloxides complexed to bis(*o*-hydroxyacetophenone) ethylenediimine (HAPENAlOMe or HAPENAlOiPr). This technology was also successfully applied to synthesize random and block copolypeptides from γ-methylglutamate- and leucine-NCAs[18].

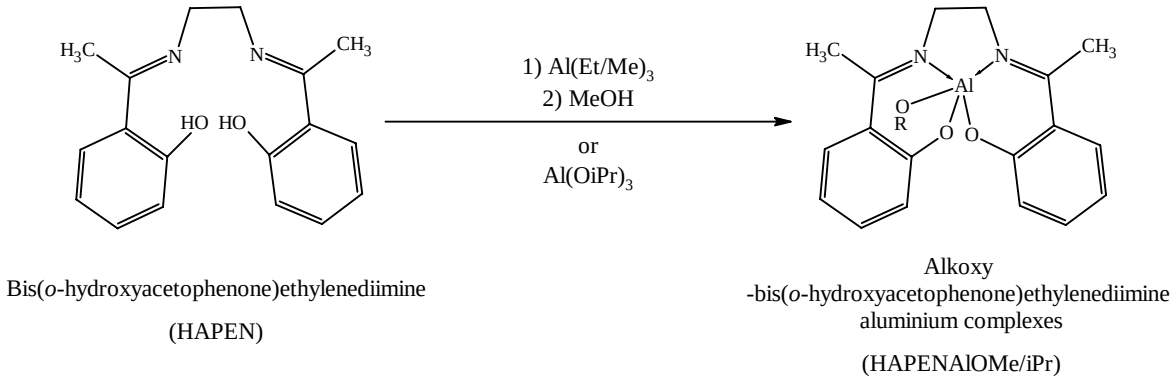

Figure 10: The alkoxy-bis(*o*-hydroxyactophenone)ethylenediimine aluminium(III) complexes used for the initiation of controlled NCA polymerization.

A two-step mechanism for the formation of a polymer initiation intermediate was proposed, which can be generated in one of two ways. The first is the coordination of the NCA N-H to the aluminium through insertion between the Al and the alkoxide. This is followed by the nucleophilic attack of the alkoxides on the $–C(O)_5$-O- NCA bond with the subsequent elimination of CO_2 to form an Al-*N*-coordinated amino acid alkyl ester as the initiating species for further polymerization through the primary amine mechanism (compare Fig. 2 with Fig. 11). The second is the nucleophilic attack of the alkoxide on the NCA $–C_5(O)$-O- in the first step followed by amine coordination to the Al. It is still not clear which of the two routes are followed[15].

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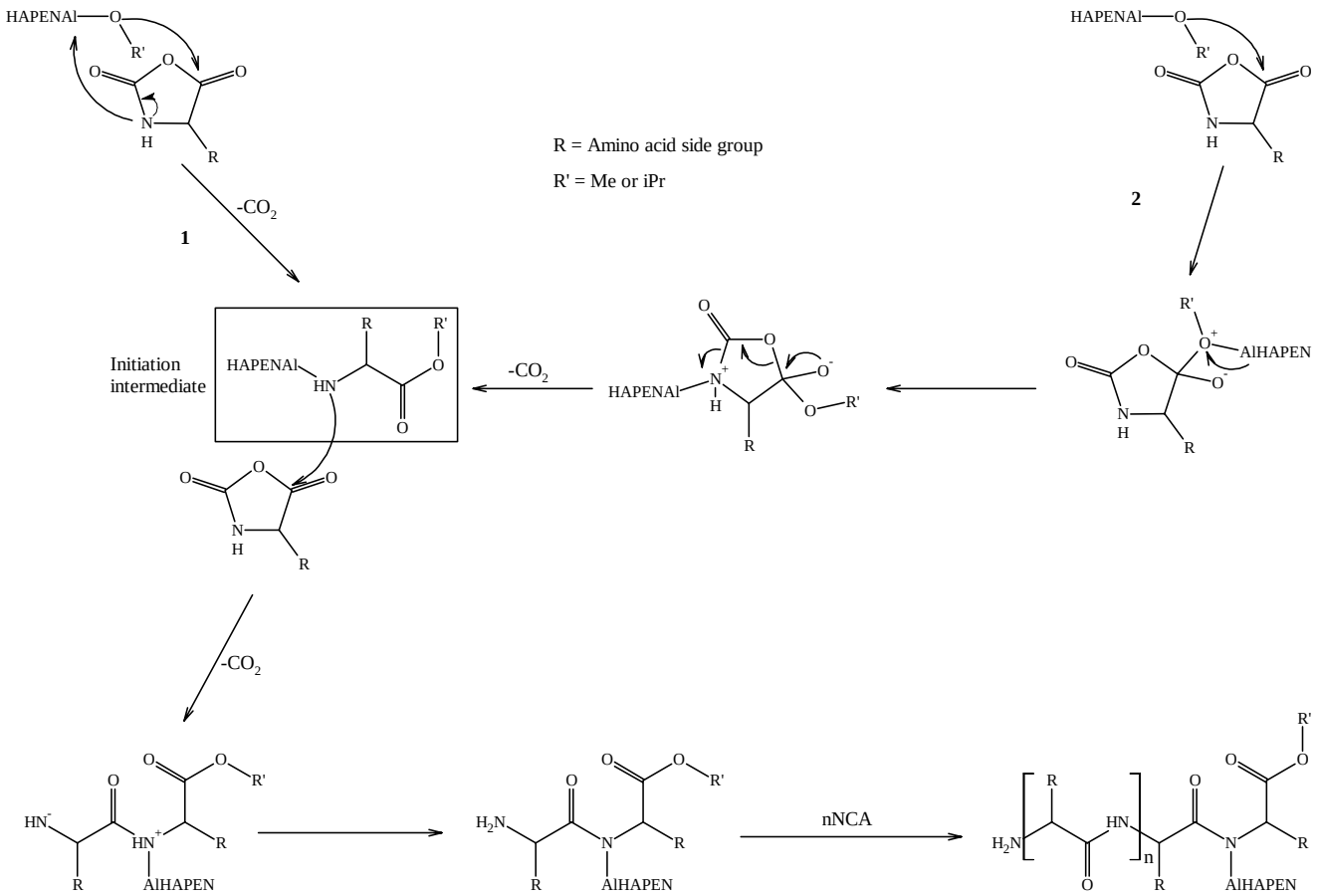

Figure 11: The possible routes to the initiation intermediate: (1) Al insertion across the –C_5(O)-O- bond followed by ester formation, or (2) nucleophilic attack by the alkoxide on the C_5(O) and the subsequent N-H coordination to the Al.

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3.4.1 Synthesis of aluminium catalysts

3.4.1.1 Synthesis of the ligand bis(*o*-hydroxyacetophenone) ethylenediimine (HAPEN)[16, 19, 20]

2-Hydroxy-acetophenone (6.02ml; 50.0mmol) was dissolved in dry ethanol under argon. 1,2-ethylenediamine (1.67ml; 25.0mmol) was added to the 2-hydroxy-acetophenone solution. The solution took on an intense yellow colour and after a few minutes of stirring at room temperature a yellow precipitate started forming. After 3h of stirring the precipitate was filtered. The mother-liquor was concentrated under vacuum and the resulting precipitate filtered. This process was repeated once more. The combined precipitates were dissolved in refluxing ethanol (75ml) with the addition of chloroform until a clear solution was obtained. Upon cooling the product crystallised. The crystals were isolated by filtration and the mother-liquor concentrated to afford more crystals. This process was repeated two more times and the crystals combined to yield HAPEN (7.168g; 40%). **EI-MS (m/z):** 296 (M^+, 40%); 161 (100%). **^{1}H NMR (400 MHz) in $CDCl_3$**: δ 7.53 (m, 2H, Arm); 7.29 (m, 2H, Arm); 6.92 (m, 2H, Arm); 6.80 (m, 2H, Arm); 3.98 (s, 4H, CH_2-CH_2); 2.39 (s, 6H, C-CH_3).

3.4.1.2 Synthesis of [bis(*o*-hydroxyacetophenone)ethylenediimine] methoxy aluminium(III) (HAPENAlOMe) [16, 19-23]

HAPEN (0.593g; 2.0mmol) was suspended in toluene (20ml), in a schlenk tube under argon. Trimethylaluminium or triethylaluminium (2M in toluene; 1.0ml; 2.0mmol) was added to the suspension with immediate gas evolution. The reaction was stirred at room temperature for 4h after which the toluene was removed under vacuum. Dry methanol (3.0ml) was added to the residue in a sealed reaction flask which was evacuated before the reaction mixture was stirred at 60^{o}C for 24h. The methanol was removed under vacuum and the residue dissolved in dichloromethane (100ml). This solution was concentrated to 20ml. The product was precipitated with the addition of pentane. The precipitate was allowed to settle and the solvent removed by a filter-tipped cannula and the product washed with pentane (2x5ml). The product was dried

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under vacuum. Yield HAPENAlOMe (0.588g; 83%). **EI-MS (m/z):** 353 (M^+, 25%); 321 (100%). **^{1}H NMR (400MHz) in $CDCl_3$:** δ 7.45 (m, 2H, Arm); 7.23 (m, 2H, Arm); 6.87 (m, 2H, Arm); 6.57 (m, 2H, Arm) 3.90 – 3.15 (m, 4H, CH_2-CH_2); 2.78 (s, 3H, OCH_3); 2.37 (s, 3H, C-CH_3); 2.31 (s, 3H, C-CH_3).

3.4.1.3 Synthesis of [bis(*o*-hydroxyacetophenone)ethylenediimine] isopropyloxy aluminium(III) (HAPENAlOiPr)[24]

HAPEN (0.883g; 3.0mmol) was added to a suspension of aluminium tri-isopropyloxide (0.611g; 3.0mmol) in toluene (15ml) in a sealed reaction flask which was evacuated before the reaction was heated to 70°C and stirred at this temperature for three days. The product was isolated by filtration and washed with toluene (2x5ml) and dried under vacuum. Yield HAPENAlOiPr (1.147g; 100%). **EI-MS (m/z):** 380 (M^+, 30%); 321 (100%). **^{1}H NMR (400MHz) in CD_2Cl_2:** δ 7.60 – 7.45 (m, 1H, Arm); 7.40 – 7.10 (m, 3H, Arm); 6.80 – 6.45 (m, 4H, Arm); 4.05 – 3.30 (m, 5H, O<u>C</u>H$(CH_3)_2$, CH_2-CH_2); 2.45 – 2.20 (m, 6H, C-CH_3); 1.34 – 1.10 (m, 6H, -CH(C<u>H</u>$_3)_2$).

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3.5 Conclusion

Various NCA initiators and catalysts were discussed in this chapter. The best known and most applied initiators – the organic bases, especially the protic and non-protic amine bases, were focused on. Highlighted was that the nature of the organic base determined the polymerization mechanism. Primary amine following predominantly the *amine* or *normal mechanism* by nucleophilically attacking the NCA at the -C_5(O)- position. Secondary amines, depending on the steric nature of their alkyl groups can either have more base-like properties or otherwise be a stronger nucleophile. Depending on the balance between the basic and nucleophilic character, the amine can either follow an amine- or *active monomer mechanism* (AMM) route for initiating NCA polymerization. Tertiary amines follow almost exclusively the AMM route.

Zero valent Ni and Co catalysts were introduced next as catalysts for living NCA polymerization. There is a distinctly different solvent preference between the $Co(PMe_3)_4$ and the BipyNiCOD – the Co catalyst working very effectively in THF while DMF is the preferred solvent for the Ni catalysts. Both the Ni and Co catalysts follows the same polymerization mechanism, through the consumption of three NCA monomers to form the *active amido-amidate propagating intermediate*. Addition of more NCAs contributed directly to the chain growth with the metal catalyst coordinated at the living end.

The last initiator complex series investigated and synthesized was that of aluminium methyl- and isopropyl alkoxides with bis(*o*-hydroxyacetophenone)ethylenediimine (HAPEN) ligands – HAPENAlOMe and HAPENAlOiPr. Two possible routes to the initiation intermediate are proposed. Firstly Al insertion across the –C_5(O)-O- bond followed by ester formation and secondly nucleophilic attack by the alkoxide on the -C_5(O)- and the subsequent N-H coordination to the Al. At this stage it is still unclear which of the two routes is the correct one.

All three classes of NCA initiators or catalysts, mentioned above, have been applied during this study where their application will be described in detail.

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References

1. Kricheldorf, H. R., *α-Amino acids-N-carboxy-anhydrides and related heterocycles: Syntheses, properties, peptide synthesis, polymerization*. Springer-Verlag, Berlin, Heidelberg, **1987**.

2. Penczek, S.; Kricheldorf, H. R.; Le Borgne, A.; Spassky, N.; Uryu, T.; Klosinki, P., *Models of biopolymers by ring-opening polymerization*. CRC Press, Inc.: Boca Ranton, **1990**.

3. Deming, T. J., Facile synthesis of block copolypeptides of defined architecture. *Nature* **1997,** 390, 386-389.

4. Deming, T. J., Amino acid derived nickelacycles: Intermediates in nickel-mediated polypeptide synthesis. *J. Am. Chem. Soc.* **1998,** 120, 4240-4241.

5. Goodwin, A. A.; Bu, X.; Deming, T. J., Reactions of α-amino acid-*N*-carboxyanhydrides (NCAs) with organometallic palladium(0) and platinum(0) compounds: structure of a metallated NCA product and its role in polypeptide synthesis. *J. Organomet. Chem.* **1999,** 589, 111-114.

6. Deming, T. J., Cobalt and iron initiators for the controlled polymerization of α-amino acid-*N*-carboxyanhydrides. *Macromolecules* **1999,** 32, 4500-4502.

7. Nowak, A. P.; Breedveld, V.; Pakstis, L.; Ozbas, B.; Pine, D. J.; Pochan, D. J.; Deming, T. J., Rapidly recovering hydrogel scaffolds from self-assembling diblock copolypeptide amphiphiles. *Nature* **2002,** 417, 424-428.

8. Deming, T. J.; Cutin, S. A., Chain initiation efficiency in cobalt- and nickel-mediated polypeptide synthesis. *J. Am. Chem. Soc.* **2000,** 122, 5710-5717.

9. Schunn, R. A., Bis(1,5-cyclooctadiene)nickel(0). *Inorg. Synth.* **1974,** 15, 5-9.

10. Damian, J. K.; Mackenzie, P. B., A new convenient preparation of bis(1,5-cyclooctadiene)nickel(0). *J. Org. Chem.* **1990,** 55, 4229-4230.

11. Eish, J. J.; Hallenbeck, L. E.; Han, K. I., Desulfurization of dibenzothiophene by nickel(0) complexes: Evidence for electron transfer in oxidative additions. *J. Org. Chem.* **1983,** 48, 2963-2968.

12. Klein, H.-F., Tretrakis(trimethylphosphine)cobalt(0): Preparation and reactions. *Angew. Chem. Int. Ed.* **1971,** 10, 343.

Stellenbosch University http://scholar.sun.ac.za

13. Klein, H.-F.; Karsch, H. H., Methyltetrakis(trimethylphosphin)kobalt und seine derivate. *Chem. Ber.* **1975,** 108, 944-955.

14. E-mail communication with the referenced author, Hans-Friedrich Klein.

15. Bhaw-Luximon, A.; Jhurry, D.; Belleney, J.; Goury, V., Polymerization of γ-methylglutamate *N*-carboxyanhydride using Al-Schiff's base complexes as initiators. *Macromolecules* **2003,** 36, 977-982.

16. Bhaw-Luximon, A.; Jhurry, D.; Spassky, N., Controlled polymerization of DL-lactide using a Schiff's base Al-alkoxide initiator derived from 2-hydroxyacetophenone. *Polym. Bull.* **2000,** 44, 31-38.

17. Jhurry, D.; Bhaw-Luximon, A.; Spassky, N., Synthesis of polylactides by new aluminium Shiff's base complexes. *Macromol. Symp.* **2001,** 175, 67-79.

18. Goury, V.; Jhurry, D.; Bhaw-Luximon, A.; Novak, B. M.; Belleney, J., Synthesis and characterization of random and block copolypeptides derived from γ-methylglutamate and leucine *N*-carboxyanhydrides. *Biomacromolecules* **2005,** 6, 1987-1991.

19. Prasad, R. N.; Tandon, J. P., Schiff base complexes of aluminium(III). *J. Inorg. Nucl. Chem.* **1974,** 36, 1473-1477.

20. Yang, J.; Yu, Y.; Li, Q.; Cao, A., Chemical synthesis of biodegradable aliphatic polyesters and polycarbonates catalyzed by novel versatile aluminium metal complexes bearing salen ligands. *J. Polym. Sci., Polym. Chem.* **2005,** 43, 373-384.

21. Dzugan, S. J.; Goedken, V. L., Factors affecting Al-C bond reactivity of tetradentate Schiff-base organoaluminium complexes. *Inorg. Chem.* **1986,** 25, 2858-2864.

22. Gurian, P. L.; Cheatham, L. K.; Ziller, J. W.; Barron, A. R., Aluminium complexes of *N*,*N'*-ethylenebis(salicylideneimine) - (H_2salen). X-ray crystal structures of [{Al(salen)}$_2$(μ-O)].MeCN and [Al($OC_6H_2Me_3$-2,4,6)(salen)]. *J. Chem. Soc. Dalton Trans.* **1991**, 1449-1456.

23. Atwood, D. A.; Jegier, J. A.; Rutherford, D., A new class of aluminium cations based upon tetradentate (N_2O_2) chelating ligands. *Inorg. Chem.* **1996,** 35, 63-70.

24. Ovitt, T. M.; Coates, G. W., Stereoselective ring-opening polymerization of *meso*-lactide: synthesis of syndiotactic poly(lactic acid). *J. Am. Chem. Soc.* **1999,** 121, 4072-4073.

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Chapter 4

Random Copolymers of Arg, Gly and Asp, as well as Glu, Cys and Asp

4.1 Introduction

Intrigued by the method, developed by Deming et al.[1, 2] and discussed in chapter 3, for the controlled living polymerization of NCAs led to the establishment this method in our research labs and to investigate other structural alternatives and applications for these zero valent metal catalyzed NCA polymerizations.

Using these metal catalysts, Deming and co-workers synthesized various poly(amino acid) structures. These were mainly diblock polymers consisting out of a charged, hydrophilic block and a neutral, hydrophobic block. The hydrophilic polyelectrolyte blocks were made up from either the hydrogen bromide salts of polylysine or the sodium salts of polyglutamate which constituted either a poly-cation or a poly-anion respectively[2]. Polyleucine was exploited to make up the hydrophobic blocks but other neutral amino acids were also investigated, these include alanine, phenylalanine, isoleucine and valine[3]. These amphiphilic polymers would self-assemble to form stable hydrogels at low concentrations (0.25 – 2.0%)[1, 2, 4, 5].

Polymerization is usually started with the hydrophilic block to improve the solubility of the growing polymer followed by the hydrophobic block. This is because of the solubility contribution of the benzyl and benzyl derived protecting groups used for the lysine and glutamic acid monomers that make up these blocks[1, 6, 7].

The charged hydrophilic blocks must be of a critical minimal length to provide sufficient interstrand charge repulsion to help force the hydrophobic blocks onto each

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other which in turn aid in stable gel formation. Hydrophilic block length variances above this critical length was shown to play a lesser role in the self assembly of these polymers while the length and narrow block dispersities of the hydrophobic blocks proved to be a prominent driving force in the self assembly[2, 5].

Monomers that make up the hydrophobic blocks can be divided into two groups – those that would form α-helixes and those that form β-sheets. α-Helix forming amino acids monomers investigated were leucine, phenylalanine and alanine while the β-sheet forming monomers include those of isoleucine and valine. The hydrophobe content must make up at least 20%, of the overall polymer, to ensure gelation at low concentrations. A minimum of 20 hydrophobic units is necessary to ensure a stable secondary structure of the second block which contributes to the self assembly[2, 3, 5].

The following conclusions were made from the study of polylysine.HBr–*block*–polyleucine copolymers with varying block lengths in aqueous solutions[5].

- These materials form hydrogel materials at low concentrations (0.25 – 2.0%)
- The formation of the hydrogels relies mostly on the interstrand hydrophobic interaction of the polyleucine block. This interaction is very similar to the 'leucine zipper' mechanism found in natural proteins whereby hydrophobic blocks interact through heptad sequences. These are seven amino acid residue repeats containing at least one leucine residue which is spaced along an α-helix such that the leucine residues are aligned and that apposing leucine residues are able to interact through hydrophobic interaction and thus effectively shielding the hydrophobic protein sections from contact with polar solvents, like water. This allows the helices to coil around each other to form protein dimers through the formation of coiled coils[8, 9].

- The way in which these amphiphilic polymers pack is of interest. They form structures similar to β-sheet fibrils. Fibrils are usually formed through the H-bonding between β-sheet folded protein sections, assembling diagonally to the length of the fibril. In the case of these amphiphilic

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polypeptides fibril-like assembly takes place between the hydrophobic polyleucine blocks, through hydrophobic interaction ('leucine zipper'-like association) and not through H-bonding.

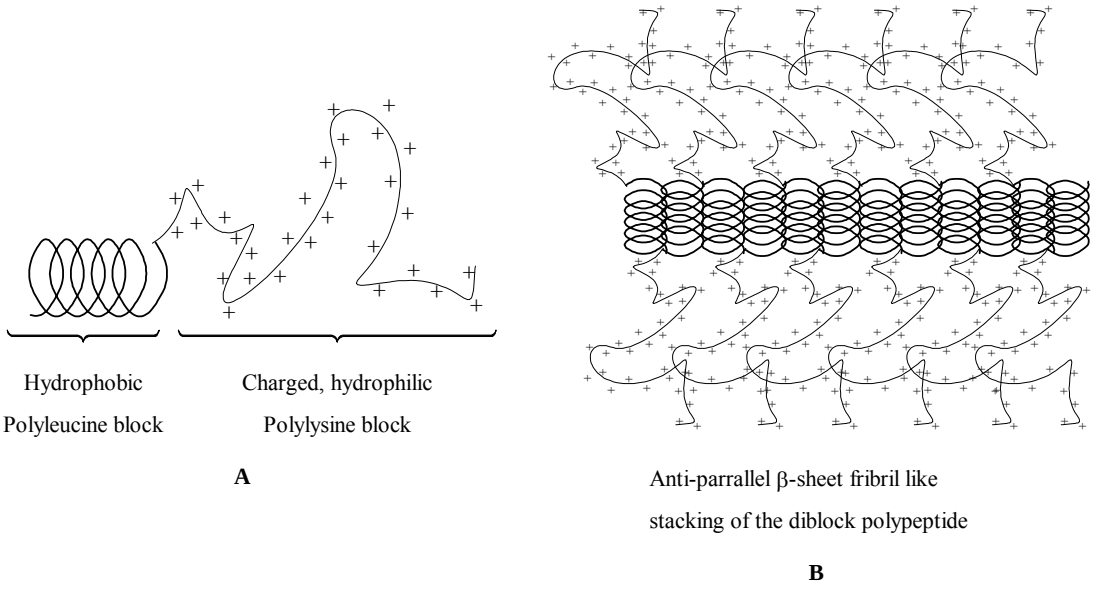

Figure 1: Diblock copolymers of leucine and lysine. (A) Indicates the α-helix structure of the polyleucine block and the random coil structure of the charged lysine block. (B) Indicates the antiparallel packing of the hydrophobic leucine blocks to form β-sheet fibril-like structures.

- The polyleucine block must be at least 20 units long to afford stable hydrogel formation. These blocks must also be of very narrow polydispersity – unequal block lengths will lead to a disruption in the fibril assembly and ineffective gel formation. Because these blocks are made up of leucine only, the hydrophobic interaction is much stronger than that found in natural proteins, the assembled structures of which may be denatured at relatively mild temperature increases, whereas it was found that some of these synthetic polymer hydrogels were stable under autoclave conditions. These hydrogels also recover quickly from shear forces due to the large area of hydrophobic interaction.
- Longer polyelectrolyte sections of the polymers lead to stronger gel formation due to an increased interstrand charge repulsion.

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- The properties of the hydrogels can be tailored by varying the different block lengths, the architectures (diblock *vs*. triblock) or by mixing polymers made up from varying block lengths.

Deming and his research group have covered most of the foundational copolymer structures i.e. diblock and triblock structures. The key to hydrogel formation is the presence of a charged, hydrophilic block and a neutral, hydrophobic block. When considering natural L-amino acids, there are only four charged amino acids available for use viz. lysine or arginine, to provide a positive charge and the negative charge can be provided by either aspartic or glutamic acid. Alanine, valine, leucine, isoleucine and phenylalanine are the available natural hydrophobic amino acids.

The major advantage of this technology is the ability to synthesise poly(amino acid) block copolymers with controlled block lengths. To form hydrogels, through self assembly, the hydrophobic blocks are essential. The properties of these hydrophobic blocks have been investigated in some detail by Deming and co-workers. The hydrophilic blocks on the other hand are still open to further investigation. If the polymer should perform any function – that ability would be determined by the nature of the hydrophilic block. This block can be further investigated, by considering different amino acid combinations.

The objective of the work, to follow in the next sections, was to take this technology as a starting point for the application driven investigations into similar structures by varying the amino acid composition.

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4.2 Random copolymers of arginine, glycine, aspartic acid and leucine

4.2.1 Why arginine, glycine and aspartic acid?

Synthetic polymers are very often used as scaffolds for tissue engineering or modification of surfaces to make them biologically more compatible through better interaction at the surface and tissue interfaces. An effective means to facilitate this interaction is to have the synthetic polymer mimic the extra cellular matrix (ECM). This is usually achieved through the covalent attachment of biological molecules that have specific interaction with the growing tissue. One of the most well known examples of these molecules is the cell-ligand tripeptide sequence Arg-Gly-Asp (RGD) either on its own or as part of a longer polypeptide[10-19].

A very important constituent of the ECM is the protein fibronectin. Fibronectin joins together and surrounds animal cells and also anchors other proteins and carbohydrates of the ECM to the cells. Each of the fibronectin's peptide subunit can be organized into several domains. Fibrin binds to the *N*-terminus, a second domain binds collagen and heparin binds to a *C*-terminal domain. There are several domains (8^{th}, 9^{th} and 10^{th} type III repeats) to which integrins bind to fibronectin. The interaction between the cells and fibronectin takes place through these integrin proteins found in the cell membrane. These are α,β-heterodimeric transmembrane proteins. There are at least 16 different α and 8 β subunits making up at least 22 distinctly different α,β heterodimers. Not only are these proteins involved in the anchoring of the cells to the extracellular matrix but is also actively involved in communication between the cytoskeleton and external proteins. One of the anchoring mechanisms of the cells, via the integrins to the fibronectin, is through an Arg-Gly-Asp (RGD) tripeptide sequence found in the type III repeats of the fibronectin[8, 20].

As stated earlier in this chapter (4.1) the intention was to apply the metal catalyst systems to synthesize new polypeptide structures. Seeing that the concept of block

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copolymers with blocks consisting of only one type of amino acid per block were already investigated it was necessary to look for other alternatives.

It was decided to test this technology in the synthesis of a three component random copolymer of arginine, glycine and aspartic acid – a block polymer with a random poly-RGD block followed by a polyleucine (L) block. Statistically, distributed along this random copolymer, the correct RGD sequences should occur that could allow for cell binding.

4.2.2 Poly-(Arg-Gly-Asp) (poly-RGDs)

Here are the factors considered, assumptions made, the envisaged structural and physical properties and possible applications for the poly-RGD and block copolymers with polyleucine. The following points were taken into account when this investigation was started.

1. Amino acid and protecting groups
   - NCA synthesis
   - Bulkiness of the protecting groups
   - Availability
   - Quantity
   - Cost
2. Solvent, catalyst and polymerization kinetics
3. Structural and physical characteristics of the final polymer
4. Applications

4.2.2.1 Amino acid protecting groups

Most amino acids are trifunctional molecules and for synthetic purposes at least one of the functional groups should be protected, but in most applications two protecting groups are necessary. When working with more than one protecting group the chemistry involved for each group should be selective to avoid unwanted deprotection

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reactions. NCA synthesis is a very important factor to consider when choosing appropriate protecting groups. As was shown in chapter two the α-amino protecting groups play a dual role in NCA synthesis. The first is to bring insoluble amino acids into solution in less polar solvents and the second involves the mechanism of NCA formation and reaction conditions. Thus either no α-amino protecting group or tBoc- or Z- protecting groups may be used in the synthesis of the NCAs.

Amino acid side chain protecting groups should not be influenced by NCA formation reactions such as side reactions, or being cleaved during the process. These protecting groups should also aid in the dissolution of NCAs and the growing polymer. The protecting group(s) should not interfere with the catalyst or initiator. Although no clear precedent could be found in literature on the influence of the protecting group's bulkiness on the polymerization reactions, the assumption was made that too bulky protecting groups would have a negative influence through steric hindrance. When different protecting groups are used on the different amino acids that make up the polymer, deprotection of these groups should take place under similar conditions to simplify the isolation of the final polymer. Because of the size (0.5 – 1g) of the reactions, careful consideration should be given to the type of protection on the amino acids. Most of the commercial amino acids have specialized protecting groups to optimize solid phase peptide synthesis (SPPS), thus not only making them very expensive, but also wasteful for larger scale work. This is because of the great molecular weight contribution of the protecting groups, and in some cases the counter ion, to the overall molecular weight of the protected amino acid.

Taking into account the considerations above the following amino acid derivatives were chosen:

tBoc-NH-Gly-OH: Glycine is not soluble in THF or ethyl acetate, therefore tBoc was chosen as α-amino protecting group to aid in its dissolution and subsequent NCA formation via the reaction with triphosgene in the presence of TEA in ethyl acetate.

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NH_2-Asp(Bzl)-OH: This benzyl ester derivative of aspartic acid is readily drawn into solution when reacted with triphosgene in THF to generate the Asp(Bzl)-NCA. Also the presence of the benzyl group should contribute in keeping the growing polymer in solution.

tBoc-NH-Arg(NO_2)-OH: The tBoc group is needed for dissolution since NH_2-Arg(NO_2)-OH is not soluble in organic solvents. The nitro group, protecting the guanidyl group of arginine, satisfies most of the criteria outlined above. It is a relatively compact protecting group and is also economical to work with. The benzyl ester of aspartic acid and the nitro group of arginine can be simultaneously cleaved via hydrogenation[21]. The only concern with the nitro protecting group is the potentially limited contribution it makes to the solubility of the growing polymer. The greatest contribution to the solubility is probably made by the benzyl protecting group.

NH_2-Leu-OH: The Leu-NCA is synthesised by the reaction of NH_2-Leu-OH with a third molar equivalent of triphosgene in THF.

4.2.2.2 Solvent, catalyst and polymerization kinetics

The arginine and glycine are both fairly polar molecules, this is why DMF was chosen as solvent for the polymerization reactions. Because DMF was used as solvent the BipyNi(COD) was the catalyst to be used to polymerize these NCA mixtures. A factor that needed to be tested in these polymerization kinetics was whether the same contributing factors that influence polymerization initiated through amines could be extrapolated to metal catalyzed polymerizations.

In all probability the most important factor in the kinetics of polymerization is the electron density in the NCA heterocycle of the different monomers. The inductive effect (I) of the amino acid side chain has great influence on the electron density. Positive (+I) groups increase the electron density in the heterocycle and reduce the electrophilic nature at the -C_5(O)- position where the nucleophilic attack by a primary amine would take place. This makes the heterocycle more stable and less reactive.

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An electron withdrawing (-I) side chain (or no side chain – like glycine) would have an opposite effect making the heterocycle less stable and increasing the reactivity towards primary amines[22].

4.2.2.3 Structural and physical characteristics of the final polymer

The established technology was used as guide to overall polymer length and the individual block length. The overall length of polymers with desirable properties was in general between 160 and 200 amino acids long. In order to achieve gelation at low concentrations the leucine block should make up at least 20% of the polymer length with a minimum of 20 amino acids[2, 5].

With these parameters in mind it was decided, as a starting point, to make the overall length of the polymer 200 amino acids long. The 150 amino acids of the polyRGD section would be made up of 50 amino acids each of the three types of amino acids, respectively. The leucine block, making up the difference, would be 50 amino acids long, making it slightly longer than the 20% (40 amino acids in this case) minimum.

As discussed in 4.1, the length, and thus the amount of charge per polymer block also plays a role in gel formation. The longer this charged hydrophilic block the greater the repulsion between similar charges, thus enhancing the gel forming capabilities. In this case the charge profile is completely different and therefore a longer hydrophobic block might be needed to compensate for the lack of interstrand charge repulsion. In fact the poly-RGD might have a 'poly-zwitter ion' nature with the guanidine acting as a strong organic base (pKa = 12.48 for the free amino acid[23]) and carrying a positive charge by deprotonation of the acid function of aspartic acid (pKa = 3.90 for the free amino acid[23]) resulting in a negative charge carried by the aspartic acid. This might result in a net zero charge with the positive ions cancelling out the negative ions. The result of this could be that no interstrand repulsion between similarly charged polymer strands might take place. On the other hand, with al the functional groups ionised the polymer might remain highly hydrated in an aqueous medium. In an aqueous medium

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the hydrophobic polyleucine blocks will most probably aggregate and because of this gel formation may still be possible.

4.2.2.4 Applications

Other than the possibility of gel formation of the poly-RGD-*block*-poly-L, the potential use for poly-RGD random copolymers, without the leucine blocks, exists by mixing with or covalently coupling them to other synthetic or natural gel forming material. These materials can also be tested for cell binding abilities. Electro-spinning of poly-RGD or poly-RGD mixed with other polymeric material could yield functional materials for tissue scaffolding or wound dressings. Hydrophobic surfaces can be modified though hydrophobic adsorption of the poly-leucine block of poly-RGD-*block*-poly-L.

