# CONTRIBUTION TO PREPARATION OF POLYMER CONJUGATES CONTAINING FERROCENE DERIVATIVES

BY

MBULELO GEOFFREY NOKWEQU

[B. Sc. Hons.]

SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

MASTER OF SCIENCE

IN THE SCHOOL OF CHEMICAL SCIENCES

IN THE FACULTY OF NATURAL AND APPLIED SCIENCES

AT THE UNIVERSITY OF THE NORTH

QWAQWA CAMPUS

PRIVATE BAG X13

PHUTHADITJHABA

SUPERVISOR PROF J BARIYANGA

CO-SUPERVISOR PROF E W NEUSE

DECLARATION

I DECLARE THAT THE DISSERTATION FOR THE DEGREE MASTER OF SCIENCE AT THE UNIVERSITY OF THE NORTH HEREBY SUBMITTED HAS NOT PREVIOUSLY BEEN SUBMITTED BY ME FOR A DEGREE AT THIS OR AT ANOTHER UNIVERSITY, AND THAT IT IS MY WORK AND THAT ALL MATERIAL CONTAINED HEREIN HAS BEEN DULY ACKNOWLEDGED.

**Mbulelo G Nokwequ**

**November 2000**

## DEDICATION

This work is dedicated to the two special women in my life,
my mother and Dibakiso Mashiloane, who have been supportive
throughout my studies

## ACKNOWLEDGEMENTS

I wish to express my profound gratitude to the following people :

* my supervisor and co-supervisor, Prof J Bariyanga and Prof E W Neuse respectively, for their guidance and support.
* my friends : Papiki Moji, Robert Lentsoane, Teboho Pitso, Mahlomola 'Dlamini' Daemane, Teboho Phakoa, Tumelo Khumalo, Molefi Thekisoe, Pat Rachidi and others ...

I also wish to express my special thanks to all who supported and criticized this project

Last, but not least, the National Research Foundation (NRF) and the University of the North(QwaQwa) for their financial support.

"I conceive thoughts. I am inspired by visions.

There is an unending fountain from which more will flow."

B.Z. Bokser

## SUMMARY

Cancer is one of the major killer-diseases in the whole world. It is a well known fact that the causes of cancer vary from person to person, they include, *inter alia,* exposure to carcinogenic substances like smoking, heredity, chronic irritation of body areas like exposure to radiation[1]. The alleviation of this disease is now one of the major challenges in the medical world. To this end, many drugs have been synthesized with the aim of curing this disease. The most widely used is cisplatin and its derivatives and it is more recently that metallocenes have also been employed as anticancer drugs. Recent studies have also shown that anchoring such drugs to biodegradable polymers improves their activity[2].

In this study ferrocene derivatives containing a BOC (*tert*-butoxycarbonyl) protected polyamine which is susceptible to platination were synthesized with a view of anchoring these to a suitable carrier polymer. Ethylenediamine, propylenediamine, and diethylenetriamine were first protected using BOC. These protected polyamines were characterized using NMR ($^1$H nuclear magnetic resonance) spectroscopy, FAB-MS (fast atomic bombardment mass spectroscopy), FTIR (Fourier transform infrared) spectroscopy, and EA (elemental analysis). The protected polyamines were then attached to the ferrocenemonocarboxylic acid using a well known coupling agent DCC (*N, N'*- dicyclohexylcarbodiimide). These ferrocene derivatives were also analyzed using techniques such as NMR, MS, FTIR, and EA and they were found to have the expected structures.

In parallel to this, a carrier polymer exhibiting a carboxylic acid side chain was synthesized from a poly(ethylene oxide) diamine and characterized by NMR, FTIR, GPC (gel permeation chromatography), ES-MS (electrospray mass spectroscopy), and EA . This polymer was later found to be water-soluble and has a long shelf life. This polymer was tested for biodegradability by digesting it with two endopeptidase enzymes, namely papain and proteinase A at 37 °C. Changes in molecular weight distribution of this polymer were then

followed. The polymer was found to be slowly biodegradable.

## ABBREVIATIONS

To help the reader, the following abbreviations which are frequently used herein are explained below :

| | |
|---|---|
| BOC | Di-tert-butyl dicarbonate |
| cisplatin | *cis* - diamminedichloroplatinum (II) |
| carboplatin | *cis* - diammine-1,1-cyclobutane-dicarboxylatoplatinum (II) |
| DCC | *N, N'* - dicyclohexylcarbodiimide |
| dien | Diethylenetriamine |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic acid |
| EA | Elemental analysis |
| EDA | Ethylenediamine |
| ESMS | Electrospray mass spectroscopy |
| FABMS | Fast atomic bombardment mass spectroscopy |
| FTIR | Fourier transform infrared |
| GPC | Gel permeation chromatography |
| HSU | *N* - hydroxysuccinimide |
| MS | Mass spectroscopy |
| NMR | Nuclear magnetic resonance |
| PDA | Propylenediamine |
| PEO | Poly(ethylene oxide) |
| THF | Tetrahydrofuran |
| transplatin | trans-diamminedichloroplatinum (II) |

## TABLE OF CONTENTS

## 1. List of tables



## 2. List of figures

## 3. List of schemes

# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

## 1.1 INTRODUCTION

Cancer has been one of the major killers in many families all over the world. References to cancerous tumors in humans date back to ancient Egypt and Greece (2000-1500 B.C) [1]. A tumor is a new abnormal tissue growth which is both uncontrolled and progressive. A benign tumor or cancer does not spread out to other body parts, but it spreads locally by expansion whilst on the other hand a malignant tumor spreads to other parts of the body by metastasis [2]. Cancers can arise in any part of the body tissues, for instance, the epithelial tissue - skin, mucous and serous membranes.

Metastasis involves relocation of malignant tissue cells by transport in the blood and lymphatic systems to other body parts or lymph nodes. The metastatic cells re-attach themselves to a new site, reproduce, an in this way establish a colony which may finally exceed the parent growth in size and destructiveness [3]. This new growth may occur in a specific organ, such as neck, breast, stomach or rectum, or throughout the body as in blood cancer (leukemia). However, the most common sites of metastatic cells are lymph nodes close to the original tumor, the lungs, spine, liver, skin, and brain. There is a lot of information gathered thus far concerning the qualitative causes of cancer. Among the known qualitative causes of cancer are [4]:

1. Heredity
2. Chronic irritation of body areas- like exposure to radiation.
3. exposure to carcinogenic substances.
4. Presence of pre-existing conditions which include, for example, white patches on the tongue or vulva, large burn scars, and rectal polyps, among others.

Attempts in ancient times to cure or alleviate this disease involved application of corrosive pastes over affected areas [5]. It is until fairly recently that various forms of cancer therapy resulting from a process of intuition have been applied. Current cancer therapy essentially represents the empirical knowledge amassed by professionals over a long time, including many years of research in laboratories. The probable cause of cancer at cellular level was first described by a German pathologist, Rudolf Virchow in 1880 and it was Frederick Sanger and coworkers in the 1970's who took this a step further by unraveling the structures of the RNA and DNA [6].

In prevention of cancer, a great deal of success has been achieved by anti-smoking campaigns and ridding the environment of carcinogenic chemicals. Over the past few decades, great sums of money have been invested all over the world on cancer prevention and treatment and thus far chemotherapy has been the major method of cancer treatment. The first advance in cancer chemotherapy came in 1941 when the female sex hormone, oestrogen, was found to be useful in treatment of prostatic cancer. In 1948 the first antifolate aminopterin was reported to be effective in the treatment of leukemia and from 1961 the activity of cisplatin [*cis*-diamminedichloroplatinum(II)] (figure1) and its derivatives against cancerous tumors was explored [2].

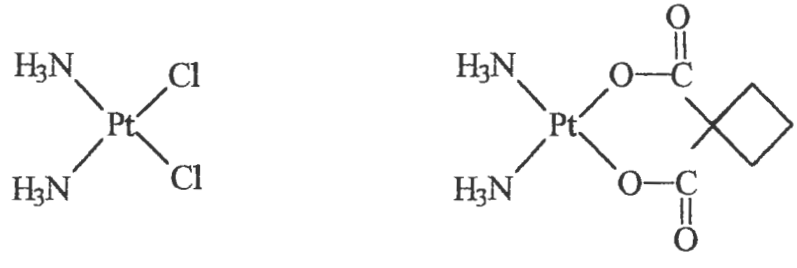



**Figure 1** : Structures of cisplatin and carboplatin

It is believed that cisplatin and its analogues interact with the nuclear DNA by intra-strand cross-linking, thus upsetting the replication of DNA of the cancer cell with the ultimate death

of the cell [7,8]. Cisplatin is predominantly used in chemotherapy of ovarian and cervical cancers and carboplatin [*cis*-diammine-1,1-cyclobutane-dicarboxylatoplatinum (II)] ( figure1) is presently one of the leading drugs clinically administered against small cell and non-small cell cancers of the lung. The 1,1-cyclobutanedicarboxylate ligand of carboplatin differs from cisplatin by being more highly bound to platinum via chelation than the chloride of cisplatin, which results in different pharmacokinetics due to their different properties in hydrolysis. protein binding, passive diffusion through the cell membrane, and binding to DNA.

In the past research was basically focused on producing more cisplatin derivatives and, in few cases, replacement of platinum with other central metals (these however were done in structural analogy to the active cisplatin compounds and their activity was compared to that of the cisplatin compounds). It has been recently realized that the clinical requirements as to the activity of new antitumor agents are not necessarily characterized by the need to synthesize compounds that are more active than cisplatin, but by the need for compounds that act completely differently [9]. It is for this reason that other non-platinum compounds have been synthesized and tested for antitumor activity, for instance, metallocenes like ferrocene and titanocene [10].

The rapid development of metallocene chemistry in the 1960's led to the formation of diverse applications in the field of organometallics . In the light of the success of cisplatin derivatives against certain types of tumor, the approach to the synthesis of potentially antitumor-active derivatives of metallocenes is only logical and completely natural [11]. Ferrocene compounds in particular have been tested for antitumor properties where one, in particular, has shown a high degree of inhibition of tumor growth (by 50%) and the life span of the animals increased significantly. The compound is L-ferrocenylethyl-1-N-benzotriazole (figure2).

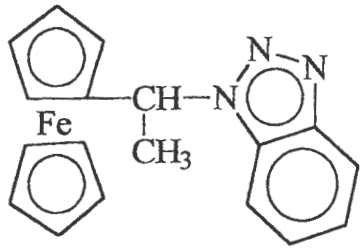



**Figure 2** : The structure of L-ferrocenylethyl-1-N-benzotriazole

The toxicity of the drug was very low compared to platinum derivatives. It has been concluded that the presence of a positive charge is a prerequisite for the manifestation of anticancer activity and in this case the azolyl residue can acquire a positive charge by protonation of one of the nitrogens which is not bonded to the L-ferrocenyethyl group [12].

The problem, however, with ferrocene is its lack of water-solubility and as such presents some problems in the administration and distribution within the body. It has been argued that one way of solving this problem is by anchoring the drug onto the water-soluble macromolecules. This will in turn ensure maximum therapeutic effect of the drug and minimal adverse effects. Methotrexate, 5-fluorouracil, cisplatin type complexes and a variety of bioactive peptides and proteins are some of the examples of such conjugates [13]. To achieve this goal the drug has to be made available at the site of action and its access to non-target tissue limited. Therefore, site-specificity is required in any form of chemotherapy, and it has been shown that non-site specific chemotherapy is less effective. Drug targeting can be achieved by attaching the drug to a polymer carrier system. To this end, some carrier systems have been synthesized from poly(ethylene oxide) because of its high water-solubility and biocompatibility. One can also take advantage of the molecular size, lipophilicity and electric charge to achieve site specificity and prolonged release of the drug [13].

In order to address the above requirements, this study will contribute to the syntheses of some conjugates with ferrocene derivatives. It is assumed that ferrocene will act by destroying those cells which have developed some kind of resistence to platinum compounds.

## 1.2 OBJECTIVES OF THE STUDY.

The main objective of the study is to contribute to finding a potential drug against cancer which presents less toxicity and high level of effectiveness. The specific objectives are :

1. Preparation of a precursor of an anti-cancer drug containing a ferrocene moiety susceptible to coordination with platinum
2. Preparation of the carrier polymer exhibiting a carboxylic side-chain for anchoring the drug.
3. Enzymatic degradation tests of the carrier polymer.

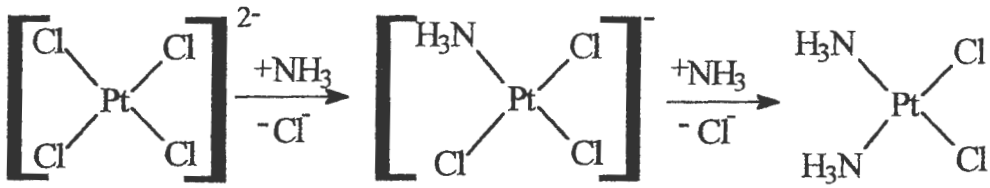
## 1.3 LITERATURE REVIEW

### 1.3.1 Platinum and Ferrocene compounds in Cancer Chemotherapy.

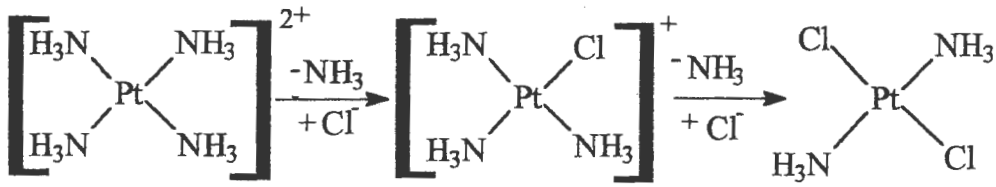
#### 1.3.1.1 Platinum Compounds in Cancer Chemotherapy

Acceptance of metal complexes as drugs was advanced by the use of Pt(II) complexes as anticancer drugs. Rosenberg and co-workers reported that some group VIIIb metal complexes can inhibit cell growth and this led to more studies being done on the complexes of Pt(II) to determine their activity as potential anticancer agents [14]. In general these complexes are square planar and even though inert with regard to kinetics of ligand exchange, they do undergo reaction slowly [14,15]. Since the discovery of the anticancer properties of *cis*-diamminedichloroplatinum(II) (cisplatin) many research groups have investigated the structure-activity relationship. There are basically two ways of forming diamminedichloro-platinum(II) using the trans-effect of the ligand [16]:

1. by the displacement of $Cl^-$ from $[PtCl_4]^{-2}$ by $NH_3$




2. by the displacement of $NH_3$ from $[Pt(NH_3)_4)]^{+2}$ by $Cl^-$.




In general, the trans-effect of some groups is ordered as follows : $C_2H_4 > CH_3^- > HSO_3^- > NO_2^- > Br^- > Cl^-$ > pyridine > $RNH_2 > NH_3 > OH^- > H_2O$. Three isomers of aminebromochloro pyridine platinum(II) have also been isolated using the sequence similar to the one given in the above example. The trans-effect can be explained by π-bonding theory. For instance, the strong π-bonding of tertiary amine directs the weak π-bonding for the trans ligand, therefore the substitution will take place there.

The active compounds are neutral and become positively charged on hydrolysis. Two cis-leaving groups are required whereas trans isomers are almost inactive [17]. The usage of carboxylate and dicarboxylate ligands which are less labile reduces the toxicity of the platinum complexes. The ligands trans to the leaving groups are more inert and are almost always primary amines [18]. Complexes with more than one tertiary amine ligand are normally inactive. In terms of biological activity the order is as follows : $NH_3 > RNH_2 > R_3N$ and most importantly the variation of R group has yielded drugs which are active even

against the cells that had developed some resistence to cisplatin [18]. The diaminocyclohexane analogue in particular, has been the most widely studied complex including its mechanism of activity against certain cancer cells [19,20].

There are still many platinum compounds which are currently undergoing clinical trials although some of them have poor solubility. Monofunctional compounds usually do not display activity although activity of $[Pt(dien)Cl]^+$ has been reported recently. Platinum-dien chelate rings are considered stable, and reactions of $[Pt(dien)Cl]^+$ are usually studied using cisplatin as a model [21]. It has been shown that $[Pt(dien)Cl]^+$ binds to methionine through the thioether sulfur, and S-bound thioether can be displayed intra- or intermolecularly by nitrogen(N-7) of guanine at physiological pH levels [22]. Given the fact that guanine is known to be a major target for anticancer platinum drugs, it stands to reason from the statements above that $[Pt(dien)Cl]^+$ works similarly. Members of the class of complexes having the general formula $[Pt(NH_3)_2AmCl]^+$, where Am is a derivative of pyridine, purine, and aniline have also shown activity against Sarcoma 180 tumors [22,23]. A proton NMR spectroscopic analysis of the reaction products of some of these triamine species indicated that only monofunctional adducts are formed. Interestingly,these thiamine complexes do not conform to the classical rules for active platinum anticancer drugs, suggesting the possible development of active platinum complexes with new coordination spheres.

A series of binuclear platinum complexes has been synthesized and charecterized with the aim of developing compounds that will form DNA lesions resistant to DNA repair and therefore be more effective against resistant tumors. Complexes with the general formula $[\{PtCl_m(NH_3)_{3-m}\}_2(\text{diamine})]^{2(2-m)+}$, where m = 0 - 3 and the diamine is usually $H_2N(CH_2)_nNH_2$, can form mono-, di-, and trifunctional complexes with the DNA, depending on the number of exchangeable chloride ligand present [24]. The most widely studied complexes belong to the series

$[\{cis\text{-}PtX_2(NH_3)_2\}_2H_2N(CH_2)_nNH_2\}]$, where $X_2 = Cl_2$ or malonate, n = 4-9, and can

potentially form two difunctional lesions with the DNA. These lesions are structurally similar to those formed by cisplatin. Furthermore, compounds of this class have shown better activity in comparison to the parent cisplatin against resistant cell lines [25].

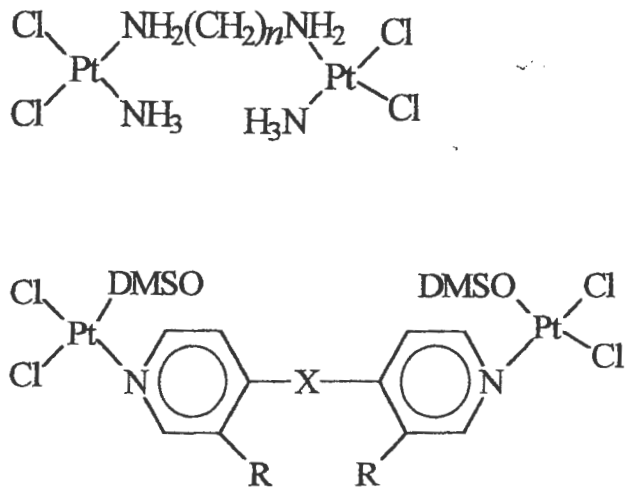



**Figure 3 :** Structures of bis-platinum(II) complexes

However, binuclear platinum complexes containing aromatic ligand have not been fully explored. On the other hand, the combined use of cisplatin with sodium selenite or seleneous acid has been found to greatly reduce the lethal toxicity and nephrotoxocity of cisplatin without necessarily masking its antitumour activity in certain tumors, and sulfur-containing ligands are known to reduce the toxic effects of the Pt(II) drugs [26]. For example, platinum complexes containing thiopyridyl triazine derivatives have been reported to show good antitumour activity. Based on this consideration, other new dinuclear platinum(II) complexes bridged by dipyridyl selenides or sulfides have been synthesized and their antitumour activity was studied. This was done because ligands in these complexes have biological properties, and their donor properties are similar to purine and pyrimidine bases of biological interest.

