Study of injectable depots for drug delivery

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Wang Liwei

SCHOOL OF MATERIALS SCIENCE & ENGINEERING

NANYANG TECHNOLOGICAL UNIVERSITY

2005

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A thesis submitted to the Nanyang Technological University
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ABSTRACT

In this research project, a novel long-duration drug delivery system was studied. The system is a solution of a biodegradable polymer [poly (lactide-co-glycolide, PLGA)] in benzyl benzoate (BB). Such solution forms a semi-solid depot when injected into body fluids, and delivers incorporated drug over time.

The study consists of three main parts: (a) the mechanism of depot formation upon injection into buffer, as well as the ageing characteristics; (b) the degradation kinetics upon ageing and upon buffer injection, and (c) the release kinetics of low and high molecular weight model drugs from such depots.

The mechanism of depot formation is investigated using rheometry, gel permeation chromatography (GPC) and differential scanning calorimetry (DSC). Nuclear magnetic resonance (NMR) of ^1H and ^{13}C was used for detailed structure characterization. Gel formation is shown to be related to the blockiness of the –GA unit in the copolymer, i.e. only polymers that have a certain average block length for glycolide units, form gels and depots.

The details of the gelation process are also solvent-dependent. For those copolymers with average block length of –GA unit not less than 2.9, gel formation is also found in ethyl benzoate (EB), ethyl acetate (EA), tetrahydrofuran (THF), and dichloromethane (DCM); while in other solvents such as n-methyl-2-pyrrolidone (NMP), triacetin, acetonitrile, no gel formation occurs. This is rationalized on the

basis of three-dimensional structure formation between GA units in the blockier polymers.

When evaluating various PLGAs in benzyl benzoate, we have found that only those that gel upon ageing also form gel depots in buffer. This indicates that depot formation in this system is due to the same mechanism of gelation during ageing, and the gelation/ depot formation in PLGA/BB system is exclusive to copolymers with certain microstructures which is fundamentally different from the non-solvent induced phase inversion depot formation that has been observed for PLGA in water-miscible solvents.

The nature of the encapsulated drug shows considerable effect on the hydrolysis of PLGA, reflecting clear catalytic effects of base drugs such as metoclopramide or lidocaine.

The in vitro drug release kinetics from PLGA/BB system is controlled both by diffusion and erosion, with the base form of the drug being always released faster than its salt form. This is due to base-catalyzed hydrolysis and better solubility of base drug in the depot. Distinct deviation is found in the release amount of proteins (bovine serum albumin and chicken egg lysozyme) quantified by size exclusion chromatography (SEC) and bicinchoninic acid (BCA) protein assay, especially in the later stage, indicating PLGA hydrolysis-induced instability of proteins. Protein drug also displays incomplete release. Low molecular weight neutral salts such as sodium chloride, or sugar such as glucose and sucrose can increase the release rate and completeness of release by several times, while additives that accelerate PLGA degradation have little effect.

Chapter 1

INTRODUCTION

1.1 Background

Chronic conditions require drug delivery over prolonged periods of time. While drug delivery from an oral or transdermal system is limited from 24 hours up to 7 days (using current technology). Several commercially available implants (e.g., Norplant[®], DUROS[®]) are designed to release drug over months and years¹. The gap between 7 days and 12 months of delivery has been bridged by a few microsphere- or hydrogel- based injectable systems, such as TAP Pharmaceuticals' Lupron Depot[®], OncoGel[®] or Regel[®] from MacroMed Inc. Lupron Depot[®] is an injectable microsphere product and available for release of leuprolide acetate over 1, 2, and 3 months; OncoGel[®], which uses Macromed's ReGel[®] system and contains paclitaxel for intratumoral injection, followed by a continuous drug release over a period of 6 weeks; ReGel[®] has also been tested for the parenteral delivery of insulin. After one injection in rats, elevated plasma insulin levels were maintained for at least 15 days².

A recent series of patents³⁻⁵ and publications⁶⁻²² has disclosed a novel delivery approach that potentially can provide prolonged zero-order release for this duration. These innovative systems, which are easier to administer and better accepted by patients than existing delivery solutions, are prepared by dissolving biodegradable polymers in biocompatible organic solvents^{2-4, 13-17}. Drug is added into the polymer solution, where it forms a homogenous solution or a suspension depending on its

solubility. The drug suspension or solution is injected subcutaneously, forming an “in-situ implant”, which slowly releases the drug over time.

Several formulations have been studied using polymers such as polylactide, poly (lactide-co-glycolide), or poly (ϵ -caprolactone) in solvents such as N-methyl-2-pyrrolidone (NMP)^{7,8,12,19}, dimethyl sulfoxide (DMSO)^{6,12,21}, glycofurol [polyethylene glycol monotetra-hydrofurfuryl ester]^{13,14}, triacetin^{11,22}. These solvents are fully or partially water miscible, and hence the in situ gels or implants are formed by a nonsolvent induced phase separation (NIPS) mechanism^{7,19}. The mass transportation (drug release) kinetics is associated with the rate of phase inversion and the gel/implant morphology. Due to this mechanism, many of these systems display an initial burst. The initial burst can be problematic with potent drugs that have a narrow therapeutic window, such as human growth hormone and many other protein drugs.

Researchers at ALZA Corporation have refined the system to minimize the so-called “burst effect,” or rapid release of drug in the first 24 hours, which is accomplished through control of the internal depot morphology and constant drug dissolution rates. Ethyl benzoate^{8,15,19,20}, and benzyl benzoate (BB)^{15,19} were introduced as solvents. It was reported that solutions of low molecular weight PLGA polymers (M_w 12,000 to 20,000; L/G 50/50) in BB provide stabilization of macromolecules and a long-lasting delivery profile without initial burst¹⁹. The PLGA/BB system is claimed to be a slow phase inversion system, and the slower phase inversion kinetics leads to membranes that are very smooth and with much smaller pore size than with water miscible solvent systems (the NMP membranes are sponge-like in comparison). This

morphological difference is believed to account for the observed lower burst effect in the PLGA/BB system.

1.2 Problems

Biodegradable polymer solutions have shown great promise in the area of delivering protein and peptide drugs as well as lower molecular weight drugs. However, the properties of such solutions are poorly characterized, although these properties directly affect the final administration. Specifically, for injectable drug carriers, the requirements for the solution properties, besides biocompatibility (defined as the ability of a material to perform with an appropriate host response in a specific application²³), include: (a) a proper viscosity for injection, (b) good stability during storage or transportation (since most pharmaceutical products must be designed to be stable in storage over a 2-year period), and (c) for drugs which do not dissolve, the suspension should be viscous enough to maintain a homogenous dispersion of drug, and (d) ability to form semi-solid depot upon injection.

Furthermore, the key to the success of this system is controlling the so-called initial “burst” effect. Most published formulations depict the problem of initial burst. Published work focuses mostly on polymer solutions in fully water-miscible solvents such as NMP and DMSO, or partially water-miscible solvents such as triacetin, glycofurol (polyethylene glycol monotetra-hydrofurfuryl ester). Little work has been done on the system with solvents having less water miscibility, such as ethyl benzoate and benzyl benzoate, while theoretically, such systems can offer nonaqueous polymer solutions for the stabilization of macromolecules, decrease exposure of the entrapped drug (especially those protein and peptide drugs which are

easily hydrolyzed) to water until it is released into the body, and hence produce a greatly reduced initial burst and prolonged sustained release.

Most in-situ formed depots are formed via non-solvent induced phase separation (NIPS) mechanism. However, in view of the limited solubility of BB in water, the mechanism of depot formation may not exactly be the same as in NIPS.

It should be mentioned that biocompatibility, in this context, is the lack of local and systemic cytotoxicity, at the doses used for the administration. N-Methyl-2-Pyrrolidone (NMP), although considered to be fairly toxic, has been approved for use in an injectable system (Eligard™) manufactured by Atrix Labs. Although no BB or EB-containing products have been approved, it is expected that these solvents are comparable to NMP in cytotoxicity.

1.3 Objective

The aim of the study is to evaluate the effect of several variables (molecular parameters of polymer, type of solvent, type of drug, polymer concentration, molecular weight, drug concentration, additives, time and temperature etc.) on:

- Mechanism of gelation during ageing and the mechanism of depot formation upon injection into buffer.
- Drug release kinetics
- Storage stability and in vitro degradation of the formulations.

in an in-situ gelling system.

1.4 Outline of This Work

In order to achieve the above objective, the work undertaken is outlined as follows:

- a) Study the properties of PLGA solutions in selected solvents, during ageing:
 - Degradation of PLGA solutions during ageing
 - The nature of the gel formed during ageing
- b) Study the “in vitro” properties of PLGA solutions upon buffer injection:
 - Degradation of PLGA solutions
 - Liquid-liquid demixing after buffer injection
 - Morphology of PLGA solutions in vitro
- c) Study sustained release of model low molecular weight drugs and protein drug:
 - Release of low molecular weight drugs—metoclopramide salt and base from PLGA/BB system
 - Release of low molecular weight drugs—metoclopramide salt and base from other PLGA/solvent systems
 - Release of large molecular weight drug—bovine serum albumin (BSA) from BB system
 - Release of large molecular weight drug—bovine serum albumin (BSA) from other PLGA/solvent systems

Chapter 2

LITERATURE REVIEW

The literature review is divided into the following sections:

Introduction to Controlled Drug Delivery

Protein / Peptide Drug Delivery

Biodegradable Drug Delivery Depots (in situ formation)

Non-solvent Induced Phase Separation (NIPS)

Gelation of Polymer Solutions

2.1 Introduction to Controlled Drug Delivery

The term drug delivery covers a very broad range of techniques used to get therapeutic agents (small molecule drug, protein, DNA, etc.) into the physiological environment. It may include tailoring of the release at a required rate (controlled release), delivery to the required site (targeting to specific area or organ), reducing dosing frequency (sustained release), all with the benefits of reduced danger of overdose and side effects. In the early stages of research on drug delivery, the primary objective of controlled release products was to develop sustained release, ideally zero-order release devices, which aim to maintain a constant drug concentration in blood for an extended period of time. Current technology has improved to such a level that delivery of some drugs for periods ranging from a few days to years is no longer a concern. Currently, research is focused on improved delivery systems for proteins and bioactive agents, particularly systems that deliver the agent for prolonged periods in the body. One such system is the so-called injectable depot, which is the focus of this work.

2.1.1 Release Devices, Mechanism and Kinetics

The release mechanism and kinetics usually are the most important issues considered by researchers. Mathematical modeling has been employed to simulate the release kinetics of different delivery systems²⁴⁻²⁹, which provides a scientific knowledge base concerning the drug transport kinetics.

Based on the release mechanism of the therapeutic agents, controlled release devices are usually categorized as:

- a. Diffusion-controlled release devices, including monolithic devices, membrane controlled devices, and swelling-controlled devices.
- b. Chemically controlled approaches, including degrading monolithic devices as pure degradation (bulk or surface) and combined degradation/diffusion; polymer-drug conjugates in which drug is released by chemical bond scission.
- c. Externally controlled devices with externally applied trigger to turn the release on/off: the trigger can be osmotic pressure, electronic, magnetic, and mechanical, etc.

Degradation-controlled release kinetics will be reviewed in this report, since our work deals with the drug release from a biodegradable system.

2.1.1.1 Surface degradation controlled monolithic systems

In the case of surface-degrading polymer matrices, polymer degradation is much faster than water intrusion into the polymer bulk. Degradation occurs, therefore, mainly in the outermost polymer layers. Consequently, erosion affects only the surface and not the inner parts of a matrix (heterogeneous process). In degradation-controlled monolithic systems, the drug is dispersed uniformly throughout the polymer matrix, and diffusion of the drug is slow compared with the polymer degradation. Drug release is

therefore affected by the surface-to-volume ratio and the geometry of the devices³⁰. Hopfenberg³¹ analyzed the drug release mechanism from surface-degrading devices in 1976.

If M_t were the amount of drug released from a sphere of radius r in the time t , then the kinetic expression describing release would be given by:

$$dM_t / dt = k_0 4\pi r^2 \quad (2.1)$$

Where k_0 is a rate constant. Since the rate-determining erosion process occurring at position r is carried out at an ever-decreasing radius, the drug release rate dM_t / dt decreases continuously. From the mass balance, M_t is also given by:

$$M_t = (4\pi/3)C_0[r_0^3 - r^3] \quad (2.2)$$

Where C_0 is the uniform initial concentration of drug dispersed throughout the device and r_0 is the initial radius at time $t=0$. Substitution of Equation (2.2) into Equation (2.1) and differentiation gives the following relationship:

$$r = r_0 - (k_0 / C_0)t \quad (2.3)$$

Substituting Equation (2.3) into Equation (2.2) yields

$$M_t = (4\pi/3)C_0\{r_0^3 - [r_0^3 - (k_0 / C_0)t]^3\} \quad (2.4)$$

Chapter 2

diffusion-controlled systems. The situation becomes more complex if the drug is released during bulk degradation, since the matrix is gradually loosened by degradation.

Siepmann and Göpferich²⁶ have thoroughly reviewed the mathematical modeling of drug release from bioerodible polymer systems based on Monte Carlo and non-Monte Carlo models. Here we introduce some non-Monte Carlo based models for the drug release controlled by a combination of diffusion/degradation.

Heller and Baker³² developed a mathematical model describing drug release from water-insoluble polymers that undergo hydrolytic backbone cleavage. Their model is based on the following assumptions:

- i. Polymer matrices undergoing bulk erosion, degradation can be described by first-order kinetics:

$$\frac{dM}{dt} = -kM \quad M(t) = M_0 e^{-kt} \quad (2.8)$$

- ii. Starting point for their mathematical analysis was the classical Higuchi Eq.³³ describing purely diffusion-controlled drug release from non-degradable slabs. The drug is initially homogeneously dispersed:

$$\frac{dM_t}{dt} = \frac{A}{2} \sqrt{\frac{2DC_s C_0}{t}} \quad (2.9)$$

for $C_0 \gg C_s$,

$$\frac{dM_t}{dt} = \frac{A}{2} \sqrt{\frac{2PC_0}{t}} \quad (2.10)$$

- iii. In contrast to the assumption of Higuchi's original model, the permeability in biodegradable polymeric systems is not constant, but increases with time. Thus another assumption was made on the relationship between the drug permeability at time t , P_t , and the initial drug permeability, P_0 :

$$\begin{aligned} P_t / P_0 &= (\text{initial number of bonds}) / (\text{number of bonds remaining}) \\ &= N / (N - Z) \end{aligned} \quad (2.11)$$

From the assumption that polymer bonds are cleaved according to first-order kinetics, thus:

$$dZ / dt = k(N - Z) \quad (2.12)$$

Where k is the first-order degradation rate constant. This leads to

$$\ln[N / (N - Z)] = kt \quad (2.13)$$

Substitution of Equation (2.11) and (2.13) into Equation (2.10) yields

$$\frac{dM_t}{dt} = \frac{A}{2} (2P_0 e^{kt} C_0 t)^{1/2} \quad (2.14)$$

Couarraze and coworkers³⁴ recently presented a mathematical model quantifying drug release from bulk eroding polymer films, considering polymer degradation and drug diffusion simultaneously. The theoretical

calculations were compared with experimental results obtained with mifepristone-loaded poly (lactic-co-glycolic) (PLGA) matrices. Similar to the classical Higuchi model³³, they used a pseudo-steady state approach, for initial drug loadings well above the solubility of the drug within the matrix. Furthermore, they assumed a homogeneous matrix erosion process and that the polymer chain cleavages follow first-order kinetics:

$$M_t = M_0 e^{-kt} \quad (2.15)$$

and the diffusion coefficient D depends on the polymer molecular weight M_t by:

$$\frac{D_0}{D_t} = \frac{M_t}{M_0} \Rightarrow D = D_0 e^{kt} \quad (2.16)$$

where M_t and D are the polymer molecular weight and diffusion coefficient of drug at time t , M_0 and D_0 are the polymer molecular weight and diffusion coefficient of drug at $t = 0$, and k is the constant of degradation rate.

The following equation for the cumulative absolute amount of drug released, Q , versus time was derived:

$$Q = S \sqrt{\frac{2C_0 C_s D_0 (e^{kt} - 1)}{k}} \quad (2.17)$$

Where S is the surface area of the film exposed to the release medium; and C_0 and C_s are the initial drug concentration and the solubility of the drug in the system.

The authors also calculated the resulting release kinetics based on the classical Higuchi equation and found the Higuchi model only agrees with the experimental data during the early stages of drug release, when the effect of polymer degradation on drug release is not pronounced.

At early time, $e^{kt} \approx 1 + kt$

$$Q = S\sqrt{2C_0C_sD_0t} \quad (2.18)$$

Model developed by Thombre, Joshi and Himmelstein³⁵⁻³⁸ applied a comprehensive theory quantifying polymer degradation from poly (ortho ester) based delivery systems containing an acid-producing species to accelerate matrix hydrolysis. The model was developed for slab geometry, assuming perfect sink conditions. The diffusion of four different species is considered: water, acid generator, acid and drug as A, B, C and E respectively. The diffusion of these four compounds is described with single partial differential equations.

To account for the increasing permeability of the system upon polymer degradation, the following equation was applied:

$$D_i = D_i^0 \exp\left[\frac{\mu(C_D^0 - C_D)}{C_D^0}\right] \quad i = A, B, C, E \quad (2.19)$$

D_i^0 is the diffusion coefficient of species i in the polymer when the polymer is not hydrolyzed; C_D is the concentration of drug at time t and μ is a constant. From equation 2.19, one can find the diffusion coefficient of drug

is not directly related to the polymer degradation, but related to the release rate.

2.2 Protein / Peptide Drug Delivery

2.2.1 Prospect and Problems of Protein / Peptide Drugs

In recent years, there have been enormous advances in the field of protein and peptide engineering and an increased understanding of the way in which biological response-modifiers function in the body. It is now possible, through the use of recombinant DNA techniques or by solid phase protein synthesis, to produce on a commercial scale a large variety of regulatory agents that are therapeutically applicable³⁹. The continually expanding list of such agents includes interferons, interleukins, monoclonal antibodies, colony-stimulating factors, human growth hormones, human insulin of recombinant DNA origin, anticoagulants, and agents that have potential in inflammation and contraception. Nowadays second-generation products have been developed, such as second generation insulins, thrombolytics and antibodies, etc., which display either an altered amino acid sequence (achieved via protein engineering or site directed mutagenesis), an altered glycocomponent (in the case of some glycosylated biopharmaceuticals) or a covalently attached chemical moiety such as polyethylene glycol⁴⁰. The biopharmaceutical industry has grown rapidly; by 2003 some 140 biopharmaceuticals had gained marketing approval in some world region at least⁴¹, commanding an estimated

global market of some \$30 billion⁴². Approximately 250 million patients had been administered these products and currently in the region of 1 in 4 new molecular entities approved for medical use are biopharmaceuticals. An estimated 500 candidates are in clinical trials, ensuring continued approval of new biopharmaceuticals into the future⁴⁰.

However the protein and peptide drugs are difficult to process, formulate, store, and deliver, and they are highly unstable, both chemically and physically. Chemical degradation may result from hydrolysis, deamidation, oxidation, racemization, disulfide exchange, or photodecomposition. Physical instability is limited to large polypeptides and proteins, which possess high level of structural order, and involves denaturation (conformation rearrangement), adsorption at interfaces, aggregation, and precipitation. The biological activity of peptide and protein drugs can be lost as a result of chemical degradation or physical structure change. The effective drug concentration may also be reduced as a result of these changes. Care must therefore be taken during the processing, handling, and packaging of these compounds, in order to eliminate or minimize chemical and physical degradation. Generally peptides and proteins are stable at low temperature and in the solid state³⁹.

The delivery of peptides and proteins into the body is difficult owing to the poor absorption of these large molecular weight compounds and their rapid enzymatic degradation in the body. Peptidases are present in the gastrointestinal tract and in most mucosal membrane tissue in the body,

including rectal, buccal, nasal, dermal, vaginal and ocular. Thus, peptide administration has been largely confined to the parenteral routes. However, the oral and alternative routes, such as buccal, nasal, rectal, vaginal, and transdermal routes, are under investigation, and it has been shown, at least in the laboratory situation, that significant bioavailability can be achieved for polypeptide and protein drug delivery via all the foregoing routes³⁹.

2.2.2. Delivery of Protein / Peptide Drugs

This section will summarize the possible methods for releasing protein / peptide drugs. What should be considered while choosing the delivery method for different protein / peptides includes properties of the protein such as stability, dose, toxicity profile, clinical indication including the duration of treatment required, and cost of production and formulation, which is directly impacted by the bioavailability achieved by the delivery route.

In contrast to small molecular weight drugs, which are easily delivered and are often formulated as a pill, in an inhaler, or a skin patch for delivery by the oral, alveolar or transdermal routes, delivery of protein / peptide drugs is significantly limited despite the many attractive features that proteins / peptide have as therapeutic agents. Because of their relatively large size, proteins /peptides have low oral and transdermal bioavailability, and are thus currently administered by frequent injection or infusion⁴³. This has not prevented researchers from placing considerable emphasis on exploring non-injectable methods of protein delivery over the last decade, including the oral⁴⁴, vaginal⁴⁵ rectal⁴⁶ buccal⁴⁷ and dermal⁴⁸ routes.

2.2.2.1 Oral Route

It's hard to deny that, given the option, patients prefer to simply swallow a drug. They find that route of administration easiest and most convenient. However, for proteins and other macromolecules, the oral route is by far the hardest to accomplish. The oral route is problematic for protein / peptide drugs, either in stability of the drugs, or in the effective absorption. Because the gastrointestinal tract (GIT) is the necessary route for oral drugs, the chemical and enzymatic environment of gastrointestinal tract (GIT) is problematic for protein or peptide drugs. They will be degraded by chemical and enzymatic action in GIT, and the molecular weight of proteins or peptides (approximately 500—100,000 Dalton)³⁹ inhibit their abilities to cross the intestinal epithelia. Furthermore, any peptide or protein drugs that are absorbed intact may undergo first-pass metabolism in the liver. All these will lead to low bioavailability. A few small bioactive peptides, such as cyclosporine and thyrotropin-release hormone (TRH), have been successfully formulated for oral use during the past decade⁴⁹⁻⁵¹. However Hichens⁵² and Yokohama⁵³ et al claimed the bioavailability of these small peptides (TRH) in oral route is only a few percent.

The oral bioavailability of peptides and proteins can be improved by the coadministration of peptidase inhibitors, by saturation of the intestinal peptidases, by coating, by employing penetration enhancers, or by avoiding first-pass metabolism. But the above methods for improving the effect of

oral delivery are mostly in their experimental stage. We may hope there will be more oral products in the near future.