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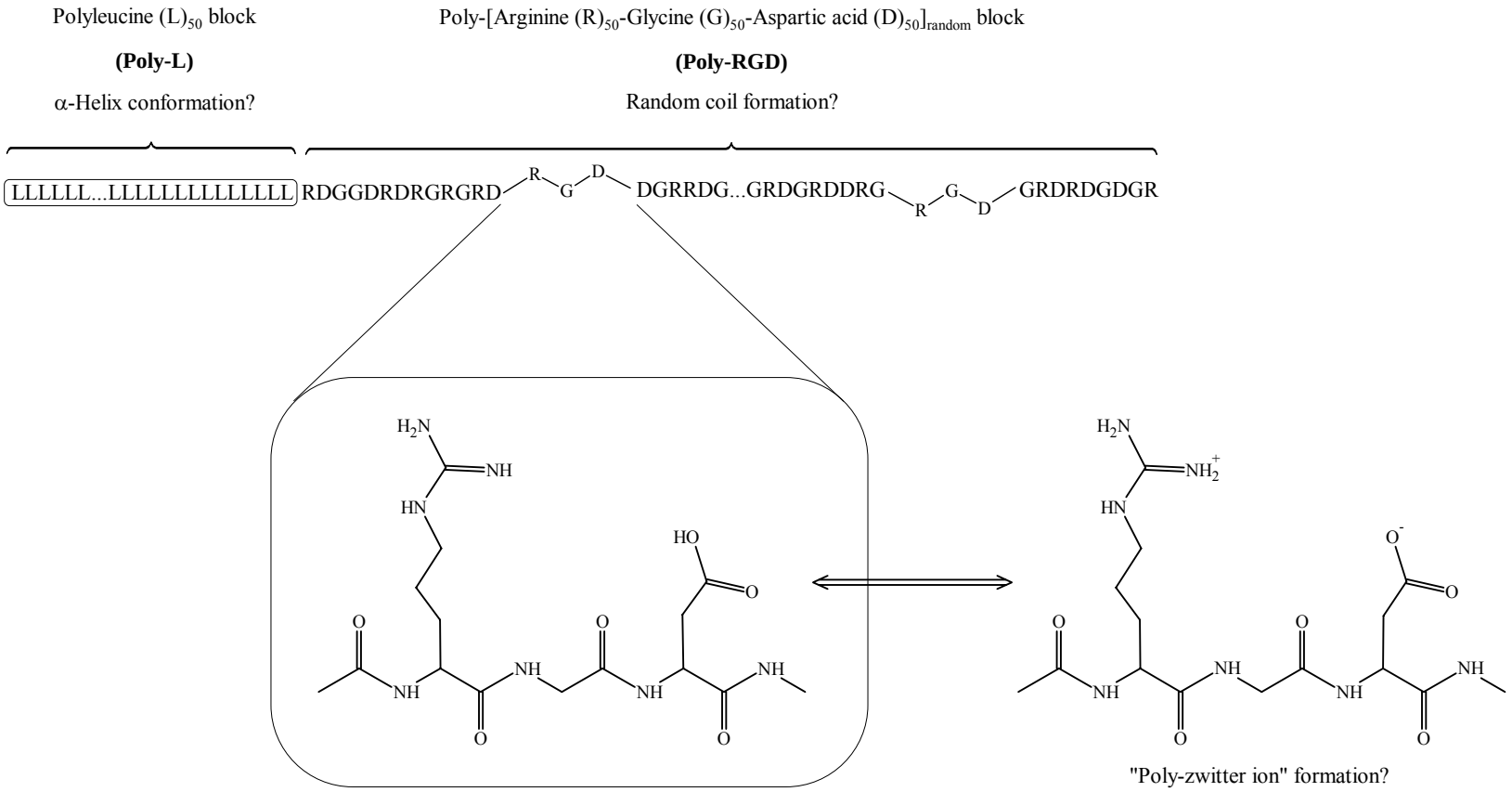

Figure 2: Envisaged Poly-(Arg-Gly-Asp)$_{random}$-*block*-poly(Leu) copoly(amino acid) with the cell binding RGD tripeptide sequence highlighted. Also indicated is the potential 'poly-zwitter ion' formation resulting from the aspartic acid deprotonation by the basic guanidyl functional group of arginine.

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4.2.3 Monomer synthesis

Asp(Bzl)-NCA: The synthesis of the aspartic acid NCA monomer was a straight forward reaction of NH_2-Asp(Bzl)-OH with a third molar equivalent triphosgene followed by repeated recrystallizations. (See chapter 2, section 2.5.1).

Glycine NCA: Glycine NCA was synthesized through the reaction of tBoc-Gly-OH with a third molar equivalent triphosgene and an equimolar amount of TEA. The triethylammonium chloride salts were removed by repeated crystallization and filtration steps. The glycine NCA was then isolated by multiple recrystallizations from THF and hexane. (See chapter 2, section 2.5.2)

Arg(NO_2)-NCA: The synthesis of the arginine NCA monomer proved to be more challenging. As discussed in 4.2.2.1 the tBoc-Arg(NO_2)-OH amino acid derivative was decided upon to synthesize the corresponding NCA monomer. The available technology described the use of triphosgene and TEA for the NCA synthesis. The major complication here was the formation triethylammonium adducts (as seen in NMR spectra) along with the NCA monomer. Various recrystallisation procedures, different solvents, reaction conditions with different bases like di-isopropyl ethylamine (DIPEA) and column chromatography were tried – all proving unsuccessful in removing or preventing these adducts from forming. Each time the procedures resulted in a yellow, highly viscous product.

A final Arg(NO_2)-NCA product free of organic base contamination was achieved by replacing the TEA/DIPEA with a DIPAM-resin (a di-isopropyl amino methyl functionalized styrene resin). This prevented adducts from being isolated because all of the base and its hydrogen chloride salts and any adducts are removed by filtration of the resin during workup. Evaporation of the solvent yielded the clean Arg-NCA as a white crystalline powder (see 4.2.5.1).

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4.2.4 Polymer synthesis

The established technology was used as a guide for initial reaction conditions such as: reaction times and temperatures, concentrations of the reaction mixture and concentrations of the catalyst and monomer stock solutions [1, 2, 6, 7, 24].

The four monomers (Arg-, Gly-, Asp- and Leu-NCA) were weighed out separately and dissolved in DMF to a fixed monomer concentration. An aliquot of the Arg, Gly and Asp monomer solutions, equal to 50 monomers per amino acid, were mixed together.

The BipyNiCOD solution was prepared the day before the execution of the polymerization reactions. The $Ni(COD)_2$ was weighed out under an inert atmosphere. A predetermined Bipy/DMF solution was added to the $Ni(COD)_2$ to make up a deep purple, fixed concentration BipyNiCOD catalyst solution. This mixture was stirred overnight at room temperature to allow for all of the $Ni(COD)_2$ to dissolve. An aliquot of this catalyst solution (a calculated amount for a catalyst to monomer ratio of 1:200) was added to the mixture of the Arg-, Gly- and Asp-NCA monomers.

After stirring for 16h at 25ºC, under an inert atmosphere an aliquot of the Leu-NCA solution (equal to 50 amino acid molecules per polymer) would be added to the polymerization reaction to complete the synthesis.

4.2.5 Experimental procedures

4.2.5.1 Synthesis of Arg(NO_2)-NCA

The general procedures laid out in Chapter 2 (section 2.5) can be applied for the synthesis of Asp-NCA, Gly-NCA and Leu-NCA.

tBoc-Arg(NO_2)-OH (2.088g; 6.54mmol) was dissolved in refluxing acetonitrile (50ml) and then quickly cooled to room temperature. Triphosgene (0.776g; 2.62mmol) dissolved in acetonitrile (3x5ml) was added directly to the solution. Di-

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isopropyl amino methyl (DIPAM) resin (3.27mmol amine/g) (2.00g) was then added to the solution. The reaction was stirred for 18h at room temperature before the resin was filtered off and the acetonitrile concentrated under vacuum. The concentrated solution (5ml) was added slowly to cold diethyl ether (50ml) causing the product to precipitate. The product was filtered off and recrystallised from acetonitrile and diethyl ether. Yield Arg(NO_2)-NCA (1.604g; 100%). **^{1}H NMR (300MHz) in TFA-d_1.** δ 4.57 (m, 1H, NH-C<u>H</u>-CO); 3.54 (m, 2H, CH_2-C<u>H_2</u>-NH-CN); 2.16 – 1.76 (m, 4H, CH-C<u>H_2</u>-C<u>H_2</u>-CH_2). **^{13}C NMR (75MHz):** 173.30 (CH-<u>C</u>O-O); 158.26 (NH-CO-O-); 154.42 (NH-CN-NH); 59.83 (NH-<u>C</u>H-CO); 43.76 (CH_2-<u>C</u>H_2-NH); 29.85 (CH-<u>C</u>H_2-CH_2); 24.77 (CH_2-<u>C</u>H_2-CH_2).

4.2.5.2 <u>Procedure used for the synthesis of poly-RGD-*block*-poly-L</u>

All the work performed in this synthesis was done using schlenk techniques working under an argon atmosphere using glassware that was dried overnight in an oven at 110ºC and cooled under high vacuum. Please refer to chapter 6 for the solvent preparation.

General procedure.

Catalysts solutions: A solution of bipyridyl in DMF was made to a known concentration. Ni$(COD)_2$ was then inertly weight out (usually 30 – 50mg). The calculated volume of the Bipy/DMF solution was added to the Ni$(COD)_2$ in a ratio of 1:1 Bipy:Ni$(COD)_2$ to make up a catalyst solution at a fixed concentration (40.0mM). The colour of the solution changed from clear to deep purple. This solution was stirred overnight to dissolve all the Ni$(COD)_2$.

NCA monomer solutions: All the polymerisation reactions were done in triplicate. NCA monomers were separately weight out, enough for 3.5 reactions. The monomers were subsequently dissolved in DMF to a known concentration (0.20M). An aliquot of each of the NCA solutions were combined in one schlenk tube. The correct amount of the catalyst solution (100µl) was then added to the NCA mixture.

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Attempted synthesis of poly-($R_{50}G_{50}D_{50}$)$_{random}$-block-poly-(L_{50})

The values for the masses, volumes, concentrations and ratios used in the following example of a typical procedure for the polymerization reactions are summarized and calculated with the aid of spreadsheets as shown in Table1 and Table 2.

$Ni(COD)_2$ (25.9mg; 25μmol) was weighed off under an inert atmosphere using a V-shape adapter joining the two schlenk tubes. Bipy (33.9mg; 0.22mmol) was dissolved in DMF (5.43ml) to make up the Bipy/DMF solution (40.0mM; 6.25mg/ml). The $Ni(COD)_2$ was then dissolved in the Bipy/DMF solution (2.35ml). The solution's colour changed from clear, light yellow to a deep purple. The reaction was stirred at room temperature overnight or until all the $Ni(COD)_2$ dissolved to make up the catalyst solution (40mM).

The Arg(NO_2)-NCA (0.2246g; 0.70mmol), Asp(Bzl)-NCA (0.1745g, 0.7mmol), Gly-NCA (0.0708g; 0.70mmol) and Leu-NCA (0.1101g; 0.70mmol) were separately weight off, each in their own Schlenk tube. They were subsequently dissolved in the calculated amount of DMF: Arg(NO_2)-NCA (3.52ml), Asp(Bzl)-NCA (3.50ml), Gly-NCA (3.50ml) and Leu-NCA (3.51ml) to make the monomer solutions (0.20M). An aliquot (1.00ml) of each of the first three monomer solution were combined in three separate Schlenk tubes to perform the reactions in triplicate.

The BipyNi(COD) catalyst solution (100μL) was added to each of three monomer solutions. This was in a molar ratio 1:200, catalyst:total amount of monomer. The reaction flask was placed in a thermostated oil bath at 25^{o}C. Shortly after addition of the catalysts the growing polymer started to precipitate.

(The next step, after 16h stirring at 25^{o}C, in the synthesis would have been the addition of an aliquot (1.00ml) of the Leu-NCA monomer solution and stirring for another 8h followed by the isolation of the product polymer).

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Table 1: Example of the Excel spreadsheets used to calculate and summarize the reaction conditions for the polymerization reactions. A summary of the catalyst solutions.

Date:

Polymer Code: **Template: RGDL001: Cat-Sol = 0.04M (100 µl/Rx); Ratio 1:200; MonomerSol = 3.5 ml @ 0.2M (1ml monomer Sol/Rx); Total Volume = 4ml @ 0.2M**

Catalytic Solution:

Bipy (Mr = 156.19; 0.025g)

Weighed (g)	# moles	Conc. (M)	Vol DMF (ml)	Conc. (mg/ml)
0.0339	2.2E-04	0.040	**5.43**	6.25

Ni(COD)2 (Mr = 275.05; 0.030g)

Mass Before (g)	Mass After (g)	Difference (g)	# moles	Conc. (M)	Vol Bipy/DMF (ml)	# moles Bipy	# mg Bipy	Vol Bipy/DMF (ml)
115.5285	115.5544	0.0259	9.4E-05	0.040	**2.35**	9.4E-05	14.71	2.35

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Table 2: A continuation of Table 1 – a summary of the NCA monomer solutions.

Monomer Solutions
(Rx in Triplicate = 3.5 ml solutions)

Arg(NO2)-NCA (Mr = 319.32; 0.2235g)

Weighed (g)	# moles	Conc. (M)	Vol DMF (ml)	Conc. (mg/ml)
0.2246	7.0E-04	0.20	**3.52**	63.864

Asp(Bzl)-NCA (Mr = 249.20; 0.1745g)

Weighed (g)	# moles	Conc (M)	Vol DMF (ml)	Conc. (mg/ml)
0.1745	7.0E-04	0.20	**3.50**	49.84

Gly-NCA (Mr = 101.07; 0.0708g)

Weighed (g)	# moles	Conc (M)	Vol DMF (ml)	Conc. (mg/ml)
0.0708	7.0E-04	0.20	**3.50**	20.214

Leu-NCA (Mr = 157.20; 0.1101g)

Weighed (g)	# moles	Conc (M)	Vol DMF (ml)	Conc. (mg/ml)
0.1105	7.0E-04	0.20	**3.51**	31.44

4.2.6 Discussion

The synthesis of this poly-RGD or poly-RGD-*block*-poly-L polymers were unsuccessful. This was due to immediate precipitation of the forming polymer as soon as the catalyst was added. The precipitation can be attributed to a couple of likely causes:

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Firstly, incorrect protecting groups, probably on the arginine's guanidyl functional group and the absence of protecting groups on glycine. The nitro protecting group may be too polar to contribute to the solubility of the forming polypeptide and once the Arg-NCA heterocycle is 'opened up' causes the amino acids to precipitate. Glycine has no protecting groups to contribute to the solubility of the growing poly-peptides, also glycine in itself is very insoluble in organic solvents – for this reason poly-glycine is unobtainable, except for very short polypeptides. The solubility contribution of the benzyl protecting group of the aspartic acid is not sufficient to compensate for the lack in solubility contribution from Arg and Gly to keep the growing polymer in solution.

A monomer such as Gly-NCA may react faster or preferentially with the metal catalysts than the other amino acids and this will cause precipitation to occur. There is no information, in literature, on the reactivity of the different NCAs towards the metal catalysts.

Arginine's guanidyl group might not be sufficiently protected to avoid all side reactions. A question could be asked if one of the nitrogens in this protected functional group can still act as a base to react with the highly reactive/unstable Gly-NCA to initiate polymerization but not basic enough to reacted with itself during NCA synthesis and work-up. The inductive effect of the amino acid side group or lack thereof could play a role in the reactivity of the NCA toward bases.

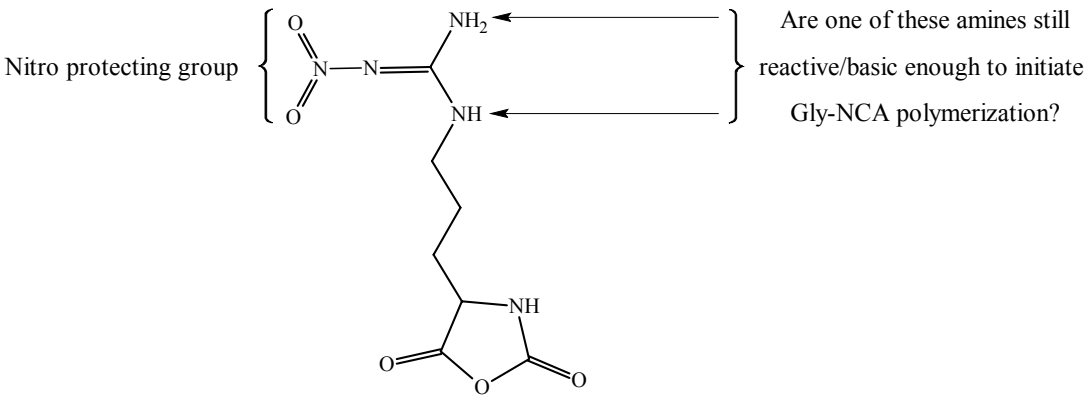

Figure 3: Arg(NO_2)-NCA as a possible initiator for Gly-NCA polymerization.

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These polymerization reactions are also plagued by a host of other practical problems which include:

- Catalytic decomposition of $Ni(COD)_2$ (Chapter 3), while in storage or while being handled (weighing out).
- Commercial unavailability of $Ni(COD)_2$
- Decomposition of the BipyNi(COD) while preparing the catalytic solution. When this happens a day is lost, because the catalytic solution should be stirred for at least 16h for all of the $Ni(COD)_2$ to dissolve.
- To have all the NCA monomers freshly prepared at the same time. The Gly-NCA is especially reactive (even with it self) and unstable and can not be stored for extended periods of time. Sometimes when the Gly-NCA is dissolved an insoluble precipitate forms due to auto-polymerization of the Gly-NCA[22].
- The polymerization reactions had to be repeated numerous times to get to a point where the catalyst could be added to the NCA-monomer mixture without something going wrong beforehand, and then catalyst decomposition can still take place during the reaction, which can be seen by the precipitation of a black residue.

From the outset of this project the aim was to find a working polymer using the technology established by Deming and co-workers. For this reason the work on poly-RGD and poly-RGD-*block*-poly-L was stopped without further investigation or characterization due to too many variables, uncertainties and practical difficulties which did not warrant any more time spent to solve or find explanations for these problems.

Still committed to the concept of a random polymer block of different amino acids followed by a homopoly(amino acid) block, a simpler combination of amino acids was tried next.

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4.3 Random copolymers with glutamic acid, cysteine, aspartic acid and leucine or phenylalanine

Alternative polymeric structures were required to replace the poly-RGD-*block*-poly-L model and find solutions to the main and limiting problem of insolubility of the growing polymer.

4.3.1 Why glutamic acid, cysteine and aspartic acid

Glutamic acid (Glu; E) – cysteine (Cys, C) – aspartic acid (Asp; D) (ECD), is also a naturally occurring cell-ligand, tri-peptide sequence[20]. This sequence presented a viable and simpler alternative and, because of the protecting groups used in the synthesis, should circumvent the solubility problem.

Similar to the RGD tri-peptide sequence, ECD has also been identified as a crucial element in certain disintegrins, which also binds to cell membrane anchored integrins to mediate certain cell to cell interactions and the interaction of certain proteins other than the ECM with the cell surface. One of most studied examples of these cell to cell interactions is that of the interaction between mammalian sperm- and egg cells. This interaction is through a sperm's membrane bound protein called fertilin β. Fertilin β belongs to the ADAM (A Disintegrin And Metalloproteinase domain) group of proteins which are implicated in many cellular functions[20, 25]. It is through an ECD sequence in the disintegrin domain of fertilin β (also know as ADAM2) that interaction between sperm and egg takes place[25-27].

Metalloproteinases and disintegrins are also important components of viperid and crotalid snake venoms like that of *Bothrops jararaca* which is part of the pit viper family[28, 29]. Large metalloproteinases are referred to as MDC enzymes which are composed of an *N*-terminal Metalloproteinase domain, a Disintegrin domain and a Cysteine rich *C*-terminus making them structurally very similar to the cell membrane anchored ADAM proteins[28]. There exists a high degree of structural homology between the MDC's disintegrin domain and that of disintegrins, although it lacks the

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RGD sequence usually found in disintegrins. It still possesses the ability to recognize and bind to integrins. Jararhagin, an MDC protein, in the venom of *Bothrops jararaca* contains an ECD sequence in the disintegrin domain which competitively binds to cell membrane integrins to disrupt platelet-collagen interaction while the metalloproteinase enzymatically damages the endothelium[29]. Alternagin-C (Alt-C), a disintegrin protein which contains an ECD sequence, was isolated from the venom of *Bothrorps alternatus.* Alt-C showed a potent chemotactic effect for human neutrophils[30].

Shown here are several examples where the ECD acts as a cell-ligand tri-peptide sequence mainly as part of a larger disintegrin domain in ADAM and MDC proteins or as part of disintegrin peptides. The interaction with the cell is through integin proteins in the cell membrane. By exploiting these cell binding properties of ECD like that of RGD, the hope is to synthesise polymeric material to act as scaffolds for tissue cells.

4.3.2 Poly-(Glu-Cys-Asp) (poly-ECDs)

The objective here was to use the same architectural approach as with the poly-RGD, to construct poly-ECD polymers with a charged, hydrophilic random poly-ECD peptide block followed by a neutral, hydrophobic homopolypeptide block made up from the α-helix forming leucine or phenylalanine (Phe; F) amino acids.

The same factors were considered for the synthesis of the poly-ECD polymers as with the poly-RGD polymers.

4.3.2.1 Amino acid protecting groups

NH_2-Glu(Bzl)-OH and **NH_2-Asp(Bzl)-OH:** The γ and β benzyl ester derivatives for Glu and Asp, respectively, are cost effective to work with in larger quantities and are readily cleavable under the same conditions – hydrogenation or via acid cleavage using glacial acetic acid saturated with HBr[21].

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tBocNH-Cys(Trt)-OH: The important factor here is the compatibility of the deprotection method for γ– and β–benzyl protecting groups of Glu and Asp and that of the triphenylmethyl- (trityl-) of cysteine's sulphur group. Both protecting groups can be cleaved under similar acidic conditions with the use of HBr/acetic acid[1, 21]. The protecting group and its method of deprotection should also be balanced against cost effectiveness and commercial availability.

4.3.2.2 Solvent and catalyst

Because of the more lipophilic nature of the protecting groups (benzyl- and trityl-) the polar solvent DMF was substituted with THF as solvent for the polymerization reactions. Under these conditions the $Co(PMe_3)_4$ was chosen as an appropriated catalyst for reasons discussed in Chapter 3 (section 3.3).

4.3.2.3 Structural and physical characteristics of the final polymer

From an architectural point-of-view the poly-RGD-*block*-poly-L and poly-ECD-*block*-poly-L/F are very similar – the major difference being that the poly-ECD will be a poly-anion once deprotected because of the presence of the carboxylic acid functional groups on both the glutamic acid and the aspartic acid moieties. This fact would probably contribute to the gel-forming ability of the block polymer because of similar charge repulsion between the polymer strands. This would be comparable to the poly-Glu-*block*-poly-Leu, synthesized by Deming and co-workers, which shows this gel forming behaviour[2].

Not only will the polyleucine block be tested in this synthesis but also a polyphenylalanine block to make up an α-helix forming hydrophobic block as an alternative to the leucine[3]. This would indicate whether there is a similar interlocking ('leucine zipper') behaviour between the phenyl rings of polyphenylalanine as observed with the isopropyl groups of polyleucine[5].

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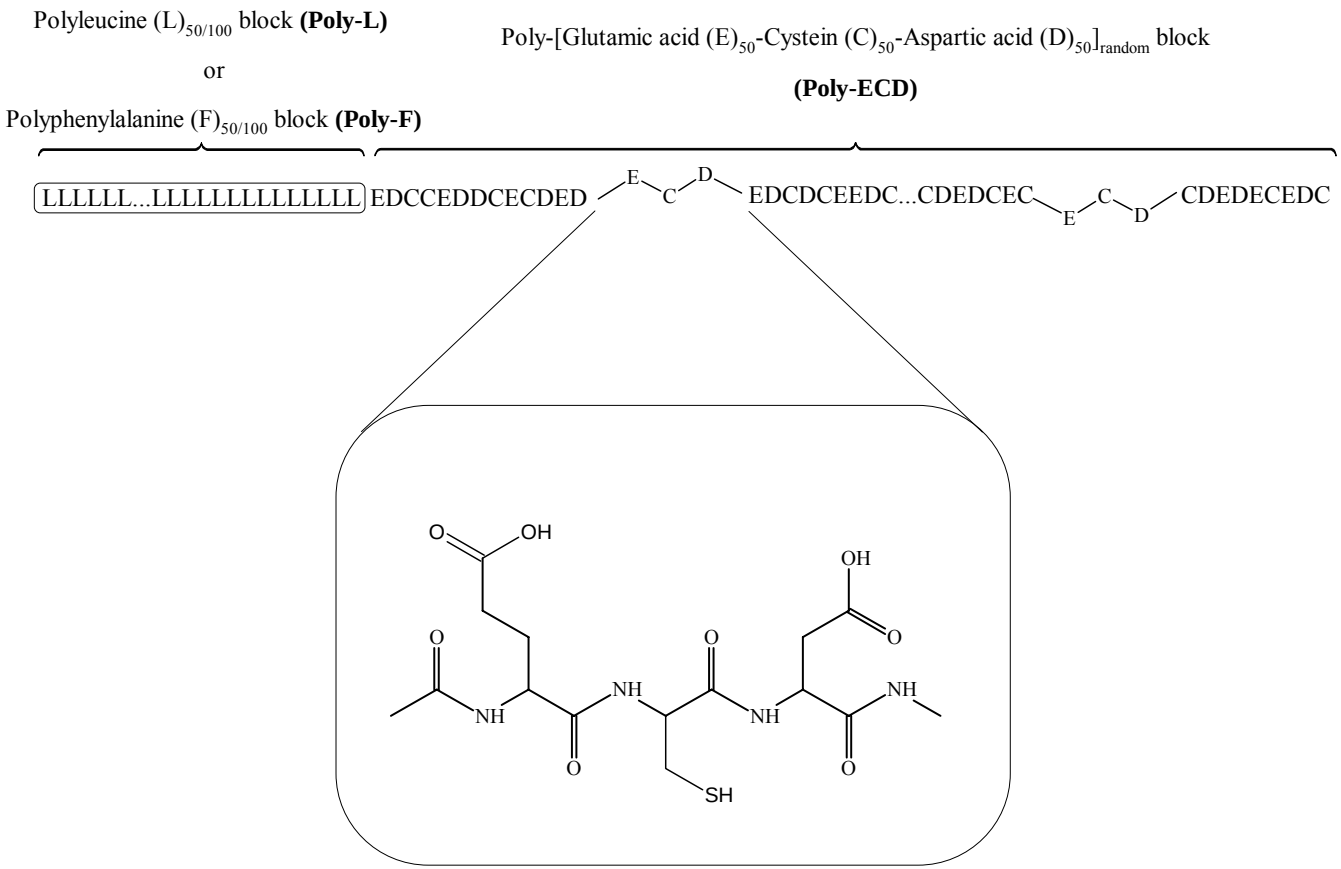

Figure 4: Envisaged Poly-(Glu-Cys-Asp)$_{random}$-*block*-poly-(Leu/Phe) copoly(amino acid) with the cell binding ECD tripeptide sequence highlighted.

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4.3.2.4 Applications

The choice of ECD was made for the possible cell ligating capability of this tri-peptide, thus possible applications would be, like that of the poly-RGD in the field of hydrogels, as tissue scaffolds, surface modifications through adsorption of the hydrophobic polymer block onto hydrophobic surfaces and wound dressing through electro-spinning.

4.3.3 Monomer synthesis

Glu(Bzl)-NCA and **Asp(Bzl)-NCA:** The approach for the NCA synthesis from these two amino acid derivatives would be exactly the same – the reaction of the amino acids with a third equivalent triphosgene in THF at 40 – 50ºC under an inert atmosphere.

Cys(Trt)-NCA: The commercially available tBoc-NH-Cys(Trt)-OH was first deprotected by selective removal of the tBoc protecting group. Because of the high solubility of Cys(Trt)-NCA the reaction conditions as set out in the general synthesis for tBoc protected amino acids, is unfavourable for isolating the pure NCA by crystallisation/precipitation. Trace amounts of $Et_3N.HCl$ was present in the final product. The use of the DIPAM-resin, as used in the synthesis of Arg(NO_2)-NCA, was also unsuccessful because the reactions was incomplete. It was therefore easier to first remove the tBoc protecting group and then follow the procedure used for the α-*N* unprotected amino acids.

Leu-NCA and **Phe-NCA:** The Leu- and Phe-NCA can be synthesised by the reaction of NH_2-Leu-OH and NH_2-Phe-OH respectively with a one-third molar equivalent of triphosgene in THF (Chapter 2, section 2.5.1).

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4.3.4 Polymer synthesis

Essentially the same procedures and similar reaction conditions were used here in the synthesis of poly-ECD-block-poly-L/F as with the poly-RGD polymers. (Section 4.2.4)

The five monomers (Gly(Bzl)-, Cys(Trt)-, Asp(Bzl)-, Leu- and Phe-NCA) were weighed out and dissolved in THF separately to make up solutions with a specific monomer concentration. Aliquots of each of the Gly(Bzl)-, Cys(Trt)-, Asp(Bzl)-NCA monomer solutions, equal to 50 amino acid units, respectively, were mixed together.

The catalyst solution was prepared by dissolving $Co(PMe_3)_4$ in the appropriate volume of THF. An aliquot of this catalyst solution (a calculated amount for a catalyst to monomer ratio of 1:200) was added to the mixture of the Gly(Bzl)-, Cys(Trt)-, Asp(Bzl)-NCA monomers.

After 16h of stirring the polymerization reaction, at 25ºC under an inert atmosphere an aliquot of the Leu-NCA (or Phe-NCA) solution (equal to 50 amino acid units) would be added to complete the synthesis.

4.3.5 Experimental procedures

4.3.5.1 Synthesis of Cys(Trt)-NCA

The tBoc- protecting group of the commercially available tBoc-NH-Cys(Trt)-OH was first removed, using a 2N HCl in ethyl acetate solution, and the deprotected NH_2-Cys(Trt)-OH reacted with triphosgene to yield the Cys(Trt)-NCA.

2N HCl/ethyl acetate solution

In a Schlenk tube, a solution of acetylchloride (14.22ml; 0.2mol) in ethyl acetate (50ml) at 0^oC, was treated by the slow addition of dry ethanol (13.00ml). The

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reaction mixture was stirred at 0°C for 10 min, before the solution was allowed to warm to room temperature. This solution was transferred to a 100ml volumetric flask, where it was made up to 100ml with dry ethyl acetate.

Deprotection of tBoc-NH-Cys(Trt)-OH

tBoc-NH-Cys(Trt)-OH (4.634g; 10.00mmol) was treated with 2N HCl/ethyl acetate solution (30ml) at 0°C for 1h after which the solvent was removed under vacuum. The residue was redissolved in a minimum amount of ethyl acetate and precipitated by its slow addition to cold diethyl ether. The product was filtered off, reprecipitated (by the same technique), isolated and dried under vacuum. Yield NH_2-Cys(Trt)-OH.HCl (3,476g; 87%). **^{1}H NMR (300MHz) in DMSO-d_6:** δ 7.54 – 7.25 (m, 15H, Arm); 3.12 (dd, 1H, J (Hz) 7.6, 5.5, NH_2-C<u>H</u>-CO); 2,85 (m, 2H, CH-C<u>H_2</u>-S). **^{13}C NMR (75MHz):** δ 170.13 (CH-<u>C</u>O-O); 145.30(Arm); 130.73 (Arm); 129.73 (Arm); 128.41(Arm); 68.59 (CH_2-<u>C</u>-Ph_3); 52.85 (NH_2-<u>C</u>H-CO); 32.81 (CH-<u>C</u>H_2-S).

Synthesis of Cys(Trt)-NCA

NH_2-Cys(Trt)-OH.HCl (1.995g; 5.00mmol) was suspended in THF (50ml) and the reaction mixture heated 40°C – 50°C. Triphosgene (0.742g; 2.50mmol), dissolved in THF (15ml), was added drop-wise to the suspension. After 30min of stirring the solution became clear. The reaction was stirred for a total of 3h before the solution was concentrated in vacuo to about 10ml and layered with hexane to precipitate the final product which was filtered off and reprecipitated using the same technique, filtered and dried under vacuum. Yield Cys(Trt)–NCA (1.821g; 94%): **^{1}H NMR (300MHz) in DMSO-*d*6:** δ 7.54 – 7.25 (m, 15H, Arm); 3.53 (dd, 1H, J (Hz) 8.2, 4.2, NH-C<u>H</u>-CO); 2.77 (m, 2H, CH-C<u>H_2</u>-S). **^{13}C NMR (75MHz):** δ 168.33 (CH-<u>C</u>O-O); 151.62 (NH-<u>C</u>O-O); 144.21 (Arm); 129.80 (Arm); 128.75 (Arm); 128.72 (Arm); 68.04 (S-C-Ph_3); 56.70 (NH-<u>C</u>H-CO); 33.38 (CH-<u>C</u>H_2-S).

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4.3.5.2 Synthesis of Poly-$(E_{50}C_{50}D_{50})_{random}$-block- (L_{50})

The $Co(PMe_3)_4$ catalyst solution was prepared by taking an amount slightly more than what would be needed and dissolving it in pentane (5ml). The solution was filtered and the pentane evaporated under vacuum. The residue, pure $Co(PMe_3)_4$ (29.1mg; 80μmol), was dissolved in THF (4.00ml) to make up the catalyst solution (20mM)

Each of the four NCA monomers, Cys(Trt)-NCA (0.6816g; 1.75mmol), Glu(Bzl)-NCA (0.4361g; 1.75mmol), Asp(Bzl)-NCA (0.4116g; 1.75mmol), and Leu-NCA (0.2750g; 1.75mmol), were dissolved in THF (3.50ml). An aliquot (1.00ml) of each of the Cys, Glu and Asp monomers were combined in three different Schlenk tubes to make up the mixed monomer solutions (3.00ml) in triplicate. The $Co(PMe_3)_4$ (500μl) was added to each and the Schlenk tubes sealed under argon. After 16h of stirring the Leu-NCA (1.00ml) would have been added to each and stirred for another 8h.

