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DEVELOPMENT AND SYNTHESIS OF NOVEL
POLY(β-AMINO ACID) DRUG DELIVERY SYSTEMS

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PING CAO

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DEVELOPMENT AND SYNTHESIS OF NOVEL POLY(β-AMINO ACID) DRUG DELIVERY SYSTEMS

By

PING CAO

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ABSTRACT

DEVELOPMENT AND SYNTHESIS OF NOVEL POLY(β-AMINO ACID) DRUG DELIVERY SYSTEMS

By

Ping Cao

A new drug delivery system based on a poly(β-amino acid) has been synthesized from 2(5H)-furanone and N, N–dimethylethylene diamine by a simple, one step polymerization. The resulting polymer was purified by Bio Gelp-P60 size exclusion chromatography and auto separated into high, medium and low molecular weight fractions. The molecular weight distribution was controlled by adjusting the reaction temperate and reaction time. The drug delivery properties of the polymers were evaluated by studying the transfer of the anticancer drug doxorubicin to mouse embryonic fibroblasts cells and the transfer of telomerase antisense RNA (with sequence GCG CGG GGA AAA GCA) and a 4.2 kb plasmid containing the green fluorescent protein gene into the same cells. Best results were obtained using the high molecular weight polymer fraction. This result proves that this polymer is a very promising delivery system which can be used for those anticancer drugs with serious side effects and also successfully bring gene or plasmid which is hardly enter the cells by themselves before.

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Thanks to all my friends both in USA and in China, you always make my life so exciting. Last but not least, I want to say thanks to my family for their love always makes me full of confidence and strength.

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Chapter 1 Literature review

1.1 Introduction

Beginning with the botanical phase of early human civilizations, through the synthetic chemistry age in the middle of 20th century, and finally to the biotechnology era at the dawn of the 21st century, drug research has evolved and matured[1, 2, 3]. In spite of this progress, it is still common to find someone who has suffered the pain of losing a friend or relative because of an incurable infection or cancer. Thus, scientists still search for better therapeutics for human beings. Some may think better medical treatment means stronger medicine; however the effectiveness of drugs strongly depends on how drugs are introduced into the human body. For example, many anti-cancer drugs effectively kill cancer cells, but without an appropriate carrier to sequester them before they are released to the cancer site, the drugs can be very toxic to human beings. The term "drug delivery system" (DDS) refers to both the methods of administration and the delivery vehicles, which are as significant as the pharmacological activities of the drug itself.

Drug delivery is becoming an increasingly important field in the pharmaceutical industry. It strongly affects patient compliance, cost efficiency and the development of new drugs.

Patient compliance

When people fail to comply with medication regimen, they mainly complain about the inconvenience of taking drugs and their concern about adverse long-term effects[4]. DDSs address three critical issues; the desire of patients to take the lowest number of drug administrations, the patients' favorite administration route, and minimizing side effects (by preventing the exposure of drugs to entire untargeted tissues) with the same efficiency.

These techniques drive the drug delivery market in the most beneficial way, which is estimated to be more than $50 billion worldwide[5].

Cost efficiency

The average development cost of a new drug remains $400-650 million and a time consuming 10-15 years[2]. Facing this risky process, pharmaceutical companies are under constant pressure to maximize the full potential of a drug candidate at an early stage of its life cycle. Fortunately, this aim can be accomplished by incorporating the drug into different kinds of drug delivery systems, which lead to extended drug patent life and more convenient dosage forms that overcome the administration problems. Specifically, a new DDS only costs around $80-130 million and requires less than 5 years of development allowing pharmaceutical companies to make the best use of their investment. DDSs give old drugs a second life with increased efficiency and patient satisfaction. For example, Cardizem DDS [6], a simple once-daily form of deltiazem had $4 billion in sales after the native composition of matter patent expired.

Development of new drugs

Developments in genetics, immunology, biochemistry, molecular biology, and information technology have made it possible to decipher the whole human genome. Meanwhile, the focus of therapeutics has moved from mainly symptomatic treatment to curing and preventing the cause of disease. Therefore, gene delivery has become a promising therapeutic method, as well as a new challenge, since larger molecules such as antisense DNA/RNA or plasmids pose greater problems in drug absorption and distribution.

What are ideal DDSs?

1. Target function

Optimal DDSs sequester and carry drugs efficiently to the appropriate part of the body (some times even to particular cells and/or a special organelle in the cell). Without the protection afforded by a DDS, the immune system, the reticuloendothelial system (RES) and a variety of enzymes can easily break, degrade, and clear drugs when they circulate within the human body. Even if the drugs are not functional during blood circulation, they still should be enveloped to prevent harmful side effects to normal tissues. The required DDS must be sufficiently inert, biocompatible, and mechanically strong. After being modified by a specific antibody or biochemical agent, the ideal DDS will only release drugs at the specific site.

2. Intelligent release

Some polymer materials are called 'smart' DDSs because they are self-regulated. They keep the drug sequestered and release it only when triggered by abnormal /disease signals (stimuli) from the human body.

3. Programmable release

One kind of DDS carries several different chemical reagents into the human body at one time and when prompted by either physician or the patient releases them selectively in a controllable manner.

4. Simple to administer

The methods of administration should be comfortable and convenient for patients.

5. Sustainable and constant release

Both patients and physicians wish medicine could be given the minimum number of times.

Ideal DDSs not only are capable of high drug loadings, but also release the drug at a controlled and constant rate to maintain the concentration in the blood within a desired range for an extended period of time. (See figure 1.1) Depending on the formulation and the application, this time may be anywhere from 24 hours (Procardia XL) to one month (Lupron Depot) to five years (Norplant)[7].

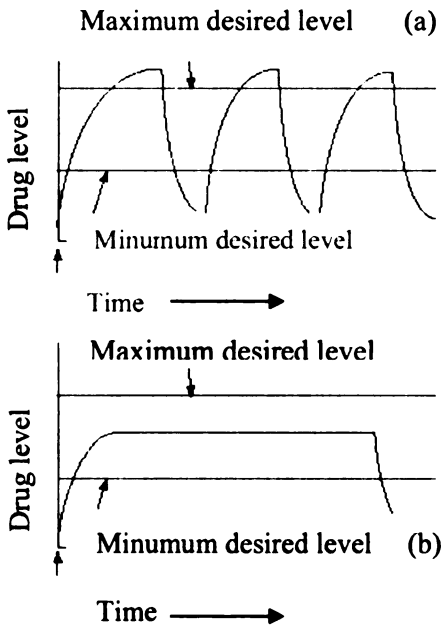


Figure 1.1 Drug levels in the blood with
(a) Traditional drug dosing
(b) Controlled-delivery dosing

Figure is adapted from Ref [7]

6. Biodegradability

After their delivery, DDSs are broken down into biologically acceptable molecules that can be absorbed or removed via normal metabolic pathways without adverse effects to human body.

7. Easy to fabricate and sterilize

In addition to having all these desirable properties, DDSs should be easy to produce and process. Therefore, if we find a simple platform for DDS synthesis that effortlessly produces a series of DDSs for different drugs, the goal will be accomplished.

Those issues are not trivial and they are as complex as drug discovery.

In summary, drug delivery is becoming a core technology that always operates alongside earlier aspects of drug development.

1.2 Administration methods

As early as four thousands years ago pills, ointments, and salves were employed by Egyptian physicians. In 1665, intravenous injections were first performed in humans. Wood introduced subcutaneous injections in 1853. Luer developed the modern hypodermic syringe in 1884. Since then, a large variety of administration methods have evolved [1]. Saltzman reviewed the current administration methods shown in Table 1.1[1].

Table 1.1 Common routes of drug administration

Route of Administration	Example	Advantages	Disadvantages
Intravenous injection (i.v.)	Antibiotics for sepsis	100% bioavailability	Discomfort to patient Requires health care-provider Risk of overdose or toxicity Risk of infection
Intravenous infusion	Heparin for anti-coagulation	100% bioavailability Continuous control over plasma levels	Requires hospitalization Risk of infection
Subcutaneous injection (s.c.)		Usually high bio-availability	Discomfort to patient
Intramuscular injection (i.m.)	Insulin for diabetes	Usually high bio- availability	Discomfort to patient
Oral (p.o.)	Many	Convenient Self-administered	Drug degradation before absorption Limited absorption of many drugs
Sublingual or buccal	Nitroglycerin for angina	Avoids first-pass metabolism in liver	Limited to lipophilic, highly potent
Ophthalmic	Pilocarpine for glaucoma	Local delivery Self-administered	Discomfort to some patients for frequent administration

Topical	Antibiotic ointments	Local delivery Self-administered	Limited to agents that are locally active
Intra-arterial injection		Control of vascular delivery to specific regions	High Risk
Intra-arterial injection		Direct delivery to brain	Limited drug penetration into brain tissue
Rectal		Avoids first-pass metabolism in liver; Self-administered	Discomfort leads to poor compliance in some patients
Transdermal	Nitroglycerin patches for angina	Continuous, constant delivery Self-administered	Skin irritation Limited to lipophilic, highly potent agents
Vaginal	Spermicides	Self-administered	Discomfort leads to poor compliance in some patients
Controlled-release implants	Norplant for contraception	Long-term release	Requires surgical procedure

* Table is taken from Ref [1]

The aim of any drug administration methods is always to combine the tissue absorption and distribution of the drug with patients' compliance in a maximum beneficial way. Among them, oral dosage forms are always preferred since they are painless, uncomplicated and self –administered. However, DDSs intended for oral administration require more consideration of gastrointestinal physiology. Otherwise, most drugs will be easily degraded within the gastrointestinal tract (GIT) or cleared by macrophage (Kupffer cells) of the liver and spleen, and not absorbed in sufficient quantity to be effective. Therefore, protein pharmaceuticals are mostly administered by parenteral delivery in order to quickly achieve the efficient concentration in the blood, while avoiding the "first

pass elimination". This occurs when drug molecules enter the circulation through a mucosal surface, and then circulate through the liver, where they can be metabolized, before distribution throughout the rest of the body[1]. However, frequent parenteral injections are poorly accepted by the elderly and children, plus they are not well supplied to some undeveloped countries and urban areas.

Besides parenteral and oral delivery methods, there are other modes of administration, such as transdermal delivery, inhalation delivery, sublingual delivery, rectal delivery, topical delivery, etc. Each delivery scheme has special attributes. See figure 2.1[8]. Inhalation delivery is fit for treating diseases of the lungs and respiration system, and it requires that the DDS be dispersed as well as the drugs. Sublingual delivery is usually used for acute cardiac dysfunction because it is faster than oral delivery, convenient for patients, and it requires the DDS to protect drugs from degradation by saliva enzymes. Topical delivery is more efficient than systemic delivery since a drug can be administered directly to the target organ or tissue. In all of the various regional deliveries, the design of the DDS always needs to take into consideration the environmental situations where the drugs will be released, such as pH, enzymes, and different barriers for further absorption and distribution in the body.

The drug concentrations in the blood realized by these administrations are different over time because all need to be absorbed (except in the case of intravenous injections) before entering the blood. It is therefore useful to know that the rate of absorption by these administration methods from the fastest to slowest is: inhalation, sublingual, rectal, intramuscular, subcutaneous, oral, and transdermal. Thus, delivery of identical amounts of drug to tissue sites with different administration routes

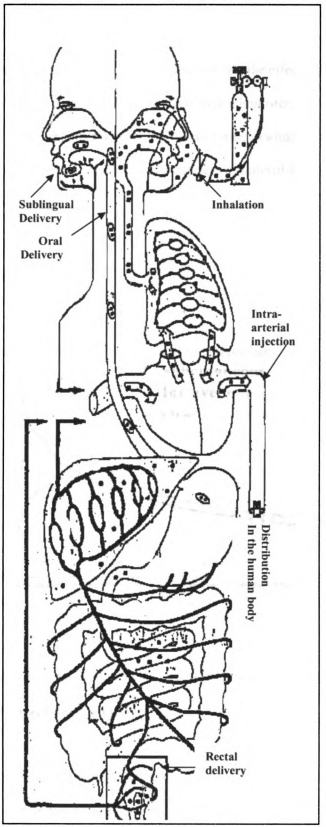


Figure1.2 Drug Delivery Methods *Figure is adapted from Ref [8]

can result in measurable differences in the drug concentration in the blood within the same period of time. In addition, the route of drug administration will influence the kinetics of biodistribution and elimination and thus the effectiveness of the therapy. For example, human immunoglobulin G (IgG) were administered orally, subcutaneously, intramuscularly, and intravenously to human patients (without delivery systems) (Figure 1.3)[9]. These different methods led to different patterns of IgG concentrations in the plasma over time.

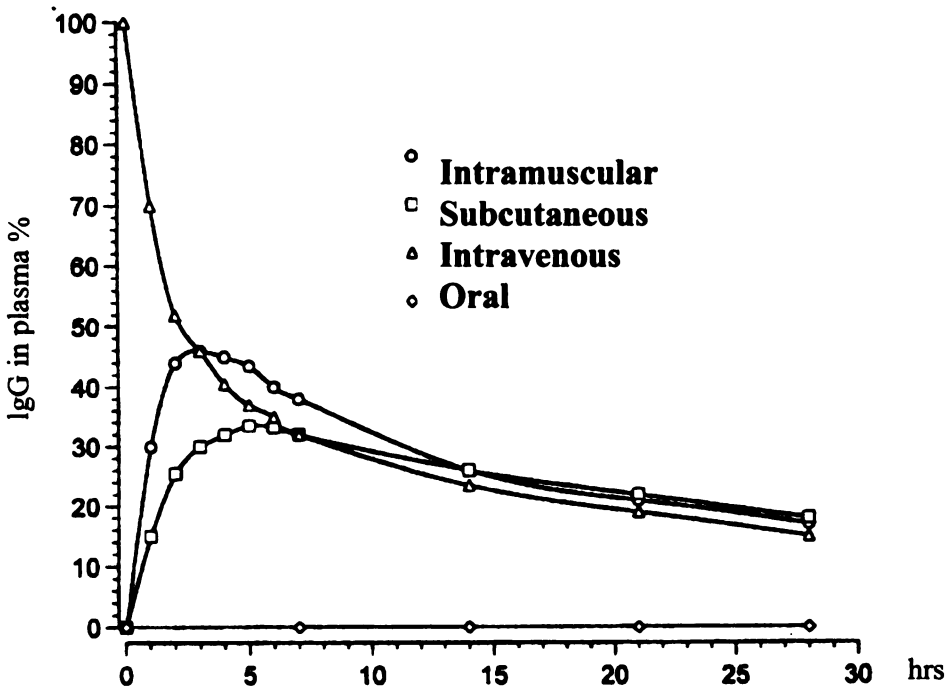


Figure 1.3 Plasma IgG following different administration routes in humans

*** Figure 1.3 was taken from Ref [1]**

Figure 1.3 shows that without the protection of a DDS, the IgG cannot be delivered orally. Although the intravenous injections give the highest initial drug concentration in the blood, the concentration decreases sharply during the first 5 hours. With an ideal DDS the

delivery could be accomplished in a controlled manner; the initial drug concentration is not too high (nontoxic), and also the rate of drug release is kept constant. Subcutaneous and intramuscular injections show smoother changes in drug concentration in the blood, which is more like controlled delivery. However, DDSs are still needed to help improve the rate at which the drug concentration in the blood reaches the efficient level.

Table 1.2 Methods for Hormones Delivery (*Adapted from Ref [10])

	Advantages	Disadvantages
Injection	Less liver stress than oral delivery. Inexpensive	Less steady hormone level. Pain and slight infection risk from hypodermic needle usage.
Oral	Convenience. Possibly more beneficial for blood cholesterol levels than other methods.	Increased stress on the liver since it has to process the hormones multiple times, resulting in an increase in clotting factors.
Transdermal film	Less liver stress than oral delivery. Hormone level more steady than injections.	Inconvenience and skin irritation. Multiple simultaneous patches required for pre-op dosage. Expensive.
Sublingual/ Buccal	Uncoated tablets can be placed under the tongue or between the cheek and gum. Less liver stress than oral delivery.	Some is also dissolved in the saliva and swallowed. The taste is not good.
Cream, Suppositories, and Pessaries	Less liver stress than oral delivery.	Absorption through a mucosal membrane is best; absorption through scrotal skin is not as good as mucosal, but better than through other skin (need more data about typical doses and absorption).

A medicine commonly has a number of delivery methods, which provides patients more opportunities to get the most suitable administration method, such as the delivery of hormones (see table 1.2). Therefore, it becomes more critical for scientists to develop DDSs that can be used for different administration routes with the lowest side effects and the same therapeutic efficiency.

1.3 Vehicles of drug delivery

1.3.1 Inorganic vehicles

In the beginning of drug delivery development, inorganic materials were unnoticed because of their lower biocompatibility. However, the excellent adsorption properties of calcium hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$[11,12] and activated charcoal[13,14] have made them successful carriers of antitumor agents for the local treatment of metastatic lesions of lymph nodes after surgical removal of the main tumor, and for the treatment of solid tumors by inhibiting cancer sell growth (Dunn osteosarcoma cells).

Another exciting development of inorganic materials for DDSs is the use of microchips,[15] as controlled drug–delivery devices. (See figure 1.4) This patented technology is based on tiny silicon chips containing hundreds of micro–reservoirs capped by noble metal membranes that open when electronically activated. Each reservoir can be filled with any combination of bioactive agents and hermetically sealed to protect the contents. Complex biochemical delivery can be achieved by opening the reservoir caps on demand in response to a preprogrammed clock, biosensor feedback, or a wireless signal from a physician or patient. This product is still waiting for the evaluation *in vivo*. However, the versatility of the technology offers tremendous potential for future development.

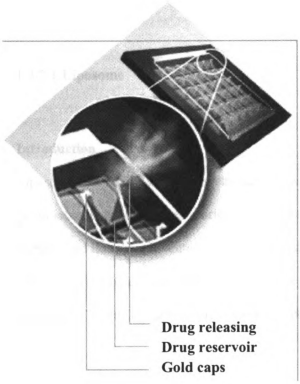


Figure 1.4: MicroCHIPS Technology (taken from Ref [15])

FeRx Corporation [16] is developing magnetically targeted systems, which make use of elemental iron magnets with drug adsorbed to the surface of carriers. Carriers that localize within tumors because of the applied magnetic field are undergoing trials for the treatment of metastatic liver cancer. Although research on DDSs still focus mainly on organic material carriers, in the future, it is believed that inorganic materials will get greater attention from drug developers.

1. 3. 2 Organic vehicles

1.3.2.1 Liposome

Introduction

Liposomes are colloidal, vascular structures based on (phospho) lipid bilayers which can be unilamellar or multilamellar oriented around an aqueous core. Since Gregoriadis, Ryman, and Bangham invented the liposome drug delivery systems (LDDS) in the early 1970s[17-20], liposomes have been under extensive investigation as DDS for a broad spectrum of agents including drugs, antibodies & antigens, genetic materials, etc.

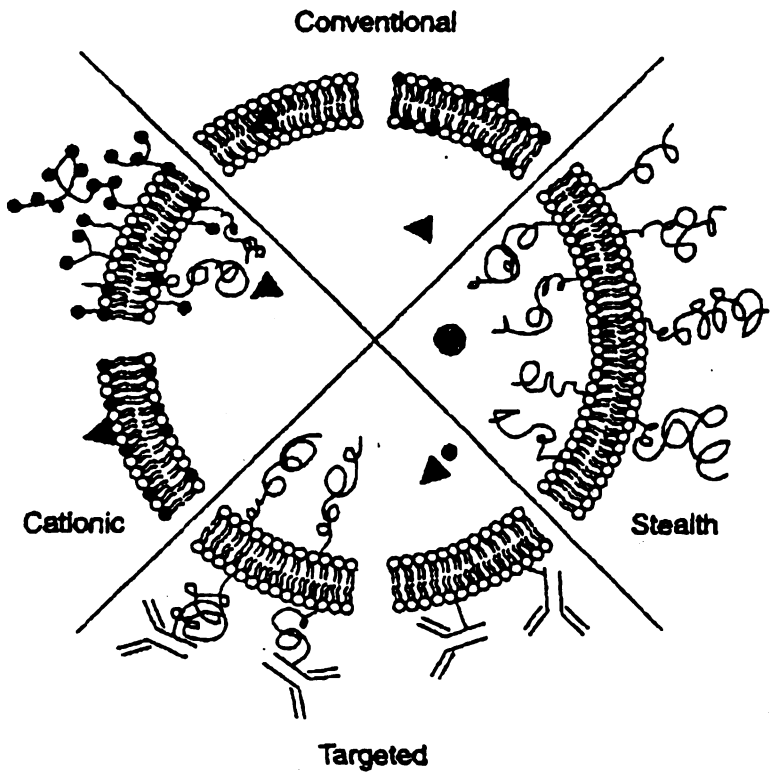


Figure 1.5 Classifications of Liposomes (adopted from Ref [21])

As drug delivery materials, liposomes may solubilize lipophilic drugs that would otherwise be difficult to administer intravenously. Through interaction with cells in various ways, liposomes can be successfully used for passive and active target delivery with additional surface modifications. Liposomes not only protect encapsulated drugs from degradation by metabolizing enzymes, but also prevent the non-targeted part of the body from being exposed to the full dose of drugs.

Classification of Liposomes

There are four major liposome types that can be broadly distinguished on the basis of composition and *in vivo* application [21] (See Figure 1.5). These four classes include: conventional liposomes, long-circulating liposomes, immunoliposomes, and cationic liposomes which will be described below.

Conventional liposomes refer to the liposomes composed of only phospholipids (neutral and/or negatively charged) and/or cholesterol. Such non-surface modified liposomes are characterized by a relatively short blood circulation time because they either disintegrate in the blood stream or circulate and then are picked up predominately by macrophages (Kupffer cells) in the spleen and liver. According to this behavior, conventional liposomes are good candidates for the delivery of antimicrobial agents to infected macrophages.

