CHAPTER 1

INTRODUCTION AND PROJECT AIM

In South Africa, a formidable burden is placed on the national health services by the overwhelming occurrence of cancerous diseases. Cancer is the third-most common cause of death among the black adult population, and the second-most common cause for the remaining population groups. South African rates for certain malignancies such as mesothelioma, cervical, oesophageal, and skin cancers rank among the highest world-wide. Cancers of the lung and prostate rate among the top six cancers with white and non-white males, and melanoma represents one of the top six cancers with white females. Extraordinarily high levels of life-time risk for females and males of the various South African population groups coincide with high age-standardized incidence rates. With increasing urbanization expected to add to the environmental and dietary factors conducive to carcinogenesis and dissemination, the demands, already overstressed, on the national health services will grow even further in the coming years, compounded by the finding that cancerous afflictions occur in approximately one-third of all male and female adult breadwinners in the age bracket of 15-54 years. The rapid spread of AIDS adds to the problem. Immunodepressed patients, at special risk to develop early neoplastic lesions, will be the marked targets for virus-related malignancies. Despite much progress in the chemotherapy of cancerous diseases it is an accepted fact that antitumor drugs in current clinical use suffer from serious deficiencies, placing severe limitations on the general administration of such drugs.

It has been the principal aim of the here-described project to develop macromolecular anticancer drug systems displaying enhanced therapeutic effectiveness as a consequence of strategically modified pharmacokinetic

pathways allowing for increased drug bioavailability, while circumventing the pharmacological shortcomings of the currently available non-polymeric drugs. Chapter 2 will provide a more detailed discussion of the topic.

CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

This section covers the topics of cancer chemotherapy, polymer-drug conjugation, and selected drug models. These topics have been the centre of many researches and publications so that only a small number of original papers will be cited in the present section, while preference has been given to review articles.

2.1. Therapeutic treatment of cancer

Surgery, radiotherapy, photodynamic therapy, and chemotherapy are the four principal types of therapy used in cancer treatment. Cancer consists of a proliferation of cells of a type different from the normal complement of the organism, and is one of the major causes of mortality in South Africa and Worldwide.[1]

I. Surgery is the oldest modality of cancer treatment; it still forms the mainstay of treatment of solid tumors. It is most effective in the treatment of localized primary tumors and associated regional lymphatics.[1]
II. Radiotherapy is a field involved in the treatment of benign and malignant diseases with ionizing radiation.[2]
III. Photodynamic therapy is an emerging modality for the treatment of neoplastic and non-neoplastic diseases, based on the activation of certain chemicals, called *photosensitizers*, that have been localized in the target tissues.[3, 63]
IV. Chemotherapy is the use of chemical agents to destroy affected tissues.[4, 5, 6]

Chemotherapy and radiotherapy can induce disabling and life-threatening side effects because these modalities destroy indiscriminately normal and healthy tissues.[7] In the body, the defence mechanism always recognises chemotherapeutic agents as poisons and tries to remove them as fast as possible by excretion, and this is detrimental in chemotherapy because, after administration, it causes a large variation of the concentration of the chemotherapeutic agent in the body, from values larger than the IC_{50} (drug concentration that induces 50% cell killing) to smaller values per day at which the drug has no effect on the disease. Cisplatin, one of the most successful chemotherapeutic drugs of the past decades, suffers from this phenomenon.[8] Many of the negative side effects related to chemotherapy find their cause in the way of excretion. In addition, some of the chemotherapeutic agents are moderately carcinogenic by inducing cancer in patients over a period of 15 years.[9]

Numerous anti-tumor agents exist. They are classified in many groups, depending on their mode of action. They include alkylating agents, antimetabolites and antibiotics. Some of these agents interact directly with the tumor cells, while others exist as pro-drugs to be activated in either the tumor cell or the liver to produce their expected effects.[10, 11]

Alkylating agents are a heterogeneous group of agents that share the following distinguishing characteristic: They have the ability to combine with cellular nucleophiles, primarily N- and O- nucleophilic groups on proteins and nucleic acids. This feat permits them to impair DNA replication during mitosis. A drug that functions in this fashion is the alkylating agent, nitrogen mustard; this drug contains a reactive ethylamine site which binds to N7 of the guanine molecule preventing the unwinding of the DNA double helix and its subsequent replication. In clinical practice these drugs are used to treat acute and chronic leukaemia, myeloma, non-Hodgkin's lymphoma and Hodgkin's disease.[12,13]

Antimetabolites are synthetic substances playing the role of inhibitors by analogy to natural substances required in the metabolism of normally proliferating cells. As drugs, they achieve their effect by mimicking an important molecule, inhibiting therefore a biological process. This is the case when methotrexate, a prototype antimetabolite, anchors to the dihydrofolate reductase, stopping the production of reduced folic acid and thereby inhibiting the synthesis of thymidylate and the production of DNA, which is necessary for cell replication. Antimetabolites are used in the treatment of various lymphomas.[14]

Antibiotics are naturally occurring substances obtained from bacteria or plants. As anti-tumor agents, their mode of action is wide including:

- binding to the enzyme topoisomerase II and stimulating strand breakage or sealing
- poisoning the microtubule that forms during the process of mitosis
- inhibiting RNA and thus blocking protein synthesis.

These anti-tumor drugs find their use in the treatment of acute lymphoblastic leukaemia, myeloid, small cell lung cancer, ovarian cancer and a wide variety of haematological malignancies.[15]

2.2. The polymer-drug conjugation concept

Among the modalities used by the oncologist in the fight against malignancies, chemotherapy, *i.e.* the treatment of cancer with antineoplastic drugs, has proved effective against certain cancers both *per se* and in combination with primary surgery and other techniques. However, presently used anticancer drugs suffer from a multitude of severe pharmacological deficiencies, all of which continue to contribute to their generally poor performance spectrum in clinical practice. These include: (i) *Excessive systemic toxicity*, which grossly diminishes therapeutic drug effectiveness and oftentimes necessitates premature termination of therapy; (ii) Lack of long-term effectiveness because of *induced drug resistance* with consequent need for premature discontinuation of drug-specific therapy; (iii) *Lack of cell specificity*, with ensuing drug distribution into

both normal and transformed cells. In consequence, drug application will be excessively wasteful, and healthy tissue will be exposed to toxic side effects; (iv) *Inadequate water solubility*, hampering swift and efficacious drug distribution in the body's aqueous fluid system, with resulting enhanced exposure to macrophage activity; (v) *Decreased serum half-life* as a consequence of catabolism, protein binding, capture by the reticuloendothelial system, or efficacious excretion mechanisms; (vi) Monophasic *salt-like* or *charged structure*, inhibiting membrane penetration and cell entry through normal passive diffusion. As a consequence, only a small fraction of the medicinal agent will successfully enter intracellular space for interaction with nuclear DNA or proteinaceous constituents. It thus holds for the great majority of cancerous, chemotherapeutically treated afflictions that periods of remission are limited and relapses frequent.

The concept of polymer-drug conjugation, pioneered *inter alia* by Ghose[16] and successfully advanced by Ringsdort[17], is based on a carrier model comprising a linear polymer chain composed of subunits bearing water-solubilizing groups, other subunits equipped with functional groups suitable for reversible drug binding (*anchoring*), and still other subunits comprising a homing device, *i.e.* some molecular entity capable of directing the conjugate molecule selectively to the target tissue. The conjugate nearly always represents a prodrug from which the active agent is released in the predestined biological environment (generally the cytoplasm for anticancer agents) by hydrolysis or through enzymatic action. Benefits to be derived from drug binding to a suitably constructed carrier polymer include the following[18-20] (i) The intravenously or intraperitoneally administered drug, even if inherently hydrophobic and insoluble in water, will be carried immediately into the aqueous phase of the central circulation system or the intraperitoneal cavity. This will ensure rapid drug distribution in the fluid systems; (ii). The polymer-drug conjugate will experience facilitated cell entry by endocytosis, thus circumventing potential problems caused by drug polarity or ionicity. This mode of entry may assume unique significance where resistance

has been developed by the target cells; (iii) Proper design of the polymer-drug connective link (spacer) may provide delayed and controlled drug release, thereby ensuring restriction of drug serum concentrations well within pharmacologically dictated limits with concomitant reduction of organ toxicity. Where drug action occurs in intracellular space, polymer conjugation of the drug may incorporate features allowing for conjugate stability in the plasma and drug release upon endocytotic cell entry. With highly toxic agents of the kinds administered in the chemotherapy of malignancies the last-named mechanism may be exploited to great advantage; (iv) While in transit in the central circulation system, the polymer-bound drug will experience temporary protection from enzymatic attack, serum protein binding, and other depletion processes. This will lead to reduced renal clearance and prolonged serum life time with substantially enhanced drug effectiveness; v) Enhanced drug affinity for the transformed cell may be brought about by incorporation of a homing device, *i.e.* some molecular entity capable of directing the conjugate molecule selectively to the target tissue, for example, certain sugar moieties or (at the stage of ultimate perfection) specific monoclonal antibodies. In addition, polymer molecules *per se* may be preferentially retained by tumorous tissue for lack of lymphatic recovery (EPR effect;[21]).

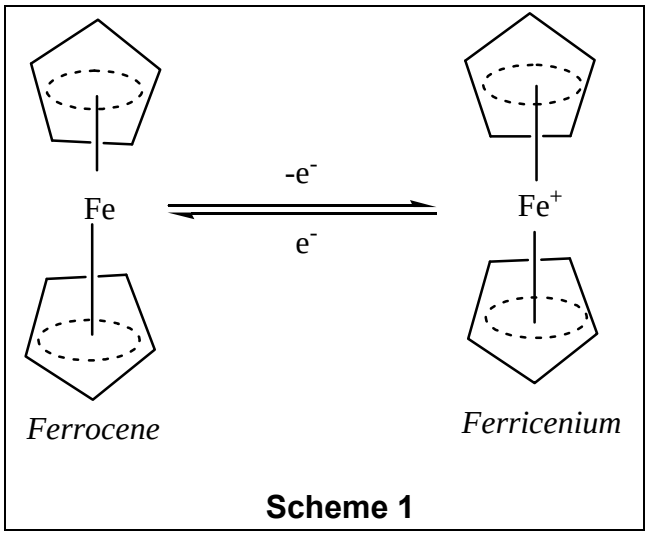
It stands to reason that the aforementioned pharmacological advantages of polymer-anchored medicinal agents should have prompted extensive development work worldwide utilizing the polymer-drug conjugation approach. This is indeed the case; numerous publications dealing with drug anchoring have appeared notably during the past 10-15 years, and two recent papers reviewing these efforts may be singled out [22, 23]. Numerous reports deal with the polymer anchoring of *methotrexate, daunomycin, 5-fluorouracil* and other “classical” anticancer agents. The carriers utilized fall predominantly into the non-biodegradable vinyl polymer class; less frequently, natural polymers, including dextran or antibodies and other proteins, as well as biodegradable synthetic polyamides and polyesters, have been used. Biomedical *in vitro* and *in vivo*

evaluation has generally revealed a spectrum of promising activities against a large number of murine and human cancer lines, provided that the drug species are anchored *via* biofissionable links (preferably oligopeptide and other spacers). An overriding finding has been a marked reduction in toxicity, frequently coupled with enhanced cancer cell specificity and extended circulation half-lives. Selected conjugates have entered clinical trials, among these a highly promising neocarcinostatin derivative. Numerous existing problems, however, including (i) lacking backbone degradability, (ii) backbone toxicity, immunogenicity, or instability, (iii) poor solubility, and (iv) incapability of being isolated in the solid state for redissolution and use, all have combined to demonstrate that the art of polymer-drug anchoring is indeed far as yet from delivering the expected results. This leaves a wide field open for challenging exploratory research.

2. 3. The drug models

2.3.1. Di (η^5-cyclopentadienyl)iron(II). Better known under the name of *ferrocene*, this drug model, while still at an experimental stage, has shown remarkable antiproliferative activity in numerous murine and human cancer cell tests and offers a great challenge to the cancer researcher because of expected low toxicity relative to most known anticancer agents, paired with low cost and ready availability of suitable starting materials. It has therefore been included in the present study as a co-conjugation agent so as to test its potential as a synergy-producing co-drug. The *ferrocene* complex features remarkable oxidation-reduction behavior; it converts readily to its one-electron oxidation product, the *ferricenium* ion, a radical cation of eminent stability. The reaction is reversible, and the inherent electron transfer has implications for biological reactions. Electron transfer and free radical reactions play vital parts in biological processes. It stands to reason, therefore, that the *ferricenium-ferrocene* couple should attract interest in biochemical and biomedical research, and numerous investigations of the behavior and functioning of *ferrocene* compounds in biological environments have indeed been reported. Pertinent reactions include

enzymatically controlled oxidation of *ferrocene* by hydrogen peroxide, *ferricenium* cation reduction by NADH and metalloproteins, and, most significantly, recombination of the *ferricenium* system with attacking free radicals, leading to substituted *ferrocenes* upon proton elimination. *Ferricenium* cation reaction with the biologically important superoxide anion radical proceeds with regeneration of *ferrocene* and dioxygen. A reverse electron transfer reaction comprising oxidation of the *ferrocene* complex to its *ferricenium* counterpart, shown in Scheme 1, is also feasible, in which ferrocenylcarboxylates interact with the aggressive hydroxyl radical, transforming the latter into harmless hydroxyl anion. Free-radical chemistry is known to play a major role in cancer etiology and in various phases of growth and control of neoplasia. The two superoxide and free radical-scavenging reaction types involving *ferrocene,* hence, might lend themselves as effective inhibition and detoxification processes in the cancerous organism. A further contribution to the retardation of cell proliferation could well arise from deactivating recombination of *ferrocene* in its oxidized state with the free-radical form of ribonucleotide reductase, an important enzymatic link in DNA synthesis. These considerations prompted *in vitro* screening *ferrocene* work with selected *ferricenium* salts against ascetic murine tumors and several human tumor clonogenic cultures[34-36]. The screens showed no activity for those salts tested that were as insoluble in water as the (biologically inactive) parent. On the other hand, *ferricenium* salts possessing water solubility with high saturation limits were found to be active[24-25]. These observations prompted polymer conjugation work in the polymer research laboratory with highly promising results[26-33]. For classical surveys of the *ferrocene* molecule and its polymeric derivatives, two books [60, 62] and three book chapters [37-39] are available, and more recent publications may be consulted as well.[40, 41]



Scheme 1

2.3.2. Methotrexate (MTX). This compound, a deoxyfolic acid derivative, has found clinical application for many years as a highly potent anticancer agent. Folate-dependent enzymes play a vital role in the provision of nucleotides required for the build-up of DNA by both normal and transformed (*i.e.* cancerous) cells, but predominantly so in the latter type because of their excessive rates of metabolism. *MTX* acts as a tight-binding inhibitor of dihydrofolate reductase, the key enzyme in intracellular folate metabolism. As a result of this process, folate cofactors accumulate in their inactive dihydrofolic acid form, which in turn inhibits purine and thymidylate synthesis, leading to cell death. This process is somewhat selective, antifolate action being stronger in transformed (*i.e.* cancerous) tissues than in normal ones. Intracellular polyglutamylation of *MTX* (and other antifolates) contributes beneficially to the drug's accumulation in the tumor cell. *MTX* is active against choriocarcinoma and acute lymphocytic leukemia, in combination with other drugs (including *cisplatin*) also against osteosarcoma and, most importantly in the context of this project, carcinomas of the lung, cervix, ovaries, bladder and other organs. As a typical cytotoxic agent, *MTX* causes highly toxic side effects and development of resistance; its uptake by the tumor cell proceeds by an active transport mechanism, which appears to be inhibited as resistance is building up. The compound should thus be an

excellent candidate for polymer conjugation, and this prediction was reduced to practice in our laboratory. For reviews of MTX activity and clinical performance, see Refs [43-44]

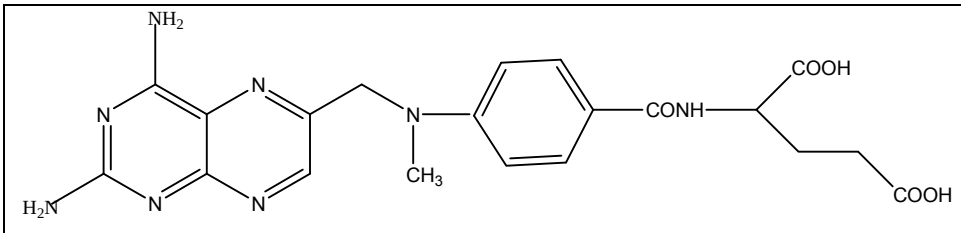


Figure 1: Methotrexate

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CHAPTER 3.