2.2.2.2 Nonconventional Routes

In comparison with oral route, nonconventional routes of administration (such as transdermal, nasal, ocular, buccal, rectal, intrauterine, and vaginal) have fewer problems concerning the gastrointestinal enzymes and hepatic first-pass metabolism. Absorption of large-molecular-weight peptides and proteins across the various epithelial barriers associated with these alternative routes are as much of a problem as in the gastrointestinal tract. Several reports⁵⁴⁻⁶⁰ have been published, and more advanced techniques and a wide variety of excipients and production processes⁶¹⁻⁶³ have been used especially for inhalation or nasal formulation, which has been thoroughly reviewed by A. H de. Boer et al.⁶⁴ Table 2-1 summarizes a number of issues that need to be considered when peptide or protein formulations for inhalation therapy are developed.

In order to increase the delivery effects, excipients that preserve the protein in its preferred state of hydration were also used to stabilize the protein in these nonconventional routes. Several studies^{62,65-66} described the role of different excipients (often in combination with production process) in the stabilization of proteins, which are classified as carbon hydrates, polyols, surfactants, water soluble polymers, proteins/amino acids, salts and chelators et cetera. However additives such as peptidase inhibitors, penetration enhancers, surfactants, possibly have side effects. For example,

some penetration enhancers such as the surfactant laurth-9 for nasal route can damage the nasal mucosa and may be toxic. Some others may cause skin irritation or permanent membrane damage.

Table 2-1 Different issues to consider for peptide or protein inhalation formulations.

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- Particle size morphology and surface characteristics
 - Moisture absorption behavior
 - Stability in dried state and when dissolved
 - Tendency to form aggregates
 - Charge of the molecule, isoelectric point
 - Solubility and dissolution behavior
 - Crystallinity and crystal form
-

In brief, each route has limitations, although peptide and protein delivery has been achieved for all of them. It seems the problems may be alleviated if milder and less irritating penetration enhancers are used, for the case of mucosal transport.

2.2.2.3 Parenteral Route

Parenteral drug delivery systems include those intended for intravenous, intra-arteriolar, intramuscular, subcutaneous, intraperitoneal, intra-articular, and intrathecal use. The simplest form of delivery system for peptides and

proteins is drug in buffered aqueous solution. The appropriate dose is then injected intravenously or subcutaneously according to the desired time pattern. However, the therapeutic use of most peptides and proteins is hampered by short half-lives of the order of seconds to hours in vivo⁶⁷, metabolic liability, immunological reactions, and the inability to achieve sufficient concentrations at the active site. Thus, targeted, prolonged-release carrier systems capable of protecting the drug and the body from unwanted reactions are being developed. These systems include soluble carrier systems such as chemically modified drugs, drug conjugates, and hybrid proteins^{67,68}, particulate carrier systems including liposomes, microspheres, nanoparticles, emulsions, red blood cells, and viruses. Among these, liposomes have attracted a lot of attention, as their structure resembles that of cell membranes; liposomes are tiny spheres that are formed when phospholipids are combined with water. However liposomes are usually unstable, have low drug-carrying capacities, and tend to leak entrapped drug substances. Viruses are extensively used as vehicles of genes for gene replacement, correction, or augmentation. Microparticles and nanospheres are also used, which are similar but have different particle sizes. They are usually formed by emulsification techniques followed by solvent evaporation, spray drying, freeze drying or third solvent extraction³⁹.

The microencapsulation into microparticles and nanospheres made of biodegradable polymers represents a practical and promising approach. Biodegradable microparticles and nanospheres can be injected

subcutaneously or intramuscularly and release the entrapped protein or peptide for extended periods of time. The design of biodegradable injectable microspheres made of polylactic-co-glycolic acid (PLGA) copolymers has had a great deal of success. This is mainly due to the accumulated information in terms of safety and biodegradability of these polymers. Examples of formulations of PLGA microspheres on market are: Lupron Depot®, Enantone Depot®, Decapeptil and Parlodel LA® etc. The review of preparation, characterization, and drug delivery application of the biodegradable PLGA microsphere was also given⁶⁹, which discussed in detail the preparation methods, means of analysis and characterization, especially the factors that would affect the properties of the microspheres and their release kinetics. Some researchers also attempted to improve the release activity by comparing the effect of different additives⁷⁰, while work of other researchers focused on the injectable suspending solution for microspheres⁷¹. Nevertheless, in spite of the high potential of PLGA microspheres for the controlled release of macromolecules, there are several difficulties, which explain, to a certain extent, the limited number of formulations on the market. These include: low encapsulation efficiency due to large molecular weight of proteins or peptides and high water solubility, protein inactivation during manufacturing process because most fabrication processes are too harsh to protein or peptide molecules, and difficulties for controlling the release of the active protein, which is why in many cases, protein loaded microspheres possess an inconsistent release profile with a significant burst and incomplete release even if the polymer

itself degrades sufficiently. These problems become even more profound in the case of the nanospheres in which the entrapment of a significant amount of proteins is even nowadays, a difficult task.

Briefly, successful formulation and process development needs to be aware that the bioactivity relies on the 3-dimensional integrity of protein and peptide. The stability of the folded state involves the interplay of a large number of both stabilizing and destabilizing interactions, and the net stabilizing interaction usually amount to only a few hydrogen bonds, hydrophobic patches, or ion pairs^{72,73}. The free energy of stabilization of the biologically active folded state in aqueous solution is, on average only about 50 KJ/mol^{72,74}. Because disruption of proper folding does not require a large input of energy, seemingly subtle aspects of manufacturing such as stirring rate can have substantial impact on the critical attributes of the final product. In addition, these quality attributes must be maintained during storage of at least 18 months despite numerous routes of both physical and chemical degradation of the proteins. As a result, it is more complicated and requires more time to develop a safe, effective, and reliable dosage form of a protein and peptide than traditional drug compounds.

2.3 Biodegradable Drug Delivery Depots (in situ formation)

The recent development of several protein-based drug therapies has spurred renewed interest in the design of effective drug delivery devices, in order to improve the efficiency and duration of peptide/protein drug delivery. The performance of these devices is evaluated primarily in terms of their release kinetics and overall ease of administration. Devices that release drug with zero-order kinetics (a time independent rate) for an extended time period are usually considered optimal. Polymeric implants with different geometric shapes have been reported to have such release property⁷⁵, which have been proposed for the sustained delivery of several therapeutic classes, including: contraceptive steroid⁷⁶, peptide hormone^{77, 78}, prostaglandins⁷⁹, narcotic antagonists⁸⁰, anti-arrhythmics⁸¹ and anti-cancer agents⁸². Although the therapeutic potential for implant systems is immense, the commercialization of these systems has been limited mainly by the difficulty of administration, i.e., needing surgical incision for implanting, and in some cases explanting, which raises medical costs; patient discomfort is another factor that has limited the widespread use of pre-formed implant devices.

Biodegradable injectable systems that form implants or depots in situ, provide an attractive alternative. These systems are injectable fluids that can be introduced into the body in a minimally invasive manner prior to solidifying or crosslinking/gelling as a result of either a physical or chemical change of the system. These devices may present attractive approaches for protein delivery and could possibly extend the patent life of protein drugs⁸³. A number of advantages,

such as ease of administration, less complicated fabrication, and less stressful manufacturing conditions for sensitive drug molecules etc. have been considered in the controlled release of bioactive macromolecules from these (semi-) solid in situ forming systems⁸³. Several polymeric drug delivery systems with the ability to form a drug reservoir at the injection site are under investigation.

2.3.1 In situ Phase Separation System

A recent series of patents by Dunn and co-workers^{3,4} described a novel approach which potentially can provide prolonged zero order release along with much greater ease of administration and have greater acceptance by patients than surgically implanted systems. When compared with those systems fabricated as microspheres, which can be injected with a syringe and therefore also avoid surgical implantation, this new system does not have such inherent disadvantages such as: 1) the need for reconstitution before injection, 2) the inability to remove the dose once injected, and 3) relatively complicated manufacturing procedures to produce a sterile, stable, and reproducible product.

These systems are prepared by dissolving water insoluble and biodegradable polymers in biocompatible organic solvents. When the polymer solution is injected into the body, the organic solvent dissipates into the surrounding tissue as the water permeates into the implant. This process leads to phase separation and subsequent coagulation of the polymer to form an implant in situ. Drugs are added into the polymer solution to form a homogenous solution or a suspension depending on the solubility of the drugs. And if the system of

polymer, solvent and drug is unstable due to interaction of the components, the product can be prepared as a two-part system (A/B). In this system, one syringe contains an appropriate amount of polymer solution, whereas the other syringe contains the drug as a dry powder.

Though not as popular as nanospheres or microspheres, several research groups have studied the possibility of these injectable solution systems that will solidify or increase viscosity to form an implant upon injection.

Dunn et al are one of the first researchers who proposed this kind of delivery system as early as 1990. In their patent³, they claimed two kinds of systems, thermoplastic and thermosetting systems, which may be used in drug delivery or orthopedics. The thermoplastic system to form the said implant involves the steps of dissolving a non-reactive polymer in biocompatible solvent to form a liquid, injecting the liquid into the animal and allowing the solvent to dissipate to produce the implant. When this system was used for drug delivery, active agent is added to the polymer solution where it is either dissolved to form a homogeneous solution or dispersed to form a suspension or dispersion of drug within the polymer solution. An alternative, a thermosetting system comprises mixing together effective amounts of a liquid acrylic-ester terminated, biodegradable prepolymer and a curing agent, placing the liquid mixture within an animal and allowing the prepolymer to cure to form the implant. Both systems provide a syringeable, biodegradable delivery system by the addition of an effective level of biologically active agent to the liquid before injection into the body. Biodegradable polymers used in the thermoplastic

system may be polylactides, polyglycolides, polycaprolactones, polyanhydrides, polyorthoesters, polyethylene glycol, and copolymers, terpolymers, or combinations or mixtures of the above materials. The preferred polymers are those, which have a lower degree of crystallinity and are more hydrophobic. While the recommended solvents for the biodegradable polymers are non-toxic, and otherwise biocompatible. Water-miscible solvents especially N-methyl-2-pyrrolidone, dimethyl sulfoxide are preferred since they can diffuse quickly into the body fluids and allow water to permeate into the polymer solution and cause it to coagulate or solidify.

Although the liquid polymeric systems as described above have proven to be beneficial in many respects, they do not enable variable control of release rate; in particular, control so that the rate is slower. Consequently, there is the need for a liquid composition in which the rate of drug delivery can be more readily controlled especially for a drug that requires longer-term release. To this end, Dunn and his coworkers then improved composition⁴ comprising a rate modifying agent selected from the group consisting of an ester of a mono, di or tricarboxylic acid, a polyhydroxy alcohol, a fatty acid, a fatty acid ester, an epoxidized oil, a sterol, a higher alkyl alcohol, and any mixture thereof, which are less water soluble than the organic solvent. The release rate can be up to 100 fold slower relative to the rate from the same matrix without the rate-retarding agent. Among these modifying agents, water-insoluble liquid “plasticizers” are used to “soften” the copolyester and cause its diffusion coefficient to increase, thereby enhancing the diffusion of nonionic drugs, while water-soluble “plasticizers” are used to create a water-swollen

microporous structure, by leaching slowly from the copolyester, to make the composition more permeable to drugs.

Practically, Dunn et al⁶ used cisplatin (platinol as brand name), a chemotherapeutic agent as model drug to investigate the release kinetics in vivo and clinic pathology. This time solutions of poly (DL-lactide-co-glycolide) (PLGA) and poly (DL-lactide-co-caprolactone) (PLC) with dimethyl sulfoxide (DMSO) were employed. Both polymer formulations gave sustained release of cisplatin as long as 30 days. Lower peak serum levels of platinum were obtained with the implant formed in situ than with intravenous injection of the same quantity of drug. This research suggests the polymer formulations afford the potential for much more effective use of cisplatin in treating cancer patients while alleviating many of its toxic effects.

In another paper of Ravivarapu et al¹⁸, a formulation of PLGA and another solvent, N-methyl-2-pyrrolidone was used for releasing of Leuprolide acetate, a potent luteinizing hormone releasing hormone (LH-RH) analog, they also made a comparison between a commercial product LUPRON® and their own ATRIGEL®. Both products gave a high initial release of drug, but the levels with the ATRIGEL® formulation were higher than the LUPRON® microspheres initially. It shows that the exchange of water and solvent between the ATRIGEL® implant and the body fluids during the initial solidification phase eliminates the lag period and provides a more sustained and elevated release of drug until polymer erosion occurs. The inherent property of high initial release from the ATRIGEL® system can be termed

advantageous or disadvantageous only on a case specific (drug and therapeutic window, application and need for a loading dose) basis.

Among the researchers of this kind of system, A.J. McHugh et al placed their emphasis on the mechanism of phase inversion after polymer solutions were injected into buffer (in vitro) or body (in vivo) in spite of the release kinetics and overall ease of administration. This research is tied to their previous work on dynamics of membrane formation⁸⁴, which may have the same mechanism as that of the delivery system made up of hydrophobic polymer and hydrophilic organic solvent in contact with water.

The basic principle comes from the dynamic interactions that take place in the latter process in polymer membrane formation by non-solvent-induced phase separation. The ensuing solvent / nonsolvent counter diffusion lowers the polymer solubility, eventually causing the solution to phase separate into a polymer-rich matrix surrounding dispersed polymer-lean droplets. The exact arrangement of the two-phase structure determines the final membrane morphology and thus its characteristics. Different variables including coagulation bath composition, polymer solution composition, additive composition, polymer type can be tailored in order to get desirable performance depending on the final usage of the membrane. Numerous studies have demonstrated that the dynamics of non-solvent-induced phase inversion can be quantified in terms of the system ternary phase diagram (i.e., polymer, solvent and non-solvent) coupled with models for the mass transfer dynamics during the quenching period⁸⁵⁻⁸⁷. Generally speaking, conditions leading to

rapid phase separation also promote the formation of thin skins at the surface of the membrane and finger-like porous cavities in the body of the film. Conversely, changes leading to more delayed precipitation; promote thicker skins and more uniform, spongy sub layers in the body of the film. In either case, the final morphology has a direct influence on the resulting membrane transport characteristics.

A feature that may distinguish injectable drug delivery systems from the above process is that a critical aspect of its performance (its initial drug release rate) occurs simultaneously with the formation of the device by phase inversion.

So McHugh et al⁷ first examined the link between the critical aspects of the in vitro gel formation kinetics and the resulting drug release properties of injectable implant, by investigation the formulations for delivering model drug chicken egg lysozyme. The formulations are made of PLGA Resomer RG502 and NMP / triacetin, with polyvinylpyrrolidone used as an additive to the formulation. Dark ground, video imaging technique was used for recording the diffusion fringe when water penetrates into the solution, which provided a fundamental basis to understand the effects that changes in the phase inversion dynamics have on the drug release kinetics. Scanning electron microscope was employed to characterize the morphology changes. Their results showed that the release rate of protein is directly related to the rate of phase inversion as well as the morphological characteristics of the formed membrane. Additives that increase the phase separation rate, such as hydrophilic high molecular weight polyvinylpyrrolidone (PVP), increase the initial drug release rate (burst

effect) at constant morphology. Conversely additives that slow the phase separation rate, such as a cosolvent with a much lower aqueous miscibility (triacetin), lead to a reduced initial drug release rates and more dense sponge-like morphologies. Consequently, protein release was postulated to occur primarily by diffusion through the interconnected polymer-lean phase that exit in the two-phase, gelled structure.

In their later research^{8, 19}, they further investigated the role of solvent properties on the phase inversion dynamics and in vitro protein release kinetics of polylactic-co-glycolic acid (PLGA) solution, in particular the role of external environment, i.e. bath side composition. This time ternary phase diagrams of PLGA, water and different solvent (NMP, triacetin, ethyl benzoate and benzyl benzoate) were created in order to make clear the effect of solvent on phase inversion, and consequently the drug release kinetics. The role of triacetin and deoxycholate, a biosalt found in the human body, derived from oxen bile, were examined as bath side additives. They concluded that protein release from depots based on high solvent-non-solvent affinity is controlled primarily by the phase inversion dynamics that govern the rapid formation of the interconnected polymer-lean phase through which transfer occurs. Depots based on relatively weak solvents (triacetin, ethyl benzoate and benzyl benzoate) with low solvent-non-solvent affinity undergo much slower phase inversion and release protein more uniformly. In the latter case, an appreciable, interconnected porous phase is not available, and protein release is determined more by the mass transfer kinetics within the continuous, polymer-rich phase. Furthermore, the addition of a small chain triglyceride or

organic salt to the aqueous receptors bath evokes a significant increase in the mass transfer rate of protein from the low solvent / nonsolvent affinity depots.

The properties of the polymer itself, such as the role of crystallization in the phase inversion dynamics and protein release kinetics were also investigated by I.R. Desnoyer and A.J. McHugh, by comparing the systems made up of poly (ϵ -caprolactone) (PCL) and poly (D, L-lactide) (PDLA) with ethyl benzoate¹². They suggested that crystallizable polymer solution-based systems could be divided into two classes. Systems with high degrees of crystallinity are characterized by porous morphologies indicative of solid-liquid phase separation. In addition, the drug release profile exhibits two distinctive regions: an initial lag period of slow release, followed by a short period of accelerated release. Alternatively, depots with low degree of crystallinity have dense morphologies that formed by mild liquid-liquid de-mixing and more uniform drug release rates that are governed by the mass transfer characteristics of the polymer-rich matrix. It is also suggested that by tailoring the semi-crystalline/amorphous polymer ratios, it is possible to affect transformations from solid-liquid de-mixing to liquid-liquid de-mixing which produce release kinetics suitable for a variety of applications, including vaccination and extended-release therapies.

Among other researchers, Rom E. Eliaz and Joseph Kost¹⁴ developed a new system, in which a new water miscible solvent glycofurol [polyethylene glycol monotetra-hydrofurfuryl ester] was used. A typical initial protein burst characterized the release profiles of this system, and the authors attributed the

reason to the burst effect during the delay period from injection to solid implant formation. Increasing the polymer concentration and molecular weight can increase the viscosity of the solution, and thus decrease the diffusion rate of proteins into the medium. The release rate as well as the extent of the burst also can be adjusted by the co-monomer ratio in the copolymer; due to its additional methyl groups, poly (lactic acid) (PLA) is more hydrophobic than poly (glycolic acid) (PGA). Hence, the protein release rate and initial burst decreased with the increase in lactic acid co-monomer content of the implant. Overall, their study provided significant insight into formulation variables that can be adjusted to achieve a variety of release rates and burst effects.

In other research by Eliaz et al, plasmid DNA was mixed and sustained released from PLGA/ glycofurol⁸⁸. This in situ formed implant system allowed DNA to be completely released in a continuous pattern: a rapid release due to convection and diffusion of solvent out of the polymer matrix, followed by a slower phase of diffusion through a polymer “skin”, and a second rapid phase due to bioerosion of the polymer skin. Degradation and linearization were not seen in released DNA and the transfection activity retains 100 ± 20 % in vitro. In vivo experiments also showed good results on sustained protein expression and biological response mediation.

This new delivery system was also used for delivering sp55-R (the human recombinant protein)/or a mixture of sp55-R and BSA freeze dried product¹³, Fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) for

release study²¹, Plumbagin (2-methyl, 5-hydroxy, 1,4 naphthaquinone) to reduce the toxicity and enhance antitumor efficacy¹¹, and growth hormone¹⁹.

In addition, a new system developed by Genentech and Southern BioSystems should be mentioned here, which used the same phase inversion (precipitation) strategy but with a solution of a non-polymeric material, sucrose acetate isobutyrate (SAIB), a noncrystalline, viscous compound, which dissolves in solvents such as DMSO and ethanol, to form a solution having the same viscosity as vegetable oil (50–200 cP). SAIB is reported to be bioerodible and essentially insoluble in water. Upon injection of SAIB dissolved in, for example, ethanol at 50% concentration, into tissue, viscosity of the solution increases dramatically as the solvent diffuses away. The SAIB then forms a depot, which sustains the release of the drug. The modification of the sucrose acetate isobutyrate (SABER™) technology of Southern Biosystems with ethanol and benzyl alcohol also allowed the incorporation of PLA as a release modifier. This modification of SABER led to a reduction of the drug burst. Drugs that have been examined for release by this system include human growth hormone⁷¹, gonadotropin hormone⁴¹, chlorhexidine, doxycycline, diclofenac, flurbiprofen, naproxen, and theophylline⁴².

The above in-situ gelling systems show promise for prolonged delivery of drugs. However, a burst effect has been commonly observed with hydrophilic macromolecular drugs especially from systems of water miscible solvents such as NMP. The local or systemic toxicity of this system is always a concern as an organic solvent is used. The injection of such solvents is said to be

painful and may lead to local myotoxic effects, while some other researchers claimed the usage of the organic solvents was within the systemically acceptable range. Also, the organic solvent used to solubilize the polymer may physically denature proteins.

2.3.2 Thermally Induced Hydrogel Systems

Aqueous polymeric solutions that can form implants in situ have gained increasing attention. Gelation can occur in situ by ionic cross-linking⁸⁹ and or after a change in pH⁹⁰ and/or temperature⁹¹. The latter approach exploits temperature-induced phase transition, ideally at temperatures between ambient and body temperatures. Several systems have been reviewed⁹², including systems of polysaccharides^{91,93}, *N*-isopropylacrylamide (NIPAM) copolymers⁹⁴, poly(ethylene oxide-*b*-propylene oxide-*b*-ethylene oxide) (PEO-PPO-PEO) and its copolymers⁹⁵⁻¹⁰¹, known as poloxamers or Pluronics®, poly(ethylene oxide)/(d,l-lactic acid-*co*-glycolic acid) (PEO/PLGA) block or graft copolymers¹⁰²⁻¹⁰⁴.

Among the above systems, poly-NIPAAm unfortunately is not suitable for biomedical applications due to its well-known cytotoxicity and the non-degradability of poly-NIPAAm. Pluronics® solutions lead to notable cytotoxicity¹⁰⁵ at high concentration and, furthermore, they increase the plasma cholesterol and triglyceride levels in rats after intraperitoneal injection¹⁰⁶. PEO and PLA/PLGA copolymers are one promising system that has entered the market.

MacroMed developed thermosensitive biodegradable polymers based on ABA and BAB triblock co-polymers, in which A denotes the biodegradable polyester block and B denotes the hydrophilic poly ethylene glycol (PEG) block. The copolymers are designed to low molecular weight, which are water soluble and capable of enhancing the solubility of drugs and hydrophobic drugs in particular, in water, to form a drug solution. Such solution undergoes a temperature-dependent physical reversible gel–sol transition.

The carrier formulation of MacroMed, ReGel® has entered the market, and OncoGel®, a lead product of MacroMed, utilizes ReGel® drug delivery system for local treatment of solid tumors. The gel release paclitaxel continuously for six weeks and concentrates and prolongs the cytostatic effects of paclitaxel in the tumor, while avoiding the side effects. ReGel® also exhibited sustained release kinetics for protein drugs.