Long-circulating liposomes or stealth liposomes are produced by covalently attaching a hydrophilic polymer, polyethylene-glycol (PEG), to the outer surface. PEG-modified lipids prevent plasma protein adsorption to the liposome surface and thus the subsequent recognition and uptake by RES[22]. It also reduces the opportunities for attack of multiple reactive groups by shielding the membrane surface of lipids[23-29]. This protection leads to a

longer circulation time, which consequently enhances the opportunity for liposomes to take advantage of "the leaky endothelium effect" attributed to the tumor site and inflammatory sites and leave the vascular system.

Immunoliposomes have specific antibodies or antibody fragments covalently attached to their surfaces to enhance target site binding. With the help of PEG coating, immunoliposomes are given a greater chance to reach targets other than macrophages. In addition to antibodies, glycolipids, proteins and vitamins have also been used to target specific cells via cell surface receptors. Moreover, immunoliposomes can be used for antibody-directed enzyme pro-drug therapy (ADEPT) designed to generate a high concentration of anticancer molecules only in close proximity to tumor cell membranes.

Cationic liposomes represent the pioneering form of liposome drug delivery systems. The cationic lipid components interact with, and neutralize, the negatively-charged DNA, thereby condensing it into a more compact structure. The resulting lipid-DNA complex, rather than DNA encapsulated within liposomes, offers the protection and improves cellular internalization and expression of the condensed plasmid.

Requirements of liposome gene delivery system *in vivo* [30]

1. Liposomes should be targeted to endocytic receptors to increase the rate of endocytosis.

2. Fusion processes should be optimized to enable efficient escape from the endosome and entry into the cytoplasm.

3. Cytoplasmic stability and nuclear targeting of the plasmids should be ensured.

Mechanism

Liposome encapsulation can alter the spatial and temporal distribution of drugs in the body, which may significantly reduce unwanted toxic side effects and increase the efficacy of the treatment. These exciting drug delivery applications result from the physicochemical and colloidal characteristics as well as their biological interactions with the cells. Liposomes resemble cell membranes in their structure and composition and have four major interactions with cells[17].

1. ***Lipid exchange*** involves the exchange of liposome lipids for the lipids of various cell membranes and depends on the mechanical stability of the bilayer.

2. ***Adsorption onto cells*** occurs when the attractive forces exceed the repulsive forces.

3. ***Endocytosis*** delivers the liposome and its contents into the cytoplasm indirectly via a lysosomal vacuole in which low pH and enzymes may inactivate the encapsulated agent.

4. ***Fusion*** is the process in which the liposomes' contents are delivered directly into cells as the liposomal lipids merge into the plasma membrane.

Problems of liposomal DDSs

There are still some difficulties[21, 31] in the development of liposome drug delivery systems (LDDS). First of all, the qualities of the raw material (phospholipids) such as phosphatidylcholine (PC), phosphatidylglycerol (PG), and phosphatidylethanolamine (PE) are poor. They all have a source-dependent composition of acyl chains. Ester hydrolysis and peroxidation also make their quality vary considerably. Because liposome behavior *in vivo* strongly depends on size, bilayer rigidity, charge and morphology, poor

characterization of the physicochemical properties of the liposomes becomes a more serious problem. Further, low drug-loading capacities and leakage, which leads to poor stability, are serious problems for DDSs.

1.3.2.2 Polymer vehicles

The combination of polymer science with pharmaceutical science has led to a quantum leap in design and development of novel drug-delivery systems. Bioadhesive polymers were first used to improve the residence time and efficacy of the DDS through their intimate contact with the epithelial cell layer. Biodegradable polymers were also recognized as capable of accomplishing drug delivery functions without surgical removal of delivery materials. Now 'smart' hydrogel break-throughs have launched a promising field of self –regulated drug delivery in response to an environmental stimulus. Compared with other materials used for DDSs, polymers have great advantages for achieving either temporal or spatial control of drug delivery[32-36].

Polymeric materials used in DDSs can be naturally occurring, synthetic or a combination of both. The main classes of natural polymeric materials used in DDSs are summarized in Table 1.3 [33] according to their origin, properties and principal applications. Although naturally derived polymers are abundant and usually biodegradable, their structural complexity often makes modification and purification difficult. In addition, significant batch-to-batch variations occur because of their bio 'preparation' in living organisms (plants, crustaceans).

Table 1.3 A summary of the main properties of applications of natural polymers in DDSs.		
Polymer	**Main applications and comments**	**Refs**
Proteins and protein-based polymers:	Absorbable, biocompatible, nontoxic, naturally available.	
Collagen	Used as drug delivery micro-spheres.	37
Albumin	Used in cell and drug micro-encapsulation.	38
Poly (amino acids):	Nontoxic, nonantigenic and biocompatible.	39
Poly (α, L-lysine) Poly (α, L-glutamic acid) Poly (aspartic acid)	Used as oligomeric drug carriers.	40,41
Polysaccharides and derivatives:		
Carboxymethyl cellulose	From vegetable sources, pH responsive and water-soluble.	39,42
Sodium Carboxymethyl cellulose	Widely used in oral and topical pharmaceutical formulations because of its viscosity–increasing properties. Also used as a tablet binder and to stabilize emulsions.	43,44, 45
Alginate	From marine sources, algae, excellent gel-formation properties; relative biocompatibility; microstructure and viscosity are dependent on the chemical composition (batch to batch) variations. Used for controlled release of bioactive substances, injectable microcapsules for treating neurodegenerative and hormone-deficiency diseases.	46,47
Carrageenan	Excellent thermoreversible properties. Used for microencapsulation.	48
Dextran	From human and animal sources. Excellent rheological properties. Widely used as sustainable DDSs, particularly for injectable and colon–specific DDSs.	49,50
Chitosan	Biocompatible, nontoxic, excellent gel-and film-forming ability, good absorption-enhancing, natural polycation, pH-responsive, controlled release as well as bioadhesive properties. The degree of deacetylation and derivation with various side chains can be a source of manipulation for specific drug-delivery applications (e.g., gels, membranes, micro-spheres).	51,52

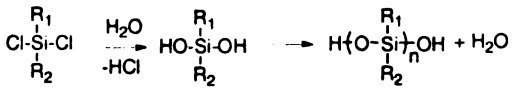
Table is from Ref. [33]

Synthetic polymers overcome many of the limitations of natural polymers. They are available in a wide variety of compositions with readily adjusted properties. Additionally, processing, copolymerization and blending provide a simultaneous means of optimizing a polymer's delivery properties. The following is a representative list of synthetic polymers used in DDSs. It is mainly divided by biodegradable polymers and non-biodegradable polymers [33, 35, 36, 53].

Non-biodegradable polymers

Silicones

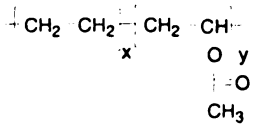
Silicones, mostly referred to as polysiloxanes, are a unique class of non-deformable polymers possessing good low-temperature flexibility, remarkable biocompatibility, excellent electrical properties and water repellency features that are not common with hydrocarbon polymers. They are usually synthesized by hydrolysis of alkylsilicon or arylsilicon halides[53]. Chemical modification commonly involves introducing constituents in place of one or both of the pendent methyl groups in the structure shown below:



Because of their ease of fabrication and high permeability, polydimethyl siloxanes (PDMSs) are used for water-soluble drugs and for the delivery of steroids through long-acting DDSs such as subdermal implants and intravaginal systems. In addition, dimethylsilicones can be copolymerized with methyl methacrylate and ethylene oxide to create a series of polymers with controlled permeability to hydrophilic or hydrophobic steroids, enhanced mechanical properties, and improved adhesion to tissues[54-56].

Poly [ethylene-*co*-(vinyl acetate)] (EVAc)

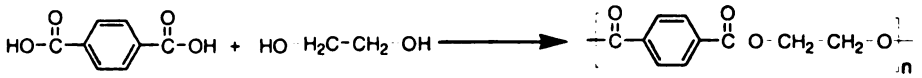
The most commonly used copolymer contains 40% vinyl acetate and has the general structure:



The copolymer is synthesized by free–radical polymerization of vinyl acetate and ethylene. Since EVAc is one of the most biocompatible polymers that has been tested as implant materials [57], it has been widely used as a matrix for controlled drug delivery.[58, 59] As stimuli-responsive hydrogels, EVAc can be used to deliver drugs using different environmental stimuli such as magnetic fields, ultrasonic radiation and changes in glucose concentration [60-62].

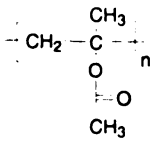
Poly(ethylene terephthalate)

Commonly used linear polyester is poly(ethylene terephthalate) (PETE), which is synthesized by condensation of ethylene glycol and terephthalic acid:



Acrylic polymers:

Poly(methyl methacrylate) (PMMA) is the one of the most popular acrylic polymers, and is prepared in large quantities commercially using free-radical polymerization. PMMA has exceptional transparency and physical strength (structure as follows).

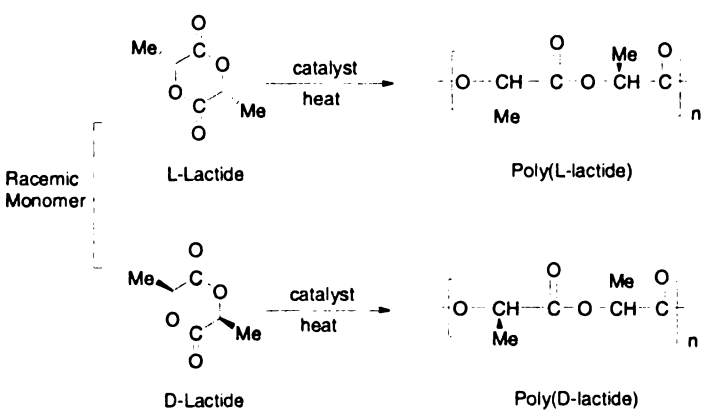


Biodegradable synthetic polymers

Aliphatic polyesters

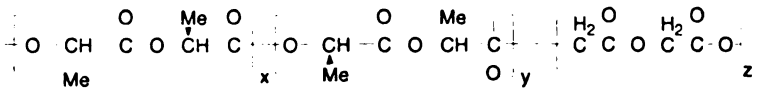
Polylactide (PLA)

Lactic acid exists as two optical isomers, D and L. L-lactide is the cyclic dimmer of naturally occurring isomer, and DL-lactide or meso lactide is the synthetic blend of D-lactide and L-lactide. The homopolymer of L-lactide (LPLA) is a semicrystalline polymer with high tensile strength and slow degradation time. Poly(DL-lactide) (DLPLA) is an amorphous polymer exhibiting a random distribution of both isomeric forms of lactic acid, and accordingly low tensile strength and more rapid degradation time[36, 53].



Poly(lactide-*co*-glycolide) (PLGA)

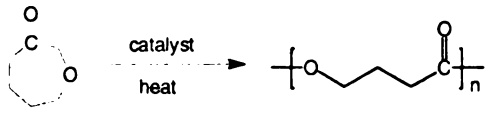
Copolymers of glycolide with both L-lactide and DL-lactide have been developed for biodegradable drug delivery systems. Adjusting the percentage of monomers can be used to regulate the degradation time of copolymers (PLGA). Although PLGA represents the 'gold standard' of biodegradable polymers (exemplified by more than 500 patents), the disadvantage of PLGA is that increased local acidity due to degradation can lead to irritation at the site of polymer application[41]. Moreover, the increased local acidity may be detrimental to the stability of protein drugs[35].



Poly(L-lactide) Poly(D-lactide) Poly(Glycolide)

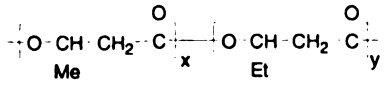
Poly(ε-caprolactone)

The ring opening polymerization of ε-caprolactone yields a semicrystalline polymer with a melting point of 59-64 °C. The polymer has been regarded as a tissue compatible and biodegradable DDS. Copolymers of ε-caprolactone and DL-lactide have yielded materials with more rapid degradation rates.



Poly(hydroxybutyrate-*co*-hydroxyvalerate)

The poly(hydroxy butyrate) is crystalline and brittle, whereas the copolymers of poly (hydroxybutyrate-*co*-hydroxyvalerate) are less crystalline, more flexible, and easier to process. These polymers typically require the presence of enzymes for biodegradation but can degrade in a range of environments and are under consideration for several biomedical applications[36].

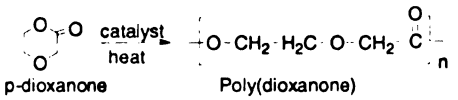


Poly(hydroxybutyrate-co-hydroxyvalerate)

Poly(dioxanone) (a polyether-ester)

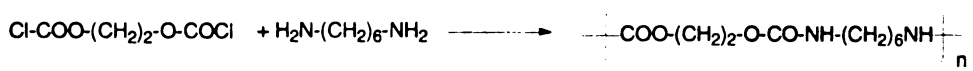
Poly(dioxanone) is synthesized by the ring-opening polymerization of *p*-dioxanone. This material is approximately 55% crystalline, with a glass transition temperature of –10 to 0

°C. The polymer should be processed at the lowest possible temperature to prevent depolymerization back to the monomer. Poly(dioxanone) has demonstrated no acute or toxic effects on implantation and it is re-absorbable after being broken down by hydrolysis[36].



Polyurethanes

Polyurethanes have excellent mechanical properties, making them suitable for many different biomedical applications. The biocompatibility of polyurethanes appears to be determined by their purity, i.e., the effectiveness of removing catalyst residues and low molecular weight oligomers from the polymer[63]. Polyurethanes can be formed by reacting a bis-chloroformate with a diamine[53],

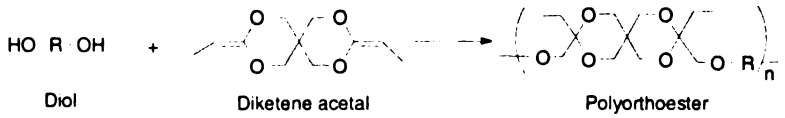


or by reacting a diisocyanate with a dihydroxy compound. For example, ethylene glycol and hexanediisocyanate react as shown[53].

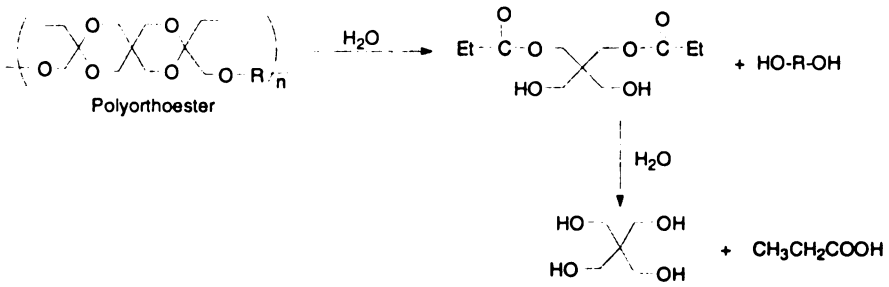
As DDSs, the surfaces of polyurethanes can be surface modified to produce materials that are resistant to thrombosis or that interact with cells and tissues in specific ways[53, 64-69].

Polyorthoester

Polyorthoesters have been synthesized by the addition of diols to diketene acetals. The mechanical properties of polyorthoesters can be readily varied by choosing appropriate diols or a mixture of diols in their synthesis[70].



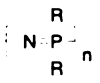
The degradation mechanism of polyorthoester is shown below.



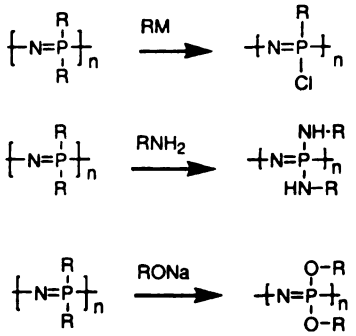
Degradation rates of polyorthoesters can be controlled by incorporating of esters of short-chain alpha-hydroxy acids such as esters of glycolic acid, lactic acid or glycolic-*co*-lactic acid copolymer into the polymer chain and by variation of the amount of these esters relative to the polymer as a whole[35, 53]. At room temperature, polyorthoesters can be made as an ointment, which is appropriate for a variety of topical and periodontal applications. Polyorthoesters can also be obtained as a viscous liquid at room temperature. Proteins and other labile molecules can be mixed into the polymer, as well, without using solvents or increasing temperature[53].

Phosphorous-based polymers

Polyphosphazenes have the general structure



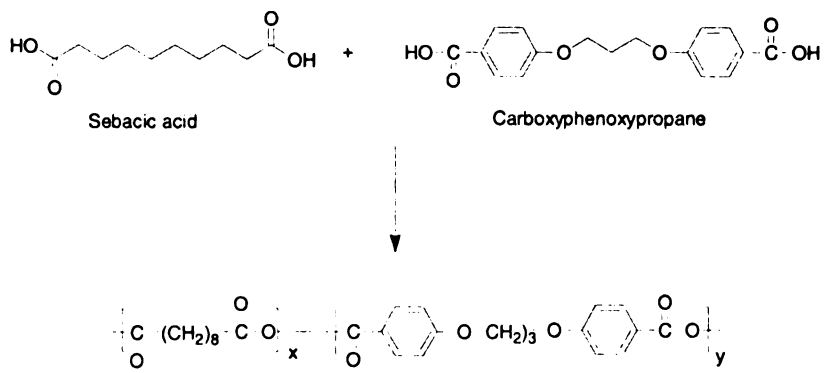
Polymers with a variety of physical, chemical, and biological properties can be produced by performing substitution reactions on the base polymer, poly(dichlorophosphazene)[53]:



This basic structure provides for considerable flexibility in the design of biomaterials, by selection of the side groups on the polymer chain[71]. The resulting polymers can be hydrophilic, hydrophobic or amphiphilic. In addition, a variety of bioactive compounds can be linked to the backbone to make multifunctional DDS.

Polyanhydrides

Polyanhydrides are characterized by their excellent biocompatibility and fast degradation through hydrolysis. They can be synthesized via the dehydration of diacid molecules through melt polycondensation as is the case with poly(sebacic acid)[53]. By selecting appropriate monomers with different degrees of hydrophobicity, the rate of drug release can be controlled from days to weeks. For example, copolymers of sebacic acid, a hydrophilic monomer, with carboxyphenoxypropane, a hydrophobic monomer can be made into a controlled drug delivery system. By adjusting the monomer ratio during copolymerization, the degradation of the resulting polymer can be modulated in a controlled manner[53, 72, 73].



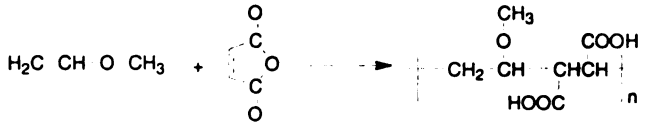
Sebacic acid

Carboxyphenoxypropane

1, 3-Bis (p-carboxyphenoxy) propane

Copolymers of methyl vinyl ether and maleic anhydride

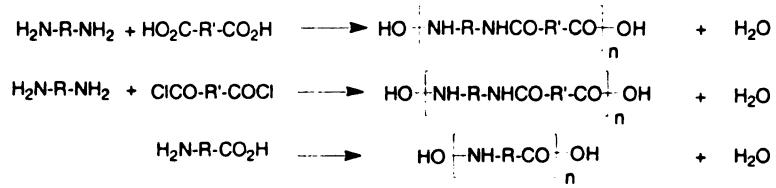
Most materials such as biodegradable polyesters erode in a disorderly pattern: defects, cracks, and holes that initially appear grow in size with time throughout the material. To provide better control of polymer matrix erosion, materials that erode homogeneously have been designed. In particular, for materials that erode from the surface only, the kinetics of dissolution and the release of incorporated drugs can be precisely controlled. A copolymer of maleic anhydride and methyl vinyl ether was synthesized for this goal. The following is obtained after partial esterification[53]:



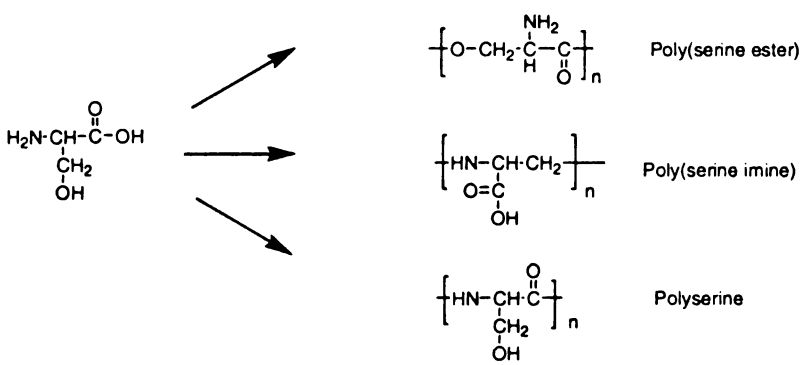
When the copolymer was placed in an aqueous environment, the carboxylic acid groups on the polymer-water interface become ionized, thus the erosion of polymer is limited to the polymer surface, and the rate of erosion strongly depends on pH. (The rate increases when pH drops). The disadvantage of this DDS is the erosion products are macromolecules and not easily metabolized or excreted by the body[53].

Polyamides

A common strategy in the design of biodegradable polymers for medical applications is to use naturally occurring monomers, with the hope that these polymers will degrade into non-toxic components. For example, poly(lactide-*co*-glycolide) degrades into lactic and glycolic acid, normally occurring metabolites. Thus, amino acids are an obvious choice as monomers for the production of polymeric biomaterials. Three conventional synthetic methods are shown below,



Poly(amino acid) have good biocompatibility for the delivery of low molecular weight compounds. Unfortunately, amino acids polymerized by conventional methods usually yield materials that are extremely antigenic and exhibit poor mechanical properties which make them difficult to process[41]. Because of their high crystallinity, the degradation of pure poly(aminoacids) is relatively slow[35, 41]. To circumvent these problems, several approaches have been developed. A few amino acids, like glutamic acid and lysine, can be modified through their side chains to produce polymers with different mechanical properties. Copolymers of L-glutamic acid and γ-ethyl L-glutamate have been used to release a variety of drugs, and the variation of the ratio of monomers in the polypeptide influences the rate of degradation of the resulting polymers[74]. Due to the stability of the peptide bond in water, the biodegradation of these implants occurs by dissolution of intact polymer chains and subsequent enzymatic hydrolysis in the liver. Alternatively, amino acids can be polymerized by linkages other than the conventional peptide bond, yielding pseudopoly(amino acids)[75]. For example, the amino acid serine can be used to produce poly(serine ester), poly(serine imine), or conventional polyserine[53]:



Dendrimers (PAMAM)

Relative newcomers to the collection of biodegradable materials used for DDSs are dendrimers, a type of highly branched macromolecule. The name "dendrimer" is derived from the ancient Greek word "dendron" (tree), and from the Greek suffix "-mer" (segment).