A second method of polymer synthesis

The following polymerisation reactions were attempted at higher dilution:

A. Poly-$(E_{50}C_{50}D_{50})_{random}$
B. Poly-$(E_{50}C_{50}D_{50})_{random}$-*block*- (L_{50})
C. Poly-$(E_{50}C_{50}D_{50})_{random}$- *block* - (L_{100})
D. Poly-$(E_{50}C_{50}D_{50})_{random}$- *block* - (F_{50})
E. Poly-$(E_{50}C_{50}D_{50})_{random}$- *block* - (F_{100})

Glu(Bzl)-NCA (1.3705g; 5.50mmol), Asp(Bzl)-NCA (1.2935g; 5.50mmol) and Cys(Trt)-NCA (2.1421g; 5.50mmol) were dissolved together in THF (82.50ml). Five aliquots (15.00ml each) of the monomer mixture solution were placed in five separate Schlenk tubes (A, B, C, D, E). The $Co(PMe_3)_4$ catalyst solution (1.00ml; 20mmol) was added to each of the five monomer solutions.

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These solutions would have been stirred for 16h before the second-block monomers would be added.

Leu-NCA (0.5504g; 3.50mmol) and Phe-NCA (0.6690g; 3.5mmol) were dissolved separately in THF (7.00ml). Leu-NCA solution (2.00ml) would have been added to B and (4.00ml) to C. Phe–NCA solution (2.00ml) would have been added to D and (4.00ml) to E.

Solubility testing

Glu(Bzl)-NCA (0.2492g; 1.00mmol), Asp(Bzl)-NCA (0.2352g; 1.00mmol) and Cys(Trt)-NCA (0.3895g; 1.00mmol) were each dissolved in THF (5.00ml). $Co(PMe_3)_4$ catalyst solution (1.00ml; 20mM) was added to each of the monomer solutions. The catalyst to monomer ratio was 1:50.

4.3.6 Discussion

Like the poly-RGD polymerization reactions the syntheses of the poly-ECD polymers were unsuccessful due to precipitation of the forming polymer shortly (within 5 minutes) after addition of the cobalt catalyst. Through separate polymerization reactions of the three monomers it was determined that Cys(Trt) was responsible for the precipitation. The homopolymerization of Glu(Bzl)- and Asp(Bzl)- remained soluble whereas the homopolymerization of Cys(Trt)-NCA precipitated shortly after addition of the catalyst. This problem of precipitation persisted even when the reactions were repeated at much lower concentrations.

Again the incorrect choice of amino acid derivative proved to be the ‘Achilles Heel’ in this type of polymerization reaction. These fundamental aspects of NCA reactivity, influenced by their functional and protecting groups, during polymerization using nickel and cobalt catalysts would be the key to broadening the applications of this technique beyond copolymers of lysine and glutamic acid with hydrophobic

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polyamino acid blocks. After all, the potential application of polyamino acids lies with the nature of the functional moieties of the amino acid side chains. It amounts to a balancing act between solubility of the growing polymer, as determined by the nature of the protecting group and the reactivity of the NCA, most probably determined by the nature of the amino acid side chain.

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4.4 Conclusion

This chapter deals with the implementation of a recently established technology for the living polymerization of NCAs through the use of zero-valent Ni and Co catalysts. This technology finds application in the successful synthesis of narrow polydispersed polyamino acid block copolymers. These polymers consist mainly of charged hydrophilic blocks and neutral hydrophobic blocks. It was shown how the different physical properties such as conformation, block lengths and charge played important roles in the behaviour of these co-polyamino acids. The most important of these was the 'leucine-zipper'-like self-assembly mechanism of the hydrophobic blocks to form hydrogels. The ability to copolymerize exact α-helical hydrophobic blocks onto the hydrophilic block is probably the most important contribution of this technology and this is what sets it apart from conventional but very well established base initiated NCA polymerizations.

In this work an attempt was made to expand this technology, by focussing on the hydrophilic block, using different amino acid combinations and going beyond the established homo poly-electrolyte blocks of lysine and glutamic acid but still incorporating the hydrophobic α-helix block.

The first amino acid combination was that of arginine (R), Glycine (G) and aspartic acid (D) (RGD). RGD is a naturally occurring cell-ligand tri-peptide. The choice to make the hydrophilic block, a random copolymer of RGD, was made to give this block specific function other than being a poly-electrolyte. The point of this technology is to form hydrogels through self assembly of the hydrophobic polyamino acid blocks. By potentially giving cell-binding capabilities to the hydrophilic blocks, the self assembled hydrogels can find medicinal applications in the field of tissue engineering.

Unfortunately the random BipyNi(COD) catalyzed copolymerization of RGD in DMF failed due to polymer precipitation shortly after the addition of the catalyst. This is

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most probably because of an inappropriate choice of protecting group for arginine and the fact that glycine does not have a protecting group to contribute to the solubility of the growing polymer. Also highlighted was a host of practical difficulties involved with this polymerization technique.

Committed to the concept of a functional random copolypeptide hydrophobic block, a simpler alternative to poly-RGD was investigated. It took the form of a random copolymer consisting of glutamic acid (E), cysteine (C) and aspartic acid (D). ECD, like RGD, is a naturally occurring cell-ligand tripeptide. This sequence is simpler in the sense that both Glu and Asp's carboxylic acid side chains are benzyl-protected. Homopolymers of these protected amino acids have been shown to be soluble in THF. Cysteine's sulphur group was protected by triphenylmethyl (Trt) protecting group. Because of the all-round lipophilic nature of the amino acid protecting groups, THF chosen as solvent with $Co(PMe_3)_4$ as catalyst.

Again polymerization failed because of polymer precipitation. Through individual polymerization of the three amino acids it was shown that Cys(Trt) was the precipitating polymer component.

Future work in this field will demand very careful consideration in the choice of amino acids and especially their protecting groups. Finding the correct combination of amino acids for the hydrophilic block might lead to very interesting and useful gel-forming materials.

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References

1. Deming, T. J., Facile synthesis of block copolypeptides of defined architecture. *Nature* **1997,** 390, 386-389.

2. Nowak, A. P.; Breedveld, V.; Pakstis, L.; Ozbas, B.; Pine, D. J.; Pochan, D. J.; Deming, T. J., Rapidly recovering hydrogel scaffolds from self-assembling diblock copolypeptide amphiphiles. *Nature* **2002,** 417, 424-428.

3. Wyrstra, M. D.; Cogen, A. L.; Deming, T. J., A parallel synthetic approach for the analysis of membrane interactive copolypeptides. *J. Am. Chem. Soc.* **2001,** 123, 12919-12920.

4. Kopeček, J., Swell gells. *Nature* **2002,** 417, 388-391.

5. Deming, T. J., Polypeptide hydrogels via a unique assembly mechanism. *Soft Matter* **2005,** 1, 28-35.

6. Deming, T. J., Cobalt and iron initiators for the controlled polymerization of α-amino acid-*N*-carboxyanhydrides. *Macromolecules* **1999,** 32, 4500-4502.

7. Deming, T. J.; Cutin, S. A., Chain initiation efficiency in cobalt- and nickel-mediated polypeptide synthesis. *J. Am. Chem. Soc.* **2000,** 122, 5710-5717.

8. Metzler, D. E.; Metzler, C. M.; Sauke, D. J., *Biochemistry - The chemical reactions of living cells*. Second ed.; Harcourt Academic Press: Boston, New York, **2001**, Vol. 1, P70

9. Nelson, D. L.; Cox, M. M., *Lehninger principels of biochemistry*. Worth Publishers: New York, **2000**, P1084

10. Hersel, U.; Dahmen, C.; Kessler, H., RGD modified polymer: biomaterials for stimulated cell adhesion and beyond. *Biomaterials* **2003,** 24, 4385-4415.

11. Rowley, J. A.; Madlambayan, G.; Mooney, D. J., Alginate hydrogels as synthetic extracellular materials. *Biomaterials* **1999,** 20, 45-53.

12. Quirck, R. A.; Chan, W. C.; Davies, M. C.; Tendler, S. J. B.; Shakesheff, K. M., Poly(L-lysine)-GRDS as a biomimetic surface modifier for poly(lactic acid). *Biomaterials* **2001,** 22, 865-872.

Stellenbosch University http://scholar.sun.ac.za

13. Burdick, J. A.; Anseth, K. S., Photoencapsulation of osteoblasts in injectable RGD-modified PEG hydrogels for bone tissue engineering. *Biomaterials* **2002,** 23, 4315-4323.

14. Woerly, S.; Pinet, E.; De Robertis, L.; Van Diep, D.; Bousmina, M., Spinal cord repair with PHPMA hydrogel containing RGD peptides (NeuroGel™). *Biomaterials* **2001,** 22, 1095-1111.

15. Lieb, E.; Hacker, M.; Tessmar, J.; Kunz-Schughart, L. A.; Fiedler, J.; Dahmen, C.; Hersel, U.; Kessler, H.; Schulz, M. B.; Göpferich, A., Mediating specific cell adhesion to low-adhesive diblock copolymers by instant modification with cyclic RGD peptides. *Biomaterials* **2005,** 26, 2333-2341.

16. Sawyer, A. A.; Weeks, D. M.; Kelpke, S. S.; McCracken, M. S.; Bellis, S. L., The effect of the addition of a polyglutamate motif to RGD on peptide tethering to hydroxyapatite and the promotion of mesenchymal stem cell adhesion. *Biomaterials* **2005,** 26, 7046-7056.

17. De Long, S. A.; Moon, J. J.; West, J. L., Covalently immobilized gradients of bFGF on hydrogel scaffolds for directed cell migration. *Biomaterials* **2005,** 26, 3227-3234.

18. Ho, M.-H.; Wang, D.-A.; Hsieh, H.-J.; Liu, H.-C.; Hsien, T.-Y.; Lai, J.-Y.; Hou, L.-T., Preparation and characterization of RGD-immobilized chitosan scaffolds. *Biomaterials* **2005,** 26, 3197-3206.

19. Park, K.-H.; Na, K.; Jung, S. Y.; Kim, S. W.; Park, K. H.; Cha, K. Y.; Chung, H.-M., Insulinoma cell line (MIN6) adhesion and spreading mediated by Arg-Gly-Asp (RGD) sequence conjugated in thermo-reversible gel. *J. Biosci. Bioeng.* **2005,** 99, 598-602.

20. Evans, J. P., Fertilin β and other ADAMs as integrin ligands: insights into cell adhesion and fertilization. *BioEssays* **2001,** 23, 628-639.

21. Green, T. W.; Wuts, P. G. M., *Protective groups in organic synthesis*. 3rd ed.; John Wiley & Sons. Inc., **1999**, p596

22. Kricheldorf, H. R., *α-Amino acids-N-carboxy-anhydrides and related heterocycles: Syntheses, properties, peptide synthesis, polymerization*. Springer-Verlag, Berlin Heidelberg, **1987**.

23. Zubay, G., *Biochemistry*. Third Ed. ed.; Wm. C. Brown Publishers: Dubuque, Melbourne, Oxford, **1993,** p51.

Stellenbosch University http://scholar.sun.ac.za

24. Deming, T. J., Amino acid derived nickelacycles: Intermediates in nickel-mediated polypeptide synthesis. *J. Am. Chem. Soc.* **1998,** 120, 4240-4241.

25. Gupta, S.; Li, H.; Sampson, N. S., Characterization of Fertilin β-disintegrin binding specificity in sperm-egg adhesion. *Bioorg. Med. Chem.* **2000,** 8, 723-729.

26. Zhu, X.; Evans, J. P., Analysis of the roles of RGD-binding intergrins, α_4/α_9 integrins, α_6 integrins, and CD9 in the interaction of the fertilin β (ADAM2) disintegrin domain with the mouse egg membrane. *Biol. Reprod.* **2002,** 66, 1193-1202.

27. Zhu, X.; Bansal, N. P.; Evans, J. P., Identification of the key functional amino acids of the mouse fertilin β (ADAM2) disintegrin loop for cell-cell adhesion during fertilization. *J. Biol. Chem.* **2000,** 275, 7677-7683.

28. Kamiguti, A. S.; Zuzel, M.; Theakston, R. D. G., Snake venom metalloproteinases and disintegrins: interactions with cells. *Braz. J. Med. Biol. Res.* **1998,** 31, 853-862.

29. Moura-da-Silva, A. M.; Línica, A.; Della-Casa, M.; Kamiguti, A. S.; Ho, P. L.; Crampton, J. M.; Theakston, R. D. G., Jararhagin ECD-containing disintegrindomain: Expression in *Escherichia coli* and inhibition of the platelet-collagen interaction. *Arch. Biochem. Biophys.* **1999,** 369, 295-301.

30. Mariano-Oliveira, A.; Coelho, A. L. J.; Terruggi, C. H. B.; Selistre-de-Araújo, H. S.; Barja-Fidalgo, C.; De Freistas, M. S., Alternagrin-C, a nonRGD-disintegrin, induces neutrophil migration via integrin signaling. *Eur. J. Biochem.* **2003,** 270, 4799-4808.

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Chapter 5

Functionalized PEG-sheathed Polylysine

5.1 Introduction and background to PEG and poly(amino acid) copolymers

Composites of poly(ethylene glycol) (PEG) and poly(amino acid)s are well known in the literature, especially in the field of biomaterials. This first part of the introduction is a summary focusing specifically on this subject of PEG and poly(amino acid) conjugates within the field of biomaterials. The aim is to provide a background to place the research work done here in context, to highlight the differences between and similarities with the already known examples of PEG poly(amino acid) materials.

PEG is used extensively, as a synthetic polymer, in materials for bio-applications. It has a number of favorable characteristics that make it such an ideal candidate – these include: its very low toxicity toward living systems, because of its chemical inertness and controlled degradation. The ability of PEG to trap water makes it a very favourable delivery vehicle, by providing a protective environment, for hydrophilic molecules such as peptides and DNA[1]. PEG's reactive hydroxyl end-group allows for chemical modifications under mild conditions, which give access to block and graft co-polymers[2], various cross-linking options (both chemical and physical)[3, 4] and the attachment of active molecules[5-7]. Through end-group modification PEG has found applications in hydrogels as polymeric networks, micelles, micro- and nano-spheres for use in tissue engineering and localized drug delivery[8-12].

When considering composites that are comprised of PEG and poly(amino acids) or peptides it can be broadly grouped in a couple of basic architectures. To follow are a few examples of how these composites are constructed.

1. Di-block or multi-block AB-type copolymers can be synthesized using terminally functionalized PEGs as a macro-initiator for the ring opening polymerization of α-*N*-amino carboxyanhydrides (NCAs) (Fig. 1)[13-17].

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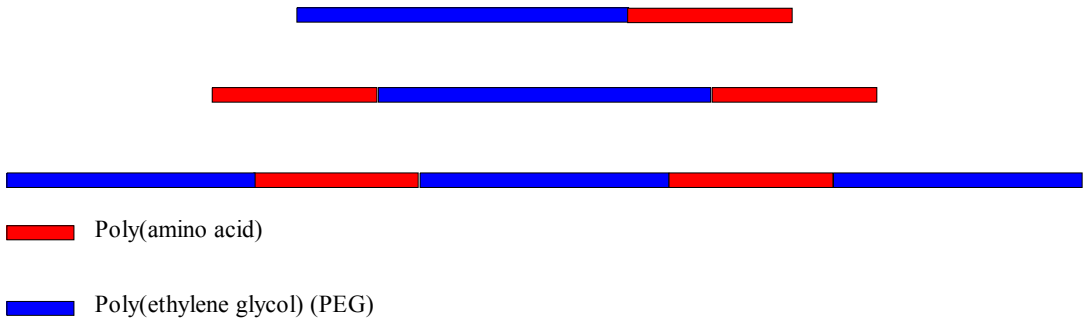

Figure 1: Linear AB-type PEG-*block*-poly(amino acid) constructs.

2. ABC type co-polymers consisting out of PEG-, poly(amino acid)- and another synthetic polymer can be constructed using the PEG-*block*-non-poly(amino acid) polymer as a macro-initiator for NCA polymerization - similar to the first point (Fig. 2)[18-20].

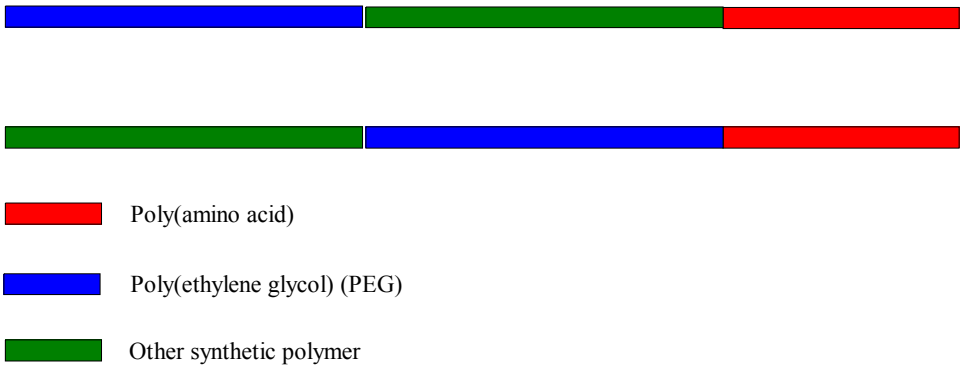

Figure 2: Linear multi-block ABC-type polymers consisting of PEG, poly(amino acids) and other synthetic polymers.

3. ω–Methyl ether terminated PEG (mPEG), ω-functionalized PEG or the normal hydroxyl terminated PEG can be grafted onto an existing poly(amino acid) such as poly-L-lysine by means of the reaction of a calculated percentage of the lysine ε-amino groups with carboxylic acid functionalized PEGs[21, 22]. The remainder of polylysine's ε-amino groups are also available for reaction with other biological active molecules such as phenylboronic acid[23]. In the case of the hydroxyl terminated PEG the hydroxyl group is open to further reactions, such as the coupling of peptides (RGDs) or other molecules such as biotin (Fig. 3)[24-26].

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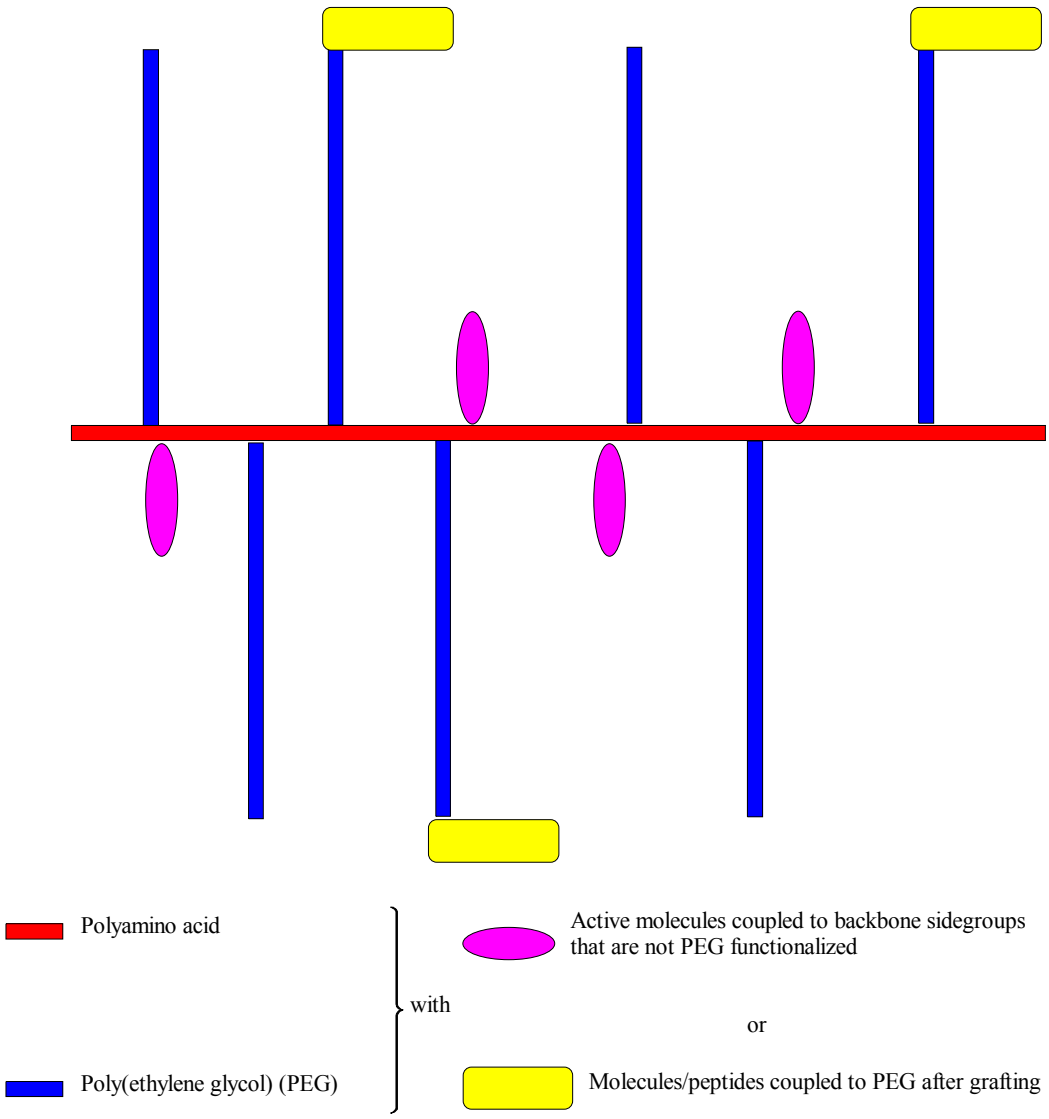

Figure 3: PEGs grafted onto a poly(amino acid) backbone. Also indicated are active molecule/peptides coupled either directly to the backbone or to the PEG end-groups. Note that the PEG grafts are spaced unevenly to indicate partial grafting according to a predetermined ratio of PEGs to poly(amino acid).

4. PEG can be coupled to peptides and drugs to alter their physical and chemical properties such as, solubility, biological absorbencies, and resistance to enzymatic degradation which increases the blood circulation lifetime (Fig. 4)[5]. Multiple PEG attachments onto protein subunits such as that of fibrinogen may lead to stimuli-induced hydrogel formation (Fig. 5)[27].

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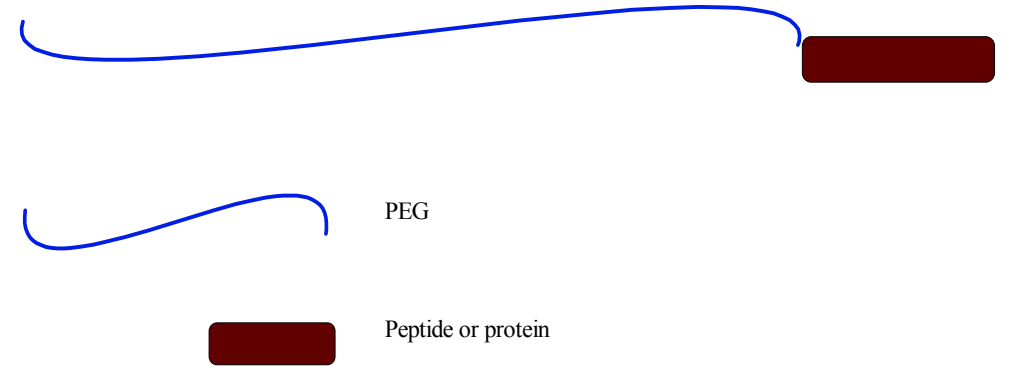

Figure 4: High molecular weight PEG coupled to a peptide or protein.

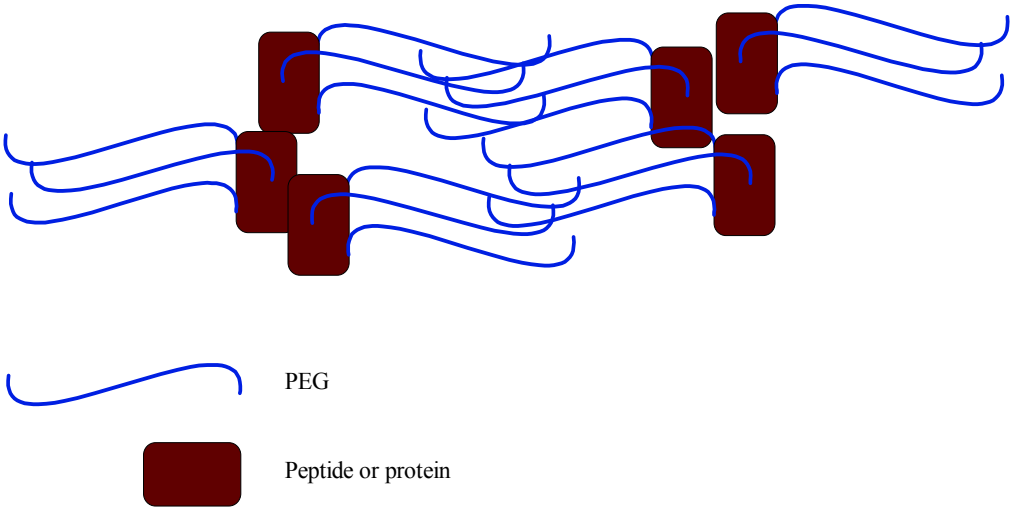

Figure 5: Multiple PEG attachments to proteins or protein fragments which aggregate and with the PEGs entwined - this forms hydrogels.

5. The arms of PEG-star polymers can be functionalized with peptides that can, when correctly triggered, crosslink in situ forming stable, bio-compatible hydrogels (Fig. 6)[28].

In the first two examples poly(amino acid)s are grown, in a linear fashion, onto an existing synthetic polymer. The synthetic polymer is used as a macro-initiator for the polymerization reactions. The poly(amino acid) block-lengths are thus determined by the macro-initiator to monomer ratios. Poly(amino acid)s have physical and chemical properties which can be exploited in the synthesis of biomaterials. In the case of linear copolymers, characteristics such as secondary structure – such as the formation of α-helixes or β-sheets can be exploited.

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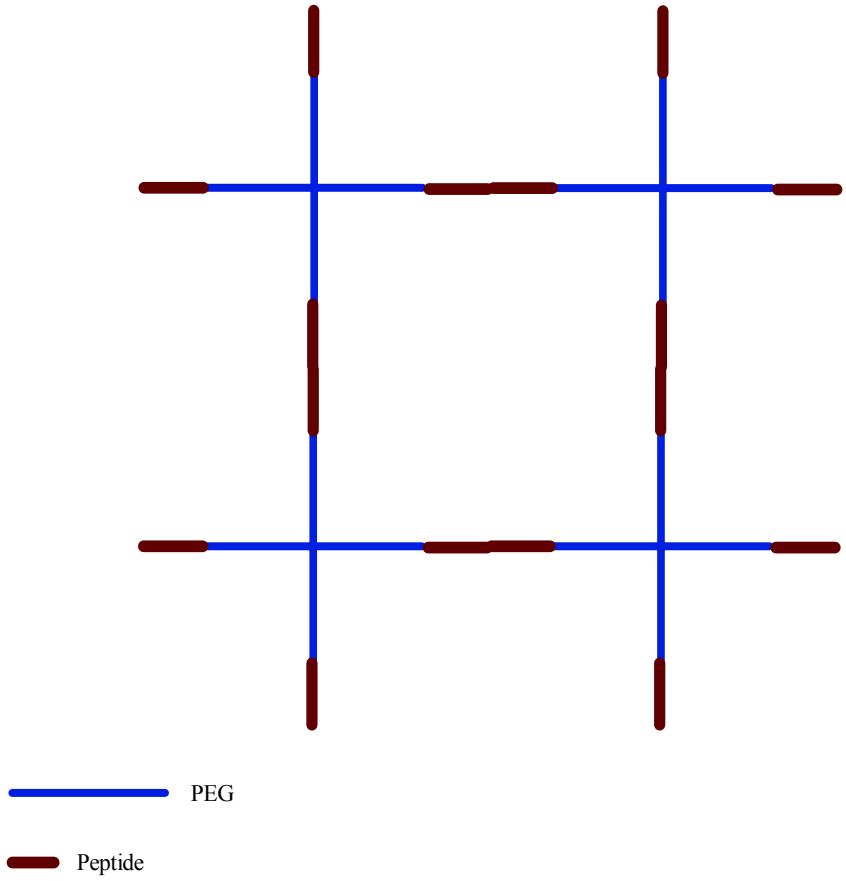

Figure 6: Peptides acts as bio-degradable/cleavable crosslinkers between the arms of PEG star polymers.

Characteristics such as hydrophobicity (polyalanine/polyleucine), hydrophilicity (polylysine/polyglutamic acid) or the charge, once the poly(amino acid) is deprotected ((+) for polylysine and (-) for polyglutamic- or aspartic acid), can also be applied to contribute to the desired function of the final polymeric product.

The third point illustrates another valuable quality of poly(amino acids) - here synthetic polymers, such as PEG, are grafted onto the poly(amino acid) backbone, in various quantities, by reaction with the amino acid side chains. Carboxylic acid terminated PEGs, for example, are reacted with the pendant ε-amino groups protruding from a polylysine backbone. Only a certain amount of the side chains are usually reacted with PEGs, leaving the unreacted, positively charged amino groups to have electrostatic interaction with an oppositely charged surface or substrate. These unreacted amino groups can also be used for the attachment of other biologically important molecules.

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In the last two scenarios PEGs are reacted with peptides or protein fragments. Peptides and proteins comprised of amino acids. Appropriately functionalized PEGs can be attached to the ends of short peptides or accessible amino acid side chain functional groups ($-NH_2$, -COOH, -SH) on the protein surface.

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5.2 Functionalized, PEG-sheathed polylysine

This work also involves the synthesis of poly(PEG-amino acid) conjugates, more specifically functionalized poly(PEG-L-lysine) polymers. From a conceptualization point-of-view the final product should be a fully functionalized-PEG grafted polylysine but in this case PEG is build into the monomer instead of being grafted onto an existing polylysine.

In this case a carboxylic acid terminated PEG is coupled to the ε-amino group of lysine and the α-amino acid-*N*-carboxyanhydride (NCA) of the functionalized lysine synthesized. This functionalized lysine-NCA is thus activated for ring opening polymerization (ROP). This can be compared to a similar concept for the polymerization of a methacrylate functionalized poly(ethylene glycol) methyl ether (mPEGMA)[29].

Yu et al. synthesized methylated mono- and diethylene glycol functionalized lysine-NCAs and polymerized these NCAs to form nonionic, α-helical, water-soluble poly(amino acid) polymers[30]. This was the only known related publication, on this approach of synthesizing poly(PEG-amino acid) conjugates at the time of completion of this study[31].

In this study the concept was taken two steps further: firstly by making the PEG segment longer and secondly, replacing the distal, 'inert' methyl ether group with a protected ester functionality. This ester could then be deprotected, after polymerization, to avail carboxylic acid groups on the surface of the PEG sheath surrounding the polylysine backbone. The distal acid groups may be used as handles for attaching biologically active peptides/molecules. Cross-linking, ionically, through divalent cations or diamines or covalently as amides or ester through diamines or diols, respectively, would also be possible through these surface acid moieties.

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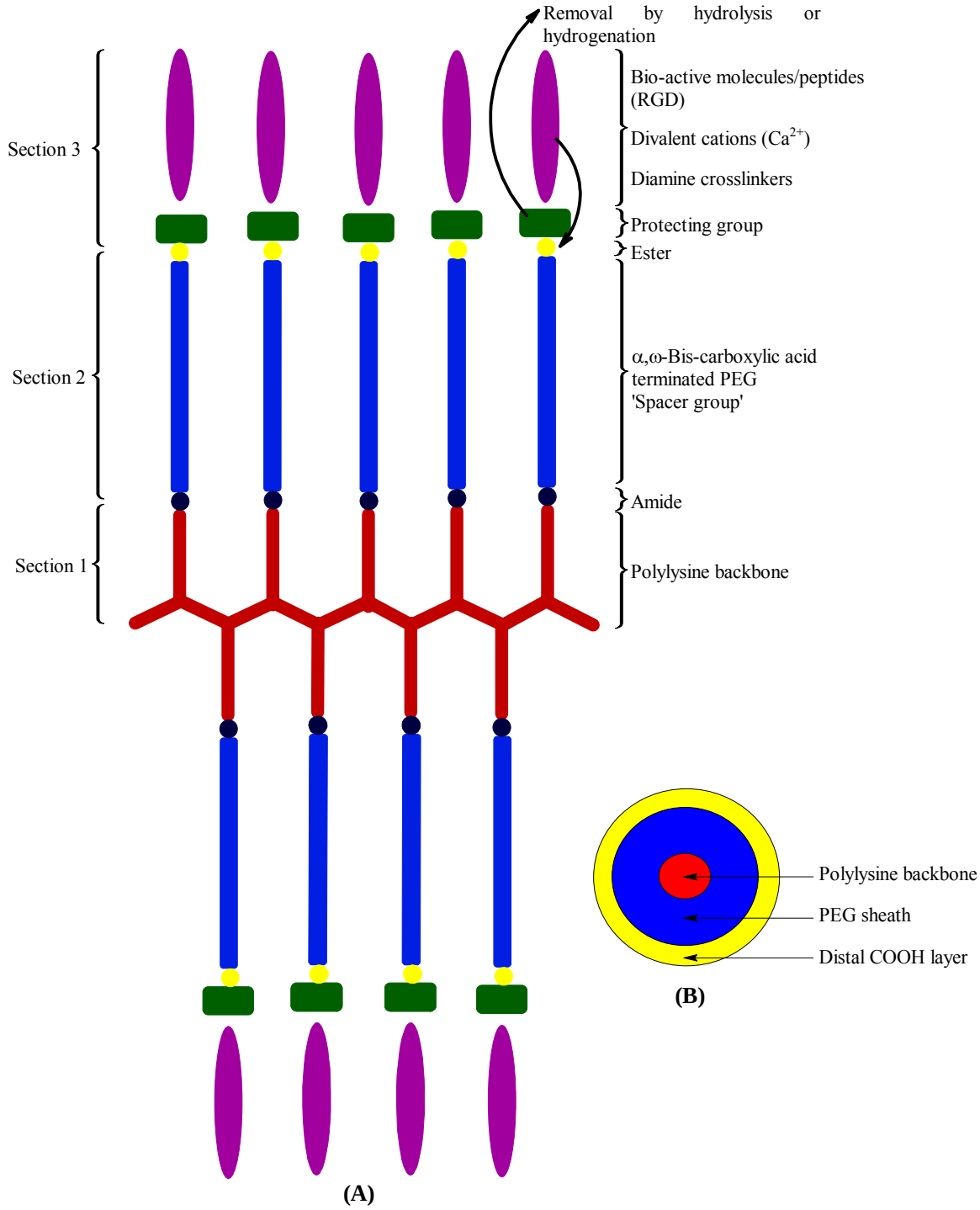

Figure 7: (A) Diagrammatic representation indicating the different components of the envisaged final product. (B) An end-on view of the final polymer once the protective distal esters have been removed.