RESULTS AND DISCUSSION

In accordance with the original research proposal, the dissertation was executed within the framework of the following three work phases:

Phase I: Synthesis of carrier polymers, both known and novel, by preparative methods developed in previous projects and improved where necessary.

Phase II: Conjugation of the carriers obtained in Phase I with selected antineoplastic drug models, these models to be taken from the ferrocene-type and MTX classes of anticancer agents.

Phase III: Submittal of drug conjugates prepared in the preceding phase to a collaborating outside institution for *in vitro* and limited *in vivo* screens. Evaluation of these biomedical results for assessment of structure-performance relationships in preparation for a subsequent doctoral thesis project. These topics are covered in detail in the following subsections.

Phase I: Synthesis of carrier polymers

In view of the most stringent structural and functional performance requirements for the macromolecular carrier components of the polymeric conjugates, the first work phase occupied the predominant part of the dissertation study. The significance of this task derived from the requirement of providing compounds that would possess an optimal combination of structural features necessary to facilitate drug transport and release in accordance with the polymer-drug conjugation concept introduced in Section 2.2. Although biologically inactive *per se*, the carrier represents a vital component of the conjugate as its structure dictates the conjugate's pharmacokinetic pathway in the body and, thus, the success or failure of the conjugate to reach the target tissue. The synthetic approaches, accordingly, were chosen so as to tailor the polymers exactly to the following structural specifications:

(i) A *nontoxic chain construction* amenable to biodegradation will ensure catabolic elimination of the polymer upon drug release.

(ii) A highly *flexible backbone* and the presence of intrachain-type or side group-attached *solubilizing groups* will ensure conjugate solution in aqueous media required for rapid dissipation in the central circulation system, even if the conjugated drug itself is insoluble. These features are of overriding importance.

(iii) The presence of *functional groups as binding sites* will ensure proper drug attachment and subsequent release in the biological environment.

(iv) The presence of *cationic functions* will cause facilitated cell entry by endocytosis, thus circumventing potential problems caused by drug polarity or ionicity. This mode of entry will assume unique importance where drug resistance has been developed by the target cells.

(v) Proper design of the *polymer-drug connective link* (spacer) will provide delayed and controlled drug release, thereby ensuring restriction of drug serum conjugate concentrations well within pharmacologically dictated limits with concomitant reduction of organ toxicity. Where drug action occurs in intracellular space, polymer conjugation of the drug may incorporate features allowing for conjugate stability in the plasma and drug release upon endocytotic cell entry. With highly toxic agents of the kinds administered in the chemotherapy of malignancies the last-named mechanism may be exploited to great advantage.

(vi) Enhanced *drug affinity* for the transformed cell may be brought about by incorporation of a homing device, *i.e.*, some molecular entity capable of directing the conjugate molecule selectively to the target tissue.

(vii) *Molecular masses* substantially in excess of 25 000 so as to retard conjugate depletion through kidney excretion, but preferably below 100 000 to avoid toxic side effects.

In agreement with these structural prerequisites, aliphatic polyamide backbone structures have been chosen as the principal carrier design elements, a schematic structure being shown below.

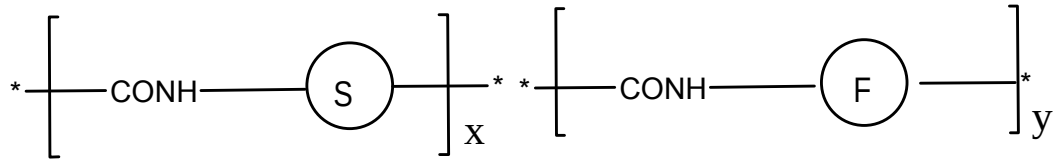


Figure 2: Structure of polyamide-type carrier

In this model, **S** stands for an extrachain- or intrachain-type hydrosolubilizing moiety. The group **F** represents or comprises an extrachain- or intrachain-type functionality capable of forming a link with the drug species. The main chain contains additional aliphatic segments (not shown), and other subunits of predetermined structures. Polyamides of this type are represented by the major class of poly-α,β-DL-aspartamides and other linear DL-peptide polymers, as well as non-peptidic polymers containing amide links in the backbone. Synthesis methods include ring opening reactions performed on poly-DL-succinimide in addition to Michael-type polyaddition, these techniques having been developed in previous programs in terms of basic methodology. The following considerations support the choice of these polymer types:

(i) The (mass average) molecular masses, generally in the range of 20000-60000, are sufficiently low to suppress (chainlength-dependent) inherent polymer toxicity, yet high enough to retard renal clearance.

(ii) The amide groups in the chain permit gradual backbone cleavage for ease of catabolic elimination of the spent polymer. The presence of D-configurated CH groups and β-peptide units in the chain precludes unduly rapid α-peptidase-mediated "unzipping", ensuring this fragmentation to be an appropriately retarded process.

(iii) Polyaspartamides are essentially nontoxic, and immunogenicity of these synthetic, peptide-type homopolymers is expected to be appreciably lower than commonly observed with high-molecular proteinaceous biopolymers.

(iv) The **S**-modified subunits can be introduced advantageously as the majority component (x > y), thus ensuring effective insulation of the **F**-modified

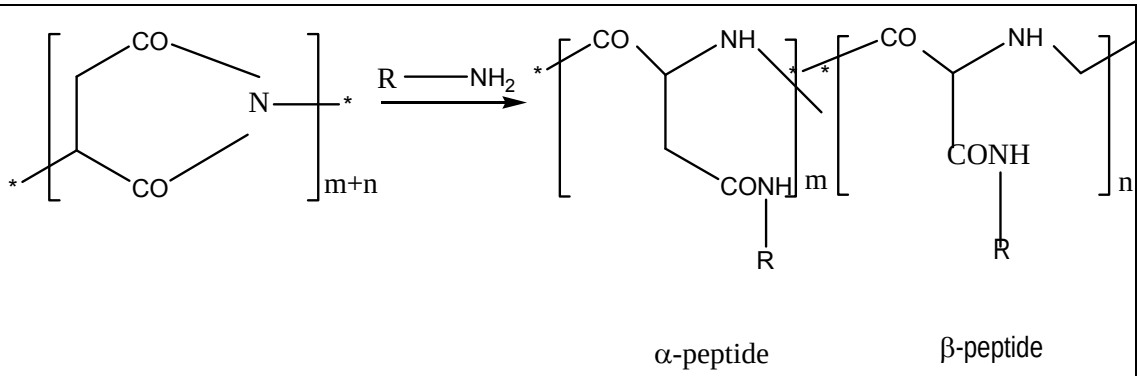
subunits from each other. This will reduce the risk of intramolecular interaction of adjacent metal complexes.

(v) *tert*-Amine functions can be introduced as side groups, providing cationic sites capable of directing the conjugate preferentially to the cancerous target cells, many types of which display negative surface charge resulting from excessive glucose metabolism.

(vi) While inherently water-soluble with **S** comprising selected amine, hydroxyl, or carboxyl functions, the polyaspartamides can be made to acquire additional solubility in selected organic solvents through the expediency of incorporating poly(ethylene oxide) side chains as additional solubilizing groups. This added solubility feature will offer advantage in certain follow-on reactions necessitating alcoholic media.

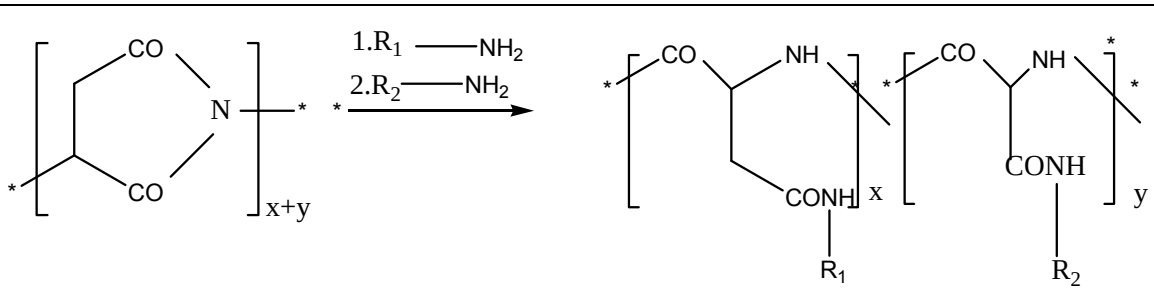
The two principal polyamide-type carriers synthesized in this work are individually discussed below.

Polyaspartamides. The potential value of polysuccinimide-derived aspartamide polymers as carriers for medicinal agents was pointed out in the Seventies by Neri *et al.*[45] and by Drobnik and co-workers [46, 47], and their proposal was subsequently translated in our laboratory into the reality of a class of eminently functional carriers for a variety of antineoplastic drug systems. Accordingly, the predominant share of carrier polymers synthesized in the dissertation project has been based on this class. The polyaspartamides are readily prepared from poly-DL-succinimide by aminolytic ring opening in anhydrous, dipolar aprotic medium, such as N,N-dimethylformamide (DMF), thereby arising as racemic (D- and L-enantiomeric) forms possessing both α and β-peptidic repeat units [45] (Scheme 2).



Scheme 2

In the following structural representations of polyaspartamides, only the α-peptidic form will be shown for simplification. This ring-opening reaction has proved to be an outstandingly versatile process, allowing for copolymer generation through stepwise use of two or more different amine reactants in given stoichiometric feed ratios. Thus, sequential participation of amines R_1-NH_2 and R_2-NH_2 will lead to copolyaspartamides in which randomly placed subunits feature the R_1 and R_2 residues in predetermined ratios (Scheme 3):



Scheme 3

When properly executed, the experimental procedure can be modified so as to accommodate another side group R_3, although this was not required in the present project. For the purpose of this dissertation, R_1 was chosen to represent a tertiary amine side group convertible *in vivo* to a cationic site, and R_2

represented a side chain terminated by a primary amino group or a hydrazide functionality suitable for drug connection. As part of the dissertation project the following polyaspartamides, with structural representations shown in Figure 3, have been synthesized in accordance with the compositional data given in Table1.

Table 1: Compositional data for Polyaspartamide1-Polyaspartamide 4

Designation	**R_1**	**R_2**	**x/y**
Polyasp 1 (80:20)	$Me_2N(CH_2)_3-$	$H_2N(CH_2)_3-$	4
Polyasp 1 (90:10)	$Me_2N(CH_2)_3-$	$H_2N(CH_2)_3-$	9
Polyasp 2 (80:20)	$Me_2N(CH_2)_3-$	$H_2N(CH_2)_2NH(CH_2)_2-$	4
Polyasp 2 (90:10)	$Me_2N(CH_2)_3-$	$H_2N(CH_2)_2NH(CH_2)_2-$	9
Polyasp 3 (80:20)	$Me_2N(CH_2)_3-$	H_2N-	4
Polyasp 3 (90:10)	$Me_2N(CH_2)_3-$	H_2N-	9
Polyasp 4 (80:20)	$Me_2N(CH_2)_2$-	$H_2N(CH_2)_3$-	4
Polyasp 4 (90:10)	$Me_2N(CH_2)_2$-	$H_2N(CH_2)_3$-	9

Note: In these and related structural representations the individual repeat units are randomly distributed along the polymer chain.

The synthetic procedure comprises two steps. In the first, polysuccinimide, prepared from aspartic acid as described, [45-47] is treated in aprotic solvent with

R_1-NH_2 (5-20h, RT) in the required stoichiometric ratio. In the second step, the ensuing solution is added slowly, at ice bath temperature, to a dilute solution of excess R_2-NH_2 (typically 20-24h, 0°C, 10-20h, RT). Careful control of the reaction conditions of this step is crucial in order to prevent crosslinking (with resultant insolubility) caused by substitution of the second $-NH_2$ group of the amine nucleophile. The product polymers so prepared, purified and fractionated by a series of operations involving precipitation, washing, and dialysis, are isolated by freeze-drying as water-soluble solids. Proton magnetic resonance spectroscopic analysis confirm the structural assignments. Table 2 contains experimental data for these polymers, and the spectroscopic data are listed in Table 3.