Dendrimers consist of a series of chemical shells built on a small core molecule. The synthesis begins with a simple seed molecule such as ethylene diamine and ammonia, which normally has two or three reaction sites. With an excess of the first monomer molecule reacting with all of the reaction sites of the seed molecule, the first branches are raised. This first monomer molecule has two distinct reactive groups, one at each end. After one end reacts, the other end will provide reaction sites for the next layer of the shell [76-79]. For example, polyamidoamine (PAMAM) dendrimers are synthesized from an ethylenediamine core with branching units containing tertiary amine and amide functionality. This core is reacted with the double bond in acrylic acid to produce a tertiary-acid molecule. This tertiary-acid is reacted with ethylene diamine to produce a tertiary-amine (G0). This tertiary-amine is reacted with acrylic acid to produce an Oct-acid, doubling the number of acids in this half-generation (G0.5). Next, another round of ethylene diamine is reacted with the G0.5 to give a G1 molecule with Oct-amines, twice the number at G0. This alternation of acrylic acid with ethylene diamine continues until the desired generation is reached. (See figure 1.6)

Dendrimers are like ordinary organic molecules for the first three generations. By G4 they are beginning to have a preference of three-dimensional structure and to become spherical. By G5 they have a consistent and specific three-dimensional structure. Then they are highly structured spheres[76]. The spherical surface of a dendrimer acts like a microscopic form of Velcro, and a variety of bioactive agents can bind to the surface.

Dendrimers have a high drug-carrying capacity because of their multivalency[76-79]. The consistency of structure makes dendrimers ideal building blocks for creating biologically active nano-materials, which can target structures less than 5 nm in diameter. This provides an excellent drug delivery system that can get through vascular pores and into tissue more efficiently than larger carriers[76]. Another advantage of dendrimers is that their synthesis results in a single molecular weight rather than a distribution of sizes, which is critical for controlled DDSs[77]. So far, all the excellent properties of dendrimers make them promising materials for controlled drug delivery systems[77].

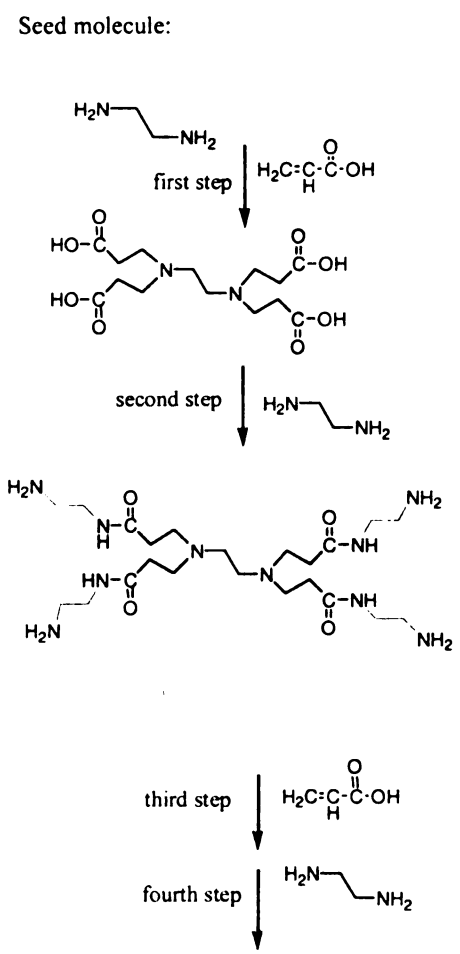


Figure 1.6 Synthesis of PAMAM Dendrimer (I)

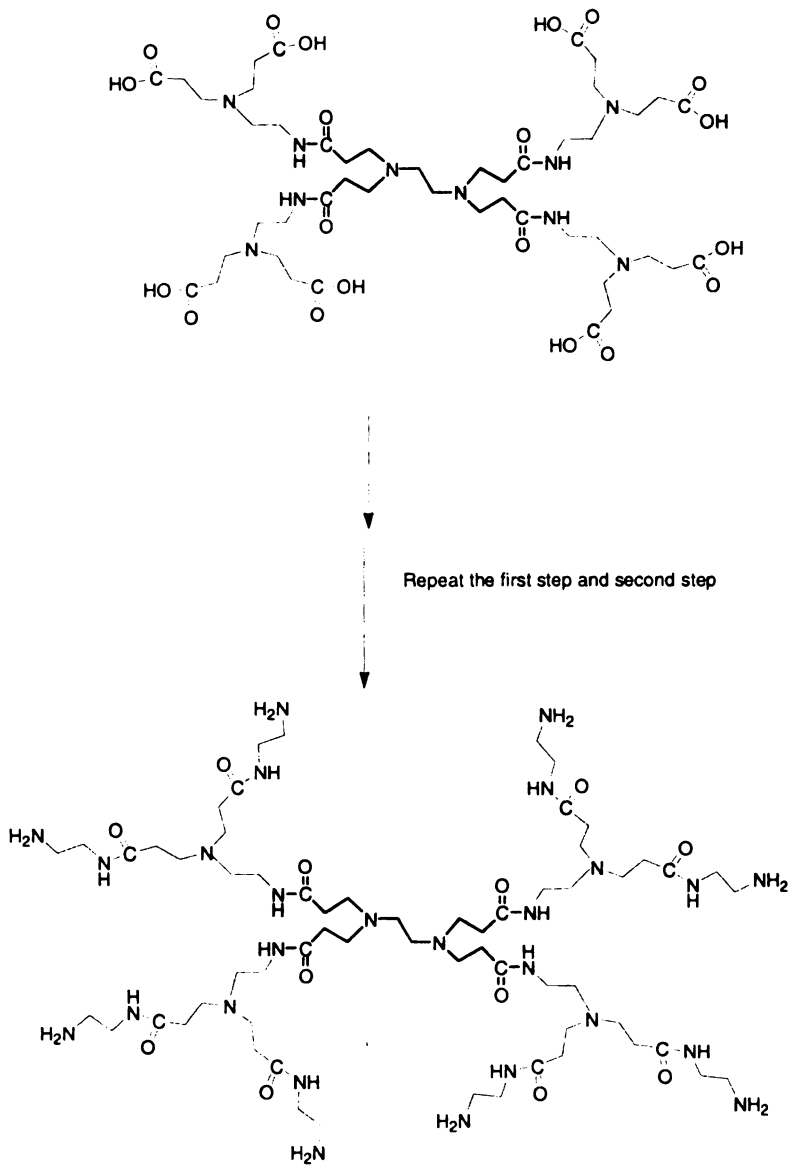


Figure 1.6 Synthesis of PAMAM Dendrimer (II)

Factors which affect degradation of polymers

A great deal of attention and research effort is being concentrated on biodegradable polymers because biodegradable polymers provide a better opportunity for controlled drug delivery without the concerns of removal of the extraneous delivery system. However, measuring the degradability of polymers is not a simple question since there are so many factors to be considered[34]. These factors include

Chemical composition and structure

1. Distribution of repeat units in multimers.
2. Presence of ionic groups.
3. Presence of unexpected units or chain defects.
4. Configuration structure.

Physical factors

1. Molecular weight.
2. Molecular weight distribution.
3. Shape and size changes.
4. Variations of diffusion coefficients.
5. Mechanical stress, stress- and solvent-induced cracking, etc.
6. Morphology (amorphous/semicrystalline, microstructures, residue stressed).

Physicochemical factors (ion exchange, ionic strength, pH).

Sites of implantation

Absorbed compounds (water, lipids, ions, etc.).

Mechanism of hydrolysis (enzymes versus water).

As well as the factors mentioned above, the processing conditions (annealing, sterilization, etc.) and storage history also affect the degradation properties of polymers.

Thus, designing controlled–release drug delivery system using biodegradable polymers is still time-consuming.

Mechanisms of drug release from polymeric DDSs

There are four primary mechanisms by which active agents can be released from polymeric drug delivery systems: diffusion, solvent-activated release, degradation, and swelling followed by diffusion. Any or all of these mechanisms may occur in a given release system[34, 80].

Diffusion

Diffusion occurs when a drug or other active agent passes through the polymer. There are two diffusion-controlled release systems, matrix and reservoir, which are shown in Figure 1.7 and Figure 1.8.

In a matrix system (See Figure1.7) a polymer and active agent are mixed to form a homogeneous system. Diffusion occurs when the drug passes from the polymer matrix into the external environment. With this type of system, the rate of drug release normally decreases, since the active agent has a progressively longer distance to travel and therefore requires a longer diffusion time to release.

In a reservoir systems (See Figures 1.8 a and 1.8 b) the drugs (pure or in a dilute or highly concentrated solution) are surrounded by a polymer film or membrane that controls the diffusion rate. Since this polymer coating is essentially uniform and of a nonchanging thickness, the diffusion rate of the active agent can be kept fairly stable throughout the lifetime of the delivery system.

When talking about diffusion-controlled systems, the drug delivery device is understood to be fundamentally stable in the biological environment and does not change its size either through swelling or degradation. In these systems, the combinations of polymer matrices and bioactive agents chosen must allow for the drug to diffuse through the pores or macromolecular structure of the polymer upon introduction of the delivery system into the biological environment without inducing any change in the polymer itself.

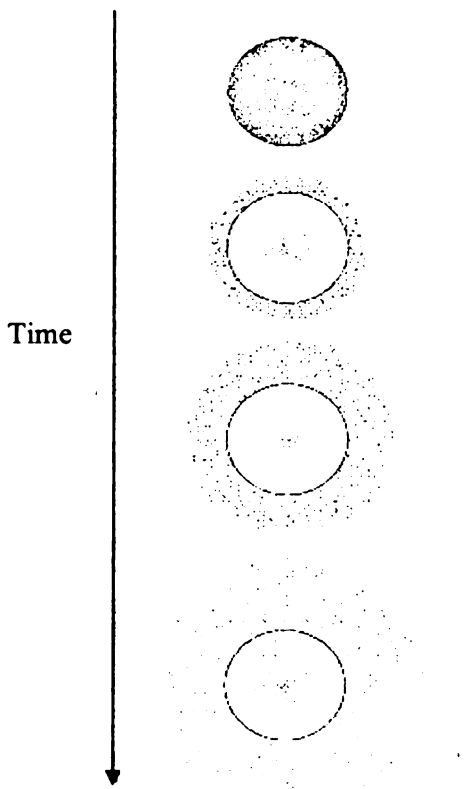


Figure 1.7:
Drug delivery from typical matrix delivery systems.

*Figure adapted from Ref [34]

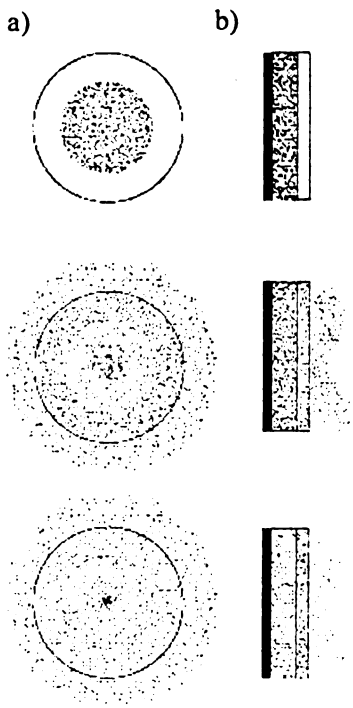


Figure 1.8:
Drug delivery from typical reservoir devices: a. Implantable or oral systems b. Transdermal systems

*Figure adapted from Ref [34]

Solvent-activated

Solvent-activated systems usually employ a semi-permeable membrane containing a small, laser-drilled hole. Within the membrane there is a high concentration of an osmotic agent, either the drug itself or a salt, which causes water to enter through the membrane. The drug is then forced out through the hole because of the increased pressure. Drug release could be kept at a constant rate in solvent-activated systems.

Degradation

There are three types of degradation mechanisms. A) Water-soluble polymers are made insoluble by cross-linking. When the cross-links are broken at some point in the body, the polymer will dissolve. B) Water-insoluble polymers are made soluble by hydrolysis or ionization of side groups. C) Insoluble polymers are broken into smaller soluble molecules with an environmental stimulus. One or all of these mechanisms can be used in degradation of controlled release systems.

There are two forms of degradation, bulk degradation and surface degradation. (See Figure 1.9 a and 1.9 b) Bulk degradation occurs throughout the polymer structure in a rather random fashion[80]. The rate of release is unpredictable, and entire dose dumping can often occur[81]. Surface degradation delivery systems eliminate the problems of bulk degradation systems by using hydrophobic polymers, which contain water-labile linkages. Thus, diffusion of water into the matrix and internal degradation are minimized.[81] Examples include polyanhydrides and polyorthoesters. The degradation occurs only at the surface of the polymer, resulting in a release rate that is proportional to the surface area of the drug delivery system. With proper surface geometry design, zero order degradation kinetics is possible[80].

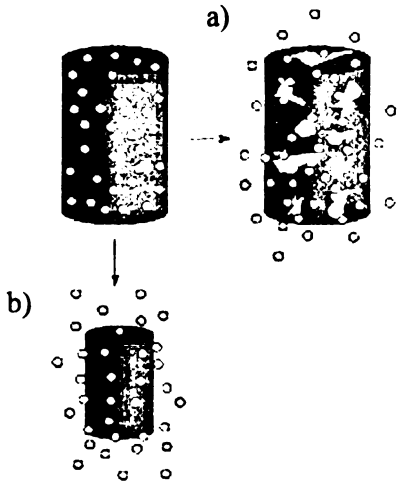


Figure 1.9:
Drug delivery from:
a) Bulk degradation controlled system.
b) Surface degradation controlled system.

*Figure adapted from Ref [34]

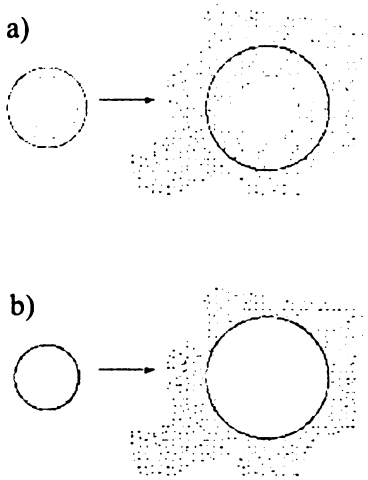


Figure 1.10:
Drug delivery from swelling–controlled release system:
a) Reservoir polymeric DDS
b) Matrix polymeric DDS

*Figure adapted from Ref [34]

Swelling-controlled release systems

Swelling-controlled release systems are initially dry and, when placed in the body, will absorb water or other bodily fluids and swell. The swelling increases the aqueous solvent content within the formulation as well as the polymer mesh size, enabling the drug to diffuse through the swollen network into the external environment[34]. Examples of these types of devices are shown in Figures 1.10 a and 1.10 b for reservoir and matrix systems, respectively.

1.4 Gene therapy

1.4.1 Introduction

Gene therapy is a technique for correcting defective genes responsible for disease development. There are two main approaches for correcting faulty genes, gene addition and antisense delivery techniques.

Gene Addition: A normal gene which is lacking or dysfunctional in patients is inserted into the human body (see figure 1.11).

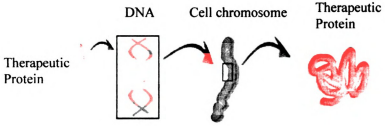


Figure1.11 Gene addition technique (taken from Ref [82]) Images in this thesis are presented in color.

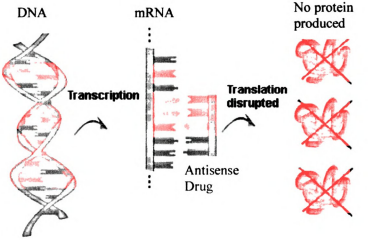


Figure 1.12 Antisense Technology (taken from Ref [82])

Antisense technique: A technology of interrupting mRNA translation by using an antisense strand to hybridize with a specific messenger to block the expression of disease-related genetic code (see figure 1.12).

Actually, gene delivery is the introduction of specific polynucleotides into human cells with the aim of altering the production of a specific protein. The changes in protein expression result in the reduction or elimination of disease[83]. In the future, all diseases including cancer[84], infectious disease[85], vascular disease[86], inflammatory disease[87], neurological disorders [88]as well as inheritable genetic abnormalities[89, 90] have the potential to be cured by this promising strategy.

From the beginning of gene therapy (1990), researchers have mostly used viruses as the gene delivery system[91]. In this method, disease-causing genes are removed and therapeutic genes are inserted into a virus. The virus vector carries and unloads the therapeutic human gene into the target cell to accomplish therapy. However, in 1999, the death of 18-year-old Jesse Gelsinger who was participating in a gene therapy trial for ornithine transcarboxylase deficiency (OTCD) caused gene therapy to suffer its first big setback[104]. Since it is believed that the boy's death was triggered by a severe immune response to the adenovirus carrier, researchers began to notice the high risk of virus delivery methods. Viruses present a variety of potential problems to the patient: toxicity, immune and inflammatory responses, and gene control and targeting issues. Further, there is unavoidable concern that the viral vector, once inside the patient, may recover its ability to cause disease.

Achieving success in gene therapy is not easy. It not only depends on the accurate expression of the therapeutic agent, but also strongly depends on efficient delivery

into target cells, successfully overcoming the subcelluar barriers such as crossing membranes of cells, drugs escaping from lysosomes, and targeting and entering the nucleus[83, 92, 93]. Now it is crucial to develop an improved delivery system for gene therapy since compared with conventional small molecules, polynucleotides exhibit different chemical and physical properties that are not well suited to cell delivery.

1.4.2 Antisense human telomerase RNA (GCG CGG GGA AAA GCA)

An average human chromosome contains a single molecule of DNA of about 150 million nucleotide pairs. The DNA molecules in eukaryotic chromosomes are linear with two ends, which are called telomeres. Telomeres are crucial to the life of the cell[94]. They keep the ends of the various chromosomes in the cell from becoming entangled and sticking to each other, and also assist in the pairing of homologous chromosomes and crossing over during prophase of *meiosis* I. Human telomeres lose about 100 base pairs from their telomeric DNA at each mitosis. With this rate, after 125 mitotic divisions, the telomeres would be completely gone. Therefore, the steady shrinking of telomeres imposes a finite life span on cells. Most cancers arise from somatic cells, but one of the specific features is their ability to divide indefinitely[95]. It turns out that cancer cells have the ability to synthesize telomeres and, thus, to compensate the shortening of their telomeres. The reason cancer cells can be distinguished from normal somatic cells is that they have telomerase, an enzyme that can add telomere repeat sequences to the end of DNA strands during the DNA replication to make cancer cells immortal[96, 97].

Telomerase is a ribonucleoprotein. Its single RNA molecule provides an AAUCCC template to guide the insertion of TTAGGG. Thus telomerase is a reverse transcriptase[98], synthesizing DNA from an RNA template. The sequence GCG CGG GGA AAA GCA is complementary to the sequence between residues 76 and 94 of human telomerase RNA. Using this antisense telomerase RNA (GCG CGG GGA AAA GCA) can interrupt the telomerase production with the aim to shorten the life span of cancer cells[99].

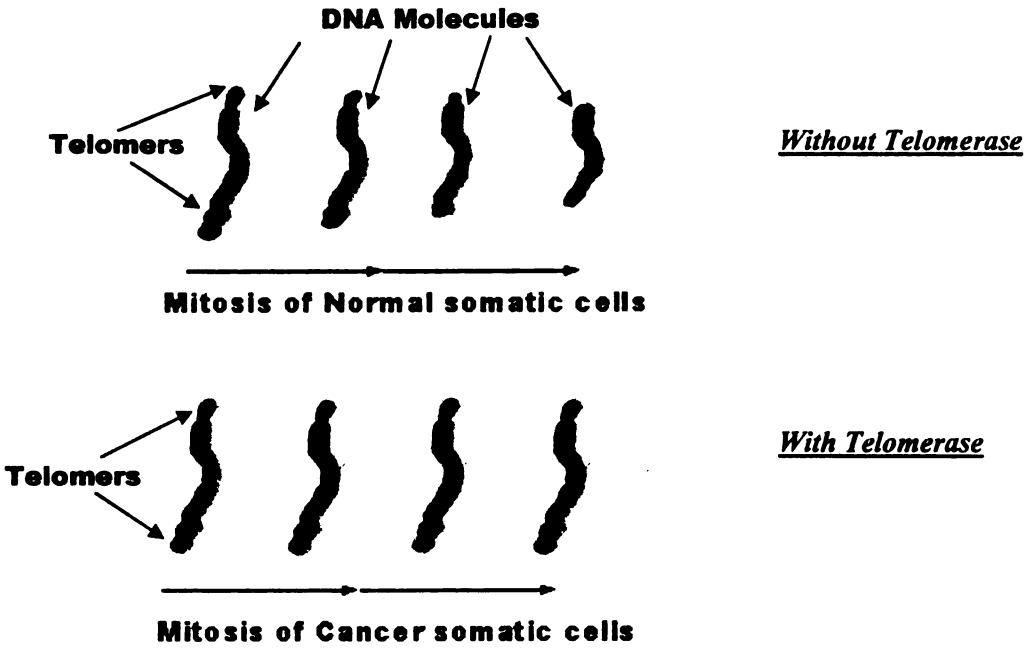


Figure 1.13 A Comparison of Mitosis of Normal Somatic Cells with Cancer Somatic Cells

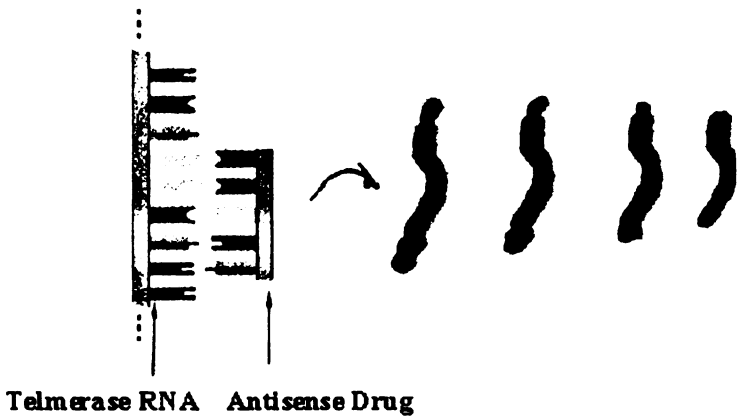


Figure 1.14 Cancer Cell Treatment with Antisense Drug (GCG CGG GGA AAA GCA)

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Chapter 2 Design of Cationic Poly-β-amino acid DDS

2.1 Introduction

In the past 30 years, liposomes have been the most commonly used drug delivery material. However, even with several improvements and diverse modifications[1, 2, 3] to overcome their shortcomings, liposomes still have many drawbacks. Some are due to the poor quality of raw materials, low drug-loading capacity and high instability [4, 5]. Therefore, the development of efficient and reliable polymeric drug delivery systems has become imperative.