It was also found that although the divalent compound cis-$[Pt(NH_3)_2Cl_2]$ (cis-DDP) and

related neutral cis-bis(ammine)platinum(II) and platinum(IV) complexes were active, the trans isomers were not [27]. The amount of $\pi$-bonding in trans-DDP is reduced by half due to replacement of two $Cl^-$ ligands with two $NH_3$ ligands, because $NH_3$ is not capable of $\pi$-

bonding and on the other hand, $\psi_{Cl}$ non-bonding orbitals must be reduced by half (with $[PtCl_4]^{-2}$ electrons in $\pi$-bonds are delocalized, while with trans-DDP they are not, due to loss of $\pi$-bonding from a pair of trans-chlorides). The remaining $\pi$-molecular orbitals are now closer in energy to the remaining $\psi_{Cl}$ non-bonding orbital , thus it is easier for bonding electrons to access non-bonding orbitals and enter into $S_N1$ reaction pathways [28]. Moreover, since there is no $\psi$ non-bonding for $NH_3$ and the hydration energy of $Cl^-$ is larger than that of $NH_3$, $Cl^-$ is the leaving group and not $NH_3$ from trans-DDP. In the case of cis-DDP, the interaction of chlorides trans to each other is lost; resulting in further reduction of $\pi$-electron delocalization. The degeneracy of the $\pi$-molecular orbitals is reduced, with one $\pi$-molecular orbital moving to a higher energy.

The energy of this orbital is therefore closer to $\psi_{Cl}$ than that of transplatin, thus, the cis-isomer is expected to be more reactive toward ligand substitution than transplatin. This is supported by the fact that the equilibrium

$$I\textit{cis}\text{-}[Pt(NH_3)_2Cl_2 + H_2O \leftrightarrow [Pt(NH_3)_2Cl(H_2O)]^+ + Cl^-$$

lies to the right while that of the trans isomer does not (see Table 1). This means that the replacement of $Cl^-$ from the cis isomer opens cis-$[Pt(NH_3)_2Cl_2$ to further reactivity [29].

In $[Pt(NH_3)(amine)Cl_2]$ complexes, the environments of the two chlorides are not identical and as such the degeneracy of all $\pi$-molecular orbitals is removed and these are much energetically closer to $\psi_{Cl}$ non-bonding orbital. This means that the ligand substitution will be enhanced compared to cis-DDP [17]. Furthermore, amines and $NH_3$ differ as electron donors [18], therefore, the energy of $\pi$-bonds compared to $\psi_{Cl}$ orbitals in

[$Pt(NH_3)_2$(amine)Cl] is expected to vary with different amine ligands [19]. The $\pi$-bonding in *cis*-$Pt(NH_3)_2$(amine)$Cl]^+$ complexes is further reduced by fewer chlorides.

**Table 1** : Comparison of *cis*- and transplatin reaction equilibria.

| Cis-Pt(II) | Trans-Pt(II) |
|---|---|
| $Pt(NH_3)_2Cl_2 + {}^{36}Cl^- + H_2O \rightarrow Pt(NH_3)_2$ $(H_2O)^{36}Cl^- + Cl^-$ $K_1 = 2.3$ exp -3 | $Pt(NH_3)_2Cl_2 + {}^{36}Cl^- + H_2O \rightarrow Pt(NH_3)_2$ $Cl(^{36}Cl) + Cl^-$ $K_1 = 2.3$ exp -3 |
| $Pt(NH_3)_2Cl_2 + H_2O \rightarrow Pt(NH_3)_2(H_2O)Cl^- + Cl^-$ $K_1 = 2.3$ exp -3 (complete substitution $t_{1/2}$ =5h) | $Pt(NH_3)_2Cl_2 + H_2O \rightarrow$ no change $K_1 = 0$ |
| $Pt(NH_3)_2Cl(L) + L + H_2O \rightarrow Pt(NH_3)_2L_2 + Cl^-$ $K_1$ = slow | $Pt(NH_3)_2Cl(OH) + L + H_2O \rightarrow$ $Pt(NH_3)_2(OH)L + Cl^-$ $K_1$ = slow |
| $Pt(NH_3)_2(H_2O)Cl^+ + C_5H_5N + H_2O \rightarrow$ $Pt(NH_3)_2Cl(C_5H_5N) + H_2O$ $K_1$ = 1.5exp-3 | $Pt(NH_3)_2Cl_2 + C_5H_5N + H_2O \rightarrow$ $Pt(NH_3)_2Cl(C_5H_5N)^+ + Cl^-$ $K_1$ =5.7exp-3 |

Hollis, Amundsen and Stern prepared and studied [$Pt(NH_3)_2$(aryl-amine)$Cl^-$] complexes and found, unlike [$Pt(amine)_2Cl_2$], that both cis and trans isomers were active although the cis isomer was more active [20].

The transition state associated with the loss of amines from [$Pt(NH_3)$(amine)$Cl]^+$ is difficult compared with the one involving $Cl^-$. The one involving the $Cl^-$ occurs much more readily due to the existence of a $\psi_{Cl}$ non-bonding orbital and the more favorable solvation energy of $Cl^-$ [21,22]. It is not unexpected, however, for a weak ligand such as aryl-amine to undergo substitution during the transition states. Payet, Leng and Dalbies reported the release of

arylamines from cis isomers of $[Pt(NH_3)(amine)Cl]^+$ complexes when they were allowed to react with DNA with cis-$[Pt(NH_3)(dGuanine)H_2O]^{+2}$ as a product where guanine is a stronger ligand than arylamines [23].

One of the important clinical problems with the use of cisplatin and its analog carboplatin is tumour resistance to these drugs. This kind of resistance is reduced by complexes of the type $[Pt(NH_3)(amine)Cl_2]$ and $[Pt(NH_3)(amine)Cl]^+$ [23,24].There are various reasons for tumour resistance to these drugs, namely : reduced uptake of the cisplatin by cells, enhanced DNA repair, sequestering of Pt(II), and transport of Pt(II) out of the cells. The role of thiol and thioether binding to Pt(II) in these mechanisms of resistance has received a lot of attention because of the ability of thiols to sequester and remove metals from the cells [25,26]. This has made thiols candidates for explaining resistance to cisplatin and its analog carboplatin

To this end, cis-$[Pt(NH_3)_2Cl_2]$ (*cis*-DDP) and its analog carboplatin have been the most widely used anticancer agents because of their success in treating bladder, lung, head and neck, cervical, testicular, and ovarian cancers [27,28]. Unfortunately, these drugs are toxic, and as mentioned before, suffer from tumour resistance and this limits their clinical use. Hence, *cis*-DDP analogues with reduced toxicity and a broader range of therapeutic activity have been developed . These so called second generation drugs display activity similar to *cis*-DDP but are less toxic.

Gust, Bernhardt, et al have recently [29] reported that the replacement of the two ammine groups on carboplatin by the carrier ligand *rac*-1,2-bis(4-fluorophenyl)ethylenediamine leads to an increased drug concentration in human MCF-7 breast cancer cells - 20 times compared to carboplatin. This led to the development of a cationic complex type drug with enhanced water-solubility, namely, [*meso*- and *rac*-1,2-bis(4-fluorophenyl)ethylenediamine]-chloro[sulfinylbis(methane)-S]platinum(II) (see figure 4*)* which possesses the desired properties. This new complex possesses the following biological and physiochemical

properties required for application in breast cancer therapy [30] :

1. It surpasses carboplatin in its activity against breast cancer.
2. It is stable in the presence of nucleophiles, a prerequisite
   a) for drug levels in the extracellular fluid of tumour tissues, sufficiently high to cause cytostatic effects;
   b) for a tolerability which is comparable to that of carboplatin.
3. The solubility and stability of this complex in physiological NaCl solution meet requirements for the development of appropriate formulations for continuous drug infusion.

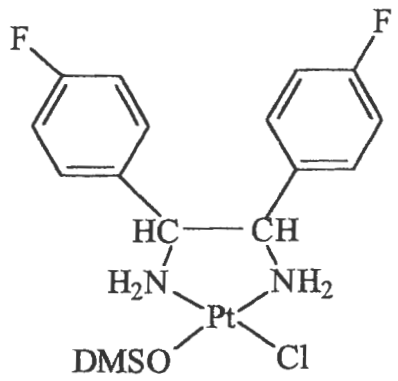



**Figure 4** : Structure of *rac*-4F-PtCl(DMSO)

The complex *rac*-4F-PtCl(DMSO) displays pharmacokinetic behavior and the same molecular mode of action as carboplatin. The antitumuor activity of this complex is markedly more than that of carboplatin owing to its carrier ligand *rac*-1,2-bis(4-fluorophenyl) ethylenediamine [29].

There is a growing interest in six-coordinate Pt(IV) complexes because of their anticancer activity, more so since they are active against cisplatin-resistant tumours.The anticancer activity mechanism of Pt(IV) complexes has not been adequately explored. It is generally believed that since Pt(IV) compounds are inert to ligand substitution as compared to their

Pt(II) analogues, they must first be reduced to the latter before binding to DNA. There are numerous experiments, however, that cannot be explained by the conventional reduction of Pt(IV) to Pt(II) prior to DNA binding [31,32]. This is corroborated by the fact that some Pt(II) complexes do not display any of the activity or selectivity displayed by their Pt(IV) counterparts. Some Pt(IV) complexes can bind to DNA fragments, guanosine 5'-monophosphate (5'-GMP), methylhypoxanthine, or methylxanthine without being necessarily reduced to their Pt(II) analogues. Roalt et al. [33] reported that although this is the case, reactions between 9-methylxanthine and Pt(IV) with ammine, organic amine, and chloro-ligands are catalyzed by addition of small amounts of Pt(II) analogous complexes. Some complexes of Pt(IV) can undergo a reasonably fast substitution reaction especially in the presence of a Pt(II) analogous complex, depending on the nature of the ligand and the reaction conditions [34].

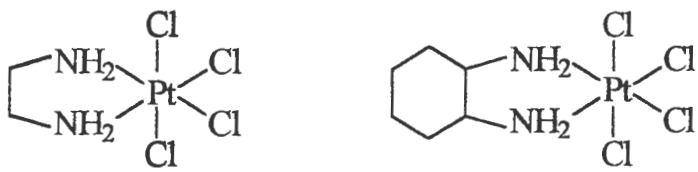



**Figure 5** : Molecular structure of some of Pt(IV) complexes, $Pt(en)Cl_4$ and $Pt(dach)Cl_4$, respectively

In conclusion, the clinical success of cisplatin and its analogues and some Pt(IV) complexes together with the progress made towards understanding their mechanism, improved efficacy, and toxic side effects are encouraging. However, there is still much room for improvement in the application of platinum-based drugs in cancer chemotherapy.

### 1.3.1.2 Ferrocene in Cancer Chemotherapy

Bis(cyclopentadienyl)metal complexes are genuine organometallic compounds in that they

have direct carbon to metal bonds. The first organometallic main-group metal species called cacodyl was synthesized in mid 1700's and its characterization was done 60 years later by Bunsen. The important breakthrough occurred when the bis(cyclopentadienyl)iron complex "ferrocene" was discovered independently by Pauson and Miller. Investigations by Fischer and Wilkinson pointed to the unusual sandwich structure of this compound and a new type of dative $\pi$-bonding between the central metal atom and the unsaturated organic ligand [35,36].

Since this discovery a number of cyclopentadienyl compounds (metallocenes) have been prepared. These can be classified into two types, namely;(i) those which do not contain other ligands,and (ii) complexes which include both cyclopentadienyl ligand and convalently bound ligand such as halides,CO, NO, $PR_3$.

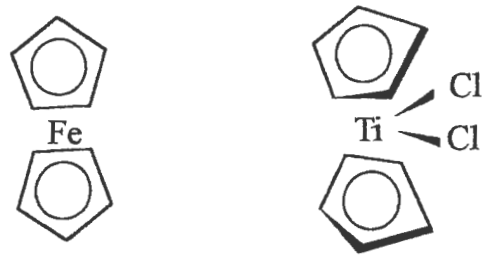



**Figure 6** : Examples of metallocenes, ferrocene and titanocene dichloride respectively.

Many metallocenes have been prepared thus far, mainly owing to the stabilizing influence of the cyclopentadienyl ring ligand allowing the isolation of quite a number of metal-carbon-bonded compounds. It has become clear over the years that some organic compounds have properties of interest for industrial and biological applications. It is for this reason, for example, that organoaluminium-titanium compounds are used as Ziegler-Natta catalysts for the polymerization of olefin and on the other hand tetraethyl and tetramethyl-lead compounds are still used as antiknock additives in gasoline, and as biocides [37].

Metallocenes have had a wide scope of biological applications although they are more toxic

to organisms than their inorganic precursors. This toxicity is the basis of their use in cancer chemotherapy. Antitumor activity of neutral bis(cyclopentadienyl)metal (metallocene) compounds has been reported and these compounds are represented mainly by metallocene diacido complexes $(C_5H_5)_2MX_2$ containing early transition metals Ti, V, Nb, or Mo in the +4 oxidation state as the central metal atom and two cyclopentadienyl ligands, with the ring planes in a tilted arrangement [37]. Ehrlich ascites tumor is considered not to be responsive to conventional cisplatin drugs. However, in the case of bis(cyclopentadienyl)metal complexes this kind of tumor appeared to be sensitive. It was also observed that metallocene chlorides containing the first and second row transition metals Ti, Mo, Nb as the central metal atom cause a dose dependent tumor inhibition.

Notwithstanding these ionic metallocene complexes, antitumor activity was displayed by a number of diverse ferricenium salts, for example, $[(C_5H_5)_2\ Fe]^+X^-$ with $X = SbCl_6$, which display relatively better watersolubility. These compounds contain iron in +3 oxidation state as the central metal iron. Ferrocene and ferricenium complexes lack neutral ligand bound covalently to the central metal and contain two cyclopentadienyl ring ligands in a parallel sandwich arrangements above and below the central metal atom, and therefore they are metallocene compounds [38].

Ferrocene containing compounds have been widely studied owing to their potential in catalysis, material sciences, hydrometallurgy, and as antitumor agents, and many ferrocne derivatives bearing diverse ligating groups with different donor atoms have been successfully tested for this purpose. Ferrocene derivatives containing atoms with good donor abilities have attracted additional interest, since the coordination of a metal to these heteroatoms produces multicentre molecules, in which the presence of proximal metals in different environments may influence the mutual cooperation of these metals in a variety of processes.

The structure of ferrocene has been a subject of investigation since its discovery. In one of

these investigations evidence was advanced for two possible rotational isomers of ferrocene, I and II (figure 7). The recent X-ray crystallographic studies have confirmed that the cyclopentadienyl ligand have a prismatic arrangement ( conformation II) around Fe [39]. These ligands also display a high level of unsaturation but the complex does not exhibit properties characteristic of polyolefins.

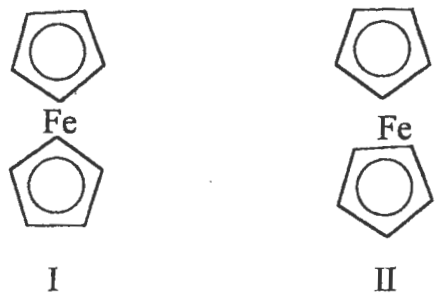



**Figure** 7 : Two possible conformations of ferrocene

The failure of ferrocene to undergo Diels-Alder addition points to the fact that it has a high level of resonance stabilization. This is further supported by the fact that it does undergo Friedel-Crafts substitution, for instance, in the formation of acetylferrocene.

The first investigations on the replacement of phenyl groups of the already known antitumour agents with ferrocenyl fragment were conducted by Frank in 1963. For example, diphenylbenzaldehyde was reported to show activity against certain kinds of cancer cells but this activity diminished with the replacement of the phenyl group with ferrocenyl fragment [40]. Another attempt to increase the anitumour activity of sarcolysine by incorporating ferrocene led to a compound which did not show a clinically meaningful antitumour activity. Therefore, early attempts to obtain antitumour agents based on metallocene led to discouraging results, namely: introduction of a ferrocenyl fragment into compounds, known to possess great antitumour effects, minimized this effect.

In principle, this conclusion was natural in that the original active compounds were

completely water-soluble and therefore it is not unexpected that the introduction of a non-polar hydrophobic ferrocenyl residue worsened the pharmacokinetic characteristics of the compound, hindering its delivery to the target tissues [41]. However, it is important to note that on the other hand this kind of modification led to a decrease in acute side-effects. Investigations in chemical carcinogenesis led to the hypothesis concerning the potential antitumour activity of rigid bulky structures with geometric parameters similar to distances between planes of DNA nucleic bases. It was assumed that an antitumour activity with low toxicity can be achieved with mono or binuclear ferrocene derivatives in neutral or ferricinium form [42]. The activity of the ferricinium salts was first reported in 1984. A correlation between activity of ferricinium salts with their solubility in water was observed and neutral ferrocene did not show any activity due to its hydrophobic nature[43]. However, ferrocene is still susceptible to biologically controlled oxidation-reduction reactions due to its low formal potential : $E_f = 0.127V$ versus standard calomel electrode(SCE) in aqueous solution at 25 $^0$C.

It is also worth mentioning that this observed activity could not be explained by referring to structural analogy with transition-metal halides like titanocene bichloride and cisplatin. It is assumed that the acquisition of a positive charge is a prerequisite for the activity of ferrocene derivatives although anionic derivatives, which are sparingly soluble in water, have been reported to be active. In the light of the cytostatic properties of ferricinium salts against certain types of tumours, it thus became of interest to combine the biooxidizable ferrocenyl group with the structural framework of cisplatin and its derivatives [44,45]. Conjugates of ferrocenylamines and platinum(II) are of particular interest because they may be selective molecular carriers possessing the antineoplastic properties of both ferrocene derivatives and the well known cisplatin.

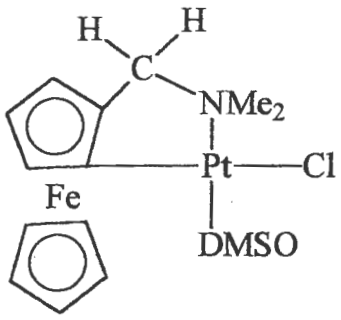



**Figure 8** : Structure of a cycloplatinated ferrocenylamine

Toxicity tests were performed on the compound in figure 8 and they have shown that cycloplatinated ferrocenylamines exhibit kidney rather than liver dysfunction and that they have reasonable toxicity [46]. However this compound was active against cisplatin resistant cells. Incorporation of carboxylate moieties into the cycloplatinated ferrocenylamines increases water-solubility necessary for drug use.

Neuse et al. reported a new class of metallocene-containing platinum complexes as potential antitumour agents, particularly dichloro(1,6-diferrocenyl-2,5-diazahexane)platinum(II) (1) and cis-dichlorobis(1-ferrocenylethylamine)platinum(II) (2) (see figure 9) [47]. This study was encouraged by the observation that the ferricinium ion possess cytostatic properties and as it has been mentioned above, some ferricinium salts have indeed shown activity against certain kinds of tumors.

The problem with most of the drugs discussed herein is that they lack site specificity and as such there is still a risk of these drugs attacking healthy cells upon administration. It has therefore become important for the drug to be carried and made available at the target site.

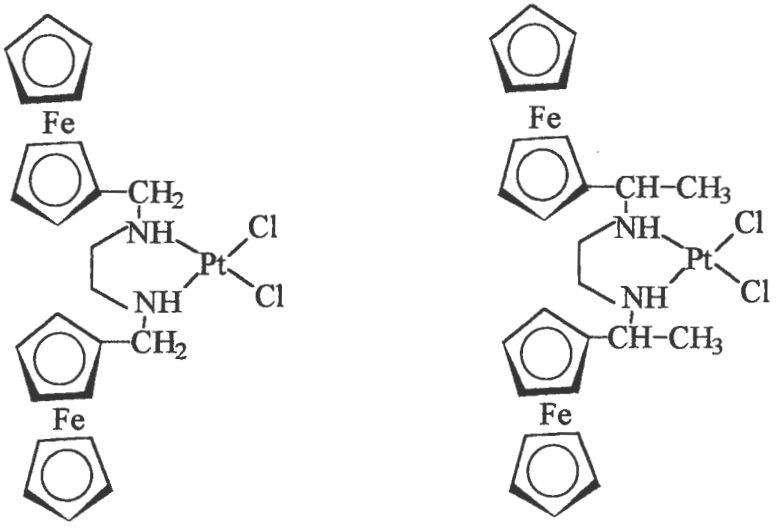



**Figure 9** : Structures of dichloro(1,6-diferrocenyl-2,5-diazahexane)platinum(II) (1) and cis-dichlorobis(1-ferrocenylethylamine)platinum(II) .