Table 2: Experimental conditions for the preparation of carriers Polyasp 1-Polyasp 4

Reactants in feed (mol %)[a]			Reaction conditions[c]	Polyaspartamides	
R_1-NH_2	R_2-NH_2	Mole ratio x/y [b]		Yield (%)[d]	Designation
DMP (80)	**PDA (20)**	4	8 h RT; O/N 0º C and 24h RT	60	**Polyasp 1 (80:20)**
DMP (90)	**PDA (10)**	9	" : " and "	62	**Polyasp 1 (90:10)**
DMP (80)	**DET (20)**	4	8 h RT; O/N 0º C and 40h RT	58	**Polyasp 2 (80:20)**
DMP (90)	**DET (10)**	9	" ; " and "	54	**Polyasp 2 (90:10)**
DMP (80)	**HY (20)**	4	" ; " and "	51	**Polyasp 3 (80:20)**
DMP (90)	**HY (10)**	9	" ; " and "	45	**Polyasp 3 (90:10**
DME (80)	**PDA (20)**	4	24 h RT; O/N 0º C and 24 h RT	52	**Polyasp 4 (80:20)**
DME (90)	**PDA (10)**	9	" ; " and "	55	**Polyasp 4 (90:10)**

a. Parenthetic numbers indicate the number of moles of amino compound per 100 base moles of poly-D, L-succinimide.

b. Mole ratio of hydrosolubilizing to drug-anchoring groups

c. RT = room temperature. O/N = overnight.

d. Polymer yield after ultimate (25 000 molecular mass-cut-off) dialysis

DME: 2-(N,N –dimethylamino) ethylamine; DET: Diethylenetriamine; DMP: 3-(N, N-dimethylamino) propylamine; HY: Hydrazine

Table 3: NMR data for Polyasp 1-Polyasp 4

Polyaspartamides			**Number of protons counted [C] (expected) [d] at given chemical shift**					
Polymer designation	**η_{inh} (mL/g) [a]**	**base molecular mass [b]**	**δ4.7-4.5**	**δ3.5-3.0**	**δ2.8-2.5**	**δ2.4-2.2**	**δ2.2-2.0**	**1.8-1.5**
Polyasp 1 (80:20)	8. 00	970. 21	4 (5)	9 (10)	10(12)	7(8)	22 (24)	9 (10)
Polyasp 1(90:10)	8. 95	1966. 47	9 (10)	18 (20)	20 (22)	(18)	49 (54)	18 (20)
Polyasp 2 (80:20)	6. 80	1012. 28	5 (5)	9 (10)	14 (16)	7(8)	22 (24)	6 (8)
Polyasp 2 (90:10)	8. 78	2008. 55	9 (10)	18 (20)	6 (26)	16 (18)	50 (54)	16 (18)
Polyasp 3 (80:10)	7. 39	926. 13		7 (8)		6 (8)	22 (24)	6 (8)
Polyasp 3 (90:10)	7. 13	1922. 39		18 (18)		16 (18)	52 (54)	16 (18)
Polyasp 4 (80:10)	9. 22	914. 10		3 (5)		11 (10)	30 (24)	2(2)
Polyasp 4 (90:10)	8. 67	1840. 23		10 (20)		9 (18)	26 (54)	2 (2)

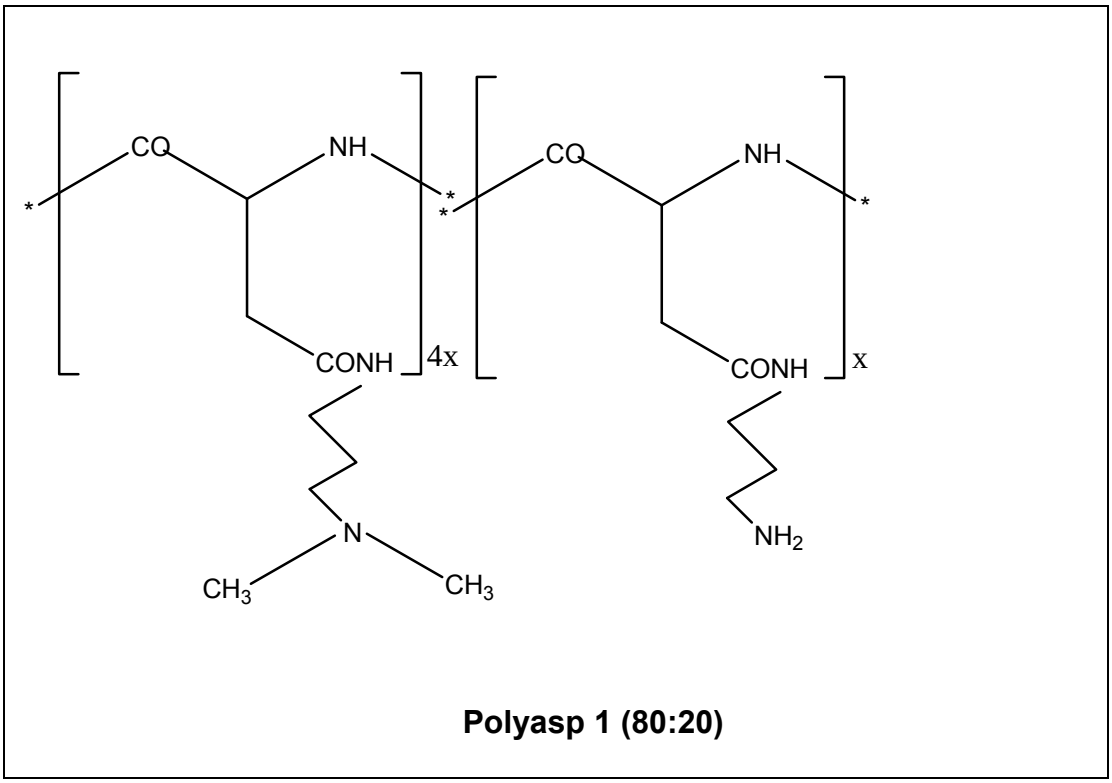
a. At 30.0 ± 0.5°C, in deionized H_2O; concentration c = 2 mg/mL.

b. Molecular mass of recurring unit structure , normalized to y = 1

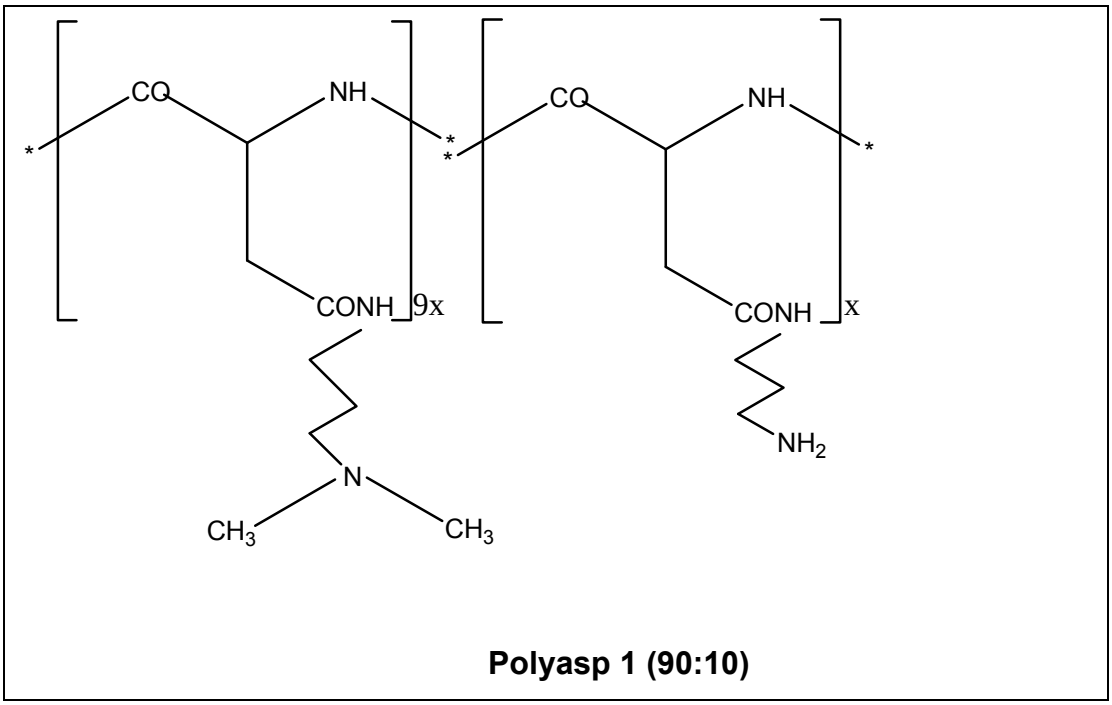
c. In D_2O, pH10-11. Chemical shifts, δ/ppm, referenced against internal sodium 3-trimethylsilyl-2,2,3,3-d_4-propionate; integration error limits ±15%.

d. Expected count for composition in accordance with recurring unit .

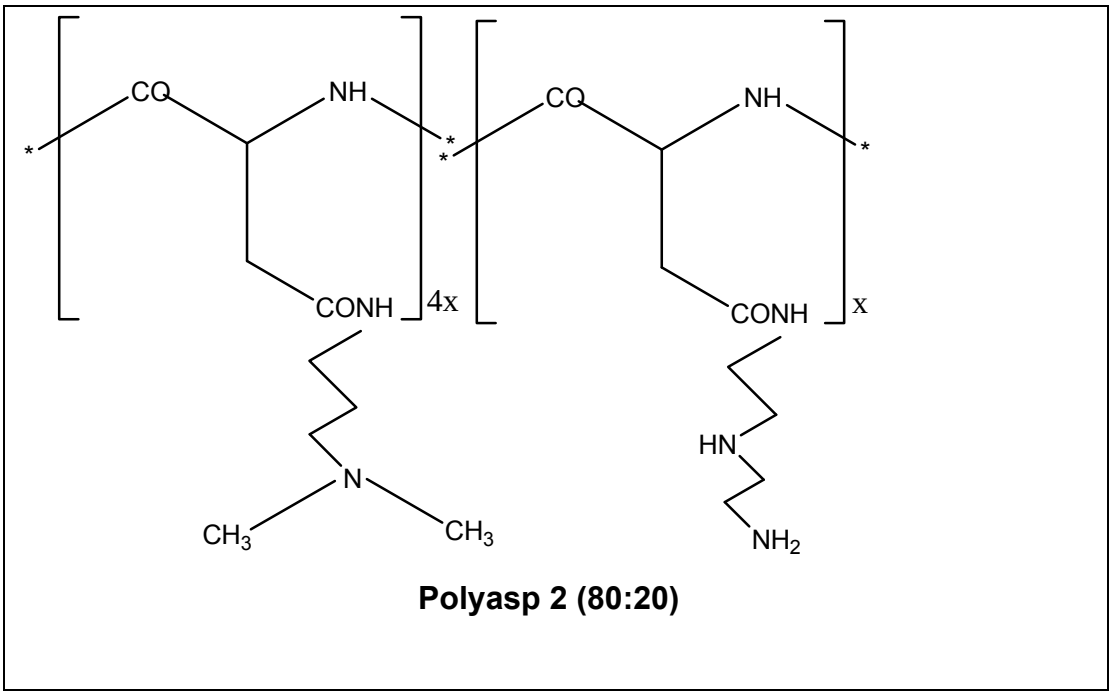
Figure 3: Structures of Polyasp 1-Polyasp 4



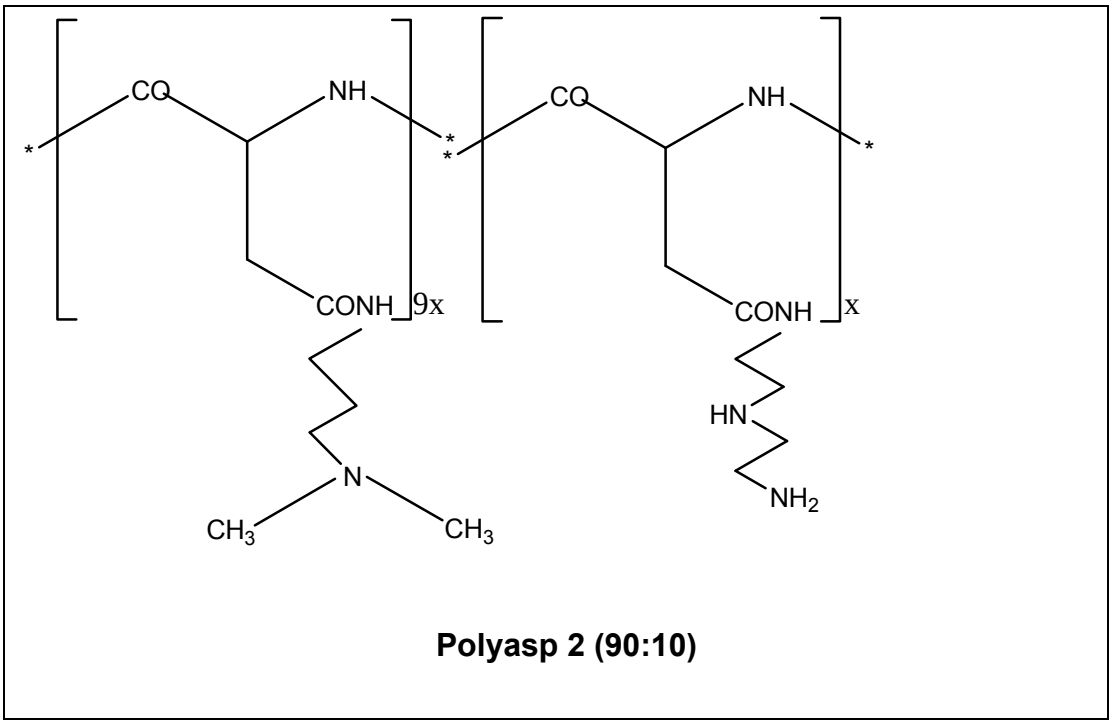
Polyasp 1 (80:20)



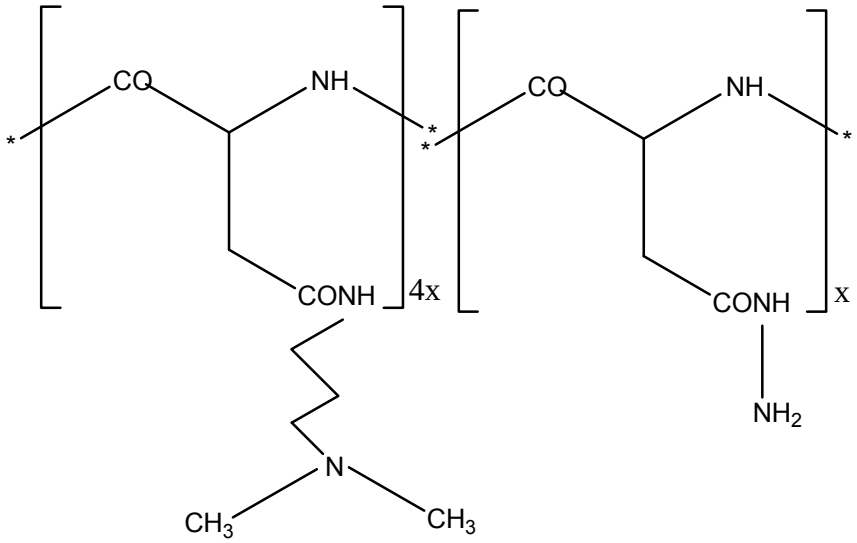
Polyasp 1 (90:10)