Generally, there are a few requirements for designing a prospective polymeric drug delivery system:

1. Biocompatibility—stable in blood circulation, good for administration and distribution
2. Drug loading/binding capability
3. Ability to penetrate cell membranes
4. Biodegradability: Drug delivery systems can biodegrades into safe, physiologically benign fragments.
5. Stimuli release: with different environmental stimuli, drugs can be accordingly freed from the drug delivery systems, especially within endosomes.
6. Target-ability—with antibody fragments to accomplish target-specific cell or tissue delivery.
7. To be traceable, with a molecular beacon, reporter group or label.

2.2 Two main elements of the design

2.21 Molecular Weight Control

Why do we need to control the molecular weight of polymers?

With oral administration, most macromolecules cannot be absorbed from the gut and also small molecules may be easily modified or degraded by glucuronyl, sulfate, acetyl, glutathione, or glycine conjugation [6] upon the first pass or subsequent exposure in the liver. Further, most animal research studies and early clinical trials are performed by direct injections. Therefore, the development of injectable dosage forms of new drugs (anticancer-drugs, proteins and genes) are more likely to succeed than alternative routes of delivery. The molecular weight control of parenteral drug delivery systems is the key element for the success of the delivery.

Regarding parenteral delivery, we need to understand several barriers in the human body. First, at the systemic level, the reticuloendothelial system (RES) and renal filtration are mainly responsible for the rapid clearance of drug delivery system from the systemic circulation[7]. The RES mainly gets rid of larger hydrophobic particles over smaller, more hydrophilic ones; while the kidney filtration selectively removes smaller molecules from the circulation in favor of larger molecules. So the hydrophilic polymeric drug delivery system with appropriate molecular weight will have a longer circulation time, which helps them to distribute drugs to different compartments or be retained at the target site.[8]

Macromolecules cross the normal vascular endothelium slowly, as evidenced by the appearance of serum proteins in the lymph. However, this process is also sensitive to molecular weight. Higher molecular weight polymers are found at lower concentration in the extracellular fluid, relative to serum, than lower molecular weight polymers[8]. This is true except for the special organs like the liver or spleen where the endothelium is fenestrated or discontinues, or the tumor site where the enhanced permeability and retention effect (EPR) [9, 10, 11] can allow high molecular weight polymers to readily diffuse through. Higher MW polymers do not reach the same concentration in the extracellular medium of normal tissue as is as found in serum. Because of this, the size of the drug delivery system needs to be considered.

2.22 Ability to traverse membranes

Among the requirements for the design of polymeric drug delivery systems, their ability to penetrate cell membranes while carrying large molecules such as proteins, genes, etc. is the most critical element. The significant discovery of protein-transduction domains (PTDs) opens a promising scene in this field.

Protein-transduction domains (PTDs) refer to special polyamino acid sequences that can efficiently translocate a variety of bioactive agents into living cells[12-18]. (See table 2.1[18], the protein-transduction domains include human immunodeficiency virus (HIV)-1 Tat (48-46), drosophila antennapedia (Antp)-(43-58), and herpes simplex virus1(HSV) VP22.)

Table 2.1: Amino acid sequence of characterized protein-transduction domains

PTDs	Amino acid sequence
HIV-1 TAT	Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg
HSV VP22	Asp-Ala-Ala-Thr-Ala-Thr-Arg-Gly-Arg-Ser-Ala-Ala-Ser-Arg-Pro-Thr-Glu-Arg-Pro-Arg-Ala-Pro-Ala-Arg-Ser-Ala-Ser-Arg-Pro-Arg-Arg-Pro-Val-Glu
Antp	Arg-Gln-Iso-Lys-Iso-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys

From table 2.2[13] intracellular delivery of various molecules using PTDs, we can see there is no limit on the type of molecules that can be transduced into cells with a PTD: enzymes, antibodies, oligonucleotides, peptides, full-length proteins, etc[19, 20, 21-16].

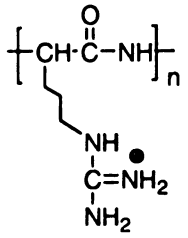
Table 2.2: intracellular delivery of various molecules using PTDs

Molecules	Carrier peptides	Conjugation
Synthetic peptides	Antp, Tat, Oligoarginine	Direct attachment, disulfide
Protein	Tat, Oligoarginine, Antp	Chemical cross-link genetic
Megnet beads	Tat	Chemical cross-link
liposomes	Tat	Chemical cross-link
Antisense oligoDNA	Tat, Antp	Disulfide
Radioisotopes	Tat	Chelate
Natural products	Oligoarginine	Chemical cross-link

Currently, the mechanism of PTDs is not known. However, most scientists presume that the arginines play a more critical role than lysine or other amines, especially exemplified in the Tat PTD[12, 13, 15]. Further, some scientists deduce that the direct penetration of the lipid bilayer of the cell membrane is mainly caused by the localized positive charge of the PTD[18].

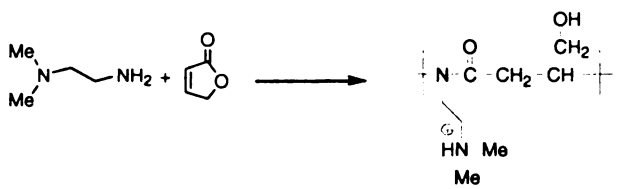
No matter what the mechanism is, it is not easy to produce or extract PTDs[12]. In addition, there is still a concern that PTDs from viruses may become reactive again *in vivo*. So the focus has been moved to preparing some polymers that have a similar structure to Tat. Oligomers of lysine, and L/D- arginine[12] were synthesized by Wender et al. A limitation to this approach is that such synthesis requires many steps and most of the steps need to be repeated several times during the synthesis.

Structure of Polyarginines:



2.3 The construction of poly(β-amino acid) drug delivery system

Amid linkages and hydroxyl groups are characteristic functionalities in biomolecules. Ways of preparing polymers containing predominately these functionalities were explored because they would be expected to have a high degree of biocompatibility. Simplicity in the synthesis of this material was also very desirable. With this in mind, the structure shown below was designed and a synthetic route was developed.



2.4 Prospective advantages of the novel poly(β-amino acid) drug delivery system

1. This drug delivery system has a poly(β-amino acid) backbone structure that resembles the poly α-amino acids (peptide), but should be more stable to biodegradation compared with peptides.

2. This drug delivery system has dimethylaminoethyl side chains where the positive charges resemble polyarginine.

3. The molecular weight of this drug delivery system can be controlled by adjusting the time and temperature of the synthesis.

4. With the positive charge, the polymer will be able to bind and mediate the uptake of a variety of drugs such as the anticancer drug–doxorubicin, antisense RNA, plasmid DNA, etc.

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Chapter 3.0 Experimental

3.1. Synthesis of Poly(β-amino acid)

3.1.1. Materials and characterization

All chemical materials used were obtained from Aldrich Company and were analytical grade unless otherwise noted. NMR measurements were made on a Varian VXR 500 MHZ Spectrometer with CD_3OD as solvent. The FT-IR spectra were obtained with a Nicolet 710 FT-IR spectrometer. Mass Spectra were taken with a MALDI Voyager-DETM STR. The molecular weight of different polymer fractions were estimated by means of gel permeation chromatography (GPC) using Shodex Asahipak GF-310 HQ column (exclusion limit is 40,000 Daltons) and using Mili-Q water as solvent with a flow rate of 0.7ml/min.

3.1.2. Synthesis of Poly(β-amino acid)

N, N- dimethyl ethylene diamine (0.01 mole, 0.9279 g) was dissolved in cold methanol (4ml) in a 50 ml vial (A). 2(5H) Furanone (0.01 mole, 0.8579g) was dissolved in cold methanol (4ml) in another 50 ml vial (B). The solution in vial (B) was quickly added to vial (A) at 0° C. Cold methanol (2 ml) was used to rinse vial (B) several times, and the rinsate was added to vial (A). The reaction mixture was left at 0° C for 4 hours. And then the reaction temperature was changed to 25° C for another 4 hours. Reaction mixtures were heated to different temperatures (60° C, 70° C, 80° C, 90° C, 120° C, 140° C, etc) and different polymerization time (0.5 hours, 1 hour, 2

hour, 4 hours, 6 hours, 8 hours, 12 hours, 24 hours, 36 hours, etc) were allow the production of polymers with a variety of molecular weight.

3.2 Purification and separation

A) Using Bio Gelp-60 Size exclusion chromatography

The purification, characterization and application experiments are described to the polymer synthesized at 120 °C for 3.5 hours. Polymer (0.2 g) was dissolved in 10 ml Mili-Q water. The polymer water solution was injected onto the Bio Gelp-60 Size exclusion column (3X80 cm) with water as the elutant. The fractions of polymer were collected by the Auto Fraction collector at a flow rate of 0.1 ml/minute with 5 ml in each test tube. Polymer solutions (200 µl) were taken from each tube of the Auto Fraction collector and dropped into the corresponding cells of an 8*12 cm plate. The UV absorbance values of polymer fractions were measured by µ Quant Universal Microplate Spectrophotometer at a wavelength of 220 nm. High (I), medium (II), low MW (III) fractions of the polymer were separated and collected according to UV absorbance results. The solvent was removed with a rotary evaporator and each polymer fraction was kept in a freeze dryer for 72 hours.

B) Precipitation and extraction

The polymers (0.2 g) were dissolved in 0.5 ml hot water (60 °C), and then 3.5 ml acetone (poor solvent) was added into the polymer water solution. The mixture was left in a 10-ml separation funnel for 24 hours. Then, the first fraction (high molecular

weight) was collected. Medium molecular weight fraction and low molecular weight fractions were collected by repeating this step two times, respectively.

3.3 Measurement of drug delivery properties

I. Anticancer drug–doxorubicin delivery*

Mouse embryonic fibroblasts (MEF) were grown in Dulbecco's Modification of Eagles Medium (DMEM) at 37^0C, 5% CO_2. The cells were plated on cover slips. One cover slip (24 hours later) was taken and flipped over onto a microscope slide which has a droplet of 5 μl of the stock solution of drug Doxorubicin (0.0002 g) and labeled polymer (0.0040 g) dissolved in 1ml phosphate buffered saline (PBS). The samples were prepared right before visualizing with the Laser Scanning Confocal Microscope.

Labeling polymer with fluorescein isothiocyanate (FTIC)

Fluorescein isothiocyanate (0.004 g) was dissolved in 40 μl acetonitrile. Polymer (0.01 g) was added along with 200 μl of water and 0.005 g of sodium bicarbonate ($NaHCO_3$). The sample was sonicated for 2 minutes and stirred overnight. On the second day, the sample was diluted with 200 μl of water and the excess FTIC was separated by passing the solution through a SEP-PAK cartridge C18. The FTIC labeled polymer solution was then dried using a lyophilizer.

II. RNA Antisense (GCG CGG GGA GCA AAA GCA) delivery*

Mouse embryonic fibroblasts (MEF) were grown in DMEM at 37^0C, 5% CO_2.
The cells were plated on cover slips. One cover slip (24 hours later) was taken and flipped over onto a microscope slide which has a droplet of 5 μl of stock solution of Cy5 labeled oligonucleotide (GCG CGG GGA GCA AAA GCA) (0.0002 g) and polymer (0.0040 g) dissolved in 1 ml phosphate buffered saline (PBS). The samples were prepared immediately before visualizing with the Laser Scanning Confocal Microscope.

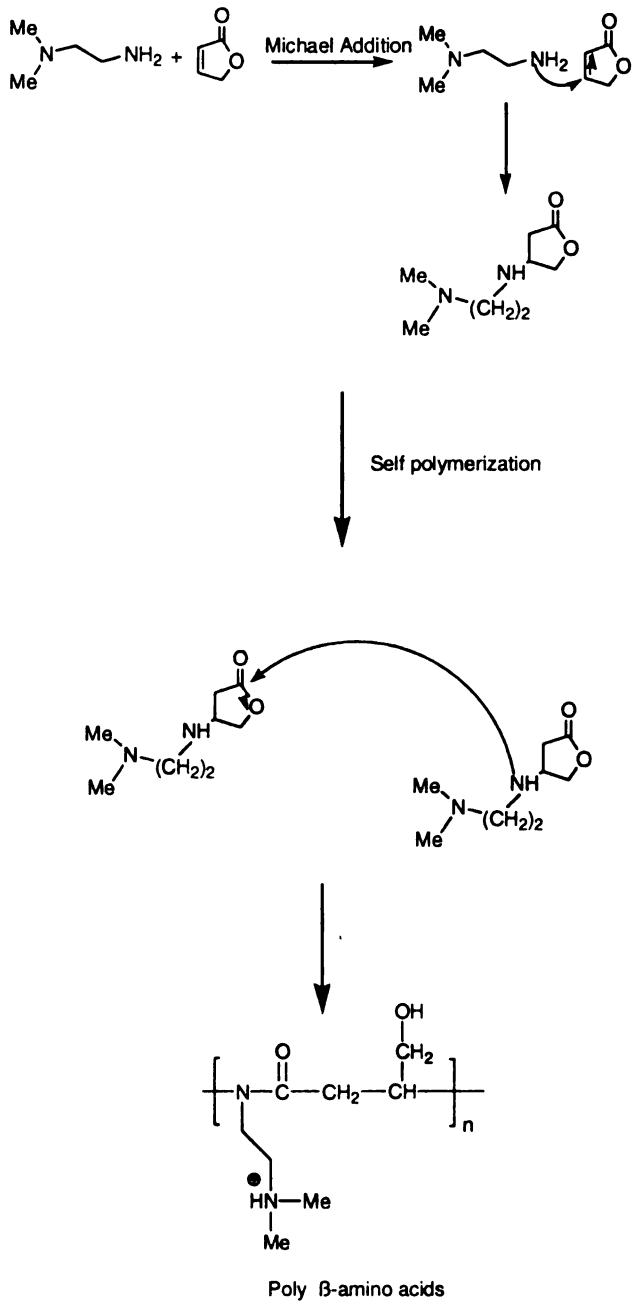
III. Plasmid GFP gene delivery*

Mouse embryonic fibroblasts (MEF) were grown in DMEM at 37^0C, 5% CO_2. The cells were plated on cover slips. Phosphate buffer saline (PBS) (1 ml) containing 2 μg of plasmid with the green fluorescence protein gene (GFP) and 0.0040 g of non-labeled polymer were added to the MEF cells in DMEM and left for 12, 13, 18, and 33 hours before visualizing with the Laser Scanning Confocal Microscope. Phosphate buffer saline (PBS) (1 ml) containing 2 μg of plasmid with the green fluorescence protein gene (GFP) and 0.0040 g of non-labeled Super Fect ^{Tm} were added to the MEF cells in DMEM and left for 12 and 17 hours before visualizing with the Laser Scanning Confocal Microscope.

*Method provided by Felicia Codrea.

Chapter 4 Results and Discussion

4.1 Synthesis of poly(β-amino acid) and MW control of Poly(β-amino acid)



Scheme 4.1 Synthesis of poly(β-amino acid)

The route of synthesizing poly(β-amino acid) is outlined in Scheme 4.1. This route can be used for a series of drug delivery systems. For example, a mixture of different primary amines can be used such that the total molar amount of these amines is equal to the molar amount of furanone used. By regulating the relative amounts of polar vs non-polar amines, charged vs neutral groups, anionic vs cationic groups etc, the pharmacological and physicochemical properties of the material can be controlled. By adjusting the temperature and/or reaction time, the molecular weight of the polymers can be controlled.

The extent of the reaction to produce poly(β-amino acid) was monitored by ^{1}H-NMR spectroscopy. Performing the exothermic Michael addition at low temperatures is important because it ensures that the addition has taken place completely before appreciable amide formation begins. After 2 hours at 0 °C, the disappearance of the signals for furanone at fields higher than 5 ppm indicated that the Michael addition was complete. The spectra indicated that the dimer product from the Michael addition was the major product. There was no evidence of polymerization (Figure 4.1 a & b).

Increasing temperature and time aided polymerization as shown in Figure 4.2 A-C (proton NMR spectra of the mixture after reaction at 70 °C for 1 hour, 4 hours and 8 hours). The signals for the methylene protons at the 4-position of the lactone ring (4.17 ppm and 4.42 ppm) disappeared indicating complete reaction of the dimer. However, the narrow linewidths of the signals in the spectra indicated that the product was composed mainly of oligomeric species. It was also found that increasing the time at 70 °C slightly increased the degree of polymerization. This is indicated by the increased width of peaks especially those between 2.0 ppm to 2.6 ppm. Figure 4.3 A-

B (proton NMR spectra of polymer reacted at 80°C for 4 hours and 10 hours) and Figure 4.4 A-C (proton NMR spectra of polymer reacted at 90 °C for 2, 4, and 8 hours showed the same trend as above. The spectra show that oligomeric species were the main products at 90°C or lower. Even though increasing the reaction time showed some benefits, the temperature was still too low for polymerization. If high molecular weight products were being formed, a broad envelope of signals will be observed instead of sharp peaks. When the reaction temperature was increased to 120 °C, the NMR results were very different (Figure 4.5 A-C). The linewidths of signals between 2.0 ppm and 3.4 ppm were significantly broadened. All of the sharp monomer signals disappeared indicating that polymerization had occurred. The spectra also show that the longer the reaction time, the better the polymerization. Analyses were done from 3.5 hours to 9.5 hours.

When the reaction was carried out at 140 °C for 0.5, 1, 2 and 4 hours, respectively, the relationship between the molecular weight (broad NMR lines) and the reaction time was still the same; however, a few sharp peaks between (3.2-3.4ppm) were found in the spectra. These were attributed to degradation to low molecular weight species as shown in Figure 4.6 (A-D). Therefore these results indicate that the polymerization is best conducted at 120°C is and that lengthening the reaction time will accordingly increase the molecular weight of the polymers. Therefore, all of the following tests such as purification, characterization, and application experiments were done on the polymer synthesized at 120 °C for 3 hours.

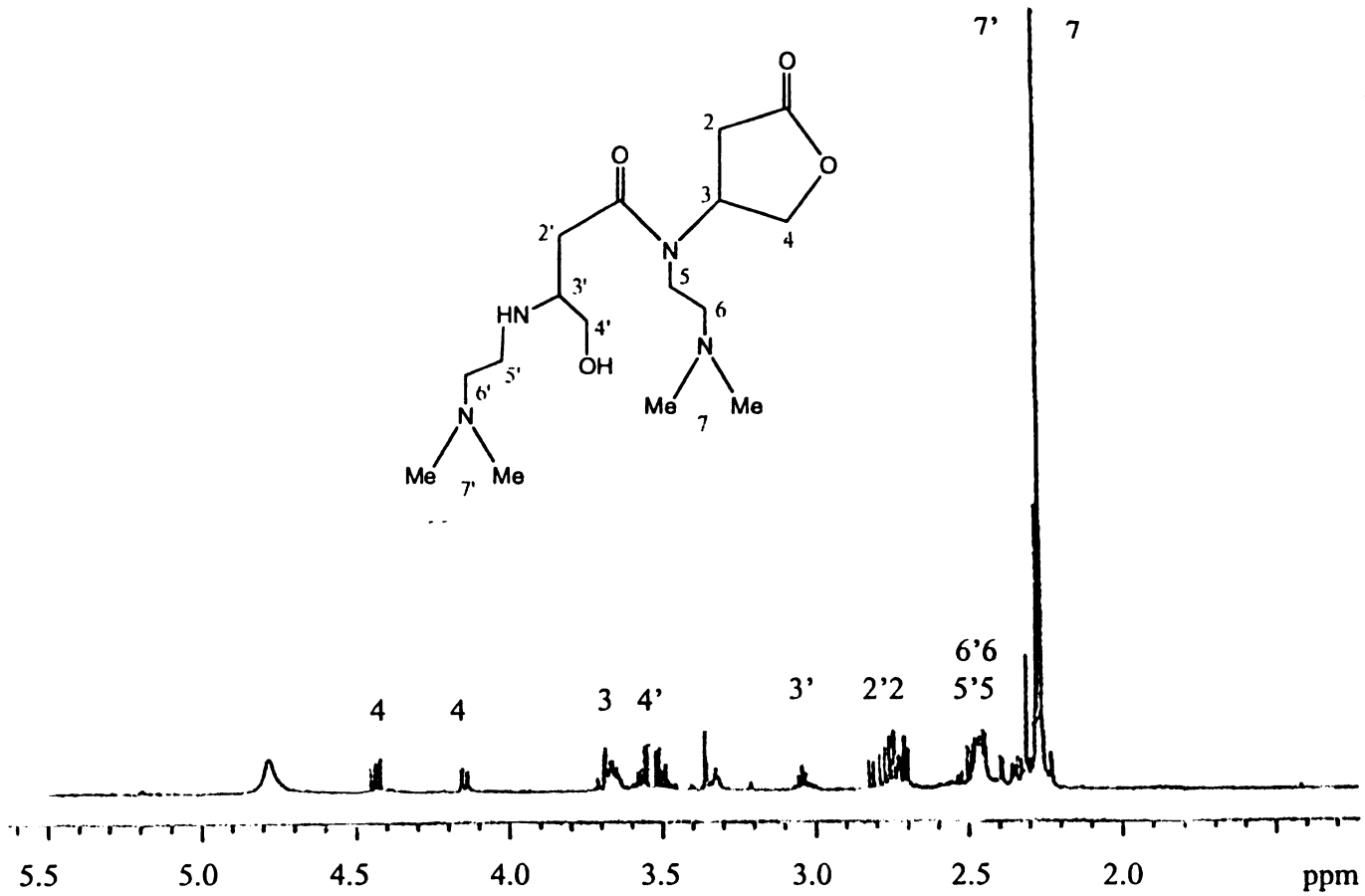


Figure 4.1(a) ^{1}H-NMR spectrum of reaction mixture at 0°C after 2 hours.
Signals are consistant with predominately dimer formation under this condition. See annotation on structure drawn in inset.