The above ABA and BAB triblock copolymers are very attractive, because of the ease of administration (aqueous solution) using conventional syringes, and the non-problematic biocompatibility and toxicity. However the physicochemical properties of these thermosensitive gel systems are critically dependent on the molecular structure of the copolymers, slight changes can have drastic effects on micelles properties¹⁰⁷. Another aspect deserves some consideration, namely the loading of the gels with hydrophilic drugs and proteins, and the release kinetics of such drugs since the system's volume will contract at the sol–gel transition state, leading to the expulsion of the aqueous phase in which proteins are dissolved. This effect will cause some initial drug

burst. Further the stability of proteins in the aqueous polymer solutions is also of concern, so that the shelf life of the formulations may be limited⁸³.

2.3.3 In situ Cross-linking Polymer Systems

The formation of a cross-linked polymer network is advantageous, because of the possibility to control the diffusion of hydrophilic macromolecules. Such a system could ideally release peptides and proteins over a prolonged period of time⁸³. Crosslinked polymer networks can be formed in situ in a variety of ways, such as free radical reactions initiated by heat (thermosets) or absorption of photons, or ionic interactions between small cations and polymer anions¹⁰⁸.

One potentially useful application is the biodegradable hydrogels produced from the macromer, PEG-oligo-glycolyl-acrylate with at least two free radical –polymerizable regions, using a photo-initiator, such as eosin and a light source (ultraviolet or visible light)^{109,110}. The controlled release of proteins was observed over a period of several days.

Photo-polymerization has also been proposed to prepare in situ cross-linking implants. However, photo-polymerization normally requires the presence of a photo initiator at the gelation site, which can be toxic. The cross-linking of thiol functional groups, a process that does not require radical-initiated polymerization was also studied, such as immobilizing thiol groups on water-soluble chitosan and deacylated gellan gum (DGG) for in situ hydrogel formation^{111,112}. Unfortunately the gelation requires several hours to occur, probably too long for in situ use. The penetration capacity of the radiation

source also limits the number of application sites, and the reaction can evoke enough heat to damage surrounding tissues.

An ion-mediated gelation of aqueous nanoparticle suspensions is considered to be a very promising approach, which is recently developed by T. Kissel et al¹¹³. This ion-mediated gelation is assumed due to the positive surface charge of nanoparticles, which are formulated using polymers consist of a poly (vinyl alcohol)-backbone modified with amines (DEAPA: diethylaminopropylamine, DMAPA: dimethylaminopropylamine, DEAEA: diethylaminoethylamine) using N,N'-carbonyldiimidazole (CDI) linker chemistry. The preparation of the nanoparticles in a one-step process is relatively easy, comprising only short-term or no contact of the bioactive agent with organic solvents. No additional cross-linking reagents are necessary. Besides, the injection of an aqueous suspension is a considerably less problematic process compared to the injection of viscous polymer solutions in organic solvents⁸³.

2.3.4 Thermoplastic Pastes

Thermoplastic pastes are polymer systems, which can be injected as a melt and form a depot upon cooling to body temperature. The requirements for such in situ forming depot include low melting or glass transition temperatures in the range of 25–65°C and an intrinsic viscosity in the range of 0.05–0.8 dl/g at 25°C¹¹⁴⁻¹¹⁶. Below the viscosity threshold of 0.05 dl/g no delayed diffusion could be observed, whereas above 0.8 dl/g the material was too viscous to be easily administered using a needle. At injection temperatures slightly above their melting point (normally above 37°C but below 65°C), these polymers

behave like viscous fluids that solidify to highly viscous depots. Therapeutic agents are incorporated into these viscous polymer systems by simple mixing procedures without the use of organic solvents. Thermoplastic pastes allow local drug delivery at sites of surgical interventions for the delivery of antibiotic or cytotoxic agents. Alternatively, they can be used to generate a subcutaneous drug reservoir from which diffusion occurs into the systemic circulation⁸³.

Intratumoral injection of Taxol™ or applications of the paste beside tumors or within tumor resection sites are examples of the application of these thermoplastic paste systems. Efforts have been made in development of semi-solid polymers such as polyanhydrides like poly (bis(*p*-carboxyphenoxy)propane-sebacic acid)¹¹⁷, polycaprolactone (PCL)¹¹⁸ and ABA triblock copolymers PLA-PEG-PLA, however these all have the drawback of very slow release kinetics. Another noteworthy problem confronted was the high melting temperature (>60°C) requiring injection temperatures greater than 60°C. This led to very painful injections¹¹⁹ and necrosis at the injection site. The encapsulation of the depot by scar tissue therefore inhibited paclitaxel diffusion.

Most disadvantages of this system have been overcome by the new generation of poly (ortho esters) (POE) developed by AP Pharma⁸³. In developing semi-solid formulations for drug release, two formulations are under development. One formulation is based on triethylene glycol, which produces somewhat hydrophilic semi-solids and the other on 1, 10-decanediol, which produces

hydrophobic semi-solids. In order to allow direct injection of such materials, their molecular weights were limited to no higher than about 5 kDa¹²⁰. The most significant advantage of these semi-solid materials is that therapeutic agents can be readily incorporated by mixing at ambient temperature and without the use of solvents. Such conditions are mild enough to incorporate even the most sensitive therapeutic agents. Mixing can be accomplished on a small scale by using a mortar and pestle, but on a somewhat larger scale it is better carried out using a three-roll mill¹²¹.

2.4 Introduction to Biodegradable Polymers

An appropriate selection of the polymer matrix is necessary in order to develop a successful drug delivery system. The polymer could be non-degradable or degradable. A major disadvantage with non-degradable polymers is that a surgery is required to harvest these polymers out of the body once they are depleted of the drug. Hence, non-degradable polymers can be used only for “permanent” applications (such as an ocular implant). Degradable polymers on the other hand do not require surgical removal and hence are preferred for drug delivery applications. However, since they degrade to smaller absorbable molecules, it is important to make sure that the monomers are non-toxic in nature. Biodegradable polymers have become increasingly important in the development of drug delivery systems. These polymers can either function as a matrix to control diffusion of the drug, followed by polymer biodegradation and elimination of the degradation products from the body, or they can participate in and control the rate of drug release by polymer hydration and degradation¹²². A great number of biodegradable

polymers have been developed and evaluated for drug delivery usage, but the most widely investigated biodegradable polymers and systems achieving the most success at this time are lactic/glycolic acid polymers. The major reasons are their reputation as safe suture materials and their degradation to products that are normal body metabolites¹²³.

Lactic/glycolic acid polymers are biodegradable, biocompatible, and bioabsorbable. Their biodegradation products are nontoxic, noncarcinogenic, and nonteratogenic. In general, polylactide, polyglycolide and the copolymer have demonstrated satisfactory biocompatibility and absence of significant toxicity by serious of in vitro and in vivo studies¹²⁴⁻¹²⁶, although some reduction in cell proliferation has been reported¹²⁷.

These polymers are easily processed, have good mechanical properties, and are sterilizable. Rates of biodegradation and mechanical properties can be custom-tailored by changing polymer composition (i.e., the copolymerization ratio of lactic acid and glycolic acid) and molecular weight. In addition to these advantages, the monomers for the synthesis of these polymers can be derived from many sources, including renewable plant sources, petroleum, coal, and natural gas¹²⁸.

Lactic/glycolic acid polymers can be categorized into low molecular weight polymers or oligomers and high molecular weight polymers. The low molecular weight polymers have a molecular weight range of a few thousand Daltons, whereas the molecular weight of high molecular weight polymers is usually defined as over 10,000 Daltons. Two polymerization processes have been employed in synthesis of lactic/glycolic acid polymers or copolymers, i.e.,

condensation from lactic acid and glycolic acid and ring opening from lactide and glycolide. Condensation polymerization is employed for low-molecular weight polymer, while ring opening is used to synthesize high molecular weight polymer. The formation and structure formula is listed below.

The polymerization method, mechanism and kinetics have been thoroughly reviewed¹²³.

PLA, PGA, and PLGA structure:

Synthesis by condensation polymerization:

Synthesis by ring-opening polymerization:

Lactic acid or lactide, the basic repeat units in these polyesters, is a chiral molecule that exists as two optical isomers, D and L. The ratio of D to L enantiomers in the polymer has a profound effect on the crystallinity, which in turn dramatically influences the material properties such as modulus, strength retention time, and degradation time in the body.

PLGA polymers had much success in drug delivery formulations, for their biocompatibility, and “tunable” biodegradability. The degradation can be tailored from weeks to years, depending on the composition of the polymer as well as the method of preparation and formulation.

The degradation of PLGA polymers in contact with aqueous in vivo fluids or media has been the subject of much research aimed at determining whether the mechanism is purely chemical or enzyme-dependent. Currently, the degradation of aliphatic polyesters in contact with living tissues or with enzyme-containing body fluids is regarded by most people as resulting from abiotic hydrolysis of ester bonds only¹²⁹.

To summarize, once a PLGA device is placed in contact with an aqueous medium, water penetrates into the specimen and the hydrolytic cleavage of ester bonds starts. The carboxyl end groups generated, in principle, can catalyze the hydrolytic reaction of other ester bonds¹³⁰. On the other hand, as soon as the molecular weight of some partially degraded macromolecules becomes low enough to allow dissolution in the aqueous medium, diffusion starts within the whole bulk, with the soluble compounds moving slowly to and off the surface while they continue to degrade. This process combining diffusion, chemical reaction, and dissolution phenomena results in a differentiation between the rates of the degradation at the surface and the interior of the matrix.

Table 2-2 presents the various factors that have been identified as influential on the behavior of PLGA polymers in aqueous media and on drug release characteristics as discussed in detail in recent reviews^{131,132}.

Table 2-2 Factors affecting the hydrolytic degradation of aliphatic polyesters

Chemical composition
Molecular weight
Molecular weight distribution
Distribution of co-units
Distribution of chiral units
Chain defects
Presence of low molecular weight compounds (monomer, oligomers, solvents, initiators, drugs)
Processing history
Morphology (annealing, quenching, microstructures, residual, stresses, porosity)
Storage history
Shape and size
Ageing conditions (temperature, ionic strength, ion exchange, PH)
Absorption and adsorption of compounds (water, liquids, ions)
Physical property changes (shape, size, diffusion coefficients, stress, cracks)
Presence of enzymes

The effect of polymer chemical composition, molecular weight and the distribution, crystallinity, glass transition temperature, and size, shape and porosity are now well documented in the literature¹³³⁻¹³⁸. Here we wish to focus the

comments on some more recent findings dealing with other factors like additives, impurities, and even the entrapped drugs.

According to the heterogeneous degradation mechanism, any electrostatic interaction between a PLGA matrix and acidic, basic, or amphoteric molecules can dramatically affect the degradation characteristics by changing the natural acid-base equilibrium of the matrix due to the presence of chain end carboxyl groups. For acidic molecules, one can expect faster hydrolysis of ester bonds. In contrast, in the case of basic molecules, two effects were observed by Li and Vert¹³⁹ et al: base catalysis of ester bond cleavage when the base drug is in excess with respect to acid chain ends, and decrease of degradation rate in the contrary. Kishida¹⁴⁰ et al have studied the effect of drugs on the biodegradation rate of poly (L-lactic acid). They found that addition of a basic drug (thioridazine) to a film of polymer greatly enhanced the biodegradation rate, and the more drug that was added, the faster the poly (L-lactic acid) degraded. Cha and Pitt et al^{141,142} also found the hydrolysis of PLLA microspheres was catalyzed by the incorporation of tertiary amine-containing drug. They found that the incorporation of methadone, promethazine, and meperidine increased the rate of microsphere hydrolysis in phosphate buffer. Meperidine produced the greatest increase in hydrolysis followed by methadone, and promethazine. Naltrexone did not have any catalyzed hydrolysis.

In summary, it appears that base and acid pH environment accelerate the degradation of PLLA and PLGA polymers. The mechanism of acid and base catalyzed hydrolysis has been well reviewed, and complies with the mechanism of ester hydrolysis under acidic and basic conditions^{123,143,144}.

Acid-catalyzed hydrolysis:

Base-catalyzed hydrolysis:

2.5 Non-solvent Induced Phase Separation (NIPS)

Phase inversion is by far the most common method used in generation of asymmetric structures in membranes. The phase inversion process consists of the induction of phase separation in a previously homogeneous polymer solution either by temperature change (thermally induced phase separation or TIPS) or by exposure to a non-solvent bath (non-solvent induce phase separation or NIPS)¹⁴⁵.

Non-solvent induced phase separation (NIPS) is based on the immersion of polymer solution into a non-solvent bath. Practically, the polymer solution is cast or spun in the form of a thin film or thin-walled hollow fiber and quenched in a non-solvent bath to induce liquid demixing and structure formation. Fig. 2-1 shows immersion of a polymer film, cast onto a suitable support, into a non-solvent bath. The solution will separate into two phases due to the solvent diffusion out of the polymer film to the precipitation bath and to the non-solvent diffusion from the precipitation bath to the film.

Figure 2-1 Schematic diagram of the immersion precipitation technique.

The diffusion of non-solvent and solvent in and out of the polymer solution film results in the formation of a ternary system, consisting of polymer (P), solvent (S) and non-solvent (NS). Such a system can be represented by a ternary composition phase diagram schematically presented in Fig. 2-2. In this diagram, the binodal curve represents the projection of the equilibrium compositions and separates single-phase and two-phase regions. The spinodal curve separates the metastable and unstable composition regions. The shadowed area stands for the composition region where the viscous effects characteristic of the polymer solution may occur.

Depending on S and NS mass transfer rates, the polymer solution will achieve the two-phase region or will go into the viscous effect region. When the binodal is first reached, the polymer solution demixes into two liquid phases, rich and lean in polymer. During membrane formation, the polymer rich phase continues to increase in extent until it reaches the viscous effect region. The polymer rich phase will form the membrane matrix, while the polymer lean phase will form the membrane pores.

Figure 2-2 Schematic representation of a ternary phase diagram.

Morphologies ranging from highly asymmetric films exhibiting columnar macrovoids (fingers) underneath a dense, thin skin, to a skinless symmetric sponge can be formed. The dynamics of the phase inversion depends on the thermodynamic and mass transfer characteristics of the polymer/solvent/non-solvent system, which is complicated by compositional changes that occur in both space and time because of mass transfer at the film-bath interface.

Nevertheless, experimental and theoretical studies have successfully correlated observed membrane morphologies with the film-bath mass transfer dynamics. Reuvers et al¹⁴⁶ developed a model for the description of mass transfer during the immersion step. Following from this model, two types of demixing occur during immersion precipitation: instantaneous demixing and delayed demixing. Delayed and instantaneous demixing result in formation of different morphologies, sponge-like or finger-like cavities. Thus by varying the parameters involved in membrane preparation, i.e., the choice of polymer, solvent, non-solvent and precipitation bath composition, the demixing kinetics can be adjusted and as a result, different morphologies can be obtained.

A combination of factors is responsible for the formation of macrovoids/sponge-like morphologies, and this topic has been well reviewed in the literature^{147,148}. For practical purposes, the predominance of a sponge-like or a macrovoid structure can be induced in different ways. Basically, the sponge-like structure is favored by:

- Increasing the polymer concentration of the casting solution
- Increasing the viscosity of the casting solution
- Changing the solvent
- Additives in the polymer solution

Non-solvent phase separation (NIPS) has been widely used in membrane technology, most commercial fiber and flat-film asymmetric membrane production is based on non-solvent induced phase inversion process. Membranes are used for a variety of applications, including water purification. In the biomedical field,

membrane filtration is utilized in artificial kidneys to care for the survival of the innumerable people suffering from kidney disease¹⁴⁵.

As stated above, in the non-solvent phase separation process, the phase inversion kinetics and the final morphologies are tailorable, by changing the solvent or employing additives, etc. Applying this technique in drug release, an in-situ formed implant was developed, which we reviewed in the former section. The kinetics of drug release is affected both by the process of phase inversion and the final formed morphologies.

Recently, this non-solvent phase separation technique was also used in preparation of a scaffold¹⁴⁹. Porous PLLA/bioglass composite was prepared by immersing the PLLA/bioglass/1,4-dioxane suspension into a water bath. For application as scaffold for cell culture in tissue engineering, pre-sieved water-soluble salt or sugar can be homogenized in the above polymer solution; additive leaching-out will occur as well as phase separation. The former produces the large pores for cell growing in, while the latter forms the interconnected pore structure.

2.6 Gelation of Polymer Solutions

2.6.1 Gels and Gelation

In a number of key industries, including the pharmaceuticals, photographic, paper, and food industries, gels and gelling processes have for many years occupied a position of prime importance, fulfilling many functions such as

controlled release, surface coating, and consumed product. It is accepted that gels are notoriously difficult to define as a class of materials. They represent a state of matter intermediate between solid and liquid, and they are usually considered as showing solid character overall, although some features of a liquid may also be present¹⁵⁰.

Gelation is the conversion of a liquid to a disordered solid by formation of a network of chemical or physical bonds between the molecules or particles composing the liquid¹⁵¹. The gels formed include irreversible, chemical gels, which can be formed in a number of ways, including cross-linking high molecular weight linear chains, either chemically or by radiation, by the polymerization of oligomeric multifunctional precursors, or by end linking reactant chains with branching units, usually in presence of a diluent. In this section, we only discuss physical gels.

2.6.2 Physical Gel and the Gelation Mechanisms

A thermoreversible physical gel is a three-dimensional network of polymer chains “crosslinked” by physical junctions instead of chemical bonds in that the latter are covalent attachments between two molecules, physical gelation can arise either as the result of phase transition or through some specific molecular association or as a result of entanglement. The intermolecular associations are weak, reversible bonds or clusters produced by van der Waals forces, electrostatic attraction, or hydrogen bonding. De Gennes¹⁵² identifies three types of interactions that can lead to physical gelation: (1) local helical structures whereby one molecule winds around another; (2)

microcrystallite formation; (3) nodular domain formation, in which the chains are chemically heterogeneous, and association occurs at preferred sites along the chain.

Helical structure formation has been implicated in the gelation of many polymers. Poly (methyl methacrylate) (PMMA)¹⁵³⁻¹⁵⁷ is one example: it is able to form gels in many solvents and such gelation process is demonstrated to be a two-step gelation mechanism: a coil-to-single helix transition, followed by association of helices into larger multichain association units. The conformational transition is also largely affected by a complicated interplay of polymer-polymer and polymer-solvent interactions. Polymer-solvent complexes are shown to be able to stabilize the helix structure.

This helix structure has also been reported in polystyrene. Isotactic polystyrene¹⁵⁸ can form gel in carbon disulfide, cis-decalin, trans-decalin, and 1-chlorodecalin systems, while syndiotactic polystyrene is also reported to form gel in bromoform¹⁵⁹. Natural polymers such as DNA, polypeptides, polysaccharides also undergo coil-helix transition, e.g., agarose in water/DMSO¹⁶⁰, carrageenan¹⁶¹ in water, and polypeptides like gelatin¹⁶² in water.

Theoretical studies on thermoreversible gelation driven by aggregation of helices formed on the polymer chains have been carried out by Tanaka¹⁶³ based on the experimental data of optical rotation in ι-carrageenan solutions at different polymer concentrations. Two fundamentally different cases of (i) multiple association of single helices, and (ii) association by multiple helices

with multiplicity k (such as double helices ($k = 2$), triple helices ($k = 3$), etc.) are treated on the basis of different equations. The helix length distribution on a polymer chain (or an assembly of chains for multiple helices) is derived as a function of polymer concentration and temperature. It obeys a power law of the parameter:

$$t = 1 - \nu / (1 - \theta) \quad (2.20)$$

where the physical meaning of t is the probability for a randomly chosen monomer to be in the random-coil sequence, θ is the helix content per chain and ν is the average number of helices on a chain divided by the total number n of the repeat units. The sol/gel transition point is found on the temperature-concentration plane. It is found that, in the case of k -ple helices, the condition $n\theta/\xi = k/(k-1)$ is fulfilled at the gel point, where ξ is the average helix length. Hence, the independent measurement of θ and ξ gives the multiplicity k .

Polymers that form ***nodular domains*** have specific groups, or “stickers” attached to them that physically bond with each other, thereby producing physical networks¹⁵¹. One class of such associating polymers consists of water-soluble polymers containing hydrophobic groups that huddle together to shield themselves from their aqueous environment.

A notable example is the Pluronic® surfactant family with the structure of PEO-PPO-PEO⁹⁵⁻¹⁰¹. One of the most interesting features of such terpolymers is the self-association in aqueous solution and the resultant phase behavior. At

low polymer concentrations and low temperatures, the triblock copolymer in water appear to be single-coils, called unimers, which form a PPO hydrophobic core surrounded by the hydrophilic PEO blocks¹⁶⁴. At higher concentrations or temperatures, critical micellization concentration (CMC) or temperature (CMT) is observed when the aggregation of PEO-PPO-PEO block copolymers occurs and leads to the formation of thermodynamically stable intermolecular micelles. At even higher concentrations and temperatures, the copolymer chains and micelles can form ordered phases. Gelation of Pluronic® solutions occurs at high polymer concentrations.

Recently a biodegradable hydrogel system was reported by a group led by Kim¹⁰²⁻¹⁰⁴. The hydrogel is formed by self-assembly via a micellar aggregation mechanism. The micelles consist of hydrophobic cores and hydrophilic coronas. Block copolymers of poly (ethylene glycol) (PEG) and either poly (L-lactide) (PLLA) or poly (lactide-co-glycolide) (PLGA) were synthesized with PEG segments on the two ends to facilitate micelle formation, e.g. PEG-PLGA-PEG. Upon heating, the copolymers in water were shown to undergo fluid-to-gel transitions, followed by gel-to-fluid transition at even higher temperatures. By tailoring the polymer composition and concentration, this type of polymer can form liquid solution at ambient conditions but gel upon physiologic conditions. The in-situ gelation property can be exploited in many clinical applications, including injectable drug delivery depot and a gentle means of cell encapsulation¹⁶⁵.

Conversely one can have hydrophobic polymers to which hydrophobic groups are attached. Aqueous suspensions of the reversed block copolymer architecture, PPO-PEO-PPO¹⁶⁶, show rather different phase behavior as PEO-PPO-PEO. But also this system is dominated by spherical micelles in a wide region of the phase diagram. Due to the hydrophobic blocks situated at both ends, however, the block copolymer associates into a network of micelles rather than the independent micelles in PEO-PPO-PEO systems. The hydrophobic PPO group is always short relative to the PEO block.

Research on above water-soluble block polymers has been extended to many other block copolymers, such as Polystyrene-*b*-polyisoprene-*b*-polystyrene (SIS)¹⁶⁷, syndiotactic poly (methyl methacrylate)(sPMMA)-polybutadiene (PBD)-sPMMA^{168,169}, polystyrene-*b*-poly (ethylene/butylenes)-*b*-polystyrene^{170,171}, etc, and their association behavior has been categorized as symmetrical triblock copolymers ABA or BAB in selective solvents for block A while nonsolvent of block B, or vice versa.