### 1.4 Polymeric Drug Carriers

In recent years the use of synthetic polymers as polymeric drugs or drug delivery systems has received increasing attention. The term polymeric drug includes both polymers that contain a drug or a chemotherapeutic unit as part of the polymer backbone. The need for targetable drug carriers has long been realised, and it was Paul Ehrlich in 1906 who coined the phrase " the magic bullet" drugs which might be selectively directed to their specific site of action [48]. Ringsdorf described a new theoretical model for the development of synthetic polymers which could serve as drug carriers, and the important characteristics he outlined provide us with a firm basis for the synthesis of these kind of polymers [49].

Most clinical agents are of low molecular weight which means that they will penetrate into

all cell types and even be rapidly excreted from the body. This implies that heavy, and sometimes lethal, doses of the drugs are required to maintain the therapeutic effect of the drug. On the other hand the attachment of the drug to a carrier polymer ensures that it may release of the drug at the target site and markedly reduces the rate at which the drug is excreted. There are problems however in relation to the development of polymeric drug carriers. For instance, drugs which have cell-surface receptors may be attached to a carrier polymer via a non-hydrolyzable linkage so that the receptor- binding site of the drug remains accessible to the cell surface [50].

However, drugs meant for intracellular spaces must ultimately be liberated from the carrier polymer so that they can reach their site of action, and hence these drugs have to be attached to the carrier polymer via a hydrolyzable linkage [51]. Another point worth mentioning is that the design of drug-carrier linkages which are only cleaved intralysosomally allows the development of a carrier complex which would be stable in the extracellular environment, but release active drugs inside of the cell. It is worth noting that the interior of the cell lysosome has a mildly acidic environment with pH ranging from 5 to 6 depending on the cell type, and therefore pharmacological agents which are unstable under these conditions cannot be used [52].

A look at the destiny of a polymer within the body is in order at this stage. It is common knowledge that the body is divided into different compartments, and the ability of the polymer to access each compartment, and hence the cells in that area, depends on many factors including its ability to penetrate intracompartmental barriers, and as such it is these factors that must be taken into account when designing a potential drug carrier [53]. Modification of synthetic polymers allows for synthesis of tailor-made polymeric carriers and may yield carriers suitable to deliver drugs to target sites and thus combat a wide range of different diseases.

Site-specific drug delivery systems have been categorized into first-order, second-order, and third-order targeting. First-order targeting refers to the delivery of drugs to the specific tissue or organ. Second-order targeting refers to the delivery of drugs to specific cell types and third-order refers to delivery to predetermined intracellular sites [54]. First-order targeting can be achieved, for example, by formulating a drug carrier complex small enough so that it be administered to the target site intravenously. For example, prolonged therapeutic effect of an antitumor agent against brain cancer can be achieved by injecting the drug carrier complex directly into the brain at the tumor site [55,56].

When formulating polymers which will act as potential drug carriers, the following properties must be displayed by the polymer [54]:

* The polymer must be soluble and easy to prepare; it must exhibit low polydispersity.
* It should exhibit drug attachment/release sites
* The polymer should display the ability to be directed to target cells.
* It should be compatible with the biological environment, i.e. non-toxic, non-antigenic, not provocative in any other respect.
* It should be biodegradable or eliminated from the organism after having fulfilled its function
* The drug, carrier, and complex should be stable during its shelf life
* The carrier must protect the drug from the bioenvironment while waiting to be released.

Single chain polymeric carriers must display a spacer necessary to permit enzyme access to the biodegradable linkage. A cell-specific targeting residue may as well be attached in order to enhance target cell membrane interaction. This targeting residue must also be separated from the polymer backbone by a spacer in order to allow the interaction of the targeting moiety with the membrane receptor [57].

to undergo bulk erosion and run the risk of releasing drugs at any site at any time. On the other hand polyanhydrides have very good physicochemical properties. They can be melded into any form that befits the target site and they can be used to prepare microspheres in ordinary organic solvents like methylene chloride [59]. One of the advantages of polyanhydrides is that the desired rate of biodegradation can be obtained by using the appropriate composition of diacid monomers. This therefore means that the required drug release rate can be obtained by choosing a carrier polymer whose degradation properties are known.

Synthetic polymers are widely used as drug carriers since they are more prone to structural modifications than natural macromolecules. Moreover, natural macromolecules have a limited number of functional groups suitable for drug binding. The molecular weight of a synthetic polymer is one of the most important parameters in the determination of its biological activity. The rate of uptake of the polymer by cells and its excretion from the body is influenced by both the molecular weight and the molecular weight distribution, particularly the latter [64]. The molecular weight distribution, that is, the polydispersity of a polymer, is defined by the heterogeneity index $M_w$ / $M_n$ (the ratio of weight average molecular weight and the number average molecular weight). For example, if all the molecules are of the same size, then this value is 1. The higher this value is, the broader the molecular weight distribution. As indicated earlier a synthetic polymer with a narrow molecular weight distribution is a better candidate for a drug carrier system.

Polymers with the C-C backbone are generally not biodegradable. In this case biodegradability can be achieved by linking short polymer chains using bonds susceptible to enzymatic attack. The advantage of a polymer prepared in this fashion is that it a specific number of bonds susceptible to enzymatic attack can be selected through structural modifications in order to control the rate of bond cleavage [60]. Otherwise, biodegradable synthetic polymers can be obtained by synthesizing polymers analogous to natural

macromolecules. A synthetic polymeric drug carrier must display drug binding sites such as -COOH, -CHO, $-NH_2$ -OH and more importantly the binding reactions should be simple and, as far as possible, must not show any side reactions.

The toxic effects of synthetic polymers include inhibition of hepatic microsomal oxidase enzymes, anaemia, thymic involution, and the decrease in bone marrow cell growth. Fortunately, many neutral synthetic polymers do not show these side-effects. In general, the toxicity of charged polymers, like polyanions, increases with molecular weight especially for molecular weight greater that 50 000 [61]. The molecular weight and the molecular weight distribution of synthetic polymers are not the only parameters which determine the biological properties of a polymer; tacticity is one of them. For example, isotactic poly(acrylic acid)s display far more antiviral activity than atactic ones.

Research on synthetic carrier polymers has been concentrated in the field of cancer chemotherapy. The utilization of cytotoxic drugs that are active in killing tumour cells in the treatment of cancer disorders is often limited by their additional cytotoxicity towards normal cells [60]. Macromolecular drug carrier systems have been developed in an attempt to enhance the selectivity of action of cytotoxic drugs by attaching such drugs to carriers with expected affinity to target tissues. In tumour tissue, maleic anhydride bound to plasma albumin , for instance, can penetrate the vascular endothelial cells and accumulate in the tumour tissue. Therefore, the polymer conjugate improves the plasma half-life of the drug; offers a higher accumulation in the tumour tissue than in the normal tissue; and in general, reduces bone marrow toxicity. The pharmacokinetic fate of an antitumour agent may be altered markedly through the expediency of binding the drug reversibly to a water soluble polymeric carrier[61]. The agent so anchored may experience cell entry through pinocytosis, thereby overcoming internalization problems caused by polar or salt like structural peculiarities.

Neuse et al. report that the grafting of poly(ethylene oxide) (PEO) onto carrier polyaspartamides improves their water-solubility and biocompatibilty. O,O'-bis(2-aminopropyl)poly(ethylene oxide), which is commercially available as Jeffamine ED-600 with a nominal molecular weight of 500, served as the source for PEO grafts [62]. Proton NMR and microanalytical results supported the following structure.

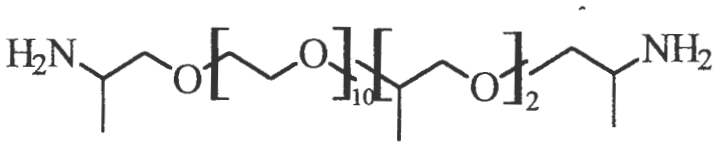


**Figure 11** : Structure of O,O'-bis(2-aminopropyl)poly(ethylene oxide)

These polyaspartamides carriers contained amine-functionalised groups apart from PEO grafts. These amino groups provide binding sites for drugs and the PEO grafts protect the drug from binding and capture by reticuloendothelial system. The main disadvantage of this protective layer of PEO is that it limits the exposure of the polymer-drug bonds to proteolytic enzymes which are necessary for the release of the drug from the carrier [62].

The polymer synthesised in this project consists of amide bonds (i.e peptide bonds ) in its backbone which in a sense makes it a (synthetic) polypeptide. As can be seen from this discussion, biodegradability is very important for a polymer to be considered as a carrier [54]. The Edman degradation is a good method of analysis for biodegradability. The best method currently available makes use of the enzyme carboxypeptidases to cleave the C-terminal amide bond in the peptide chain. Some of the enzymes employed are papain (endopeptidase), proteinase A (endopeptidase),and aminopeptidase(exopeptidase). The analysis is carried out by incubating the polypeptide with one of the enzymes and watch for the changes in the molecular weight distribution of the polymer. In this case the polymer is going to be treated with papain (endopeptidase) and proteinase A (endopeptidase) at 37 °C in a phosphate

buffered solution. The changes in the molecular weight distribution of this polymer are going to be followed using GPC.

The objective of this study is to prepare a precursor of an anticancer drug exhibiting a ferrocene moiety susceptible to platination. It is common knowledge that peptide synthesis is complicated. It is for this reason that before a polyamine can be coupled with an acid it must be protected first so that the desired product can be obtained. Amino groups are usually protected as their tert-butoxycarbonyl amide (BOC) derivatives.

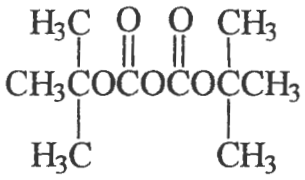


**Figure 12** : Di-tert-butyl dicarbonate

The BOC protecting group is easily introduced by reaction of the amino group with the di-tert-butyl dicarbonate in a typical nucleophilic acyl substitution reaction, and is removed or deprotected by brief treatment with a strong acid like trifluoroacetic acid [63]. The protected polyamide will be attached to the ferrocenemonocarboxylic acid using DCC and deprotected later as described above. This ferrocene derivative is going to be anchored to the biodegradable carrier polymer described above, and later be coordinated to platinum.

# CHAPTER 2

# EXPERIMENTAL

## 2.1 INTRODUCTION

In this chapter experimental procedures for the syntheses of BOC-protected polyamines and the coupling thereof to ferrocenemonocarboxylic acid are outlined. The procedure for the synthesis of a water-soluble poly(ethylene oxide) derivative polymer bearing a carboxylic side-chain function is also outlined. The analytical techniques employed for analysing the compounds are also briefly discussed.

## 2.2 REAGENTS

**Table 2**

| REAGENTS | SUPPLIER | PURITY |
|---|---|---|
| Acryloyl chloride | Sigma-Aldrich Chem Co. | 96% |
| β-alanine | Fluka AG | 98% |
| Cellulose dialysis tube, $M_w$ cutoff 12 000 | Sigma-Aldrich Chem Co. | |
| Diethylenetriamine | Riedel-de Haen AG | Riedel-de Haen AG |
| Di-tert-butyl dicarbonate | Sigma-Aldrich Chem Co. | 97% |
| *N,N'*-Dicyclohexylcarbodiimide | Merck | 99% |
| Ethylenediamine | Sigma-Aldrich Chem Co. | Riedel-de Haen AG |
| Ferrocenemonocarboxylic acid | Aldrich Chemical Co. | Riedel-de Haen AG |
| *N'*-Hydroxysuccinimide | Merck | 98% |
| Jeffamine-ED600 | Fluka AG | Riedel-de Haen AG |
| Polyethylene glycol standards | Fluka AG | 99% |
| Propylenediamine | Riedel-de Haen AG | Riedel-de Haen AG |

All reagents were used as received. Di-tert-butyl dicarbonate was kept in the freezer to avoid decomposition. All solvents were dried over molecular sieves for 24h prior to use.

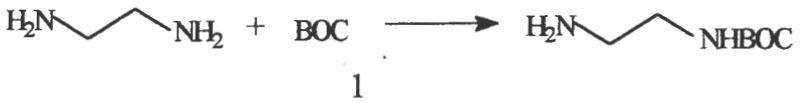
## 2.3 Apparatus

All the glassware was washed with Extran soap solution, rinsed with clean water and acetone, and dried in the oven at 100 °C . All the ferrocene reaction vessels were covered with aluminum foil to avoid decomposition from light and protected from moisture using calcium chloride drying tube. A Gallenkamp vacuum oven was used to dry all samples for 24h at 40 °C and 500 mbar. A Flexi-Dry freeze-drier was used to freeze-dry the polymer at -53 °C and 156 mT.

## 2.4 Experimental procedure

### 2.4.1 BOC protection of polyamines

#### i) Synthesis of BOC-ethylenediamine




1

BOC= $(CH_3)_3C—O—CO—$

**Scheme 1**

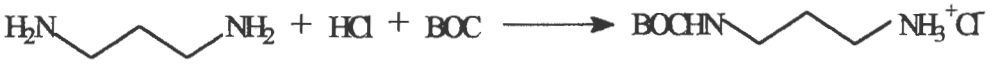
A solution of BOC (8 g, 0.036 mol) in 50 mL dioxane was added dropwise over 3 hours to a solution of ethylenediamine (17 g, 0.284 mol) in 60 mL dioxane. The resulting solution was stirred for another 22 hours at room temperature. This solution was then concentrated under vacuum at 50 °C and 80 mL of distilled water was added to the residue. The white precipitate formed after the addition of water was filtered off and the filtrate was extracted three times with 30 mL dichloromethane. The extracts were combined and concentrated under vacuum

at 30 °C and the resulting oily residue was left to crystalize. The resulting crystals were dissolved in 20 mL distilled water and filtered. The filtrate was left on air to evaporate and white crystals were formed after two weeks.

mp : 105 -108 °C

% yield : 20%

**ii)Synthesis of BOC-propylenediamine**




2

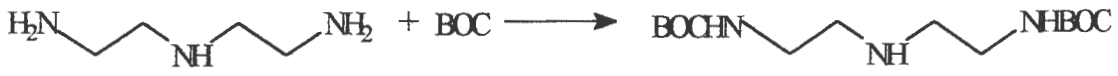
**Scheme 2**

Propylenediamine (14.81 g, 0.2 mol) was dissolved in 40ml dry methanol. To this solution, 18 ml hydrochloric acid was added dropwise with stirring. A solution of BOC (8.76 g, 0.04mol) in 20 ml dry methanol was added to this solution over a period of 3 hours. The resulting solution was stirred for 2 days at room temperature. The solvent was evaporated under vacuum at 30 °C. Distilled water (40 mL) was then added and the white precipitate (which is a bis-BOC protected product) was filtered off and the filtrate was put in a column packed with silica gel and eluted with ethyl acetate : petroleum ether (1:2) solution. The first purple band was collected and the collected fractions were left on air to evaporate. The resulting crystals were re-crystalized from ethyl acetate : petroleum ether (1:2) solution.

mp : 150 °C

% yield : 50%

**iii) Sythesis of bis(BOC)-diethylenetriamine**




3

**Scheme 3**

A solution of BOC ( 13,32 g, 0.061 mol) in 40 mL methanol was added to a solution of diethylenetriamine (19.27 g, 10.19 mol) in 50 mL methanol over 3h. This solution was stirred for 22h and it was concentrated on the rotary evaporator at 40 °C. Water (40 mL) was then added to the residue to dissolve the bis-protected product. This aqueous phase was extracted three times with 30 mL of methylene chloride, and the combined organic phases were washed two times with 25mL water . The organic layer was concentrated on the rotary evaporator at 30 °C and distilled under vacuum. At 120 °C, 4 g of a clear oily product was collected.

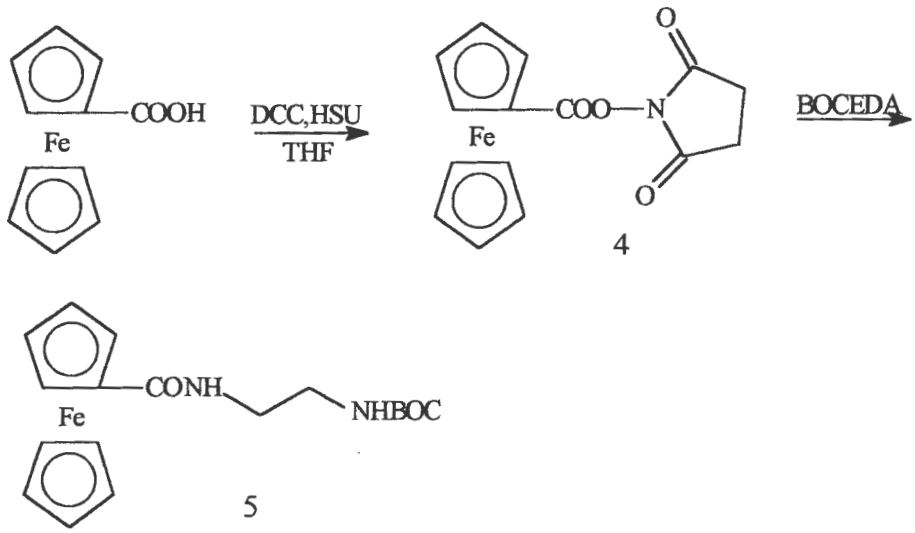
This oil was dissolved in minimum amount of water and the pH of this solution was 10.2. A white insoluble solid precipitated from this solution. This was the tris-protected diethylenetriamine as we shall find out in the results. The mother liquor was left in air to evaporate and a crystalline white water-soluble solid (bis-protected) was formed as we shall see in the results section.

mp : 80 °C

% yield : 26%

### 2.4.2 Synthesis of ferrocene derivatives

### i) Synthesis of ferrocene-BOCethylenediamine



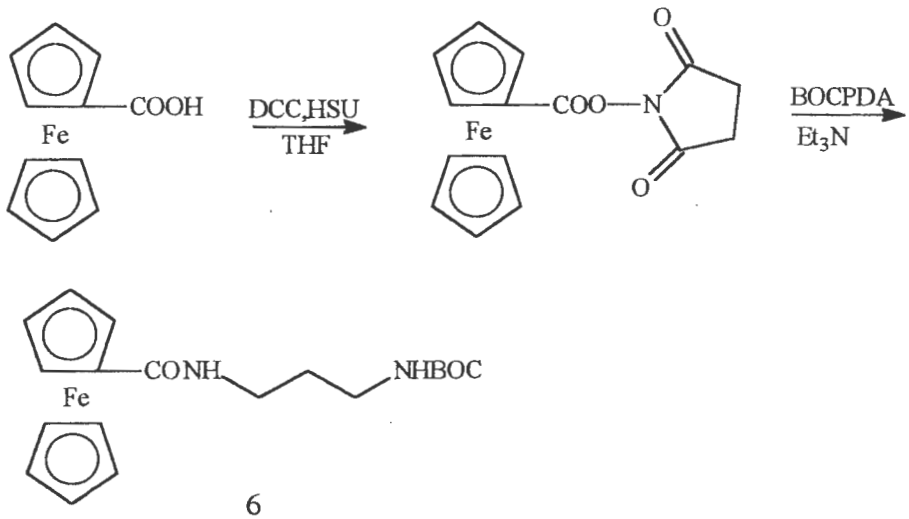

**Scheme 4**

*N,N'*-dicyclohexylcarbodiimide (3.44 g, 0.02 mol) was added to a solution of ferrocenemonocarboxylic acid (4 g, 0.02 mol) in 30 mL tetrahydrofuran. *N* - hydroxy-succinimide (2 g, 0.02 mol) was then added to this solution and the mixture was stirred for 24 hours at room temperature. The brownish solution was filtered and BOC-ethylenediamine ( 3.52 g, 0.02 mol) was then added to the filtrate followed by the addition of 1.76 g of triethylamine. The resulting solution was then stirred at room temperature for 4 days. the solution was then filtered and concentrated to dryness. The residue was dissolved in 30 mL of ethyl acetate and washed with 40 mL water, 5% acetic acid, and 5% sodium bicarbonate. The organic layer was dried over anhydrous sodium carbonate and filtered. The filtrate was concentrated under vacuum and left in the fridge for crystallization. The resulting crystals were re-crystalized from ethyl acetate.

mp : 167 - 170 °C

% yield : 28.5%

**ii)Synthesis ferrocene-BOCpropylenediamine**




6

**Scheme 5**

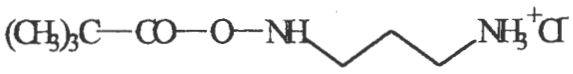
*N,N'*-dicyclohexylcarbodiimide (3.4g, 0.02mol) was added to a solution of ferrocenemonocarboxylic acid (4g, 0.02mol) in 30mL tetrahydrofuran. *N* - hydroxysuccinimide (2g, 0.02mol) was then added to this solution and the mixture was stirred for 24 hours at room temperature. The brownish solution was filtered and BOC-propylenediamine (4.08 g, 0.02 mol) was then added to the filtrate followed by the addition of triethyleneamine (2 g,0.02 mol). The resulting solution was then stirred at room temperature for 4 days. The solution was then filtered and concentrated to dryness. The residue was dissolved in 30mL ethyl acetate and washed with 40 mL water, 5% acetic acid, and 5% sodium bicarbonate. The organic layer was dried over anhydrous sodium carbonate and filtered. The filtrate was concentrated under vacuum and left in the fridge for crystallization. The resulting crystals were re-crystalized from ethyl acetate.

mp : 155 °C

% yield : 18%

The above calculations were based on the following structure :

$C_8H_{19}O_2N_2^+Cl^-$



### 3.2.3. Synthesis of bis(BOC)-diethylenetriamine

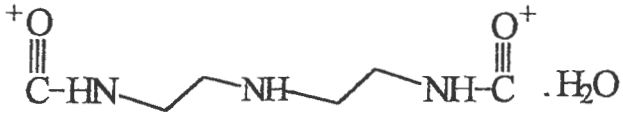
This reaction gave a mixture of two products, namely; bis and tris- protected diethylene triamine. However bis-protected product was the target product which was isolated by using differences in water solubility of the two products. The analytical results of both compounds are given below.