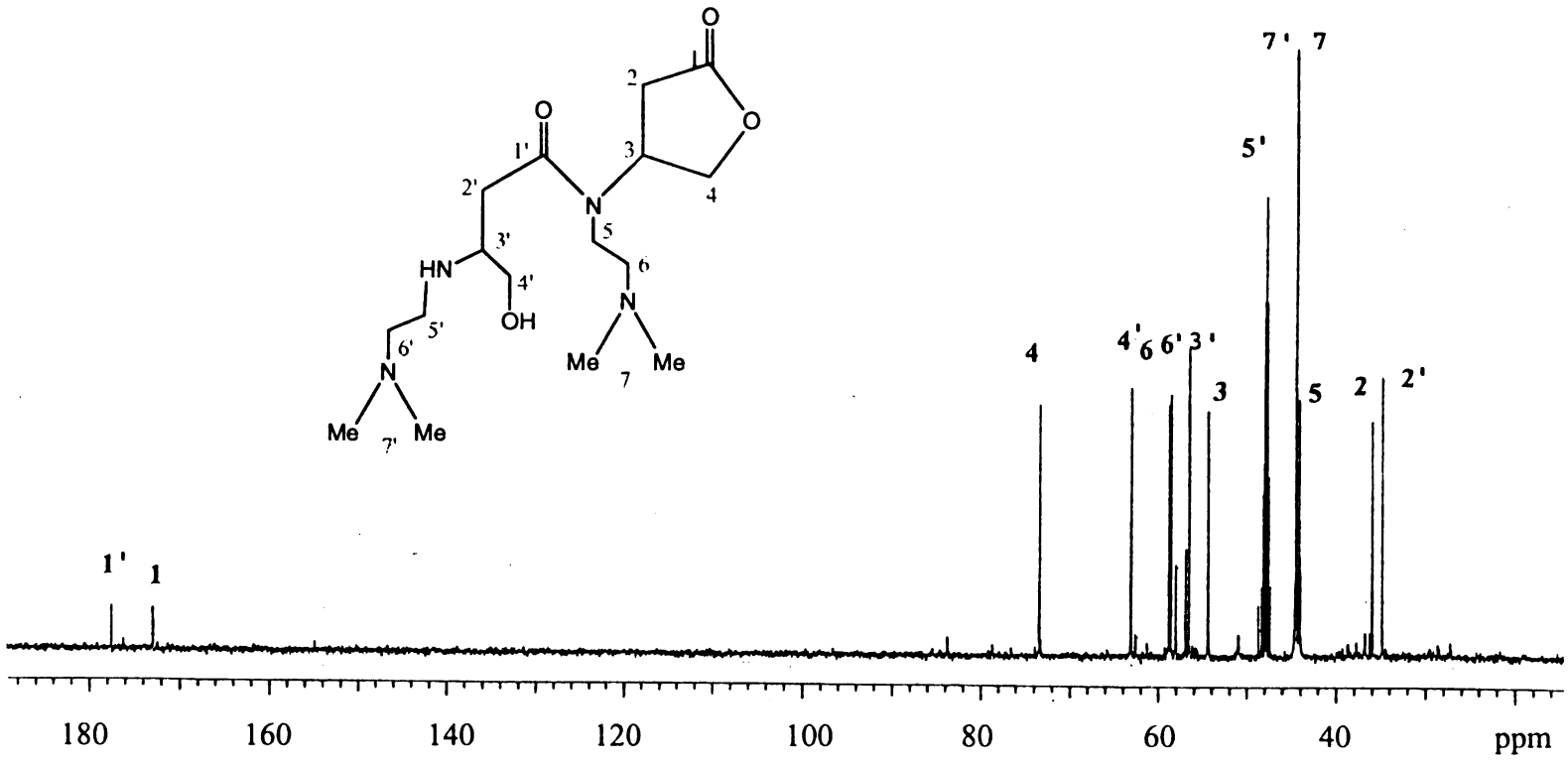


Figure 4.1(b) ^{13}C-NMR spectrum of reaction mixture at 0°C after 2 hours.
Signals are consistant with predominately dimer formation under this condition.
See annotation on structure drawn in inset.

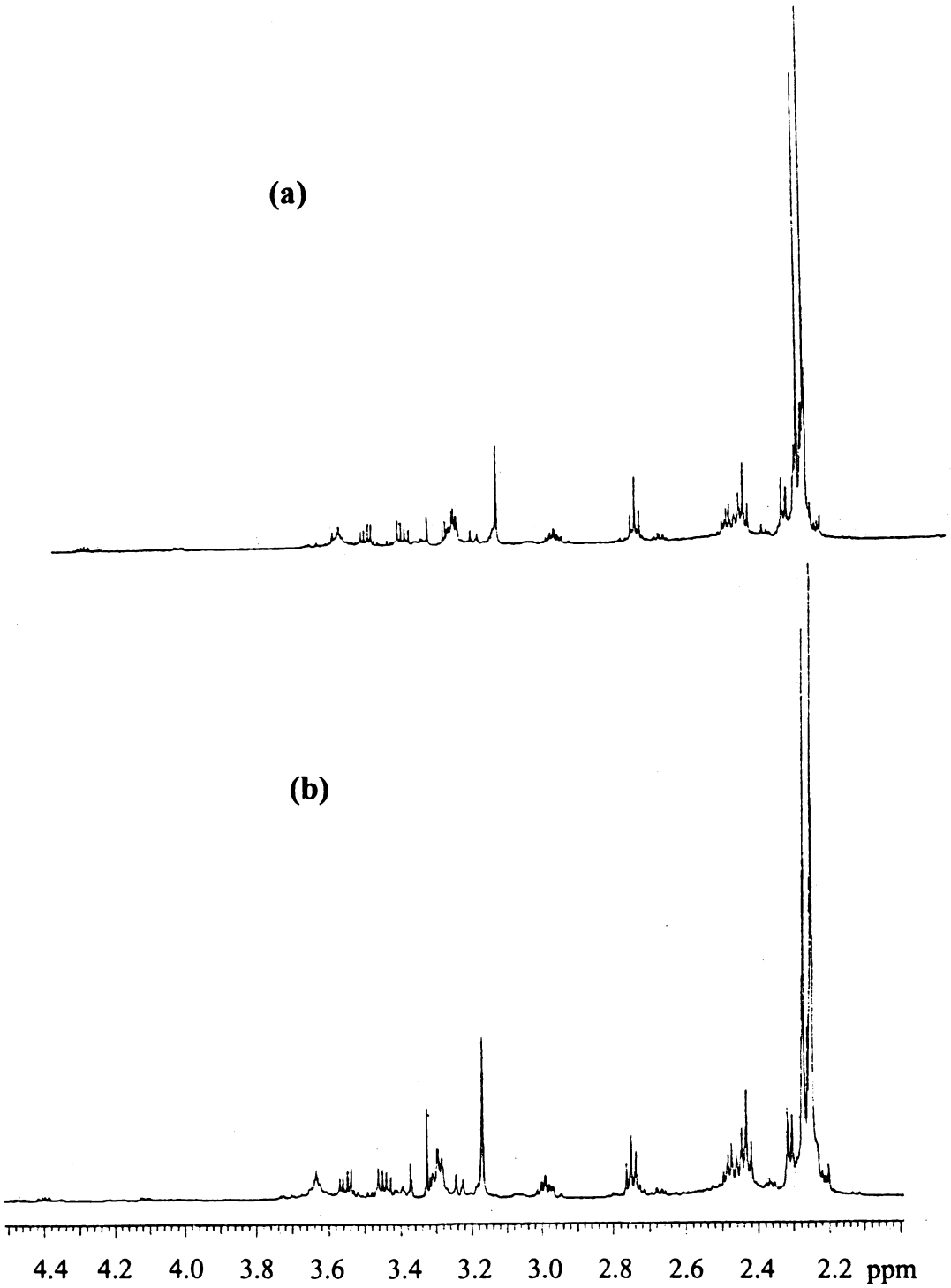


Figure 4.2 ^{1}H-NMR spectrum of reaction mixture at 70°C after 1 hour (a) and after 4 hours (b).

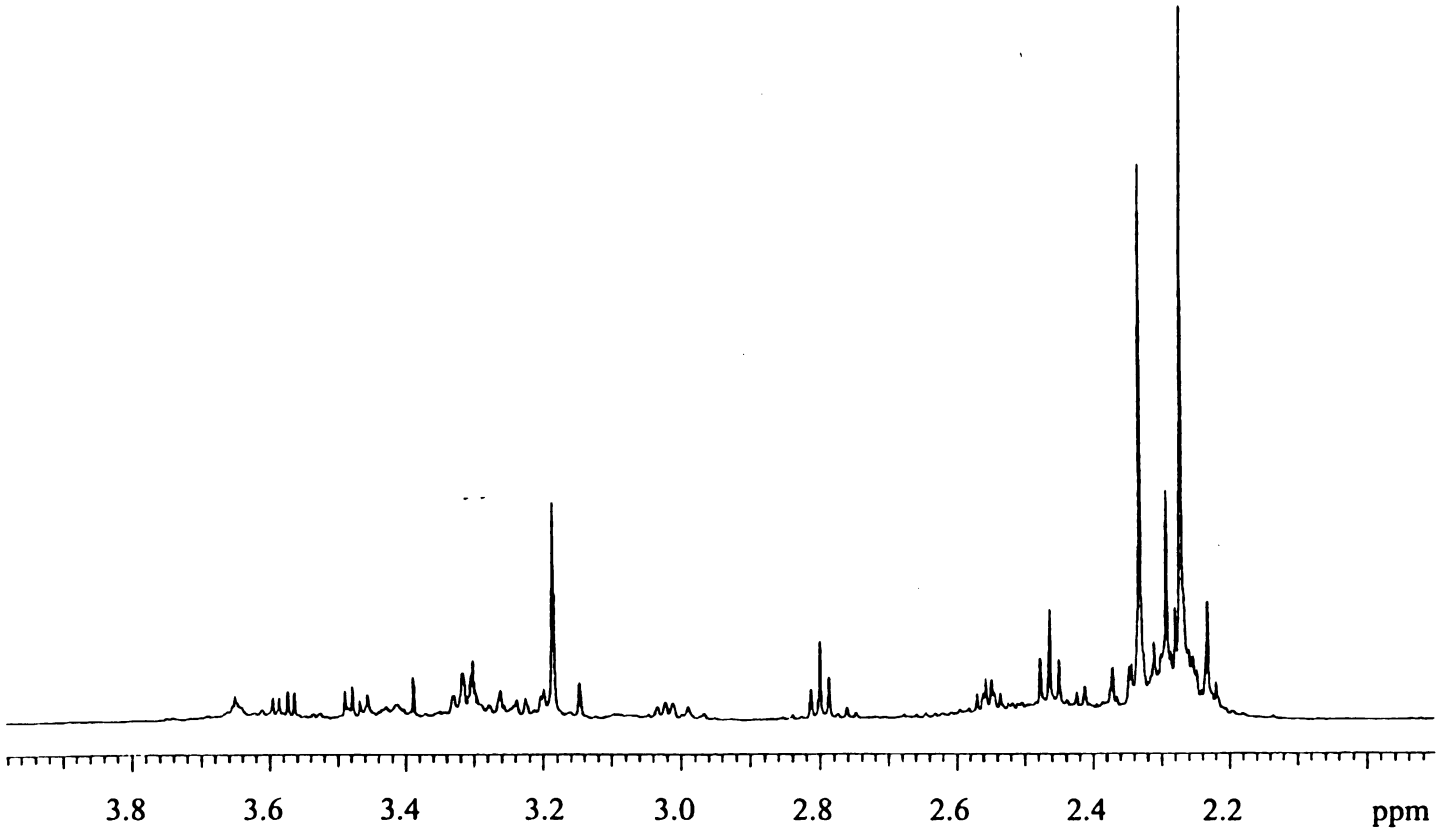


Figure 4.2(c) ^{1}H-NMR spectrum of reaction mixture at 70°C after 8 hours.

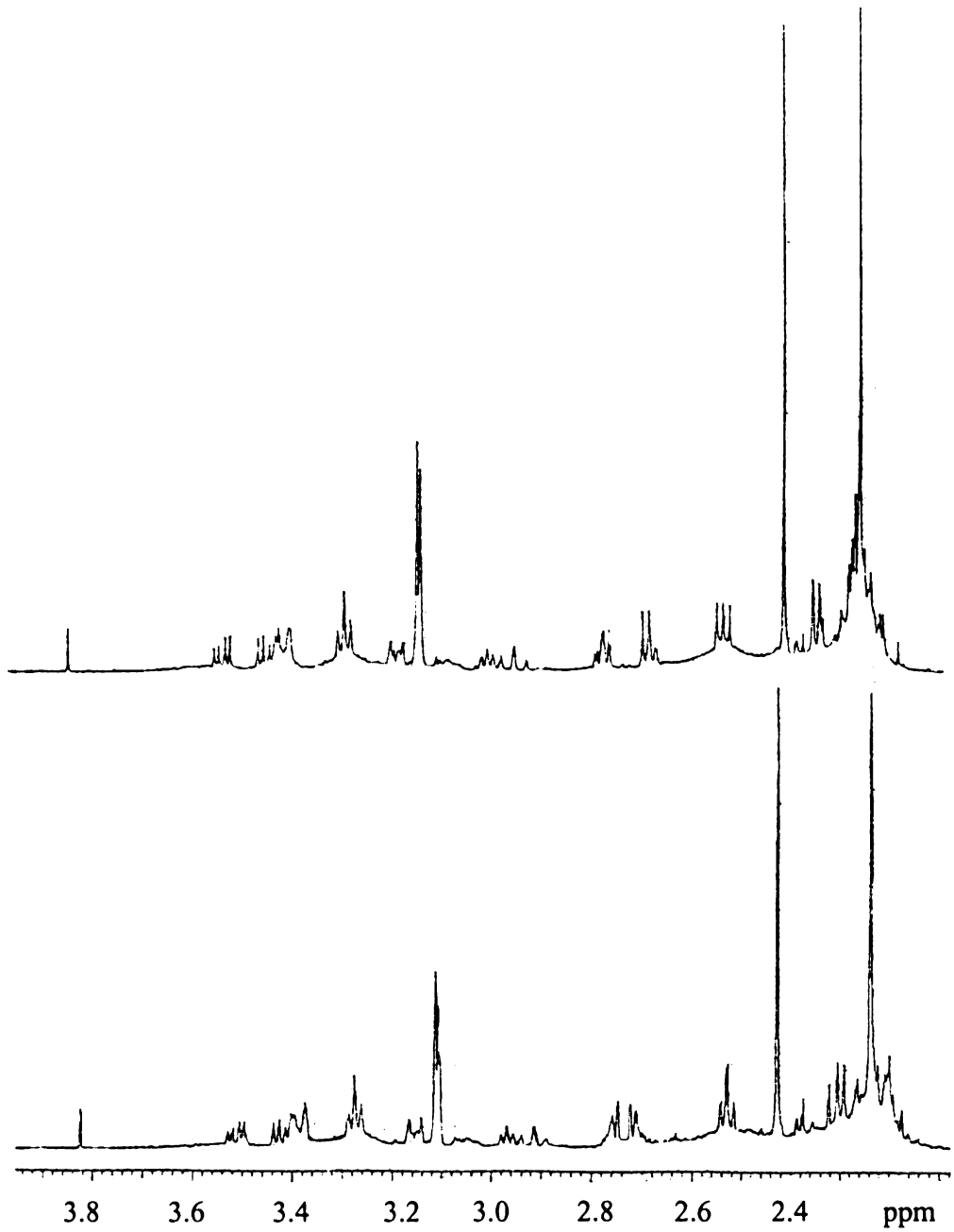


Figure 4.3 ^{1}H-NMR spectrum of reaction mixture at 80°C after 4 hours (a) and after 10 hours (b).

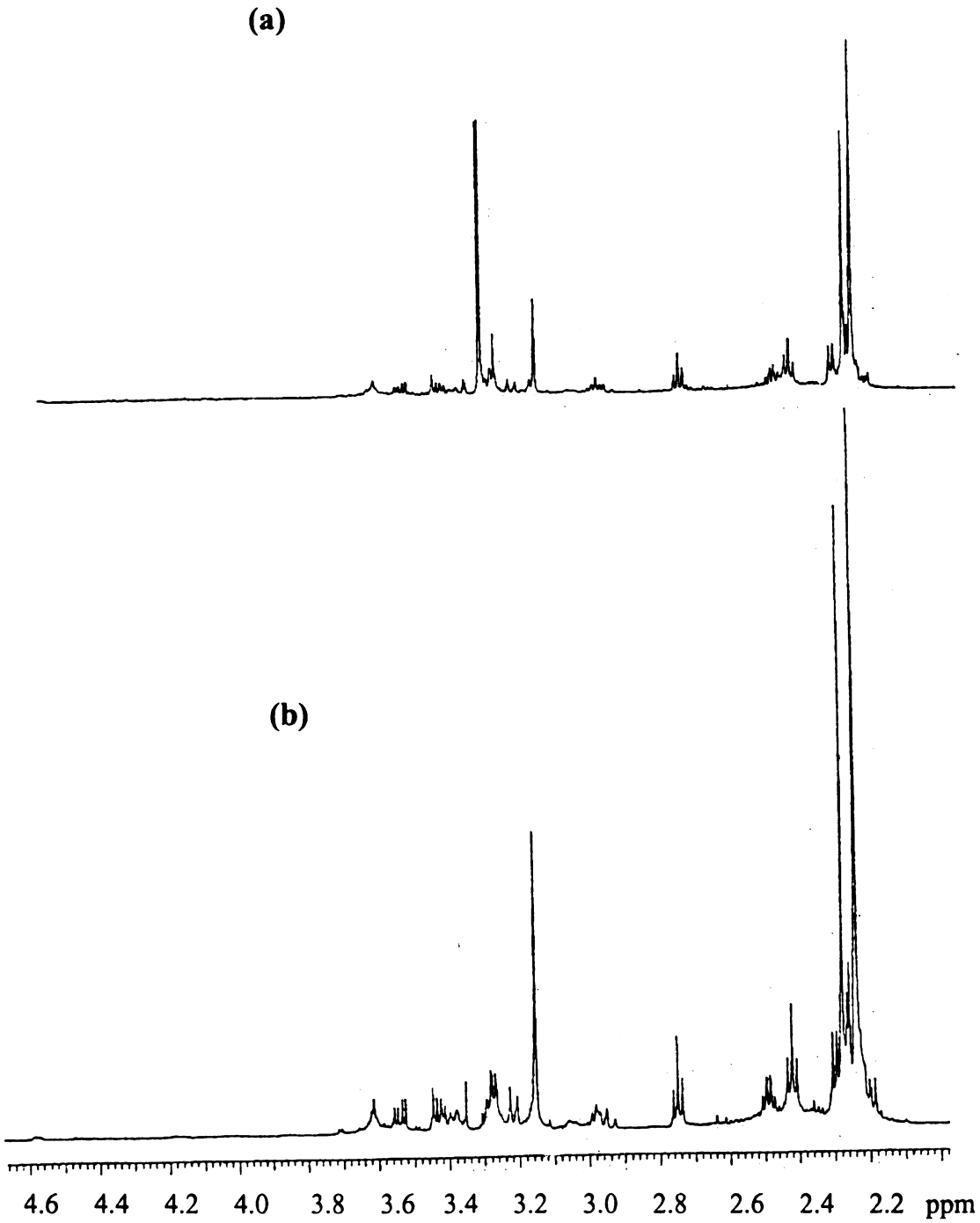


Figure 4.4 ^{1}H-NMR spectrum of reaction mixture at 90°C after 2 hours (a) and after 4 hours (b).