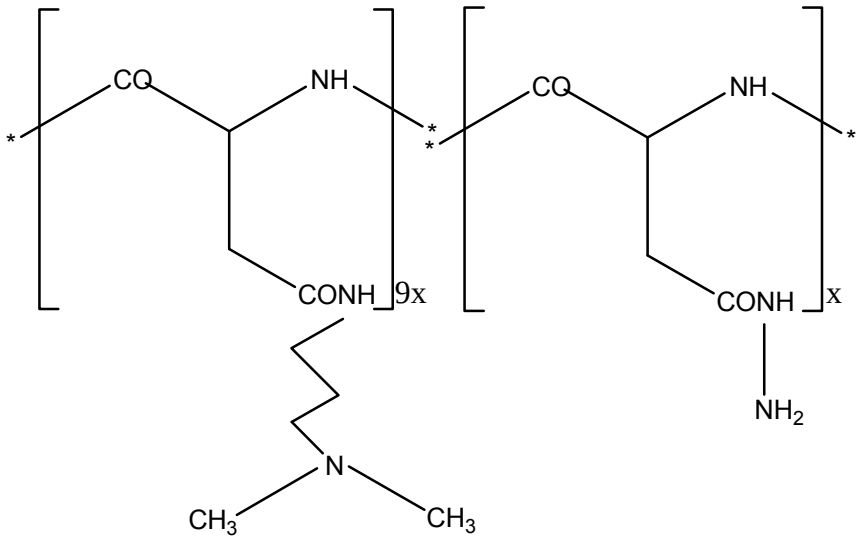
Polyasp 2 (80:20)



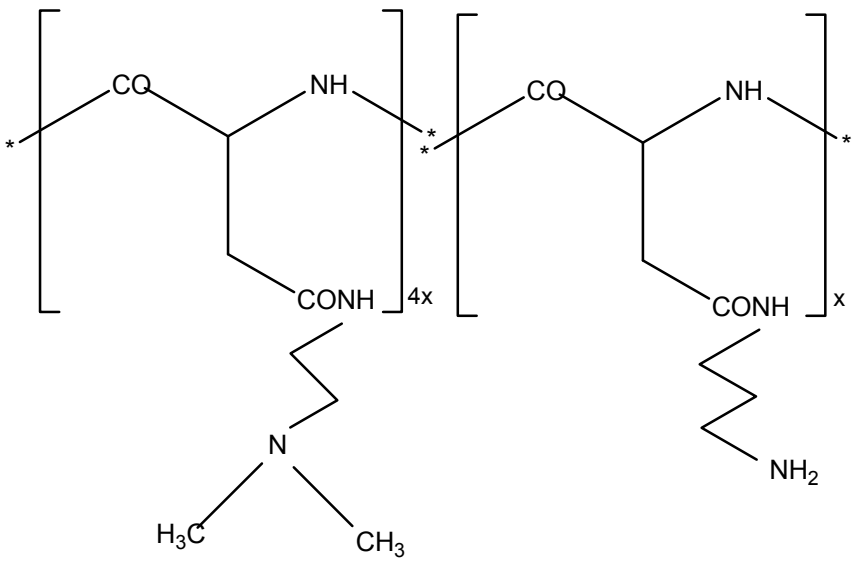
Polyasp 2 (90:10)



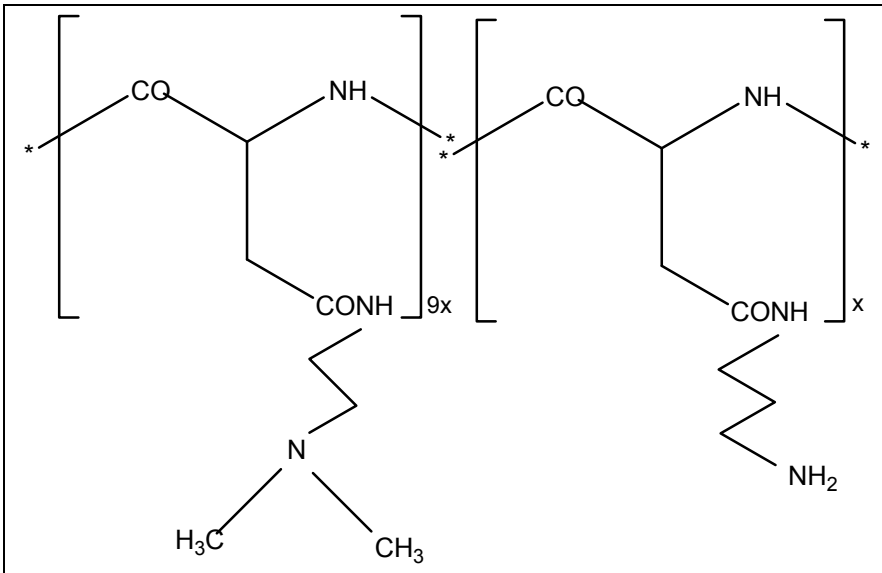
Polyasp 3 (80:10)



Polyasp 3 (90:10)

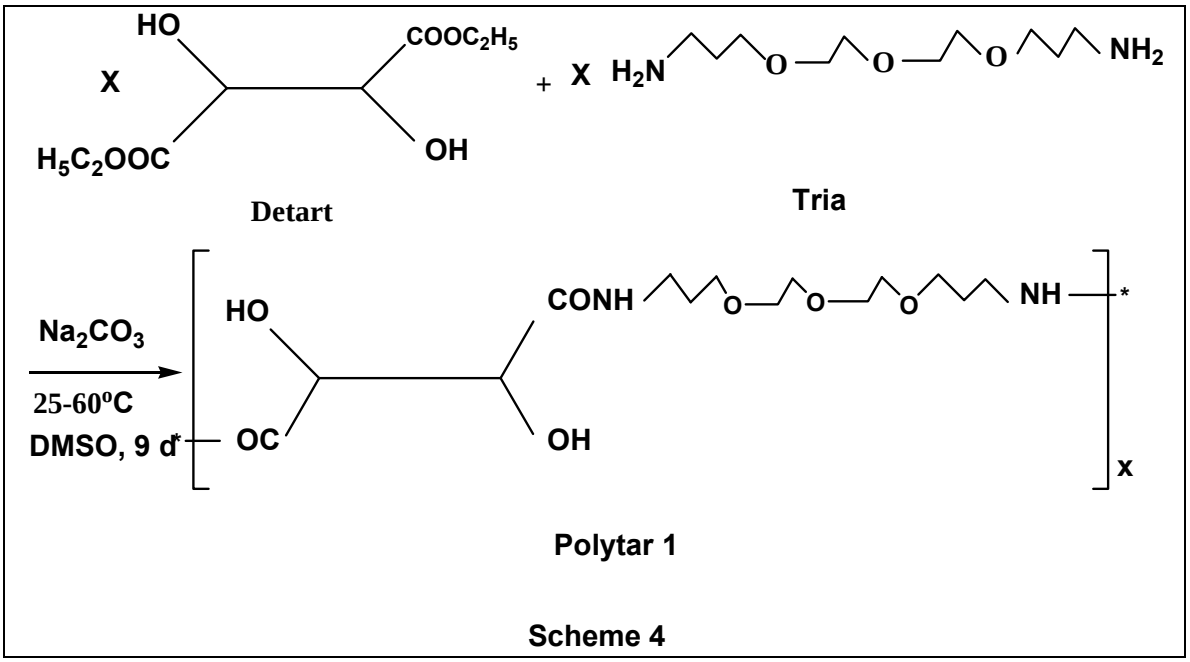


Polyasp 4 (80:20)

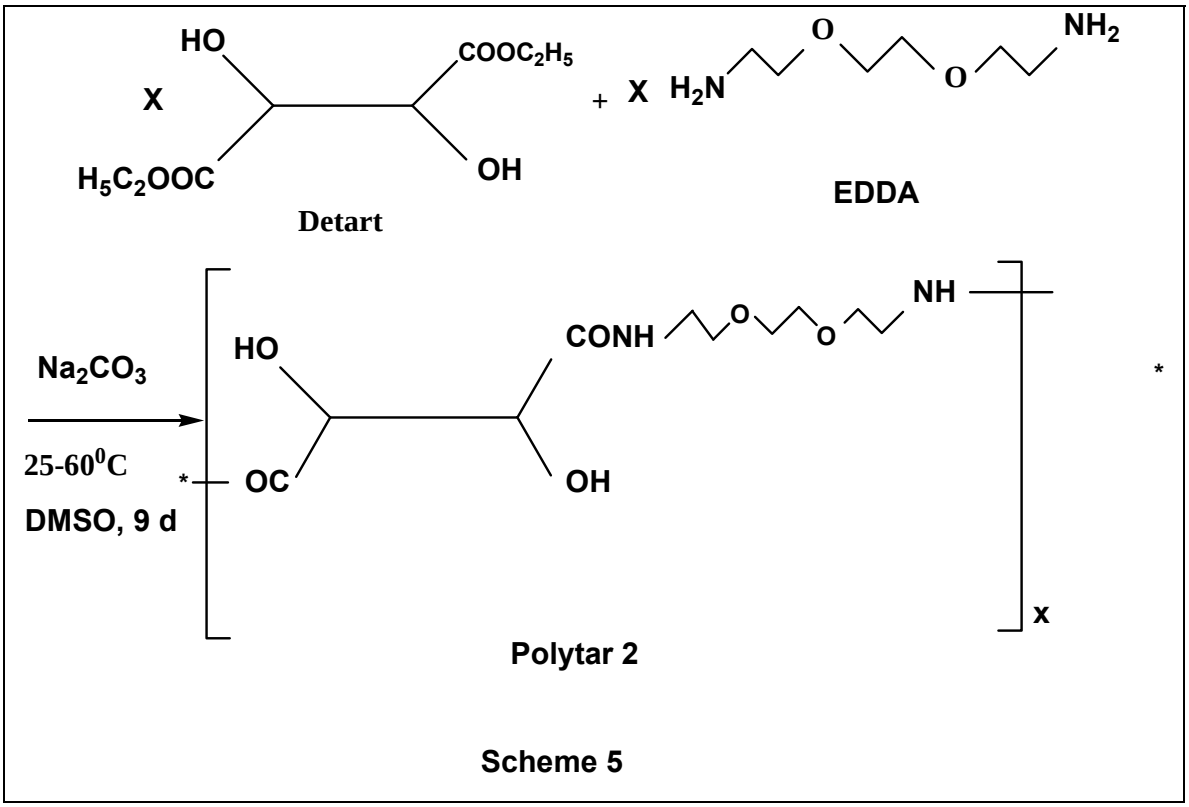


Polyasp 4 (90:10)

*0ther polyamides.*These polymers, prepared by ester-amine polycondensation, were synthesized previously as useful carrier candidates for antineoplastic drug anchoring [29]. As part of the dissertation, aliphatic polyamides of this kind were obtained by base-catalyzed polycondensation of diester and diamines in bulk or solution polymerization. The reaction was conducted in the presence of 20% anhydrous sodium carbonate at temperatures ranging from ambient to 65°C, initially in undiluted state. The addition of aprotic solvent such as DMSO at later stages served to maintain sufficiently low viscosity for proper homogenization. In the present project equimolar quantities of diethyl L-tartrate (TART) and 4, 7, 10-trioxa-1, 13-tridecanediamine (TRIA) were copolymerized to form a polymer **Polytar 1** (Scheme 4), and the combination of diethyl L-tartrate with 2, 2'-(ethylenedioxy)diethylamine (EDDA) as a diamine monomer produced the straight-chain polyamide **Polytar 2** (Scheme 5). Use of diethyl L-tartrate leads to polyamides possessing hydroxyl groups used for drug attachment. The water-soluble polyamides were crudely fractionated by aqueous dialysis (12000-25000 molecular mass cut-off) and collected by freeze-drying in a yield range of 18-45%. The water-soluble carriers possessed viscosities of 8-12 mL/g. Individual reactant combinations, mole ratios, as well as reaction conditions and yields are shown in table 4. NMR spectroscopy (Table 5) confirmed the composition of polyamide structures**.**



Scheme 4



Scheme 5

Table 4: Experimental conditions for the preparation of carriers Polytar 1 and Polytar 2

Polymer designation	Reactant in feed (mmol)					DMSO (mL)	Reaction conditions	Yield (%)
	TART	TRIA	EDDA	2-Hydroxy-pyridine	Na_2CO_3			
Polytar 1	10	10		1	9	6	24 h RT; 9 d 65ºC	57
Polytar 2	10		10		9	6	24 h RT; 20 d 57ºC	52

TRIA: 4, 7, 10- Trioxa-1, 3- tridecanediamine

TART: Diethyl L- tartrate

DMSO: Dimethyl sulfoxide

EDDA: 2, 2'-(ethylenedioxy)diethylamine

Table 5: NMR data for carriers polytar 1 and 2

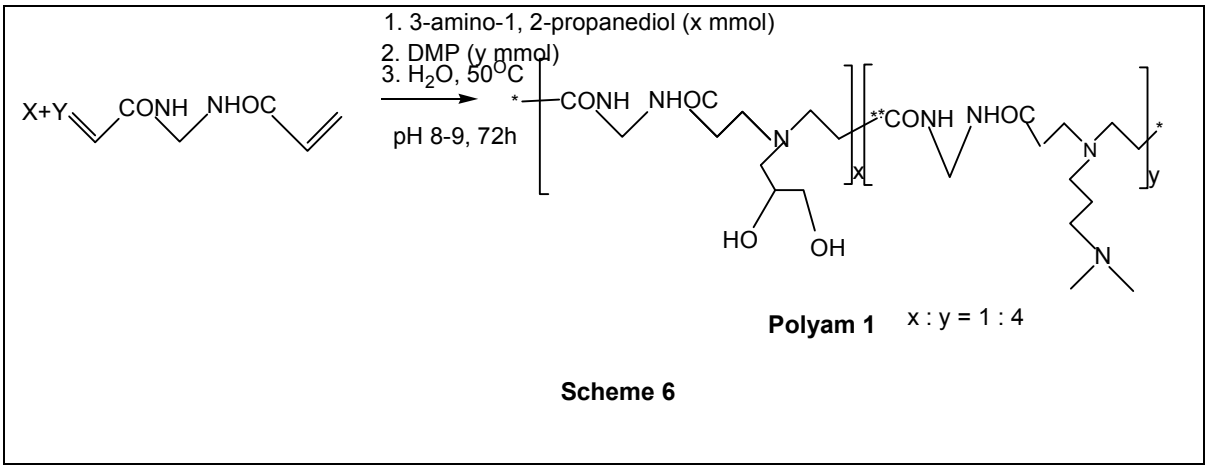
Polymer designation	η_{inh} (mL/g) [b]	Number of protons counted (expected) in ^{1}H NMR [a]				
		δ[c] 4.5-4.4	δ3.8-3.5	δ3.3-2.8	δ1.8-1.7	δ1.4-0.8
Polytar 1	20	1.8 (2)	9 (12)	3 (4)	2.6 (4)	2.8 (4)
Polytar 2	17.3		1.6 (2)	10 (12)	3 (4)	

a. In D_2O, pD 10. Chemical shifts, δ/ppm, referenced against internal sodium 3-(trimethylsilyl) -2, 2, 3, 3-d_4-propionate, integration error limits ± 15%

b. At 30.0 ± 0.5ºC, in deionized H_2O, concentration c = 2mg/mL

c. Range of chemical shifts, δ/ppm.