Among the molecular mechanisms involved in gel formation, gelation occurring as a consequence of the *crystallization* of polymers from dilute and moderately dilute solution was recognized a long time ago^{172,173}. Generally, it has been assumed that the gelation of crystalline polymers is accompanied by the formation of microcrystallites. In this type of gelation, a three dimensional network is formed where primary molecules of finite molecular weight are bound together, or connected, by crystallites. When gelation occurs as a

consequence of crystallization, a very fluid polymer solution is converted to a rigid medium of infinite viscosity.

Based on the detailed studies of the gel properties of homopolymers and copolymers of ethylene, Mandelkern et al¹⁷⁴ gave a summary of the mechanism of gelation formed from crystallization: in very dilute solution, a molecule by necessity is restricted to participate in only one crystallite. However, as the concentration is increased further, some chains will be free to leave the crystallite of origin. They will then be in a position to participate in the formation of another crystallite. A partitioning of sequence will then take place between those that remain in the crystallite and those that leave, transverse the non-crystalline region, and are in a position to participate in yet another crystallite. The structural basis for gel formation by crystallization thus becomes clear. Because of the limited number of sequences that are available for crystallization in copolymers, which also gel due to the same mechanism, a relatively higher critical concentration c^* will be required for gelation, which is defined based on the point of Takasashi¹⁷⁵ that in order to develop a network structure a chain, on the average, must pass through at least two different crystallites. The value of c^* varies systematically with molecular weight, temperature, etc.

Despite several experimental and theoretical efforts, it is still recognized that the nature of the physical junctions involved in thermo-reversible gel is not well understood, especially compared to chemical gelation. The mechanism of thermoreversible gelation varies from system to system, and assigning one of

the above De Gennes' three main types of association to a particular gel system is usually not definitive. Consider for instance the case of PVC-based gels, a system exhaustively studied for over 50 years, which is still the subject of controversy. Hydrogen bonding, crystallite formation, liquid-liquid separation, and solvent-polymer interaction were all postulated by different researchers to be the driving force for the gelation^{176,177}.

Another distinctly controversial system is from atactic polystyrene. Wellingshoff et al first reported that chemically inactive polymer noncrystalline atactic polystyrene (aPS) could produce a thermoreversible gel in various solvents¹⁷⁸. Since then, many investigations of the molecular structure of the aPS gel were carried out by thermal analysis^{179,180}, neutron scattering¹⁸¹, infrared spectroscopy¹⁸² etc. The gelation has been confirmed to be thermoreversible and to be a fairly universal phenomenon that takes place in a wide variety of solvents, while the mechanism of cross-linking remains a topic of discussion. One series of studies postulates^{179,183} the existence of short crystallizable stereoregular segment sequences on polymer chains, even if they are atactic, which are responsible for the formation of microcrystalline junctions. Another studies^{184,185} reach a different conclusion that cross-linking takes place by forming stoichiometric compounds involving solvent molecules.

Independent of the details of the association, the attractive interactions in polymer solutions can promote both gelation and macro-phase separation¹⁵¹. If physical associations are to produce gelation, rather than phase separation, one

essential point must be remembered: the junctions between molecules that are formed by such association must not be able to enlarge very much. Thus, there must be some means of frustrating the growth of these associating domains, so that their size is limited. For instance, in “microcrystal” gel system, one important factor blocking the growth of the association regions is tacticity¹⁸⁶. If the chains are not stereoregular, they cannot crystallize (or build up helices) over long lengths, then gel formation is natural.

2.6.3 Characterization of Gels and Sol-gel Transitions

Characterization of gels and sol-gel transition covers a wide range of methods and techniques in chemistry, physics, material science and engineering, especially polymer materials.

Test tube tilting is the conventional method for determining T_{gel} involves a stopped test tube, containing a sample in the gel phase, upside-down at a controlled temperature, normally in a well-stirred water bath. The bath is warmed up and the temperature at which the sample falls to the bottom of the test tube is taken to be T_{gel} . Gel melting temperature T_m can be obtained by a contrary operation. As a simple method, test tube tilting is often the method by which one can get a primary idea of the occurrence of sol-gel transition. This method works reasonably well for samples that form stiff gels. However, for a soft gel, the polymeric crosslinks (almost physically) may split under the stress produced by gravity, due to the upside-down movement, and the gel may flow like a liquid even before it turns into a sol¹⁸⁷.

Ball drop or falling ball test is a characterization method that is almost unused recently. Detailed operation had been described by Takashashi et al.¹⁸⁸. The measurement for sol-gel transition includes: known quantities of polymer and solvent were introduced along with a steel ball in a glass tube and the tube were kept to allow for homogenization prior to any measurements. The ball was then allowed to sink to the bottom of the tube, and the gel was formed by slowly cooling. The tube was upside-down after the gel formation. The temperature was raised at a controlled rate. A magnetic field was applied from time to time to make sure that the ball was still supported by a gel-like material and not by a viscous solution. The temperature at which the ball fell through the sample under a magnetic field was taken as the gel melting temperature¹⁸⁹.

The dynamic oscillatory rheology has become nearly routine in characterization of gels over the last two decades. For a temperature sensitive sol-gel transition, a simply oscillatory temperature ramp by a temperature-controlled rheometer is used to measure the sol-gel transition. Storage and loss modulus, and the dynamic viscosity are recorded across the temperature range, usually at a low frequency such as 1 Hz, the samples are heated/cooled at certain rate. The abrupt onset of viscosity and storage modulus jump indicate the sol-gel transition^{190,191}. Further gelation is a kinetic process. It is very simple to follow the gelation kinetics by modern rheometry. Ungelled solution is subjected an oscillatory time ramp at certain frequency with controlled stress or deformation, the behavior of storage modulus G' and loss modulus G'' are followed as a function of time. The trace observed shows a

characteristic shape “cure” curve of $\text{Log } (G' \text{ and } G'')$ against time. For the fluid state, G'' will be greater than G' . Subsequently both G'' and G' begin to increase, after a given time, there should be a crossover where G' becomes greater than G'' , sometimes associated with the gelation time. Subsequently G' continues to increase before leveling off, whereas G'' ideally passes through a parabolic maximum and then decreases to zero¹⁵¹.

The thermo-analysis (DSC or micro-DSC) is another powerful approach in the study of gels and sol-gel transition, which is used to determine the solutions sol-gel points, gel formation (T_{GL}) or gel melting (T_{m}), enthalpies for these transitions, and time/temperature dependence for these transitions.

Spectrometries, including Fourier transform infrared (FTIR), Raman, NMR of ^1H and ^{13}C are used to provide molecular based information in gelation. They are used to measure the change of conformation of polymer, solvent, which is associated with sol-gel transition.

Other characterizations, such as SEM, TEM, polar microscopy, LANS, SAXS, neutron diffraction, and XRD etc, have been used to study the morphology, domain, crystallinity of gels whenever possible, from these information, the mechanism of gelation are evaluated.

Chapter 3

MATERIALS AND EXPERIMENTAL

3.1 Raw Materials:

- **Polymers**

Biodegradable Poly (d, l-lactide-co-glycolide)s and poly (d, l-lactide) were used in this experiment, which were purchased from different companies. Detail information is listed in Table 3-1.

- **Solvents**

Reagent-grade benzyl benzoate (BB) from Aldrich Chemical Company Inc., N-methyl-2-pyrrolidone (NMP) from Lab-Scan Analytical Sciences, and triacetin from Eastman Chemical Company were used in PLGA depot formulations with respective character in drug release kinetics.

Further ethyl benzoate (EB) from Aldrich, reagent-grade ethyl acetate (EA), HPLC-grade dichloromethane (DCM), tetrahydrofuran (THF), and acetonitrile from Tedia were used in investigation on the mechanism of gelation in PLGA solutions.

Table 3-1 Copolymers/homopolymer used for the preparation of formulations

Polymer	Manufacturer	L/G* ratio	I.V. (dl/g) [#]	Mw (g/mol) ⁺	D ⁺ Mw/Mn
PLGA/PDLA					
<u>Resomer RG502</u> **	Boehringer	52/48	0.22	17,000	1.83
Resomer RG502H	Ingelheim	53/47	0.19	14,500	1.74
A137-28	Absorbable Polymer Technologies	100/0	0.20	15,000	1.94
A137-29		75/25	0.19	15,000	1.88
<u>A137-31</u>		54/46	0.22	11,100	1.52
APT071603-1		53/47	0.19	16,000	2.60
PURASORB PDLG	PURAC	52/48	0.13	10,100	1.98
PURASORB DL 655FK	Biochem bv.	53/47	0.47	50,000	1.56

* as specified in certificate analysis.

Inherent viscosity as specified in certificate analysis, in chloroform.

+Molecular weight and the polydispersity as determined by GPC in lab.

** Polymer as a present from ALZA Corporation, Palo Alto, California.

Resomer RG502 is terminated with ethyl ester

A137-31 is terminated with lauryl ester

All others terminated by –COOH

• Model drugs

Metoclopramide monohydrochloride (salt) from SIGMA-ALDRICH, and metoclopramide base (made in the laboratory from the salt) are used as low molecular weight model drugs. The chemical information is listed below:

The two model drugs have different solubility in water, which may represent the hydrophilic drug and its opposite hydrophobic counterpart. Thus they are used to compare the release kinetics of two different types of low molecular weight drugs.

Lidocaine (base) and lidocaine hydrochloride (salt) from SIGMA-ALDRICH are used for comparison:

Bovine serum albumin (BSA) fraction V Sigma is used as model protein drug. Chicken egg lysozyme chloride from TCI Tokyo is used for comparison.

• Additives

Following low molecular weight sugar and salt were used as additives in drug release experiment:

Alpha-D-(+)-glucose anhydrous from Aldrich

Alpha-D-lactose monohydrate from Sigma

Sodium chloride from Merck

salt as specified above

● **Buffer solution**

Phosphate buffer with pH 7.00 (20 °C) from MERCK was used as the in vitro receptor liquid. The buffer contains 3.533g Potassium dihydro-phosphate and 7.265g sodium chloride phosphate (a mixture of sodium phosphate and sodium chloride in 1:1 mol ratio) per liter.

3.2 Experimental Procedures

3.2.1 Preparation of Solutions

For studies of the flow property, degradation and drug release kinetics, polymer solutions of different composition and concentration were prepared. All solutions were prepared by weight percentage. As the polymers used in this study are all amorphous and with low molecular weight, solutions readily formed in NMP, ethyl acetate and dichloromethane at room temperature in half an hour. In order to effect solubilization with solvents like benzyl benzoate, mixtures were stirred with spatula immediately to achieve a preliminary homogeneity, and then kept at 37°C incubator (overnight if necessary), stirred with spatula every 1 hour until a transparent solution was achieved. This solution was then tightly capped and put into 65°C oven to remove trapped air bubbles. Since the ageing process led to structure

formation, and the structure formation may be minimized or eliminated by either storing PLGA/BB solutions at low temperature such as $\sim 10^{\circ}\text{C}$ in refrigerator, or by briefly heating them above gel melting temperature (heat in 65°C oven for half an hour in this study) to eliminate the gel structure before administration.

For in vitro release and degradation experiments, the drug was mixed into the above solution. The drug was milled and sieved to a geometric diameter less than $100\mu\text{m}$, dispersed in the PLGA/BB solution, and homogenized with spatula- mixing.

3.2.2 Rheological Experiments

A Carri-Med CSL500 and a ThermoHaake Rheostress 300 rheometers with temperature control are used for measuring the steady shear viscosity and the dynamic flow properties of the PLGA solutions, in order to characterize the flow property of different PLGA solutions, the degradation and gelation kinetics upon ageing and after injection into buffer, and the time and temperature effects etc.

Dynamic flow properties of PLGA/NMP, PLGA/EB, and PLGA/BB solutions were measured by oscillatory frequency “sweep”. The experiments were performed on a cone-and-plate fixture having a diameter of 20mm and a cone angle of 1 degree. The linear visco-elastic range of each sample was first determined by an oscillatory stress sweep, and within which the strain amplitude ($\gamma = 0.005\text{—}0.02$) or the stress amplitude ($\tau = 10\text{Pa}$) was kept low

enough so that structures formed, if any, were not disrupted by measurement. Since gelation slowly raised viscosity of the PLGA/BB solution upon storage, in order to characterize the effect of the gelation process on viscosity, the PLGA/BB solutions were aged at 25°C in plastic (PE) syringes with i.d of ~20mm, and the front tips were cut off. The syringes were sealed with Parafilm and stored together with a pack of silica gel to keep dry. Measurements were taken at different time points by carefully cutting proper-sized samples and loading them directly onto the cone-plate of the rheometer, without any further heat treatment.

For PLGA/BB solutions that were aged, the oscillatory frequency “sweep” also conducted in another way. The sample was heated up to 75°C directly on the cone-plate in order to remove bubbles and especially eliminate the contribution of gelation on the flow properties, then cooled to the test temperature (25°C), a oscillatory frequency “sweep” was immediately started to measure the dynamic viscosity, the storage and loss modulus. The change of the viscosity with time is correlated with the polymer molecular weight change during ageing.

Second, an in vitro rheological study was conducted to PLGA/BB solutions with/ without drug, where the dynamic rheological properties as a function of time upon injection into buffer were monitored. The formulations were injected into 10 ml buffer. The study was carried out at 37 °C and the buffer solutions were periodically changed as was done for the drug release studies (3.2.4). For this study, samples in buffer were periodically removed and the

rheological properties measured as follows: after absorbing the surface water with filter paper, the sample was loaded directly onto the cone-plate of the rheometer, without any further heat treatment.

Except the measurements for temperature effect, all the other rheological studies were performed at 25°C.

The experiments were repeated 3 times, and the average and standard deviation were calculated.

3.2.3 Degradation Studies

The degradation of lactic/glycolic acid polymers has been characterized in term of change in molecular weight and molecular weight distribution, weight loss, water uptake, change of morphology, glass transition or crystallinity change, and mechanical strength etc. For this PLGA/solvent system, most characterizations are much more difficult than clean and clear polymer film, tablet, or spheres, and the results are sometimes ambiguous. In this report the degradation was mainly characterized by molecular weight information.

A gel permeation chromatography Agilent series 1100 GPC was used to determine the molecular weight and the polydispersity of polymers at different time points. The following conditions were adopted: Plgel 5 μ m MIXED-C column with length / I.D. (mm) of 300*7.5 was used; mix-solvent of 80:20 tetrahydrofuran and dichloromethane was the mobile phase at a flow rate of 1.0 ml/minute. The mobile phase was chosen to achieve good solubility for a wide range of homopolymers of lactide or its copolymers with glycolide. The

elution was conducted at 35 °C. RID signal is detected at 254 nm. Samples were dissolved in the mixed-solvent at a concentration of ~0.5mg/ml, filtered with 0.2 micro filters, and then filled into 2 ml vials, which were used for auto injection. The injection volume is 50 µl. Weight average molecular weight (M_w), number average molecular weight (M_n) and the polydispersity index (D) were calculated based on a calibration curve generated with 3 Agilent Polystyrene EasiCal vials, each containing a mixture of 4 narrow polydispersed polystyrene samples. The 12 polystyrene samples have number average molecular weight (M_n) from ~162 to 4,592 K (g/mol).

Degradation studies were conducted on the ageing PLGA solutions (with or without drug) and the samples immersed in buffer solution for in vitro study.

PLGA/BB solutions (50/50 by weight) without drug were aged at 10 °C in a digital controlled ventilated refrigerator, and at 25 °C and 37°C in two incubators. Molecular weights of samples at different time points were determined by gel permeation chromatography.

PLGA/BB solutions loaded with drugs (metoclopramide salt and base) at a weight ratio of 50/50/5 were aged at 10 °C and 25 °C, and molecular weight of fresh prepared samples and those aged for 43 days were determined by the same method as above. A pure solution without drug was also aged under the same condition and determined at the same time for comparison.

For in vitro degradation test, PLGA solutions with/ without drug (about 250 mg) were loaded into 10 ml pH 7.00 phosphate buffer. The study was carried

out at 37 °C in a Grant Sub14 water bath, the buffer solutions were periodically changed and the samples were taken out at various time points, absorbed with filter paper, dried under vacuum at ambient temperature immediately and then subjected to GPC analysis.

The experiments were repeated 3 times, and the average and standard deviation were calculated.

3.2.4 Model Drug Release

For the two drug systems, i.e. low molecular weight model drugs including salt and base form metoclopramide and high molecular weight model drugs including Bovine serum albumin and chicken egg lysozyme, formulation variables including solvent and drug type, drug concentration, polymer concentration, polymer molecular weight, L/G ratio and terminating groups, additives and ageing effect, etc. were studied.

Polymer solutions containing drug (~500mg) was injected into 12ml vials in which 10ml pH 7.00 phosphate buffer would be added thereafter. The vials were kept at 37 °C using a Grant Sub14 water bath. The study was carried out statically.

Receptor solution was sampled and refreshed at intervals of approximately 1 hour, 12 hours, 24 hours, 48 hours, etc. The drug concentration and the amount of solvents diffused out were determined by UV-Vis spectrophotometer, size-exclusion chromatography (SEC), and Reverse-phase HPLC, which we will describe in the following paragraphs.

For metoclopramide salt and base, the drug concentration was determined by UV-Vis spectrophotometer (UV-2501, SHIMADZU). Fresh buffer was used as reference. A control sample (pure PLGA/solvent solution without drug) was also sampled and analyzed at the same time point for comparison. Calibration curves were obtained using five drug solutions in buffer with concentration ranging from ~5 to 100 µg/ml, readings of absorbance at 309.4 nm were recorded, which is the characteristic absorbance of metoclopramide drugs unaffected by solvent, degraded PLGA monomers and oligomers diffused out from the depot. Linear regression was applied to the absorbance at 309.4 nm according to the Beer's law.

$$A = \varepsilon bc \quad (3.1)$$

where **A** is absorbance (no units, since $A = \log_{10} P_0 / P$).

ε is the molar absorptivity with units of L mol⁻¹ cm⁻¹.

b is the path length of the sample-that is, the path length of the cuvette in which the sample is contained.

c is the concentration of the compound in solution (mol/L).

Here we use weight/volume concentration (µg/ml), and combined the molar absorptivity “ε” and the cuvette parameter “b” into “ε*” (ml µg⁻¹).

$$A = \varepsilon^* c \quad (3.2)$$

The calibration curves of metoclopramide salt and base are shown in Figure 3-

1. Release amounts of drug were calculated based on this calibration.

Figure 3-1 Calibration curves of Metoclopramide salt and base in pH 7.0 buffer at wavelength of 309.4nm, measured by UV-Vis spectrophotometry.

For protein drug, two quantitative methods were used. Bicinchoninic acid (BCA) protein assay kit from Sigma-Aldrich was used to analyze the total protein released, and size-exclusion chromatography (SEC) was used to detect the protein aggregation and the amount of protein released.

The principle of the bicinchoninic acid (BCA) assay relies on the formation of a Cu^{2+} -protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+} . The amount of reduction is proportional to the protein

present. The macromolecular structure of proteins, the number of peptide bonds and the presence of four amino acids (Cysteine, cystine, tryptophan, tyrosine) have been reported to be responsible for color formation in protein samples when assayed with BCA. Compounds with groups similar to those of cysteine, cystine, tryptophan or tyrosine, are known to react with BCA¹⁹².

The purple-colored reaction product of this assay is formed by the interaction of two molecules of BCA with one cuprous ion (Cu^{+1}). This complex is water-soluble and exhibits a strong absorbance at 562nm, allowing the spectrophotometric quantification of protein in aqueous solution.

The BCA-Protein reaction scheme is described as:

The measurement was performed according to a modified Sigma standard assay protocol. Disposable polystyrene cuvettes, which are designed to be used under wavelength from 350 to 900 nm, are used for BCA—protein reaction and UV-Vis spectrometry. 0.2 ml (instead of 0.1 ml in standard protocol, in order to increase the sensitivity) of protein sample is mixed with 2 ml BCA working reagent. The assays are performed at 37°C in incubator for 30 minutes with the cuvettes sealed with parafilm. The absorbance at 562 nm is recorded by UV-Vis spectrophotometer (UV-2501, SHIMADZU) and the concentration is calculated based on a calibration curve. The calibration curve

was created during each assay from five fresh prepared protein solutions, and the protein concentration ranges from 10 to 350 $\mu\text{g/ml}$, which are approximately the range of the sample concentration from drug release experiment, which was estimated according to the results of SEC. Satisfactory linear regression within this range was achieved as shown in Figure 3-2.

Figure 3-2 Calibration curves of Bovine Serum Albumin (BSA) and chicken egg lysozyme (lysozyme) reacted with BCA kit and measured by UV-Vis spectrophotometry at wavelength of 562nm, calibration curve were done before each analysis.

Size-exclusion chromatography (SEC) separates proteins on the basis of effective size of the sample molecule, relative to effective pore diameter of the column packing material. Molecular size of sample molecule is related to its molecular weight. For molecules of a given conformational state (i.e.,

spherical, coiled, rod-like), the retention time is inversely proportional to the logarithm of molecular weight.

Figure 3-3 Calibration curves of Bovine Serum Albumin (BSA) and chicken egg lysozyme (lysozyme) measured by Size-exclusion chromatography (SEC) at wavelength of 210nm, pH 7.0 buffer was used as mobile phase.

An Agilent ZORBAX GF-250 column is utilized as a size exclusion column, which is connected with Agilent 1100 Series instrument equipped with a VW detector. The operating conditions were as follows: sample volume 20 μ l, mobile phase flow-rate 1.0 ml/min, λ =210 nm. Phosphate buffer with pH 7.00 is used as mobile phase. Quantitation was carried out by integration of the peak areas (peaks at ~2.2 minute for BSA and ~5 minute for lysozyme) using external standardization method. Calibration curve was created with fresh BSA/buffer solutions, and a satisfactory linear regression was achieved as

shown in Figure 3-3. The released amounts of protein were calculated based on the calibration constant.

Figure 3-4 Calibration curves of benzyl benzoate (BB), triacetin, and 1-methyl-2-pyrrolidone (NMP), measured by reverse-phase HPLC and size-exclusion chromatography (SEC). Triacetin and NMP: SEC at wavelength of 210nm, peaks at ~3.1 minute (triacetin), and ~3.3 minute (NMP) were integrated. BB: reverse-phase HPLC at 278nm, peaks at ~6.5 minute were integrated.