#### I) bis(BOC)-diethylenetriamine

The NMR spectrum shown in figure 3 suggests that there was a bis-protected product. This is supported by the following observations from the spectrum :

A singlet at 1.4 ppm accounts for 18 protons from six BOC methyl groups and this is typical of BOC protons to appear in this region [64, 65, 66]. A singlet at 1.5 ppm accounts for 1 proton of the unprotected NH group and according to Krapcho et al. [64] this proton is usually found in this region and sometimes, depending on its environment, it overlaps with BOC. A triplet at 2.75 ppm accounts for 4 -$CH_2$NH protons, and again this is comparable to the results of the mono-BOC protection of dipropylenetriamine by Krapcho et al. [64], where it was found that a triplet due to 4 protons from -$CH_2$NH appeared at 2.65 ppm. A multiplet at 3.2 ppm accounts for 4 protons from two -$CH_2$ groups bonded to BOC protected NH groups.

A peak at 175.14 corresponds to the following fragment :




Note that the molecular weight of this fragment is 175 and 157 without the water molecule. A peak at 101 with relative abundance of 55% corresponds to the above fragment without $H_2O$ and CO. The presence of this water molecule will be supported by the elemental analysis of this compound.

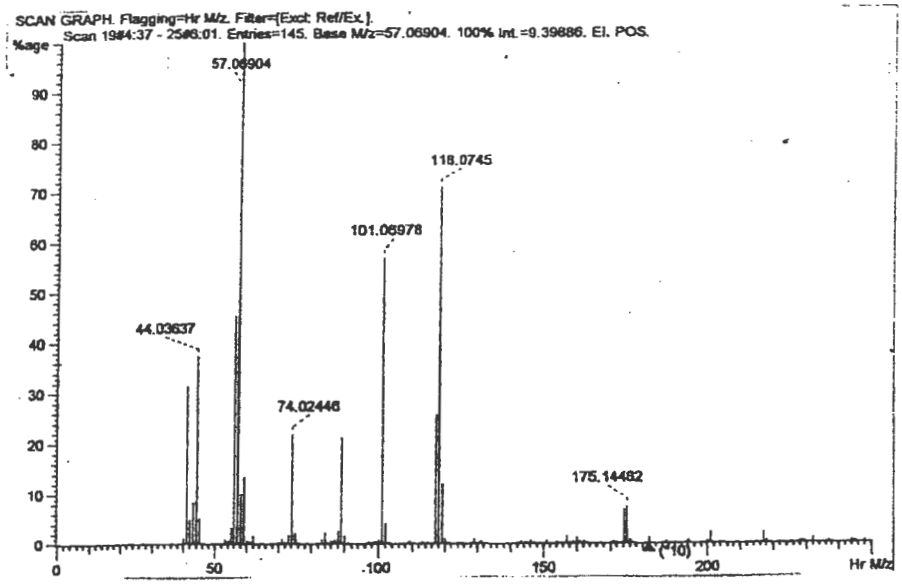



**Figure 17** :Mass spectrum of bis(BOC)-diethylenetriamine

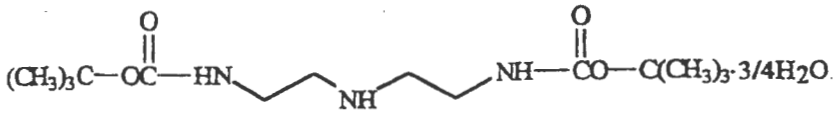
**Table 5** :Analytical results of bis(BOC)-diethylenetriamine

| **Element** | **Calculated %** | **Found %** |
|---|---|---|
| C | 53.08 | 52.13 |
| H | 13.27 | 14.31 |
| N | 9.63 | 9.48 |
| C/N | 5.51 | 5.50 |

From the elemental results above, it is clear that this compound is of acceptable purity since the difference in calculated and found percentages is within reasonable range.

The calculated percentages were based on the following structure, which will be supported by the MS results :

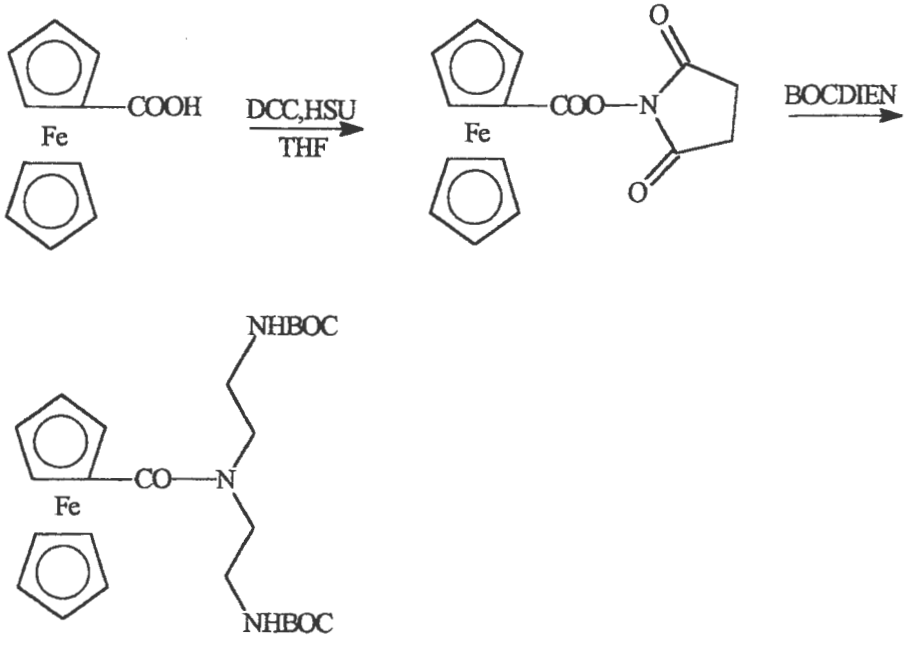
$C_{14}H_{30.5}O_{4.75}N_3$




**II) tris(BOC)-diethylenetriamine**

This compound was insoluble in water and hence the assumption that it is tris-protected as it is typical of tris-protected amines. The following observations were made from the NMR spectrum, shown in figure 18, of this compound : A singlet at 1.4 ppm accounts for 27 protons due to six BOC methyl groups and this is typical of BOC protons to appear in this region [64]. A multiplet at 3.2 ppm accounts for 8 protons from the four $CH_2$ groups bonded to BOC protected NH groups. These $CH_2$ groups are now the same, hence the protons from them all appear in the same region. It is also worth noting that a multiplet at 1.65 ppm is also present in this case, which is probably due to contamination by the monoBOC-protected compound.

**iii) Synthesis of ferrocene-bis(BOC)diethylenetriamine**




7

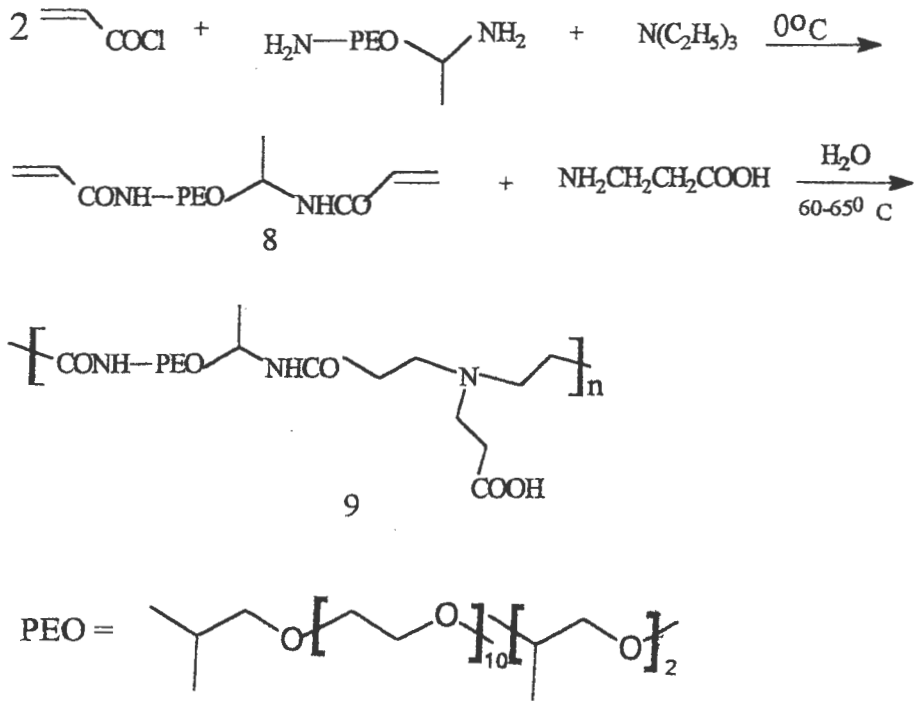
**Scheme 6**

Ferrocenemonocarboxylic acid (1g, 4.3 mmol) , DCC (0.86 g, 4.3 mmol), and HSU (0.495 g. 4.3 mmol) were dissolved in 20 mL THF and stirred for 24h. The *N,N'*- dicyclohexylurea was filtered off and washed with THF (5 mL). The filtrate was mixed with 6 mmol of bis-BOC-diethylenetriamine and stirred for another 24h. The solution was left overnight and the urea was filtered off. This solution was evaporated under vacuum at 40 °C to dryness. Ethyl acetate ( 40 mL) was added and this solution was washed two times with 20 mL water followed by 5% sodium hydrogencarbonate and 5% glacial acetic acid . The organic phase was dried with anhydrous magnesium sulphate. This solution was filtered and concentrated to 5 mL. This solution was then left in the fridge for crystallization.

mp : 135 °C

% yield : 15%

### 2.4.3 Synthesis of the carrier polymer




**Scheme** 7

Acryloyl chloride (0.02 mol, 1.84 g) was dissolved in 50mL of acetone and flushed with nitrogen. To this solution, jeffamine (0.01 mol, 6 g) and triethylamine(0.01 mol, 1.0 g) were added and this solution was stirred for 1hour in icebath. β-Alanine(0.01 mol) was then added, followed by additon of 50 mL distilled water. This was then stirred in an icebath for 3h and left overnight at room temperature. This solution was then stirred at 60-65 °C for 3 days. The solution was the concentrated on the rotary-evaporator and the resulting residue was washed three times with 25 mL of acetone : ether (1:1) solution. The yellowish residue was dissolved in 30 mL of distilled water and dialyzed against distilled water for 2 days with water being changed after 24h. This solution was freeze-dried to obtain a brick-red sol.

**i)Enzymatic Digestion**

A phosphate-buffered solution of the polymer was equilibrated 37 $^0$C and mixed with 4mg papain enzyme (10 units/mg)or 1 mg of proteinase A enzyme(15 units/mg). The final concentrations of the polymer and the enzyme in the reaction mixture were 5g.L$^{-1}$ and 0.04 g.L$^{-1}$ respectively. In the course of the reaction samples were withdrawn from each reaction mixture and the enzyme activity was stopped by heating the samples on boiling water bath for 5 minutes. The buffer solution was prepared as follows :1 L of 0.06 M $NaH_2PO_4.2H_2O$, 1 L of 0.06 M $NaHPO_4.2H_2O$, and 1 L of 0.2 M NaCl were mixed to give 3 L solution of 0.06 M NaCl buffered with 0.02 M phosphate of pH 7.8. This solution was filtered using the Millipore filtration kit before use.

The control samples were prepared in the same way but without the polymer to detect products due to enzyme autodigestion. It was found that the amount of the products of enzyme autodigestion was negligible as compared to the amount of polymer fragments, and as such they could not interfere with the analysis. Heating the polymer at 37 °C and 100 °C for 5 minutes did not have any impact on the polymer degradation.

**2.5 Measurements**

The following techniques were used to characterize the ferrocene-bisBOCdiethylenetriamine derivative and the polymer :

$^1$H NMR was taken on a Bruker Avance DPX - 300MHz in $D_2O$ for polymer 9 and BOCpropylenediamine and in $CDCl_3$ for all other compounds. The infrared was run on an Impact 410 FTIR spectrophotometer with resolution 8 cm$^{-1}$ and 200 scans and the photoacoustic device was used for polymer 9. Omnic software was used to treat the results. Elemental analysis was done by the Microanalytical Services at the University of Cape Town in duplicate for consistency and the results averaged.

The GPC analysis was done on a Waters 150C ALC/ GPC, with the refractive index detector

using a phosphate buffer solution of pH 7.8 as an eluent with a Waters Ultrahydrogel Linear column at 0.5 $mL.min^{-1}$ flow rate. The calibration curve was obtained using polyethylene glycol narrow standards with the following molecular weights : 940, 4350, 8560, 12000, 24800. The concentration of these standards in phosphate buffer solution was also 5 $g.L^{-1}$. The Millennium 2000 software from Waters was used for data acquisition. The working temperature for the column, injector, and the detector components was optimized at 50 °C.

Mass spectrometry was performed using a VG 70 SE spectrometer and the samples ionized using electron ionization(EI) directly at 70 eV with a 200 °C source. Electrospray mass spectra for the polymers were recorded in positive-ion mode on a VG Quattro triple quadripole spectrometer. Samples were dissolved in 100 µL of 50% (v/v) acetonitrile. Ten µL of the final solution was diluted to 100 µL with 50% acetonitrile containing 0.1% formic acid. Ten µL of the final solution were injected through Rheodyne injection valve and the carrier solvent was the solution of 50% acetonitrile at a flow rate of 20 $\mu L.min^{-1}$. Spectra were made of 10 scans and recorded using cone voltages in the range of 5 V to 20 V. The moving mean smoothing method was used for removing the noise peaks which are much narrower than the real peaks. The smoothing factors used were 5 for methoxypolyethylene glycol 5000 p-nitrophenyl carbonate and 10 for the polymer 9.

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1 INTRODUCTION

In the discussion that follows, results of attempts to synthesize potential candidates for anticancer drugs containing a ferrocene moiety are presented and discussed. Results of synthesis and characterization of a potential polymeric drug carrier exhibiting a carboxylic binding site are also presented and discussed. The results of enzymatic degradation of the this polymer are also presented and discussed. The FTIR and GPC results have been treated with the Microcal Origin 6.0 program to achieve readable curves. All NMR results given in this section are $^{1}H$ NMR, unless otherwise specified .

### 3.2 Syntheses of BOC-protected polyamines

### 3.2.1 Synthesis of BOC-ethylenediamine

The isolated crystals of the abovementioned compound were submitted for analysis using NMR spectroscopy in $CDCl_3$ . The NMR spectrum of this compound is shown in figure 13. The following observations from the spectrum support the fact that the compound obtained is an expected one : A singlet at 1.4 ppm accounts for 9 protons from six BOC methyl groups and it is characteristic of BOC protons to appear in this region [64]. A triplet at 2.8 ppm for 2 $CH_2NH_2$ protons. A triplet around 3.2 ppm is due to 2 $CH_2NHBOC$ protons which is comparable with the results by Krapcho et al.[64]. A small peak at 5.45 ppm is due to 1 proton from NHBOC group[65]. The peak due to 2 unprotected $NH_2$ protons overlaps with the BOC signal at around 1.4 ppm. A singlet at 4.2 ppm may be due to water still trapped in the crystals as they were re-crystalized from water. The $^{13}C$ NMR confirms that there are only the following types of carbon atoms (see figure 14) : 1 carbon from C=O at 170 ppm, 3 carbons from the 3 $-CH_3$ groups at around 28 ppm, 1 carbon from C-O group at around 76 ppm - all

of these carbons are from the BOC group. Finally, 2 carbons from the primary amine C-N groups which appear around 40 ppm [66].