*Polyamidoamines.*These compounds comprise amine and amide functional groups together in the backbone. They contain hydrolysable bonds in their main chain allowing degradation in aqueous media, and additional functional groups may be introduced as side substituents. Their synthesis is based on a Michael-type nucleophilic polyaddition of an amino group across an activated double bond.This synthesis method has been pioneered by Ferruti's group [48, 49], which demonstrated that the reaction of equimolar quantities of primary monoamines or secondary diamines with bisacrylamides in aqueous solution could lead to polymerization giving linear, water-soluble polyamidoamines comprising secondary amide and tertiary amine functions in the main chain [50-52]. In this dissertation, work was concentrated on the polyamidoamines containing the dihydroxyl group arrangement as a side chain terminal. The reasons for choosing these functional groups are that dihydroxyl-functionalized water-soluble carriers have shown special promise in this laboratory as carriers of diaminocyclohexanediaquaplatinum(ll) salt (DACH-Pt aq), a drug system which has antiproliferative effectiveness against the human HeLa carcinoma cell line [53]. Such carriers could also provide hydroxyl groups for ferrocene and methotrexate drug binding by esterification. Two synthetic strategies have been adopted in the present dissertation for the synthesis of polyamidoamines. The first one, involving the simultaneous addition of 3-(N, N- dimethylamino)propylamine (DMP) and 3-amino-1, 2-propanediol (APD) to the vinyl group of methylenebisacrylamide (MBA), resulted in the polymer **Polyam 1** (Scheme 6), while Scheme 7 displays the structure of **Polyam 2** arising from a simultaneous addition of 2, 2'-(ethylenedioxy)diethylamine (EDDA) and ethylenediamine (EDA) to the vinyl group of MBA.

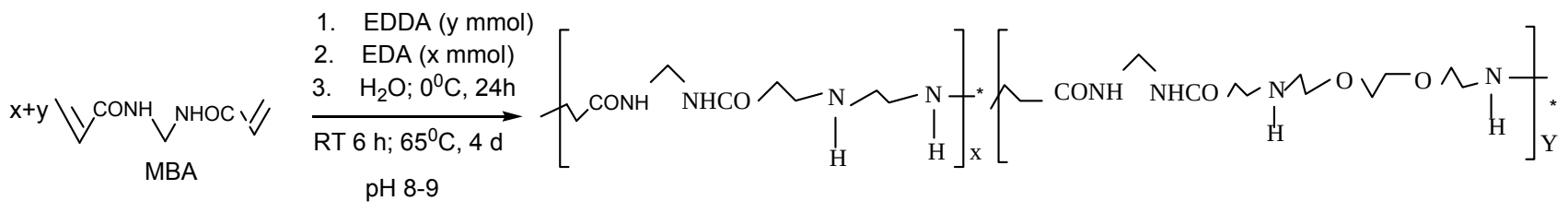


Polyam 1 x : y = 1 : 4

Scheme 6

Reaction took place in aqueous solution under weakly basic conditions at 50ºC for 3 days, the pH was brought to 7-7.5 during fractionation and purification by dialysis in membrane tubing possessing molecular mass cut-off limits of 25000. The polymers were ultimately collected by freeze-drying in yields of typically 10% as solids possessing excellent solubility in water. The reason for these low yields

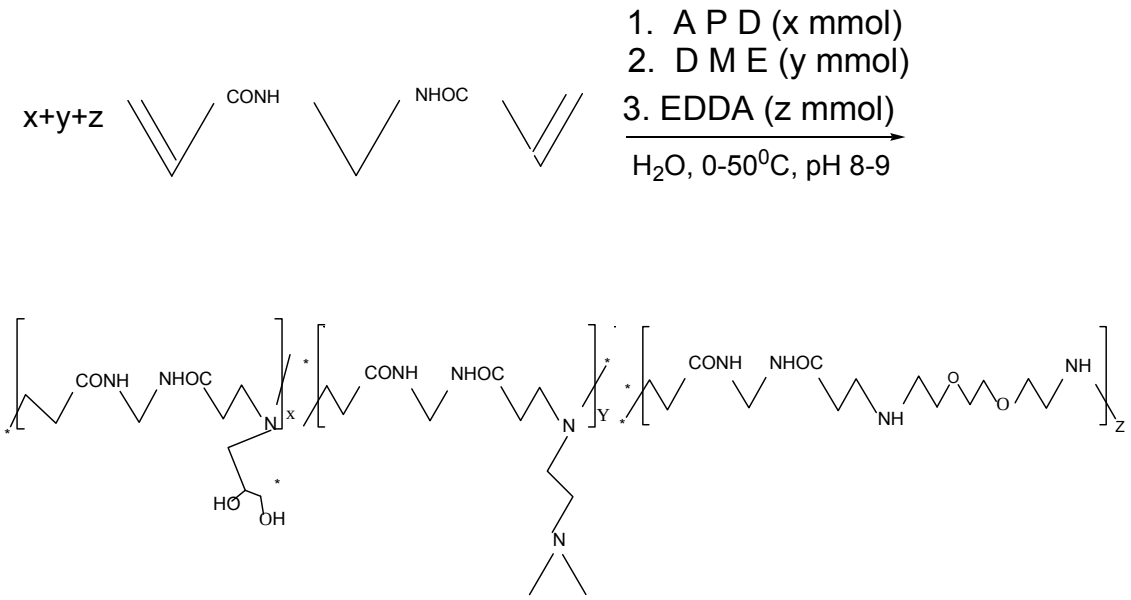
is hydrolytic backbone cleavage proceeding simultaneously with propagation in the aqueous reaction medium; it had to be tolerated, though, as the price to be paid for obtaining clean polymer fractions of the required molecular size. Inherent viscosities were found, $\eta_{inh,}$ 8mL/g for **Polyam 1** and $\eta_{inh,}$ 9 mL/g for **Polyam 2**. NMR spectroscopic data (Table 6) agree with the given structure (Scheme 6), and the reaction conditions are tabulated in Table 7.



Polyam 2

Scheme 7

The second strategy involved the incorporation of 2, 2'-(ethylenedioxy)diethylamine (EDDA) as the diamine in addition to the dihydroxyl-functionalized 3-amino-1, 2-propanediol (APD) and the monoamine DME, which replaced DMP. This kind of carrier displays a structure comprising dihydroxyl and amino groups in the same polymer and provides the advantage of different drug-binding sites and solubilizing groups. The synthesis involved mixing MBA, APD and DME in aqueous solution and allowing reaction to take place at room temperature, then at 50ºC for another day; upon dilution with H_2O, this solution was added drop-wise to an EDDA solution at ice bath temperature followed by one day of stirring at this temperature. Fractionation and purification took place by dialysis in membrane tubing possessing molecular cut-off limits of 25000. The polymer was collected by freeze-drying in yields of typically 26% as a water-soluble solid, $\eta_{inh,}$ 9 mL/g. NMR spectroscopic data (Table 6) agree with the given structure of **Polyam 3** (Scheme 8), and the reaction conditions are displayed in Table 7.

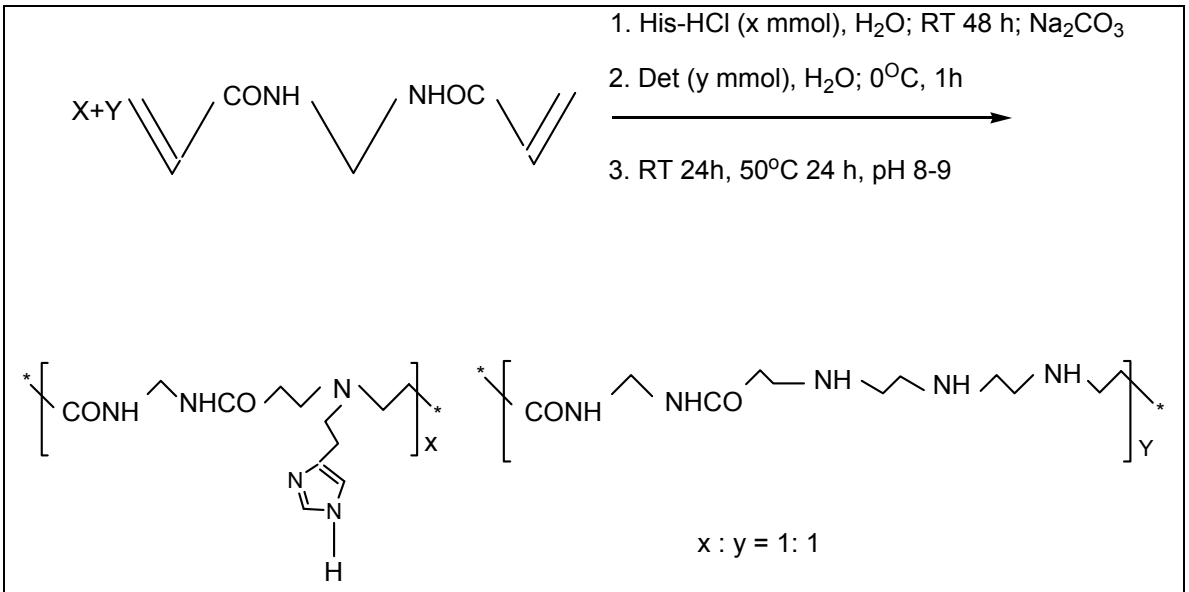


x : y : z = 1 :4 : 1

Polyam 3

Scheme 8

As our contribution to the toxicological study performed in our laboratory, the last methylenebisacrylamide (MBA)-based polyamidoamine to be designed in this work is **Polyam 4,** synthesized by adding histamine hydrochloride (His.HCl) predissolved in H_2O to MBA predissolved in warm H_2O, then cooled at RT. The histamine residue, like DME, gives the advantage of presenting a cationic site that could serve as both solubilizing group and homing device. After addition of Na_2CO_3, the clear solution was stirred for 48 h at RT then added dropwise at 0ºC to diethylenetriamine (DET) dissolved in H_2O and stirred overnight at this temperature under N_2. The solution was then stirred at RT for 24 h and at 50ºC for another 24 h. Water was removed from the clear solution by rotoevaporation. The product was then washed with hot acetone before purification and fractionation by dialysis in membrane tubing possessing a molecular cut-off limit of 25000. Throughout the process, the pH was adjusted to 8-9 with NaOH. The polymer was collected by freeze-drying in yields of typically 10% as a water-soluble solid, $\eta_{inh,}$ 7.5 mL /g. NMR spectroscopic data (Table 6) agree with the given structure of **Polyam 4** (Scheme 9), and the reaction conditions are displayed in Table 7.



Polyam 4

Scheme 9

Table 6: NMR data for carriers Polyam 1 to Polyam 4

Polymer Designation	η_{inh} (mL/g) [b]	Number of protons counted (expected) in ^{1}H NMR [a]					
		δ7.8-7 [c]	δ4.7-4.2	δ3.8-3.5	δ3-2.6	δ2.8-2	δ1.8-1.1
Polyam 1	8		9.7(10)	4.3(3)	22.8(20)	37(32)	8(8)
Polyam 2	9		4(4)	6.8(8)		22.7(24)	
Polyam 3	9		20.4(20)	19.3(11)	118(120)	48(48)	
Polyam 4	11	2(2)	4(4)		25(28)		

a. In D_2O,p D 10-11

b. At 30.0± 0.5ºC. in deionized H_2O, concentration c= 2mg/mL

c. Range of chemical shifts, δ/ppm

Table 7: Experimental conditions for the preparation of carriers polyam 1 to 3

Polymer Designation	H_2O (mL)	Monomer in feed (mmol)									Reaction conditions	Yield (%)
		His. HCl	DET	MBA	DMP	DME	APD	EDDA	EDA	EA		
Polyam 1	20			5	4		1			0.25	2 d RT, 2 d 50ºC, 2 h 50ºC	19
Polyam 2	12.5			2				1	1	0.1	24 h 0ºC, 6 h RT, 4 d 65ºC	20
Polyam 3	70			10		8	1	1			1 d RT, 1 d 50ºC, 1 d 0ºC	18
Polyam 4	18	1	1	2							2 d RT, 12 h 0ºC, 1 d RT, 1 d 50ºC	18

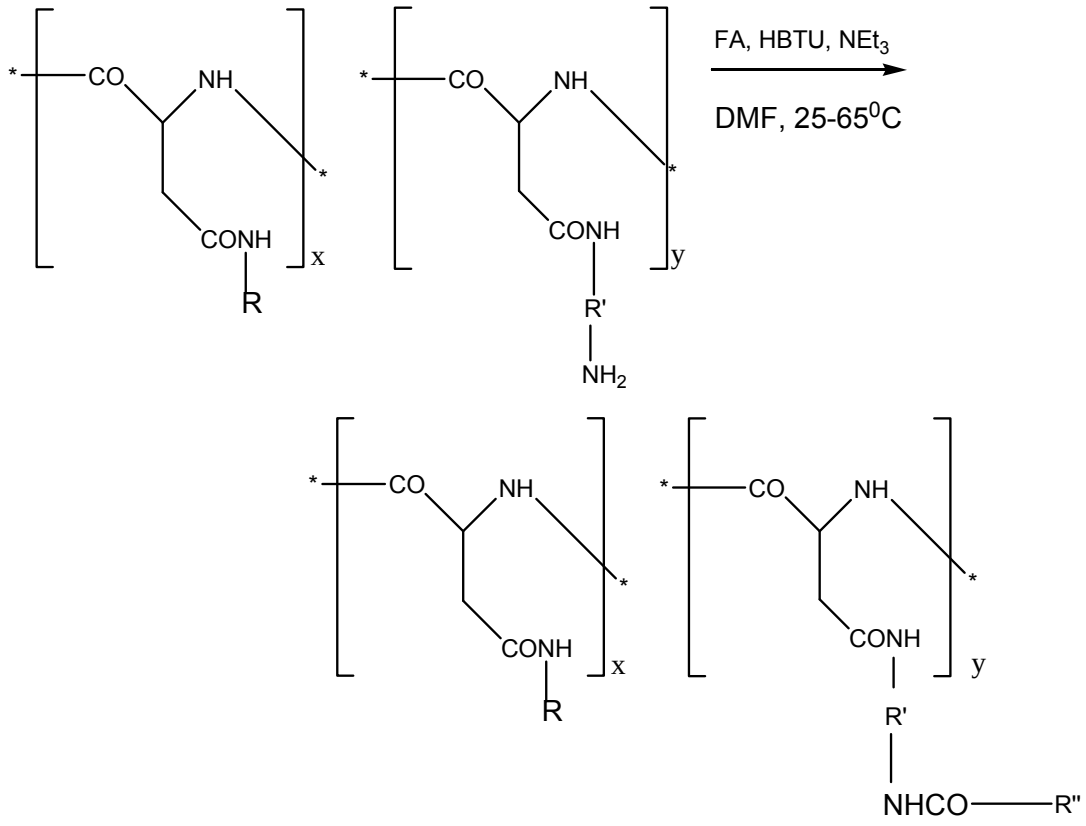
Phase II: Conjugation of carriers with selected antineoplastic agents

The carriers synthesized in Phase I of this work have been used for conjugation studies. These include carriers of the polyaspartamide-type with complete water-solubility. Other polyaspartamides and polyamidoamines have been kept for future work. As mentioned in Section 2, the two selected antineoplastic agents were *ferrocene* and *methotrexate*. The following copolyspartamide carriers: **polyasp 1 (80:20)**, **polyasp 1 (90:10)**, **polyasp 2 (80:80)**, **polyasp 2 (90:10)**, **polyasp 3 (80:20)** and **polyasp 3 (90:10)** were used for drug anchoring within the scope of the present work. While the conjugation strategy involving these bioactive agents had been previously developed in a number of projects in this laboratory [40-42, 53-55], the compositions of the polymers presently synthesized and used are different, and that may serve to demonstrate the wide range of applicability of the polymer-drug conjugation technology.