This monomer would also have the potential of being used in the synthesis of co-polymers together with the more conventional NCA monomers such as that of Lys(Z)-NCA, Glu(Bzl)-NCA, Asp(Bzl)-NCA, Leu-NCA, Ala-NCA, etc.

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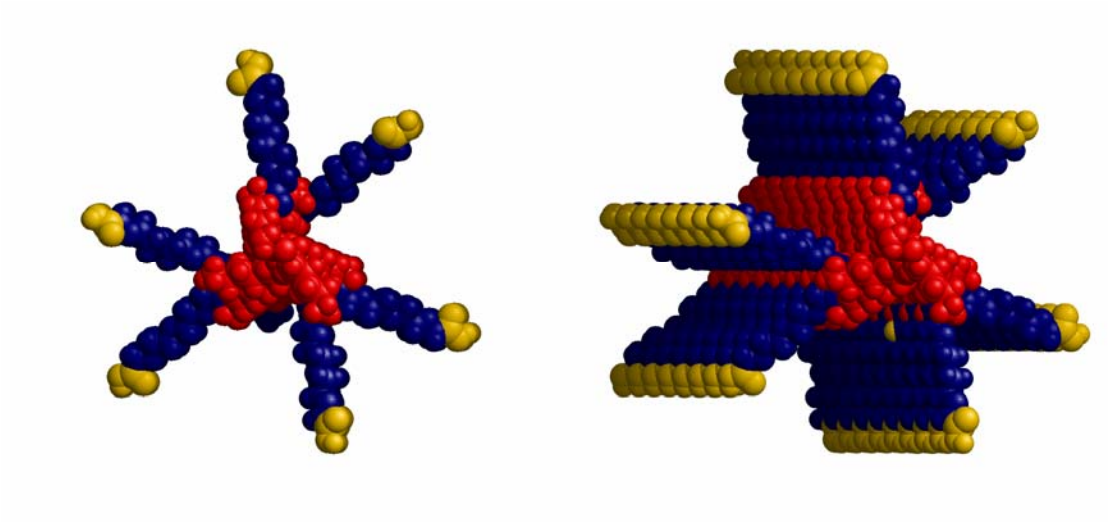

Figure 8: The final deprotected polymer is drawn in Hyperchem and renderd in RASMOL - assuming that the poly-L-lysine backbone (red) forms a stable α-helix. The PEG spacer is indicated in blue and the distal carboxylic acids groups in yellow.

5.2.1 The aim of the study

This study was aimed toward the synthesis and characterization of a novel PEG-lysine NCA monomer and the polymerization thereof. The functionalized, distal end (the end of the PEG which is not coupled to the amino acid – in this case the ε-NH_2 group of lysine) should be appropriately protected and be easily removable only after polymerization.

The envisaged application of this monomer is in the synthesis of biocompatible polymers – for this reason the monomer synthesis should be efficient and scaleable to multi-gram quantities in order to produce reasonable amounts of polymer.

5.2.2 Some of the questions asked in this study

1. How long should or could the pendant PEG group be and still achieve successful polymerization.
2. What are the reaction conditions to asymmetrically functionalize the PEG di-acid?
3. What would be an appropriate protecting group for the distal carboxylic acid?

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4. Will it be possible to cost-effectively produce the monomer in a large (multi-gram) scale?
5. How would polymerization be initiated and under which conditions?
6. Can this polymer be copolymerized with other NCA monomers?

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5.3 Design aspects of the functionalized PEG-lysine macromonomer

Like as in most polymers, the monomers contain most of the functional components that the polymer is comprised of. In this case the monomer can be divided into three sections as in the final polymer product (Figs. 7 and 9), and are as follows:

1. Lysine-NCA moiety: Polymerization would take place via the NCA moiety. This will form the poly-L-lysine backbone.
2. PEG pendant spacer molecule – α,ω-bis carboxylic acid poly(ethylene glycol) PEG-$(COOH)_2$. This group should make the polymer more water soluble with out being charge dependant. It should protect the polymer backbone from enzymatic attack. It should act as a spacer group between the polymer backbone and the active groups.
3. The distal -COOH handle, protected as an ester. After polymerization, depending on the nature of the ester and the solubility of the protected polymer, deprotection can take place through either hydrolysis or hydrogenation.

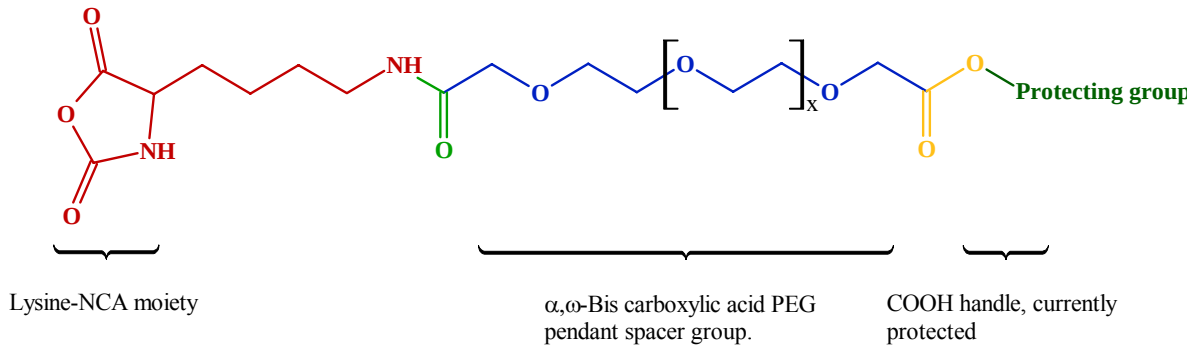

Figure 9: A detailed outline of the different components that make up the macromonomer.

5.3.1 The NCA moiety

Three methods for NCA synthesis were evaluated. Firstly NCAs can be synthesized with the use of bis(trichloromethyl)carbonate (triphosgene) reacting with α-*N* unprotected amino acids[32] or with *tert*-butyloxycarbonyl-α-*N* protected amino acids in the presence of an organic base such as triethylamine[33]. Dichloromethyl methyl ether (DCMME) is a very effective reagent for NCA synthesis when benzyloxycarbonyl-α-*N* protected amino acids are used as substrate[30, 34].

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5.3.2 The PEG side chain

How long should the PEG be? A too bulky monomer could give complications during monomer synthesis, polymerization and disrupt the conformation of the growing polypeptide. Furthermore, Whitesides et al. have shown that a high density of short, as little as two ethylene glycol units, are just as effective in protecting surfaces from protein adsorption as high molecular weight PEGs[35]. A slightly longer PEG was chosen, in light of the fact that ethylene glycol- and diethylene glycol methyl ether have been proven effective in making the neutral polylysine derivative water soluble[30]. The commercial availability of a short doubly functionalized PEG also played a role in the choice of PEG used.

For this purpose the shortest available, α,ω-bis carboxylic acid PEG [PEG-$(COOH)_2$] was acquired – poly(ethylene glycol) bis(carboxymethyl) ether was commercially available from Sigma-Aldrich (MW = 250, CAS #: 39927-08-7, catalogue #: 406996). From NMR it was determined that there are two ethylene oxide units between the terminal carboxymethyl groups (Fig. 10). Thus the oligoPEG$(COOH)_2$ has MW = 222.19g/mol, this was also confirmed by EI-MS.

5.3.3 Ester protection of the distal carboxylic acid 'handle'

The choice of alcohol used to protect the acid, had to be done taking into consideration the existing protecting groups on the amino acid and under what conditions they would be cleaved[36]. The synthetic method and final product separation used to synthesize the ester were also important factors in the choice of a suitable alcohol.

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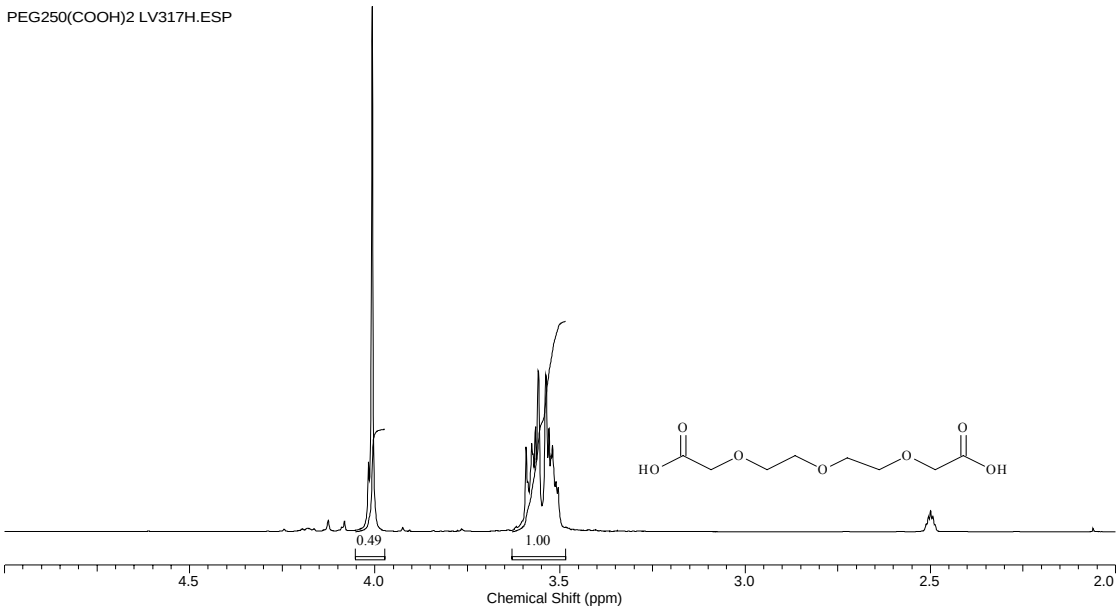

Figure 10: 400MHz ^{1}H NMR in DMSO-d_6: Tri(ethylene glycol) bis(carboxymethyl) ether (MW = 250 g/mol) (PEG-(COOH)$_2$).

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5.4 Synthesis of the functionalized PEG-lysine

Two approaches were investigated for the problem of selectively and asymmetrically functionalizing the two ends of the PEG-$(COOH)_2$, which lies in the centre of the molecule and is central to the synthetic challenge.

The first approach would be to react, in excess, activated PEG-$(COOH)_2$ with an appropriately protected lysine, as the first step and in a second step, react the remaining distal acid group with a suitable alcohol to protect it during further reactions and polymerization.

5.4.1 First synthetic approach

Solid phase synthesis was attempted first as a means to synthesize the final product free of potential cumbersome separation and purification steps (Fig. 11).

Functionalization of the lysine would be through the ε-NH_2 group. To achieve this, the lysine needs to be coupled to the resin through the carboxylic acid group and the two amino groups differentially protected. Once the resin is loaded with the protected lysine, the ε-NH_2 can be deprotected and coupled to the activated PEG-$(COOH)_2$.

Z-L-Lys(Fmoc)-OH was synthesized for this procedure and coupled to the resin (Nova-Syn TGA, 0.25mmol/g, hydroxyl terminated resin) using PyBOP (benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate)[37]. The ε-NH-Fmoc protecting group was subsequently cleaved, making available the lysine's ε-amino group for reaction with *N*-hydroxysuccinimide (HOSu) activated PEG-$(COOH)_2$ [PEG-$(COOSu)_2$]. Once reacted, the excess PEG-$(COOSu)_2$ was washed away, methanol was added to react with the now terminal PEG succinimide ester to yield the methyl ester.

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Methanol was decided on because as an alkyl ester it would be resistant towards hydrogenation, used in the removal of the α-NH-Z protecting group and once the polymer is synthesized the methyl ester may be hydrolyzed under basic or acidic conditions, leaving the carboxylate/carboxylic acid on the polymer surface.

This route proved to be very ineffective, because of the low effective loading (<10%, based on the cleaved fulvene's UV absorbance) (Equation 1) of Z-Lys(Fmoc)-OH the resin.

$$\text{Loading (mmol/g)} = \frac{\text{Fulvene absorbance at 301 nm } (A_{301})}{\text{Molar absorbance of fulvene } (\varepsilon)} \times \frac{\text{Volume 20\% pipiridine/DMF solution (ml)}}{\text{Amount of resin (g)}}$$

$$\varepsilon_{(\text{Fulvene})} = 7800\ \text{M}^{-1}\text{cm}^{-1}$$

Equation 1: Calculating the effective loading of the amino acid on the resin. This is calculated from the concentration of the cleaved fulvene as determined by its UV absorbance at 301nm[38].

A similar approach was tried next, with a higher capacity, Wang-resin (1.2mmol/g, hydroxyl terminated resin). Z-Lys(Fmoc)-OH was first converted to the acid chloride, the acid chloride reacted with 1-hydroxybenzotriazole (HOBt) in the presence of *N*-methylmorpholine (NMM) and the resin[37]. After coupling and Fmoc deprotection, HOBt, instead of HOSu, activated PEG-$(COOH)_2$ solution was added to the resin. This was again followed by washing, reaction with methanol and cleavage from the resin. HOBt, which is a stronger activating group than *N*-hydroxysuccinimide (HOSu), was used in an attempt to improve the loading of the amino acid to resin as well as improving the coupling to PEG-$(COOH)_2$.

This route was also characterized by low loading (14%, based on cleaved fulvene UV absorbance) of the Z-Lys(Fmoc)-OH to the resin.

This low coupling might be ascribed to the Fmoc protecting group, being a steric hindrance because it is situated on the side chain of lysine and not on the α-amino group as is the case in normal solid phase peptide chemistry and thus being too close to the resin surface and hindering the coupling reaction between the resin hydroxyl group and the carboxylic acid group of the lysine. NMR of the cleaved product

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showed mainly PEG-$(COOH)_2$ indicating that the activated PEG-$(COOH)_2$ coupled to the resin in the second step.

At this point it was obvious that solid phase route might not be the best way to approach the problem. A solution phase process was therefore considered as alternative.

Z-Lys(HTos)-OBzl was synthesized to 'mimic' solid phase conditions, with the ε-amino group now open to react with a PEG-$(COOSu)_2$. The α-amino- and carboxylic acid groups are also now fully protected as would be the case in solid phase synthesis. The added molecular weight to the product, through the addition of the benzyl ester, should help with the separation of the final PEG functionalized product through precipitation (Fig. 12).

PEG-$(COOSu)_2$ was reacted, in excess, as a first step with the Z-L-Lys(HTos)-OBzl in the presence of pyridine in THF. The second step followed with the in-situ treatment of the reaction mixture with an excess methanol to yield a distal methyl protected acid functionality. Because of complex reaction mixtures, separation of the final product proved difficult. Separation could not be achieved through extraction, precipitation or flash chromatography.

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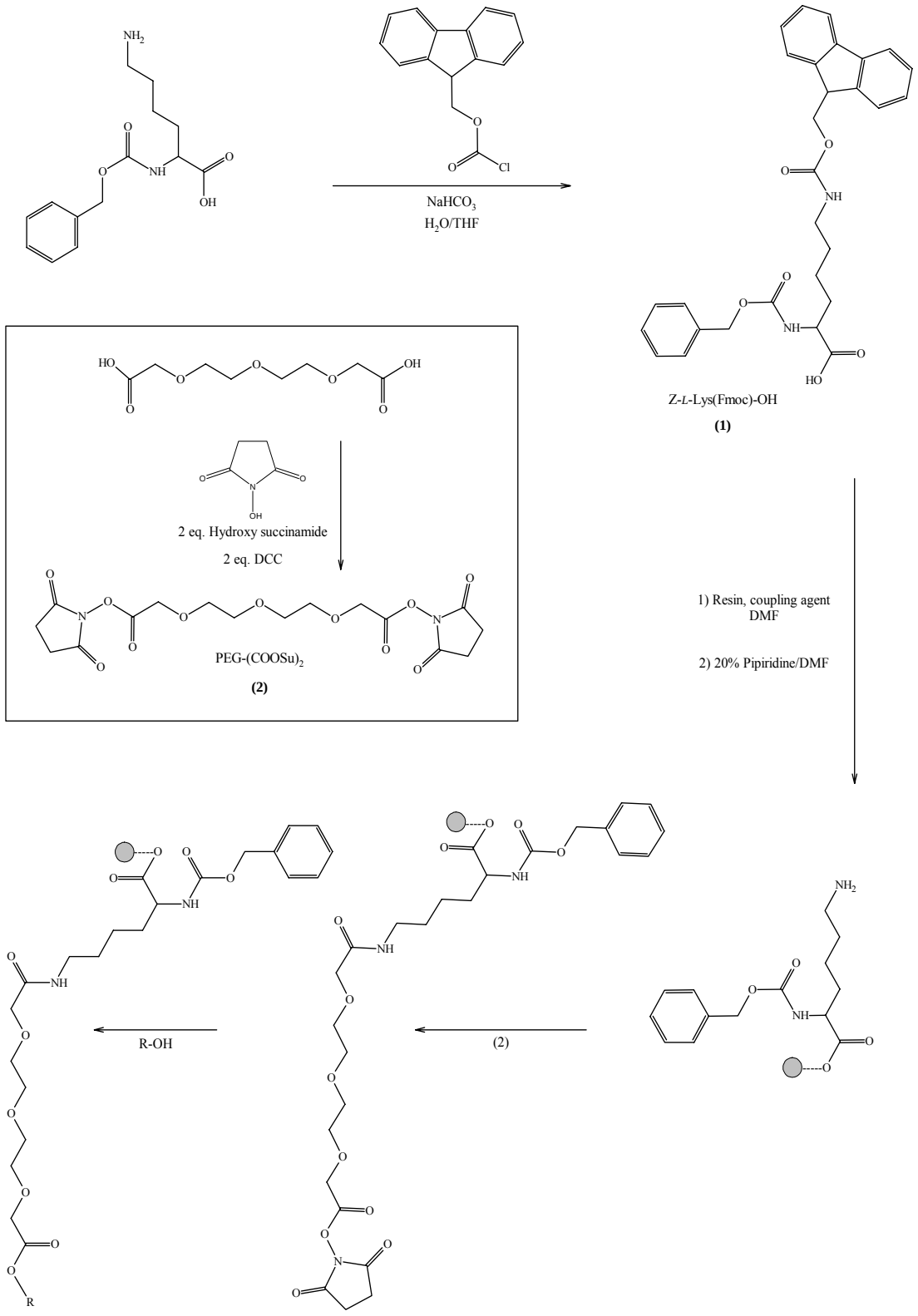

Figure 11: Solid phase synthethis (SPS) strategy for the synthesis of an alkyl ester functionalized PEG-Lysine. It also shows the synthesis of Z-L-Lys(Fmoc)-OH (1) and PEG-(COOSu)$_2$ (2).

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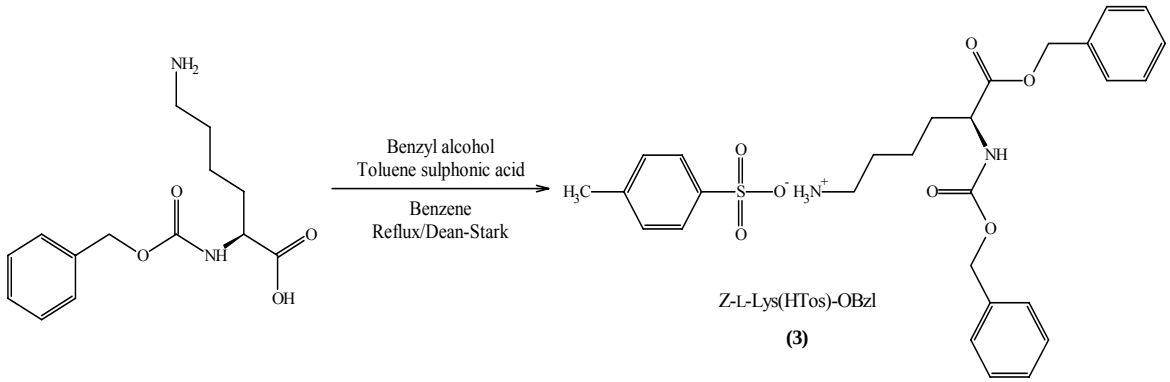

Figure 12: Synthesis of Z-L-Lys(HTos)-OBzl

A different, more streamlined, approach was needed at this stage - there were practical problems working with the current reaction sequence (amino acid + excess PEG-$(COOSu)_2$ + excess alcohol) being clumsy and wasteful - a more streamlined approach was necessary.

Because of these complications a second approach was needed in the reaction conditions and separation of the final product. An improved approach would be to asymmetrically protect the PEG-$(COOH)_2$ with an ester on one side, while leaving the other carboxylic acid unreacted, as a first step. The second step will be the 1:1 reaction of the (HOOC)-PEG-(COOR) with an appropriately protected lysine.

5.4.2 Second synthetic approach

$$\text{PEG-(COOH)}_2 \xrightarrow[\text{Step 1}]{\text{R}_1\text{-OH}} \text{(HOOC)-PEG-(COOR}_1\text{)} \xrightarrow[\text{Step 2}]{\text{Protected lysine}} \text{Protected Lysine-PEG(COOR}_1\text{)}$$

With the correct choice of alcohol it should be possible to separate the mono-protected PEG-$(COOH)_2$ from the reaction mixture and be stable during removal of the benzyl ester (Bzl)- and benzyloxycarbonyl (Z)- protecting groups on the lysine.

2-Phenyl ethanol (PhEt-OH) was chosen for synthesizing the asymmetric (COOH)-PEG-(COOPhEt) for the following reasons:

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1. As an ester it would contribute significantly to the overall molar mass of the functionalized PEG final product and the chromatographic behavior. This should make it possible to separate the three reaction products from each other.
2. The phenyl group is a chromophor, which will make the products clearly visible under UV light when using TLC to monitor the reaction.
3. It is a high boiling alcohol.
4. The assumption was made that the phenylethyl ester would have an alkyl- in stead of a benzyl-character making it more resistant to the hydrogenation conditions used to cleave the Z and benzyl protecting groups; while on the other hand it would hydrolyze under basic or acidic conditions (alkyl character), once the polymer was formed[36].

PEG-$(COOH)_2$ was treated with 2-phenyl ethanol in the presence of toluene sulphonic acid as condensation catalyst in refluxing benzene to remove the water azeotropically using a Dean-Stark apparatus. This reaction was refluxed for at least 3h or until the formation of the water condensate ceased.

The toluenesulphonic acid could not be completely removed during the workup of the final product and was eventually replaced by a strong cation exchange resin as acid catalyst for this reaction which simplified the work-up procedure considerably, since the resin could be removed by filtration (Fig. 13).

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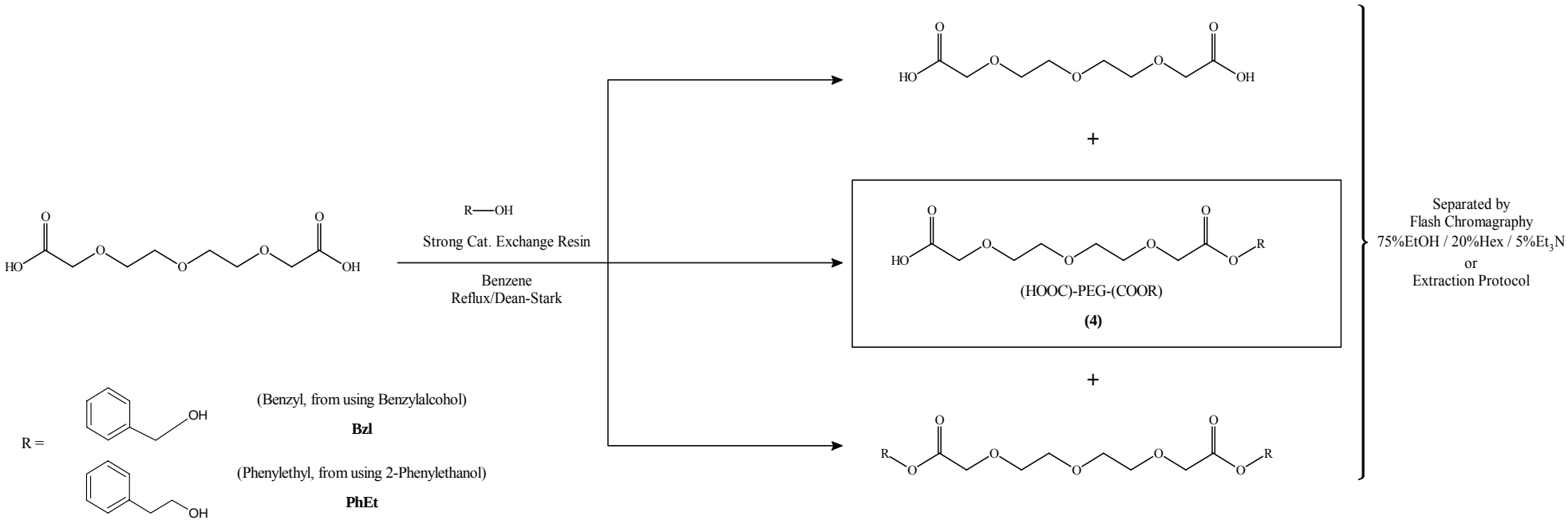

Figure 13: Synthesis of (HOOC)-PEG-(COOPhEt/Bzl) and the product separation from the di-ester and unreacted PEG-$(COOH)_2$ reaction by-products.

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The three components of the product mixture could be successfully separated by flash chromatography (7.5:2:0.5 ethanol:hexane:triethylamine) with the PEG-$(COOR)_2$ (R_f = 0.79), (HOOC)-PEG-(COOR) mono-ester (R_f = 0.26) and the unreacted PEG-$(COOH)_2$ (R_f = 0.05).

Later on in the project an extraction protocol was developed for the successful separation of the three components. This extraction protocol allowed for larger scale synthesis. The capacity of the silica columns are the limiting factor for the scale of synthesis. A 150g silica column can separate a 6 - 7g of reaction mixture using about 2L of solvent mixture. With the extraction protocol the same results can be achieved with less that 0.5L ethyl acetate.

The (HOOC)-PEG-(COOPhEt) was then coupled to the Z-Lys(HTos)-OBzl, using DCC, and pyridine (pyridine is an effective base to react with the toluene sulphonic acid – it precipitates out of solution as an insoluble salt).

The next step in the synthesis involved the hydrogenation of the Z- and Bzl-protecting groups [36, 39, 40]. Catalytic transfer hydrogenation with 10% Pd/C and ammonium formate in methanol was very effective, but removing the ammonium formate salts proved to be problematic even after freeze drying several times there were still some traces of ammonium formate left[41]. Hydrogenation with 10% Pd/C and hydrogen (in a balloon) was also successful and rapid, the main advantage of it being a clean reaction. Catalytic transfer hydrogenation using formic acid as the hydrogen donor and solvent was not successful in this case[42].

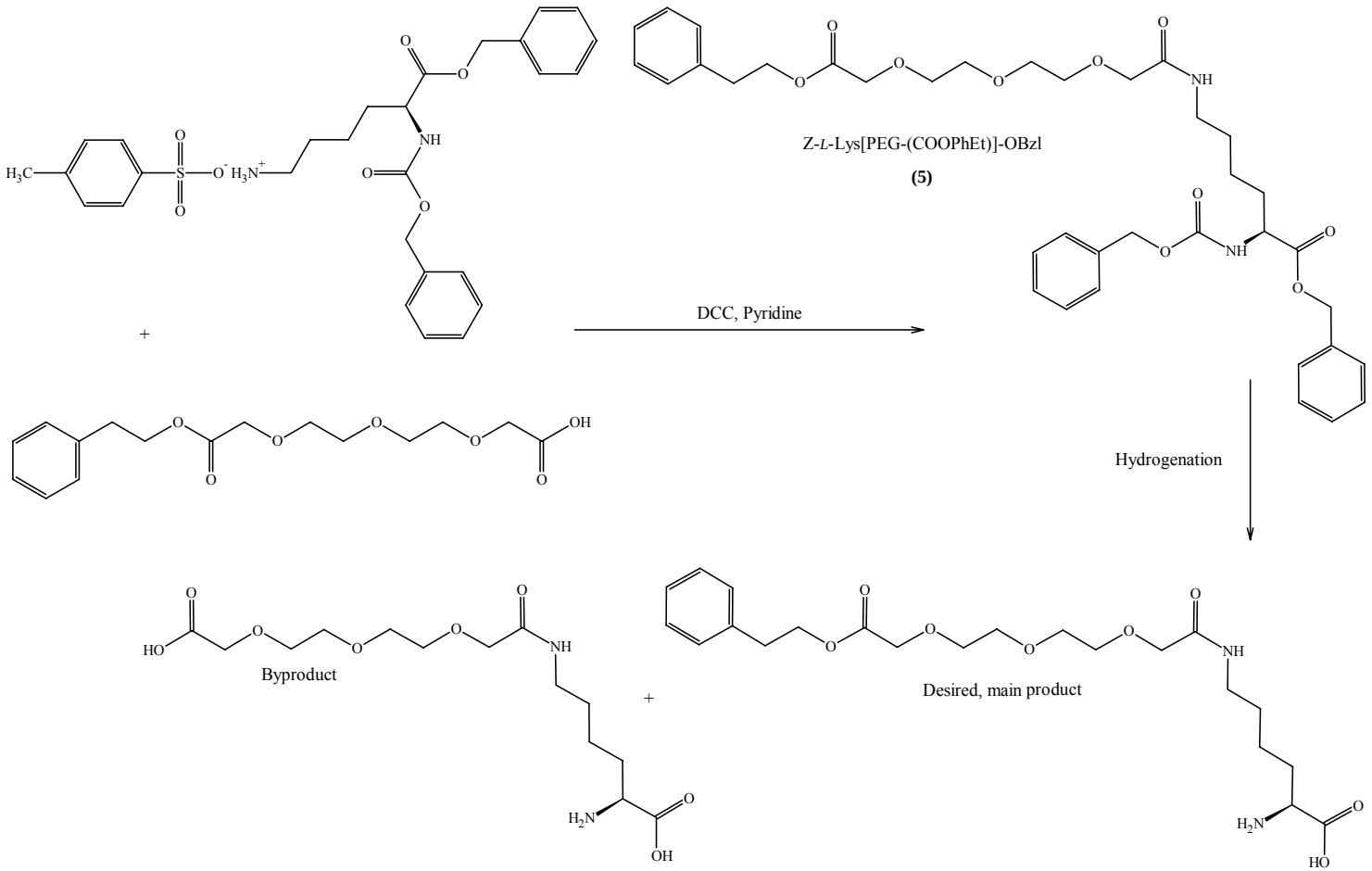


Figure 14: Synthesis of Z-L-Lys[PEG-(COOPhEt)]-OBzl followed by hydrogenation and indicating the hydrogenation product mixture. The unwanted deprotected (phenylethyl ester hydrogenated) by-product is present in quantities of up to 50% depending on hydrogenation conditions.

A major complication was that partial hydrogenation (up to 50%) of the phenylethyl ester took place at the same time during the removal of the protecting groups. This was visible in the NMR with the loss of intensity of the phenylethyl peaks and the appearance of the -O-C$\underline{H_2}$-COOH at 3.90 ppm in the ^{1}H NMR and the appearance of an extra carbonyl peak at 170.65 ppm in the ^{13}C spectra. Duplication of peaks is clearly visible in both the ^{1}H and ^{13}C spectra when the concentration of the hydrogenated byproduct is high enough. The amount of hydrogenation side reaction went down when the amount of catalyst used was reduced, nevertheless there was always hydrogenated byproduct present in the final product (TLC 5:3:1 chloroform:methanol:acetic acid. $R_{F\{Lys[PEG\text{-}(COOPhEt)]\text{-}OH\}} = 0.62$ and $R_{F\{Lys[PEG\text{-}(COOH(\text{-}PhEt)]\text{-}OH\}} = 0.13$).

The synthetic procedure was now further modified to exclude the necessity of the α-carboxylic acid benzyl ester protecting group. This was done by reacting the succinimide ester of the (COOH)-PEG-(COOPhEt) with Z-Lys(NH_2)-OH to yield Z-Lys[PEG-(COOPhEt)]-OH. This also eliminated the need for hydrogenation because the final NCA product can be formed from the reaction of α-NH-Z protected amino acids with dichloromethyl methyl ether (DCMME)[30, 34]. The byproducts are benzylchloride and HCl.