Reverse-phase HPLC was used to quantify the amount of benzyl benzoate released from the PLGA/BB depot into the bath side. The chromatographic analysis is performed on an Agilent 1100 Series instrument equipped with a VW detector and a ZORBAX 300SB-C18 column. The operating conditions were as follows: sample volume 20 μ l, mobile phase flow-rate 1.0 ml/min, λ =278 nm. 60/40 v/v acetonitrile/water with 80 mM trifluoroacetic acid (TFA) is used as mobile phase. The calibration curve was measured with

BB/acetonitrile solutions since the solubility of BB in buffer (H₂O) is too low to accurately weigh and dissolve in reasonable volume of buffer. Quantitation was carried out by integration of the peak areas (peaks at ~6.5 minute). Calibration curve is shown in Figure 3-4.

Figure 3-4 also shows calibration curves of triacetin and NMP for calculating the amount of solvents released from PLGA/triacetin and PLGA/NMP depots into buffer. The two calibration curves were determined by SEC, using the method described above. Fresh buffer solutions of triacetin and NMP were used for calibration.

The experiments for drug and solvent release were repeated 3 times; the average and standard deviation were calculated.

3.2.5 Thermo-analysis

3.2.5.1 Thermo Gravimetric Analyzer (TGA)

TGA measures weight changes of material as a function of temperature (or time) under a controlled atmosphere. Its principal applications include measurement of a material's thermal stability and composition. A High resolution TGA-2950 thermal gravimetric analyzer, from TA Instruments was used in this study.

The thermo-gravimetric analysis was done for the raw polymers. The following steps were taken during the process: samples (10-20mg) were heated from room temperature (~20°C) to 400°C at a heating rate of

5°C/min. The resulting weight loss versus temperature provides information about the thermal stability and composition of the material.

The measurements were conducted with dry nitrogen as purging gas at a flow rate of 50 ml/min.

In this report, TGA was also used to quantify the weight loss of PLGA/BB solution presumably due to solvent evaporation in the air. The PLGA/BB solution (50/50) was equilibrated at 25°C, followed by an isothermal scan at 25 °C for six hours under dry nitrogen atmosphere. The weight loss versus isothermal time was recorded.

3.2.5.2 Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) measures temperatures and heat flows associated with thermal transitions in a material. Properties measured include glass transitions, "cold" crystallization, phase changes, melting, crystallization, product stability, curing kinetics, and oxidative stability, etc.

Modulated differential scanning calorimetry (DSC 2920 Modulated, TA Instruments, USA) equipped with refrigerated cooling system (RCS) was used in this study. Baseline, cell constant, and temperature calibrations are checked once a month, or when cell is cleaned or purge changed. The baseline calibration is performed by heating an empty cell through its temperature range (e.g. -20 to 200 °C) at a heating rate (e.g. 10°C/min), this program is to calculate the slope and offset values needed to flatten the baseline and zero the heat flow signal; DSC cell constant and temperature

calibrations are done with Indium (with known heat of fusion (28.46 J/g) and onset of melt (156.6°C), measured at a heating rate of 10°C/min.

The glass transition temperatures (T_g) or melting points (T_m) of raw polymers were measured. The following steps were taken during the process: samples (~5mg,) were equilibrated at -20 °C, and then were heated to 100 °C at 10 °C/min, after which it was held isothermally for 2 min. The samples were cooled back to -20 °C at the same rate. After 2 min of isothermal stage at -20°C, the second heating cycle proceeded at 10 °C/min to 200 °C. The T_g of polymer was obtained by taking the mid-point of the slope during glass transition. In the present research, two heating cycles were conducted. As all polymers used in this study are amorphous, no T_m has been detected.

The measurements were conducted with dry nitrogen as purging gas at a flow rate of 50ml/min. standard aluminum pan was used.

An example thermogram of Resomer RG502H is shown in Figure 3-5, the glass transition temperature is taken as the midpoint of glass transition from the second run.

To characterize the PLGA gels, fresh and aged PLGA solutions (~10mg) were weighed and sealed in aluminum hermetic pans in order to prevent solvent evaporation, the samples were equilibrated at 0°C for 2 minutes; the sample was then heated up to 80°C at a heating rate of 5°C/min. For the dried “gel”, the sample was heated up to 120°C at the same heating rate.

Dry nitrogen is used as purging gas at a flow rate of 50 ml/min. The peak temperature and the heat of fusion were recorded.

The measurements were repeated 3 times; the average and standard deviation were calculated.

Figure 3-5 DSC thermogram of PLGA (Resomer RG502H).

3.2.6 Microstructure Analysis by ^1H and ^{13}C Nuclear Magnetic Resonance (NMR)

The ^1H and ^{13}C NMR spectra of the copolymers and homopolymer were recorded on a Bruker DRX 400 spectrometer in 5 mm o.d. sample tubes.

Samples were prepared by dissolving approximately 50 mg of polymer in ~0.6 ml Dimethyl sulfoxide- d_6 (one ampoule) from Sigma Aldrich.

^{13}C NMR spectra were measured at 300 K according to quantitative ^{13}C -NMR spectroscopy (INVGATE method) issued by Boehringer Ingelheim. The acquisition time was 0.65 s, pulse width 19.9 μs and delay between pulses 6.00 s. A total of 2000 transients were accumulated. For ^1H NMR spectra, 16 transients were accumulated.

^1H NMR spectra were used to determine the L/G (lactic acid moieties to glycolic acid moieties) of PLGA copolymers, which is based on the distinct behavior of involved hydrogens in a magnetic resonance. The mol fraction of lactic acid moieties to glycolic acid moieties is calculated from the integrated peaks at 4.8 ppm (a) and 5.2 ppm (b), which arise from hydrogens in the following groups¹²³:

Assigning A to the peak area of proton (b) and B to one-half of the peak area of protons (a), the following relationships exist:

$$\text{molar fraction of lactic acid} = A/(A+B) \quad (3.3)$$

$$\text{molar fraction of glycolic acid} = B/(A+B) \quad (3.4)$$

$$\text{molar ratio of lactic acid to glycolic acid} = A/B \quad (3.5)$$

For ^{13}C NMR spectra, the NMR signal of the carbonyl C-atoms of the glycolic acid units appears at approximately 167 ppm and is split into a double peak. The left part of the signal (measured at 167.57 ppm) and the right part of the signal (measured at 167.47 ppm) individually arise from the sequences: GGGG (167.57 ppm) and GGLL (167.47 ppm), where L stands for the lactyl unit $-\text{CH}(\text{CH}_3)-\text{CO}-\text{O}-$, and G for the glycolyl unit $-\text{CH}_2-\text{CO}-\text{O}-$. The two peaks were integrated in order to compute the average length of a glycolide unit in the poly (d,l-lactide-co-glycolide) random copolymers^{193,194}.

Data were collected from two repetitions. The average and the deviation were calculated.

3.2.7 Other Characterizations

- **Moisture/ water content determination**

A Mitsubishi Moisture Meter Model CA-06 Karl Fisher Titration was used to determine the moisture in solvents and the in vitro samples from different time points.

The Karl Fischer Method is an accurate moisture measurement method utilizing the quantitative reaction of water with iodine:

In coulometric titration, electrolytic oxidation is carried out in the anolyte, which consists mainly of iodide, sulfur dioxide, and solvents such as

pyridine—alcohols. Electrolytic oxidation proceeds when a sample is added to the anolyte. The Karl Fischer reaction occurs to produce iodine:

Iodine is produced in proportion to the quantity of electricity according to Faraday's Law, so the water content can be estimated based on the coulombs required electrolytic oxidation.

$$1\text{mg of water} = 10.71\text{ coulombs} \quad (3.6)$$

Based on this principle, the water content in the sample can be determined by quantity of electricity required for electrolysis. Coulometric reagents do not require standardization.

Approximately 0.2 ml liquid samples were directly injected into the measuring cell. For the solid in vitro samples (samples after buffer injection), in order to ensure that the water inside the samples is completely titrated, the samples were dissolved in a water miscible solvent such as NMP. The moisture content of the solvent and the solutions were determined. The water content is then calculated from above results.

The measurements were repeated 5 times; the average and standard deviation were calculated.

- **Scanning electron microscopy (SEM)**

A JEOL JSM-5410 LV scanning electron microscope (SEM) was used to study the morphologies of hardened depots from PLGA/NMP system.

Samples were prepared by casting PLGA/NMP solutions with/without drug onto a glass slide at a thickness of 200 μm and then immersed in pH 7.0 buffer at 37°C. The samples were taken out after 72 hours, fractured in liquid nitrogen, and vacuum dried at room temperature for at least 24 hours. Samples were sputter coated with gold using SPI-Module™ sputter coater to improve their electrical conductivity.

The SEM micrographs were observed at 10 KV, magnification was chosen from 100X to 1,000X.

- **X-ray diffraction**

Polymers that form gels during ageing were scanned using a Shimadzu 6000 X-ray diffraction (XRD) to check the existence of crystallites in pure polymers and dried gels. Scans were run over a 2θ spectrum of 10°-90° at a scan rate of 2°/min⁻¹, and Cu K α radiation was employed.

Chapter 4

RESULTS AND DISCUSSION

The main focus of this study was the PLGA/BB type of injectable depot, which has been reported to have favorable drugs/protein release characteristics in vivo or in vitro^{15,19}. This system forms a semi-solid depot upon injection into the subcutaneous space of normal rats or buffer; the mechanism is claimed to be a slow NSIP mechanism¹⁹.

In our studies, we sought answers to the following questions:

- a) Is the mechanism of depot formation for PLGA/BB fundamentally different from NSIP?
- b) What is the stability of the PLGA/BB system upon storage at various temperatures? Does it form a semi-solid upon ageing? Do all PLGAs in BB form this structure?
- c) What is the nature of this gel or semi-solid?
- d) Do all PLGAs in BB form a semi-solid depot/gel upon injection into buffer? Is there a correlation between those that gel upon ageing and those that gel upon buffer injection? If so, why?
- e) How does hydrolytic degradation affect the gel formation?

- f) What sort of in vitro drugs release profiles are obtained upon injection into buffer and why?

4.1 Basic Characterizations for the Study of Gel/Depot Formation and Drug Release.

4.1.1 Solubility of PLGAs:

The solubility of various PLGA polymers can be affected by their crystallinity, molecular weight, composition including the ratio of lactide to glycolide and the randomness of the monomeric units in the copolymer^{21,123}. The calculated solubility parameters of lactic/glycolic acid polymers range from $19.9 \times 10^{-3} ([J/m^3]^{0.5})$ for poly(d, l-lactide) to $18.3 \times 10^{-3} ([J/m^3]^{0.5})$ for poly (d, l-lactide-co-glycolide) with L/G ratio of 58/42 (calculated according to Hoy's data^{123,195}) or from 16.4 to $16.8 \times 10^{-3} ([J/m^3]^{0.5})$ (determined experimentally¹⁹⁶). Table 4-1 lists some solvents that we studied for the solubility of PLGAs.

According to the experimental observation on the solubility of polymers listed in Table 3-1, we could divide the polymers into two types. One type includes PDLA (L/G 100/0), PLGAs (L/G 75/25), and some PLGAs with L/G ratio of ~50/50 (A137-31 and PURASORB DL 655FK as shown in Table 3-1), which showed good solubility in all solvents listed in Table 4-1 over a wide range of concentration, with no sol-gel transition observed. The other type includes most PLGAs with L/G ratio of ~50/50, to which the listed solvents show different dissolving behaviors: a). In ethyl benzoate (EB) and benzyl benzoate

(BB), a lower limit of concentration is found, which is approximately 45% by weight, while in other solvents, no such limit has been found. b). In ethyl acetate (EA), tetrahydrofuran (THF), ethyl benzoate (EB), benzyl benzoate (BB), and methylene chloride (DCM), the solutions form thermoreversible gels (sol-gel transition will be discussed in the following section), while no sol-gel transition has been found in the solutions in triacetin, N-methyl-2-pyrrolidone (NMP) and acetonitrile (ACN). The experimental data of solubility of PLGAs in different solvents are shown in Table 4-2.

Table 4-1 Solubility parameters of some solvents used with PLGA polymers, at room temperature (20°C)

<i>Compound</i>	$\delta \times 10^{-3} \text{ (J/m}^3\text{)}^{0.5}$
Ethyl acetate (EA)	18.6*
Tetrahydrofuran (THF)	18.6*
Ethyl benzoate (EB)	18.6 [#]
Benzyl benzoate (BB)	19.7 [#]
Methylene chloride (DCM)	19.8*
Triacetin	21.1 [#]
N-methyl-2-pyrrolidone (NMP)	23.1*
Acetonitrile (ACN)	24.3*

* data from “Polymer Handbook”¹⁹⁵.

[#] data calculated from Hoy’s data.

Table 4-2 Solubility of PLGAs in different solvents, at room temperature (20 +/- 0.5°C).

<i>Solvent</i>	<i>Polymer**</i>	<i>Concentration range (wt %)</i>
Triacetin	Type A & B	0 to 70%# sol
N-methyl-2-pyrrolidone (NMP)	Type A & B	0 to 70%# sol
Acetonitrile (ACN)	Type A & B	0 to 70%# sol
Ethyl acetate (EA)	Type A Type B	0 to 70%# sol sol to gel
Tetrahydrofuran (THF)	Type A Type B	0 to 50%# sol sol to gel
Methylene chloride (DCM)	Type A Type B	0 to 50%# sol sol to gel
Ethyl benzoate (EB)	Type A Type B	0 to 50%# sol sol to gel
Benzyl benzoate (BB)	Type A Type B	0 to 50%# sol sol to gel

The solubility is determined by visual observation.

The gelation is determined by test tube tilting (2.6.3), the c^* for gelation will vary depending the characteristics of solvent and polymer.

** Type A polymers refer to: A137-28, A137-29, A137-31, and PURASORB DL 655FK; Type B polymers refer to: Resomer RG502, Resomer RG502H, APT071603-1, and PURASORB PDLG. Details see Table 3-1.

The range quoted here represents the range we studied in our experiments; the solubility is higher than the upper limit quoted.

-- All polymers are poly (d,l-lactide) or the copolymer poly (d,l-lactide-co-glycolide); poly (l-lactide) or poly (l-lactide-co-glycolide) not included.

The above results indicate that the quality of solvents toward the copolymers is mainly governed by the glycolide portion, and may not be determined by the solubility parameter concept or the principle of “dissolving the similar”, at least for PLGAs with high glycolide content.

4.1.2 Solubility of Drugs in PLGA/BB Solution/Buffer

As mentioned in chapter three, salt and base forms of metoclopramide are chosen since they have distinctly different solubility in water. the salt form conveniently represents the hydrophilic drug and the base form its hydrophobic counterpart, and thus they are used to compare the release kinetics of two different types of low molecular weight drugs. In this section, we measured the solubility of these drugs in the depots, also in the buffer solution used.

In making the drug-containing solutions, first the drug is milled into small particles; the solution is also designed to be relatively viscous to maintain a kinetically stable dispersion. Visually, all the drug-containing polymer solutions (PLGA/BB/drug ranging from 50/50/0.5 to 50/50/10 by weight) are above saturation, as indicated by the presence of discrete particles; however, the exact measurement of C_s in viscous polymer solutions is extremely difficult.

In this study, the solubility of metoclopramide salt and base in benzyl benzoate were determined, by adding excess drug into the solvent, magnetically stirring for 24 hours and then allowing the solution to remain

unstirred for another 24 hours to permit the precipitation of dispersed drugs. The upper liquid was carefully taken out by syringe and filtered with 0.02 μ filters to guarantee no solid drug particles are taken for measurement. The saturated solution was diluted with acetonitrile. The concentrations were determined with reverse phase HPLC (as described in 3.2.4, except that the λ is set at 309.4 nm, which is the absorption wavelength for the drugs). The solubility was calculated from 5 parallel samples:

$$C_{\text{salt}}: 0.0218 \pm 0.0012 \text{ wt\%}$$

$$C_{\text{base}}: 0.298 \pm 0.0428 \text{ wt\%}$$

As the solubility parameter of PLGA is similar to that of benzyl benzoate as discussed in above section, we assume that the salt and base forms of metoclopramide have the same solubility in PLGA and BB. We resort to this method because the more direct methods, such as making polymer/drug films by solvent evaporation and then detecting undissolved crystals microscopically or by DSC, usually do not give accurate values.

The solubility of drugs in the buffer were measured by the same method as in BB: adding overdose drug in the buffer, magnetically stirring for 24 hours and then allowing equilibration for another 24 hours to permit the precipitation of dispersed drugs. The upper liquid was carefully taken out by syringe and filtered with 0.02 μ filters. The saturated solution was diluted with fresh buffer. The concentrations were determined with UV-Vis spectrometry, which has been described in 3.2.4. The solubility was calculated from 5 parallel samples:

$$C'_{\text{salt}}: 651.72 \pm 1.79 \text{ mg/ml}$$

$$C'_{\text{base}}: 5.122 \pm 0.003 \text{ mg/ml}$$

4.2 Properties of PLGA Solutions during Ageing

Several researchers have investigated the use of polymer solutions as injectable depots for drugs, particularly for protein or peptide drugs, during the last decade. The focus was on the mechanism of phase inversion, kinetics of drug release, morphology changes and degradation in vitro or in vivo. However, except for some research on the solubility of the polymer^{8,12}, the properties of such solutions are poorly characterized, although they directly affect the final administration.

As injectable carriers for depot-based drug release, the requirements for PLGA solutions besides biocompatibility, include: (a) a proper viscosity for injection, (b) good stability during storage or transportation since most pharmaceutical products must be designed to be stable in storage over a 2-year period, and (c) a high enough viscosity to ensure homogeneity of suspension (if the drug is not dissolved but only dispersed), and (d) the capability to form a semi-solid or solid depot upon injection.

Since the stability of solution is an important issue, in this section, we investigated the degradation of the PLGA solutions while ageing under different storage conditions. An Agilent 1100 series GPC was used for molecular weight determination.

We also investigated the flow properties, especially the dynamic flow property of the delivery systems that we used for injectable drug delivery. Carri-Med CSL500 and ThermoHaake Rheostress 300 rheometers with temperature control were used in these studies. Solutions made up of PLGA with L/G ratio 50/50 in benzyl benzoate (BB) and N-methyl-2-pyrrolidone (NMP) were tested for ageing effects, and for PLGA/benzyl benzoate system, we also investigated the gelation properties, which occurred over time of storage, by rheological experiments, MDSC, and NMR.

4.2.1 Molecular Weight Changes

- **Molecular weight of PLGA/BB solutions (without drug) in storage:**

The degradation kinetics of PLGA (Resomer RG502)/benzyl benzoate solution were followed over a period of approximately 6 months. The solution has a weight concentration of 50%. The decrease of molecular weight as a function of time and temperature is demonstrated in Fig. 4-1.

The results show a lag time of degradation, i.e., the solutions are quite stable over an initial period, which obviously depends on temperature. It can be seen, within the whole period of experiment, there is nearly no change in the molecular weight for the solution aged at 10 °C. At 25 °C, the molecular weight changes very little up to 2,520 hours, and then decreases rapidly. The solution aged at 37 °C seems to have the lowest stability; the lag time of degradation is only about 2 weeks, and is followed by a decrease faster than that at 25 °C.

Figure 4-1 Molecular weight (M_w) change as a function of time for PLGA (Resomer RG502)/BB (50/50) solutions aged at 10°C, 25°C, and 37°C. Data are taken as the average of 3 repetitions, standard deviation shown as error bar.

Figure 4-2A Molecular weight and its distribution change as a function of time for PLGA (Resomer RG502)/BB (50/50) solutions aged at 25°C. Data are taken as the average of 3 repetitions, standard deviation shown as error bar.

Figure 4-2B Molecular weight and its distribution change as a function of time for PLGA (Resomer RG502)/BB (50/50) solutions aged at 37°C. Data are taken as the average of 3 repetitions, standard deviation shown as error bar.

The observed decrease in molecular weight is assumed due to hydrolytic cleavage of G-G and L-G ester bonds in the backbone of the PLGA.

Figure 4-2A showed the molecular weight and the distribution change with time for the solution ageing at 25°C, the polymer distribution was becoming broader with time initially, and then narrowed from ~2520 hours. The same tendency was also seen in solution ageing at 37°C (Figure 4-2B), inferring bulk degradation in a close system. The rate of degradation at different temperatures appears to follow an Arrhenius-type relationship, implying the need for activation energy:

$$k = A \exp (-E_a/RT) \quad (4.1)$$

where k is the rate coefficient (s^{-1}), A is a constant (s^{-1}), E_a is the activation energy ($kJmol^{-1}$), R is the universal gas constant ($8.314 \times 10^{-3} \text{ kJ mol}^{-1}K^{-1}$), and T is the temperature (in degrees Kelvin).

Theoretically, hydrolysis would not occur if there were no water in the system. However, 1) thermal-decomposition would not happen at these temperatures as per the TGA result; 2) the solutions were not subject to any sound, light, or electro-magnetic treatment, and thus no degradation would happen induced by such factors; 3) moisture present in the polymer and solvent initially and introduced during processing and storage can initiate the hydrolysis (the water contents were $\sim 0.06wt\%$ and $\sim 0.16wt\%$ in raw PLGA and BB, as measured by Karl-Fischer titration, replications = 5, standard deviation less than 10%). We believe this is why PLGA degrades in benzyl benzoate solution even without water contact.

Degradation during ageing also occurs in other systems such as PLGA/NMP system shown in Figure 4-3. Due to the more hydrophilic nature of NMP compared to BB, and due to the use of carboxyl acid terminated DL655FK instead of ethyl ester terminated Resomer RG502, the degradation is even faster in NMP system than in BB system. Since there won't be any transesterification reaction between 1-methyl-2-pyrrolidone (NMP) and PLGA, the degradation of PLGA during ageing is assumed to be hydrolysis initiated by the moisture content.

Efforts have been made to remove the moisture from the system, including drying the polymer and solvent by vacuum evaporation and molecular sieves

respectively, and storing the solutions in desiccators. Water content decreases in the fresh-treated polymer and solvent (but not to zero), but degradation still occur to the PLGA solutions, although a little improvement has been achieved.

The storage stability of PLGA/BB solution aged at 10 °C for as long as 2 years was also verified, the molecular weight (Mw) decreased from 16.8 Kg/mol to 14.3 Kg/mol.

Figure 4-3 Molecular weight (Mw) change as a function of time for PLGA (Resomer RG502)/BB solution and PLGA (DL 655FK)/NMP solution, ageing at 25°C. Data are taken as the average of 3 repetitions, standard deviation shown as error bar.

• Molecular weight change of PLGA solution (with drug) in storage

The degradation of PLGA polymers in contact with aqueous in vivo fluids or in vitro media has been the subject of many discussions aimed at determining the mechanism of degradation. According to the general degradation mechanisms of PLGA polymers, there are four main factors that condition the

diffusion-reaction-dissolution phenomena: (a) the hydrolysis rate constant of the ester bond; (b) the diffusion coefficient of water within the matrix; (c) the diffusion coefficient of chain fragments within the polymeric matrix; and (d) the solubility of degradation products, generally oligomers, within the surrounding liquid medium which is also the source of water. Additional factors, such as temperature, additives in the polymeric matrix, additive in the surrounding medium, pH, buffering capacity, size and morphology, etc, can also influence the degradation.