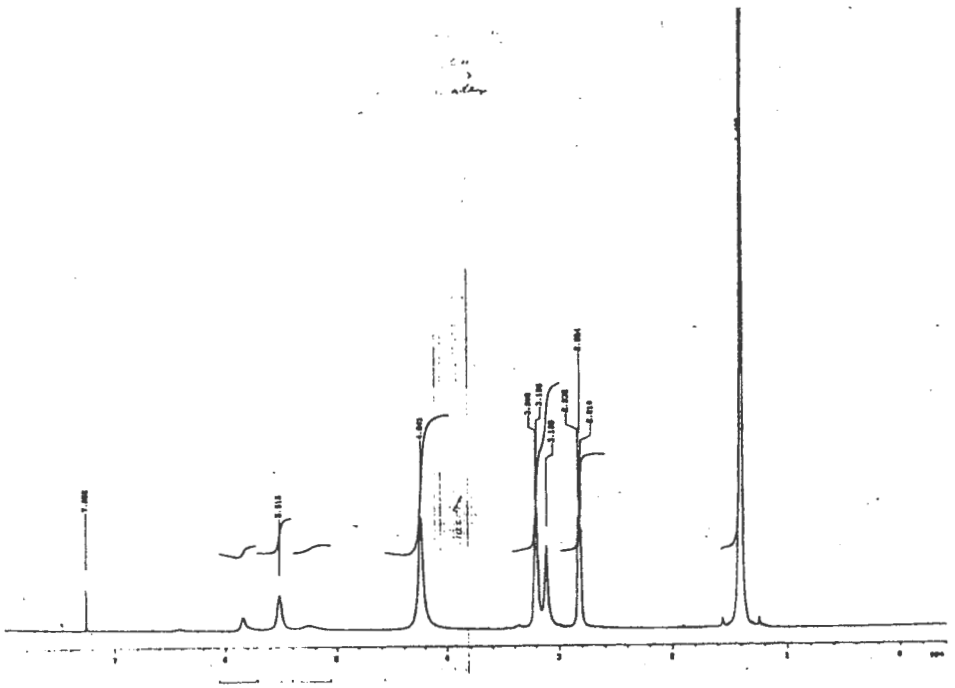


**Figure 13** : NMR spectrum of BOC-ethylenediamine

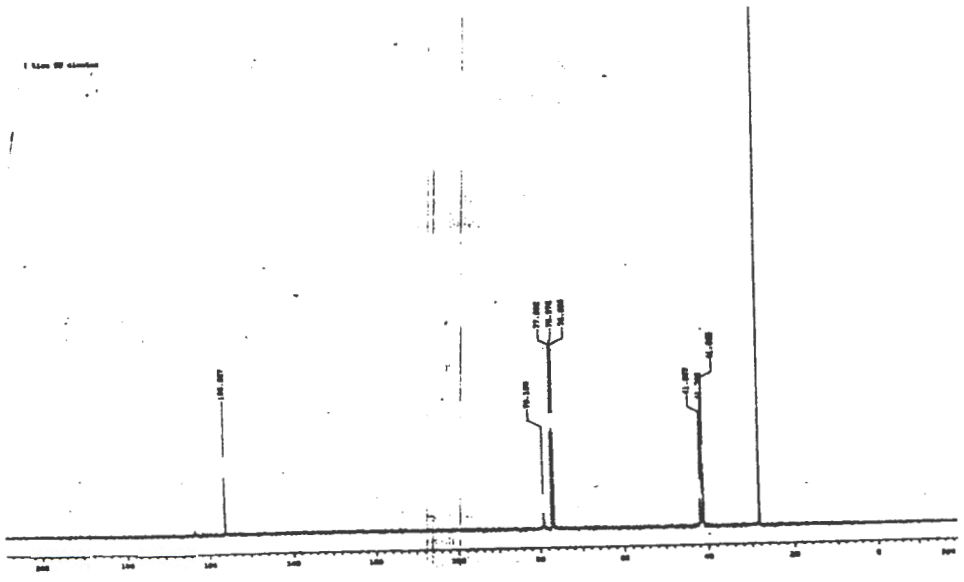


**Figure 14** : $^{13}C$ NMR spectrum of BOC-ethylenediamine

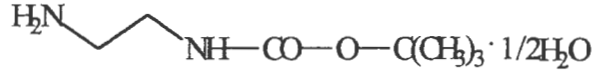
The elementary analysis shows that the composition of the product is consistent with the proposed structure (table 3 and scheme 1). Note that this compound has water as discussed in NMR results above.

**Table 3** : Analytical data for BOC-ethylenediamine

| **Element** | **Calculated %** | **Found %** |
|---|---|---|
| C | 49.70 | 49.53 |
| H | 10.00 | 9.00 |
| N | 16.56 | 15.60 |
| C/N | 3.00 | 3.17 |

The calculations above were based on the following structure :

$C_7H_{16.5}O_{2.5}N_2$




### 3.2.2. Synthesis of BOC-propylenediamine

The crystals of this compound were submitted to NMR spectroscopy in $D_2O$ because it is a salt and hence its more soluble in water than in an organic solvent. The proton NMR of this compound revealed the following information (see figure 15) : A singlet at 1.3 ppm is accounts for 9 protons from the BOC group. This is characteristic of BOC protons [64,65]. A multiplet at 1.65 ppm accounts for 2 protons from the middle -$CH_2$ group. A triplet at 2.85 ppm is due to 2 -$CH_2NH_3^+$ protons and finally, the triplet at 3.0 ppm is due to 2 -$CH_2$NHBOC protons [67]. Note however that NH groups protons are not seen here since this sample was run in $D_2O$.

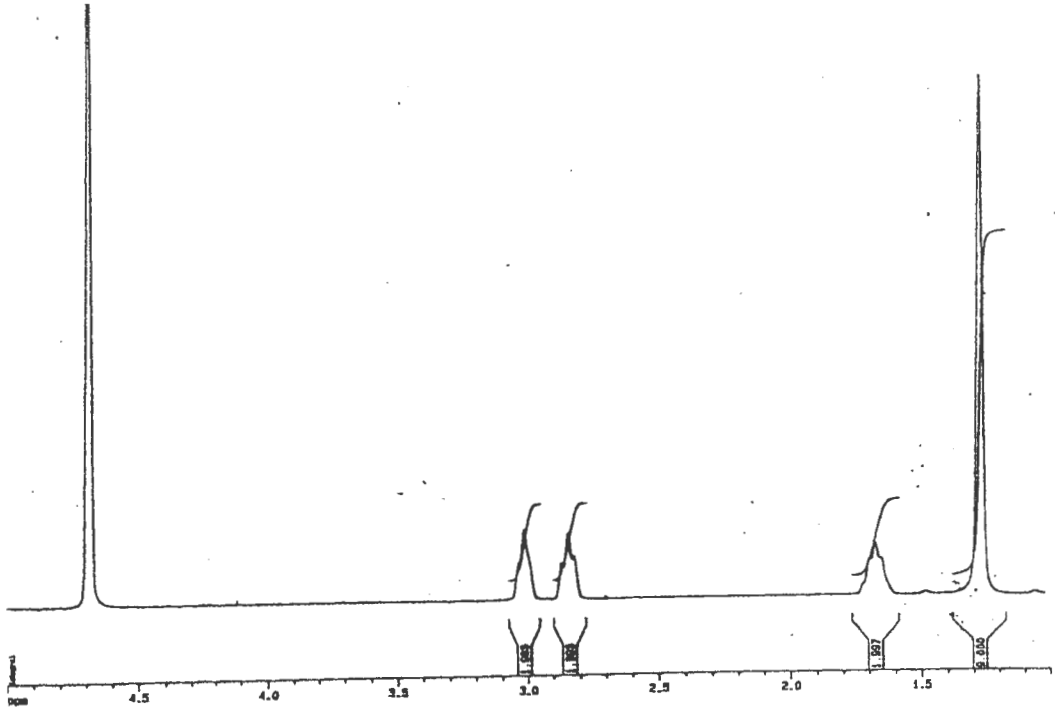



**Figure 15** : NMR spectrum of BOC-propylenediamine

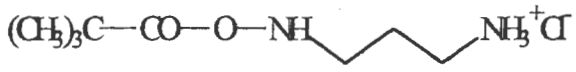
The following elementary analysis results show that the composition of this compound is consistent with the proposed structure (see table 4) :

**Table 4** : Analytical data of BOC-propylenediamine

| **Element** | **Calculated %** | **Found %** |
|---|---|---|
| C | 45.6 | 45.63 |
| H | 9.02 | 9.26 |
| N | 13.3 | 13.31 |
| C/N | 3.43 | 3.43 |

The above calculations were based on the following structure :

$C_8H_{19}O_2N_2^+Cl^-$



### 3.2.3. Synthesis of bis(BOC)-diethylenetriamine

This reaction gave a mixture of two products, namely; bis and tris- protected diethylene triamine. However bis-protected product was the target product which was isolated by using differences in water solubility of the two products. The analytical results of both compounds are given below.

#### I) bis(BOC)-diethylenetriamine

The NMR spectrum shown in figure 3 suggests that there was a bis-protected product. This is supported by the following observations from the spectrum :

A singlet at 1.4 ppm accounts for 18 protons from six BOC methyl groups and this is typical of BOC protons to appear in this region [64, 65, 66]. A singlet at 1.5 ppm accounts for 1 proton of the unprotected NH group and according to Krapcho et al. [64] this proton is usually found in this region and sometimes, depending on its environment, it overlaps with BOC. A triplet at 2.75 ppm accounts for 4 -$CH_2$NH protons, and again this is comparable to the results of the mono-BOC protection of dipropylenetriamine by Krapcho et al. [64], where it was found that a triplet due to 4 protons from -$CH_2$NH appeared at 2.65 ppm. A multiplet at 3.2 ppm accounts for 4 protons from two -$CH_2$ groups bonded to BOC protected NH groups.

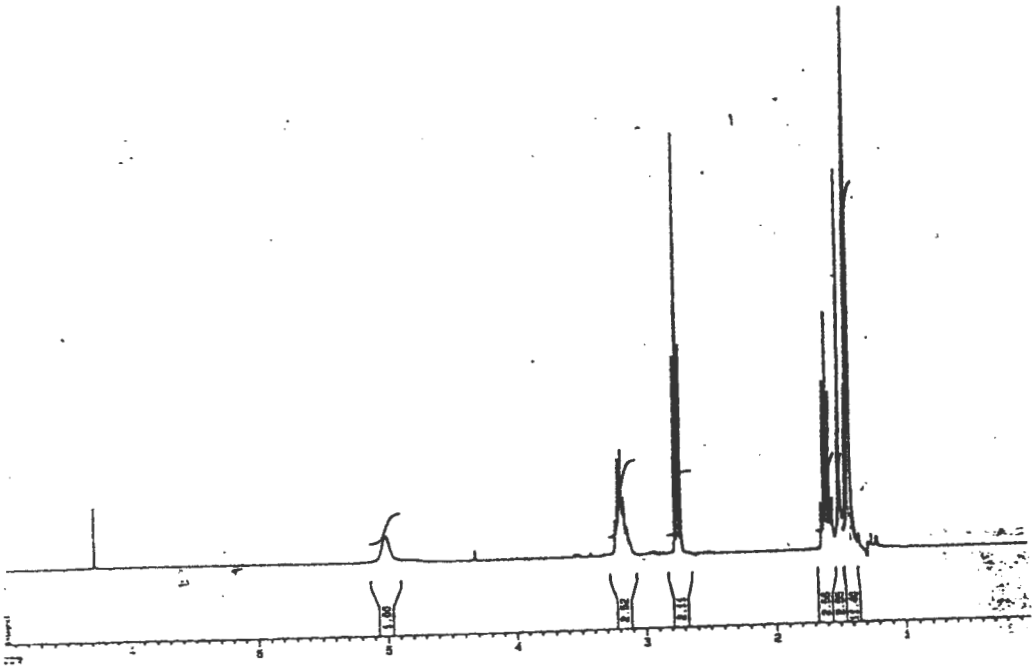
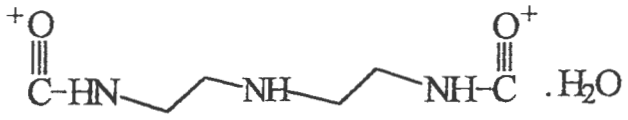


**Figure 16** : NMR spectrum of bis(BOC)- diethylenetriamine

This is in line with the results from the mono-BOC protection of ethylenediamine by Mattingly [65] where it was found that this type of proton appears in the region 3.13 - 3.19 ppm. A singlet at 5 ppm is due to the two protons of the two BOC-protected NH groups which according to Krapcho et al. usually appears at 5.2 ppm [64]. A multiplet at 1.65 ppm is probably due to contamination by monoBOC-protected compound. It is on this basis that the mass spectrum and elementary analyses of this compound were performed in order to draw a meaningful conclusion about the structure of this compound.

The figure 17 shows the mass spectrum of bis(BOC)-diethylenetriamine. This sample has a base peak at m/z = 57.07 with 100% relative abundance which corresponds to the fragment $C(CH_3)_3^+$ with a molecular weight of 57. The following peaks were also identified as important: 74.02 with relative abundance of 23% and 175.14 with relative abundance of 5%. A peak at 74.02 corresponds to the $C(CH_3)_3O^+$ fragment which has a molecular weight of 73. These two fragments referred to above come from the BOC group.

A peak at 175.14 corresponds to the following fragment :



Note that the molecular weight of this fragment is 175 and 157 without the water molecule. A peak at 101 with relative abundance of 55% corresponds to the above fragment without $H_2O$ and CO. The presence of this water molecule will be supported by the elemental analysis of this compound.

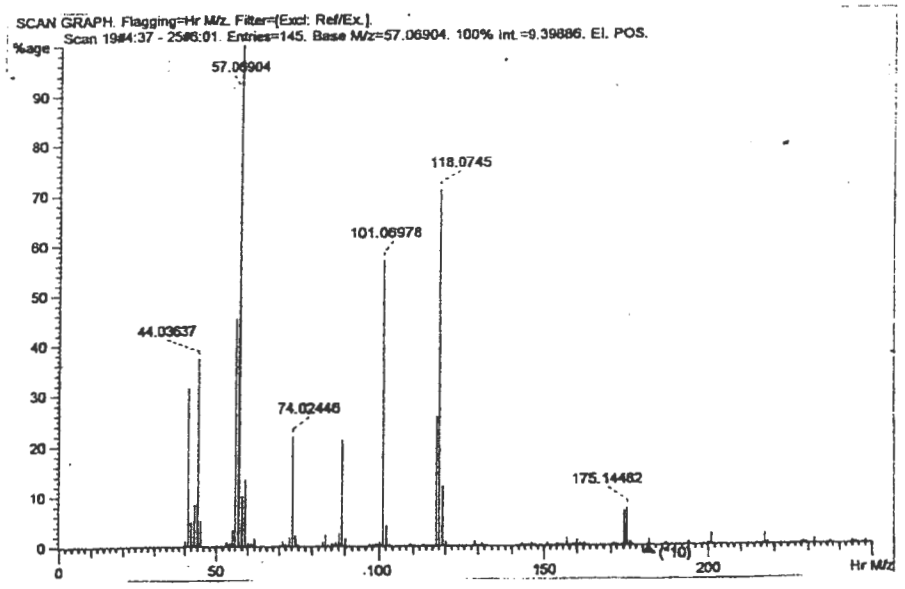



**Figure 17** :Mass spectrum of bis(BOC)-diethylenetriamine

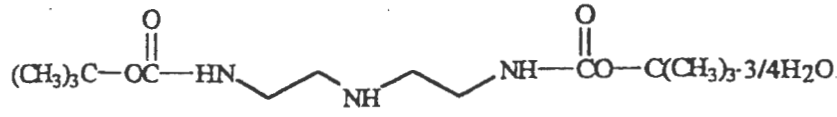
**Table 5** :Analytical results of bis(BOC)-diethylenetriamine

| **Element** | **Calculated %** | **Found %** |
|---|---|---|
| C | 53.08 | 52.13 |
| H | 13.27 | 14.31 |
| N | 9.63 | 9.48 |
| C/N | 5.51 | 5.50 |

From the elemental results above, it is clear that this compound is of acceptable purity since the difference in calculated and found percentages is within reasonable range.

The calculated percentages were based on the following structure, which will be supported by the MS results :

$C_{14}H_{30.5}O_{4.75}N_3$




**II) tris(BOC)-diethylenetriamine**

This compound was insoluble in water and hence the assumption that it is tris-protected as it is typical of tris-protected amines. The following observations were made from the NMR spectrum, shown in figure 18, of this compound : A singlet at 1.4 ppm accounts for 27 protons due to six BOC methyl groups and this is typical of BOC protons to appear in this region [64]. A multiplet at 3.2 ppm accounts for 8 protons from the four $CH_2$ groups bonded to BOC protected NH groups. These $CH_2$ groups are now the same, hence the protons from them all appear in the same region. It is also worth noting that a multiplet at 1.65 ppm is also present in this case, which is probably due to contamination by the monoBOC-protected compound.

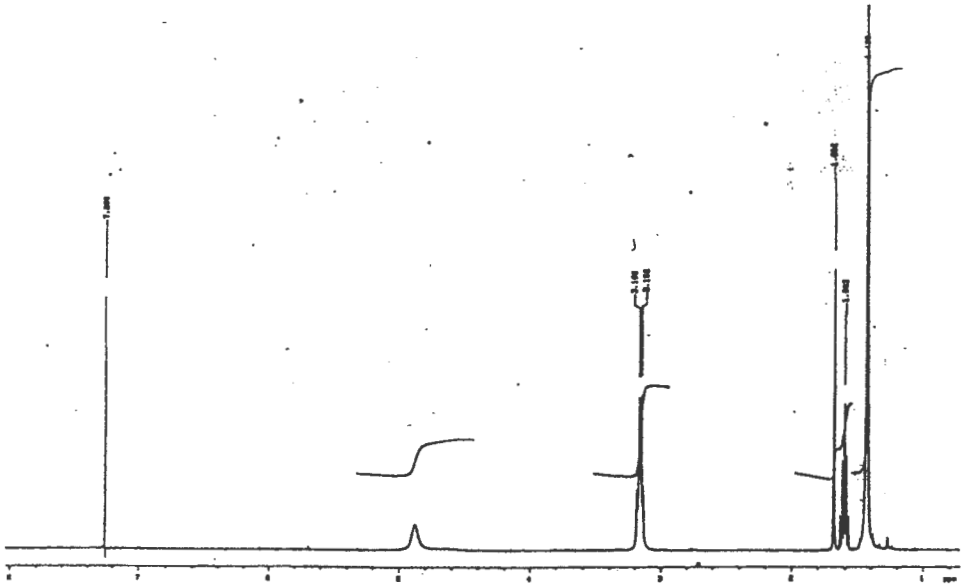


**Figure 18** : NMR spectrum of tris(BOC)- diethylenetriamine

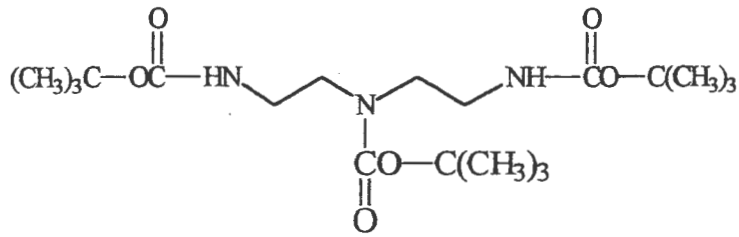
The following are the elementary analysis results of the tris(BOC)-protected diethylenetriamine (see table 6)

**Table 6** : Analytical data of tris(BOC)-diethylenetriamine

| **Element** | **Calculated %** | **Found %** |
|---|---|---|
| C | 56.43 | 56.91 |
| H | 9.18 | 9.82 |
| N | 10.42 | 10.34 |
| C/N | 5.41 | 5.50 |

These calculations were done on the basis of the following tris(BOC)-diethylenetriamine structure :

$C_{14}H_{37}O_6N_3$



In this case also, the differences in calculated and found percentages are within acceptable range.

### 3.3. Syntheses of ferrocene derivatives

#### 3.3.1. Synthesis of ferrocene-BOCethylenediamine

Scheme 4 outlines the synthesis of the above mentioned compound, where DCC and HSU were used as coupling agents. Compound 4, which is an intermediate in this reaction, was isolated and submitted for fast atomic bombardment mass spectrometry (see figure 19). The purpose of this exercise was to confirm the existence of this intermediate.