All the final products involving the PEG are very viscous oils (syrup-like). To try and avoid any by-products being trapped in the final NCA product, the final step of the synthesis must be as 'clean' as possible, with by-products being volatile and of low boiling point. There was a concern that traces of benzylchloride would be trapped, because of its high boiling point, in the final NCA product.

For this reason tBoc-Lys[PEG-(COOPhEt)]-OH was also synthesized analogously to Z-Lys[PEG-(COOPhEt)]-OH.

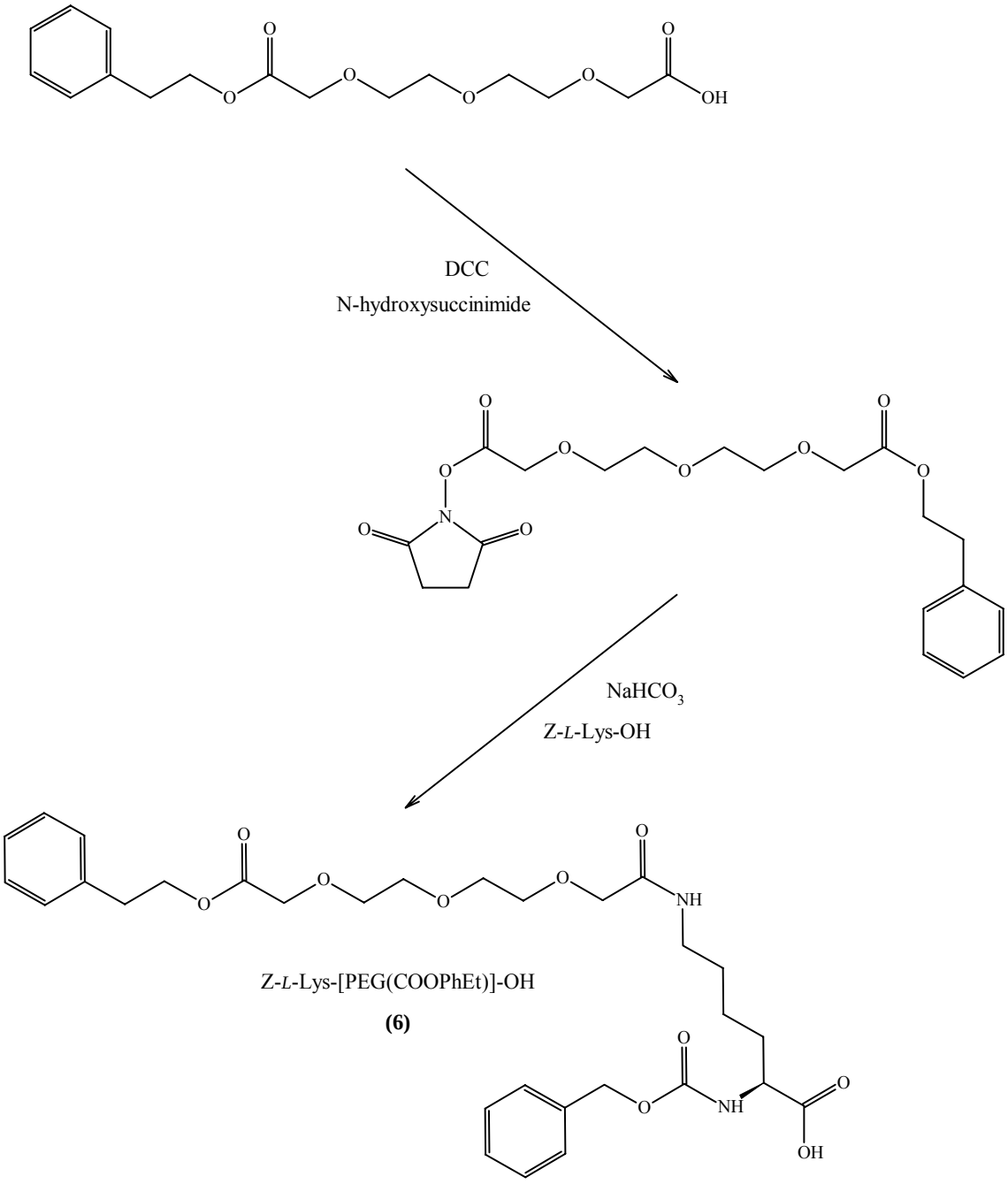


Figure 15: Synthesis of Z-L-Lys[PEG-(COOPhEt)]-OH. This synthesis is also applicable to the α–NH tBoc protected lysine and the benzyl ester functionalized PEG variants.

tBoc-Lys[PEG(COOPhEt)]-OH also reacts with DCMME to yield the NCA, but in this case the byproducts are *tert.*butylchloride and HCl. *tert.*Butylchloride boils at a much lower temperature than benzyl chloride and can be completely removed under vacuum. The use of DCMME to synthesize NCAs from tBoc-protected amino acids was not found in the literature.

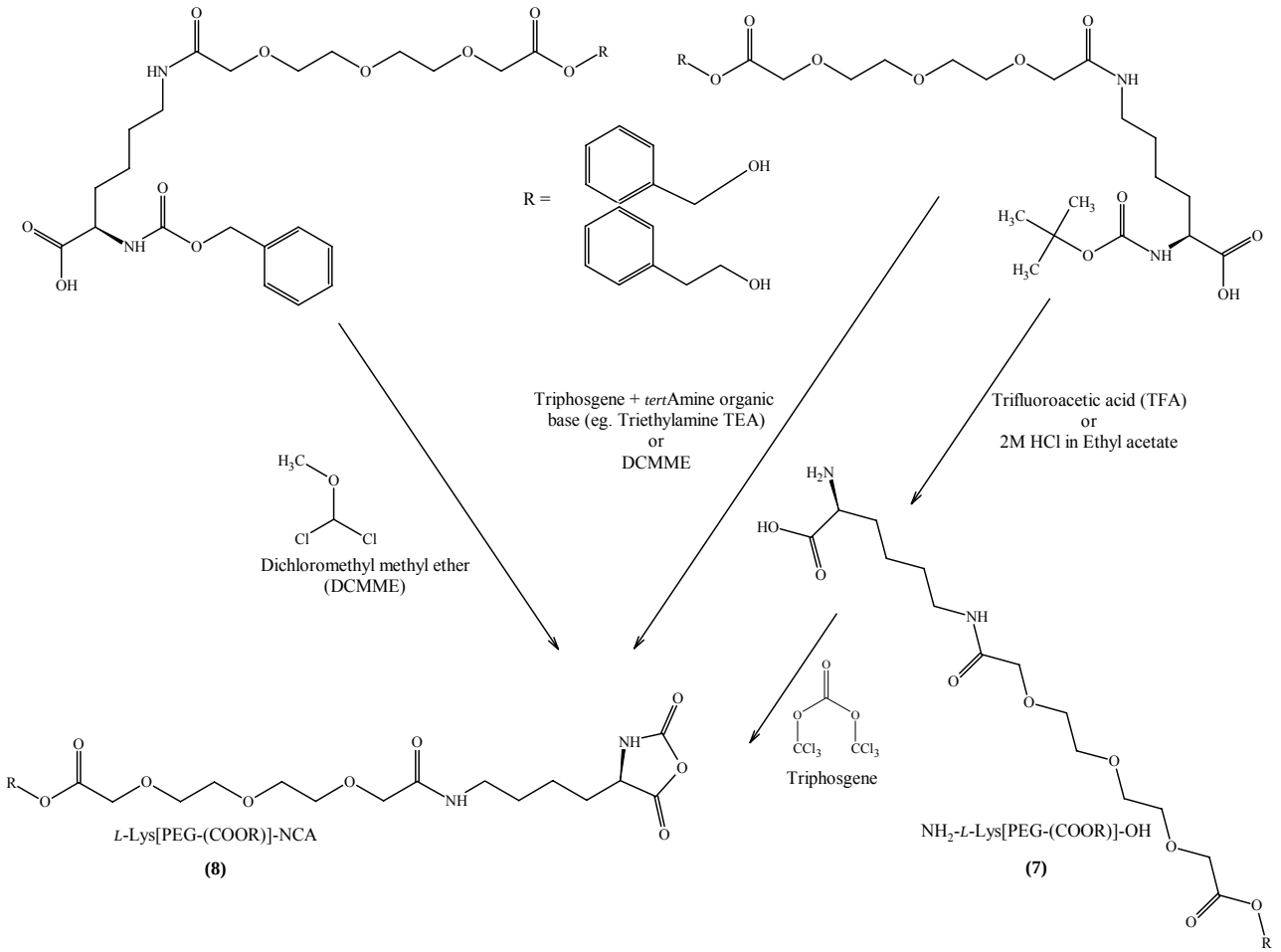


Figure 16: Synthetic routes used to synthesize the L-Lys[PEG-(COOPhEt/Bzl)]-NCA final product. The synthetic route choice depends on the α-NH protecting group.

tBoc-Lys[PEG-(COOPhEt)]-OH lends itself to other methods to get to the NCA product. Not only does the DCMME method work very well, but the following is also available:

- The tBoc group can be removed using 50% trifluoroacetic acid in DCM or 2M HCl in ethyl acetate. The second has the advantage that no extra contaminants, other than HCl, are introduced in the synthetic procedure. With the tBoc removed the unprotected amino acid will react with triphosgene (0.3 – 0.5 eq.) to form the NCA with HCl as the only byproduct.
- tBoc protected amino acids also react with triphosgene in the presence of an organic base [triethyl amine (TEA) or *N*-methyl morpholine (NMM)][33]. The risk of amine-HCl salt contaminating the final product is of concern here.

While working on optimizing reaction conditions for the removal of tBoc protecting groups to yield NH_2-Lys[PEG-(COOPhEt)]-OH, the product was freeze-dried. Upon analysis of the dried sample, by NMR, it was observed that partial hydrolysis (almost 50%) of the phenylethyl ester took place, revealing NMR spectral characteristics very similar to when the hydrogenation side reactions took place during the deprotection of Z-Lys[PEG-(COOPhEt)]-OBzl. This was probably because under these conditions the α-amino group acts as a base catalyst for hydrolysis to take place. Under the same conditions no hydrolysis took place when the amino group was protected. An acid catalyzed mechanism for hydrolysis is also possible with the presence of trapped trifluoro acetic acid in the product during freeze drying.

PhEt was initially chosen to be acid or base hydrolysable once the final polymer was formed. Since the synthetic procedure moved away from the use of Z-Lys(HTos)-OBzl to tBoc-Lys-OH or Z-Lys-OH as starting materials, the phenylethyl ester was now exposed to aqueous basic and acidic conditions during the synthesis of the PEG modified lysine. There arose a chance that hydrolysis of the phenylethyl ester (even in very small amounts) could take place and be carried through to the final NCA product, where carboxylic acids or acid chlorides can be contaminants towards polymerization.

No clear indication of the relative stability, toward hydrogenation or hydrolysis, of the PhEt-ester relative to *n*-alkyl or benzyl esters and benzyl carbamates could be found in the literature. Also a contributing factor to the hydrolysis side reactions can be the presence of an ether oxygen β to the ester carbonyl. This can make the carbonyl more nucleophilic and thus more susceptible to base catalyzed hydrolysis. Thus it seems that the PhEt ester has characteristics of both benzyl and *n*-alkyl esters being susceptible, to some extent, to hydrogenation and hydrolysis.

With the risk of hydrogenation side reactions eliminated, the phenylethyl ester was replaced with a benzyl ester. Benzyl esters are well known protecting groups for carboxylic acids, being stable under acidic and basic conditions, but easily removed by hydrogenation. Replacing the phenylethyl ester with a benzyl ester also reduces the possibility of hydrolysis side reactions taking place.

PEG-$(COOH)_2$ was reacted with benzyl alcohol under exactly the same conditions as with the PhEt-OH. Separation was also achieved under the same conditions.

5.4.3 The use of longer PEGs

5.4.3.1 PEG_{600}-$(COOH)_2$

PEG_{600}-$(COOH)_2$ was also commercially available as poly(ethylene glycol) bis(carboxymethyl) ether, from Sigma-Aldrich. This was tested under similar conditions as with PEG_{250}-$(COOH)_2$ to synthesize (HOOC)-PEG_{600}-(COOR). Although the reaction was successful, because of the smaller contribution of the ester(s) and free acid(s) towards the chemical and physical properties of the final product(s), the mixture could not be separated into its three components.

With the expectation that with increasing PEG lengths that the desired or undesired intermediates could selectively be precipitated out – an excess PEG_{600}-$(COOSu)_2$ was treated with tBoc-Lys-OH followed by the addition of benzyl alcohol. Product

separation could not be achieved either through preferential precipitation or chromatography.

5.4.3.2 PEG_{1000}-$(COOH)_2$

PEG_{1000}-$(COOH)_2$ was synthesized by the oxidation, using Jones reagent, of the terminal hydroxyl groups, of PEG_{1000}-$(OH)_2$ to the corresponding carboxylic acids[43,44]. PEG_{1000}-$(COOH)_2$ was subsequently activated towards amide formation through *N*-hydroxysuccinimide activation. PEG_{1000}-$(COOSu)_2$ was reacted in a two fold excess with the ε-amino group of tBoc-Lys-OH followed by the addition of benzyl alcohol. Here the reaction mixture could be precipitated but there was no bias towards product or byproduct, thus no separation was possible. NMR analysis of the final precipitated reaction mixture also indicated that very little of the benzyl alcohol reacted to yield the distal benzyl protected acid group.

5.5 Synthesis of the functionalized PEG-lysine-NCA and polymerization reactions

5.5.1 Lys[PEG-(COOPhEt/Bzl)]-NCA synthesis (Method 1)

tBoc-Lys[PEG-(COOBzl)]-OH or Z-Lys[PEG-(COOPhEt/Bzl)]-OH as starting compound may be reacted with DCMME. Yu et al. used this reagent in stoichiometric quantities in DCM as solvent[30], while Lange et al. used DCMME as reagent and solvent[34]. In both precedures it was Z-protected amino acids that were treated with DCMME. There are no examples in the literature where DCMME was used to form NCAs from tBoc-protected amino acids. We formed NCAs equally effectively from tBoc- and Z-protected amino acids. Incomplete reactions were obtained when stoichiometric amounts of DCMME was used in refluxing DCM for 16h. Complete reactions were obtained when DCMME was used as solvent stirring at 60^{o}C for 30 min. and 2h at RT.

5.5.2 Lys[PEG-(COOPhEt/Bzl)]-NCA synthesis (Method 2)

N-α-tBoc-protected amino acids may be treated with ⅓ equivalent triphosgene in the presence of a stoichiometric amount of organic base. Triethyl amine (TEA) and *N*-methyl morpholine (NMM) are both convenient bases when working in ethyl acetate but, when using THF as solvent NMM is the preferred base. The hydrochloride salts of these bases are insoluble in these solvents. This method is only suitable if the final NCA product can be purified by repeated crystallization from the appropriate solvents[33].

Since Lys[PEG-(COOPhEt/Bzl)]-NCA is not crystalline, traces of the hydrochloride salts still remain after repeated dissolution, filtration and evaporation of the solvent. Any form of attempted precipitation of the final product also resulted in the precipitation of the salts. The use of DIPAM resin [see synthesis of Arg(NO_2)-NCA; Chapter 4, section 4.2.3] was not successful in this case since the NCA forming reactions did not go to completion.

5.5.3 Polymerization reactions

Initial polymerization reactions were performed with NCA synthesized from Z-L-Lys[PEG-(COOPhEt)]-OH, prepared by method 1, yielding Lys[PEG-(COOPhEt)]-NCA, as described above, followed by washing with pentane (3X), before being dried for 24h under vacuum.

Catalysts to monomer ratios of 1:25, 1:50 and 1:100 were normally used in the polymerization reactions.

Initially one of the aims was to apply the living polymerization technology through Ni^0 and Co^0 metal catalysis established by Deming[45, 46] (Chapter 3).

BipyNiCOD catalyst was available and was used first, in DMF as solvent, under conditions the same as with the Poly-RDG reactions. Polymerization did not occur because of either catalyst decomposition (black precipitate forming) or the absence of any reaction after stirring for 16h at 25°C.

The lack of success with the Ni catalyst in DMF led to the investigation of polymerization using the $Co(PMe_3)_4$ catalyst in THF. Again no successful polymerization was observed – no reaction after 16h at 25°C.

With the Ni^0 and Co^0 catalysts being unsuccessful an alternative living catalyst was investigated. HAPENAlOMe catalyst was synthesized and applied to the monomer under different solvent conditions (DCM, dioxane and THF). No successful polymerization was achieved.

It was at this stage that tBoc-L-Lys[PEG-(COOBzl)]-OH was synthesized, because of the consideration that hydrolysis contamination could be preventing polymerization of the Lys[PEG-(COOPhEt)]-NCA. Method 1 was applied to prepare Lys[PEG-(COOBzl)]-NCA from tBoc-*L*-Lys[PEG-(COOBzl)]-OH. Polymerization reactions were carried out using the same procedure applied to Lys[PEG-(COOPhEt)]-NCA with BipyNiCOD, $Co(PMe_3)_4$ and HAPENAlOMe as catalysts. No polymerization occured.

When TEA and *n*-butylamine were used as initiators in the aforementioned ratios to the monomer, there was no reaction either place. With the addition of more base, a white precipitate started forming. We became aware of the possibility that there could be HCl contamination present in the monomer solution. This possibility was substantiated when a sample of the monomer, dissolved in THF, was added to an aqueous silver nitrate solution upon which a white precipitate formed.

The following measures were applied to remove the HCl contamination. Polymerization was again attempted in THF by the addition of triethyl amine (1:10) as a test to the success of the removal of the HCl.

- Reversed polarity flash chromatography (C18 with a pentane – THF gradient). No polymerization.
- Poché et al. reported that non-crystalline NCAs may be freed from HCl by dissolving the NCA in an organic solvent and washed briskly with an ice cold, dilute $NaHCO_3$ (0.5%) solution[47]. This method was applied with ethyl acetate and dichloromethane as solvent. In both cases emulsions formed, exposing the Lys[PEG-(COOBzl)]-NCA to the aq.$NaHCO_3$ for too long, resulting in hydrolysis or uncontrolled polymerization of the NCA.
- When the Lys[PEG-(COOBzl)]-NCA was dissolved in ethyl acetate and washed with water, emulsion formation was considerably less than with the $NaHCO_3$ solutions. The NCA survived the water exposure, but still tested positive for Cl^- when a sample was added to a silver nitrate solution. Polymerization was unsuccessful.
- Since the $NaHCO_3$ solution was too basic to wash the NCA and water was not basic enough, washing with a phosphate buffered solution (pH ≈ 7.5) was attempted next to remove the HCl. This also was unsuccessful.
- With the NCA dissolved in THF, filtering through or stirring over $NaHCO_3$ resulted in uncontrolled NCA polymerization. When the NCAs were treated with HCO_3-resin – polymerization took place.
- Fuller et al. showed that NMM characteristics are such that it may be used as a non-nucleophilic weak base in the presence of NCA[48]. The pKa of NMM is 7.38[49]. When 0.5eq NMM was added to a THF solution, a white precipitate

(hydrochloride salt) formed. NMR analysis revealed that uncontrolled polymerization also occured.

- Method two describes the use of an organic base with triphosgene to synthesize the NCA from tBoc-protected amino acids. When DIPAM-resin was used as the base equivalent, the reaction did not go to completion. When TEA in ethyl acetate or NMM in THF was used as base the problem of hydrochloride salt formation of these bases persisted. The NCAs decomposed on silica gel during flash chromatography when it was attempted to remove these salts.
- After the NCA was formed (method 1), it was again dissolved in DCM and refluxed for 16h with an argon flow – in an attempt to remove the excess HCl. After this time it still tested positive for HCl.

Successful test polymerization reactions (triethyl amine in THF, the same as above) were achieved with the development of method 3 for the synthesis of Lys-[PEG-(COOBzl)]-NCA.

5.5.4 Lys[PEG-(COOBzl)]-NCA synthesis (Method 3)

This method involves the removal of the *N*-α-tBoc protecting group by means of 50% trifluoroacetic acid in DCM or 2M HCl in ethyl acetate. After removal of the volatiles the deprotected, NH_2-Lys[PEG-(COOBzl)]-OH, product was isolated by repeated precipitation from cold diethyl ether. The NH_2-Lys[PEG-(COOBzl)]-OH could now be reacted with triphosgene to yield Lys[PEG-(COOBzl)]-NCA which was purified through repeated precipitation from cold ether solutions thus removing the contaminants followed by repeated precipitation from cold pentane.

With the development of method 3 as the preferred method for the NCA synthesis, method 1 fell away.

5.6 Amine-initiated polymerization of Lys[PEG-(COOBzl)]-NCA synthesized by Method 3

5.6.1 *n*-Butylamine initiated homopolymerization

After the successful test reactions with triethylamine, it was decided to do more detailed experiments using the primary amine *n*-butylamine. Primary amines follow a nucleophilic-attack-route in the initiation of polymerization reactions of NCAs resulting in amide formation at the initiating end of the polymer. Using the characteristic CH_3-triplet of the resulting *n*-butylamide in the NMR spectrum of the polymer product it is possible to estimate the degree of polymerization (DP) (Equation 2). By dividing 0.66 of the integration value of the *n*-butylamide CH_3 (triplet at 0.87 ppm) into the integration value of the benzyl ester CH_2 (singlet/doublet at 5.23 ppm) an effective initiator to monomer ratio may be calculated.

$$Degree\,of\ Polymerization\,(DP) = \frac{Integration\,value\,of\ benzyl\,ester\ CH_2\ (A)}{0.66\left(Integration\,value\,of\ butylamide\ CH_3\,(I)\right)}$$

Equation 2: Calculation of the the degree of polymerization (DP) through the use of intergration values of the benzyl ester CH_2 (A) and the *n*-butylamide CH_3 (I)

The work-up of the polymerization reactions were so designed, as to not loose unreacted monomer, but remove unreacted initiator.

Effective homopolymerization took place for initiator to monomer ratios of 1:5 and 1:10 (Figs. 17 and 18). This can be seen by the absence of the NCA α-CH multiplet at δ = 4.28 (as compared with spectra where the polymerization reactions were unsuccessful) ppm and the presence of the butyl CH_3 at 0.87 ppm with the expected integration values in relation to the benzyl CH_2 at 5.23 ppm.

No reaction was observed for the homopolymerization reaction with ratio's of 1:20 and 1:50 as can be seen by the presence of the NCA α-CH multiplet at 4.28 ppm (1:20) and 4.28 ppm (1:50) as well as the absence of the *n*-butyl CH_3 triplet at 0.87 ppm (Figs. 19 and 20)(Table. 1).

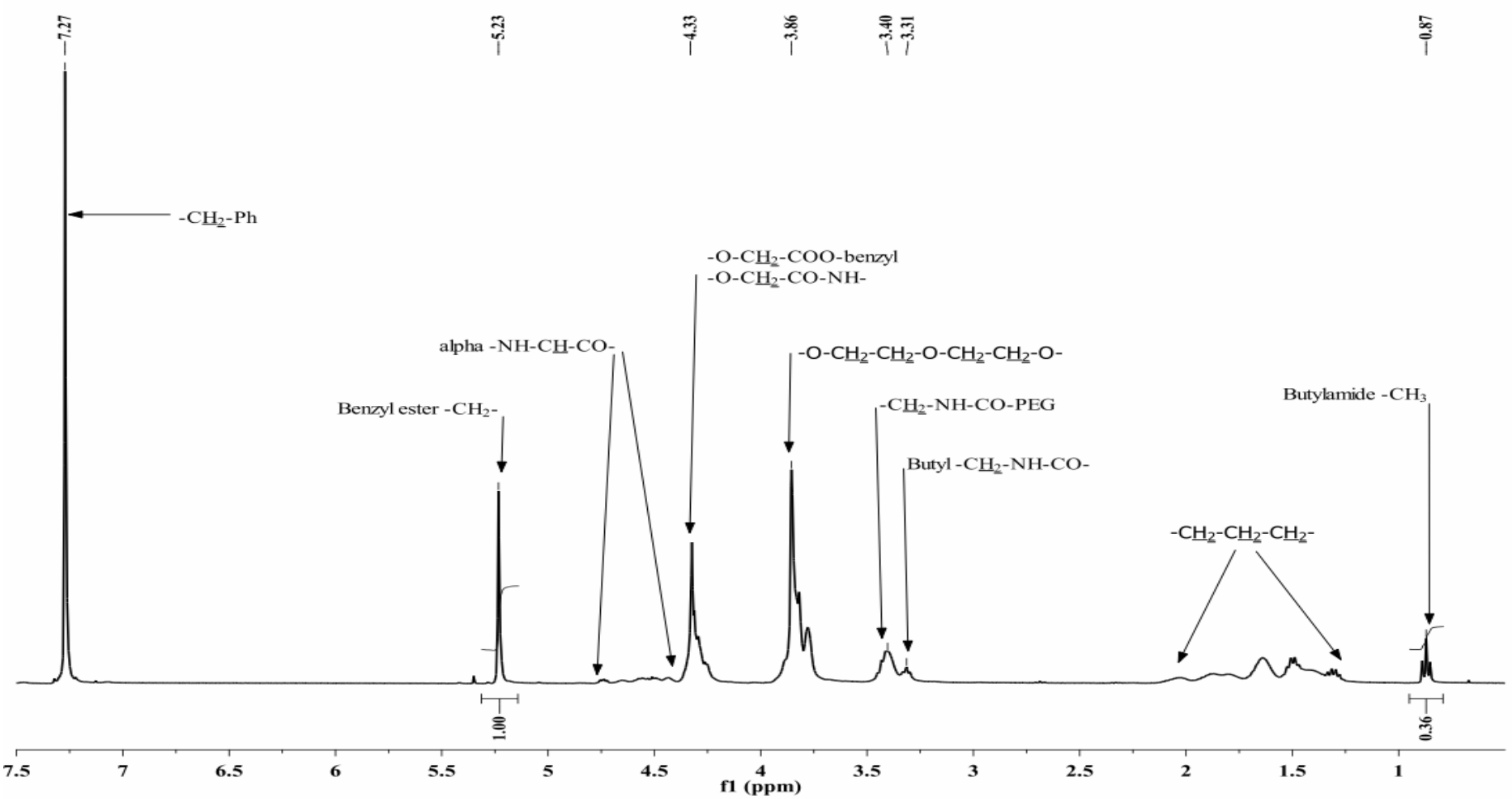


Figure 17: 400MHz 1H NMR in TFA-d_1. Lys[PEG-(COOBzl)]-homopolymer with initiator to monomer ratio (I:M) of 1:5 (found 1:4.2).

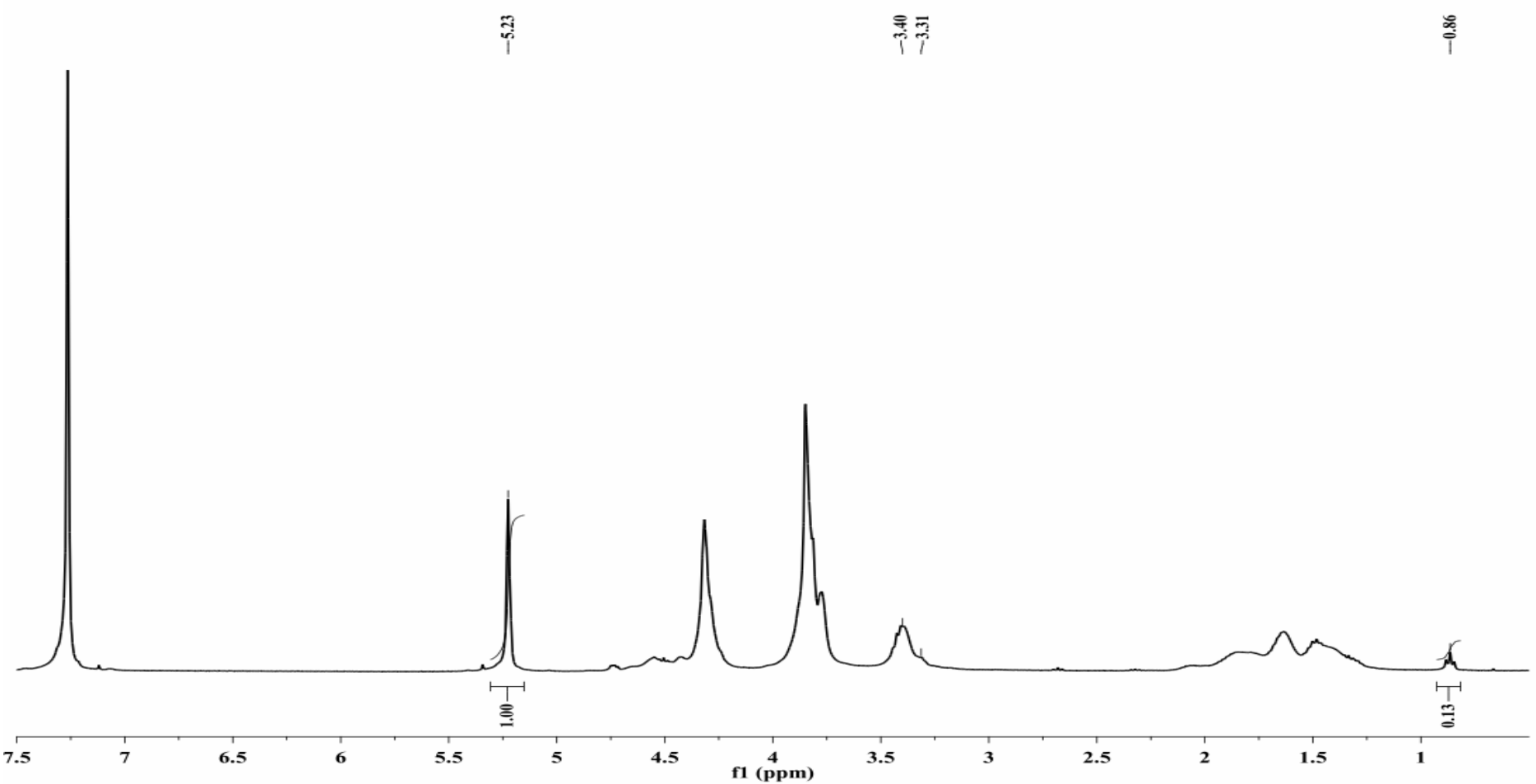


Figure 18: 400MHz 1H NMR in TFA-d_1. Lys[PEG-(COOBzl)]-homopolymer with initiator to monomer ratio (I:M) of 1:10 (found 1:11.1).

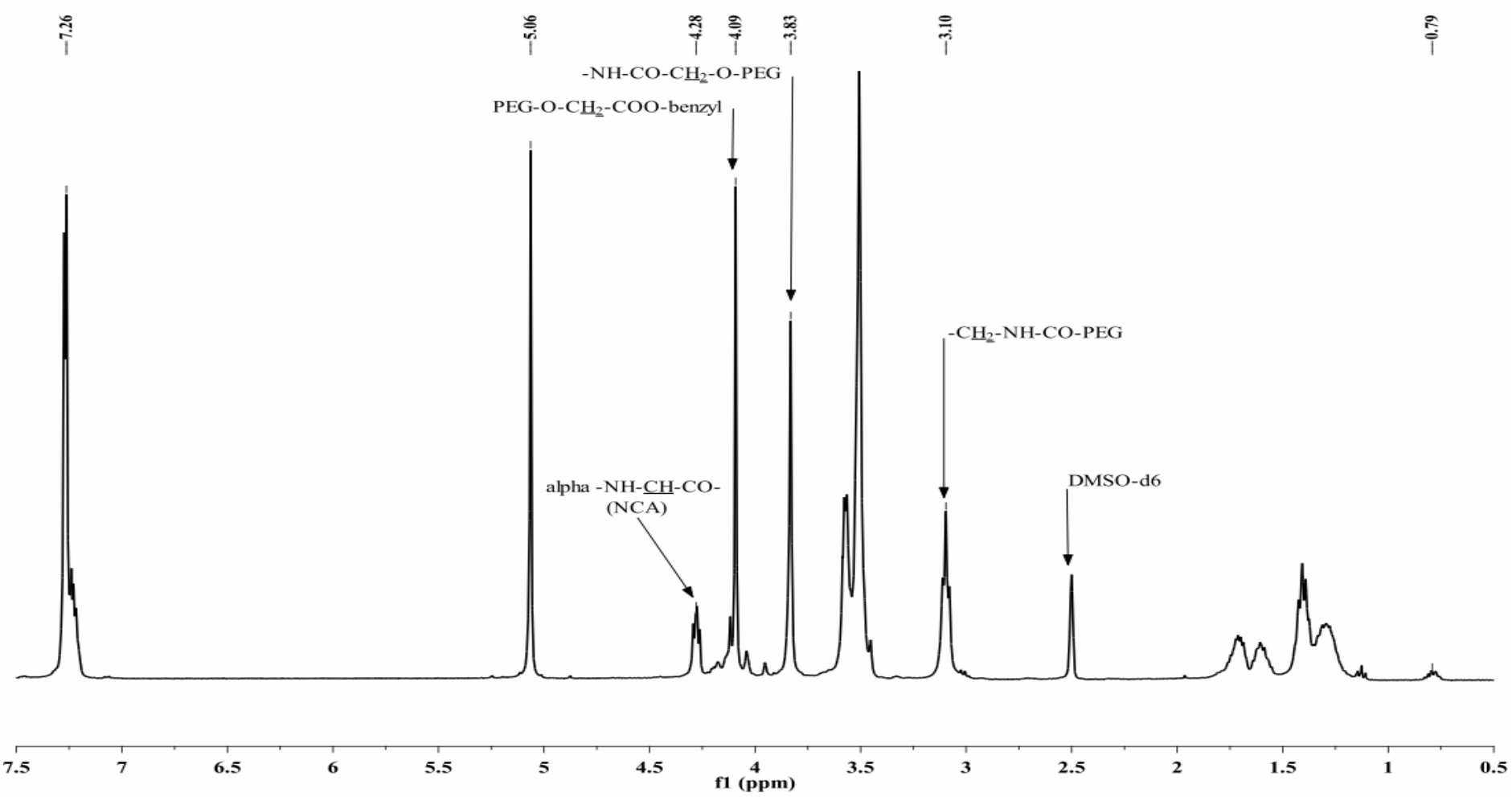


Figure 19: 400MHz 1H NMR in DMSO-d_6/TFA-d_1. Unsuccessful homopolymerization of Lys[PEG-(COOBzl)]-NCA using *n*-butylamine in a I:M ratio of 1:20.