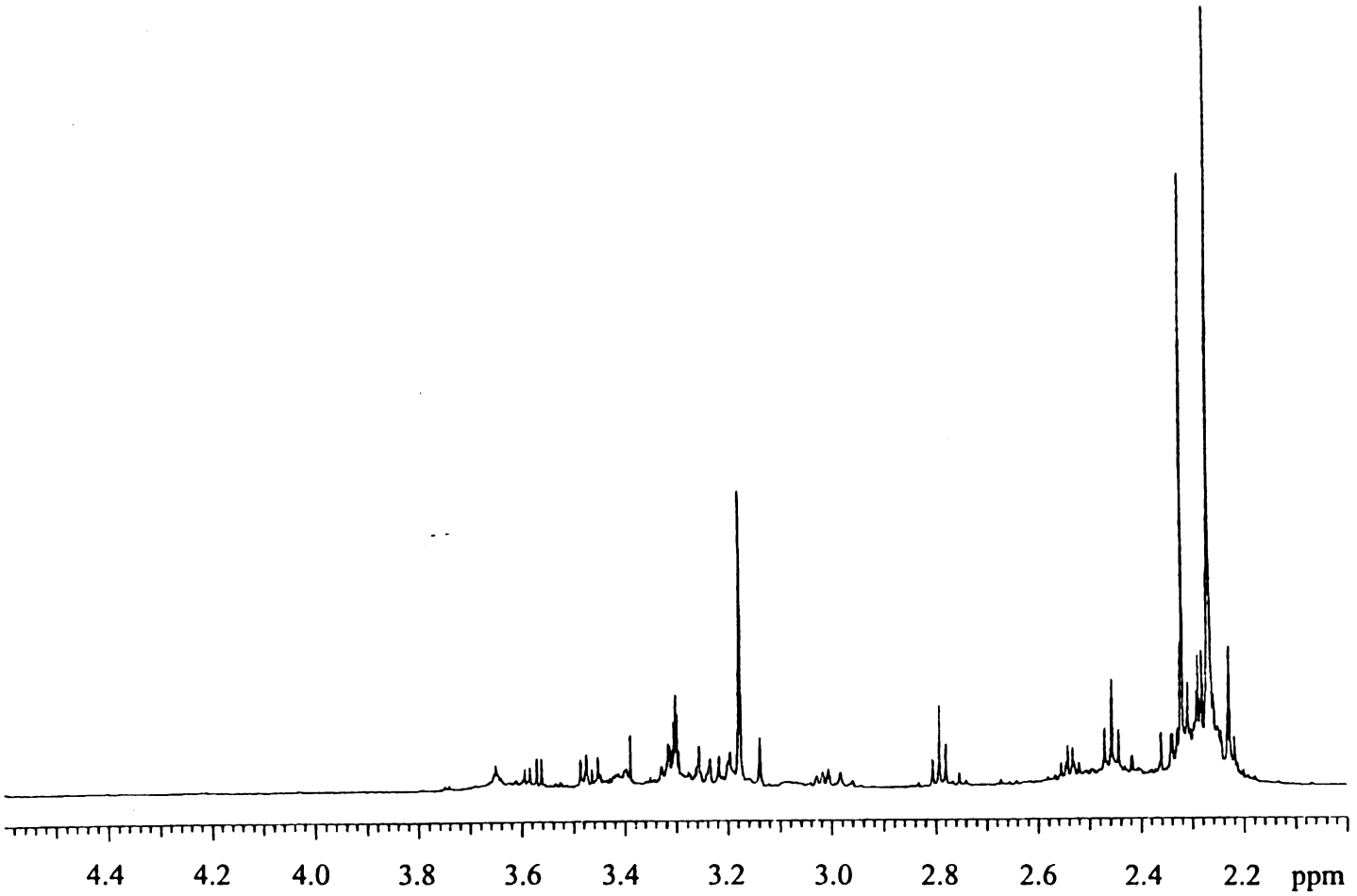


Figure 4.4 (c) ^{1}H-NMR spectrum of reaction mixture at 90°C after 8 hours.

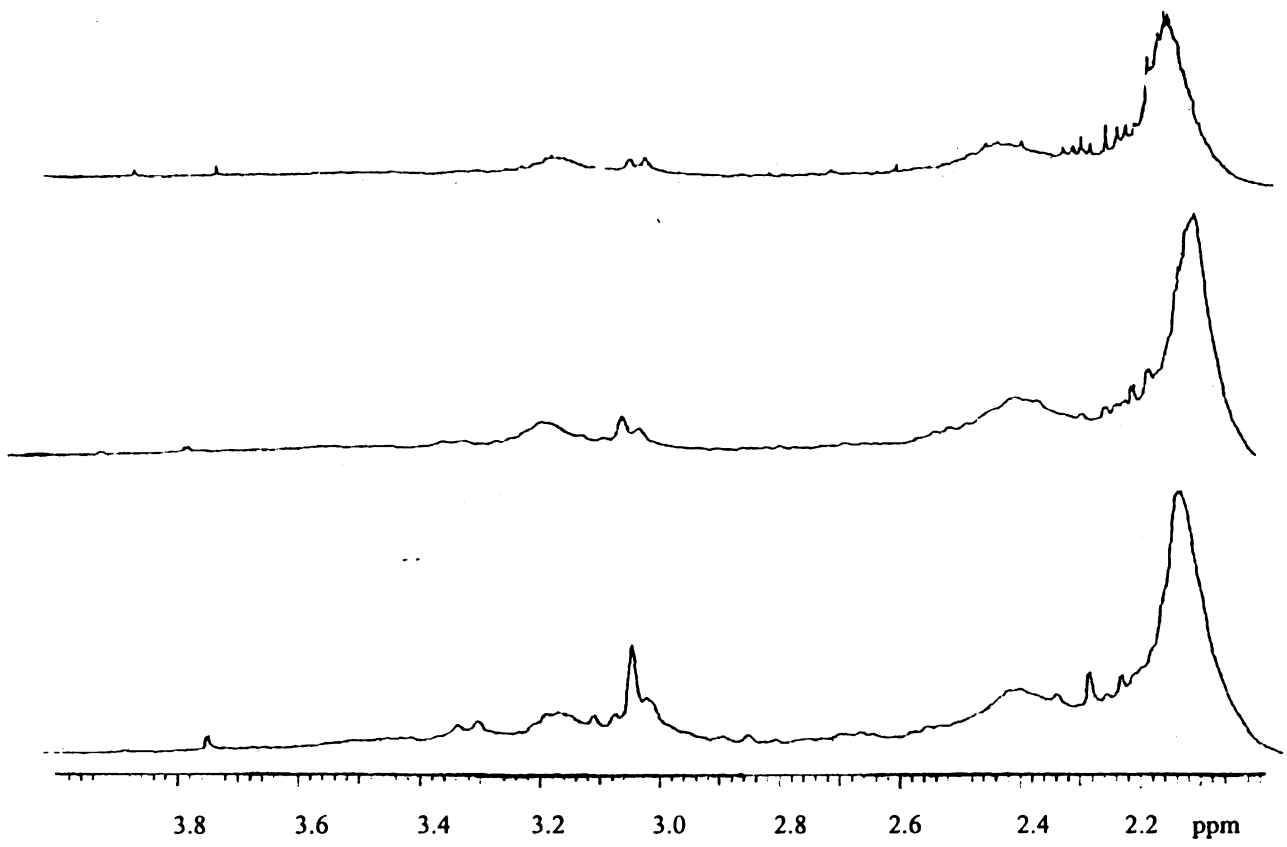


Figure 4.5 ^{1}H-NMR spectrum of reaction mixture at 120°C after 3.5 hours (a), 6.5 hours (b), and 9.5 hours (c).

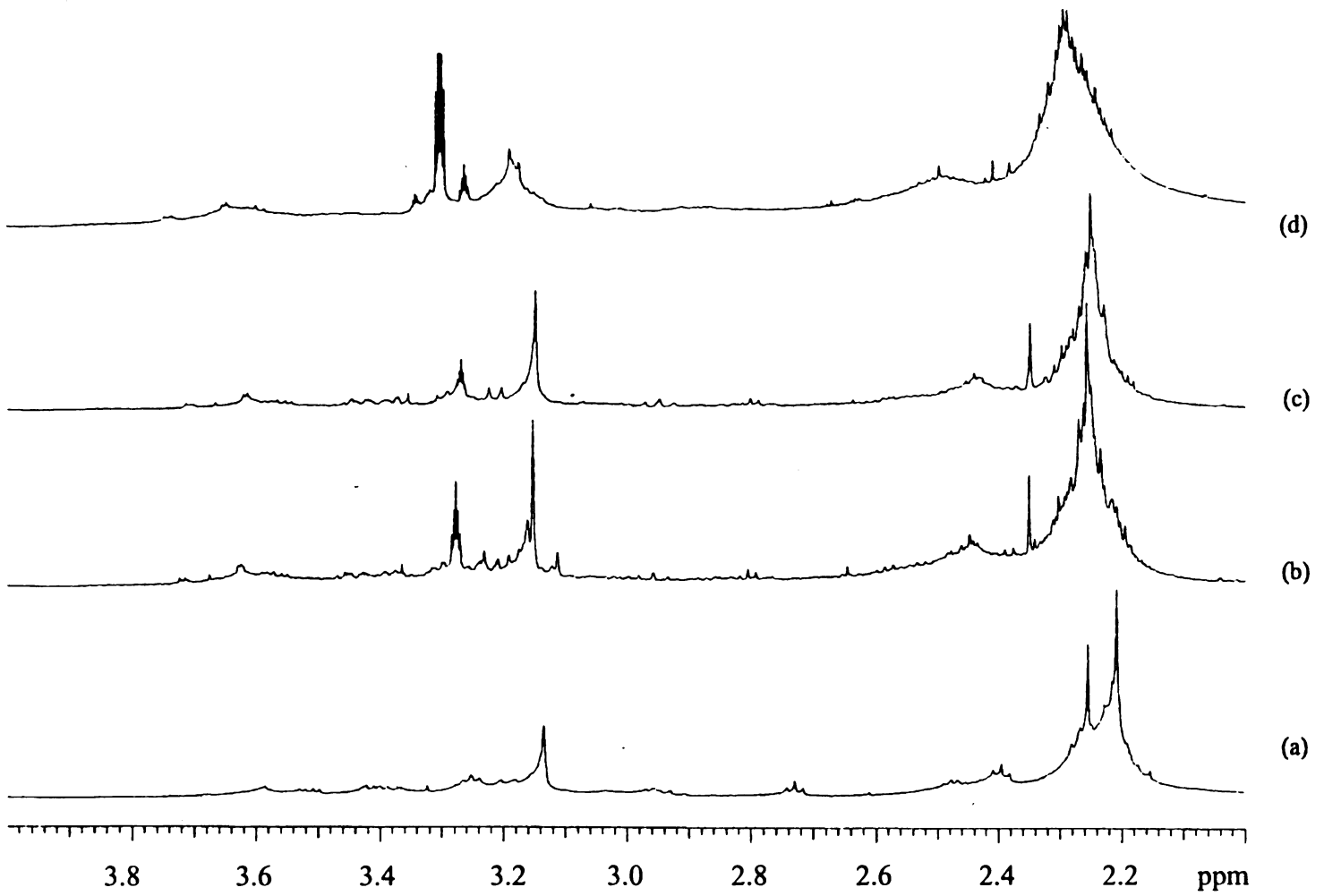


Figure 4.6 ^{1}H-NMR spectrum of reaction mixture at 140°C after 0.5 hour (a), 1 hour (b), 2 hours (c), and 4 hours (d).

4.2 Separation and estimation of molecular weight (MW) of the polymer fractions:

4.2.1 Biogel P-60 Size exclusion chromatography of the poly(β-amino acid)

During biogel P-60 Size exclusion chromatography, fractions of the poly(β-amino acid) were collected and monitored at 220 nm. In Figure 4.7, a plot of the fraction number vs UV absorption is shown.

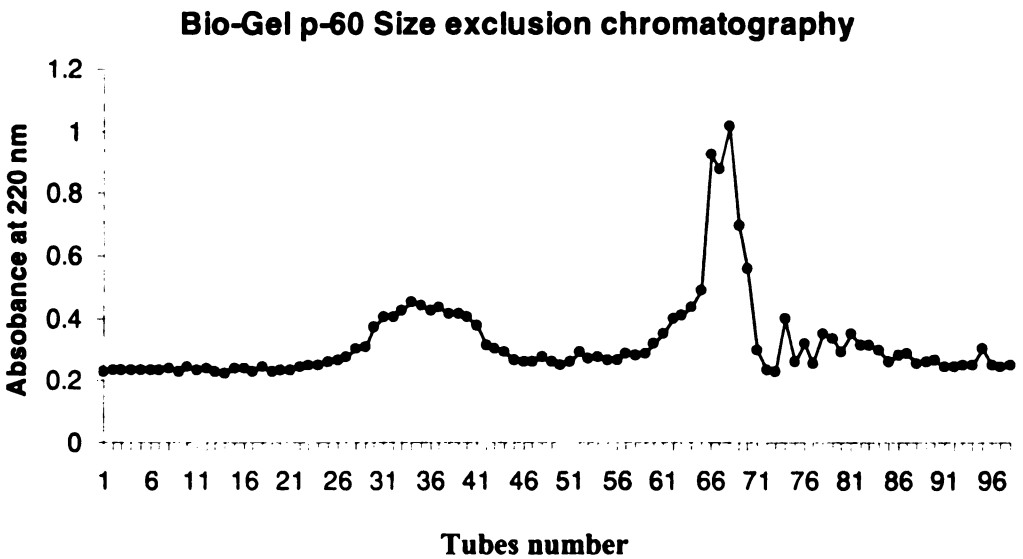


Figure 4.7 Plot of fraction number vs absorbance

Based on the plot, the polymer distribution was preliminarily assigned to a high molecular weight fraction (tubes 19-47), a medium molecular weight fraction (tubes 48-73) and a low molecular weight fraction (tubes 74-98).

4.2.2 Determination of the MW of poly(β-amino acid) fractions by Waters 1525 HPLC

The molecular weights of three polymer fractions after Biogel P-60 Size exclusion chromatography were estimated by means of gel permeation chromatography (GPC) using a column with an exclusion limit of 40,000 Daltons. The retention times (T_e) were obtained of Dextrans standards (MW 5200, 11600, 23800). Table 4.1 shows the data from the standards. Figure 4.8 shows the curve of log MW v.s. K_{av}. [K_{av}= (T_e-T_0)/ (T_t-T_0)]. T_0 (the exclusion limit, 7.05 minutes) was the retention time of Dextran with MW 686,000. T_t (12.54 minutes) was the retention time of glucose (MW=180).

Table 4.1 Data of standard curve (lgMW v.s. K_{av})

MW	log MW	T_e (minute)	K_{av} =(T_e-T_0)/(T_t-T_0)
5200	3.72	9.43	0.434
11600	4.06	8.10	0.321
23800	4.38	8.81	0.192

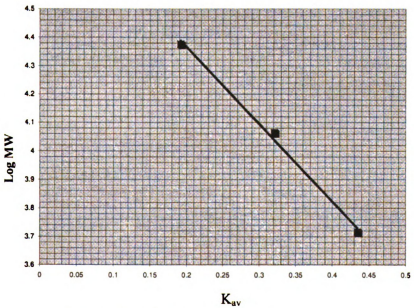


Figure 4.8 MW v.s. K_{av} calibration

The linear line fit to the data from the dextran standards is

$\log MW = -2.72K_{av} + 4.91$

The high MW fraction's retention time was 8.565 minutes (Fig 4.9 a) corresponding to a molecular weight of 15,000 Da. The medium MW fraction's retention time was 9.532 minutes (Fig 4.10 a) indicating that its molecular weight should be 5000 D. The MALDI Voyager-DE^{TM} STR spectrometer was used to obtain more accurate values of 16,986 Da and 5383 Da for the molecular weights at the peaks of the distributions (Figure 4.9 b and Figure 4.10 b). The relative yield for each MW fraction (high, medium, and low) is shown in Table 4.2.

Table 4.2 Relative yield of each fraction (percentage of recovered materials)

Fractions	**MW**	**Yield %**
High molecular weight	**16,000-17,000**	**53.9%**
Medium molecular weight	**5,000-6,000**	**31.4%**
Low molecular weight	**<1000**	**14.7%**

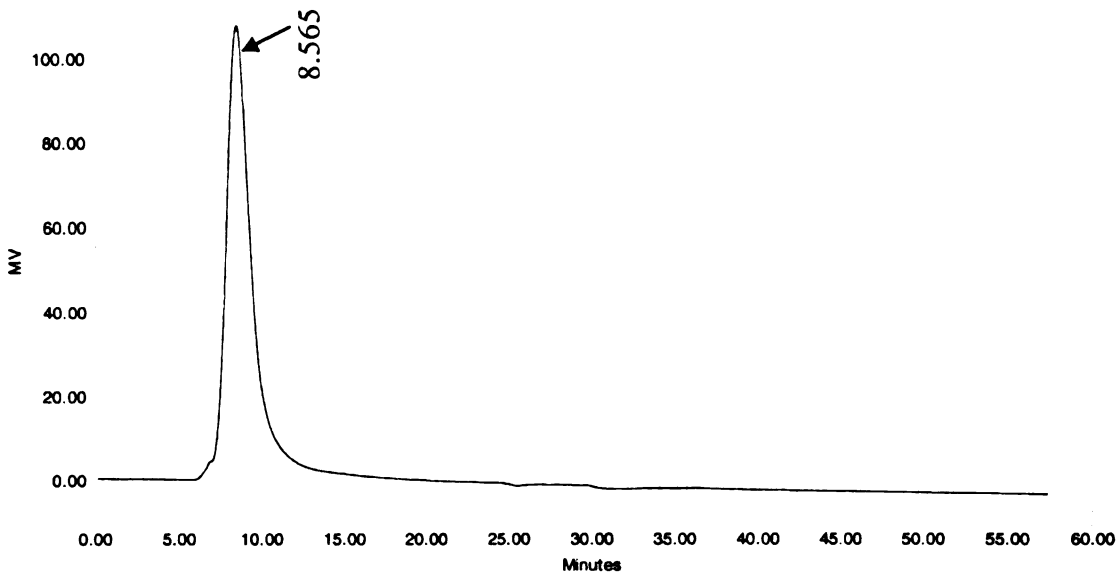


Figure 4.9 (a) GPC results for the high MW fraction isolated by Bio GelP-60 chromatography

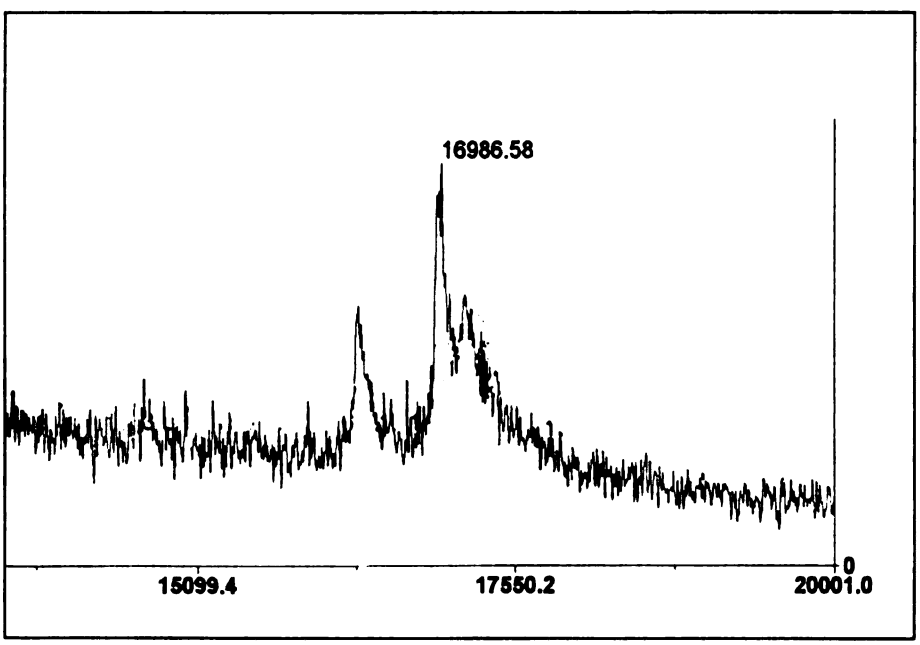


Figure 4.9 (b) Mass spectrum of the high MW fraction isolated by Bio GelP-60 chromatography

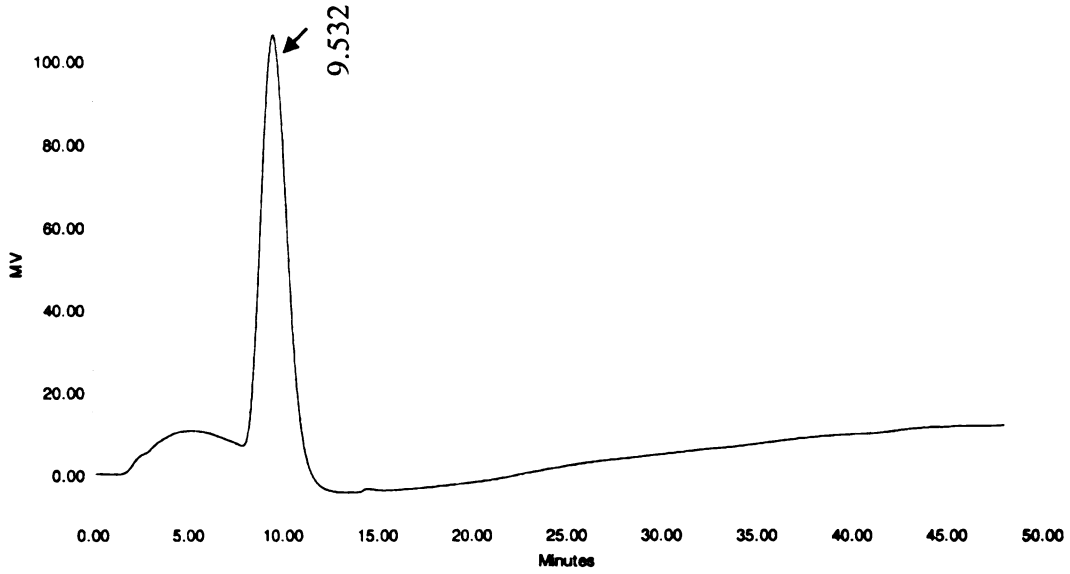


Figure 4.10 (a) GPC results of the medium MW fraction isolated by Bio GelP-60 chromatography