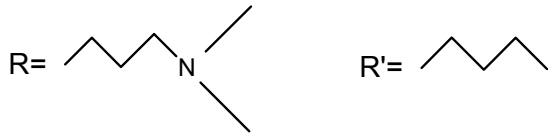
Polymer- methotrexate conjugation

This section describes conjugation of the carriers synthesized in the present project with *MTX.* Before performing *MTX* conjugation proper, a training exercise with folic acid [55] had to precede in order to familiarize ourselves with the conjugation technique and to avoid wastage of costly *MTX*. The choice of folic acid in this training exercise was motivated by its structural resemblance to *MTX* and its commercial availability at low cost, and the carriers used were chosen from the class of water-soluble polyamides. After an achievement of an incorporation of 100% of folic acid into the conjugate, *MTX* was coupled to selected polyaspartamide carriers of our program. The anchoring reactions involving *MTX* and folic acid were conducted under amidation conditions. In this task, **Polyasp 1 to Polyasp 3** were the copolymeric carriers selected to be anchored to folic acid and *MTX*. The coupling agent herein used was O-Benzotriazol-1-yl-tetramethyluronium salt (HBTU), and the reaction time was extended to three hours. The molar ratio of drug to polymer repeat unit was 1.2-

1.4. The product polymers were purified by elution on a chromatography column charged with silica gel G25, then by aqueous dialysis in tubing of 12000 molecular mass-cut-off, under controlled pH conditions. They were isolated by freeze-drying. Yields ranged from 50 to 70% (see Tables 8-11). Conjugates were characterized by ^{1}H-NMR spectroscopy (see Tables 8 and 9). This conjugation procedure is illustrated by **Scheme 10** for folic acid and **Scheme 11** for *MTX*.

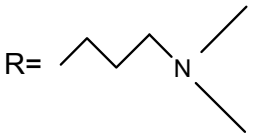


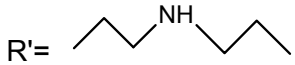
Polymer-FA



x/y= 4 **Polyasp 1 (80:20)-FA**

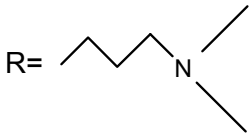
x/y= 9 **Polyasp 1 (90:10)-FA**





x/y=4 **Polyasp 2 (80:20)-FA**

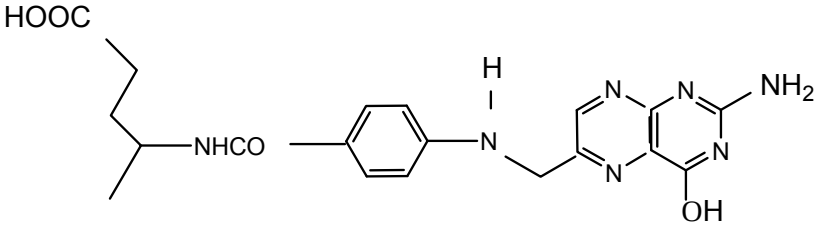
x/y=9 **Polyasp 2 (90:10)-FA**



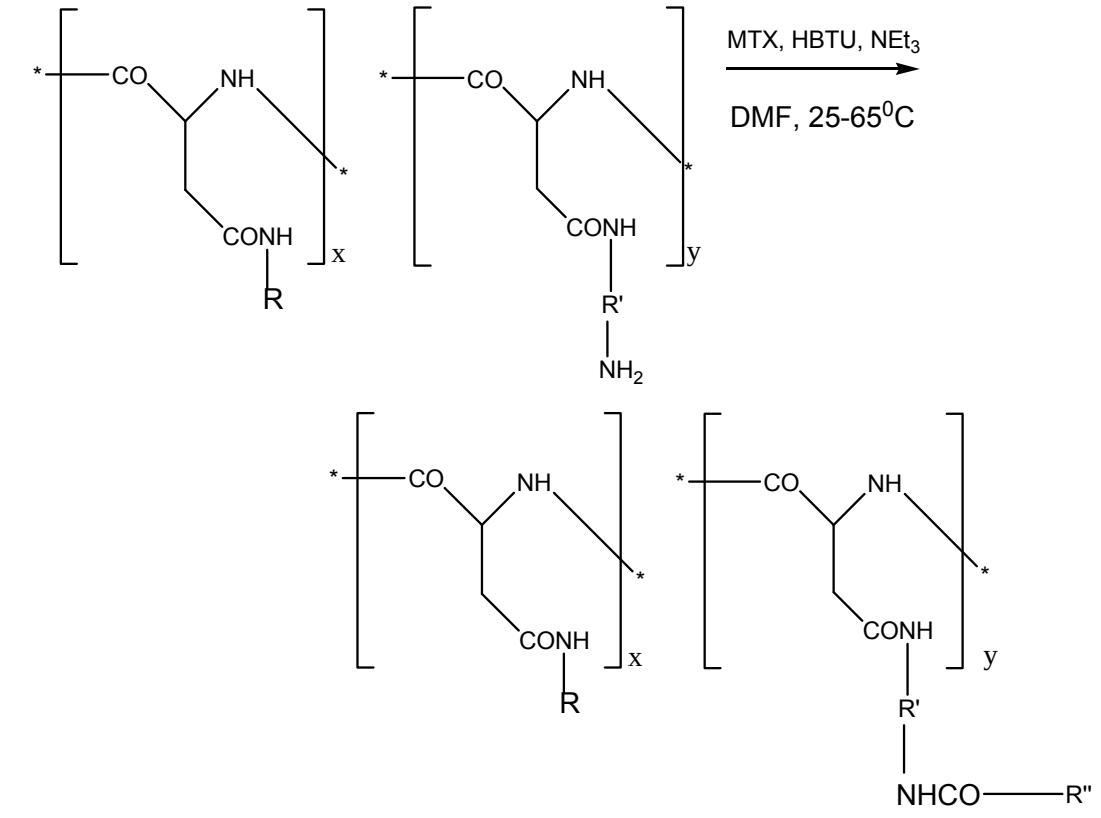
x/y=4 **Polyasp 3 (80:20)-FA**

x/y=9 **Polyasp 3 (90:10)-FA**

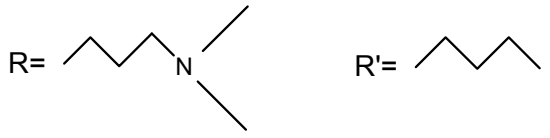
R":



Scheme 10

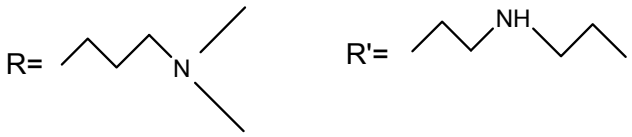


Polymer-MTX



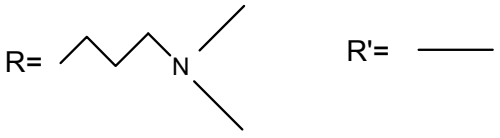
x/y= 4 **Polyasp 1 (80:20)-MTX**

x/y= 9 **Polyasp 1 (90:10)-MTX**



x/y=4 **Polyasp 2 (80:20)-MTX**

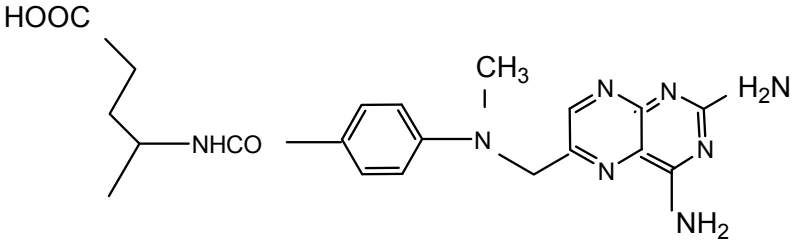
x/y=9 **Polyasp 2 (90:10)-MTX**



x/y=4 **Polysp 3 (80:20)-MTX**

x/y=9 **Polyasp 3 (90:10)-MTX**

R":



Scheme 11

Table 8: Viscometric and analytical results for folic acid conjugates studied in this project.

FA conjugates				Protons counted (expected)[d] in ^{1}H NMR				
Conjugate Designation	**η_{inh} mL/ g)[a]**	**% FA Cald [b]**	**% FA Found [c]**	δ 8.8-6.5[e]	δ 4.75-4.0	δ 3.4-2.8	δ 2.5-2.0	2.0-1.5
Polyasp 1 (80:20)-FA	13	44	43	4 (5)	4 (5)	7 (10)	27 (32)	10 (12)
Polyasp 1 (90:10)-FA	16	22	41	4 (5)	6 (10)	16 (20)	58 (72)	18 (22)
Polyasp 2 (80:20)-FA	15	42	40	3 (5)	4 (5)	8 (12)	28 (32)	11 (10)
Polyasp 2 (90:10)-FA	17	21	19	5 (5)	5 (10)	16 (22)	54 (72)	18 (2)
Polyasp 3 (80:20)-FA	11	46	42	5 (5)	5 (5)	5 (8)	26 (32)	8 (10)
Polyasp 3 (90:10)-FA	12	22	21	4 (5)	6 (10)	15 (18)	56 (72)	19 (20)

a. At 30.0 ± 0.5ºC, In deionazed H_2O, concentration c= 2mg/mL.
b. Calculated for given structures.
c. Derived from ^{1}H-NMR spectrum (error limit ± 15%).
d. In D_2O; pD 10-11.
e. Range of chemical shifts, δ/ppm.

Table 9: Viscometric and analytical results for the *MTX* conjugates studied in this project.

MTX conjugates				Protons counted (expected)[d]				
Conjugate Designation	**η_{in} (mL/ g)[a]**	**%MTX Cald [b]**	**% MTX Found[c]**	δ 8.8-6.5[e]	δ 4.75-4.0	δ 3.4-2.8	δ 2.5-2	2.0-1.5
Polyasp 1 (80:20)-MTX	12	45	42	5 (5)	3 (5)	8 (12)	25 (32)	9 (12)
Polyasp 1 (90:10)-MTX	13	22	22	5 (5)	7 (10)	17 (22)	59 (72)	17 (22)
Polyasp 2 (80:20)-MTX	14	43	41	5 (5)	3 (5)	9 (12)	24 (32)	10 (10)
Polyasp 2 (90:10)-MTX	14	22	20	4 (5)	6 (10)	18 (22)	58 (72)	19 (20)
Polyasp 3 (80:20)-MTX	10	47	45	4 (5)	3 (5)	5 (8)	22 (32)	6 (10)
Polyasp 3 (90:10)-MTX	11	23	20	4 (5)	4 (10)	17 (18)	62 (72)	17 (20)

a. At 30.0 ± 0.5ºC, In deionazed H_2O, concentration c= 2mg/mL.

b. Calculated for given structures.

c. Derived from ^{1}H-NMR spectrum (error limit ± 15%).

d. In D_2O; pD 10-11.

e. Range of chemical shifts, δ/ppm.

Table 10: Experimental conditions for folic acid conjugates studied in this project

Reactants in feed			Reaction conditions [b]	Folic acid conjugates	
Carrier designation	**Coupling agent**	**Polymer-drug-coupling agent ratio [a]**		**Yield (%) [c]**	**Designation**
Polyasp 1 (80:20)	HBTU	1: 1.2: 1.1	3 h, RT	70	**Polyasp 1 (80:20)-FA**
Polyasp 1 (90:10)	HBTU	1: 1.2: 1.1	3 h, RT	62	**Polyasp 1 (90:10)-FA**
Polyasp 2 (80:20)	HBTU	1: 1.2: 1.1	3 h, RT	65	**Polyasp 2 (80:20)-FA**
Polyasp 2 (90:10)	HBTU	1: 1.2: 1.1	3 h, RT	56	**Polyasp 2 (90:10)-FA**
Polyasp 3 (80:20)	HBTU	1: 1.2: 1.1	3 h, RT	60	**Polyasp 3 (80:20)-FA**
Polyasp 3 (90:10)	HBTU	1: 1.2: 1.1	3 h, RT	54	**Polyasp 3 (90:10)-FA**

a. Molar ratio of carrier repeat unit to FA to HBTU

b. RT=room temperature.

c. Conjugate yield after ultimate (12 000 molecular mass-cut-off) dialysis.