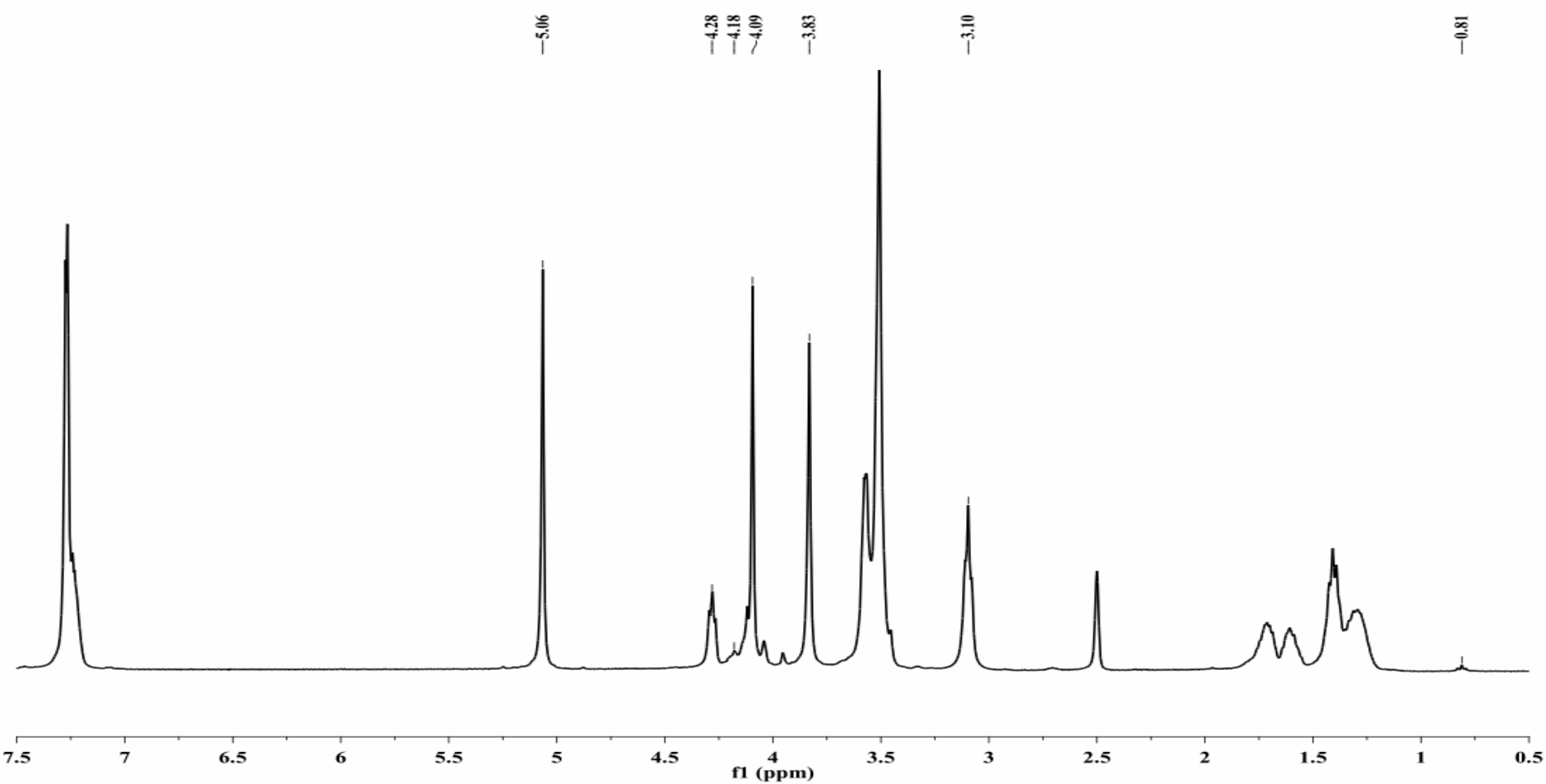


Figure 20: 400MHz 1H NMR in DMSO-d_6/TFA-d_1. Unsuccessful homopolymerization of Lys[PEG-(COOBzl)]-NCA using *n*-butylamine in a I:M ratio of 1:50.

A probable reason for the failure to polymerize at the higher I:M_1 ratio's can be ascribed to residual trace impurities still present in the monomer. At higher ratios the concentration of the initiator becomes very low and then even very little impurities can deactivate the initiator.

Table 1: Homopolymerization of Lys[PEG-(COOBzl)]-NCA using *n*-butylamine as initiator.

I.:M Ratio	A	I	0.66(I)	DP
1:5	1	0.36	0.24	4.2
1:10	1	0.13	0.09	11.1
1:20	No Reaction			
1:50	No Reaction			

Degree of Polymerization (DP) results, using equation 2 for the homopolymerization of Lys[PEG-(COOBzl)]-NCA (M^1) using *n*-butylamine as initiator (I). Results are calculated from the integration values for benzyl ester CH_2 (A) and the *n*-butylamide CH_3 (I).

5.6.2 Dendritic amine initiated polymerization[50]

A generation 1.0 dendrimer, *N*,*N*,*N'*,*N'*-tetrakis(3-aminopropyl)-1,4-butanediamine (DAB-Am-4), was also investigated as a possible macro-initiator with four primary amine groups to initiate polymerization to yield a four arm star polymer. Two initiator to monomer reaction ratios were tested: firstly five monomers to each arm and secondly 10 monomers to each arm. It is clear from 1H NMR that polymerization took place, seen by the absence of the α-CH NCA peak at 4.28ppm. Unlike the case for the homopolymers, it is not possible to find a well defined peak for the dendritic initiator against which the degree of polymerization may be determined.

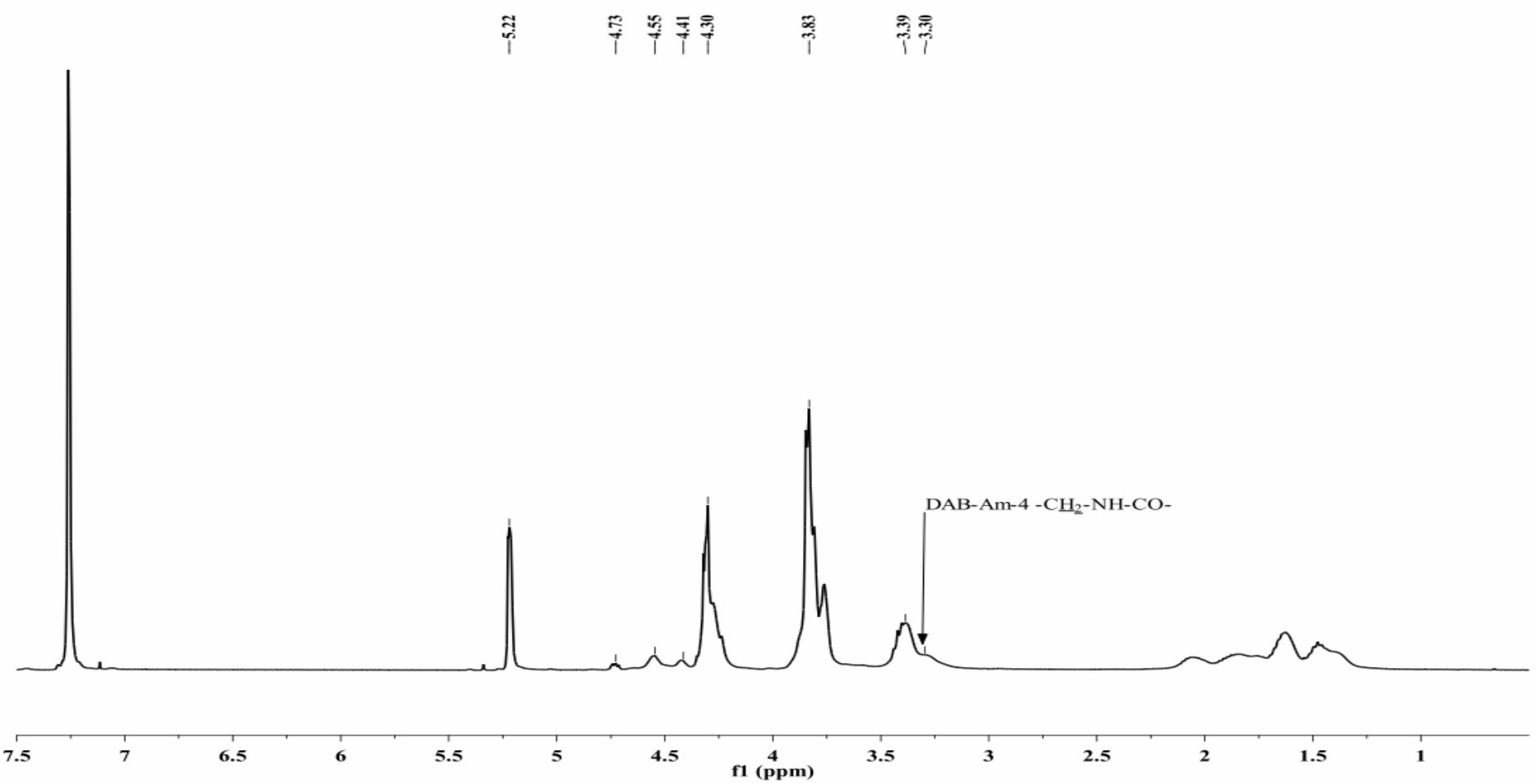


Figure 21: 400MHz 1H NMR in TFA-d_1. Dendrimer [*N*,*N*,*N'*,*N'*-tetrakis(3-aminopropyl)-1,4-butanediamine] (DAB-Am-4) initiated polymerization of Lys[PEG-(COOBzl)]-NCA using a ratio of 5 monomers per dendritic arm.

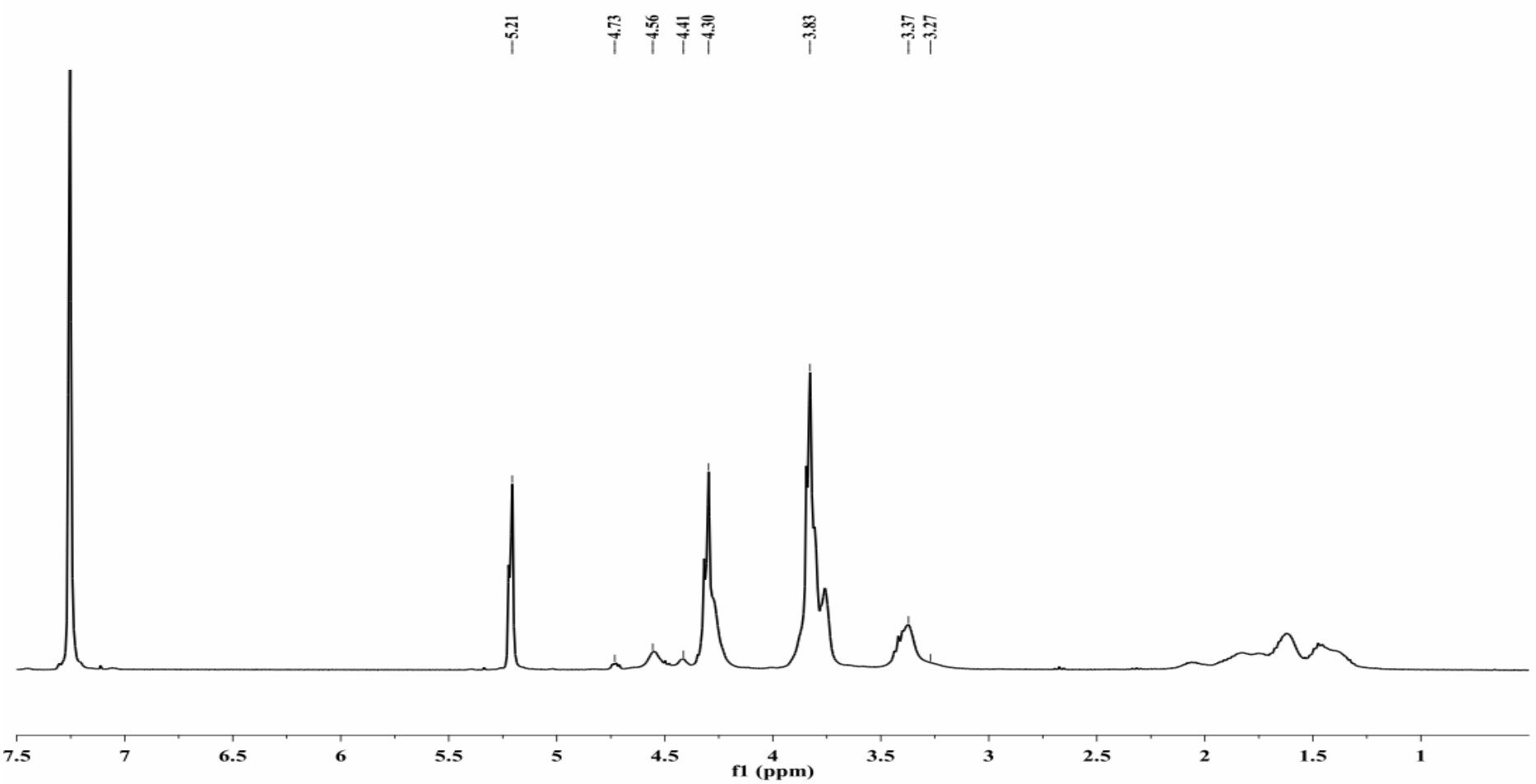


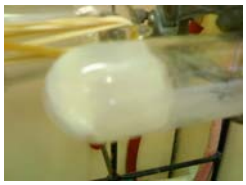
Figure 22: 400MHz 1H NMR in TFA-d_1. Dendrimer [*N*,*N*,*N'*,*N'*-tetrakis(3-aminopropyl)-1,4-butanediamine] (DAB-Am-4) initiated polymerization of Lys[PEG-(COOBzl)]-NCA using a ratio of 10 monomers per dendritic arm.

5.6.3 *n*-Butylamine initiated copolymerization of Lys[PEG-(COOBzl)]-NCA with Lys(Z)-NCA

Lastly, Lys[PEG-(COOBzl)]-NCA was tested as a monomer in the copolymerization with Lys(Z)-NCA (also synthesized using method 3, but was recrystallised three times from THF and pentane). Table 2. shows the different reaction conditions for the copolymerization. Polymerization was initiated by *n*-butylamine and the solvent was THF. An interesting observation was that after 2-3h, the reaction mixtures started to gel. The polymerization reaction containing the highest amount of Lys[PEG-(COOBzl)]-NCA was totally clear, the least rigid and dissolved the easiest in the DMSO-d_6/TFA-d_1 NMR solvent mixture. On the other hand the homopolymer of Lys(Z)-NCA was the most turbid, the most ridged and insoluble in the NMR solvent mixture (for this reason the NMR spectra was repeated in TFA-d_1 which afforded complete solubility of all the polymers and better peak resolution due to the increased solubility). The observed physical properties of the middle two reaction mixtures was in between the first and last, with an increase in turbidity and rigidity, and decrease in solubility as the Lys(Z)-NCA contents increased.

The monomer compositions of the three different copolymers compare very well with that of the calculated values. This can be seen in the NMR spectra of the polymers by dividing the integration value of the benzyl ester CH_2 (5.20 ppm) (A) of the Lys(PEG(COOBzl)) monomers into the integration value of the benzyl carbamate CH_2 (5.09 ppm) (B) of the Lys(Z) monomers.

Table 2: Summary of the Lys[PEG-(COOBzl)]-NCA and Lys(Z)-NCA composition ratios for the different *n*-butylamine initiated copolymerization.

Eq. I.	Eq. M^1	Eq. M^2	Ratio M^1:M^2	Ratio I:(M^1+M^2)	NMR Spectra	Reaction mixtures after 5H
1	5	10	1:2	1:15	Fig. 23.	
1	5	15	1:3	1:20	Fig. 24.	
1	5	20	1:4	1:25	Fig. 25.	
1	0	15		1:15	Fig. 26.	

Summary of the molar equivalents (Eq.), of the initiator, *n*-butylamine (I), Lys[PEG-(COOBzl)]-NCA (M^1) and Lys(Z)-NCA (M^2) composition ratios for the different copolymerization reactions. The photos illustrate the gelled nature of the reaction mixtures and the increased relative turbidity with an increase in the Lys(Z) composition.

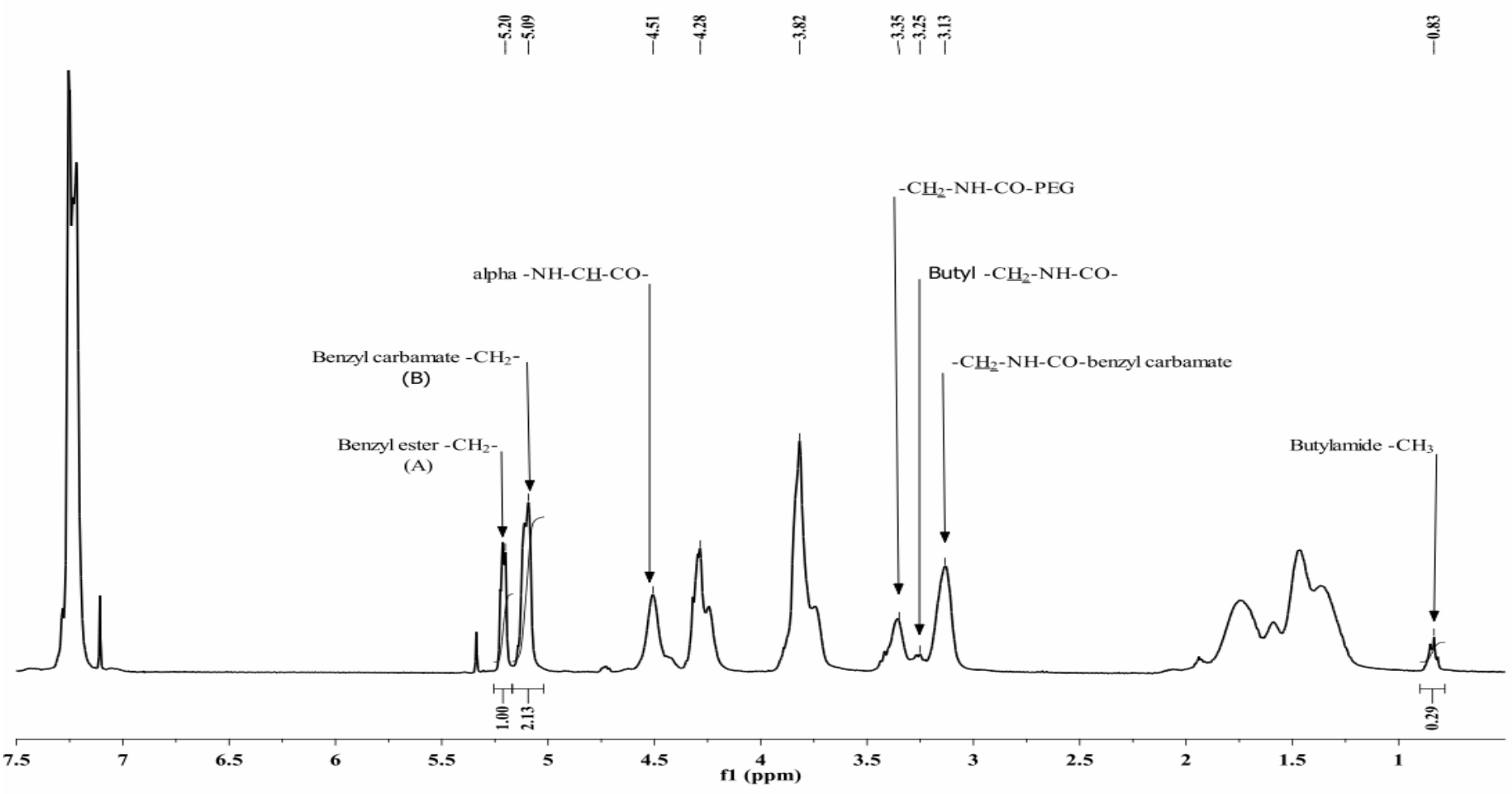


Figure 23: 400MHz 1H NMR in TFA-d_1. Copolymerization of Lys[PEG-(COOBzl)]-NCA (M^1) and Lys(Z)-NCA (M^2) in a ratio of 1:2 and with an initiator to monomer ratio [I:(M^1 + M^2)] of 1:15 (found 1:16.5).

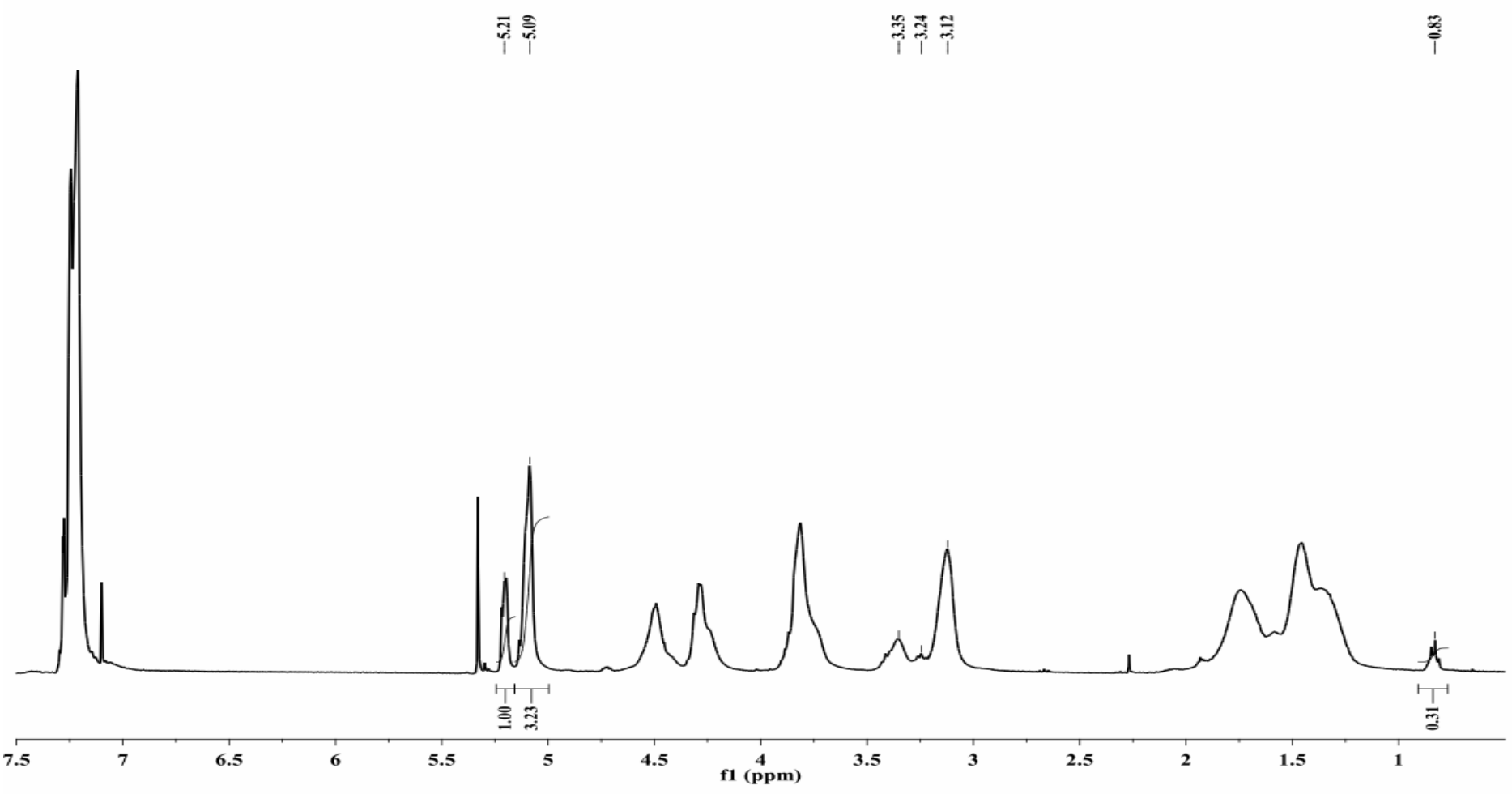


Figure 24: 400MHz 1H NMR in TFA-d_1. Copolymerization of Lys[PEG-(COOBzl)]-NCA (M^1) and Lys(Z)-NCA (M^2) in a ratio of 1:3 and with an initiator to monomer ratio [I:(M^1 + M^2)] of 1:20 (found 1:20.1).

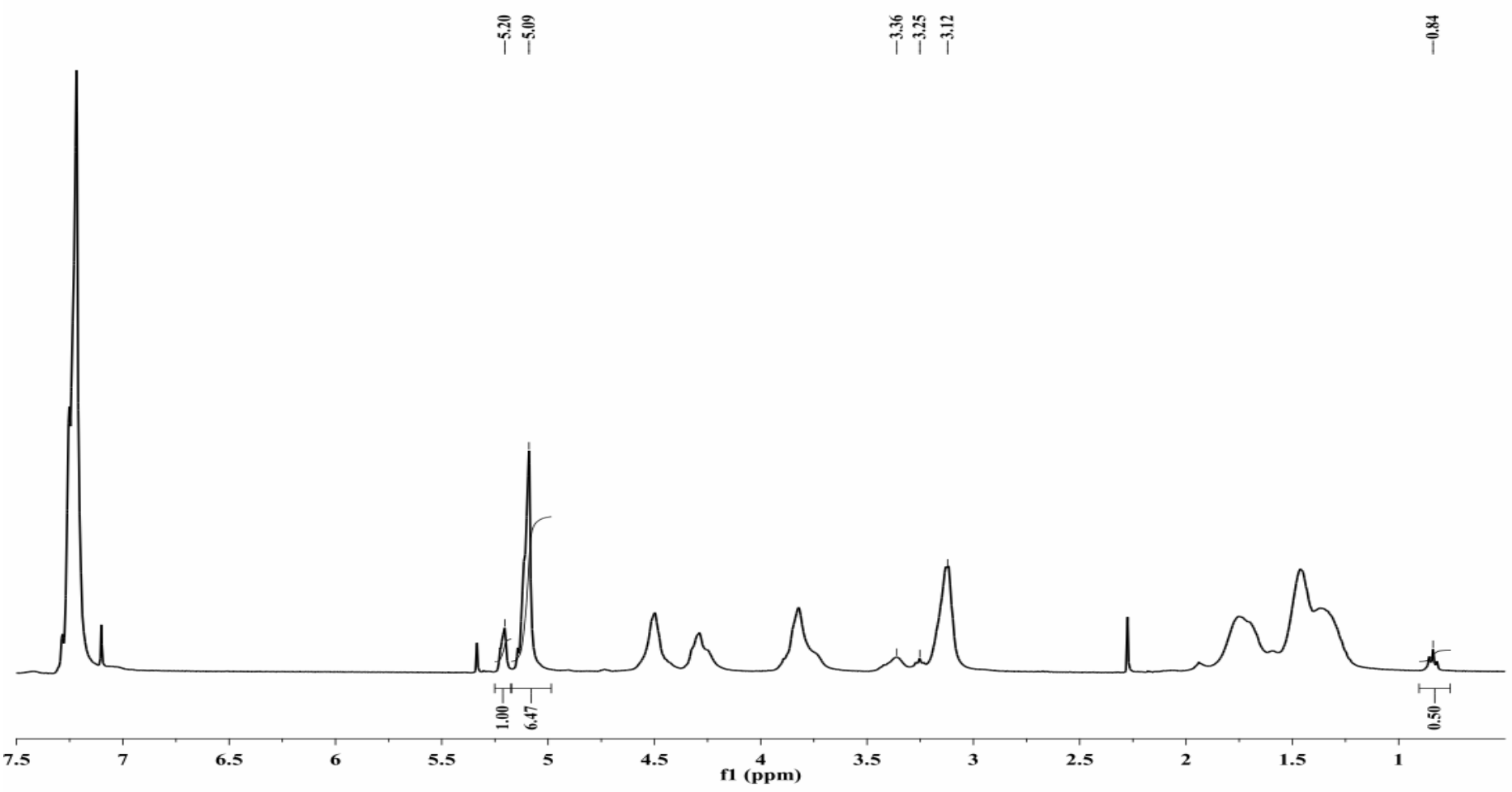


Figure 25: 400MHz 1H NMR in TFA-d_1. Copolymerization of Lys[PEG-(COOBzl)]-NCA (M^1) and Lys(Z)-NCA (M^2) in a ratio of 1:4 and with an initiator to monomer ratio [I:(M^1 + M^2)] of 1:25 (found 1:22.6).

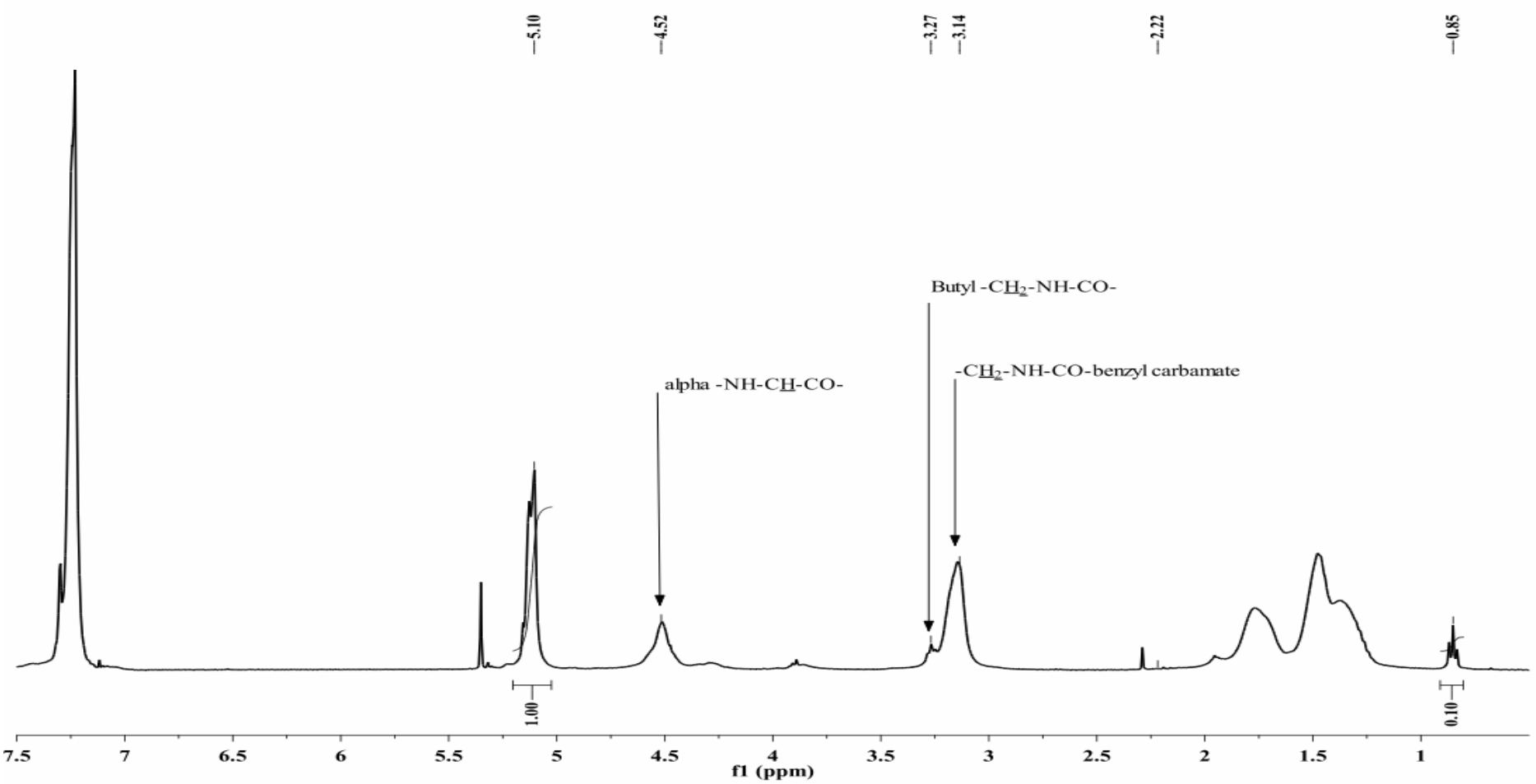


Figure 26: 400MHz 1H NMR in TFA-d_1. Homopolymerization of Lys(Z)-NCA (M^2) with an initiator to monomer ratio [I: M^2] of 1:15 (found 1:14.3).

The degree of polymerization can also be determined in the same way as with the homopolymers of Lys(PEG(COOBzl))-NCA by dividing 0.66 (integration value of *n*-butyl amide CH_3 (I) into the combined integration values of the benzyl ester CH_2 (A) and benzyloxycarbonyl (Z) CH_2 (B).

$$Degree\ of\ Polymerization\ (DP) = \frac{Integration\ value\ of\ (A+B)}{0.66\left(Integration\ value\ of\ (I)\right)}$$

Equation 3: Calculation of the the degree of polymerization (DP) through the use of integration values for the benzyl ester CH_2 (A), the benzyloxycarbonyl (Z) CH_2 (B) and the *n*-butylamide CH_3 (I)

The values in Table 3. indicate a correlation between the calculated an experimental DP values. Some peak broadening in the spectra are influencing the integration values.