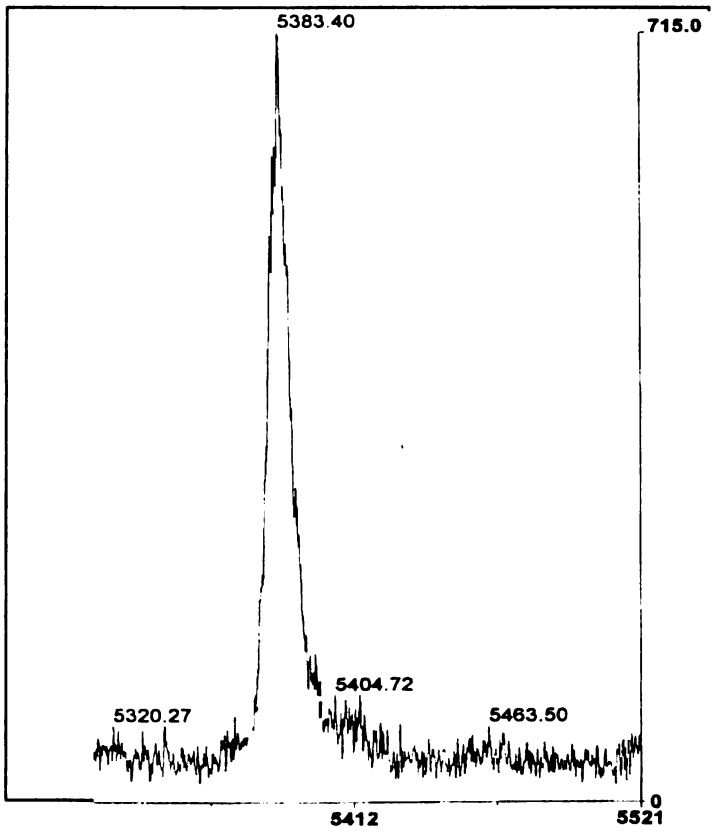


Figure 4.10 (b) Mass spectrum of the medium MW fraction isolated by Bio GelP-60 chromatography

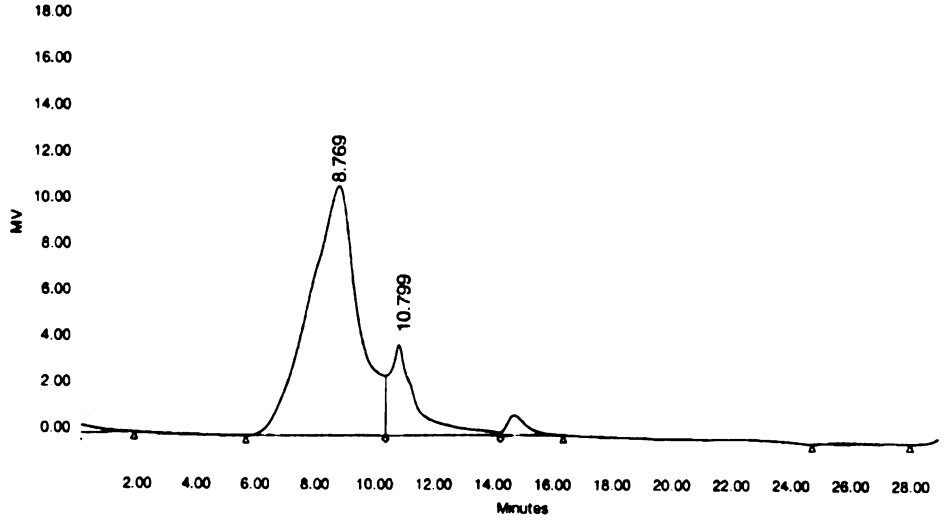


Figure 4.11 GPC result of high MW fraction isolated by acetone precipitation method

4.2.3 GPC result of high MW fraction isolated by acetone precipitation method (Figure 4.11)

From figure 4.11, we can see the high MW fraction from acetone precipitation was noticeably impure. The peak width is broad and the low MW product accounts for more than 20% of the sample. Compared with the precipitation

method of fractionation, gel filtration using size exclusion chromatography gave a much better separation. However, the acetone precipitation method allowed the processing of a much larger amount of material and could be used as an efficient initial fractionation method before final purification and sizing.

4.3. Characterization of high MW fractions of the poly(β-amino acid)

The high MW fractions of the poly(β-amino acid) were characterized by proton NMR and FT-IR spectroscopy. (Figure 4.12 and Figure 4.13). From the proton NMR spectrum, the apparently broad envelope indicated high molecular weight poly(β-amino acid). In the FTIR spectrum, the signal at 3297 cm^{-1} indicated the presence of –OH, signals at 2943 cm^{-1}, 2858 cm^{-1} and 2768 cm^{-1} indicated the structures of $-CH_2$, $-CH_3$, –CH respectively, the signal at 1660 cm^{-1} indicated –C=O, and the signal at 1042 cm^{-1} indicated –C-O.

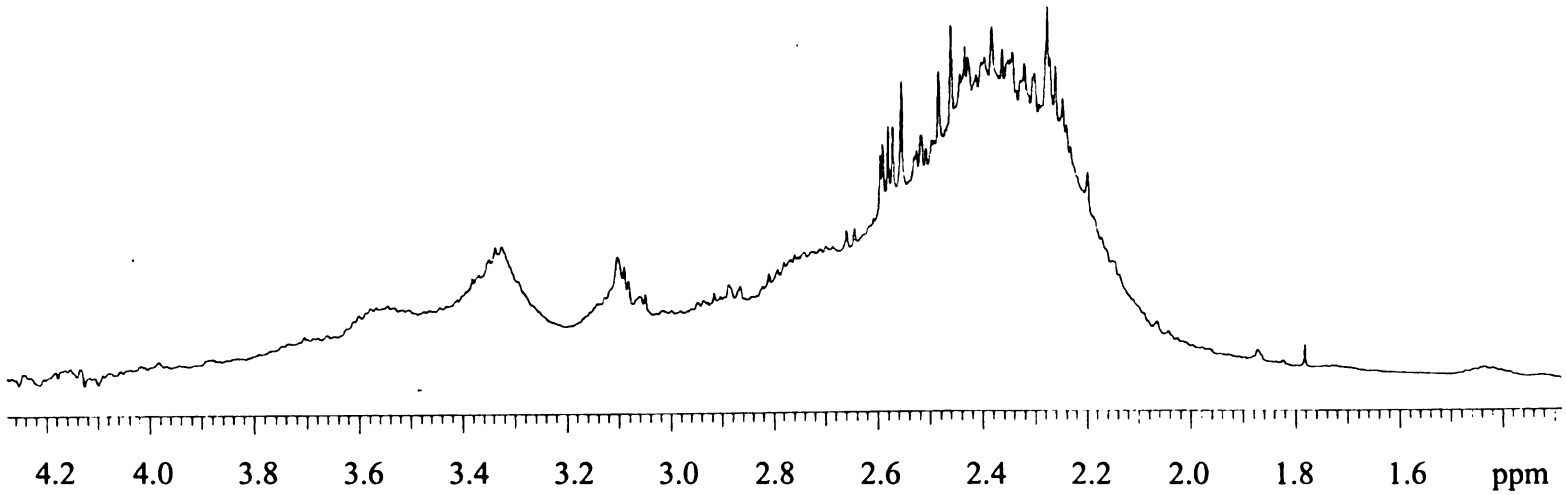


Figure 4.12 ^{1}H-NMR spectrum of high MW fraction using Bio GelP-60 chromatography

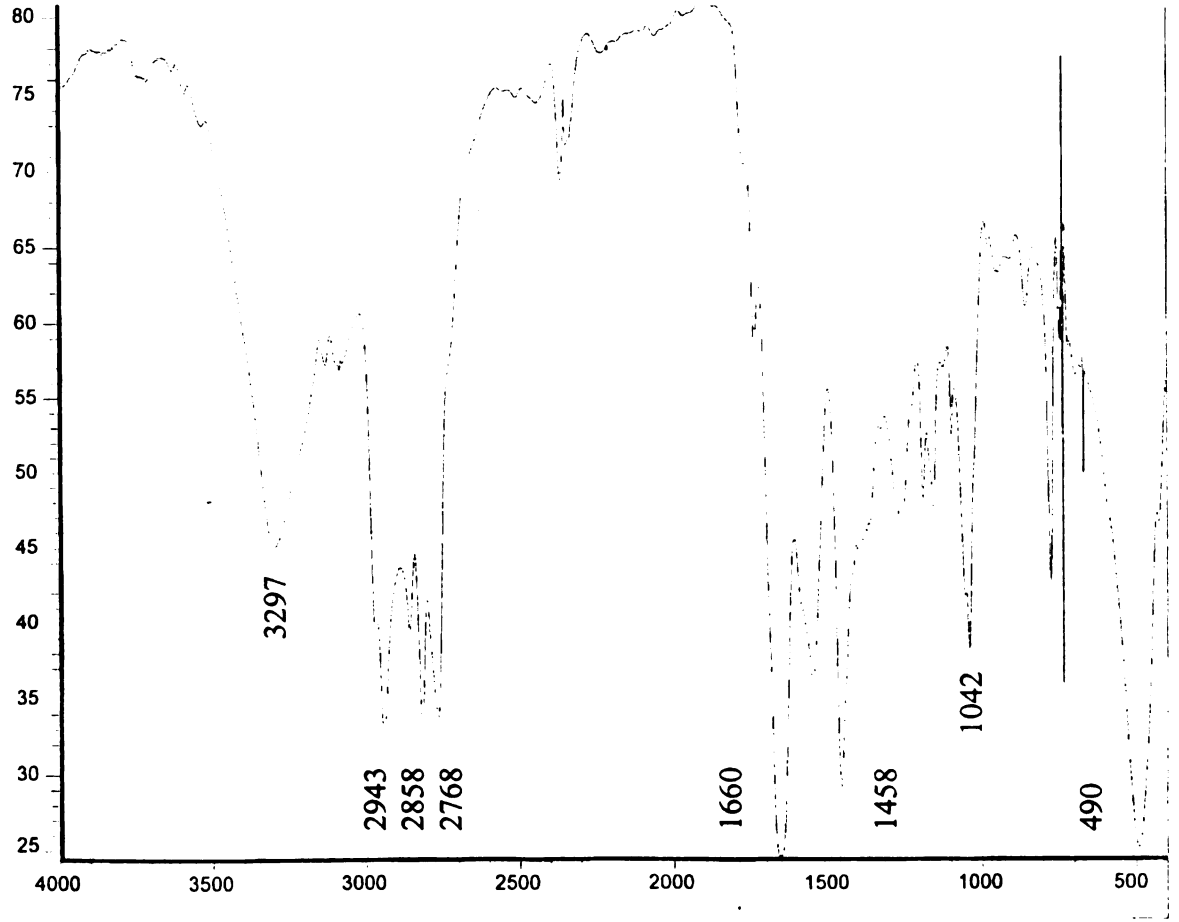


Figure 4.13 FTIR spectrum of the high MW fraction isolated by Bio GelP-60 chromatography

4.4 Drug delivery properties of poly(β-amino acid)

4.4.1 Anticancer drug delivery (doxorubicin)

There are countless drugs that are effective in killing cancer cells in the laboratory, but act differently in a living system because they do not affect only the tumor, and cause extremely harmful side-effects. Doxorubicin is one of the most widely used anticancer drugs (see figure 4.14). It is effective in the treatment of many solid tumors such as lymphomas, tumors of the breasts, lungs, ovaries, testes, prostate, cervix, head and neck. It is also a therapy for osteogenic sarcomas, Ewing's sarcoma, AIDS related Kaposi's sarcoma etc.[1-2]

Doxorubicin is an anthracycline antineoplastic agent produced by the fungus streptomyces peucetius[3]. Doxorubicin damages DNA by intercalation of the anthracycline portion of the molecule, metal ion chelation, or by generation of free radicals. It also inhibits DNA topoisomerase II, an enzyme that is critical to DNA function by making the reproduction of DNA effective.[1, 3]

Doxorubicin also is called "red death" because of its physical appearance (red color) and its extremely inherent toxicity. From the name, we could imagine how dangerous its side effects could be. Since doxorubicin is water soluble and diffuses into cells quickly and freely, it has been associated with a number of toxicities such as hair loss, nausea, vomiting, diarrhea, allopecia, stomatitis, esophagitis, cardiotoxicity and bone marrow depression which lead to anemia, greater risk of bleeding, infection etc[1, 3]. Among them, cardiotoxicity is a major concern during doxorubicin therapy. The heart contains excessive enzymes that convert doxorubicin to free radicals[4], however, unlike most

tissues, the heart has poor defense mechanisms against free radicals. The risk of cardiotoxicity is proportional to the cumulative dose of doxorubicin received[1].

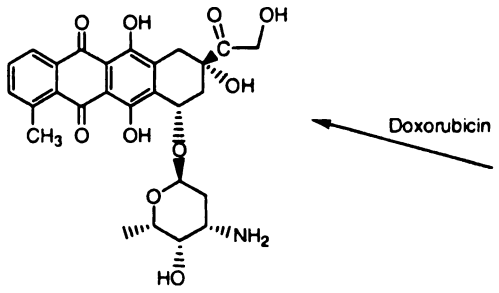
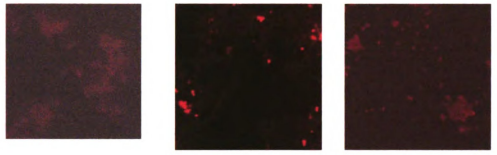


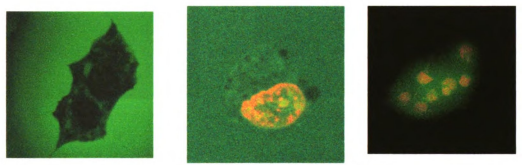
Figure 4.14 Structure of doxorubicin

Liposome delivery systems have been used to deliver doxrubicin[5], however the side effects are large[1, 3]. Developing an efficient polymeric drug delivery system with good drug binding ability is the key to solving this problem[6, 7]. The polymeric delivery system we prepared and tested was very effective at sequestering doxorubicin and releasing it inside mouse embryonic fibroblasts (MEF) cells. This is illustrated in Figure 4.15 A to F. Figure A shows a florescence micrograph of a solution of doxorubicin in phosphate buffer saline (PBS) solution. The entire field is evenly covered showing that the drug is soluble and completely diffused throughout the solution. Figure 4.15B shows a florescence micrograph of a PBS solution of doxorubicin with the high molecular weight fraction of poly (β-amino acid) as the carrier. Figure 4.15 C shows a florescence micrograph of a solution of doxorubicin with the medium molecular weight fraction of poly (β-amino acid) as the carrier in phosphate buffered saline solution. These two figures (B &C) show that for the two different polymers, the brightness of colloidal gel particles of polymers in PBS solution were different, the higher MW polymers have better binding ability to doxorubicin. Figure 4.15 D shows the high MW polymer without

doxorubicin inside of one cell which is undergoing division. The green color corresponds to the green fluorescence of poly(β-amino acids). Figure E shows that with the carrier, doxorubicin molecules were inside the nucleus of one cell. The orange color is due to the green fluorescence of the polymer plus red fluorescence of doxorubicin. Figure F shows that with the carrier, doxorubicin molecules inside several cells were trapped in a polymer colloidal gel particle (green plus red). With such good sequestering properties by the polymers, the doxorubicin can be delivered to the target in a controllable manner. As a result, the serious side effects of doxorubicin can be decreased by a large degree.



(A) (B) (C)



(D) (E) (F)

Figure 4.15 (A-F) Light micrographs showing of doxorubicin sequestration and delivery to MEF Cells.

(A) Doxrubicin buffer solution without polymer binding

(B) Doxorubicin buffer solution with polymer (MW=16978)

(C) Doxorubicin buffer solution with polymer (MW=5383)

(D) Polymer inside MEF cells without doxorubicin

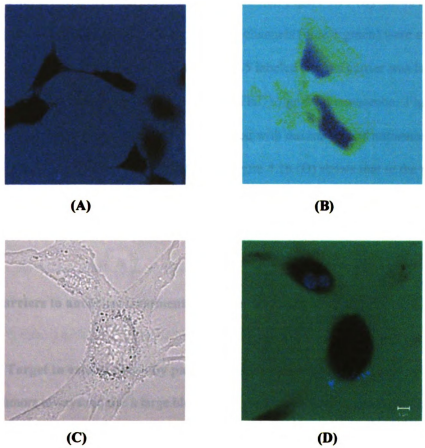
(E) Polymer in one MEF cell with doxorubicin

(F) Polymer gel particle with doxorubicin inside MEF cells

* Doxorubicin was red fluorescence, polymer was green fluorescence (labeled by FTIC) Orange is due to red plus green when polymer exists with doxorubicin.

4.4.2. Delivery of antisense human telomerase RNA (GCG CGG GGA AAA GCA)

The light micrographs of the poly(β-amino acid)polymers delivery system with the antisense drug (GCG CGG GGA AAA GCA) are shown as follows: (See Figure 4.16 A-D)



(A) **(B)**

(C) **(D)**

Figure 4.16 The light micrographs of antisense delivery

(A) MEF cells with Cy5 labeled antisense without polymers (blue fluorescence image)

(B) MEF cells with Cy5 labeled antisense with polymers (green + blue fluorescence image)

(C) MEF cells with Cy5 labeled antisense and polymers (transmission image)

(D) MEF cells with Cy5 labeled antisense and polymers (green + blue fluorescence image)

One of the barriers of antisense delivery is that unlike most small molecule drugs, antisense oligonuleotides have very high densities of negative charge which inhibit penetration of cell membranes. Hence in Figure 4.16 (A), the labeled antisense molecules are not taken up by cells in the absence of carrier. Figure 4.16 (B) shows the efficient antisense translocation by the carrier. Two channels (blue + green) were monitored. The antisense molecule fluoresces blue with Cy5 labeled and the carrier was labeled with fluorescent fluorescein isothiocyanate (FTIC) to green fluorescence. Figure 4.16 (C) is a bright field image of a cell that was treated with the carrier and antisense oligonucleotides. The cell is still intact. Figure 4.16 (D) shows that in the presence of carrier, no lysis of cells was observed and the Cy5 labeled antisense oligonuleotides (blue) were delivered into the cells and mainly localized in the nucleus.

Barriers to antisense treatment of cancer

I. Target to cancer site---by passive targeting mechanisms.

Tumors always require a large blood supply and demand highly vascularized tissue to maintain their rapid rate of growth[8] .The vasculature of tumors is extremely different from normal tissues. Unlike normal tissue, tumors have leaky capillaries, high vascular density and permeability–enhanced factors[9, 10]. Another characteristic is the dysfunction of the lymphatic system that is responsible for the drainage of macromolecules from normal tissues.[11, 12] Because of the **enhanced permeability and retention effect**, polymers can enter tumor tissues and remain there for a prolonged time, while small molecules are not retained because of their ability to return to the circulation by diffusion. Rapidly dividing cancer cells constantly ingest nutrients from their surroundings by macropinocytosis or

random gulping of extracellular fluid[12]. Because of this, the DDS can be efficiently internalized without modification with some special targeting of cell-surface receptors in certain types of cancers[13].

II. Subcellar barrier—lysosome escape

The major barrier to the subcellular level of drug delivery is whether the drug can successfully escape from the lysosomes. The lysosomes contain a number of degradation enzymes and also have a harsh environment that renders drugs ineffective too soon after the drug delivery system enters the cell. Figure 4.16 (D) proves that the antisense drug is able to successfully escape from the lysosome and reach the final target—the nucleus.

The following Figure 4.17 is the strategy for subcelluar drug delivery by the cationic poly(β-amino acid). In the delivery mechanism outlined in Figure 4.17, the positive charge of the carrier binds the drugs tightly and facilitates their interaction with the cell membrane which has an overall negative charge. The drugs are taken up into cells by the process of endocytosis within structures called endosomes. A family of enzymes called lysosomal thiol-dependent proteases catalyze the cleavage of the polymer-backbone to set the drug free. Protonation of the carrier also leads to unfolding and this also facilitates drug release. The drug /carrier complex also passes through the nuclear membrane, which is the ultimate target for drug processing.

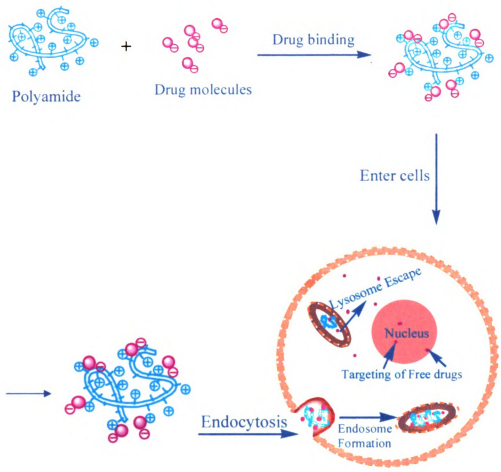
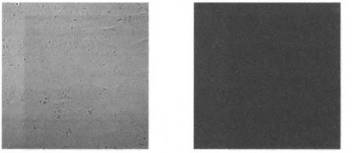


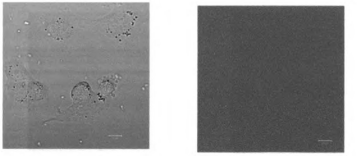
Figure 4.17 Subcellular drug delivery by poly(β-amino acid)polymers

4.4.3 Plasmid GFP gene delivery by the poly(β-amino acid)polymers

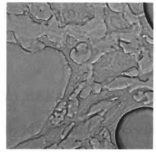
The delivery of an entire plasmid into a cell is the biggest challege in the gene therapy field. There are a few commercial products for this "transfection" process, but their efficiency is usually very low. In some cases, a large proportion of cells is damaged. Super Fect is one of these commercial products. We compared the efficiency of our carriers to Super Fect (SF) in the delivery of plasmids containing a gene for green fluorescent protein (GFP) into mouse embryonic fibroblast (MEF) cells. Light micrographs describing the results of these experiments are shown below in figure 4.18 (A-L):



(A) **(B)**



(C) **(D)**



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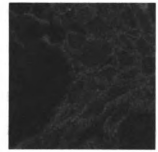

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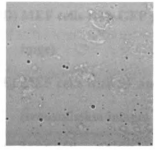

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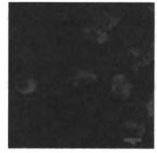

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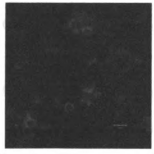

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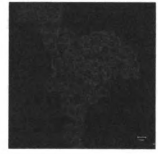

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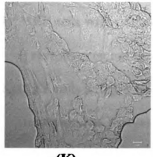

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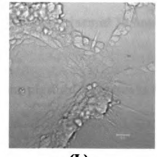

(L)

Figure 4. 18 (A—L) fluorescence and transmission light micrographs of MEF cells illustrating transfection of plasmid DNA encoding GFP mediated by the drug delivery vehicles described here and a commercial transfection agent (Super Fect ^{Tm})

(A) MEF cells 12 hours in incubation (transmission image)

(B) MEF cells 12 hours in incubation (green fluorescent image)

(C) MEF cells with GFP gene plasmid 12 hours in incubation (transmission image)

(D) MEF cells with GFP gene plasmid 12 hours in incubation (green fluorescent imge)

(E) MEF cells with SF and GFP gene plasmid 12 hours in incubation (transmission image)

(F) MEF cells with SF and GFP gene plasmid 12 hours in incubation (green fluorescent image)

(G) MEF cells with polymer and GFP gene plasmid 12 hours in incubation (transmission image)

(H) MEF cells with polymer and GFP gene plasmid 12 hours in incubation (green fluorescent imge)

(I) MEF cells with polymer and GFP gene plasmid 18 hours in incubation (green fluorescent image)

(J) MEF cells with polymer and GFP gene plasmid 33 hours in incubation (green fluorescent image)

(K) MEF cells with SF and GFP gene plasmid 17 hours in incubation (transmission image)

(L) MEF cells with SF and GFP gene plasmid 17 hours in incubation (transmission image)

Figure 4.18 A is a transmission light micrograph of mouse embryonic fibroblast (MEF) cells after 12 hours of incubation without the carrier or the plasmid. Figure 4.18 B shows the same microscope field but this time only monitoring the green channel for the green fluorescent protein (GFP). From the images, it is known that the cells have no intrinsic green fluorescence.