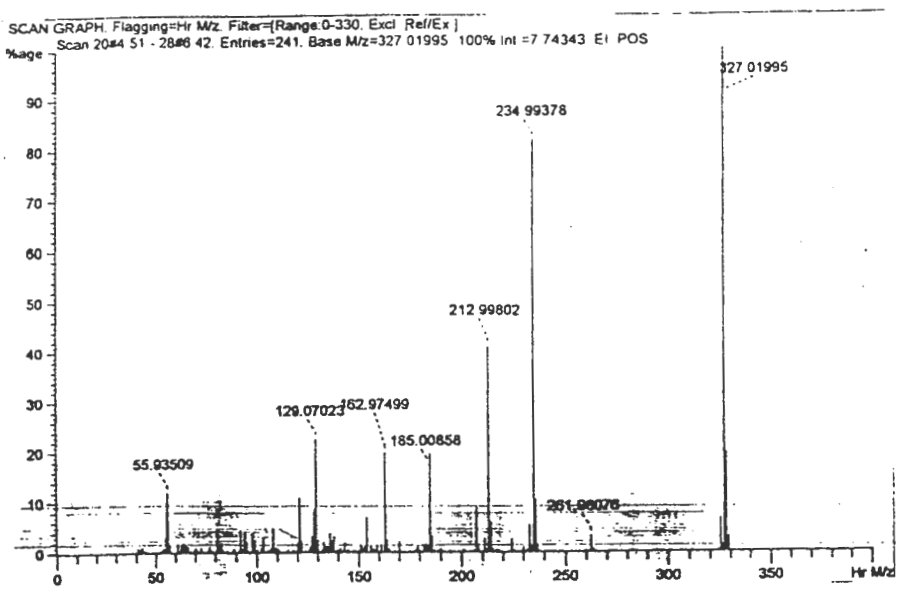



**Figure 19** : FAB mass spectrum of the intermediate (compound 4)

This mass spectrum shows a base peak at 327.02 The following peaks were identified as important in identifying this compound : 327.02with relative abundance of 100% with the

formula $C_{15}H_{13}NO_4Fe$, 55.93 which corresponds to Fe with relative abundance of 11.76, and 129.07 with the relative abundance of 22.45 which corresponds to $C_{10}H_9^+$ fragment. The calculated molecular weight of this intermediate is 327.02 which is the same as the found one. The presence of the ferrocene moiety is further corroborated by the presence of Fe-56 [68] and the two cyclopentadienyl rings with the calculated molecular weight of 129.07.

Ferrocene-BOC-ethylenediamine was submitted for NMR spectroscopy and was run in $CDCl_3$. The NMR spectrum (figure 20) of this compound gave the following information : A singlet at 1.45 ppm accounts for 9 BOC protons and a singlet at 1.65 ppm is due to one ferrocenyl-CONH proton. A triplet at 3.2ppm is due to two $CH_2$NHBOC protons and the one at 3.4 ppm is due to two ferrocenyl-CONHC$H_2$ protons. A cluster of peaks between 4 ppm and 5 ppm is due to 9 ferrocene protons and lastly a small peak at 5.1ppm is due to one NHBOC proton.

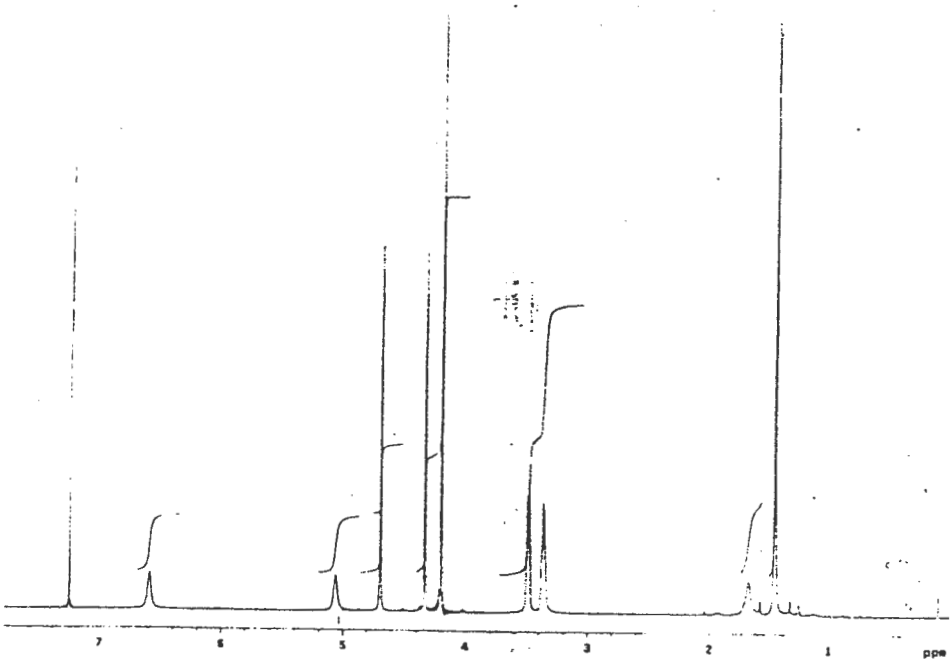



**Figure 20** : NMR spectrum of ferrocene-BOC-ethylenediamine

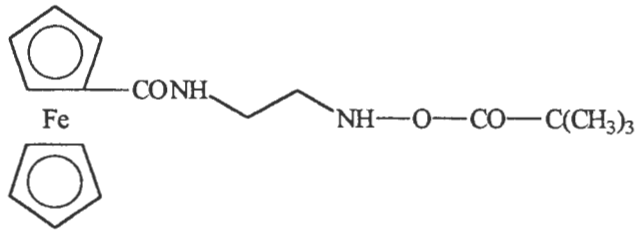
The following; elementary analysis results (see table 7) support the proposed structure of the abovementioned compound as outlined in scheme 4.

**Table 7** : Analytical data for ferrocene-BOC-ethylenediamine

| Element | Calculated % | Found % |
|---|---|---|
| C | 58.00 | 58.63 |
| H | 6.28 | 7.06 |
| N | 6.77 | 7.52 |
| C/N | 8.57 | 7.80 |

The above calculations were based on the following structure :

$C_{18}H_{24}N_2O_3Fe$




### 3.3.2. Synthesis of ferrocene - BOC-propylenediamine

This compound was also submitted to NMR spectrometry in $CDCl_3$. The NMR spectrum (figure 21) of this compound gave the following information : A singlet at around 1.45 ppm accounts, as before, for 9 protons from three methyl groups from BOC. A multiplet at 1.65 ppm is due to 2 protons from the -$CH_2$ $CH_2CH_2$- group.A triplet at 3.2 ppm is due to the 2 protons from the $CH_2NHBOC$ group and another triplet at 3.4 ppm accounts for 2 protons from the ferrocenyl-$CONHCH_2$ group. A cluster of peaks between 4 ppm and 5 ppm is due to 9 protons from the ferrocene cyclopentadienyl rings. A small peak at 5ppm is due to 1 proton of the BOC protected NH. Lastly the peak due to 1 NH proton from ferrocenyl-CONH group may be overlapping with the BOC signal.

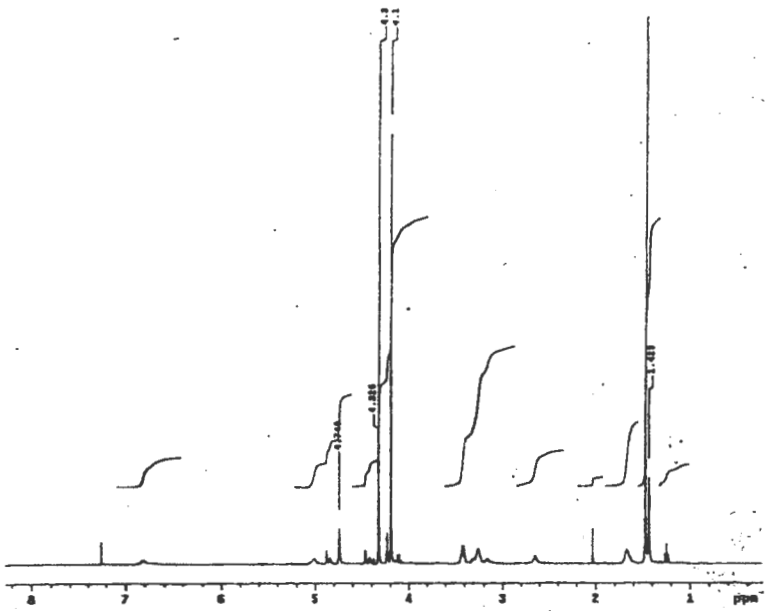


**Figure 21** : NMR spectrum of ferrocene -BOC-propylenediamine

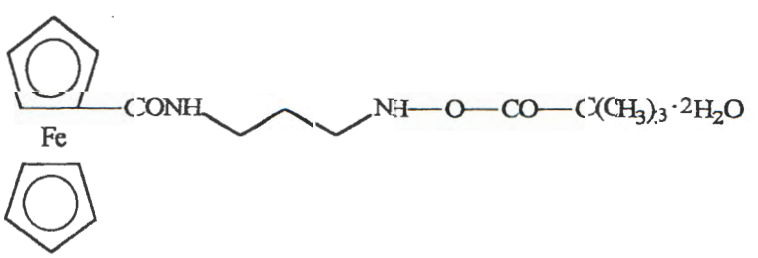
The elementary analysis results of this compound given in table 8 are consistent with the proposed structure as outlined in scheme 5.

**Table 8** : Analytical data of ferrocene-BOC-propylenediamine

| **Element** | **Calculated %** | **Found %** |
|---|---|---|
| C | 53.67 | 53.68 |
| H | 7.06 | 6.41 |
| N | 6.59 | 6.28 |
| C/N | 8.14 | 8.55 |

The calculations above were based on the following structure. Note that there is still some water in the crystals as they were re-crystalized from water.

$C_{12}H_{30}N_2O_5Fe$



### 3.3.3 Synthesis of ferrocene-bis(BOC)-diethylenetriamine

This orange crystalline compound with melting point 135°C had the following results :The NMR spectrum of this compound is shown in figure 22. The split peak at 1.4 ppm is due to 18 protons from BOC. The splitting is due to different environments experienced by the two sets of protons due to the two different ferrocene rings. The singlets between 4 ppm and 5 ppm are due to 9 protons from the ferrocene rings. A multiplet around 3.2 ppm is due to 4 protons of the two $CH_2$'s bonded to the BOC protected NH groups. A singlet at 2.6 ppm is due to 4 equivalent protons from the two $CH_2$ groups bonded to the amine. Again in this case, a multiplet around 1.6 ppm is present which is due to contamination by the monoBOC-protected compound . Lastly, two singlets at 6.5 ppm and 6.7 ppm are from protons of amide NH groups.

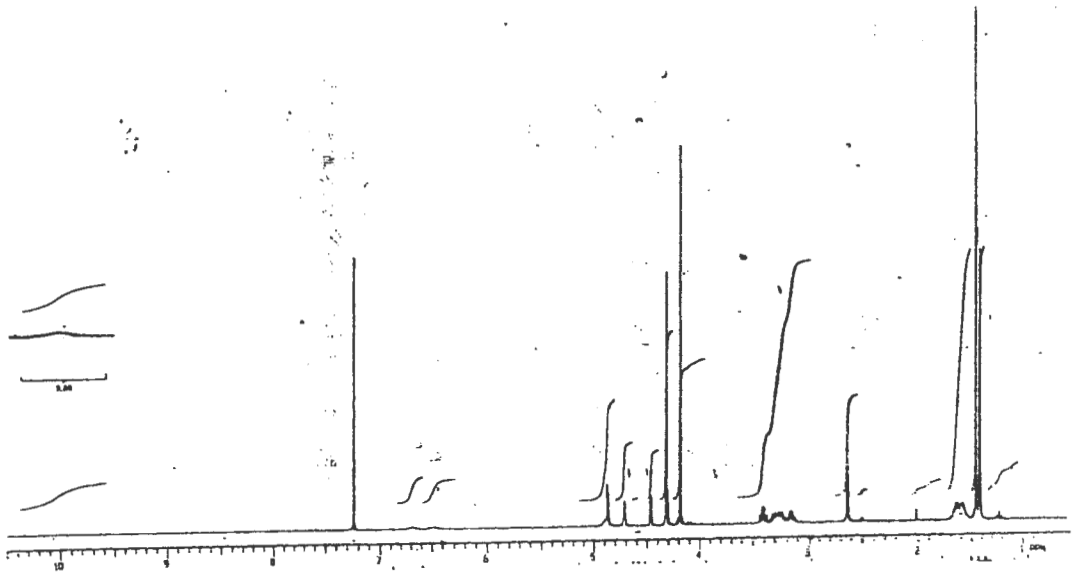


**Figure 22** : NMR spectrum of ferrocene - bis(BOC)-diethylenetriamine

The mass spectrum of this compound (figure 23) shows a base peak at 312.06. The following peaks were identified as crucial in identifying this compound : 386.13 with relative abundance of 69%, 312.05 with relative abundance of 100%, which corresponds to the following

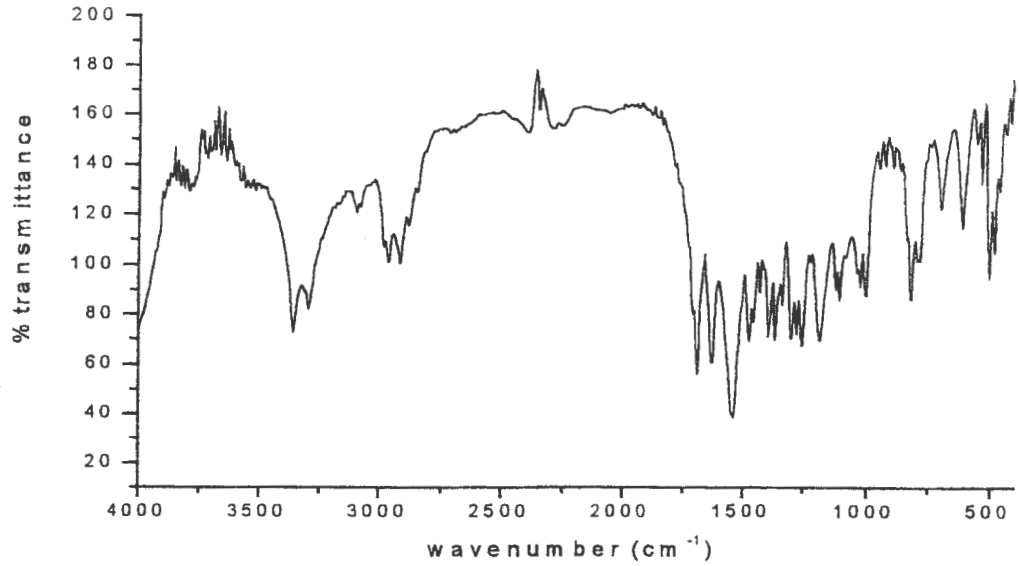



**Figure 24** : FTIR spectrum of ferrocene-bis(BOC)-diethylenetriamine

The FTIR spectrum (figure 24) of this compound reveals the following results : A peak at 1700 $cm^{-1}$ is due to an amide carbonyl group. A peak with a shoulder just above 3350 $cm^{-1}$ is due to an amide NH group and a small sharp peak around 3100 $cm^{-1}$ is due to the two cyclopentadienyl ligands from the ferrocene moiety. The broad peak above 3500 $cm^{-1}$ is due to the water molecule found in this compound.

### 3.4. Synthesis of the carrier polymer

#### 3.4.1. Analytical results of the polymer

To synthesize biodegradable, biocompatible, and polyamide-containing polyanionic polymer, O, O'- bis(2-aminopropyl) poly(ethylene oxide) was chosen as a monomer since it is biocompatible and PEO grafts protect the drug from binding and capture by reticuloendothelial system. Synthesis of the polymer was a typical Michael addition reaction and is outlined in scheme 7. The first step of the reaction consists of the addition of an acrylamide group at both ends of Jeffamine ED600. This step required very low temperatures to avoid autopolymerization of polymer 8 and the second step was the incorporation of the

carboxylic side-chain into the expected polymer carrier. To ensure correct polymerization, the temperature of the reaction mixture had to be increased progressively. The evidence of existence of polymer 8 was confirmed by isolating it by filtering the mixture after the first step in order to eliminate salts of triethylamine formed during acryloylation and leaving the solution to evaporate in the air. The resulting whitish oily substance was submitted to NMR spectroscopy in $CDCl_3$. The NMR data of polymer 8 are detailed in table 9. The NMR spectrum of this polymer is shown in figure 25 .

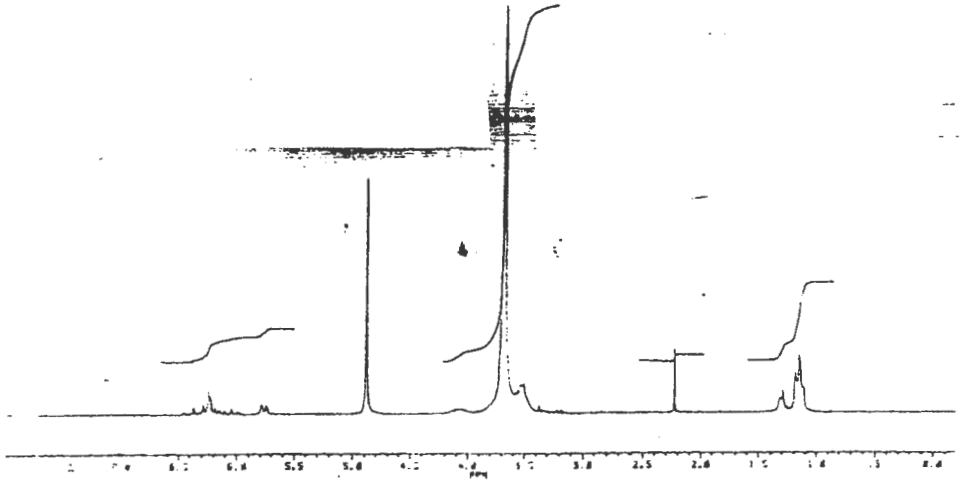


**Figure 25** : NMR spectrum of polymer 8

**Table 9** : NMR data for polymer 8

| | $CH_2$=CH (6.3-5.8ppm) | $CH_2$-O, CH-O, $NH_2CH(CH_3)CH_2O$ (3.8-3.5ppm) | $CH_3$-CH (1.1-1.3ppm) |
|---|---|---|---|
| No of protons expected | 6 | 52 | 12 |
| No of protons found | 5.4 | 51 | 12 |

A set of signals due to 5.4 protons between 6.3 and 5.8 ppm can be attributed to the 6 vinyl protons. a cluster of peaks around 3.7 ppm with a count of 51 protons is due to $CH_2$-O, CH-O, and $NH_2CH(CH_3)CH_2O$ from jeffamine ED600 and lastly the methyl group signals are found at arount 1.1 ppm. The fact that all methyl groups are above 1.0 ppm is a clear proof that acryloylation has successfully occured.

The polydispersity obtained for polymer 9 is 1.13.This is a very important feature for a potential polymer carrier as it has been suggested earlier on. The NMR data of the starting material jeffamine ED600 and the polymer are given in table 10 and 11, respectively. The NMR spectra of these two polymers are given in figure 26. The NMR spectrum of jeffamine ED600 shows two doublet peaks around 1 ppm which are from the $CH_3$-CH protons and on the other hand the peaks around the same region look different in the case of the polymer due to the reaction of the starting material with acryloyl chloride which means that the environment is progressively changing.