Table 3: NMR results for the *n*-butylamine initiated copolymerization of Lys[PEG-(COOBzl)]-NCA and Lys(Z)-NCA.

M^2/M^1	Ratio $I:(M^1+M^2)$	A	B	I	0.66(I)	DP
2	1:15	1.00	2.13	0.29	0.19	16.5
3	1:20	1.00	3.23	0.31	0.21	20.1
4	1:25	1.00	6.47	0.50	0.33	22.6
1	1:15		1.00	0.10	0.07	14.3

Degree of polymerization (DP) was determined for the copolymerization of Lys[PEG-(COOBzl)]-NCA (M^1) and Lys(Z)-NCA (M^2), initiated by *n*-butylamine (I) the using the integration values for the benzyl ester CH_2(A), the benzyloxycarbonyl (Z) CH_2 (B) and the *n*-butylamide CH_3 (I).

These initial reactions indicate that polymerization of functionalized PEG amino acids such as Lys[PEG-(COOBzl)]-NCA are indeed possible showing good results for the shorter homopolymerization reactions. The polymerization of longer (>10) homopolymers seems problematic – the most probable reasons for this is the purity of the monomer. Further purification, through preparative HPLC, should resolve this problem. Both the copolymerization with Lys(Z)-NCA and the polymerization reactions initiated by the dendritic initiators seems very promising warranting further investigation and optimization.

5.7 Experimental:

5.7.1 Z-Lys(Fmoc)-OH (1)

Z-Lys-OH (1.402g; 5.0mmol) and sodium bicarbonate (2.100g; 25.0mmol) were dissolved together in water (50ml) and THF (15ml). Once dissolved the mixture was cooled in an ice bath and the Fmoc-Cl (1.294g; 5.0mmol) dissolved in THF (50ml) was added slowly to the lysine solution. Thereafter the reaction was removed from the ice and stirred for 3h at RT. The THF was removed under vacuum and more water (50ml) was added. The solution was acidified to pH = 2 -3, using a dilute (3M) HCl solution. The aqueous solution was extracted with dichloromethane DCM (3 x 75ml). The combined DCM fractions were dried over anhydrous sodium sulphate and the DCM solution concentrated to about 10ml after which pentane (50ml) was added. A white precipitate formed which was filtered off and washed with pentane and dried under vacuum. Yield Z-Lys(Fmoc)-OH (2.513g; 94%). **^{1}H NMR (400MHz) in DMSO-d_6.** δ 7.88 (d; 2H; J = 7.5Hz; Fmoc) 7.68 (d; 2H; J = 7.4Hz; Fmoc), 7.45 – 7.22 (m; 9H; Fmoc & Bzl), 5.03 (m; 2H; CH_2-Bzl), 4.30 (d; 2H; J = 6.9; CH_2-Fmoc), 4.21 (t; 1H; J = 6.7Hz; CH-Fmoc), 3.92 (m; 1H; NH-C<u>H</u>-CO), 2.96 (m; 2H; CH_2-C<u>H</u>$_2$-NH-Fmoc), 1.79 – 1.11 (m; 6H; CH_2-CH_2-CH_2-). **^{13}C:** 173.90 (COOH), 156.13 (COOBzl), 156.01 (COOFmoc), 143.87 & 140.67 (Arm. Fmoc), 136.94 (Arm. Bzl), 128.26, 127.73, 127.65, 127.52 & 126.97 (Arm. Fmoc & Bzl), 125.07 & 120.03 (Arm. Fmoc), 65.33 (CH_2-Bzl), 65.10 (CH_2-Fmoc), 53.78 (NH-<u>C</u>H-CO-), 46.73 (CH_2-<u>C</u>H$_2$-NH-Fmoc), 30.37, 28.86 & 22.81 (CH_2-CH_2-CH_2-). **FTIR (υ in cm^{-1})**: 1718 (C=O carboxylic acid; s), 1692 (C=O Z; m), 1658 (C=O Fmoc; m).

5.7.2 SPS procedure 1: Using Nova-Syn TGA resin (0.25mmol/g)

A solid phase synthesis (SPS) reaction vessel was loaded with Nova-Syn TGA resin (0.25mmol/g; 2.00g; 0.5mmol). The resin was swollen for 1h in DMF (25ml) before being washed with DMF [3 x 10ml (bed volume)]. Z-Lys(Fmoc)-COOH (0.7539g; 1.5mmol) dissolved in DMF (15ml) was added to the resin followed by the addition of PyBOP (0.7805g; 1.5mmol) dissolved in minimum amount of DMF. This reaction was shaken for 2h at RT. The solution was filtered off and the resin washed with DMF (4 x bed volumes). The Fmoc was cleaved by the addition of a 20% piperidine

in DMF solution (25.00ml) and shaken for 1h at RT. (After this time a sample of the solution was taken to measure the coupling of the amino acid to the resin by measuring the UV absorbance of the cleaved fulvene from cleaved Fmoc protecting group (Equation 1). The measured UV absorbance was 0.0143 which calculated to 0.023mmol/g coupling of the amino acid to the resin). The resin was washed with DMF (4 x bed volumes).

In a separate flask PEG-$(COOH)_2$ (1.875g; 5.0mmol) was dissolved with *N*-hydroxy succinimide (1.151g; 10.0mmol) in DMF (10ml). DCC (2.064g; 10mmol) in DMF (10ml) was added slowly to the PEG solution. A white DCU precipitate started to form after a few minutes. This reaction was stirred for 3h at RT and left to stand for 1h in the fridge before the DCU was removed by filtration.

This PEG-$(COOSu)_2$ solution was added to the resin containing the now deprotected ε-NH lysine functionality, *N*-methyl morpholine (NMM) (140 μL) was also added to the reaction. This was shaken for 16h before dry methanol (1ml) was added to the reaction mixture and shaken for another 3h at RT. The reaction mixture was filtered off and the cleaving protocol started by first washing the resin with dichloromethane (3 x 10ml). This was followed by *tert*-amyl alcohol (10ml), acetic acid (10ml), *tert*-amyl alcohol (10ml), and diethyl ether (4 x 10ml). The resin was dried under vacuum for 16h over phosphorous pentoxide. The resin was then treated with 95% trifluoroacetic acid (TFA)/water solution (20ml) for 3h after which the resin was washed with TFA (3 x 10ml). All the TFA fractions were collected and the acid removed under vacuum before precipitated by addition to diethyl ether.

NMR showed that hardly any reaction had taken place. This was also reflected in the low value for the coupling of the lysine to the resin.

5.7.3 SPS procedure 2: Using Paramax Wang resin (1.2mmol/g)

Z-Lys(Fmoc)-COOH (1.5077g; 3.0mmol) was dissolved in DCM (20ml) after which thionyl chloride (2.18ml; 30.0mmol) was added to the solution and followed by a catalytic amount of DMF (200μl;). The reaction was stirred for 2h at RT before the DCM and other volatiles were removed under vacuum. The residue was again

dissolved in a minimum amount of DCM and added to pentane. A white sticky residue precipitated, the supernatant was decanted and the residue dried under vacuum. The residue was treated with pentane and left to stand overnight in the freezer. The supernatant was decanted from the white residue and the residue dried under vacuum.

The residue was dissolved in DMF (30ml) and added to a SPS reaction vessel containing pre-swollen Wang resin (1.2mmol/g; 0.500g; 0.6mmol), followed by the addition of hydroxybenzotriazole (HOBt) (0.4054g; 3.0mmol) and *N*-methyl morpholine (NMM) (330μL; 3.0mmol). This reaction was shaken for 2h at RT followed by washing with DMF (4 x 15ml). The Fmoc protecting group was removed by treating the resin with 20% piperidine/DMF solution (15.00ml) 1h at RT. After this a sample of the solution was taken to measure the coupling of the amino acid to the resin by measuring the UV absorbance of the fulvene from cleaved Fmoc protecting group. The measured UV absorbance was 0.0625 which calculated to 0.24mmol/g coupling of the amino acid to the resin.

Again the loading efficacy was very low – the synthesis was not continued.

5.7.4 Z-Lys(HTos)-OBzl (3)

A solution of Z-Lysine (5.016g; 18.0mmol), *p*-toluene sulphonic acid (5.140g; 27mmol) and benzyl alcohol (10ml) in benzene (100ml) was refluxed, using a Dean-Stark water trap, for 4h when no more water was eliminated. The benzene and benzyl alcohol were removed under vacuum and the residue treated with diethyl ether (100ml). An oily precipitate formed. Ethanol was added to the mixture until the solution became homogeneous. White crystals formed upon standing overnight in the freezer which were filtered off. The filtrate was reduced under vacuum and the residue dissolved in a minimum amount of ethanol and added to diethyl ether to form a white precipitate. This was repeated for a total of three times. The combined white precipitates were recrystallised by dissolution in a minimum amount of boiling ethanol and the addition of this solution to diethyl ether. The white precipitate was filtered and dried under vacuum for 16h. Yield Z-Lys(Tos)-OBzl (7.552g; 77%). **^{1}H NMR (400MHz) in DMSO-d_6.** δ 7.51 (m; 2H; Tos), 7.37 (m; 10H; Arm. Bzl & Z),

7.13 (d; 2H; J (Hz) = 7.9; Tos), 5.14 (m; 2H; CH_2-Bzl ester), 5.05 (m; 2H; CH_2-Bzl carbamate), 4.07 (m; 1H; NH-CH-CO), 2.73 (m; 2H; CH_2-CH_2-$NH_3^+Tos^-$), 2.29 (s; CH_3; Tos)1.85 – 1.25 (m; 6H; CH_2-CH_2-CH_2). **^{13}C NMR (75MHz):** 172.12 (COOBzl), 156.13 (COOBzl carbamate), 145.23 & 136.77 (Arm. Ipso C(C) Tos), 137.81 (Arm. Ipso C(C) Z), 135.85 (Arm. Ipso C(C) Bzl), 128.44, 128.36, 123.29, 128.21, 128.06, 127.99, 127.80, 127.71 & 125.40 (Arm. C(H) Tos; Z; Bzl), 65.88 (CH_2-Bzl carbamate), 65.49 (CH_2-Bzl ester), 53.79 (NH-CH-CO), 38.52 (CH_2-CH_2-$NH_3^+Tos^-$), 29.94, 26.32 & 22.28 (-CH_2-CH_2-CH_2-), 20.71 (CH_3; Tos). **FTIR (υ in cm^{-1}):** 1731 (C=O benzyl ester; m), 1689 (C=O Z; m).

5.7.5 (HOOC)-PEG-(COOPhEt/Bzl) (4)

These conditions can be applied for reaction scales from 5g to 30g PEG-$(COOH)_2$. The procedure is outlined for a 5g synthesis.

PEG-$(COOH)_2$ (5g), 2-phenylethyl alcohol or benzyl alcohol (1 equivalent) and the strong cation exchange resin, Amberlite IR120 (Plus) 4.4 meq/g, (1g) were suspended in benzene (100ml) and azeotropically distilled under Dean-Stark conditions for at least 3h by which time the water stopped condensing. The resin was filtered and the solvent evaporated under vacuum. The residue was dissolved in a minimum amount of the chromatographic elution mixture, which was 7.5:2:0.5 ethanol:hexane:triethylamine.

Work-up through flash chromatography: A flash column was prepared by packing ~50mm ID column with silica gel (230-400mesh), suspended in the elution mixture, to a height of ~20cm. The top of the column was capped with a 2cm layer of sand. The product solution was applied to the sand layer and the product mixture separated by eluting under a constant pressure of about 30mBar. Fractions were collected in 75ml portions.

The middle fractions, PEG mono-ester (R_f = 0.26) was collected and combined and the solvent removed under vacuum. The residue was dissolved in distilled water (150ml). The water layer was acidified by carefully adding concentrated HCl to a pH = 2 – 3 (universal indicator strips). At this lower pH the product starts to separate

from the water. The water layer was extracted with ethyl acetate (3 x 50ml). The combined product and ethyl acetate fractions were again washed with water (1 x 50ml) before being dried over magnesium sulphate.

Work-up through the extraction protocol: After the esterfication reaction was completed, the resin removed and the solvent evaporated, the residue was dissolved in 100ml ethyl acetate. The organic solution was washed with water (3 x 30ml) – the combined water fractions tested acidic (pH = 3) and were discarded. The organic layer was again extracted with a sodium bicarbonate solution (3 x 30ml; 0.5M). The organic layer was now discarded and the combined water fractions acidified by the careful addition of concentrated HCl to a pH 2 – 3. The water solution turned murky to white, as an emulsion formed, as the acid was added. The water solution was then extracted with ethyl acetate (3 x 30ml). The combined organic fractions were again washed with water (1 x 50ml) before being dried over anhydrous magnesium sulphate.

In both the workup procedures the yields were the same with about 3g final product starting with 5g PEG-$(COOH)_2$. **^{1}H NMR (400MHz) in DMSO-d_6.** δ For (HOOC)-PEG-(COOPhEt): 7.26 – 7.18 (m; 5H; Arm. Phenyl), 4.29 (t; 2H; J (Hz) = 6.9; CH_2-CH_2-Ph), 4.09 (s; 2H; O-CH_2-COO-PhEt), 4.02 (s; 2H; O-CH_2-COOH), 3.62 – 3.44 (m; 8H; -O-CH_2-CH_2-O-CH_2-CH_2-O), 2.90 (t; 2H; J (Hz) = 6.9; CH_2-CH_2-Ph). **^{13}C NMR (75MHz):** 171.59 (O-CH_2-COOH), 170.05 (O-CH_2-COOPhEt), 137.77; 128.79; 128.39; 126.34 (Arm. Phenyl), 69.93 (O-CH_2-COOPhEt), 69.79 (O-CH_2-COOH), 69.62; 69.66; 67.62; 67.56 (O-CH_2-CH_2-O-CH_2-CH_2-O), 64.49 (CH_2-CH_2-Ph), 34.28 (CH_2-CH_2-Ph). δ For (HOOC)-PEG-(COOBzl): 7.40 – 7.30 (m; 5H; Arm. Bzl), 5.15 (s; 2H; CH_2-Ph), 4.19 (s; 2H; O-CH_2-COOBzl), 4.01 (s; 2H; O-CH_2-COOH), 3.65 – 3.50 (m; 8H; O-CH_2-CH_2-O-CH_2-CH_2-O). **^{13}C NMR (75MHz):** 171.56 (O-CH_2-COOH), 170.02 (O-CH_2-COOBzl), 135.79; 128.38; 128.05; 126.01 (Arm. Bzl), 69.98 (O-CH_2-COOBzl), 69.75 (-O-CH_2-COOH), 69.61; 69.59; 67.66; 67.51 (O-CH_2-CH_2-O-CH_2-CH_2-O-), 65.52 (CH_2-Ph).

5.7.6 Z-Lys[PEG-(COOPhEt)]-OBzl (5)

Z-Lys(Tos)-OBzl (2.227g; 6.8mmol) and pyridine (1.10ml; 14.0mmol) were dissolved in THF (25ml) at 40^{o}C and allowed to cool to ambient temperature. (HOOC)-PEG-(COOPhEt) (3.360g; 7.0 mmol), dissolved in THF (10ml) was added to the lysine solution. DCC dissolved in THF (20ml) was slowly added, by means of a dropping funnel, to the reaction mixture. After 15 minutes a white precipitate (DCU and pyridinium tosylate) started to form. The reaction was stirred at room temperature for 4h after which it was left to stand overnight in the refrigerator. The precipitated DCU and pyridinium tosylate were filtered off and the filtrate concentrated under vacuum. Upon evaporation more DCU formed, this was again removed by filtration. The process was repeated until no more DCU formed. After filtration of the DCU a TLC (8:2 ethyl acetate : methanol; $R_{F(DCU)}$ = 0.01 and $R_{F(Product)}$ = 0.44) of the solution showed that there was still traces of DCU left in solution. The traces of DCU were removed by flash chromatography. Yield Z-Lys[PEG-(COOPhEt)]-OBzl (1.80g; 40%). **^{1}H NMR (400MHz) in DMSO-d_6.** δ 7.45 – 7.15 (m; 15H; Arm. PhEt, Z, Bzl), 5.12 (m; 2H; CH_2-Bzl ester), 5.03 (m; 2H; CH_2-Bzl carbamate), 4.28 (t; 2H; J (Hz) = 6.9; C$\underline{H}_2$-CH_2-Ph), 4.08 (s; 2H; O-CH_2-COOPhEt), 4.04 (m; 1H; NH-C$\underline{H}$-CO), 3.84 (s; 2H; O-C$\underline{H}_2$-CONH-CH_2), 3.6 – 3.47 (m; 8H; O-CH_2-CH_2-O-CH_2-CH_2-O), 3.06 (m; 2H; -CH_2-C$\underline{H}_2$-NH-CO-), 2.89 (t; 2H; J (Hz) = 6.9; CH_2-C$\underline{H}_2$-Ph), 1.80 – 1.20 (m; 6H; CH_2-CH_2-CH_2-). **^{13}C NMR (75MHz):** 172.23 (COOBzl ester), 169.99 (O-CH_2-$\underline{C}$OOPhEt), 168.91 (O-CH_2-$\underline{C}$O-NH-CH_2), 156.11 (COOBzl carbamate), 137.71 (Arm. Ipso $\underline{C}$(C); Z), 136.80 (Arm. Ipso $\underline{C}$(C); PhEt), 135.87 (Arm. Ipso $\underline{C}$(C); Bzl), 128.74, 128.33, 128.27, 127.94, 127.75, 127.66, 127.65, 126.31 (Arm. $\underline{C}$(H) PhEt; Z; Bzl), 70.12 (O-$\underline{C}H_2$-COOPhEt), 69.89 (O-$\underline{C}H_2$-CO-NH-CH_2), 69.88; 69.58; 69.37; 67.56 (O-CH_2-CH_2-O-CH_2-CH_2-O), 65.79 (CH_2-Bzl carbamate), 65.43 (CH_2-Bzl ester), 64.45 ($\underline{C}H_2$-CH_2-Ph), 53.93 (NH-$\underline{C}$H-CO), 37.69 (CH_2-$\underline{C}H_2$-NH-CO-CH_2), 34.22 (CH_2-$\underline{C}H_2$-Ph), 30.21, 28.61, 22.76 (CH_2-CH_2-CH_2-).

5.7.7 Z-/(tBoc)-Lys[PEG-(COOPhEt)]-OH and (tBoc)-Lys[PEG-(COOBzl)]-OH (6)

Reaction conditions are the same for all four variations of the PEG modified α-*N*-protected lysine, thus a general synthetic procedure is given here for a scale of 1 – 2g Z- or tBoc-protected lysine.

(HOOC)-PEG-(COOPhEt/Bzl) (1.1eq.) and *N*-hydroxysuccinimide (1.2eq.) were dissolved in THF (30ml). DCC (1.2eq.) was dissolved in THF (10ml) and added drop wise to the PEG solution. After about 5 min DCU started precipitating. The reaction was stirred at RT for 3h before the DCU was removed by filtration and the THF evaporated under vacuum, more DCU formed, which was filtered off and the filtrate concentrated – this process was repeated three times. Any remaining DCU trapped in the crude (SuOOC)-PEG-(COOPhEt/Bzl) was separated out completely in the work-up procedure of the second step of the synthesis.

α-*N*-tBoc (Z)-Lysine (1eq) and $NaHCO_3$ (2eq) were dissolved in 50% THF/water (50ml) and (SuOOC)-PEG-(COOPhEt/Bzl) (1.1eq.) dissolved in THF (20ml) was added drop wise to the lysine solution with the formation of CO_2 gas. The reaction was stirred for another 2h at RT after the gas formation ceased (as monitored by an oil bubbler connected to the reaction vessel). The THF was removed under vacuum and the resulting water solution was diluted to 100ml. (At this point the remainder of the DCU, carried through from the (SuOOC)-PEG-(COOPhEt/Bzl) synthesis, precipitate out – this was removed by filtration.) The water solution was acidified to pH = 2 – 3 by the careful addition of concentrated HCl. With the addition of the acid, the solution became murky and white as the product separated out of solution. The acidified water solution was extracted with ethyl acetate (3 x 30ml). (Note: The use of ethyl acetate was preferred over the use of dichloromethane or chloroform as extracting solvent, as the latter two tend to form emulsions that complicate and contaminate the extraction process.) The combined ethyl acetate fractions were dried over $MgSO_4$. The solvent was removed under vacuum and the product dried as a viscous, yellow oil under high vacuum at 40°C for 24h. Yields of Z-Lys[PEG-(COOPhEt)]-OH, tBoc-Lys[PEG-(COOPhEt)]-OH and (tBoc)-Lys[PEG-(COOBzl)]-OH were usually near quantitative.

^{1}H NMR (400MHz) in DMSO-d_6/TFA-d_1. δ For Z-Lys[PEG-(COOPhEt)]-OH: 7.35 – 7.10 (m; 10H; Arm. PhEt, Z), 5.00 (m; 2H; CH_2-Bzl carbamate), 4.25 (t; 2H; J (Hz) = 6.7; CH_2-CH_2-Ph), 4.03 (s; 2H; O-CH_2-COOPhEt), 3.94 (m; 1H; NH-CH-CO), 3.84 (s; 2H; O-CH_2-CONH-CH_2), 3.65 – 3.42 (m; 8H; O-CH_2-CH_2-O-CH_2-CH_2-O), 3.09 (m; 2H; CH_2-CH_2-NH-CO), 2.85 (t; 2H; J (Hz) = 6.7; CH_2-CH_2-Ph), 1.77 – 1.22 (m; 6H; CH_2-CH_2-CH_2-). **^{13}C NMR (75MHz):** 174.77 (COOH), 170.77 (O-CH_2-COOPhEt), 170.15 (O-CH_2-CO-NH-CH_2), 156.98 (COOBzl carbamate), 138.56 (Arm. Ipso C(C); Z), 137.80 (Arm. Ipso C(C); PhEt), 129.49, 128.99, 128.96, 128.43,127.03 (Arm. C(H); PhEt; Z), 71.07 (O-CH_2-COOPhEt), 70.79 (O-CH_2-CO-NH-CH_2), 70.58, 70.49, 70.25, 68.39 (O-CH_2-CH_2-O-CH_2-CH_2-O), 66.24 (CH_2-Bzl carbamate), 65.25 (CH_2-CH_2-Ph), 54.51 (NH-CH-CO), 38.52 (CH_2-CH_2-NH-CO-CH_2), 35.10 (CH_2-CH_2-Ph), 31.17, 29.41, 23.67 (CH_2-CH_2-CH_2).

^{1}H NMR (400MHz) in DMSO-d_6. δ For tBoc-Lys[PEG-(COOPhEt)]-OH: 7.31 – 7.14 (m; 5H; Arm. PhEt), 4.29 (t; 2H; J (Hz) = 6.9; CH_2-CH_2-Ph), 4.09 (s; 2H; O-CH_2-COO-PhEt), 3.85 (s; 2H; O-CH_2-CONH-CH_2), 3.80 (m; 1H; NH-CH-CO) 3.65 – 3.45 (m; 8H; O-CH_2-CH_2-O-CH_2-CH_2-O), 3.05 (m; 2H; CH_2-CH_2-NH-CO), 2.90 (t; 2H; J (Hz) = 6.9; CH_2-CH_2-Ph), 1.81 – 1.20 (m; 6H; CH_2-CH_2-CH_2), 1.37 [s; 9H; C-$(CH_3)_3$; tBoc]. **^{13}C NMR (75MHz):** 174.14 (COOH), 169.97 (O-CH_2-COOPhEt), 168.88 (O-CH_2-CO-NH-CH_2), 155.51 (COOtBoc carbamate), 137.70 (Arm. Ipso C(C); PhEt), 128.74, 128.27, 126.30 (Arm. C(H); PhEt), 77.94 [C-$(CH_3)_3$], 70.24 (O-CH_2-COO-PhEt), 70.01 (O-CH_2-CO-NH-CH_2), 69.98, 69.70, 69.66, 69.49 (O-CH_2-CH_2-O-CH_2-CH_2-O-), 64.55 (CH_2-CH_2-Ph), 53.35 (NH-CH-CO), 37.77 (CH_2-CH_2-NH-CO-CH_2), 34.24 (CH_2-CH_2-Ph), 28.12 [C-$(CH_3)_3$] 30.36, 28.73, 22.97 (CH_2-CH_2-CH_2).

^{1}H NMR(400MHz) in DMSO-d_6. δ For tBoc-Lys[PEG-(COOBzl)]-OH: 7.40 – 7.29 (m; 5H; Arm. Bzl), 5.15 (s; 2H; CH_2-Ph), 4.19 (s; 2H; O-CH_2-COOBzl), 3.84 (s; 2H; O-CH_2-CONH-CH_2), 3.81 (m; 1H; NH-CH-CO), 3.67 – 3.49 (m; 8H; O-CH_2-CH_2-O-CH_2-CH_2-O), 3.07 (m; 2H; CH_2-CH_2-NH-CO), 1.68 – 1.17 (m; 6H; CH_2-CH_2-CH_2), 1.37 [s; 9H; C-$(CH_3)_3$; tBoc]. **^{13}C NMR (75MHz):** 174.13 (COOH), 169.97 (O-CH_2-COOBzl), 168.86 (O-CH_2-CO-NH-CH_2), 155.50 (COOtBoc carbamate), 135.74 (Arm. Ipso C(C) Bzl), 128.74, 128.35, 128.03, 127.98 (Arm. C(H) Bzl), 77.82 [C-

($CH_3)_3$], 70.12 (O-$\underline{C}H_2$-COOBzl), 69.97 (O-$\underline{C}H_2$-CO-NH-CH_2), 69.87, 69.60, 69.37, 67.63 (O-CH_2-CH_2-O-CH_2-CH_2-O), 65.51 (CH_2-Ph), 53.33 (NH-$\underline{C}$H-CO), 37.74 (CH_2-$\underline{C}H_2$-NH-CO-CH_2), 28.11 [C-($\underline{C}H_3)_3$], 30.34, 28.71, 22.95 (CH_2-CH_2-CH_2).

5.7.8 Synthesis of PEG_{1000}-$(COOH)_2$

5.7.8.1 Preparation of Jones' reagent.

Chromium oxide (CrO_3) (70.0g; 0.7mol) was dissolved in distilled water (500ml) and stirred for 10 min. The reaction flask was placed in an ice bath and concentrated sulphuric acid (61ml) was added slowly.

5.7.8.2 Oxidation of PEG_{1000}-$(OH)_2$ to PEG_{1000}-$(COOH)_2$.

PEG_{1000} (5.00g) was dissolved in acetone (50ml), distilled from $KMnO_4$. Jones' Reagent (17.00ml; 1.25M) was added all at once to the acetone solution. A blue-green precipitate formed and the temperature of the solution started rising exothermically. Activated carbon (5g) was added to the solution and stirred overnight. The carbon was removed by filtration through Celite. This process was repeated until the solution became clear. The acetone was removed under vacuum after which the water phase was removed by azeotropic distillation with benzene; the benzene was then removed under vacuum. The residue that remained was dissolved in a small quantity of THF and precipitated by addition to cold diethyl ether. The ether layer was then decanted the process repeated twice before drying the final product overnight in a vacuum desiccator. Yield $PEG_{1000}(COOH)_2$ (4.23g). **^{1}H NMR (400MHz) in DMSO-d_6:** δ 4.01 (s; 4H; O-C$\underline{H}_2$-COOH), 3.51 (m; 80H; O-CH_2-CH_2-O). **^{13}C NMR (75MHz):** 172.05 (O-CH_2-$\underline{C}$OOH); 69.91 (-O-CH_2-CH_2-O); 67.67 (O-$\underline{C}H_2$-COOH). **FTIR (υ in cm^{-1}):** 1710 (C=O; vs).

5.7.9 PEG_{1000}-$(COOSu)_2$

PEG_{1000}-$(COOH)_2$ (3.625g; 3.5mmol) and hydroxy succinimide (1.611g; 14mmol) were dissolved in THF (35ml). DCC (2.889g; 14mmol) dissolved in THF (20ml) was added drop wise to the reaction mixture. A white precipitate (DCU) rapidly formed.

The reaction was stirred at RT for 4h, after which it was left to stand overnight in the refrigerator. The precipitate was filtered off and the THF removed from the filtrate under vacuum. The residue was redissolved in THF (30ml), more precipitate formed which was filtered off again and the solvent removed from the filtrate under vacuum. This process was repeated, with decreasing amounts of solvent, until no precipitate formed. The residue, dissolved in a minimum volume THF, was precipitated by addition to cold diethyl ether. This was left in the refrigerator overnight for complete precipitation. The clear, supernatant diethyl ether was decanted and the product dried under vacuum overnight. Yield $PEG_{1000}(COOSu)_2$ (4.038g; 94%). **^{1}H NMR (400MHz) in DMSO-d_6:** δ 4.63 (s; 4H; O-CH_2-COSu), 3.75 – 3.47 (m, 80H, O-CH_2-CH_2-O-), 2.85 (s; 8H; CO-CH_2-CH_2-CO). **^{13}C NMR (75MHz):** 170.37 (O-CH_2-COSu), 166.92 (CO-CH_2-CH_2-CO), 70.40; 69.79; 69.57 (O-CH_2-CH_2-O-), 65.72 (O-CH_2-COSu), 25.30 (CO-CH_2-CH_2-CO).

5.7.10 General method of NCA synthesis (0.5g scale synthesis)

5.7.10.1 Method 1

Lys[PEG-(COOBzl)]-NCA (8). Z-L-Lys[PEG-(COOPhEt)]-OH or tBoc-L-Lys[PEG-(COO-PhEt/Bzl)]-OH was dissolved in dichloromethyl methyl ether (DCMME) (3 – 5ml) in a small Schlenk tube under argon. The cap was replaced and the tube evacuated. The solution was then stirred, under vacuum, at 60^{o}C for 30min and then at RT for 2h. The DCMME was then removed under vacuum. The residue was redissolved in a minimum amount of THF followed by precipitating the product by addition to pentane. The clear, supernatant pentane was decanted and the residue and the residual pentane removed under vacuum before the residue was redissolved in a minimum amount of THF – this was repeated three times.

5.7.10.2 Method 2

Lys[PEG-(COOBzl)]-NCA (8). tBoc-L-Lys[PEG-(COO-PhEt/Bzl)]-OH (1eq.) was dissolved in THF/ethyl acetate (20ml) under argon. Triphosgene (0.33eq) dissolved in THF/ethyl acetate (10ml) was added to the reaction mixture. This mixture was cooled to 0^{o}C and NMM or TEA (1eq.) dissolved in THF/ethyl acetate (10ml) was

added drop wise, under argon, via a dropping funnel. After all the base was added the mixture was allowed to warm to RT and stirred at this temperature for 16h. The reaction mixture was again cooled in an ice bath to 0°C for 30min after which the white HCl salt of the organic base was removed by filtration. Upon evaporation of the solvent from the filtrate more salt formed which was again filtered off– this process was repeated at least 3 – 5 times to remove at least 99% of the salt.

5.7.10.3 Method 3

***L*-Lys[PEG-(COOBzl)]-OH (7)**. tBoc-*L*-Lys[PEG-(COOBzl)]-OH was dissolved in dichloromethane (5ml) and cooled to 0°C and trifluoroacetic acid (5ml) added to this solution and stirred at this temperature for 15 min and then 45 min at RT. The volatiles were subsequently removed under vacuum and the resulting residue was dissolved in a minimum of THF and added to diethyl ether in a centrifuge tube. This mixture was cooled down in liquid nitrogen until a precipitation formed. The cold mixture was then centrifuged and the supernatant removed. The precipitates from the first precipitation cycles were yellow viscous oils. After about four - five cycles the product became a white, solid gel layer with an increasing volume at the bottom of the centrifuge tube. After the supernatant was removed, the white gel layer was dried under vacuum to yield a clear amorphous final L-Lys[PEG-(COO-Bzl)]-OH product. **^{1}H NMR (400MHz) in DMSO-d_6/TFA-d_1.** δ 7.34 – 7.22 (m; 5H; Arm. Bzl), 5.10 (s; 2H; CH_2-Ph), 4.13 (s; 2H; O-CH_2-COOBzl), 3.88 – 3.79 (m; 3H; O-C<u>H</u>$_2$-CONH-CH_2 & NH-C<u>H</u>-CO), 3.66 – 3.46 (m; 8H; O-CH_2-CH_2-O-CH_2-CH_2-O-), 3.11 (m; 2H; CH_2-C<u>H</u>$_2$-NH-CO), 1.86 – 1.22 (m; 6H; CH_2-CH_2-CH_2). **^{13}C NMR (75MHz):** 171.85 (COOH), 170.90 (O-CH_2-<u>C</u>OOBzl), 170.33 (O-CH_2-<u>C</u>O-NH-CH_2), 136.74 (Arm. Ipso <u>C</u>(C) Bzl), 129.18, 128.87, 128.85 (Arm. <u>C</u>(H) Bzl), 71.20 (O-<u>C</u>H$_2$-COOBzl), 70.99 (O-<u>C</u>H$_2$-CO-NH-CH_2) 70.70, 70.61, 70.37, 68.58 (O-CH_2-CH_2-O-CH_2-CH_2-O-), 66.53 (CH_2-Ph), 52.70 (NH-<u>C</u>H-CO), 38.41 (CH_2-<u>C</u>H$_2$-NH-CO-CH_2), 30.36, 29.44, 22.52 (CH_2-CH_2-CH_2).