Figure 4.18 C is a transmission light micrograph of mouse embryonic fibroblasts (MEF) cells after they were incubated with a GFP encoding plasmid and no carrier for 12 hours. Figure 4.18 D shows the same microscope field, but only monitoring the green channel for GFP. The results show no evidence for transfection.

Figure 4.18 E is a transmission light micrograph of mouse embryonic fibroblast (MEF) cells after incubation with a plasmid encoding for the GFP gene and the commercial transfection agent Super Fect^{Tm} for 12 hours. Figure 4.18 F shows the same microscope field monitoring the green channel for GFP. The results indicate that most cells show lysis. The cells were destroyed by Super Fect during the transfection.

Figure 4.18 G is the transmission light micrograph of mouse embryonic fibroblast (MEF) cells after they were incubated with the GFP encoding plasmid and the carrier—Poly(β-amino acid) polymers for 12 hours. Figure 4.18 H shows the same microscope field monitoring the green channel for GFP. The carrier not only efficiently mediates transfection, but also does not affect the structure of the cells.

Figure 4.18 I is a green fluorescent light micrograph of mouse embryonic fibroblast (MEF) cells after they were incubated with the GFP encoding plasmid and the carrier—Poly(β-amino acid) polymers for 18 hours. From the image, a larger number of cells show

green fluorescence after 18 hours treated with the polymer. All of the cells are intact. None of cells were destroyed by the carrier.

Figure 4.18 J is a green fluoresce light micrograph of mouse embryonic fibroblast (MEF) cells after they were incubated with the GFP encoding plasmid and the carrier—poly(β-amino acid) polymers for 33 hours. Many more cells show green fluorescence after 33 hours with the carrier and still the cells are intact (none of them showed lysis). After 33 hours, the delivery ability of the poly(β-amino acid) is still very good, which gives a strong indication that this polymer has the potential to be used as a long term controlled release delivery system.

Figure 4.18 K and L are two images of transmission light micrographs of mouse embryonic fibroblast (MEF) cells after they were incubated with the GFP encoding plasmid and the commercial transfection agent Super Fect^{Tm} for 17 hours. The images show that the cells were destroyed more seriously by Super Fect after 17 hours.

Conclusion:

The experimental results show that the poly(β-amino acid) polymers are a promising drug delivery system. They successfully sequester and mediate the delivery of the anticancer drug doxorubicin into mouse embryonic fibroblast (MEF) cells. This carrier might be useful in reducing or eliminating the serious side effects of this drug. The delivery of RNA, gene or gene sequences into cells is an especially challenging task. Poly(β-amino acid) carriers described here efficiently delivered the RNA antisense (GCG CGG GGA GCA AAA GCA) drug into MEF cells without any cell lysis occurring. Thus, they provided another exciting prospect for curing cancer. Poly(β- amino acid) carriers also showed excellent suitability for the transfection of plasmids. Compared with the commercial transfection agent Super Fect^{Tm}, they also showed superb non-cell lysis properties, which will be the most significant property as a drug delivery system.

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Chapter 5.0 preparation of cationic polyelectrolytes

Introduction

Polyelectrolytes are polymers containing charged groups at regular intervals along the length of the chain. The preparation of such materials is another important application for the poly(β-amino acid) we described in the earlier chapter 3. At low pH, protonation of the dimethylaminoethyl side chain would result in the formation of a polyelectrolyte. A permanent charge can be obtained by alkylation leading to quaternization of the side chain nitrogen and the formation of a cationic polyelectrolyte. Polyelectrolytes can be used to stabilize or destabilize interactions between charged particles in numerous applications. There are several examples showing when polyelectrolytes can be used to inhibit the aggregation of particles. For instance, printing inks are suspensions of colloidal particles and the aggregation of these particles leads to loss of resolution and blockage of the ink dispenser. This can be reversed or inhibited by coating the pigment (color) particles with a polyelectrolyte so they do not associate with each other. Further, bacteria and other microorganisms are invariably negatively charged and tend to disperse in aqueous media. Polyelectrolytes are often used as flocculants[1-3] to produce aggregates from these dispersions in wastewater treatment. Polyeletrolytes are also frequently applied directly to soil as conditioning agents to maintain soil structural conditions by stabilizing the colloidal nature of the soil particles. They are also used as carrier gels for fluid drilling in the placement of pre-germinated seeds in agricultural and horticultural practice[4]. Besides other uses as thickeners, detergents[5] and coagulants, one important and exciting use of polyelectrolyte gels is in the fabrication of solid electrolyte batteries that have greater safety because they are less likely to leak toxic liquids[6]. Last, but not least,

as a biosensor, polyelectrolytes can be used for the controlled drug delivery[7-9] triggered by different bio-stimuli such as change in pH, the concentrations of enzymes, sugars, antigens, etc. In this study, cationic polyelectrolytes were prepared by protonation and by methylation of poly(β-amino acid) with dimethylaminoethyl side chains. Polyelectrolytes often form hydrogels, which make them significant materials for use in controlled drug delivery systems.

Smart polymer material—Hydrogels

Hydrogels are polymers that will absorb at least 10-20% amount of fluid and swell when placed in water or other biological liquids to form a gel. Water absorbance of hydrogels, depending on hydrophilic structure of polymers, can vary from 20% to many times their dry weight. One of the most remarkable properties of hydrogels is that the swelling or shrinking can be triggered by a change in the environment surrounding the delivery system. The swelling or shrinking of a hydrogel is reversible and repeatable after additional changes from the external environment. Depending on the different compositions of hydrogels, the environmental change can involve pH, temperature, ionic strength etc. A number of these environmentally sensitive or "intelligent" hydrogel materials are listed in Table 5.1[10]. Because variations of pH are known at several body sites[11], the pH sensitive hydrogel drug delivery system has received more attention by scientists. Hydrogels are elastic in nature because of the presence of a memorized reference configuration to which they return even after being deformed for a very long time. In addition, they consist of polymers combined with water and as such have dual characteristics. Hydrogels show a solid character due to the polymer, which make them available in a variety of structures for different drug delivery functions. They also display certain water–like properties, such as

permeability, for many water-soluble substances. When hydrogels are loaded with drugs, they can be implanted into the human body and establish the controlled drug release at specific pH values. Through further modifications, hydrogels can release drugs under different stimulations as we mentioned in table 5.1: such as different pHs, temperatures, magnetic fields, ultrasonic pulses, electric fields, etc.

Table 5.1 stimuli-responsive hydrogels in drug delivery

Stimuli	Polymer	Drug	Refs
Magnetic field	Ethylene-*co*-vinyl acetate (EVAC)	Insulin	12
Ultrasonic radiation	(EVAC); Ethylene-*co*-vinyl alcohol	Zinc bovine insulin Insulin	13
Electric field	Poly(2-hydroxyethyl methacrylate)	Propranolol Hydrochloride	14
Glucose	EVAC	Insulin	15
Urea	Methyl vinyl ether-*co*-maleic anhydride	Hydrocortisone	16
Morphine	Methyl vinyl ether-*co*-maleic anhydride	Naltrexone	17
Antibody	Poly(ethylene-*co*-vinyl acetate)	Naltrexone Ethinyl estradiol	18
pH	Chitosan-Poly(ethylene oxide)	Amoxicillin Metronidazole	19
	Poly(acrylic acid) : PEO	Salicylamide Nicotiamide Clonidine Hydrochloride Prednisolone	20
	Gelatin-PEO	Riboflavin	21
	Poly(2-hydroxyethyl methacrylate)	Salicylic acid	22
	Poly(acrylamide-*co*-maleic acid)	Terbinafine Hydrochloride	23
	N-vinyl pyrrolidone, polyethylene glycol diacrylate, chitosan	Theophylline 5-fluorouracil	24
Temperature	Poly(N-isopropyl acrylamide)	Heparin	25
pH and temperature	Poly(N-isopropyl acrylamide-*co*-butyl methacrylate-*co*-acrylic acid)	Calcitonin	26

*Table is adapted from Ref [10]

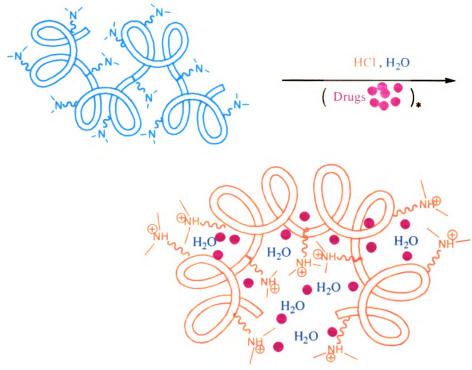


Figure 5.1 Scheme of hydrogel formation

Preparation of polyelectrolytes hydrogles by protonation

We prepared polyelectrolytes by protonating the dimethylaminoethyl side chain. A scheme illustrating hydrogel formation by the poly(β-amino acid) polymer leading to capture of drugs is shown in Figure 5.1. (* Means drug could be added into hydrogel.) The hydrogels are formed because protonated poly(β-amino acid) molecules repelled each other and then water molecules inserted into the space between the charged groups to form the hydrogels.

Experimental section

1. Polyelectrolytes by protonation

The high, medium, and low fraction polymers (0.01g) were each added to a preweighed watch glass. One drop of concentrated HCl followed by a few drops of water were added to each watch glass. The watch glass was turned over to cover the top of a 50-mL flask containing 10 mL concentrated HCl for 24 hours. (The amount of water added was controlled to prevent the polymer solution from dripping when the watch glass was turned over.) After 24 hours, each watch glass was weighed and then the three gels were dried in a vacuum oven for 4 hours to determine the amount of water absorbed by the polymer gels. (* Diameter of watch glass = 2.5 cm.) In addition, one drop of hexane and one drop of iodine were added to the gels as a stain to aid visualization, and then photographs of gels were taken.

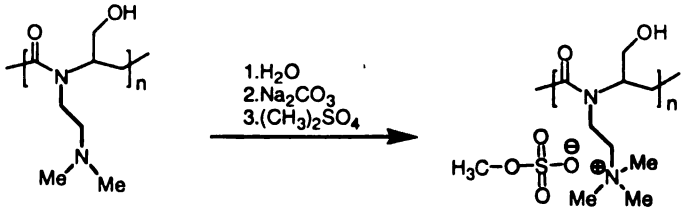
2. Polyelectrolyte hydrogels by methylation

Materials and characterization

All chemical materials used were obtained from the Aldrich Company and were analytical grade unless otherwise noted. NMR measurements were made on a Varian VXR 500 MHZ Spectrometer.

Synthesis

Na_2CO_3 (0.030 g) dissolved in 2 ml H_2O and 0.030 g of the poly(β-amino acid) dissolved in 1ml of methanol, were mixed in a 50 ml vial. Dimethly Sulfate (30μl) was then quickly added to the vial at room temperature. The reaction mixture was heated at 60 °C degree for 1 hour. The solvents were then removed by rotary evaporator. The crude polymer (0.01g) was dissolved in 1 ml of H_2O and the polymer solution was passed over an anion exchange resin (chloride form) to remove the methyl sulfate anion. The solvent (H_2O) was removed again by rotary evaporator. The final polymer solutions were lyophilized for 72 hours.



Scheme of methylation of the poly(β-amino acid)

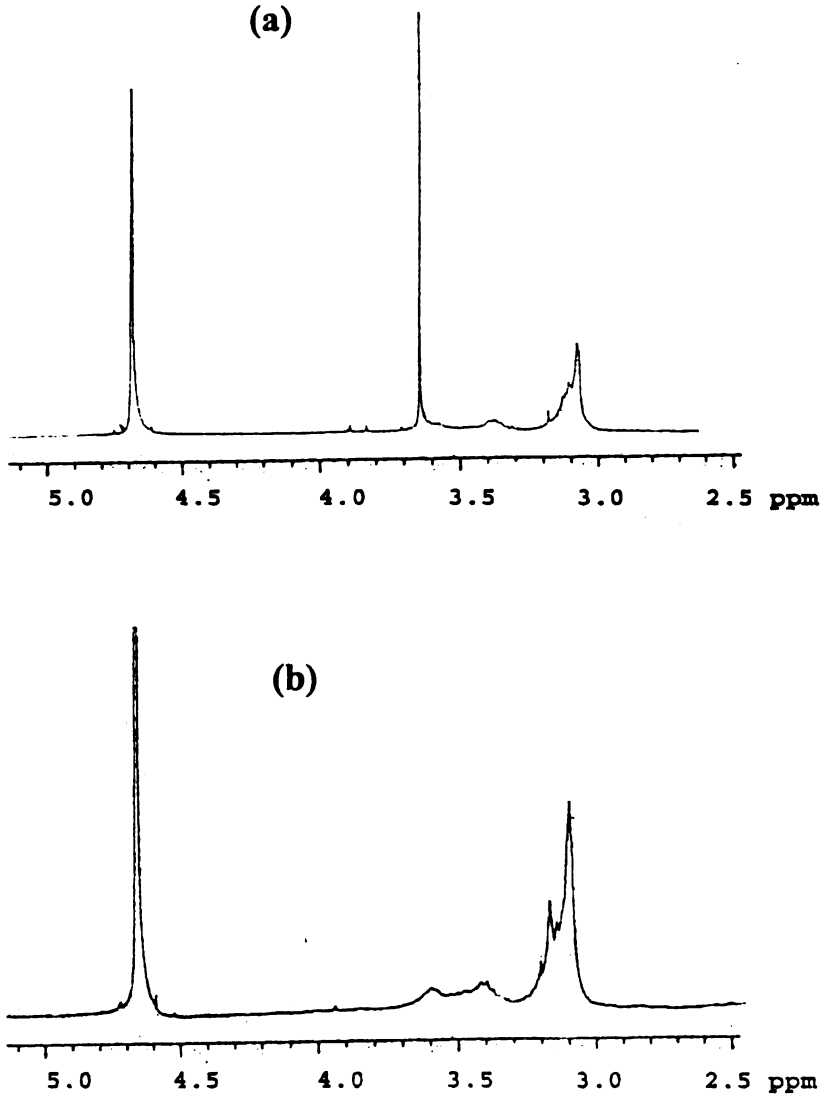


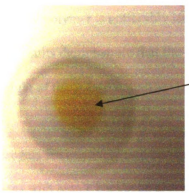
Figure 5.2 ^{1}H-NMR spectrum of methylated polymer product before ion change with chloride anion (a) and after ion change with chloride anion (b). * Solvent: D_2O

Results and discussion:

NMR spectra

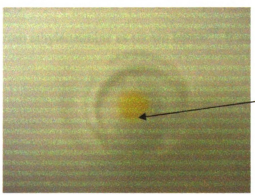
Figure 5.2 A is the NMR spectrum of the polymer product before ion exchange and Figure 5.2 B is the NMR spectrum of the polymer product after ion exchange with chloride anion. In Figure 5.2 A, the sharp peak at 3.65 ppm is the signal for the $H_3COSO_3^-$ anion. In Figure B, disappearance of the peak indicated that the $CH_3OSO_3^-$ anion was exchanged successfully.

Photographs of hydrogels of poly(β amino acid) polymer:



Iodine-stained hydrogel formed from 0.01g poly(β-amino acid) and 82% (w) water.

Figure 5.4 The hydrogel of the high MW fraction



Iodine-stained hydrogel formed from 0.01g poly(β-amino acid) and 50% (w) water.

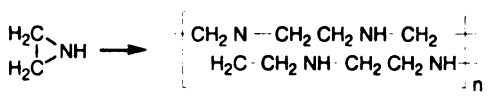
Figure 5.5 The hydrogel of the medium MW fraction.

Table 5.2 Water absorbance of different molecular weight polymer fractions

Fractions	Water Absorbance %
High molecular weight	82%,
Medium molecular weight	50%
Low molecular weight	33%

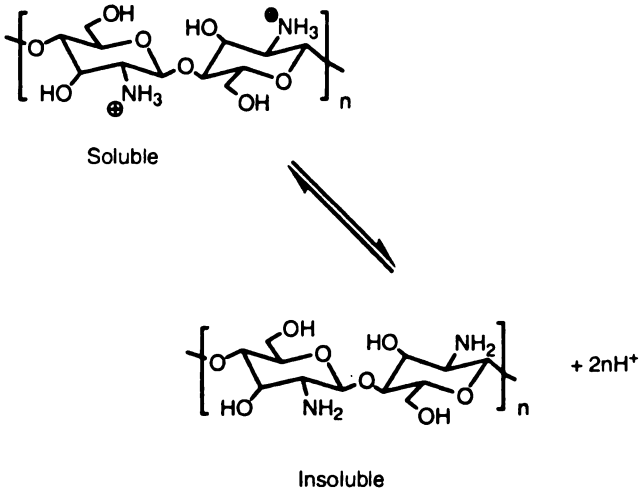
Table 5.2 shows water absorbance for the different molecular weight fractions of poly(β-amino acid) polymers. Figure 4.15 and Figure 4.16 are the photographs of hydrogels of high MW and medium MW polymer. The photographs show that the hydrogel volume from the high MW polymer is noticeably larger than that from the medium MW polymer. Experimental results demonstrate that the water absorbance is increased with the molecular weight of the poly(β-amino acid) polymers.

Cationic hydrogels are relatively rare compared to anionic and neutral hydrogels. Cationic polyelectrolytes with amide backbone are uncommon. Polylysine is one example of a cationic polyelectrolyte with a polyamide backbone, but its gel-forming ability is not well documented. The materials we described here are therefore important new contributions. Polyethyleneimine is another example of a cationic polyelectrolyte. It can be prepared from ethyleneimine as shown below[27]. It cannot be readily degraded in biological systems.



Chitosan (poly β-1,4-D-glucosamine) is the only biogenic cationic polyelectrolyte. Unlike polyethyleneimines, chitosan has good water-absorbing properties. Chitosan forms gels at high pH (>6.3)[28]. These contain as much as 98% water[28]. In contrast to the systems developed here, gel structure is maintained by a hydrogen-bonding network and not by

charge. At low pH (pH < 6), chitosan is protonated and soluble. When the pH is raised above about 6.3, the amino groups become deprotonated and this polysaccharide can form an insoluble hydrogel network [29, 30].

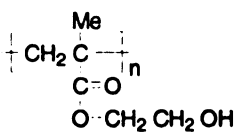


Materials that can form hydrogels under all pH conditions are desirable. This can be facilitated by converting groups that are charged in a fashion depending on pH into permanently charged groups. This was successfully accomplished in this study by methylation of the poly(β-amino acid).

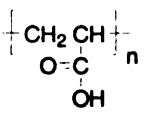
Since they were first synthesized in 1960, pHEMA (poly-hydroxyethyl methacrylate) gels have been utilized for biomedical applications. Extensive studies have been carried out on the structural, chemical properties, and applications of pHEMA. pHEMA is one of the most popular neutral hydrogels with water content of approximately 40%[31]. The water content can be regulated by copolymerization with hydrophobic or hydrophilic monomers. Currently, much research is being carried out on biomedical applications for pHEMA. This cannot be degraded enzymatically or hydrolyzed by acids or bases. One approach is to copolymerize pHEMA with maleic acid and maleic anhydride to improve their degradation, but this results in poorer

water absorption compared to pure pHEMA[32]. Compare with pHEMA hydrogels, the polymer we described here has much better water absorption properties. In addition, this polymer is biodegradable, and its poly β-peptide backbone makes it a potentially excellent biocompatible drug delivery system.

The poly(β-amino acid) system we describe also offer significant advantages over those containing polyacrylic acid (PAA) backbones, which are among the most intensely studied hydrogel systems.[33] Polyacrylic acids hydrogels are known for their ability to form extended polymer networks through hydrogen bonding. The water content of smart poly (acrylic acid) hydrogels (37.27-47.71%) prepared by Kim[34] is considerably lower than our poly(β-amino acid) system. This limits their capacity to carry drugs. Another advantage of our system is that the cationic charges on the side chains make this polymer more efficient at transporting drugs through cell membranes which have negative charges.



Poly-hydroxyethyl methacrylate



Polyacrylic acid

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