Table 11: Experimental conditions for *MTX* conjugates studied in this project

Reactants in feed			Reaction conditions [b]	MTX conjugates	
Carrier designation	**Coupling agent**	**Polymer-drug-coupling agent ratio [a]**		**Yield (%) [c]**	**Designation**
Polyasp 1 (80:20)	HBTU	1: 1.2: 1.1	3 h at RT	65	**Polyasp 1 (80:20)-MTX**
Polyasp 1 (90:10)	HBTU	1: 1.2: 1.1	3 h at RT	54	**Polyasp 1 (90:10)-MTX**
Polyasp 2 (80:20)	HBTU	1: 1.2: 1.1	3 h at RT	70	**Polyasp 2 (80:20)-MTX**
Polyasp 2 (90:10)	HBTU	1: 1.2: 1.1	3 h at RT	52	**Polyasp 2 (80:20)-MTX**
Polyasp 3 (80:20)	HBTU	1: 1.2: 1.1	3 h at RT	55	**Polyasp 3 (80:20)-MTX**
Polyasp 3 (90:10)	HBTU	1: 1.2: 1.1	3 h at RT	54	**Polyasp 3 (90:10)-MTX**

a. Molar ratio of carrier repeat unit to MTX to HBTU

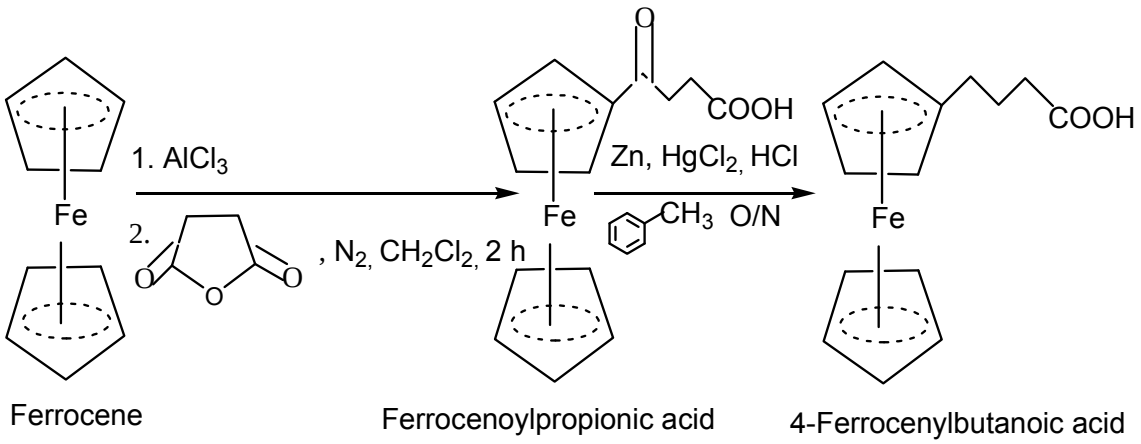
b. RT = room temperature.

c. Conjugate yield after ultimate (12 000 molecular mass-cut-off) dialysis.

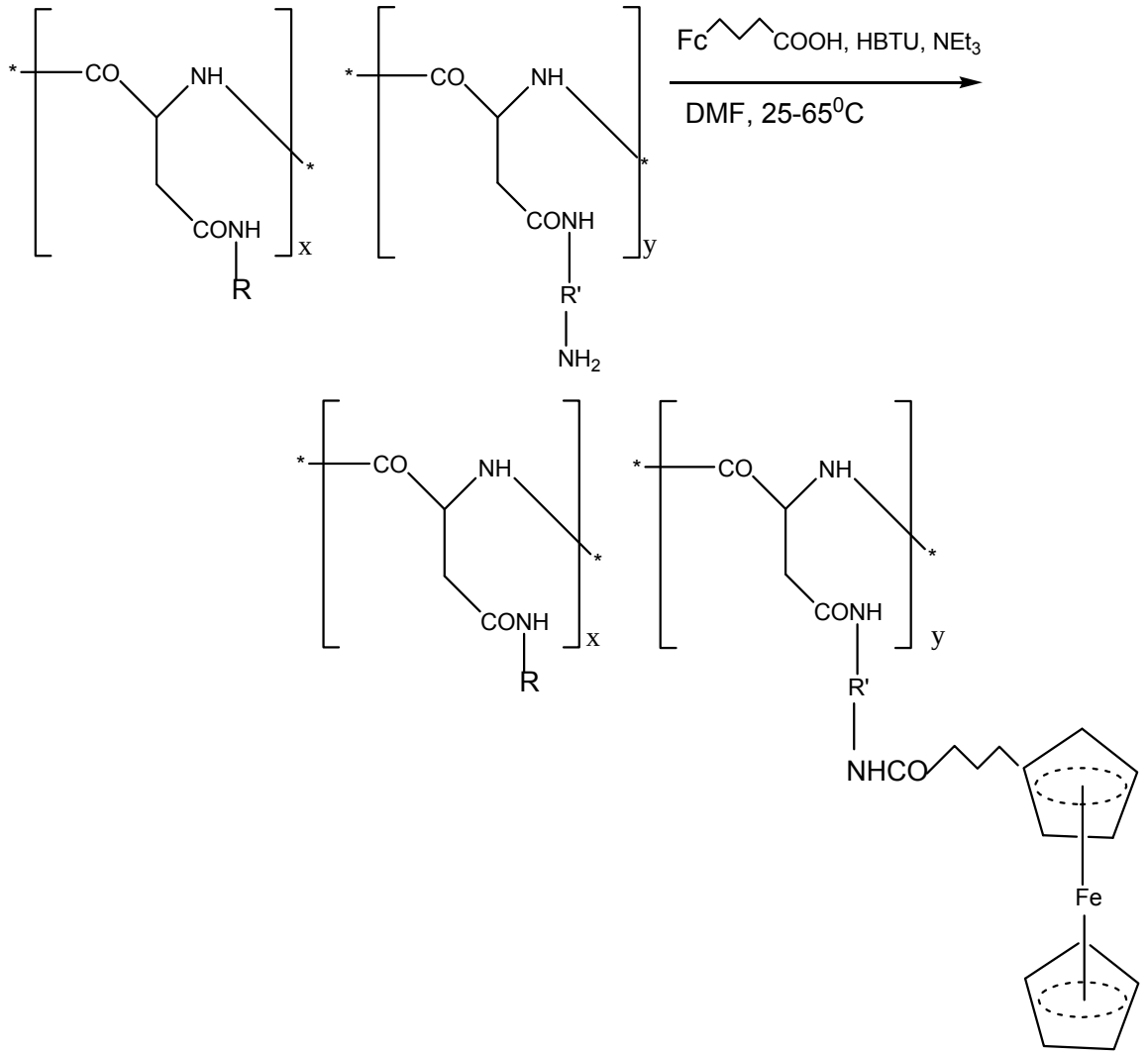
Polymer-ferrocene conjugation

Ferrocene, like some other metallocenes, has shown antineoplastic activities against tumor cells . For *ferrocene*, these activities are associated with its electronic properties rising from its structure described in Section 2. 3. Conjugation of *ferrocene* with a suitable carrier polymer provides a prodrug from which the bioactive ferrocene compound may be released in the intracellular space. Many synthetic programs were initiated in this laboratory, aiming at development and routine sythesis of such *ferrocene* conjugates for biomedical investigations . In the present work, carrier-*ferrocene* coupling was achieved *via* amide bonds.

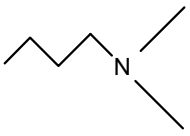
For this purpose, polyaspartamide carriers **Polyasp 1** to **Polyasp 3** comprising hydrosolubilizing *tert*-amine terminals in the side groups R_1 of **Scheme** 3, and primary or secondary amino groups as terminals in R_2 serving as drug binding sites were coupled to *ferrocene,* and the ferrocenylation agent used was 4-ferrocenylbutanoic acid (**Scheme 11**) prepared by known literature methods [56]. The coupling agent herein used was HBTU, and the reaction time was extended to three hours. The molar ratio of drug to polymer repeat unit was 1.2-1.4. The product polymers were purified by elution on chromatography column charged with silica gel G25, then by aqueous dialysis in tubing of 12000 molecular mass-cut- off under controlled pH conditions and they were isolated by a freeze-drying operation. Yields ranged from 45 to 60% (see Table 12). The conjugates were characterized by ^{1}H-NMR spectroscopy (see Table 13). This conjugation procedure is illustrated by **Scheme 12**.


1. AlCl3
2.
, N2, CH2Cl2, 2 h
Zn, HgCl2, HCl
CH3 O/N
Fe
Fe
Fe
O
COOH
COOH
O
O
O
Ferrocene
Ferrocenoylpropionic acid
4-Ferrocenylbutanoic acid

Scheme 11

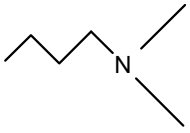
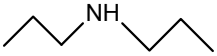


Polymer-Fc

R=  R'= 

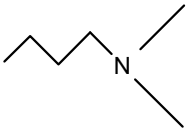
x/y= 4 **Polyasp1 (80:20)-Fc**

x/y= 9 **Polyasp1 (90:10)-Fc**

R=  R'= 

x/y=4 **Polyasp 2 (80:20)-Fc**

x/y=9 **Polyasp 2 (90:10)-Fc**

R=  R'= ——

x/y=4 **Polyasp 3 (80:20)-Fc**

x/y=9 **Polyasp 3 (90:10)-Fc**

Scheme 12

Table 12: Experimental conditions for the *ferrocene* conjugates studied in this project

Reactants in feed			Reaction conditions [b]	*Ferrocene* conjugates	
Carrier designation	**Coupling agent**	**Polymer-drug-coupling agent ratio [a]**		**Yield (%)[c]**	**Designation**
Polyasp 1 (80:20)	HBTU	1: 1.2: 1.1	3 h, RT	65	**Polyasp 1 (80:20)-Fc**
Polyasp 1 (90:10)	HBTU	1: 1.2: 1.1	3 h, RT	51	**Polyasp 1 (90:10)-Fc**
Polyasp 2 (80:20)	HBTU	1: 1.2: 1.1	3 h, RT	46	**Polyasp 2 (80:20)-Fc**
Polyasp 2 (90:10)	HBTU	1: 1.2: 1.1	3 h, RT	52	**Polyasp 2 (90:10)-Fc**
Polyasp 3 (80:20)	HBTU	1: 1.2: 1.1	3 h, RT	47	**Polyasp 3 (80:20)-Fc**
Polyasp 3 (90:10)	HBTU	1: 1.2: 1.1	3 h, RT	50	**Polyasp 3 (90:10)-Fc**

a. Molar ratio of carrier repeat unit to Fc to HBTU

b. RT = room temperature.

c. Conjugate yield after ultimate (12 000 molecular mass-cut-off) dialysis.

Table 13: Viscometric and analytical results for the *ferrocene* conjugates studied in this project.

***Ferrocene* conjugates**				**Protons counted (expected) [d] in ^{1}H NMR**				
Conjugate Designation	**η_{inH} (mL/g)[a]**	**% Fc Cald [b]**	**% Fc Found [c]**	δ 4.25-4.0 [e]	δ 3.4-3.0	δ 3.0-2.5	δ 2.4-2.1	δ 1.9-1.4
Polyasp 1 (80:20)-Fc	10	26	23	12 (14)	10(12)	7 (8)	26 (32)	11 (12)
Polyasp 1 (90:10)-Fc	10	13	10	19 (19)	19(22)	18(18)	72(72)	20(22)
Polyasp 2 (80:20)-Fc	12	25	21	12 (14)	10 (12)	7 (8)	26 (32)	9 (10)
Polyasp 2 (90:10)-Fc	13	13	10	16 (19)	24 (22)	15 (18)	62 (72)	18 (20)
Polyasp 3 (80:20)-Fc	9	27	19	10 (14)	10 (8)	6 (8)	33 (32)	10 (10)
Polyasp 3 (90:10) Fc	11	13	11	21 (19)	21 (18)	15 (18)	65 (72)	17 (20)

a. At 30.0 ± 0.5ºC, In deionazed H_2O, concentration c= 2mg/mL.
b. Calculated for given structures.
c. Derived from ^{1}H-NMR spectrum (error limit ± 15%).
d. In D_2O; pD 10-11.
e. Range of chemical shifts, δ/ppm.

Phase III: biomedical testing

The last step of this work is toxicology assessment for selected carriers and conjugates synthesized in **Phase II**. This toxicological work was performed in collaboration with Professor Connie Medlen, University of PRETORIA, department of Pharmacology. The following Methotrexate-based conjugates were the ones involved in this work stage: **Polysap 1 (80:20)-MTX**, **Polyasp 1 (90:10)-MTX**, **Polyasp 2 (80:20)-MTX**, **Polyasp 2 (90:10)-MTX**, **Polyasp 3 (80:20)-MTX** and **Polyasp 3 (90:10)-MTX.**

Though toxicological test results are still unpublished, preliminary in *vitro* tests revealed an acceptably low toxicity from all our conjugates, which means that they are potent candidates for eventual in *vivo* tests.

The following carriers synthesized in **PHASE II** of this work were also submitted to a toxicological test: **Polyasp 1 (80:20), Polyasp 1 (90:10), Polyasp 2 (80:20), Polyasp 2 (90:10), Polyasp 3 (80:20), Polyasp 3 (90:10), Polyasp 4 (80:20)** and **Polyasp 4 (90:10)**. In *vitro* toxicological work revealed here again an acceptable degree of toxicity from all carriers, although those falling in the series of **DMP (90)** are on the “watch list”, with minor toxicity effects considered possible at this time.

CHAPTER 4

CONCLUSION AND SCOPE OF FUTURE WORK

The major objective of the present dissertation project was the development and synthesis of known and novel macromolecular, water-soluble compounds to serve as carriers for biologically active compounds and coupling these carriers to selected drug systems for use in cancer chemotherapy. Drugs involved comprise the organoiron compound *ferrocene* and the antitumor drug *methotrexate*, anchored to carriers through biofissionable amide bonds for the purpose of obtaining water-soluble conjugates. Thes will be submitted to an outside institution for assessment of their cytotoxicity in order to confirm their anticipated antiproliferative properties.

When focusing on this objective, eight carriers were synthesized, all being derivatives of poly-α, β-D L-aspartamide. In this synthetic work, previously established expertise was used to develop and modify preparative methodology. All carriers synthesized met solubility and compositional requirements.

The carrier-anchoring of the ferrocenylation agent, 4-ferrocenylbutanoic acid, generally by well-established procedures, proceeded successfully, yielding water-soluble conjugates in which the metallocene was bound to the amine-functionalized *via* amide links, with drug incorporation in the range of 71-95%.

Methotrexate conjugation through amide links, using established and slightly modified procedures was successful, as the resulting conjugates were all water-soluble, and drug incorporation ranged from 83 to 100%.

Selected carriers and MTX conjugates synthesized in this work were delivered into a pool of bioactive polymers which has been submitted to the department of PHARMACOLOGY, UNIVERSITY of PRETORIA for a preliminary in *vitro* bioassessment. The cytotoxicity found in most of these polymers qualified them for prospective tests in vivo as they can be considered as potent antineoplastics.