Lys[PEG-(COOBzl)]-NCA (8). The L-Lys[PEG-(COO-Bzl)]-OH was now treated with triphosgene in THF at 40 – 50 °C for 3h. This resulted in a yellow solution which was concentrated and added to diethyl ether in a centrifuge tube and cooled in

an acetone/dry ice bath (-78°C). A yellow amorphous precipitate formed. This mixture was centrifuged cold and the supernatant collected, discarding the pellet, concentrated and again added to diethyl ether, cooled down and centrifuged. This process was repeated until the supernatant was a clear solution with no precipitate forming under these conditions, each time discarding the yellow pellet. The clear supernatant solution was concentrated to a minimum volume under vacuum and 'precipitated' with addition to pentane in a centrifuge tube. This mixture was cooled in liquid nitrogen until a precipitate formed which was collected by centrifugation. The supernatant was discarded and the pellet was redissolved in a minimum amount of THF and again precipitated by the addition to cold pentane. This process was repeated three times, each time retaining the pellet and discarding the supernatant. The residue was dried under high vacuum for 16h. **^{1}H NMR (400MHz) in DMSO-d_6/TFA-d_1.** δ 7.36 – 7.24 (m; 5H; Arm. Bzl), 5.12 (s; 2H; CH_2-Ph), 4.37 (m; 1H; NH-C<u>H</u>-CO), 4.16 (s; 2H; O-CH_2-COOBzl), 3.84 (s; 2H; O-C<u>H</u>$_2$-CONH-CH_2), 3.68 – 3.47 (m; 8H; O-CH_2-CH_2-O-CH_2-CH_2-O), 3.10 (m; 2H; CH_2-C<u>H</u>$_2$-NH-CO), 1.84 – 1.17 (m; 6H; CH_2-CH_2-CH_2-). **^{13}C NMR (75MHz):** 172.08 (<u>C</u>O-O-CO-NH, NCA), 170.58 (O-CH_2-<u>C</u>OOBzl), 169.76 (O-CH_2-<u>C</u>O-NH-CH_2), 153.16 (CO-O-<u>C</u>O-NH, NCA), 136.41 (Arm. Ipso <u>C</u>(C) Bzl), 128.89, 128.57, 128.55 (Arm. C(H) Bzl), 70.83 (O-<u>C</u>H$_2$-COOBzl), 70.65 (O-<u>C</u>H$_2$-CO-NH-CH_2) 70.42, 70.28, 70.03, 68.26 (O-CH_2-CH_2-O-CH_2-CH_2-O-), 66.17 (CH_2-Ph), 57.48 (NH-<u>C</u>H-CO), 38.16 (CH_2-<u>C</u>H$_2$-NH-CO-CH_2), 31.23, 29.07, 22.25 (CH_2-CH_2-CH_2).

5.7.11 Polymerization reactions of Lys[PEG-(COOBzl)]-NCA synthesized through method 3

5.7.11.1 <u>General procedure for the polymerization reactions</u>

All glassware was oven dried at 110°C overnight and cooled under high vacuum. Airtight syringes were used fitted with a stop-valve between the syringe and needle. All reactions were carried out, under argon, in schlenk tubes.

The THF used as solvent was first distilled under argon from sodium wire and distilled a second time, under argon, from calcium hydride and used on the same day. The *n*-butylamine used was stored on sodium hydroxide and distilled, under argon,

from calcium hydride and used on the same day. The *N,N,N',N'*-tetrakis(3-aminopropyl)-1,4-butanediamine (DAB-Am-4) was used as is from a bottle, freshly opened under an argon atmosphere.

Reaction mixtures were made up by taking the appropriate volume aliquots from the stock-solutions and combining them in argon-purged schlenk tubes. All the reactions were stirred for four days at room temperature. The reaction mixtures were concentrated, using vacuum and precipitated by the addition of dry hexane (10ml) added. The resulting precipitate was left to stand for 4h in the freezer before the solvent was decanted. The product was dissolved twice in chloroform (10ml) and the chloroform removed under vacuum. The products were dried under high vacuum for 16h at room temperature.

5.7.11.2 Monomer and initiator solutions used in the reaction mixtures

Lys[PEG-(COOBzl)])-NCA/THF solution: The Lys[PEG-(COOBzl)])-NCA (1.614g; 3.46mmol) was dissolved in THF (18.00ml) making up a 89.66mg/ml Lys[PEG-(COOBzl)])-NCA/THF solution. An aliquot (2.00ml; 179,33mg; $3{,}84.10^{-4}$mol) of this solution was used in each of the polymerization reactions.

Lys(Z)-NCA/THF solution: Lys(Z)-NCA (1.4115g; 4,61mmol) was dissolved in THF (24,00ml) making up a 58,81mg/ml Lys(Z)-NCA/THF solution.

n-Butylamine/THF solution: *n*-Butylamine (247µl; 2,50mmol) was diluted, using THF, to 25,00ml making up a 0,10M *n*-butylamine/THF solution.

N,N,N',N'-Tetrakis(3aminopropyl)-1,4-butanediamine (DAB-Am-4)/THF solution: DAB-Am-4 (824µl; 2,50mmol) was diluted, using THF, to 25,00ml making up a 0,10M solution.

5.7.11.3 Polymerization reaction compositions

The compositions for the different polymerization reactions are summarized in the tables below.

Table 4: Homopolymers of Lys[PEG-(COOBzl)]-NCA initiated by *n*-butylamine.

In. : M^1	Volume In. solution (µl)	Volume M_1 solution (ml)
1:5	768	2,00
1:10	384	2,00
1:20	192	2,00
1:50	76.8	2,00

Table 5: Four-arm star-polymer of Lys[PEG-(COOBzl)]-NCA initiated by DAB-Am-4.

Arm-NH_2 : M^1	Volume DAM-Am-4 solution (µl)	Volume M_1 solution (ml)
1:5	192	2,00
1:10	96	2,00

Table 6: Copolymer of Lys[PEG-(COOBzl)]-NCA and Lys(Z)-NCA initiated by *n*-butylamine.

In. : M^1 : M^2	M^1:M^2	Volume I. solution (µl)	Volume M^1 solution (ml)	Volume M^2 solution (ml)
1:5:10	1:2	768	2,00	4,00
1:5:15	1:3	768	2,00	6,00
1:5:20	1:4	768	2,00	8,00
1:0:15		768		6,00

I. = *n*-Butylamine initiator.
Arm-NH_2 = One of the four arms of the dendrite
M^1 = Lys[PEG-(COOBzl)]-NCA
M^2 = Lys(Z)-NCA

5.8 Conclusion

This work shows the development of an optimized and simplified multi-gram synthesis of a novel functionalized PEG-lysine NCA monomer and the subsequent polymerization thereof.

At the heart of the synthetic problem is PEG-$(COOH)_2$ – the two carboxylic acid groups need to be differentially functionalized/protected. In this work several strategies were applied to obtain a viable final product.

The initial synthetic approach was through the use of solid phase chemistry applying the following reaction sequence: coupling an appropriately protected lysine (Z-L-Lys(Fmoc)-OH) to the resin, side-chain deprotection, PEG-$(COOH)_2$ - functionalization of the ε-NH_2 group on the lysine side-chain, followed by cleaving from the resin and NCA formation. The two reaction procedures applied were both unsuccessful because of ineffective coupling of the protected lysine to the hydroxyl groups on the resin. A probable cause for this is the bulkiness of the Fmoc protecting group shielding the carboxylic acid group from reaction with a resin hydroxyl group.

Applying this reaction sequence to solution phase chemistry, Z-*L*-Lys(HTos)-OBzl was synthesized. HOSu activated PEG-$(COOH)_2$ was then reacted in excess with the lysine in the first step and in a second step the unreacted succinimide ester was reacted with methanol to protect the distal PEG carboxylic acid group. This was also a very unsatisfactory procedure because of cumbersome product separation.

The main features of these first approaches were the reaction of an excess of activated PEG in relation to the protected lysine and the use of methanol in the second step to protect the distal PEG carboxylic acid. The use of methanol was motivated by the mild basic hydrolysis of the methyl ester once the polymer was formed.

To simplify the synthetic procedure, the PEG-$(COOH)_2$ was asymmetrically protected via condensation with 2-phenylethanol under Dean-Stark conditions with benzene as solvent and a strong cation exchange resin as acid catalyst. The resulting product mixture could be easily separated with column chromatography or extraction. The

desired (HOOC)-PEG-(COOPhEt) was coupled to the ε-NH_2 of Z-L-Lys(HTos)-OBzl using DCC and pyridine to yield Z-L-Lys[PEG-(COOPhEt)]-OBzl. The PhEt ester was not completely stable under the hydrogenation conditions used to cleave the Z- and benzyl protecting groups resulting in a partial loss of the ester.

To circumvent hydrogenation at this step, *N*-hydroxysuccinimide activated (HOOC)-PEG-(COOPhEt) was attached to Z- or tBoc- protected lysine to yield Z(tBoc)-L-Lys[PEG-(COOPhEt)]-OH. NCAs can be synthesized from α-NH carbamate protected amino acids. tBoc-Lys-OH was chosen as the preferred starting material because of the greater volatility of the *tert.*butylchloride byproduct during NCA formation compared to that of benzylchloride, from the benzyloxycarbonyl (Z) protecting group, which could be trapped as a contaminant.

The pentane-washed Lys[PEG-(COOPhEt)]-NCA was reacted with polymerization catalysts BipyNiCOD in DMF, $Co(PMe_3)_4$ in THF and HAPENAlOMe in DCM, dioxane and THF. None of these reactions were successful.

Optimizing the workup procedure revealed that the PhEt ester is also susceptible to hydrolysis. The suspicion arose that traces of hydrolyzed PhEt esters might contaminate the polymerization reactions. The PhEt ester was subsequently replaced by a benzyl ester which is stable under hydrolytic conditions.

The polymerization reactions were repeated, as above. Still no reaction took place. When TEA was added to the NCA solutions, white triethylammonium chloride salts precipitated. From this it was apparent that the repeated pentane precipitations was not enough to rid the final NCA product of HCl contamination due to from the NCA synthesis reactions. The NCA moiety is a reactive anhydride with limited means of purification. Various methods were investigated to rid the final product of HCl.

Method 3 emerged as the only viable method to purify this particular NCA. From the homopolymerization reactions, using *n*-butylamine, there are indications that there might still be contaminants present that neutralize the base. With initiator to monomer ratios of 1:5 and 1:10 there were successful polymerization reactions taking

place. Polymerization reactions with the ratios 1:20 and 1:50 were unsuccessful – this seems to indicate contaminants that quench the base which is present in very low concentrations. NMR evidence indicates that successful polymerization was initiated by the DAB-Am-4 initiator and that copolymerization was achieved for the different Lys[PEG-(COOBzl)]-NCA and Lys(Z)-NCA monomer combinations initiated by *n*-butylamine.

5.9 Future work

- Further purification of the NCA monomer is first and foremost - preparative HPLC may deliver the desired purity that should make longer homopolymers possible.
- Once more successful polymerizations are achieved, more in-depth characterization, other than NMR, of the protected polymer will be needed. These include MALDI-TOF analysis – determining the appropriate matrix would be key to this analytical technique. Finding suitable standards will be necessary for GPC analysis. FT-IR would be very complicated, and would probably not offer much information, because of the presence of two different amide bonds and an ester in the structure.
- Cleaving of the distal benzyl ester should be optimized. Hydrogenation of the benzyl ester was tested successfully on Lys[PEG-(COOBzl)]-OH in methanol using 10% Pd/C and H_2 gas (balloon). Due to the presence of the benzyl ester the polymer might not be soluble in solvents like methanol or ethanol. Other suitable solvents, such as DMF, may need investigation if the hydrogenation route is to be followed. Alternatively glacial acetic acid saturated with HBr will cleave the benzyl esters.
- Is the following assumption true? Because of the PEG sheath the polymer should be water soluble without being pH dependant, where the acid groups do not need to be ionized, to form salts at high pH to make the polymer water soluble.

  A question that arises here is: if an α-helical conformation is maintained by the polylysine backbone - the carboxylic acid groups on the surface of the PEG sheath would be well ordered (depending on the length of the PEG) - could this lead to cooperative hydrogen bonding between neighboring polymer strands and result in self-assembly of the polymer strands, at neutral pH, trapping water molecules while also creating channels along the polymer chains (Fig. 26)? Various microscopy techniques might be useful in answering this question.

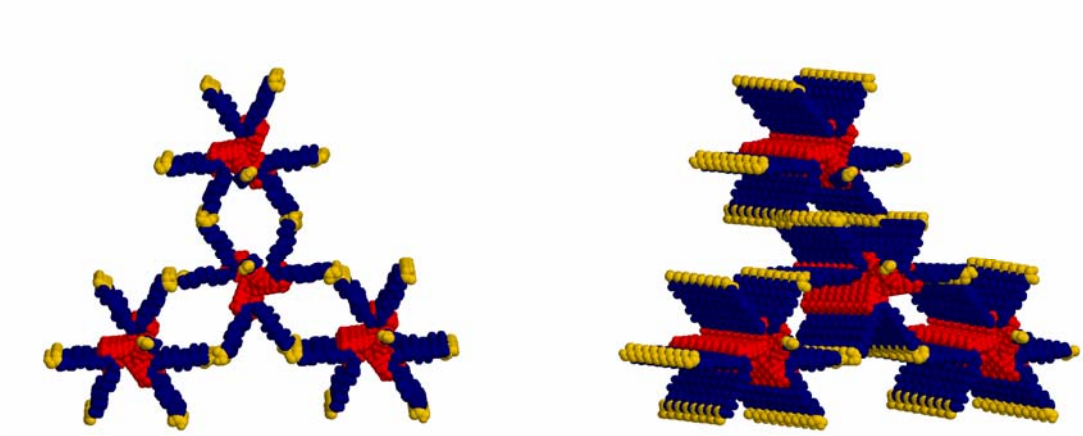


Figure 27: Possible self-assembly through cooperative hydrogen bonding between opposing pendant carboxylic acid groups. The final deprotected polymer is drawn in Hyperchem and rendered in RASMOL - assuming that the poly-L-lysine backbone (red) forms a stable α-helix. The PEG spacer is indicated in blue and the distal carboxylic acids groups in yellow.

- Synthesize various copolymers using different amino acids together with this NCA. Using different types of polymeric macro-initiators to polymerize this Lys[PEG-(COOBzl)]-NCA might lead to interesting materials
- Copolymerization with neutral, methyl ether capped PEGs (that can also vary in length), functionalized monomers to control the amount of negative charges while still maintaining the PEG sheath characteristics.
- Could the same concept be applied to amino acids like glutamic- or aspartic acid which have carboxylic acid terminated side chains? This could be achieved through ester formation using hydroxy terminated PEGs or otherwise through amides, using amine terminated PEGs.
- The envisaged application of these polymers is as hydrogels – will they form hydrogels and under what conditions? Crosslinking may be necessary – which type would be effective?
- The distal carboxylic acid functionalities was deliberately made part of the structure to act as a handle for the attachment of bio-molecules, such as cell-ligand peptides (RGDs) or molecules such as biotin. The coupling conditions for these molecules need to be determined, followed by the appropriate biological testing.

References

1. Kissel, T.; Li, X.; Unger, F., ABA-triblock copolymers from biodegradable polyester A-blocks and hydrophilic poly(ethylene oxide) B-blocks as a candidate for in situ hydrogel delivery system for proteins. *Adv. Drug Deliv. Rev.* **2002,** 52, 99-134.

2. Tirelli, N.; Lutolf, M. P.; Hubbell, J. A., Poly(ethylene glycol) block copolymers. *Rev. Molec. Biotech.* **2002,** 90, 3-15.

3. Hoffman, A. S., Hydrogels for biomedical applications. *Adv. Drug Deliv. Rev.* **2002,** 43, 3-12.

4. Hennick, W. E.; Van Nostrum, C. F., Novel crosslinking methods to design hydrogels. *Adv. Drug Deliv. Rev.* **2002,** 54, 13-36.

5. Li, J.; Kao, W. J., Synthesis of polyethylene glycol (PEG) derivatives and PEGylated-peptide biopolymer conjugates. *Biomacromolecules* **2003,** 4, 1055-1067.

6. Kao, W. J.; Lok, D.; Li, J., Preparation of heterodifunctional polyethyleneglycols: Network formation, characterization, and cell culture analysis. *J. Biomater. Sci. Polym. Edn.* **2001,** 12, 599-611.

7. Yaniç, C.; Bredenkamp, M. W.; Jacobs, E. P.; Swart, P., Adsorbed surfactants for affinity chromatography: End-group modification of ethylene glycol polymers. *Bioorg. Med. Chem. Lett.* **2003,** 13, 1381-1384.

8. Tessmar, J. K.; Goepferich, A. M., Customised PEG-derived copolymers for tissue-engineering applications. *Macromol. Biosci.* **2007,** 7, 23-39.

9. Dwawan, S.; Varma, M.; Sinha, V. R., High molecular weight poly(ethylene oxide) -based drug delivery systems. Part 1: Hydrogels and hydrophilic matrix systems. *Pharm. Tech.* **2005,** 29, 72-80.

10. Jarret, P.; Lalor, C. B.; Chan, L.; Redmon, M. P.; Hickey, A. J., Micelle formation and reaction kinetics of a bioabsorbable polyethylene glycol-oligolactide ABA block copolymer hydrogel. *Colloid. Surface. B.* **2000,** 17, 11-21.

11. Kumar, N.; Ravikumar, M. N. V.; Domb, A. J., Biodegradable block copolymers. *Adv. Drug Deliv. Rev.* **2001,** 53, 23-44.

12. Vinogradov, S. V.; Bronich, T. K.; Kabanov, A. V., Nanosized cationic hydrogels for drug delivery: preparation, properties and interactions with cells. *Adv. Drug Deliv. Rev.* **2002,** 54, 135-147.

13. Yang, Z.; Yuan, J.; Cheng, S., Self-assembling of biocompatible BAB amphilic triblock copolymers PLL(Z)-PEG-PLL(Z) in aqueous medium. *Eur. Polym. J.* **2005,** 41, 267-274.

14. Wang, L.; Wang, S.; Bei, J. Z., Synthesis and characterization of macroinitiator-amino terminated PEG and poly(γ-benzyl-L-glutamate)-PEO-poly(γ-benzyl-L-glutamate) triblock copolymer. *Polym. Adv. Technol.* **2004,** 15, 617-621.

15. Brzezinska, K. R.; Cutin, S. A.; Deming, T. J., Polypeptide end-capping using functionalized isocyanates: Preparation of pentablock copolymers. *Macromolecules* **2002,** 35, 2970-2976.

16. Cho, C.-S.; Jeong, Y.-I.; Kim, S.-H.; Nah, J.-W.; Kubota, M.; Komoto, T., Thermoplastic hydrogel based on hexablock copolymer composed of poly(γ-benzyl L-glutamate) and poly(ethylene oxide). *Polymer* **2000,** 41, 5785-5793.

17. Zhang, G.; Ma, J.; Li, Y.; Wang, Y., Synthesis and characterization of poly(L-alanine)-block-poly(ethylene glycol) monomethyl ether as amphiphilc biodegradable copolymers. *J. Biomat. Sci.-Polym. E.* **2003,** 14, 1389-1400.

18. Deng, C.; Chen, X.; Yu, H.; Sun, J.; Lu, T.; Jing, X., A biodegradable triblock copolymer poly(ethylene glycol)-*b*-poly(L-lactide)-*b*-poly(L-lysine): Synthesis, self assembly and RGD peptide modification. *Polymer* **2007,** 48, 139-149.

19. Karal-Yilmaz, O.; Kayaman-Apohan, N.; Misirli, Z.; Baysal, K.; Baysal, B. M., Synthesis and characterization of poly(L-lactic acid-co-ethylene oxide-co-aspartic acid) and its interaction with cells. *J. Mater. Sci.-Mater. M.* **2006,** 17, 213-227.

20. Tian, H. Y.; Deng, C.; Lin, H.; Sun, J.; Deng, M.; Chen, X.; Jing, X., Biodegradible cationic PEG-PEI-PBLG hyperbranched block copolymer: synthesis and micelle characterization. *Biomaterials* **2005,** 26, 4209-4217.

21. Young, H. C.; Feng, L.; Jin-Seok, K.; Young, K. C.; Jong, S. P., Polyethylene glycol-grafted poly-L-lysine as polymeric gene carrier. *J. Control. Release* **1998,** 54, 39-48.

22. Young, H. C.; Feng, L.; Jong, S. P.; Sung, W. K., Lactose-Poly(ethylene glycol)-grafted poly-L-lysine as hepatoma cell-targeted gene carrier. *Bioconjugate Chem.* **1998,** 9, 708-718.

23. Windblade, N. D.; Nikolic, I. D.; Hoffman, A. S.; Hubbell, J. A., Blocking adhesion to cell and tissue surfaces by the chemisorption of a poly-L-lysine-graft-[poly(ethylene glycol); phenylboronic acid] copolymer. *Biomacromolecules* **2000,** 1, 523-533.

24. Tossattia, S.; De Paula, S. M.; Askendalb, A.; Van de Vondele, S.; Hubbell, J. A.; Tenvall, P.; Textor, M., Peptide functionalized poly(L-lysine)-*g*-poly(ethylene

glycol) on titanium: resistance to protein adsorption in full heparinized human blood plasma. *Biomaterials* **2003,** 24, 4949-4958.

25. Van de Vondele, S.; Vörös, J.; Hubbell, J. A., RGD-grafted poly-L-lysine-graft-(polyethylene glycol) copolymers block non-specific protein adsorption while promoting cell adhesion. *Biotechnol. Bioeng.* **2003,** 82, 784-790.

26. Huang, N.-P.; Vörös, J.; Paul, S. M. D.; Textor, M.; Spencer, N. D., Biotin-derivatized poly(L-lysine)-*g*-poly(ethylene glycol): a novel polymeric interface for bioaffinity sensing. *Langmuir* **2002,** 18, 220-230.

27. Almany, L.; Seliktar, D., Biosynthetic hydrogel scaffolds made from fibrinogen and polyethylene glycol for 3D cell cultures. *Biomaterials* **2005,** 26, 2467-2477.

28. Sanborn, T. J.; Messersmith, P. B.; Barron, A. E., In situ crosslinking of a biomimetic peptide-PEG hydrogel via thermally triggered activation of factor XIII. *Biomaterials* **2002,** 23, 2703-2710.

29. Lecolley, F.; Tao, L.; Mantovani, G.; Durkin, I.; Lautru, S.; Haddleton, D. M., A new approach to bioconjugates for proteins and peptides ("pegylation") utilising living radical polymerisation. *Chem. Comm.* **2004**, 2026-2027.

30. Yu, M.; Nowak, A. P.; Deming, T. J.; Pochan, D. J., Methylated mono- and diethyleneglycol functionalized polylysines: nonionic, α-helical water-soluble polypeptides. *J. Am. Chem. Soc.* **1999,** 121, 12210-12211.

31. Keywords and chemical structure searches in Scifinder Scolar®

32. Daly, W. H.; Poché, D., The preparation of N-carboxyanhydrides of α-amino acids using bis(trichloromethyl)carbonate. *Tetrahedron Lett.* **1988,** 29, 5859-5862.

33. Wilder, R.; Mobashery, S., The use of triphosgene in the preparation of *N*-carboxy-α-amino acid anhydrides. *J. Org. Chem.* **1992,** 57, 2755-2756.

34. Lange, M.; Fischer, P. M., Efficient synthesis of differentially protected (*S*,*S*)-2,7-diaminooctanedioic acid, dicarba analogue of cystine. *Helv. Chim. Acta* **1998,** 81, 2053-2061.

35. Prime, K. L.; Whitesides, G. M., Adsorption of proteins onto surfaces containing end-attached oligo(ethylene oxide): A model system using self-assembled monolayers. *J. Am. Chem. Soc.* **1993**, 10714-10721.

36. Green, T. W.; Wuts, P. G. M., *Protective groups in organic synthesis*. 3rd ed.; John Wiley & Sons. Inc.: **1999**, p369, 494.

37. Kates, A. S.; Albericio, F., *Solid-phase synthesis - A practical guide*. Marcel Dekker: New York, Basel, **2000**, p79, 275.

38. Novabiochem, *2004/5 Catalog*. Merck.

39. Gowda, D. C.; Abiraj, K., Heterogeneous catalytic transfer hydrogenation in peptide synthesis. *Lett. Pept. Sci.* **2002,** 9, 153-165.

40. Salomon, C. J.; Mata, E. G.; Mascaretti, O. A., Tetrahedron report number 331: Recent developments in chemical deprotection of ester functional groups. *Tetrahedron* **1993,** 49, 3691-3748.

41. Anwer, M. K.; Spatola, A. F., An advantageous method for the rapid removal of hydrogenolysable protecting groups under ambient conditions; synthesis of leucine-enkephalin. *Synthesis* **1980**, 929-932.

42. ElAmin, B.; Anantharamaiah, G. M.; Royer, G. P.; Means, G. E., Removal of benzyl-type protecting groups from peptides by catalytic transfer hydrogenation with formic acid. *J. Org. Chem.* **1979,** 44, 3442-3444.

43. Lele, B. S.; Kulkarni, M. G., Single step room temperature oxidation of poly(ethylene glycol) to poly(ethylene)-dicarboxylic acid. *J. Appl. Polym. Sci.* **1998,** 70, 883-890.

44. Fishman, A.; Acton, A.; Lee-Ruff, E., A simple preparation of PEG-carboxylates by direct oxidation. *Synth. Comm.* **2004,** 34, 2309-2312.

45. Deming, T. J., Facile synthesis of block copolypeptides of defined architecture. *Nature* **1997,** 390, 386-389.

46. Deming, T. J.; Cutin, S. A., Chain initiation efficiency in cobalt- and nickel-mediated polypeptide synthesis. *J. Am. Chem. Soc.* **2000,** 122, 5710-5717.

47. Poché, D. S.; Moore, M. J.; Bowles, J. L., An unconventional method for purifying the *N*-carboxyanhydride derivatives of γ-alkyl-L-glutamates. *Synthetic Commun.* **1999,** 29, 843-854.

48. Fuller, W. D.; Cohen, M. P.; Shabankareh, M.; Blair, R. K., Urethane-protected amino acid N-carboxy anhydrides and their use in peptide synthesis. *J. Am. Chem. Soc.* **1990,** 112, 7414-7416.

49. Lide, D. R., *CRC Handbook of chemistry and physics*. 81st ed.; CRC-Press, Boca Raton, London, New York, Washington DC, 2000, sec. 8, p49

50. Aliferis, T.; Iatrou, H.; Hadjichristidis, N.; Messman, J.; Mays, J., Synthesis of 3- and 4-arm star-block copolypeptides using multifunctional amino initiators and high vacuum techniques. *Macromol. Symp.* **2006,** 240, 12-17.

Chapter 6

Materials and Instruments

The description of the solvent preparation, reagents and instruments used are general throughout the study, thus in stead of each chapter having a 'materials' section it is combined in one single chapter giving detailed descriptions.

6.1 Solvents

DMF(AR): This solvent was stored and dried for at least three day on potassium hydroxide flakes (KOH) before being distilled under vacuum (35 – 45 mBar) with an Ar bleed at 60ºC collecting only the middle fraction at a constant boiling point. For polymerization reactions the DMF was freshly distilled and used on the same day while being manipulated under Ar.

THF(AR): This solvent was stored and pre-dried for at least three days on KOH before being distilled (daily) under Ar from sodium wire with benzophenone as indicator which turned deep purple when the solvent is moisture free. THF dried in this manner was used for general synthesis. When THF was used as solvent in polymerization reactions, it was distilled a second time from calcium hydride (CaH_2) and manipulated under Ar and used the same day.

Dioxane (AR): This solvent was only used in some Al catalysed polymerization reactions. Dioxane from a freshly opened bottle was distilled from CaH_2 and manipulated under Ar.

Ethyl acetate (AR) and dichloromethane (AR): For synthetic purposes these solvents were distilled from CaH_2 under Ar. For extractions the solvents were used straight from the bottle.

Diethyl ether (Ar), pentane (AR), hexane (Ar) and toluene(AR): These solvents were pre-dried and stored on KOH for at least three day before being distilled from sodium wire under Ar with benzophenone as moisture indicator. These were also redistilled daily.

Methanol (AR), ethanol (AR): These solvents were super-dried by refluxing over iodine activated magnesium turnings (± 5g per litre solvent) until al the magnesium was used, before being distilled and manipulated under Ar and used on the same day.

Benzene (AR): This solvent was used straight from the bottle without further distillations.

6.2 Amino acids

Amino acid	Supplier
NH_2-Asp(Bzl)-OH	Sigma/GL Biochem
NH_2-Glu(Bzl)-OH	GL Biochem
NH_2-Leu-OH	Nova-Biochem
NH_2-Lys(Z)-OH	Fluka
NH_2-Phe-OH	Nova-Biochem
tBoc-Arg(NO_2)-OH	Fluka/GL Biochem
tBoc-Cys(Trt)-OH	Gl-Biochem
tBoc-Gly-OH	Fluka
tBoc-Lys-OH	Fluka
Z-Arg(NO_2)-OH	Fluka
Z-Lys-OH	Fluka

Protecting groups

tBoc-	*tert*.Butyloxycarbonyl-
Bzl-	Benzyl-
NO_2-	Nitrousoxide- (Nitro)
Trt-	Triphenylmethyl- (Trityl)
Z-	Benzyloxycarbonyl-

6.3 Reagents

Reagent	Supplier	Comment
1,3 Butadiene	Air Liquede	Supplied in lecture bottles
10%Pd on carbon	Fluka/Merck	
2,2' Bipyridyl (>99%)	Aldrich	Used as is
2-Hydroxyacetophenone (99%)	Aldrich	
2-Phenylethanol	Fluka	
33% HBr in actic acid (for synthesis)	Merk	
4-Methyl morpholine (>98%)	Fluka	
Aluminium isopropoxide (>97%)	Fluka	Used as is from a freshly opened bottle
Amberlite IR-120(Plus) resin, 4,4meq/g	Aldrich	Washed with THF and dried under vacuum.
Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP)	Nova-Biochem	
Benzyl alcohol (>99%)	Merck	Used as is
Bis(trichloromethyl) carbonate (Triphosgene)	Aldirh/Merck Fluka	
Calcium hydride (>95%)	Fluka	Used for solvent drying
Cis,cis-1,5-cyclooctadiene (99%)	Aldrich	
Cobalt(II) chloride anhydrous (for synthesis)	Merck	Used as is
Dichloromethyl methyl ether	Fluka	
Ethyl chloroformate (>98%)	Fluka	
Ethylene diamine (for synthesis)	Merk	Stored on NaOH and distilled from CaH_2 under Ar
N,*N*'-Dicyclohexyl carbodiimide (DCC)	Fluka	
n-Butylamine (99%)	Aldrich	Stored on NaOH and distilled from CaH_2 under Ar.

N-Hydroxy succinimide	Aldrich	
Nickel(II) acetyl acetonate (95%)	Aldrich	Dried by toluene distillation
Piperidine	Aldrich	
PL-DIPAM resin, 3.27mmol N/g, 150-300μm	Polymer Laboratories	
PL-HCO3 MR resin, 2.5mmol/g, 500-600μm	Polymer Laboratories	
Poly(ethylene glycol) bis(carboxymethyl) ether, Mn = 250 and 600	Aldrich	Used as is
Poly(ethylene glycol), Mn =1000	Aldrich	Used as is
Poly-propylenimine-tetraamine dendrimer, *N*,*N*,*N'*,*N'*-tetrakis(3-aminopropyl)-1,4-butanediamine Generation 1.0 (DAB-Am-4)	Aldrich	
Potassium permanganate	Merck	
Silaca Gel (Kiesel-Gel), 230-400mesh	Merck	
*Tert.*Amyl alcohol	Aldrich	
Triethyl amine (>99.5%)	Fluka	When used as initiator: Stored on NaOH and distilled from CaH_2 under Ar.
Triethylaluminium (97%)	Aldrich	Supplied in a metal flask fitted with a stop-valve
Triflouro acetic acid (for synthesis)	Merck	
Trimethyl phosphine (97%)	Aldrich	
Trimethyl phosphine, 1M solution in THF	Fluka	
Trimethylaluminium solution, 2M in Toluene	Fluka	

6.4 Instruments

Nuclear Magnetic Resonance (NMR) spectroscopy: NMR results were obtained by using either a Varian VXR 300MHz or a Varian Unity Inova 400 MHz spectrometers. (http://academic.sun.ac.za/saf/units/nmr/nmr)

Mass-Spectroscopy (MS): An AMD 604 High Resolution Mass Spectrometer was used to obtain Electron impact ionization (EI) MS results and a Waters API-Q-TOF Ultima was employed for the Electron Spray ionization (ES)-MS analyses. (http://academic.sun.ac.za/saf/units/msu/msu)

Fourier-Transform Infra-Red spectroscopy (FT-IR): IR spectra were collected using a Thermo Nicolet's Attenuated Total Reflectance (ATR) Avatar 330 spectrometer.

Appendix 1

Table 1: Summary of characteristic NMR shifts of some of the NCAs synthesized.

NCA	^{1}H		^{13}C				
	NCA α-CH	**Protecting group**	**NCA α-CH**	**NCA NH-(CO)-O**	**NCA CH-(CO)-O**	**Protecting group**	
Gly	4.41(s)		48.55	159.55	171.47		1
Ala	4.59(q)		54.07	152.88	173.06		2
Leu	4.58(d,d)		59.23	158.46	174.06		1
Phe	4.76(m)		61.85	158.04	172.81		1
Asp(Bzl)	4.78(m)	5.25(d) Benzyl CH_2	56.63	158.05	172.14	173.88 Benzyl ester CO	1
Glu(Bzl)	4.58(m)	5.21(s) Benzyl CH_2	59.40	157.94	172.52	178.05 Benzyl ester CO	1
Lys(Z)	4.55(m)	5.07(s) Benzyl CH_2	59.36	154.17	173.37	158.65 Bzl carbamate CO	2
Cys(Trt)	3.53(d,d)		56.70	151.62	168.33	68.04 (-S-C-Ph_3)	3
Arg(NO_2)	4.57(m)		59.83	158.26	173.30	154.42 (-CN-)	1

1 = TFA-d_1; 2 = Acetone – d_6; 3 = $CDCl_3$