In another words, the presence of acryloyl chloride has vastly increased the number of $CH_2$ protons. The peaks around 2.3 - 3.2 ppm in the jeffamine ED600 NMR spectrum are due to CH-$NH_2$, CH-$NH_2$,$CH_2$-O protons. Note that in the case of the polymer, the peaks in the same region look different because the terminal $NH_2$ groups have reacted with acryloyl chloride. It is however important to note that in both cases peaks due to $CH_2$-O protons are visible at 3.3 - 3.7 ppm which means that both compounds have a poly(ethylene oxide) backbone. There is a good agreement between these results and the proposed structure of polymer 9 (scheme 7)

**Table 10** : NMR data of jeffamine ED600

| Type of protons | $CH_2$-O, CH-O (3.3 – 3.7 ppm) | $CH_3$-CH (0.8 – 1.1 ppm) | CH-$NH_2$ (2.6 – 3.2 ppm) |
|---|---|---|---|
| Calculated number of protons | 50 | 12 | 6 |
| Found number of Protons | 49 | 12 | 7 |

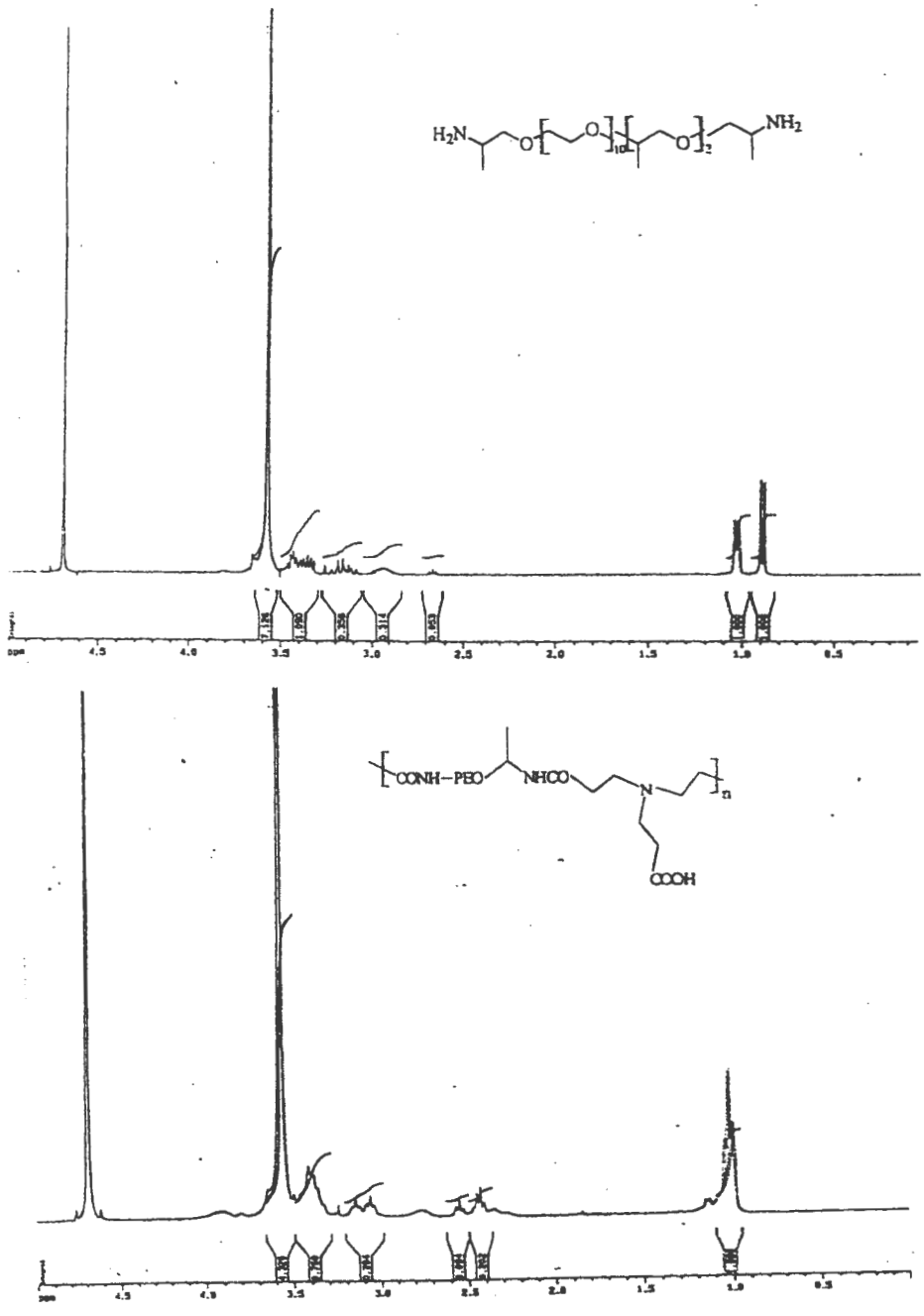



**Figure 26** : NMR spectra of jeffamine ED600 and the polymer, respectively.

**Table 11** : NMR data of the carrier polymer

| Type of protons | $CH_2$-O, CH-O (3.3 - 3.7ppm) | $CH_3$-CH (1.0ppm) | CH-NH, CH-NH, $CH_2$-O(2.3 - 3.1ppm) |
|---|---|---|---|
| Calculated number of protons | 50 | 12 | 14 |
| Found number of protons | 51 | 12 | 16 |

The FTIR (see figure 27) spectrum of jeffamine a peak of low intensity at 3600 $cm^{-1}$ and at 3375 $cm^{-1}$ which is due to N-H stretching. The region between 1590 to 1610 $cm^{-1}$ is due to N-H deformation. However, when one looks at the FTIR spectrum of the polymer, the region 3000 - 3500 $cm^{-1}$ is different from that of the starting material due to the formation of amide bonds and the presence of water which was used in the reaction. These observed peaks in this region are due to secondary amide N-H stretching. Another point worth noting is that the peaks in the region 1590 - 1610 $cm^{-1}$ are very weak to a point of disappearance due to the formation of amide bonds. That is, the amines are converted to amides. The region between 2700 - 3000 $cm^{-1}$ has a peak in both the starting material and the product, the only difference is their intensity.

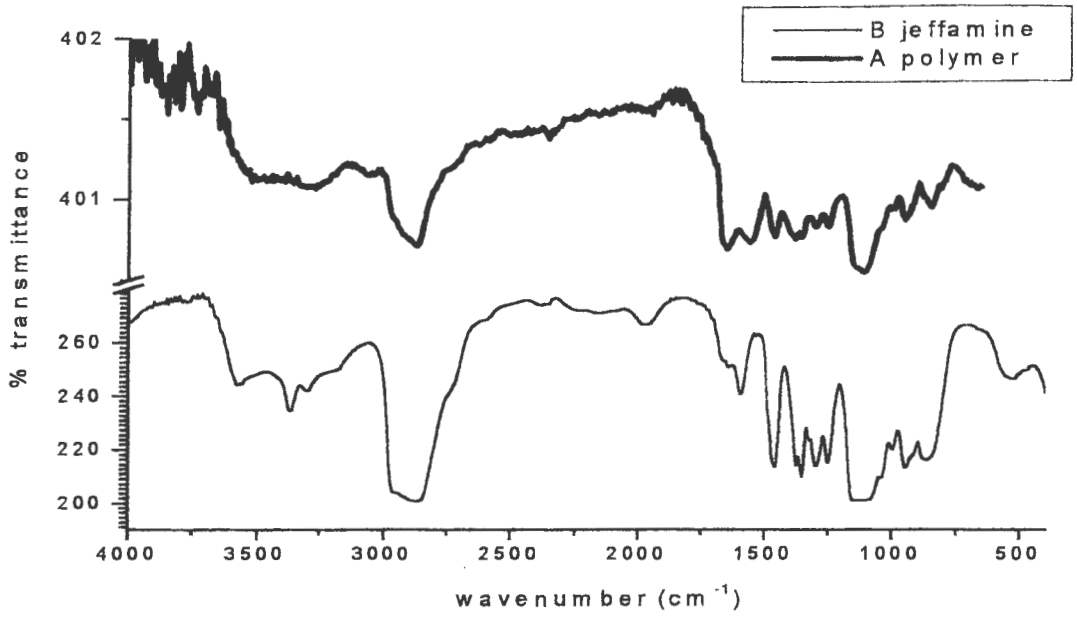



**Figure 27** : FTIR spectra of jeffamine ED600 and the carrier polymer

The peak for the starting material has a shoulder which is due to $CH_3$ and sharp peak around is due to $CH_2$- O (ether from the PEO backbone) stretching. On the other hand, the same peak for the product has three shoulders which are due to $CH_3$ and $CH_2$ and the sharp peak is from the OH of the -$CO_2H$ group. We also see a peak due to this group at 1430 $cm^{-1}$. There is a peak at 1655 $cm^{-1}$ for the polymer which is due to the amide carbonyl group and the shoulder at 1658 $cm^{-1}$ which is due to the C=O of the -$CO_2H$ group , but this is not observed in the case of the starting material. There is peak at the region 1000-1155 $cm^{-1}$ is similar in both cases and it is due to the C-O-C and $CH_2$-O of the PEO backbone.

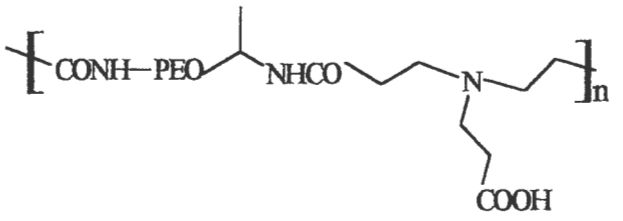
The elemental analysis results (table 12) reveals that the composition of the repeat unit of the carrier polymer is consistent with the proposed structure of polymer 9 as outlined in scheme 7.

**Table12** : Analytical data of the carrier polymer

| | CALCULATED | FOUND |
|---|---|---|
| % C | 55.58 | 54.29 |
| % H | 9.00 | 9.27 |
| % N | 4.74 | 4.71 |
| C/N | 11.72 | 11.53 |

The difference in calculated and found percentages is within acceptable range and as such these results represent the purity of the polymer. These calculations were performed on the basis of the following structure :

$(C_{41}H_{60}O_{13}N_3)_n$




### 3.4.2. Molecular weight measurement and enzymatic Degradation of the polymer.

The molecular weight of this polymer was determined by GPC in a pH 7.8 buffer solution. The problems associated with accurate GPC measurements of molecular weight distribution (MWD) of water-soluble polymers are well known and are described elsewhere [69]. We tried to determine the molecular weight distribution of this polymer by GPC relative to narrow poly(ethylene glycol) standards, but the measured molecular weight was only 1375 .This value is very low since the dialysis was performed with a cut-off limit of 12 000. This result can be explained by the fact that ionic and branched polymers act as comblike structures in solution and therefore their hydrodynamic volume is smaller than thei linear and neutral analogs. The other problem is that we were not able to obtain commercially available narrow polyanionic standards which would have been similar to this polymer.

In an attempt to determine the absolute molecular weight of polymer 9, the electrospray mass spectrometry (ESMS) was used. This technique is relatively new but powerful for the characterization of samples, which may be thermally unstable and non volatile such as biopolymers as described by Colton et al. [70 ]. During ionization, large biomolecules are normally multiply charged. The number of charges varies somewhat with specific composition of the polymer and the pH of the system. The figure 28 shows the spectrum of polymer 9 in positive mode.

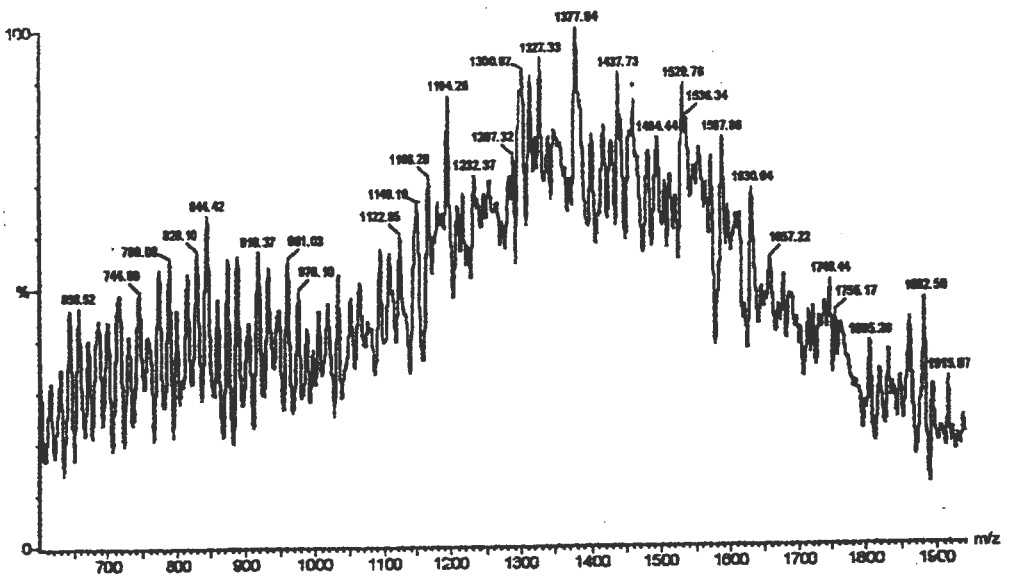



**Figure 28** : Electrospray mass spectrum of polymer 9

It is evident that it represents the molecular weight of the polymer. This spectrum has a base peak at approximately 1378. The mass of the repeating unit will be represented by the mass difference between two consecutive peaks. In this case, if the ions are considered singly charged, the mass of repeating units would be 14, 15, and 16. Clearly the mass of the repeating units are too low to be real. This spectrum does not, however, represent typical multiply charged spectra. In a multiply charged spectrum of a macromolecule, the mass differences between adjacent peaks decrease from right to left, whereas in the case of polymer 9 they are roughly equidistant across the mass range. So we decided to compare this spectrum to that obtained from methoxypolyethylene glycol 5000 p-nitrophenol carbonate in the same

conditions. The latter is shown in figure 29.

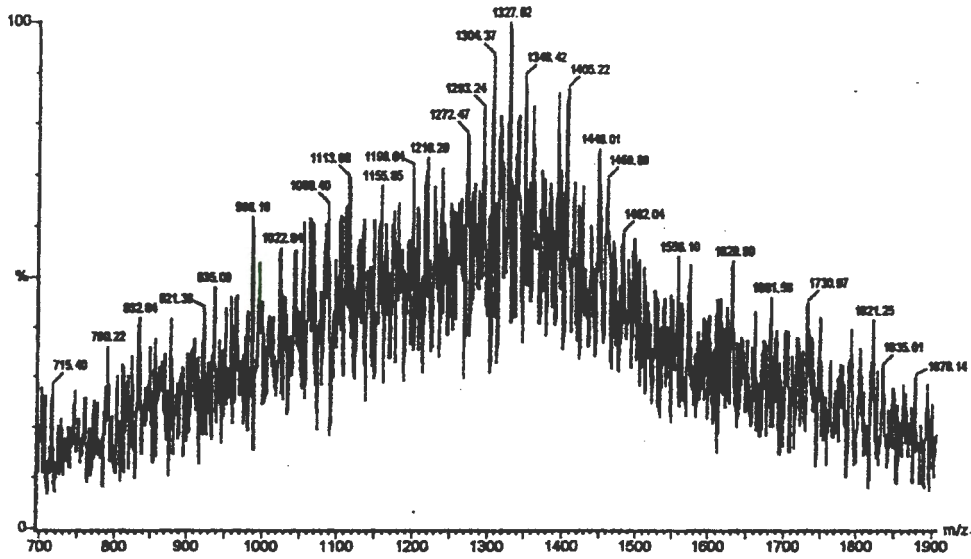



**Figure 29** : Electrospray mass spectrum of methoxypolyethylene glycol 5000 p-nitrophenyl carbonate

It has a base peak at 1327, which means that this compound has molecular weight of 1327 and notably the pattern for peaks in this case is the same as for polymer 9. Since the true molecular weight of this compound is 5000, it could be considerd as carrying 4 charges. Assuming that polymer 9 is similarly charged, its absolute molecular weight would be 5500. This polymer could go through the dialysis membrane of 12 000 cutoff limit because its molecules stick together to form aggregates and therefore behave like larger molecules.

The enzymatic degradation tests on this polymer were done using papain and proteinase, which are both endopepetidases at pH 7.8 and 37 °C . These two enzymes used in this study have been chosen because they provide a kinetic model for the endopeptidase degradation in mammals. For instance, Pytela et al report that both papain and cathepsin B proved to be effective in endopeptidase degradation of PHEG and its degradation products were found in

native kidney tissue [71].

The enzymatic degradation of the carrier polymer is shown in figures 30 and 31. We chose not to describe molecular weight changes of this polymer by $M_n$ or $M_w$ commonly used to describe the average molecular weight distributions of polymers. But the tendency toward degradation as a result of molecular weight distribution change could easily be monitored by using $M_p$ [60] In an aqueous solution of pH 7.8, it is shown in figure 30 that the polymer was degraded by papain.

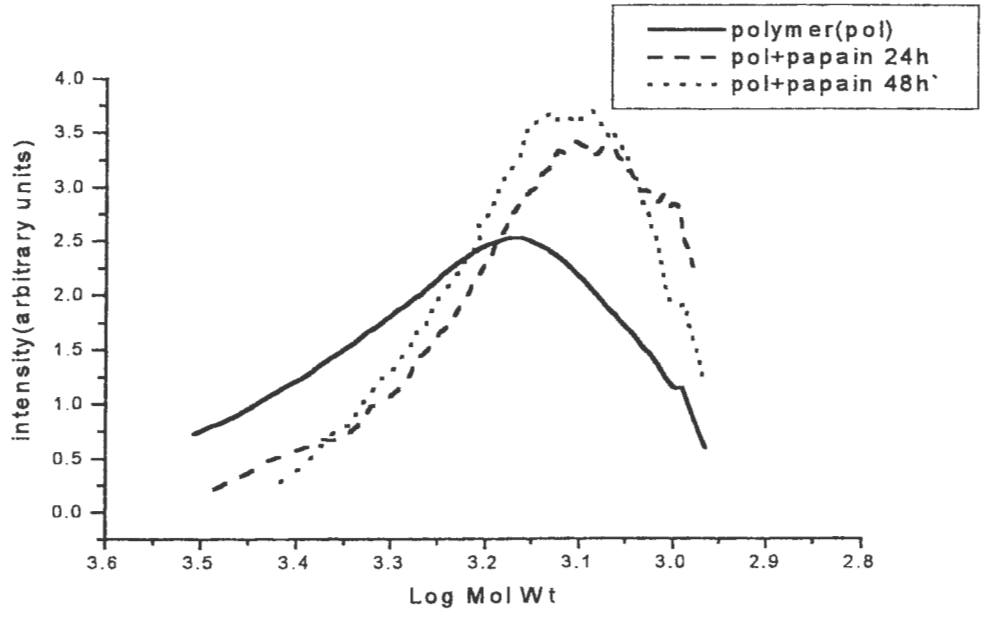



**Figure 30** : Molecular weight distribution curves of polymer 9 and the degraded products for papain activity

This enzymatic degradation of the polymer by papain becomes apparent after at least 24 hours. After 24 hours of incubation, the papain showed 81% degradation of the polymer and this remains the same after 48 hours. It is also worth noting that the polydispersity of the polymer is decreasing as the enzymatic degradation progresses. This means that the polymer is baing progressively cut into oligomers of almost the same chain lengths. The figure 31 below shows

the enzymatic degradation of the same polymer by proteinase

The conditions for this reaction are similar to the ones described above. However, the degradation proceeded relatively fast, and it is also evident that the enzyme remains active even beyond 48 hours. This can be seen from figure 31. These results for proteinase A show that there is a continuous degradation throughout as the polymer has degraded by 82% and 78% for 24 hours and 48 hours respectively. The polydispersity is decreased also in this case. This means that the polymer is being progressively cut into oligomers of the almost the same chain lengths. It is therefore clear that in both cases the polymer is degraded by the endopeptidase enzymes.