CHAPTER 5

EXPERIMENTAL

5.1. GENERAL PROCEDURES

^{1}H NMR spectra (200 or 400 MHz) were obtained in D_2O solutions, chemical shifts δ, in ppm, were referenced against sodium 3-(trimethyl)-2,2,3,3-d_4-propionate ($\delta_{HOD} \approx 4.83$ ppm; integration error limit ± 15%). In order to eliminate protonation effects, spectral solutions were adjusted to pD 10-11 with NaOH. Inherent viscosities, $\eta_{inh,}$ were determined at 30.0 ± 0.5°C in Cannon-Fenske tubes. Deionized water was the solvent (c = 0.2 g/100mL), and the results, averages of two determinations, were given in units of mLg^{-1}. Dialysis operations were performed in cellulose tubing, types Spectra/Por 4 and Spectra/Por 6 (Spectrum Industries, Los Angeles, CA), with molecular mass cut-off limits of 12000-14000 and 25000, respectively, against several batches of deionized water.

Aqueous solutions were freeze-dried in a VIRTIS Bench Top 3 freeze-drier operating at -30ºC, under 10-15 Pa. The freeze-dried polymers were routinely post-dried in a SARTORIUS Thermo Control infrared drying system. Analytical samples were additionally dried for two days at 70ºC in an Abderhalden tube.

5.2 REACTANTS, REAGENTS AND SOLVENTS

The aprotic solvent, N, N-Dimethylformamide (DMF), freshly distilled under reduced pressure, was dried over Molecular Sieves. All reactions involving its use were performed under anhydrous conditions. Deionized H_2O was used for both dialysis and viscometric operations.

The hydroxyamines and diamines, commercial grades (Fluka Chemie AG), were used as received. These included: ethanolamine (EA), 1,3-propylenediamine (PDA), 2,2'-(ethylenedioxy)diethylamine (EDDA), 3-(N,N-

dimethylamino)propylamine (DMP), diethylenetriamine (DET), ethylenediamine (EDA), and hydrazine (HY).

D L-aspartic acid, N,N'-dicyclohexylcarbodiimide (DCC); 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium fluorophosphate (HBTU); triethylamine (NEt_3), 4-(dimethylaminopyridine (DMAP); 4,7,10-trioxa-1,13-tridecanediamine (TRIA); diethyl L-tartrate (Detart) (all Fluka Chemie AG) were also used as delivered.The following solvents, diethylether (Et_2O) and petroleum ether (b.p 65-70ºC), were used as supplied. Folic acid and *methotrexate (MTX*), each of which contains 0.4 % of water, were pre-dried in an Aberhalden tube for 24h at 50ºC before use for conjugation.

5.3 EXPERIMENTAL PROCEDURES

5.3.1 Preparation of poly-D L-succinimide (PSI)

PSI was prepared by the method of Neri and Antoni [57]; products of approximately equal viscosity were pooled from several runs and thoroughly mixed to give a master batch with η_{inh} (determined in DMF) of 27 mL/g

5.3.2 Preparative procedure for 4-Ferrocenylbutanoic acid

This ferrocenylation agent was prepared according to the procedure described in the literature[58]. Several products of close melting points were thoroughly mixed to give a batch of mp. 115-120ºC (Lit [58]. 119-120ºC)

5.3.3 Preparative procedure for 3-Ferrocenylpropenal

This ferrocenylation agent, although not used in the present project, was prepared for a future project according to the procedure described in the

literature [59]. Several products of close melting points were thoroughly mixed to give a batch of mp: 95-99ºC (Lit [59]. 97-98ºC).

5.3.4 Preparative procedure for polyaspartamide carriers

These carriers were prepared according to the general method used in our laboratory [61]. For polyamides bearing a tertiary amine-terminated side chain as the hydrosolubilizing group, some modifications were introduced, concerning the reaction time in certain steps. For this kind of polyaspartamide carrier, the preceding reactant was added dropwise to the following.

Polyasp 1 (80:20): To the stirred solution of PSI, 1.94 g (20 mmol), in 20 mL of DMF, was added 3-(N, N-dimethylamino)propylamine (DMP), 1.64 g (16 mmol, in 10 mL of DMF. After saturation with N_2, the resulting solution was stirred for 8h at room temperature. 1, 3-Propylenediamine (PDA), 0.89g (12 mmol), dissolved in 10 mL of DMF, was then added dropwise at ice bath temperature and stirring continued for 12h. Solvent was partially removed under reduced pressure. The polymer was precipitated with 100 mL of a 2:1 mixture (by vol.) of diethyl ether and hexane , redissolved in 80 mL of H_2O, and dialysed successively in Spectra/Por 4 tubing for 48 h against distilled H_2O and another 48 h in Spectra/Por 6 tubing against distilled H_2O. Freeze-drying of the retentate gave a 60 % yield of **Polyasp 1 (80:20)** as a hygroscopic and water-soluble solid**,** η_{inh}, 8 mL/g.

Polyasp 1 (90:10) was synthesized using the same procedure as for **Polyasp 1 (80:20)** except that 1.84 g (18 mmol) of DMP and 0.445g (6 mmol) of PDA were used.

Polyasp 2 (80:20) and Polyasp 2 (90:20) was synthesized by the same procedure as for **Polyasp 1(80:20)** and **Polyasp 1 (90:10)** respectively**.** However, PDA was replaced by diethylenetriamine (DET) in different molar ratios in this case. The reaction time and conditions differed as well (refer to Table 2).

Polyasp 3 (80:20) and **Polyasp 3 (90:10**) were synthesized by the same basic procedure except that hydrazine (HY) was used in the second ring-opening step at required molar ratios, and the stirring time at room temperature was extended to 40h (Refer to Table 2).

Polyasp 4 (80:20) and **Polyasp 4 (90:10)** were synthesized using the same procedure as for **Polyasp 1 (80:20)** and **Polyasp 1 (90:10)** except that in the present cases, dimethylaminoethylamine (DME) was used instead of DMP at required molar ratios (refer to Table 2),

Polyasp 5 (80:20) and **Polyasp 5 (90:10)** were synthesized by the same general procedure as for **Polyasp 4 (80:20)** and **Polyasp 4(90:10).** However, diethylaminoethylamine (DEE) was used at required molar ratios in these cases in place of DME (refer to Table 2).

Polyasp 6 (80:20) and **Polyasp 6 (90:10)** were synthesized by the same procedure except that in these cases, hydrazine as the hydrate was used at required molar ratios in place of PDA (refer to Table 2).

For other polyamides, **Polytar 1** was prepared according to a procedure that has been used in this laboratory: diethyl L-tartrate, 2.062g (10mmol), 4, 7, 10-trioxa-1,13-tridecanediamine, 2,203g (10mmol), and anhydrous sodium carbonate, 0.85g (representing 20 % of total mass of reactants), were mixed. The mixture, saturated with N_2, was stirred at room temperature for 24 h, diluted with 2mL of DMSO, resaturated with N_2 and stirred continuously for 3 weeks in an incubator at 55ºC. Liberated ethanol was intermittently removed under reduced pressure at 60ºC bath temperature. Thereafter the polymer solution was concentrated on a rotary-evaporator under reduced pressure and the precipitate redissolved in 20 mL of water. The aqueous polymer solution (pH 10) was acidified to pH 7.5, then dialysed in Spectra/Por 4 tubing for 48 h. Freeze-drying of the retentate furnished

7.8g (52%) of **Polytar 1** as a yellow, fluffy and water-soluble solid, $\eta_{inh,}$ 20 mL/g. **Polytar 2** was prepared by the same basic procedure except that 2, 2'-(ethylenedioxy)diethylamine (EDDA) was used in place of 4, 7, 10-trioxa-1, 13-tridecanediamine.

5.3.5 Preparative procedure for polyamidoamine carriers

In the present project, polyamidoamines have been synthesized according the general procedure used in our laboratory.

For **Polyam 1,** 1g (6.49 mmol) of methylenebisacrylamide (MBA) and a mixture of 0.53 g (5.9 mmol) of 3-(N, N'-dimethylamino)propylamine with 0.124 g (2 mmol) of 3-amino-1,2-propanediol were dissolved together in 30 mL of warm H_2O. The clear solution was flushed with N_2, then stoppered and stirred at 50ºC for 3 d. The pH was 8.2. The solution was then concentrated on a rotary-evaporator under reduced pressure before being washed two times with hot acetone. It was dissolved in H_2O (10 mL) and dialysed in Spectra/Por 4 tubing for 48h. Freeze-drying of the retentate gave 100 mg (10%) of **Polyam 1** as a white water-soliuble solid, $\eta_{inh,}$ 8 mL/g.

Polyam 2 was prepared by the foregoing method yet 0.601g (20 mmol) of ethylene diamine (EDA) and 1.48g (10 mmol) of 2, 2'-(ethylenedioxy)diethylamine (EDDA) were used respectively in place of DMP and APD. 700 mg (26 %) of **Polyam 2** was collected as a water-soluble solid of $\eta_{inh,}$ 9 mL/g.

For the preparation of **polyam 3,** a mixture of MBA, 4.625g (30mmol), DME, 2.12g (24mmol) and APD, 0.73g (8mmol) was stirred at room temperature for 1 d then at 50ºC in the incubator for another day. After dilution with H_2O (190 mL), the mixture was added to 2, 2'-(ethylenedioxy)diethylamine (EDDA), 1.19g (8mmol) pre-dissolved in H_2O (10 m L) and heated, at ice bath temperature and stirred for 1 d at this temperature. After concentration on a rotary-evaporator to a

resinous residue state, the mixture was washed twice with hot acetone, then dissolved in H_2O and dialysed in Spectra/Por 4 tubing for 2 d with the pH checked 8.5. Freeze-drying of the retentate furnished 6g (69%) of **Polyam 2** as a colorless, water-soluble solid, η_{inh}, 9 mL/g.

Polyam 4 was prepared by mixing histamine hydrochloride (His. HCl) (3.25 mmol) dissolved in H_2O (60 mL) and MBA, 2g (12.98 mmol) dissolved in warm H_2O, then cooled to RT. Na_2CO_3, 0.69g (6.49 mmol) was then added. Under N_2 atmosphere, the clear solution was stirred at RT for 2 d. DET, 0.67g (6.49 mmol) dissolved in H_2O was added at 0º C dropwise and stirring at that temperature continued overnight under N_2 atmosphere, then at RT for 1 d and at 50º C for another d. The following purification step was done as in the foregoing.18% of **Polyam 4** was collected as a white water-soluble solid, η_{inh}, 11 mL/g.

5. 3. 6 Preparative procedures for MTX conjugates

`These conjugates were prepared using methods that have been developed in this laboratory.

Polyasp 1 (80:20)-MTX was prepared by mixing the carrier **Polyasp 1 (80:20),** 200 mg (0.206 mmol) dissolved in 5 mL of DMF to *MTX,* 54 mg (0.122 mmol) dissolved in 4 mL of the same solvent and stirring the mixtrure for 1h at RT. HBTU, 43 mg (112 mmol) dissolved in 1m L of DMF was added dropwise to the above mixture followed by NEt_3, 28 µL (0.204mmol). After stirring for 2 h, the polymer was precipitated from the yellow solution with 15 mL of a 2:1 (by vol.) mixture of diethyl ether and acetone. The precipitate was redissolved in 8mL of H_2O. The pH, approximately 7, was adjusted to 9 with NaOH. The solution was passed through a chromatography column charged with silica gel G 25 and the pH was brought to 7 with glacial acetic acid; the solution was dialyzed in Spectra/Por 4 tubing for 48 h against H_2O then in Spectra/Por 6 tubing for 48 h. During the last 6 h of dialysis, pH was adjusted to 3 for 5minutes with HCl then

brought to 6 with ammonia. Freeze-drying of the tube content gave 140 mg (42 %) of **Polyasp 1 (80:20)-MTX** as yellow water-soluble solid, $\eta_{inh,}$ 12 mL/g. **Polyasp 1 (90:10)-MTX** was prepared by the same general procedure except that **Polyasp 1 (90:10)** was used in place of **Polyasp 1 (80:20**). **Polyasp 1 (90:10)-MTX** was collected as a yellow water-soluble solid in a yield of 46 mg (22 %).$\eta_{inh,}$ 13 mL/g.

Polyasp 2 (80:20)-MTX, Polyasp 2 (90:10)-MTX, Polyasp 3 (80:20)-MTX and **Polyasp 3 (90:10)-MTX** were prepared by the same method as the foregoing except that reagents were taken at recalculated molar ratios. These reactions resulted in conjugates **Polyasp 2 (80:20)-MTX,** 41%, $\eta_{inh,}$ 14 mL/g, **Polyasp 2 (90:10)-MTX,** 20%, $\eta_{inh,}$ 14 mL/g, **Polyasp 3 (80:20)-MTX,** 45%, $\eta_{inh,}$ 10 mL/g and **Polyasp 3** (**90:10)-MTX,** 20%, $\eta_{inh,}$ 11 mL/g.

3. 5. 7. Preparative procedures for ferrocene conjugates

For the synthesis of ferrocene conjugates, procedures formerly developed in this laboratory were used.

To prepare conjugate **Polyasp 1 (80:20)-Fc**, 4-ferrocenylbutanoic acid, 34 mg (0.122 mmol), and HBTU, 43 mg (0.112 mmol), were combined, dissolved in 2 mL of DMF, stirred for 1 h and added to polymer **Polyasp (80:20)**, 200mg (0.102 mmol), pre-dissolved in 6 mL of the same solvent. Triethylamine (NEt_3), 28 µL (0.204 mmol) was added to the above mixture, which was stirred for another 2 h.. The polymer, **Polyasp 1 (80:20)-Fc,** was precipitated with 15 mL of a 2:1 (by vol.) mixture of diethyl ether and acetone, then redissolved in 15 mL of H_2O. Upon pH adjustment to 9 (NaOH), the yellow solution was passed trough a chromatography column charged with silica gel G 25. Then pH was brought to 7 with glacial acetic acid before dialysis in Spectra/Por 4 tubing for 2 d and Spectra/Por 6 for another 2 d. During the last 6 h of dialysis, the pH was adjusted to 3 (HCl) for 5 minutes, then to 6.5 with ammonia. Freeze-drying of the retentate afforded 120 mg (60%) of **Polyasp 1 (80: 20)-Fc** as a tan-colored, fluffy and water-soluble material, $\eta_{inh,}$ 10 mL/g.

The conjugates **Polyasp 1 (90:10)-Fc, Polyasp 2 (80:20)-Fc, Polyasp 2 (90:10)-Fc, Polyasp 3 (80:20)-Fc** and **Polyasp 3 (90:10)-Fc** were obtained using the preceding procedure. The polymeric carrier **Polyasp 1 (90:10)** used to prepare **Polyasp 1 (90:10)-Fc**, was replaced by **Polyasp 2 (80:20)**, **Polyasp 2 (90:10)**, **Polyasp 3 (80:20)** and **Polyasp 3 (90:10)** for the synthesis of **Polyasp 2 (80:20)-Fc**, **Polyasp 2 (90:10)-Fc**, **Polyasp 3 (80:20)-Fc** and **Polyasp 3 (90:10)-Fc** respectively.

APPENDIX