Table 4-3A GPC results of PLGA (Resomer RG502)/BB (50/50) solutions with/without drug aged at 10°C, the formulation is PLGA/BB/(drug) = 50/50/(5). Data are taken as the average of 3 repetitions.

Ageing conditions	Samples	Mw (g/mol)
Fresh preparation	PLGA/BB	17,000
Aged at 10 °C For 43 days	PLGA/BB	16,810 ± 123.4
	PLGA/BB/salt drug	17,093 ± 154
	PLGA/BB/base drug	15,694 ± 468.5

Table 4-3B GPC results of PLGA (Resomer RG502)/BB (50/50) solutions with/without drug aged at 25°C, the formulation is PLGA/BB/(drug) = 50/50/(5). Data are taken as the average of 3 repetitions.

Ageing conditions	Samples	Mw (g/mol)
Fresh preparation	PLGA/BB	17,006
Aged at 25 °C For 43 days	PLGA/BB	16,970 ± 168
	PLGA/BB/salt drug	16,873 ± 95
	PLGA/BB/base drug	12,871 ± 161

The results listed in Table 4-3a show the weight average molecular weight as a function of ageing time for solutions made up of PLGA (Resomer RG502) and benzyl benzoate with or without drug [metoclopramide salt and metoclopramide base, the drug loading (50/50/5) is much higher than their solubility in PLGA/BB solution (~0.02 wt % for metosalt and 0.30 wt % for metobase), so both drugs mostly disperse in the PLGA/BB solution], which were sealed and stored at 10 °C in a digital controlled refrigerator. It is clear that the molecular weight change of the solution without drug is negligible over the period of the experiment, and the formulation containing metoclopramide salt does not show much change either. This may due to the low reaction rate constant at 10 °C according to the Arrhenius formula. However, the solution containing metoclopramide base, a primary amino compound as shown in Table 4-3A, degrades by about 8% even at the low temperature. This general phenomenon of base catalysis of PLGA degradation has been observed in our labs with slabs with the same drug or lidocaine salt/base¹⁹⁷ and elsewhere¹⁹⁸⁻²⁰⁰. For example, Li et al reported that basic additives had two opposite effects, i.e., base catalysis of ester bond cleavage when the drug was in excess with respect to acid chain ends, and decrease of degradation rate at another aspect¹⁹⁸. Maulding et al¹⁹⁹ also reported that poly (α -hydroxyl acids) degraded much faster when they were loaded with basic drug. Thioridazine, which is a tertiary amino compound, was reported to accelerate the degradation of poly (d, l-lactide)²⁰⁰.

The solutions aged at 25°C exhibit the same tendency, i.e., the formulation containing metoclopramide base, degrades faster as shown in Table 4-3B.

The mechanism of acid and base catalyzed hydrolysis of polyesters is supposed to be:

Acid-catalyzed hydrolysis^{123,143,144}:

Base-catalyzed hydrolysis^{123,143,144}:

4.2.2 Flow Properties of PLGA Solutions during ageing

In this section, we report on the flow properties, especially the dynamic flow properties of the delivery systems that we use for injectable drug delivery. A

Carri-Med CSL500 and a ThermoHaake Rheostress 300 rheometer were used. Solutions made up of PLGA with benzyl benzoate (BB) and N-methyl-2-pyrrolidone (NMP) were tested for ageing effects.

4.2.2.1 Rheology of *PLGA/NMP* (*N-methyl-2-pyrrolidone*) Solutions during Ageing

The PLGA/NMP system is the first system developed as an injectable depot, which solidifies and forms an implant in situ upon injection. McHugh et al identified the mechanism as a theory of phase inversion. Different researchers also studied the ternary phase diagram of water, NMP, and polymer, the release kinetics of different drugs, the degradation during in vitro and the morphology related to the phase inversion^{7,8,21}.

In the following work, we report on the rheological behavior, since the flow properties are related to the preparation and stability of the delivery system, in terms of the final administration.

Dynamic flow properties of polymer solutions aged at different temperature were measured by oscillatory frequency “sweep”, as functions of ageing time. A typical rheogram of PLGA/NMP solution is shown in Figure 4-4. Over the frequency range studied, the solution exhibits nearly Newtonian behavior, with slight shear thinning observed at the higher frequencies.

Figure 4-4 Typical rheogram of PLGA/NMP (30 wt %) solution. Frequency “sweep” was conducted: $\omega = 0.1\text{--}100 \text{ rad. s}^{-1}$, $\gamma = 0.02 \text{ rad}$, at 25°C .

Normally, for linear polymer, the relationship between polymer molecular weight and viscosity is represented by:

$$\eta_0 = KM^{3.4} \quad \text{when } M > M_c \text{ or}$$

$$\eta_0 = KM^1 \quad \text{when } M < M_c \quad (4.2)$$

where M_c is the critical molecular weight.

Fig. 4-5 shows that the dynamic viscosity of 30 wt% PLGA/NMP solutions decreases with ageing time both at 25°C and 37°C ; this suggests a decrease of molecular weight since the polymer concentration, which also determines the viscosity, did not change with time. In other words, the viscosity decrease reflects polymer degradation. The tendency shown in

Fig. 4-5 shows a period during which the solutions are relatively stable, followed by accelerated decrease of viscosity. That may be caused by a slow hydrolysis in the early stage, followed by acceleration due to the catalysis by the accumulated carboxyl groups produced by hydrolysis. This “degradation” at 37 °C is faster than that at 25 °C, and the temperature effects probably indicate Arrhenius dependence.

Figure 4-5 Temperature effects on dynamic viscosity of PLGA/NMP solutions, which were aged at 25 °C and 37 °C, viscosity taken from oscillatory frequency “sweep” at $\omega = 1 \text{ rad.s}^{-1}$, at 25°C. Data are taken as the average of 3 repetitions, standard deviation shown as error bar.

In a reacting system, viscosity affects the motility of macromolecular chains, thereby influencing the reaction rate of a macromolecular reaction. Fig. 4-6 shows the concentration effects on the dynamic viscosity change as a function of time. The degradation rates of these three solutions show no difference in the hydrolysis rate. This may be attributed to the relatively

low viscosities obtained with this product, which are not high enough to affect the diffusion of the reactant species.

Figure 4-6 Concentration effects on dynamic viscosity of PLGA/NMP solutions, which were aged at 25 °C, viscosity taken from oscillatory frequency “sweep” at $\omega = 1 \text{ rad.s}^{-1}$, at 25°C. Data are taken as the average of 3 repetitions, standard deviation shown as error bar.

4.2.2.2 Rheology of *PLGA / BB* (Benzyl benzoate) Solutions during ageing

A typical rheogram of fresh PLGA/BB solution is shown in Figure 4-7. A so-called Newtonian region was observed at low frequency, followed by shear-thinning flow with a power law index of 0.9442.

Figure 4-7 Typical rheogram of PLGA/BB (50 wt %) solution. Frequency “sweep” was conducted: $\omega = 1\text{--}50 \text{ rad. s}^{-1}$, $\gamma = 0.01 \text{ rad}$, at 25°C .

Since gelation slowly raises viscosity of the PLGA/BB solution upon storage and thereby masks the effect of decreasing molecular weight, two methods were used for loading sample onto the cone-plate. In the first method, the gelation effect was eliminated by raising the temperature to 75°C within 2 minutes after sample loading; this process also served to remove air bubbles. Subsequently, the temperature was decreased to the experimental setting (25°C). In order to characterize the effect of the gelation process on viscosity, the PLGA/BB solutions were aged in plastic (PE) syringes with i.d. $\sim 20\text{mm}$. Measurements were taken at different time points by carefully cutting proper-sized samples and loading them directly onto the cone-plate of the rheometer, without any further heat treatment.

- **Heated samples**

The PLGA (Resomer RG502)/BB solution was aged at 10 °C, 25 °C and 37 °C, and oscillatory frequency sweep was conducted at different time points over a time span of approximate 70 days. Fig. 4-8 showed the time effects on dynamic viscosity of the solutions. For solutions aged at 25°C or 37°C, the viscosity decreased over time, while it remained nearly unchanged for solutions aged at 10°C.

The decrease of dynamic viscosity was caused by degradation due to the same reasons as cited for the PLGA/NMP system, i.e. at zero shear, the viscosity of polymer solution is proportional to the molecular weight as shown in Equation 4.2. Since the polymer concentration in the PLGA/BB solution does not change with aging, the decrease in dynamic viscosity seen over time thus suggests a decrease of molecular weight in the aged samples. This assumption is confirmed by the observation that the reduction in viscosity parallels that in molecular weight for the PLGA/BB solutions at all three aging temperatures (compare Figures 4-8 with 4-1). The dynamic viscosities of the solution aged at 10°C show no effect of degradation, as explained in the last section. Also it can be explained by the fact that the melting point of BB lies between 18 and 22 °C and the phase-separated “solutions” aged at 10°C may also have contributed to the constant viscosity.

Fig. 4-9 shows a faster decrease of dynamic viscosity for the solution containing added water than that without water. The result supports the

conclusion that PLGA degradation is mainly a process of hydrolysis, which is initiated by water attack.

Figure 4-8 Temperature effects on dynamic viscosity of PLGA/BB (50/50) solutions, which were aged at 10 °C, 25 °C and 37 °C. Viscosity taken from oscillatory frequency “sweep” at $\omega = 1\text{rad.s}^{-1}$, at 25°C. Data are taken as the average of 3 repetitions, standard deviation shown as error bar.

Figure 4-9 Effects of addition of H₂O on the dynamic viscosity of PLGA/BB (50/50) solutions, which were aged at 37 °C. Viscosity taken from oscillatory frequency “sweep” at $\omega = 1\text{rad.s}^{-1}$, at 25°C. Data are taken as the average of 3 repetitions, standard deviation shown as error bar.

The system containing added water also shows an earlier initiation of viscosity decrease, i.e., hydrolysis occurred earlier within this system. However, during the later stages, the difference caused by water addition disappears. This suggests that the acceleration effect of accumulated carboxyl groups produced by hydrolysis has become the main factor, which dominates the rate of hydrolysis reaction. This effect is more pronounced for the PLGA/BB system, compared to the PLGA/NMP system (Figure not shown).

The above results indicate that 10 °C or below could be a proper temperature for storage, to minimize the degradation of the PLGA solution.

- **Temperature effects on the dynamic viscosity of PLGA/BB solution**

Figure 4-10 Temperature effects on the dynamic viscosity of PLGA (Resomer RG502)/BB (50/50) solution.

Fig. 4-10 shows the relationship between dynamic viscosity and temperature. Using an Arrhenius-type relationship,

$$\eta_0 = A \exp(E^\# / RT) \quad (4.3)$$

where η_0 is the zero shear viscosity, $E^\#$ is the activation energy of viscous flow, R is the idea gas constant, and T is the absolute temperature, The activation energy of flow is calculated to be 89.5KJ/mol, and

$$\eta_0 = 8.579E-14 \exp(10771.0 \frac{1}{T}) \quad (4-4)$$

The dynamic viscosity decreases by approximately 80% from 298K to 310K; this suggests that the solution could be more easily administered by a slight increase of the temperature.

The data presented so far appears to indicate that both PLGA/NMP and PLGA/BB solutions behave similarly upon ageing. The molecular weight and the dynamic viscosity both decrease, reflecting degradation effects only. However, this similarity appears to be due to the particular method employed in the viscosity measurement for the PLGA/BB solutions, i.e. the sample was heated up to 75°C to eliminate the structure before rheological measurement. In fact, we observed an increase in viscosity upon storage of some PLGA/BB solutions visually by test tube tilting; hence we modified our sample preparation technique to get a more accurate picture of the viscosity behavior, as described below.

- Time effects on dynamic viscosity of PLGA/BB solution: sample without heating

(a) Time Sweep in Rheometer

First a series of frequency ramps was conducted on the same sample, which was allowed to age between the cone and plate of the rheometer for about six hours. Dynamic viscosities at different time points were plotted in Fig. 4-11. It shows that the dynamic viscosity increases with the ageing time, which may be attributed to two possible reasons, concentration increasing due to solvent evaporation, or creation of some structure within the system as a function of time.

Figure 4-11 Time effects on the dynamic viscosity of PLGA (Resomer RG502)/BB (50/50) at 25°C.

To exclude the possibility that evaporation of BB raised the concentration of PLGA and thus the viscosity, the weight loss of the solution over time was determined by TGA. As expected from the high boiling point of BB (323°C), the six-hour weight loss at 25°C was less than 0.05% (Table 4-4). The corresponding increase in the PLGA concentration of less than 0.1% could therefore not account for the observed rise in the dynamic viscosity of the aged PLGA/BB.

Thus the later explanation (i.e. structure formation) is preferred. A series of experiments were designed to monitor the time effect on the structure change of PLGA/benzyl benzoate system in the later work.

Table 4-4 TGA result of PLGA (Resomer RG502)/BB solution isothermal at 25 °C for six hours

Time (min.)	Weight loss (%)	Concentration (wt %)
0	0.0000%	50.000%
60	0.0032	50.001%
120	0.0122	50.003%
180	0.0202	50.005%
240	0.0297	50.007%
300	0.0355	50.009%

* The start concentration is 50wt%, and supposed the weight loss was only from solvent evaporation.

(b) Special Sample Preparation:

The PLGA/benzyl benzoate solutions were aged in plastic syringes (see 3.2.2) at 10 °C, 25 °C and 37 °C, and oscillatory frequency sweeps were conducted at different time points by cutting proper-sized samples and loading directly on the plate of rheometer. Samples were not heated.

Figure 4-12 Time effects on the dynamic viscosities of the ageing solutions made up of PLGA (Resomer RG502)/BB (50/50). The solutions were aged at 10, 25, and 37 °C. Viscosity taken from frequency “sweep” at $\omega = 1 \text{ rad.s}^{-1}$, at 25°C, and are shown as the average of 3 repetitions, standard deviation shown as error bar.

Fig. 4-12 shows that the dynamic viscosities of PLGA (Resomer RG502)/benzyl benzoate solutions aged at 25 °C and 37 °C increase with ageing time, in contrast to those samples loaded by heating. Compare with Fig. 4-8. These results also support the hypothesis that some structure formation occurs in the solutions.

The storage and loss modulus change with time as shown in Fig. 4-13. For solutions aged at 25 °C and 37 °C, the storage and loss modulus both increase and the storage modulus increases faster than loss modulus. The two modulus cross each other at approximately 250 hours at 25 °C and about 500 hours at 37 °C after ageing, which suggest that a gel structure has been formed.

By comparison of the two experimental methods (Fig.4-14), we can infer that the above gelation structure is thermally reversible. The results obtained from the former method, (i.e., loading sample with heating) reflect the effect of the molecular weight change during ageing, while the results from the new method show the combined effects of degradation and gelation. In the early stages, there is a large difference between G' and G'' and the viscosity is relatively low. At the later stages, the viscosity increases considerably and eventually starts to decrease.

The decrease occurs when the degradation becomes dominant (caused by acceleration by accumulated carboxyl groups). Fig. 4-13C and 4-14C showed the G' , G'' and dynamic viscosity increased much more slowly at the later stage, and even decreased after approximately 550 hours at 37 °C.

Ageing at 10 °C shows no changes in G' , G'' , and dynamic viscosity (Fig. 4-13A, Fig. 4-14A), since benzyl benzoate has a melting point above this temperature. The frozen system makes the movement of molecules and molecular chains impossible, thus preventing the formation of gel.

Figure 4-13A, B and C Time effects on the storage modulus and loss modulus of PLGA (Resomer RG502)/BB (50/50) solution aged at 10, 25, and 37°C. Data are taken from frequency “sweep” at $\omega = 1 \text{ rad. s}^{-1}$, at 25°C, and are shown as the average of 3 repetitions, standard deviation shown as error bar.

Figure 4-14A, B and C Comparison of the dynamic viscosities from two methods of frequency sweep, PLGA (Resomer RG502)/BB solution was aged at 10, 25, and 37 °C. Data are taken from oscillatory frequency “sweep” at $\omega = 1 \text{ rad, s}^{-1}$, at 25°C, and are shown as average of 3 repetitions, standard deviation shown as error bar.

4.2.3 Nature of the Gel

It is clear that the PLGA/BB solution behaves quite differently from a PLGA/NMP solution upon ageing. Although molecular weight shows only a decrease for both, the dynamic rheological quantities indicate an initial increase for the PLGA/BB solution. The crossover of G' with G'' points to gel formation.

Since the formation of a semi-solid (gel) has relevance to drug delivery, in the following sections, we focus on study of the mechanism of gelation by means of rheometry, and Modulated Differential Scanning Calorimetry (MDSC). ^1H and ^{13}C Nuclear Magnetic Resonance were also employed to relate the microstructure of the amorphous polymer to the gelation phenomenon.

4.2.3.1 Molecular Structure Basis for Gelation

In order to determine the mechanism of the gelation phenomenon in PLGA solution, a series of poly (d, l-lactide-co-glycolide) and poly (d, l-lactide) were purchased from three different manufacturers (all were made by ring-opening synthesis from Sn-series initiators according to their certificate analysis reports). The microstructure was characterized by ^1H and ^{13}C n.m.r. spectra and compared with the gelation kinetics of their solutions in BB.

- **Sequence length determination**

We were interested in determining whether the glycolide or lactide sequence length was a factor in the gelation process. The NMR measurements (^{13}C) were used to quantify these lengths, as described below.

By applying the probability theory to the estimation of copolymer sequence distribution²⁰¹, the instantaneous copolymer composition, the number average length of monomer runs and the measure of the deviation from random statistics can be expressed as:

Instantaneous copolymer composition:

$$y = P(A) / P(B) = (1 + r_A x) / (1 + r_B / x) \quad (4.5)$$

r_A and r_B are reactivity ratio of monomer A and B,

x is the monomer feed ratio, $[A]/[B]$.

Number average length of A or B runs:

$$L_A = P_1(A) / P_2(AB) = 1 / P(B/A) = 1 + r_A x \quad (4.6)$$

$$L_B = 1 / P(A/B) = 1 + r_B / x \quad (4.7)$$

A measure of the deviation from randomness statistics may be obtained from the parameter, χ , and defined as:

$$\chi = (r_A x + 2 + r_B / x) / (r_A x + 1 + r_A r_B + r_B / x) \quad (4.8)$$

The copolymer is:

Completely random when $\chi = 1$

Completely alternative when $\chi = 2$

Block copolymer when $\chi \approx 0$

Copolymer with an alternating tendency when $\chi > 1$

Copolymer with a “blocking” tendency when $\chi < 1$

It is evident that the reactivity ratio and the monomer feed ratio determine the final microstructure of copolymer.

An example of the ^{13}C NMR spectra of the poly (d, l-lactide-co-glycolide) is shown as Fig. 4-15: the entire copolymer exhibits two well resolved signals arising from the sequences: GGGG (167.57 ppm) and GLL (167.47 ppm), where L stands for the lactyl unit $-\text{CH}(\text{CH}_3)-\text{CO}-\text{O}-$, and G for the glycolyl unit $-\text{CH}_2-\text{CO}-\text{O}-$. Using the procedure outlined in Appendix, we can compute the average length of a glycolide unit (which is twice the length of a glycolyl unit defined above).

The average lengths of glycolide blocks are listed in Table 4-5, which were calculated from the integration of the two split signals. The detailed method is recorded in the Appendix. The calculation method has been used by the manufacturer and other researchers^{193,194}.

In the ring-opening polymerization, the glycolide monomer is reported to have higher reactivity than the lactide monomer. The reported reactivity is 2.8 for glycolide and 0.2 for lactide. Thus, the polymerization rate of

glycolide is higher than that of lactide. Glycolide has almost a 3-to-1 preference to react with a glycolide unit than with a lactide unit, and lactide has a 5-to-1 preference to react with a glycolide unit than with a lactide unit^{123,202}. When lactide is copolymerized with glycolide at a high glycolide concentration, there is a tendency for blocks of glycolide to be formed rather than a random copolymer structure of glycolide and lactide units. At the beginning of polymerization, blocks of glycolide will be separated by single lactide units, while lactide will be incorporated to ever-increasing extents as the glycolide is depleted.

The measured average block lengths of glycolide confirm this. It can be seen that even the copolymer with L/G ratio of 75/25 has an average sequence length of 2.58 for glycolide. The variation of the G-sequence length among the 50/50 copolymers is due to the fact that the reactivity ratio may be affected by other variables such as the type of initiator, reaction temperature, the solvent used, etc. Further the monomer feed ratio may also change from one process to another, and the reaction may be terminated at different conversion. All these variations in process parameters result in the different microstructure of the polymers from different manufacturers, or even from different production batches.

Further transesterification processes that take place together with the main copolymerization reaction also play important role in the microstructure of copolymer chains. Transesterification, mainly the specific second mode transesterification of lactidyl groups, causes the appearance of short

segments containing lactyl and glycolyl sequences (-GLG-, -LGL-, -GLGL-, and others similar to them). As a result of more transesterification reactions, a decrease of glycolyl block length and an increase of LGL sequence concentration will occur. All of this is reflected in the differences between polymers obtained from different vendors.

Table 4-5 L/G ratios from ^1H n.m.r. and number average sequence length of glycolide from ^{13}C n.m.r. Average data from two consistent measurements are shown.

Polymer	Number average block length of glycolide sequence	L/G ratio
	Calculated from ^{13}C n.m.r.	Calculated from ^1H n.m.r.
Resomer RG502	3.39 ± 0.12	51.7/48.3 (52/48)
Resomer RG502H	2.91 ± 0.04	52.5/47.5 (53/47)
A137-28	~	100/0 (100/0)
A137-29	2.58 ± 0.07	75.3/24.7 (75/25)
A137-31	2.50 ± 0.11	54.0/46.0 (54/46)
APT071603-1	2.91 ± 0.06	53.2/46.8 (53/47)
PURASORB PDLG	3.42 ± 0.14	54.5/45.5 (52/48)
PURASORB DL FK655	2.78 ± 0.08	53.9/46.1 (53/47)

Data in bracket refer to the specified values from suppliers.

- **L/G ratio determination**

From the results of ^1H n.m.r. spectra, the L/G ratios were calculated based on the distinct behavior of the involved hydrogens in a magnetic field⁶², which are coincident with the specified values from manufacturers (Table 4-5). In addition, we observed dominant quota of G-pentads (-GGGGG-) at 4.88 ppm in all the copolymers (Fig. 4-16)²⁰³, although the exact proportion cannot be calculated from the results because of insufficient resolution. This result further confirms the blocky structure demonstrated by ^{13}C nuclear magnetic resonance.

Figure 4-15 ^{13}C NMR (400 MHz) spectrum of poly(lactide-co-glycolide)s.