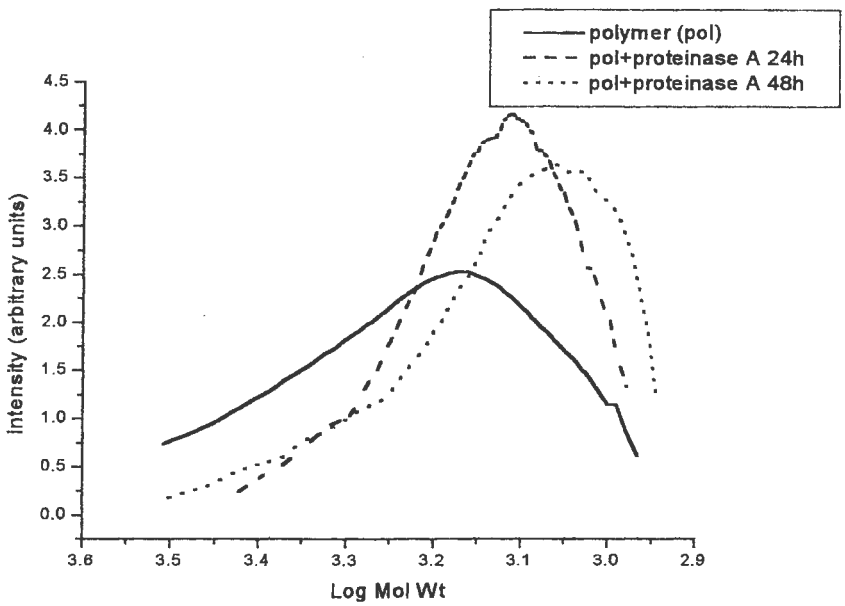



**Figure 31** : Molecular weight distribution curves of polymer 9 and the degraded products for proteinase A activity

Other studies under similar conditions showed a 40% degradation of poly(amino acid) by papain after 2 hours and a 20% degradation of poly(ethylene glycol) derivative by trisosomes after 5 hours [71]. The relative stability of polymer 9 to enzymatic degradation could be

attributed to its ionic nature (the carboxylic acid group is ionized at pH 7.81, giving a zwitterion form similar to that of amino acids). As mentioned earlier [54], low degradation is one of the important requirements for a polymer carrier since lower degradation rate leads to lower immunogenecity and thus the reduction of patient intolerance. This means that higher blood concentrations and longer residence time for the drug can be maintained.

# CHAPTER 4

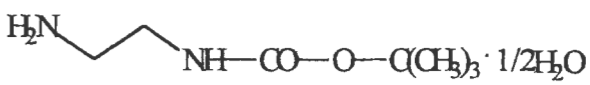
# CONCLUSIONS

## 4.1 INTRODUCTION

In the previous chapter the results were detailed and discussed without necessarily drawing any conclusions. In this chapter results will be interpreted and conclusions together with recommendations will be drawn.

## 4.2 Syntheses of BOC-protected polyamines

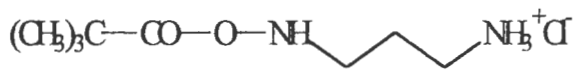
The NMR and elementary analyses have confirmed the following structure for BOC-ethylenediamine :



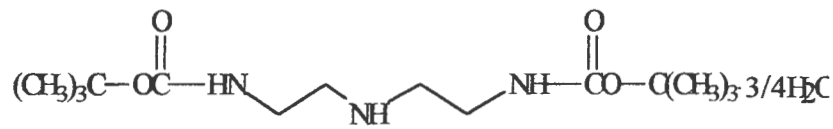

In the NMR spectrum of this compound there is a peak around 4.2ppm which could not be accounted for. So we decided to run $^{13}C$ NMR which revealed only 4 groups of carbon atoms as outlined in 3.2.1. This peak around 4.2 ppm may therefore be due to water. This conclusion is further corroborated by the elemental results of this compound which also revealed that there is water in the crystals of this compound.

The NMR results of the BOC-propylenediamine confirm that this compound has the expected structure. The elemental analysis reveals that this compound is in fact a chloride salt since the amine groups of propylenediamine were protonated using HCl prior to BOC protection. It is therefore concluded here that BOC-propylenediamine has the following

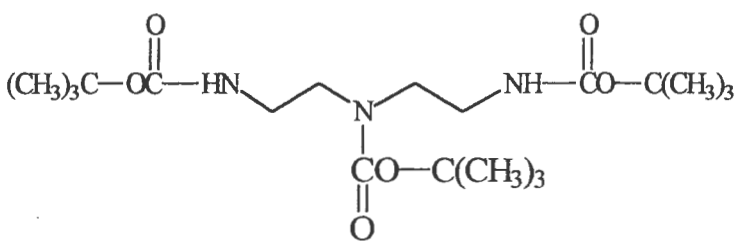
structure :



As seen from the results section, the BOC-protection of diethylenetriamine gave a mixture of bis- and tris- substituted compounds. It is however worth noting that the most abundant species was the tris-substituted one since it is the most preferred compound because it is the most stable, followed by the bis-substituted compound. The last one if present at all would be the mono- substituted compound which is very difficult to isolate. The NMR, mass spectrum, and the elemental analyses of the bis(BOC)-ethylenediamine led to the conclusion that we have the following structure :



During the preparation of the above compound a whitish insoluble compound was also collected. The NMR and elemental analyses of this insoluble product showed that it was a tris-substituted compound and the following structure was confirmed :



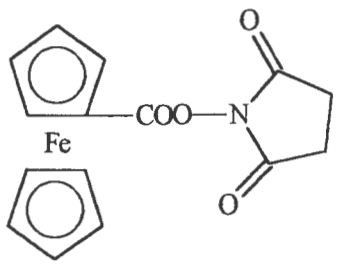
The NMR spectra of both the bis(BOC)-diethylenetriamine and tris(BOC)-diethylenetriamine

show a peak which could not be accounted for around 1.65 ppm. This is probably due to contamination by trace amounts of the mono-substituted compound.

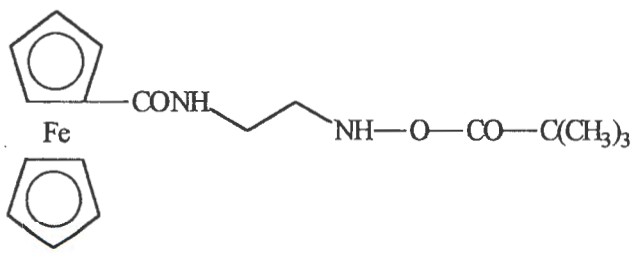
All the results of these BOC-protected polyamines are comparable to the ones in other similar published works [64,65,67].

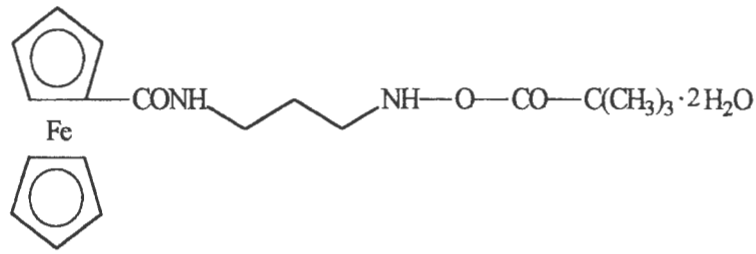
### 4.3 Syntheses of ferrocene derivatives

In coupling the BOC-protected polyamines to the ferrocenemonocarboxylic acid, DCC and HSU were used as coupling agents. The intermediate in all these coupling reactions was isolated and proved to be the same. This intermediate was isolated and submitted for FAB-MS. The analysis of these results confirmed the following structure for this intermediate with a formula mass of about 327.11:

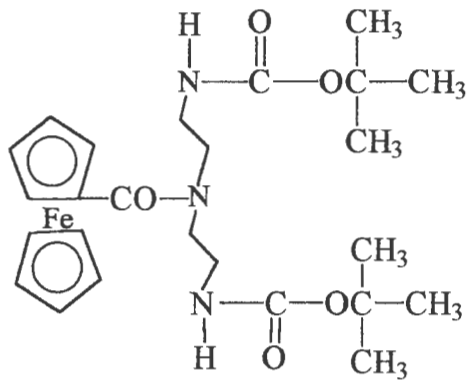



Ferrocene-BOC-ethylenediamine and ferrocene-BOC-propylenediamine were submitted for NMR spectroscopy and elemental analysis. These results confirmed the following structures for these compounds, respectively :






The ferrocene-bis(BOC)-diethylenetriamine was submitted for NMR spectroscopy and mass spectroscopy. The results from these analyses confirmed the following structure :



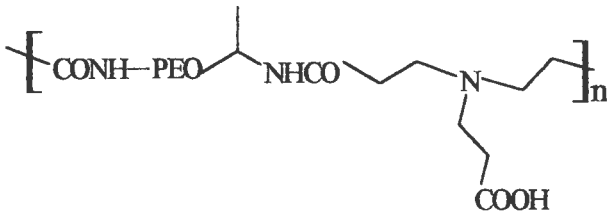

Note however that the NMR spectrum of this compound still shows an unaccounted for signal around 1.65 ppm. Again, this is probably due to contamination by trace amounts of the mono-substituted compound. The splitting of the peaks at 1.4 ppm is due to the different environment they experience due to the two ferrocene rings. There are still 18 protons that appear at around 1ppm which are due to BOC.

Generally, the yields of the compounds above are extremely low probably due to the experimental methods used. The experimental methods referred to above gave a mixture of

products, namely, mono- and bis- substituted products in the case of propylenediamine and ethylenediamine and mono-, bis-, and tris substituted products in the case of diethylenetriamine. This is due to the fact that during amine protection using BOC tris- and bis- products are preferentially formed and thus decreasing the expected percentage yield of the mono- protected products.

### 4.4 Synthesis of the carrier polymer

The elementary analysis and NMR results given and discussed in the results section confirm the following structure for the repeat unit of polymer 9 :




The GPC data for polymer 9 indicated a narrow molecular weight distribution but could not determine the absolute molecular weight. However, the results from electrospray mass spectrometry indicated a molecular weight of about 5500 for polymer 9. The biodegradability of the polymer has also been determined by GPC using papain and proteinase A, which are both endopeptidases. The polymer was found to degrade very slowly and proteinase A showed a slightly longer activity than papain. Therefore the synthesized polymer satisfies four major requirements for a polymer carrier : water solubility, spacer group, narrow molecular weight distribution, and low biodegradability. The results of this polymer are promising for application of this type of a polymer in highly water-soluble drug carrier systems.

## 4.5 Recommendations

It is recommended that other methods for the BOC-protection of polyamines that can afford better yields be employed. It is also recommended that the ferrocene derivatives prepared in this study be deprotected and be anchored to the polymeric carrier prepared in this project. In the case of ferrocene-bisBOCdiethylenetriamine this can be further platinated.

It is known that polymers when administered parenterally can be taken into the cells by endocytosis and , if they are not fully degradable, they can accumulate in the cell lysosomes. The lysosomal membrane has been found to be impermeable to polar compounds larger that a dipeptide. Although lysosomes contain a broad range of lytic enzymes, unless the endocytosed polymer can be degraded to fragments below the above threshold limit, the degradation products may remain in the lysosomes. Final products of degradation by the endopeptidases papain and proteinase A are oligomers, which may possibly be susceptible to further degradation by exopeptidases, ultimately yielding monomers [71]. It is on the basis of this consideration that it is recommended that the polymeric carrier investigated in this study be subjected to further studies that will determine whether the degradation products by papain and proteinase A of this polymeric carrier can be further degraded by an exopeptidase enzyme to fragments below the abovementioned threshold limit. In conclusion, it is also recommended that tests on this polymer be carried out *in vivo*.

**REFERENCES**


1. Considine D. M, *Cancer and Oncology,* Van Nostrad's Scentific Encyclopaedia, 515, 8th edition, New York, (1990)
2. Rauscher F. J, *Cancer*, The World Book Encyclopaedia, vol 6, 135, New York, (1988)
3. Andrews P.A, Jones J.A, *Cancer Commun.*, vol 3,1, (1991)
4. Heim M. E, *Platinum and Non-platinum Complexes in Clinical Trials*, Metal Complexes in Cancer Chemotherapy, 11, B.K Keppler (ed), John Wiley & Sons, New York, (1990)
5. Sherman S.E, Lippard S. J, *Structural Aspects of Platinum Anticancer Drug Interactions with DNA*, Chem. Rev., 87, 1173, (1987).
6. Brown S.J, Chow C. S, Lippard S. J, *Platinum Based Anticancer Drugs*, Encyclopaedia of Inorganic Chemistry, vol 6, 3305, John Wiley & Sons, New York, (1990)
7. Comess K.M, Lippard S. J in Molecular Aspects of Anticancer drug-DNA Interactions, 134, Neidle S (ed), Macmillan, London, vol 1, (1993)
8. Farrell N, Transition Metal Complexes as Drugs and Chemotherapeutic Agents, Kluwer, Dordrecht, (1989).
9. Babin V. N, Raevskii P. M, Snegur L. V, *Antitumor Activity of Metallocenes*, Mendeleev Chem. Journal, 39, 2, 17, (1995)
10. Holler E, *Antitumor Metallocenes,* Metal Complexes in Cancer Chemotherapy, 261, Keppler B. K (ed), John Wiley & Sons, New York, (1990)
11. Cooper R. L , Green M. L. H.,*Chem. Commun* J., Chem. Soc.,421, (1972)
12. Azori M., in 'Critical Reviews in Therapeutic Drug Carrier Systems', CRC Press. Boca Raton, vol. 4, 30 , (1987)
13. Caldwell G., Neuse E. W., Perlwitz A. G., *Journal of Applied Polymer Science*, 66, 911, (1997)
14. Rosenberg B, VanCamp L, Grimely E. B, Thomson A. J, The Journal of Biolog. Chem. 242, 6, 1347, (1967)
15. Rosenberg B, VanCamp L, Krigus T, *Nature* , 205, 698, (1965)
16. Schweietert C. W, McCue J. P, *Coordination Chemistry Reviews*, 189, (1999)

17. Farrell N, Kelland L. R, Roberts J. D, van Beusichem M, *Cancer Res.*, 52, 5065, (1999)

18. Reedijk J, et al. *Structure and Bonding* 67, Springer - Verlag Berlin Heidelberg, 54 - 87, (1987)

19. Evans B.D, Rajn K.S, et al. *Cancer Treat. Rep.* 67, 997, (1983)

20. Eastman A, *Chem. Biol. Interact.*, 61, 241, (1987)

21. Chen Y, Guo J, P del Socorro, Zang E, Sadler P.J, *J. Chem. Soc.,Dalton Trans.*,1503 (1998)

22. Van Boom S.S.G.E, Reedijk J, *J. Chem. Soc.,Dalton Trans.*,385, (1997)

23. Holls L.S, Sandquist W. I, Ahmed K. J, et al. *Cancer Res.*,51, 1866, (1991)

24. Montet Y, Kozelka J, *J. Inorg. Chem.*, 284, pp 103 - 106, 1999

25. Farrell N, *Platinum and other Metal Complexes in Cancer Chemotherapy*, S.B. Howell Ed., Plenum - New York, 81, (1991)

26. Zhao G, Lin H, Zhu S, Sun H, Chen Y, *J. Coord. Chem.*, 46, 79, (1998)

27.Banerjea D, Basolo F, Pearson R.G, *J. Am. Chem. Soc.*, 78, 4055, (1957)

28. Bierbach U, Hambley W.T, Peroutka J, Nguyen H. L, Doedee M, Farrell N, *Inorg. Chem.*, 38, 3535, (1999)

29. Gust R, Heinrich H, Krauser R, Schonenberger H, *Inorg. Chim. Acta*, 285, 184,(1999)

30. De Napoli L, Iacovino R, Messere A, Montesarchio D, Picialli G, Romanelli A, Roffo F, Saviano M, *J. Chem. Soc., Dalton Trans.*, 1945, (1999)

31.Abrams M. J, Giandomenico C. M., Vollano J. F, Schwartz D. A, *Inorg. Chim. Acta*, 3, 131, (1987)

32. Takahara P. M, Frederick C. A, Lippard S. J, *J. Chem. Soc.*, 118, 1230, (1996)

33. Lee Y. A, Chung Y. K, Sohn Y. S., *J. Inorg. Biochem.*, 68, 289, (1997)

34. Cushman B. M, Earnest S. E, Brown D.B., *J. Organometal. Chem.*, 159, 431,(1978)

35.Fischer E. O., Pfab W., Z. Naturforsch. 7b, 377, (1952)

36. Ruch E, Fischer E. O, Z. Naturforsch, 7b, 676, (1952)

37. Holler E, in ' *Metal Complexes in Cancer Chemotherapy*', B.K. Keppler (ed),261 ,

John Wiley & Sons, New York, (1987)

38. Gowik P, Klapotke T., *J. Organomet. Chem.*,368, 35 , (1989)

39. Rosenblum M, Woodward R. B., *J. Am. Chem. Soc.*, 74, 5443,(1958)

40. Ramani P, Ranatunge- Bandarange R, Robinson B. H, Simpson J., *Organometal.*, 13,500, (1994)

41. Ramani P, Ranatunge- Bandarange R, Duffy N. W, Johnston S. M, Robonson B. H, Simpson J., *Organometal.*, 13, 511, (1993)

42. Gallagher J. F, Kenny P. T. M, Sheehy M. J, *Inorg. Chem.*, 2, 200 - 202, (1999)

43. Popova L. V, Babin V.N, , et al., *Appl. Organometal. Chem.*, 7, 85, (1993)

44. Snegur L. V, Babin V. N, et al.,in *'Chemotherapy and Medical treatment of Oncological Diseases '* , Meditsina, Moscow, 95, (1993)

45. McGrouther K, Weston D. K, Fenby D, Robinson B. H, Simpson J., *J. Chem. Soc.,Dalton Trans.*, 20, 1957, (1999)

46. Mason R. W, McGrouther K, Ranatunge-Bandarage P. R. R, Robonson B. H, Simpson J., *Appl. Organometal. Chem.*, 13, 163, (1999)

47. Neuse E. W, Meirim M. G, Blom N. F, *Organometal.* 7, 2562, (1988)

48. Ehrlich P, in '*Studies in Immunity*', New York, Wiley, (1906)

49.Rejmanova P, et al., *Makromol. Chem.*, 10, 182, (1983)

50. Hirano T, Klesse W, Ringsdorf H, *Makromol. Chem.*, 180, 1125, (1979)

51. Duncan R, Kopecek J, in ' *Soluble Synthetic Polymers as Potential Drug Carriers*', Karel Dusek (ed), 2 ,(1983)

52. Dellian M, Fukumura D, Leunig M, et al., *Cancer Research*, 55, 3752, (1995)

53. Langer R, *Science*, 249, 1527, (1990)

54. Inoue T, Chen G, Hoffman A. S., *J. Bioactive and Compatible Polymers*, 13, 50 , (1998)

55. Yamaoka T, Tabata Y, Ikada Y, *J. Pharm. Sci.*,84, 349, (1995)

56. Ramesh D. V, et al., *J. Bioactive and Compatible Polymers.*, 14, 137, (1999)

57. Domb A, Amselem S, Shah J, Maniar M, *Polymers for Advanced Technologies.*, 3, 279, (1992)

58. Lewis D. H, in '*Biodegradable Polymers as Drug Delivery Systems*', Langer R (ed), Marcel Dekker, 1 , New York, (1990)
59. Carter D. H, Luttinger M, Gardner D. N, *J. Controlled Release,* 8, 15, (1998)
60. Lim Y, Choi Y. H, Park J, *J. Am. Chem. Soc.,* 121, 5633, (1999)
61. Neuse E. W, Caldwell G, van Rensburg C. E. J, *Appl. Organometal. Chem.,* 13, 189, (1999)
62. Neuse E. W, Caldwell G, Perlwitz A. G, *J. Appl. Polymer Science.,* 66, 91, (1997)
63. Bodanszky M, *Peptide Chemistry*, 74, 2nd Ed, Berlin, Springer-Verlag, (1993)
64. Krapcho P, Kuell C.S., *Synthesis Commun*, 20 ,16, 2559, (1990)
65. Mattingly P.G., *Synthesis*, 366, (1990)
66. McMurry J, *Organic Chemistry*, 4th Ed, Brooks/Cole, CA, (1996)
67. Benoit F, Loussouarm A, Remand P, Chatal J. F., *Synthesis*, 1113,(1998)
68. Howe I, Williams D. H, Bowen R. D, *Mass Spectrometry : Principles and Applications,* 171, 2nd Ed, McGraww-Hill Inc, New York, (1982)
69. Styring M. G, Hamielec A. E, In *Determination of Molecular Weight*, Cooper A.R, Ed; Wiley, New York, (1989)
70. Colton R, D'Agostino G, Traeger J.C., *Mass Spectrom. Rev.,* 14, 17, (1995)
71. Pytela J, Kotva R, Rypacek F, *J. Bioactive and Compatible Polymers,* 13, 198 ,(1998)