Figure 4-16 ^1H NMR (400 MHz) spectrum of poly (lactide-co-glycolide)s, region of glycolide methylene protons, and assignment of signals see reference of Kasperczyk²⁰³.

4.2.3.2 Gelation properties of BB solutions of different polymers:

Rheology

As discussed above, Resomer RG502/BB solution has clearly shown a complex flow property influenced by both gelation and degradation during ageing. In this section, the flow properties of different polymers with different molecular structure and from different manufacturers are reported.

Solutions of different polymers in benzyl benzoate were prepared at the same concentration (50wt%), and allowed to age at 25°C. When rheological measurements were made on these unheated aged samples, an increase over time was observed in the dynamic viscosity of BB solutions

prepared from RG502H (Fig. 4-17A), PURASORB PDLG (Fig. 4-17B), and APT071603-1 (Fig. 4-17C). These results indicate that some sort of thermoreversible structure formation occurred in these solutions similar to the observation for Resomer RG502. However, for the solutions of A137-28, A137-29, A137-31 and PURASORB DL655FK, only decreases in the viscosity were seen. These solutions do not form any 3-D structures, and their behavior is merely a consequence of degradation (Fig. 4-17D to 4-17E).

Gel points were calculated as the time when the storage modulus equals the loss modulus, and are shown in Table 4-6. By comparing with the results in Table 4-5, it is evident that the gelation behavior of the PLGA in solution correlates with the glycolide block length in the polymer. For polymers with average glycolide length of less than ~ 2.90 , no gelation occurs, regardless of the L/G ratio and the terminal group of the copolymers.

Figure 4-17 A, B, C, D & E Storage/loss modulus and dynamic viscosity as a function of time for five PLGA/BB (50/50) solutions aged at 25°C. Samples were not heated before measurements taken. Data are taken from oscillatory frequency “sweep” at $\omega = 1 \text{ rad.s}^{-1}$, at 25°C, and are shown as average of 3 repetitions, standard deviation shown as error bar.

Table 4-6 Gel formation kinetics of various PLGA/BB solutions ageing at 25°C

BB solution of:	Composition PLGA/BB (wt)	Gel point (hours) defined by G' crosslinking with G''
Resomer RG502	50/50	~250
Resomer RG502H	50/50	> 1100 [@]
A137-28	50/50	*
A137-29	50/50	*
A137-31	50/50	*
APT071603-1	50/50	~450
PURASORB PDLG	50/50	~6
PURASORB DL FK655	50/50	*

[@] viscosity increased slowly, gel point not detected within 2 months.

* does not gel either upon ageing or upon injection into buffer.

Gelation found in the PLGA/BB system may reflect a type of solvent-influenced association, which is dominated by the glycolide sequence length. It is well known the homopolymer poly (glycolide) is a highly crystalline polymer that does not dissolve in most common solvents, and the poly (d, l-lactide) is an amorphous polymer that has a wide range of solvents. The copolymers are usually amorphous (especially if the lactide is a racemic mixture of *d* and *l*) or of very low crystallinity. However, if the

glycolide sequence is long enough, ordering (or microcrystallite formation) might occur at local portions of the chain. When this occurs in a diluent, it promotes gel formation. These segments tend to aggregate in order to lower the energy of the system.

Gel formation occurs in these systems if the average glycolide segment is above 2.9, however, the details (including the kinetics) of the gel formation process may be dependent on the sequence length distribution, as well as on the average sequence length. That is probably why the Resomer RG502H (with a similar average block length as APT071603-1) exhibits different gelation kinetics.

4.2.3.3 Differential Scanning Calorimetry (DSC) Observations

Figure 4-18 Calorimetric thermograms of a PLGA(Resomer RG502)/BB (50/50) solution aged at 25°C at heating rate of 5°C/min; Measurements shown were taken after 3 hours (t1), 92.1 hours (t3), or 188.2 hours (t6). After 188.2 hours of aging, the solution was also measured after melting (t6 melt).

Figure 4-19 Comparison of DSC endotherm and dynamic viscosity as functions of time for a PLGA (Resomer RG502)/BB (50/50) solution aged at 25°C. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Fig. 4-18 shows the DSC curves for a Resomer RG502/BB solution aging at 25°C. The three curves correspond to data obtained at three time points during the ageing process. While no distinct endothermal peak was observed at the initial stage (up to 30 hours), a clear broad peak was observed at about 47°C for solutions aged for 92 hours (t₃). This endothermal peak is associated with structure formation in the aged solution. The increase in the peak area at the next time point (t₆) indicates growth of this gel structure. Peak position does not change with aging time implying that the structure merely grows in extent with time.

Fig. 4-18 also shows two DSC curves obtained from the gelled solution before and after melting. No endothermal peak can be detected for the

“melted” sample, suggesting that the gel structure is destroyed by heat. The increase in the enthalpy change with increasing aging time parallels that of the dynamic viscosity obtained from rheological experiments (Fig. 4-19).

Figure 4-20 Comparison of DSC endotherm and dynamic viscosity as functions of time for a PLGA (Resomer RG502)/EB (50/50) solution aged at 25°C. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

The gelation kinetics of Resomer RG502 was also studied in another solvent, ethyl benzoate (EB) (Fig. 4-20). The EB system showed faster gelation kinetics than the BB system (gel point is ~60 hours for EB solution), but the endothermal peak appeared at a comparable temperature, which indicated that similar structures are formed in the two solvent systems. The faster kinetics could be due to the relatively low viscosity of the EB solution that is favorable to the motion of molecular chains.

The variation of dynamic viscosity and enthalpy with time reflects relatively slow gelation kinetics. The transition seems to be dominated by time instead of temperature, thus the gel formation exotherm cannot be determined by a “cooling ramp”.

Figure 4-21 Calorimetric thermograms of different PLGA/BB solutions aged at 25 °C at heating rate of 5°C/min.

Thermal behavior similar to that of Resomer RG502 is also observed in the other PLGA/BB solutions, where the average sequence length of glycolide is above ~2.9, see Fig. 4-21. The transition temperature and the enthalpy reveal some differences for different copolymers. The RG502H has the lowest enthalpy, which is consistent with its slow gelation kinetics found in rheological data. The Purasorb, which has the lowest molecular weight but the largest average sequence length of glycolide shows two endotherms: a

lower melting endotherm whose onset and maximum peak position are a little lower than the other two polymers, and a small higher melting endotherm at about 56°C. This phase behavior is very similar to the thermal behavior of a “crystallizable” polymer gel system, where liquid-liquid phase separation takes place before crystallization.

A possible reason for gel formation is the existence of some sort of polymer-polymer crystallite aggregation in three dimensions. To explore this, we studied gel formation of PLGAs in a low-boiling solvent, ethyl acetate (EA). For those polymers that do form gels, their ethyl acetate solutions show relatively faster gelation than the BB system (observed by test tube tilting). After ageing for 18 days, the Resomer RG502/EA gel was analyzed by MDSC measurement, and at the same time part of the gel was vacuum dried immediately at room temperature (which is lower than the “melting” temperature of the gels). After 48 hours, the partially dried sample was measured by MDSC. The endothermal peak was still present, but shifted to a higher temperature (Fig. 4-22) for reasons to be explored below. The same phenomenon was also detected in the EB system (data not shown).

Figure 4-22 Calorimetric thermograms of Resomer RG502/EB (50/50) solution aged at 25°C for 18 days; Measurement was also done to the partially dried gel, the heating rate is 5°C/min.

The crystallinity of semicrystalline polymers generally depends on the polymerization degree and the crystal perfection²⁰⁴. Using the bulk heat of fusion for poly (glycolide), which was reported to be 11.96 KJ*(mol monomer)⁻¹ or 11.75 KJ*(mol monomer)⁻¹ for 100% crystalline poly (glycolide)^{205,206}, the PLGA “crystallinity” in our gel systems was estimated to be around 7 – 8 % of the total glycolide units. However, the “crystallites” in the gels are small due to the low molecular weight of the glycolide segment and due to the presence of solvent molecules. These two factors account for the low temperature of “melting” observed in the gels, which are about 130°C below the bulk melting point of poly (glycolide),

and about 35°C lower than T_m of diglycolide²⁰⁴. The solvent behaves as defects or an impurity, and thus lowers the melting temperature; when it is removed (as in the case of EA), the melting point shifts upward (Fig. 4-22).

We use the terms “crystallite”, “crystallinity” etc to describe the existence of an ordered “aggregate” structure due to the presence of sustained glycolide segment lengths. Although a “melting” endotherm has been detected, we have been unable to observe any X-ray diffraction patterns of crystal for these gels.

Figure 4-23 Calorimetric thermograms of PLGA (Resomer RG502) and the dried Resomer RG502/DCM gel.

The gelation properties are also solvent dependent. When studying the other solvents that might induce gelation, we found that the above polymers do not form gels in “good” solvents such as acetonitrile and N-methyl-2-

pyrrolidone but do form gels in certain other “good” solvents, dichloromethane (DCM) and tetrahydrofuran (THF). The heating curve of MDSC showed the fully dried gel (from a DCM solution) has an endothermal peak at about 76 °C as shown in Fig. 4-23, which did not exist in the raw polymer. Perhaps preferential solvents for the glycolide segment might be suppressing gel formation.

The gelation of PLGA solution is concentration dependent. However, as mentioned above, a lower solubility limit exists in the PLGA/benzyl benzoate system. As a result, PLGA/ethyl acetate (EA) system is studied below.

PLGA (RG502) is easily dissolved in ethyl acetate at room temperature, the solutions with different concentrations were allowed to age in sealed vials at 25°C. The phase behavior was observed visually and by tilting. Solutions form gel structures when $c \gtrsim 20\text{wt}\%$, and phase separation takes place when $c \lesssim 20\text{wt}\%$. The phase-separated system consists of an upper layer of clear solution and a bottom layer of polymer clusters.

The existence of this critical concentration seems to be due to the limited number of sequences that are available for association or “crystallization” in the copolymers. A relatively higher critical concentration c^* will be required for gelation, which can be defined as per Takasashi¹⁷⁵ as the minimum concentration at which each polymer chain passes through at least two different crystallites. The concentration is confirmed by the composition of the cluster layer for the phase-separated system, which is

calculated to be nearly 20wt% (less than 20wt% normally) when the top layer is carefully removed from the bottom layer, and the latter is analyzed.

Figure 4-24 Calorimetric thermograms of PLGA (Resomer RG502)/EA solutions aged at 25°C for 550 hours at heating rate of 5°C/min.

Since the gelation of PLGA solution is kinetically slow, and also affected by polymer degradation, the equilibrium phase diagram is not easy to obtain. Experimentally, a systematic DSC investigation was carried out at different time points. The melting endotherm increased with time until it reached a stable value. Fig. 4-24 shows the typical DSC traces of PLGA (RG502)/EA gels, with a broad gel melting endotherm, which suggests a first-order transition.

The gel melting temperature and the enthalpies involved in gel melting (gel melting enthalpy) are plotted as a function of concentration (Fig. 4-25). The gel melting enthalpies are given per gram of gel, and the values are measured at 5°C/min. As can be seen from Fig. 4-25, the melting enthalpies first increase linearly to a concentration $C_g \approx 50\%$ (region B1) and then decrease (region B2). The gel melting point (maximum of melting endotherm) increases monotonously as a function of polymer concentration initially until it reaches a stable value at $C \approx 50\%$. Region A represents the phase-separated region.

Figure 4-25 Melting enthalpies (per gram gel) and gel melting point as determined from heating rate of 5°C as a function of polymer concentration in PLGA (Resomer RG502)/EA gels. The data are from gels after ageing for 550 hours. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

The existence of the maximum both in gel melting temperature and the enthalpy as a function of concentration of C_g may be due to several possible reasons: 1) a stoichiometric compound of polymer-solvent exists, which is responsible for the formation of physical gel as in the aPS-CS₂ system¹⁸⁹; 2) the solution at higher concentration has not reached its thermodynamic equilibrium point due to the slow gelation kinetics; 3) microphase separation and 4) polymer self-interaction.

Considering the possibility of the existence of polymer-solvent stoichiometric compound, as in the aPS gel system, the structure will collapse when the solvent is progressively removed¹⁸⁹. However, the results shown in Fig. 4-22 and Fig. 4-23 indicate a well-preserved structure after removing the solvent, which implies that a stoichiometric polymer-solvent compound is NOT formed. However, it is true that the polymer structure forms, induced by solvent.

As we discussed above, the increase in the dynamic viscosity and the growth of endotherm in PLGA gel system both reflect a slow kinetics of gelation. In a certain polymer/solvent system, viscosity may be an important factor that affects gelation kinetics, especially the growth of gel, by affecting the mobility of polymer chains, i.e., the higher the viscosity of the polymer solution, the slower the gelation kinetics.

As a result, the decrease of enthalpy at higher polymer concentration shown in Fig. 4-25 may be due to gelation kinetics. The decrease of enthalpy with

concentration in Fig 4-25 (B2) may be due to incomplete or non-equilibrium gel formation.

There is no visual evidence of microphase separation at the higher concentrations. However, polymer self-interaction may exist due to increased concentration, and this effect should be more pronounced in long molecular chains (high molecular weight). The contribution of polymer self-interaction should not be significant in this system since the molecular weight of the PLGA is only 17,000 Dalton.

The above results indicate random association in solutions of PLGA in selective solvents, when the average sequence length of glycolide is long enough. The insoluble glycolide sequences form multiplet-like domains bridged by soluble lactide sequences. Formation of polydispersed clusters takes place at lower copolymer concentrations, while gel formation is favored at higher concentrations due to the growth of randomly multiconnected clusters linked together by insoluble domains.

To summarize, we studied the nature of the gels formed in certain PLGA/solvent systems. The microstructures of the polymers were characterized by ^1H and ^{13}C NMR. The kinetics of gel formation was quantified using rheometry, while the energetics was quantified using MDSC. The data clearly establish some interesting facts about these gel-forming systems of random copolymers:

- 1) The three-dimensional structure formed is most likely an ordered aggregate of microcrystallites.

- 2) The aggregation is thermoreversible, and is associated with an enthalpy.
- 3) The ordered structure is found to form only for polymers that have glycolide block lengths above about 2.9 (number-average). For polymers with glycolide lengths below this value, no gel formation is observed. This may imply that a certain local stereoregularity is necessary for structure formation.
- 4) The kinetics of gel formation is dependent on microstructural details, as well as on the nature of the solvent, and a critical concentration is required for gelation.
- 5) The observed melting temperatures for the gels are much lower than the melting temperatures for the homopolymer of poly (glycolide), and are attributed to the much shorter glycolide segments, and to the presence of substantial amounts of solvent. Solutions of these polymers in BB/EB form depots by an entirely different mechanism.

4.3 Depot Formation of PLGA Solutions

4.3.1 In vitro Degradation in Buffer

We studied the properties of PLGA solutions after injection into a suitable buffer. For convenience, we use the term “in vitro” to depict the study of PLGA solution in buffer media.

In vitro degradation tests were carried out in phosphate buffer (pH 7.0, 37°C). Fig. 4-26 and Fig. 4-27 show the changes in molecular weight of solutions made up of PLGA (Resomer RG502) in two different solvents (BB and NMP).

Figure 4-26 In vitro degradation of PLGA (Resomer RG502)/BB solutions with/without drug, where drug is metoclopramide in salt and base forms, the formulation is PLGA/BB/(drug) = 50/50/(5). Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Figure 4-27 In vitro degradation of PLGA (Resomer RG502)/NMP solutions with/without drug, where drug is metoclopramide salt or metoclopramide base, the formulation is PLGA/NMP/(drug) = 50/50/(5). Data are shown as average of 3 repetitions, standard deviation shown as error bar.

The molecular weight (Mw) of all the PLGA solutions decreased immediately after placement in buffer and continued to decrease throughout the time course of the study. All systems show the same tendency, i.e., the solution with metoclopramide base displayed the fastest degradation, followed by the solution with metoclopramide salt and the solution without drug. For solutions with different metobase loadings, the degradation rate increased with the drug loading (Fig. 4-28), and the degradation rate constant (within the initial 200 hours) is proportional to the drug loading that suggest a kinetics of co-existence of catalyzed reaction and non-catalyzed reaction as in a typical reaction $A \rightarrow P$, where catalyzed and non-catalyzed reaction co-exist, and

$$r = r_0 + r_c = (k_0 + k_c[C])[A] = k[A] \quad (4.9)$$

$$k = k_0 + k_c[C] \approx 0.0017 + 0.0201[C] \quad (4.10)$$

where r and k are total reaction rate and rate constant, r_0 and k_0 are reaction rate and rate constant without catalysis, r_c and k_c are reaction rate and rate constant of catalyzed reaction and $[A]$ and $[C]$ are concentrations of reactant and catalyst. k_c is much higher than k_0 .

The actual condition has the following deviation from the hypothesis:

(1). The concentration of catalyst refers to the dissolved catalyst, due to the limited solubility (~0.30 wt %) of metobase in the depot formulation, the rate constant should negatively deviate from the linear relationship when increased the drug loading (this is true initially). The above linear relationship is only

possibly true when drug completely dissolved in the depot and the drug released from different depot at similar rate by percentage.

(2). There might be another kind of self-catalysis by the terminal group, so:

$$r = r_0 + r_c + r_{c1} = (k_0 + k_c[C] + k_{c1}[B])[A] = k[A] \quad (4.11)$$

where k_{c1} represents the reaction rate constant of terminal group self-catalyzed reaction, $[B]$ represents the concentration of terminal group and is quite low.

Figure 4-28 Effect of metoclopramide base loadings on the degradation of PLGA (Resomer RG502)/BB formulations, the pseudo-first order hydrolysis shown for different drug loadings within the first 200 hours.

Compared with the systems with metosalt and without drug, where self-catalysis by terminal groups also exists and the rate constants are 0.0018 and 0.0015 individually, we believe catalysis by metobase contributes more in the

degradation of PLGA, the more drug that was added, the faster the polymer degraded.

The trends parallel those observed in section 4.2.1, where the same samples, when aged at different temperatures in closed vials (no injection into buffer) also show similar decreases in molecular weight. The extent of degradation is much less during the ageing process, presumably due to the relatively high amount of water available in the in vitro experiment.

Figure 4-29 In vitro degradation of PLGA (Resomer RG502)/BB solutions with/without drug, where drug is lidocaine in salt and base forms, the formulation is PLGA/BB/(drug) = 50/50/(5). Data are shown as average of 3 repetitions, standard deviation shown as error bar.

The molecular weight change is also consistent with the morphology change during the whole experimental process. For PLGA/benzyl benzoate system, about 213—252 hours after injection into buffer, the depot containing

metoclopramide base appeared spongy and creamy, while the same phenomenon appeared in those containing salt only from 480—560 hours after in vitro. When the solvent is changed to NMP, degradation occurs earlier in the PLGA/NMP system containing base than that with salt. See Fig. 4-27. The same base catalysis on hydrolysis of PLGA was seen in system with another drug, lidocaine salt and base, as shown in Fig. 4-29.

The composition and molecular weight of PLGA in surface layer and in the core of the depot for BB system were also investigated. The molecular weight of PLGA in the surface layer e.g., Resomer RG502/BB solution in vitro for 43 days has molecular weight of 5650 Dalton at the surface and 2650 Dalton in the core, the molecular weight distribution of PLGA in core is also broader than that of the surface polymer. Leaching of the degradation products (dissolvable monomers or oligomers) should be responsible for the difference in the molecular weight and its distribution. This could be due to heterogeneous effects as water penetrates the core.

Two model protein drugs, lysozyme and BSA, were also investigated. Fig. 4-30 shows that both depots containing model protein drug showed faster degradation than that without drug initially; however at long times, the effect disappears. Comparing the two systems with protein drug, PLGA molecular weight of depot with chicken egg lysozyme, a basic protein ($pI = 11.1$) degraded slower than that with BSA, a weak acidic protein ($pI = 5.82$).

Figure 4-30 In vitro degradation of PLGA (Resomer RG502)/BB solutions with/without drug, where drug is chicken egg lysozyme (lysozyme) and bovine serum albumin (BSA), the formulation is PLGA/BB/(drug) = 50/50/(10). Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Encapsulated proteins would increase the hydrophilicity of the PLGA/BB depot, which accounts for the faster degradation of PLGA in the early stage. While proteins are amphoteric molecules, the pI value is related to the ratio of basic to acidic residue amino acid groups in the protein molecule: the higher the pI value, the more the basic residue groups. The buffering capacity of protein might neutralize the carboxyl groups generated by hydrolysis, which then affects the degradation kinetics. This is coincident with the finding of P. Johansen et al that the buffering effect of the encapsulated protein itself at high loading may counteract a local pH drop, and BSA as an additive was suggested to act as a proton scavenger during release of tetanus toxoid from PLGA microparticles²⁰⁷. The effect of encapsulated proteins is also observed

in the results of M. Sandor et al²⁰⁸, although they claimed that encapsulated protein, regardless of molecular weight (including lysozyme and BSA) did not affect degradation or erosion of the PLGA microsphere system, their results on molecular weight loss and mass loss did show slower degradation in the initial followed by faster degradation for the control sample (without protein) than those encapsulated with proteins.

Beside the effects of entrapped drug, molecular parameters also affect the degradation rate of the polymer, the nature of the terminal group being one parameter. The RG502 used above is an ethyl ester terminated polymer. In this section we compared RG502 with another two polymers with nearly the same L/G ratio but terminated by carboxylic acid group. As expected, the PLGA terminated by carboxylic acid is more difficult to dissolve in the solvent BB; longer time and higher temperatures are needed to get the solution, which we believe to be due to the terminal group effect. As shown in Fig. 4-31, the PLGA terminated by carboxylic acid groups degraded much faster than PLGA terminated by ethyl ester. Applying first order degradation kinetics to the early stage of in vitro, the degradation rate constant of the carboxylic acid terminated PLGA was found to be about 3 times that of the ethyl ester terminated PLGA. This is consistent with the finding of Tracy et al²⁰⁹, who found the uncapped end group polymer degraded 2-3 folds faster in vitro than the capped polymer.

Figure 4-31 In vitro degradation of PLGA/BB (50/50) solutions, where PLGA are RG502 terminated by $-\text{COOC}_2\text{H}_5$, Apt071603-1 terminated by $-\text{COOH}$ and DL655FK terminated by $-\text{COOH}$. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Two possible reasons might be responsible for the faster degradation of carboxylic acid terminated PLGA, relative harsh preparation conditions and/or the function of terminal groups. By comparing the PLGA molecular weight of raw material and the fresh solution, we found the molecular weight loss due to preparation is negligible for both polymers. Thus the faster hydrolysis is due to the catalytic effect of the acidic end groups, as well as some increased hydrophilicity. The faster degradation of PLGA terminated by carboxylic acid group was also confirmed in the NMP system, in which the same preparation time and temperature was used (result not shown).

It should be stated that degradation after buffer injection should be characterized both by molecular weight information of the polymer, as well as

by the monomers and oligomers released into buffer (which is equivalent to the weight loss). The system composition in our study made the latter determination difficult. However, the GPC method we used for the polymer still gave good information on in vitro degradation.

4.3.2 Mechanism of Depot Formation

It is well known the PLGA solution was developed as an injectable in situ formed drug carrier based on the mechanism of non-solvent induced phase separation (NIPS), which has been used in explaining membrane formation. The basic idea needs a polymer that does not dissolve in the bath side liquid and a solvent of the polymer that is miscible with the bath side liquid fully or partially. Liquid – liquid demixing is a necessary step in the process that is responsible for initiation and growth of the formed solidified structure, which includes a skin at the surface and porous support structure.

To quantify the liquid-liquid de-mixing, we injected PLGA solutions (about 500mg) without drug into buffer solution. The amount of solvent migrating into the buffer was analyzed by HPLC. It can be seen from Fig. 4-32, that the NMP, a fully water-miscible solvent, showed the fastest migration kinetics, the NMP released completely within 200 hours. Triacetin migrated into buffer phase more slowly and for benzyl benzoate, due to its limited solubility in water, the total release was less than 3% within 1100 hours. For samples from drug release experiment, the concentration of BB ranges from 0.01446 to 0.01839 mg/ml, the variation is due to the released drugs (metoclopramide/protein) and the degraded PLGA.

Figure 4-32 The amount of solvent released into the buffer as a function of time when PLGA (Resomer RG502) solutions in vitro at 37°C in buffer, the formulation is PLGA/solvent = 50/50. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

On the other hand, we also monitored the water content and its distribution in the PLGA (RG502)/BB depots. The results are shown in Fig. 4-33. Although the estimation of water content in the depot is difficult, the overall trend is clear in Fig. 4-33. Water locates mostly at the surface layer of the depot, especially during the earlier stage. It seems to invade the depot as a result of leaching-out of drugs or of degraded products from the depot, and exists mostly as macro-phase separated water vesicles. Very little water diffused into the core of the depot, e.g., water content in the core of RG502/BB depot without drug is only 0.29% after immersion in buffer for approx. 300 hours. Encapsulated drugs undoubtedly increased the hydrophilicity of the depots by showing much higher content of water in the drug-loaded depot than that

without drug. For the depot containing base drug, the interface between surface layer and core disappeared early from about 250 hours, which is consistent with the aforementioned fast degradation and the drug release kinetics that we will discuss later.

Figure 4-33 water content in PLGA (Resomer RG502)/BB depots with/without drugs during in vitro, filled legends represent the water content in the whole depot (including core and surface), while unfilled $\square$ represents the water content in the core of RG502/BB/metosalt system, unfilled $\circ$ represents the water content in the core of RG502/BB/BSA system, unfilled $\diamond$ represents the water content in the core of RG502/BB system. Data are shown as average of 5 repetitions, standard deviation shown as error bar.

From above results, we can infer that the NMP and triacetin systems act in accordance with this NIPS principle.

In the case of BB system, the amount of BB diffused-out is limited, leading to a small viscosity increase in the depot due to concentration change. On the other hand, the water diffused-in contributes more to hydrolysis of PLGA that leads to a decrease in viscosity, than in inducing phase separation (polymer precipitation). As a result, PLGA/BB system may not form a depot upon injection into buffer via the NIPS mechanism. Thus most PLGA/BB solutions do not form a semi-solid depot upon buffer injection, however, some PLGA/BB solutions do form a semi-solid depot that we believe occurs via a different mechanism.

4.3.3 Depot Formation Kinetics

The PLGA/BB system has been claimed to be a slow phase inversion system when used as drug carrier. However, we found that PLGA solutions from different solvents may not all form depots after injection into a water environment. Among the different systems we investigated, all NMP solutions of PLA/PLGA can form semisolid/solid like depot after injection most certainly due to a non-solvent induced phase separation mechanism (NIPS), triacetin solutions also formed depots but more slowly. However, not all BB solutions of PLGAs form depots during in vitro, most solutions were fluid observed (by tilting the vials) within the whole period of experiment until they turn into a creamy morphology (still easily flowable), while others formed depots but without solidified skin as in NMP system. In association with the different gelation properties of PLGA/BB solutions during ageing, we

investigated the flow properties of PLGA solutions after buffer injection (“in vitro”).

Fig. 4-34 shows an increase of dynamic viscosity over time for RG502/BB solution in vitro at 37°C in pH 7.0 buffer. The amount of BB remains fairly constant, as measured by the BB concentration in the receptor liquids using HPLC, see 4.3.2. The figure shows that the viscosity reaches a maximum before decreasing. This again parallels the rheological behavior observed upon ageing (no buffer injection), except for a shift in the time scale to shorter times (for the buffer injection).

Figure 4-34 The flow properties of PLGA (Resomer RG 502)/BB solution in vitro/ageing at 37°C, the formulation is PLGA/BB = 50/50 by weight. Data are taken from oscillatory frequency “sweep” at $\omega = 1 \text{ rad.s}^{-1}$, at 25°C, and are shown as average of 3 repetitions, standard deviation shown as error bar.

In order to correlate the flow property of PLGA/BB solution after buffer injection with that during ageing, we compared the dynamic flow properties of different PLGA (PDLA)/BB solutions after in vitro injection and upon ageing as shown in Table 4-7. As is evident from the table, only those solutions that form gels during ageing also form gels upon buffer injection. We believe therefore that similar mechanisms are operating upon injection into buffer as for the aged samples, except that the time is shifted to shorter times for the buffer injections. The structure formation leads to a viscosity increase, which is eventually negated by molecular-weight decrease, so that a maximum is observed in the viscosity-time plots.

Figure 4-35 Effect of terminal group on the flow properties of PLGA/BB solution in vitro at 37°C, the formulation is PLGA/BB = 50/50 by weight. Data are taken from oscillatory frequency “sweep” at $\omega = 1 \text{ rad.s}^{-1}$, at 25°C, and are shown as average of 3 repetitions, standard deviation shown as error bar.

The viscosity of the PLGA terminated by carboxylic acid groups, in this case APT071603-1 (which has similar molecular weight and L/G ratio as RG502) was found to increase due to the structure formation and then decrease as the degradation of PLGA became dominant, but as expected from the degradation results, the maximum occurs at a much earlier time than that of RG502 system (Fig. 4-35). This result again confirms the earlier conclusion that terminal groups affect not only the degradation kinetics but also the kinetics of gel formation.

Table 4-7 Gel Formation Kinetics, PLGA/BB solutions without drug

Samples	Composition PLGA(PDLA)/BB	$t_{\eta\max.}$ (hours)	$t_{\eta\max.}$ (hours)
		In vitro	Ageing
RG502/BB	50/50	330	520
Apt071603-1/BB	50/50	90	590
RG502H/BB	50/50		~900 η^* increased slowly, but system has not gelled
DL655FK (Purac)	50/50	**	**
A137-28	50/50	**	**
A137-29	50/50	**	**
A137-31	50/50	**	**

** does not gel either during ageing or upon injection into buffer.

In contrast to the gelation behavior of above polymers, we found that those polymers that do not form gel during ageing, such as PLGA 53/47 (DL655FK

from Purac, initial Mw 45,000), also do not exhibit gel formation upon injection into buffer, from BB solutions. See Fig. 4-36. For this polymer, the viscosity decreases continuously over time, as would be expected of degrading polymers that do not form structures (or gels).

Figure 4-36 Gelation effect on the flow properties of PLGA (Resomer RG502)/BB solutions in vitro at 37°C, the formulation is PLGA/BB = 50/50 by weight. Data are taken from oscillatory frequency “sweep” at $\omega = 1 \text{ rad.s}^{-1}$, at 25°C, and are shown as average of 3 repetitions, standard deviation shown as error bar.

Flow properties of PLGA/BB solutions with drug (metoclopramide salt and base) were also investigated. As shown in Fig. 4-37, Fig. 4-38 and Table 4-8, for the solutions with drug, the dynamic viscosities of these solutions also show an initial increase followed by an eventual decrease. The onset of this decrease occurs earlier when base drug is used, indicating accelerated hydrolysis kinetics.

Figure 4-37 The flow properties of PLGA (Resomer RG502)/BB solution with/without drug in vitro at 37°C, the formulation is PLGA/BB/drug = 50/50/1 by weight. Data are taken from oscillatory frequency “sweep” at $\omega = 1 \text{ rad.s}^{-1}$, at 25°C, and are shown as average of 3 repetitions, standard deviation shown as error bar.

Figure 4-38 The flow properties of PLGA (Resomer RG502)/BB solution with/without drug in vitro at 37°C, the formulation is PLGA/BB/drug = 50/50/5 by weight. Data are taken from oscillatory frequency “sweep” at $\omega = 1 \text{ rad.s}^{-1}$, at 25°C, and are shown as average of 3 repetitions, standard deviation shown as error bar.

Table 4-8 Gel Formation Kinetics, PLGA/BB solutions with drug

Samples	Composition	$t_{\eta\max.}$ (hours)	$t_{\eta\max.}$ (hours)
		In vitro	Ageing
RG502/BB	50/50	330	520
RG502/BB/metosalt	50/50/1	300	
RG502/BB/metosalt	50/50/5	300	
RG502/BB/metobase	50/50/1	90	
RG502/BB/metobase	50/50/5	75	

Certain PLGA/BB systems that form in situ implants also undergo gelation upon ageing under ambient conditions or at 37 °C. We believe that the gel formations during ageing and upon buffer injection are due to similar mechanisms, namely “microcrystallite” formation. Correlating the results that we discussed in the previous section with the observed morphology of PLGA/BB systems after buffer injection, gelation upon ageing correlates well with in vitro depot formation, thus indicating that the depot formation mechanism is fundamentally different from phase inversion, as observed for the same polymer in a good water-miscible solvent, such as NMP, DMSO. Depot formation in these systems appears to be due entirely to the formation of an ordered three-dimensional structure, and not due to phase inversion.

Above we discussed the properties of PLGA solutions in a pure solvent. It should be mentioned the BB system is thought to form depot by the same

NIPS mechanism as in the NMP system, and the only expected difference is its slow phase inversion rate. Actually the BB system may act as a NIPS system in some circumstance, e.g., adding triacetin in the bath side during in vitro⁸ or using a mixed solvent of BB and water miscible solvent as NMP¹⁵ at proper ratios. These will lead to even more complicated kinetics especially for those PLGAs which gel at the same time in BB system.

4.3.4 Morphology of PLGA Solutions in vitro

For non-solvent induced phase separation (NIPS) system, liquid – liquid demixing directly affects the morphology of the formed structure and the latter determines the mass transport kinetics. McHugh et al have monitored the phase inversion kinetics of different PLGA solution using a dark field photograph technique, and related the phase inversion kinetics to the final morphology observed by SEM^{7,8}. Morphologies ranging from finger like macrovoids underneath a dense, thin skin, to a skinless sponge can be formed due to the dynamics of phase separation. The slower phase inversion results in sponge-like morphology and better release kinetics of the encapsulated drug (mostly protein or peptide drug), while the fast phase inversion results in finger like macrovoids and an obvious initial burst release of drug (mostly protein or peptide drug).

Figure 4-39 Scanning electron micrographs of depots made by quenching formulations with PLGA/NMP (50/50 by weight) in phosphate buffer for 72 hours: (A) A137/28/NMP (L/G 100/0) viewed at 200X (B) A137-29/NMP (L/G 75/25) viewed at 200X (C) RG502H/NMP (L/G 50/50) viewed at 350X (D) DL655FK/NMP (L/G 50/50) viewed at 750X.

Fig. 4-39 shows the morphologies of all PLGA/NMP solutions we studied for drug release. As shown in the SEM pictures, except for NMP solutions of DL655FK, all the other NMP solutions formed sponge-like morphology with small cavities under the skin. Fig. 4-39D showed large tear-like voids almost penetrating the whole film. Increased thickness of the quenched film is found in Fig. 4-39A to C, indicating swelling of the three low molecular weight PLGAs during phase inversion, while for DL655 FK, which is of relative large

molecular weight, shrinkage was found in the thickness of the formed film. The reason may be due to the molecular weight, i.e., although higher molecular weight is said to increase the viscosity of the solution that is unfavorable for diffusion, it also produces entanglements that result in shrinkage of the surface during phase inversion.

We were unable to measure the morphology of triacetin and especially BB systems primarily because the solvent exchange is slow. We found the surface of BB depot is neither a skin nor skinless sponge, but due to the degradation of PLGA, the surface layer will turn into a creamy and even softer layer than the depot core.

4.4 Controlled Release of Drugs from Depots

In this section, we report the investigations on sustained release of two model low molecular weight drugs—metoclopramide salt and base and two model protein drugs – bovine serum albumin (BSA) and chicken egg lysozyme (lysozyme), from the solutions of PLGA in three solvents, which have different water affinity. Effects of solvent type, drug type, polymer concentration, drug concentration, molecular weight, and ageing at 10 °C and 25 °C on the release kinetics were tested in vitro in phosphate buffer (pH 7.0, 37 °C). UV-Vis spectroscopy and high performance liquid chromatography were used for quantify the release of metoclopramide drug, and the protein drugs, bovine serum albumin and chicken egg lysozyme.

4.4.1 Low Molecular Weight Drugs: metoclopramide salt/base from BB system

- **Effect of drug type**

Fig. 4-40 shows the release kinetics of the salt and base form of metoclopramide into buffer. The release kinetics is affected by several factors that change the permeation parameter and permeation routes. Interestingly, the base drug shows a faster overall release kinetics compared to the salt drug.

The PLGA/BB/metobase system exhibits faster degradation than the PLGA/BB/metosalt system. On the other hand, in the PLGA/metobase system,

the drug is both dissolved and dispersed (relatively higher drug solubility in PLGA/BB, ~0.30 wt %), whereas in the metosalt case, the drug is almost completely dispersed in PLGA/BB (low drug solubility, ~0.02 wt%), and hence expected to show a greater burst effect, which is clearly not seen in this set of studies.

Figure 4-40 Release kinetics of metoclopramide salt and base from PLGA (Resomer RG502)/BB system, the formulation is PLGA/BB/drug = 50/50/5 by weight. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

According to the mathematical modeling of diffusion/degradation controlled drug release from biodegradable matrix developed by Couarraze and coworkers³⁴, considering polymer degradation and drug diffusion simultaneously, the cumulative absolute amount of drug released, Q (refers to M_t in most descriptions) versus time has the relationship as:

$$Q = S \sqrt{\frac{2C_0 C_s D_0 (e^{kt} - 1)}{k}} \quad (4.12)$$

where S is the surface area of the film exposed to the release medium; and C_0 and C_s are the initial drug concentration and the solubility of the drug in the system, respectively, and k is the first order degradation rate constant. The above equation can be also expressed as:

$$Q = A \sqrt{\frac{e^{kt} - 1}{k}} \quad \text{where } A = S \sqrt{2C_0 C_s D_0} \quad (4.13)$$

$$\text{or } Q = A_1 \sqrt{C_s} \sqrt{\frac{e^{kt} - 1}{k}} \quad \text{where } A_1 = S \sqrt{2C_0 D_0} = A / \sqrt{C_s} \quad (4-14)$$

In our case, both depots have the same drug loading C_0 (50/50/5). The 1st order degradation constants (k) are 0.0018h^{-1} for salt containing depot and 0.0058h^{-1} for base containing depot. The drug solubility in PLGA/BB depot is 0.02 wt % for metosalt and 0.30 wt % for metobase. From Equation 4.14, we can see both terms (in the square root) determined by drug solubility C_s (higher for base) and by degradation rate constant point to a faster release of the base drug than the salt drug. Such an analysis also implies that the burst effect is perhaps dominated by degradation-controlled release.

A non-linear regression analysis of the plot of the cumulative absolute release amount of drug Q versus square root of time $t^{1/2}$ is shown in Figure 4-41. Here we assume the geometry is same for salt and base depot, and the 1st order degradation rate constants were fixed as 0.0018h^{-1} for metosalt and 0.0058h^{-1} for metobase. The *Couarraze* model describes only the initial data of the

metobase system, i.e. before 100 hours as shown in Figure 4-41B, while data from the metosalt system matched well by the model in the first 800 hours (Figure 4-41A).

The values of A can be estimated from the slopes, i.e. $199.3 \mu\text{g}/\text{h}^{1/2}$ for metosalt system and $821.4 \mu\text{g}/\text{h}^{1/2}$ for the metobase system.

The discrepancy observed might be explained by usage of a single constant k to evaluate the degradation kinetics within the whole time span: actually for the PLGA/BB/metobase (50/50/5) depot, 1st order kinetics $M_t = M_0 e^{-kt}$ is observed only for the first 100 hours as shown in Figure 4-28. After 100 hours, the experimental data negatively deviate from the *Couarraze* model. We even observed a $t^{1/2}$ dependent release pattern from 100 hours to 900 hours, perhaps due to diffusion through a predominantly water-filled pore structure. The pores are caused by diffusion out of the drug molecules, low-molecular weight degradation product and/or of solvent.

For the PLGA/BB/metosalt (50/50/5) system, the experimental data positively deviate from the model after 800 hours when we used the experimental value $k = 0.0018$. By computerized optimization, we matched the experimental data with the *Couarraze* model over the whole time span (Figure 4-41C), the values of A and k thus obtained are $111.5 \mu\text{g}/\text{h}^{1/2}$ and 0.00436 h^{-1} , the 1st order degradation constant is much higher than the experimental data (0.0018 h^{-1}). When a similar fitting is carried out with the metobase system (Figure 4-41D), a negative k value is obtained, confirming the non-applicability of the model.

Figure 4-41 metoclopramide salt/base released from PLGA/BB system. PLGA/BB/drug = 50/50/5 by weight. The release kinetics was regressed with *Couarraze* model. A, B: 1st order degradation rate constant fixed; C,D: curve-fitting.

- **Concentration Dependence**

Regression analysis was also applied to the PLGA/BB depots with different drug loadings. All the metobase systems showed the same phenomenon as in Figure 4-41B, the *Couarraze* model only matched the first 100 hours' data, after that a $t^{1/2}$ linear dependent release pattern was observed. The regressions to the PLGA/BB systems with different loading of metosalt were shown in Figure 4-42 (data matched up to 800hours). The A and k values from optimized computing are listed in Table 4-9. Linear regression based on Higuchi was also calculated, as

At early time ($t < 100$ hours in this case),

$$e^{kt} \approx 1 + kt \text{ and: } Q = A\sqrt{t} \text{ --for Couarraze model} \quad (4.15)$$

$$\text{Here we used } Q(t) = a + b\sqrt{t} \text{ --for Higuchi's linear regression} \quad (4.16)$$

Both models describe the data well in the initial time points, and the calculated A and b values are shown in Table 4-9.

Table 4-9 Regressions of drug release amount versus $t^{1/2}$ based on: $Q = A\sqrt{\frac{e^{kt} - 1}{k}}$,
and $Q(t) = a + b\sqrt{t}$

PLGA/BB/metosalt Ratio by weight	t (hours)	<i>Higuchi model</i> <i>b</i> (ug/h ^{1/2})		<i>Couarraze model</i> <i>A</i> (ug/h ^{1/2})		
		experiment	R ²	matched		R ²
				<i>A</i>	<i>k</i>	
50/50/1	24 -- 800	68.384	0.994	51.9	0.0014	0.999
50/50/1.82		110.05	0.987	85	0.0016	0.997
50/50/5		228.68	0.996	175.4	0.00232 (0.0018*)	0.996
50/50/10		533.01	0.985	305.1	0.0040	0.995
PLGA/BB/metobase Ratio by weight	t (hours)	<i>Higuchi model</i> b (ug/h ^{1/2})		<i>Couarraze model</i> <i>A</i> (ug/h ^{1/2})		
		experiment	R ²	matched		R ²
50/50/1	100--900	102.59	0.998	Not matched		
50/50/5		643.03	0.997			
50/50/10		2002.2	0.998			

* experimental value.

Figure 4-42 metoclopramide salt released from PLGA/BB system with different drug loadings, the release kinetics was fitted with *Couarraze model*.

The Couarraze model fits the release data, for the metosalt, up to 800 hours, but in the initial time points (up to 100 hours), the model reduces to a diffusion model, similar to the Higuchi model. From the Couarraze model fit to the metosalt data, it is possible to extract values for the first-order degradation constant k . for the PLGA/BB/metosalt (50/50/5) sample; this k value is comparable to the experimentally determined value.

- **Effect of polymer concentration**

Figure 4-43 Effects of polymer concentration on release kinetics of PLGA (Resomer RG502)/BB/drug formulation. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Due to the narrow adjustable range of polymer concentration in BB system, we altered the PLGA concentration from PLGA/BB 50/50 to 45/55. Fig. 4-43

showed the release kinetics of metoclopramide salt and base. The release rate increased as the polymer concentration decreased, and this agrees with the above discussion that the drug release at the early stage is mainly controlled by diffusion in such systems, and permeability of the system depends on the viscosity. The higher is the polymer concentration, the higher the viscosity and thus the lower the diffusion coefficient.

- **Effect of L/G ratio of polymer**

As stated in the above section, not all BB solutions of PLGAs can form depot during in vitro, most solutions were flowable (by tilting the vials) within the whole period of experiment before turning into a “creamy” morphology. Among the polymers we used in experiment, polymers with L/G ratio above 50/50 could not form depot. The release kinetics of metoclopramide salt and base were shown in Fig. 4-44 and 4-45. The release was much faster than that in RG502/BB (gelled) system. The release mechanism is similar to the metoclopramide release from a newly published poly (ortho ester) system²¹⁰, since both matrices are injected as flowable liquid, which are subjected to degradation but without further solidification that may due to gelation or polymer precipitation under phase inversion.

Figure 4-44 Metoclopramide salt released from PLGA solutions made up of PLGA with different L/G ratio, the formulation is PLGA/BB/drug = 50/50/5 by weight. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

- Effect of terminal group of polymer

We discussed the degradation and “in vitro” dynamic viscosity change of PLGA/BB solutions with different terminal groups in above section. The carboxylic acid terminated PLGA showed faster degradation and earlier decrease of dynamic viscosity than that terminated by ethyl ester. Fig. 4-46 shows release kinetics of metoclopramide salt from these two systems, i.e., APT071603-1/BB system in which APT071603-1 is terminated by carboxylic acid group, and RG502/BB system in which RG502 is terminated by ethyl ester.

Figure 4-46 Metoclopramide salt released from PLGA solutions made up of PLGA with different terminal group, the formulation is PLGA/BB/drug = 50/50/5 by weight, the APT071603-1 is terminated by $-\text{COOH}$, and RG502 is terminated by ethyl ester. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

As expected, the acceleration phase due to polymer degradation appeared much earlier in APT071603-1/BB system, and the total drug was released out within a period approximately half of that in RG502/BB system.

Up to 250 hours (before polymer degradation phase), the release of drug is slower from the APT071603-1 system compared to the RG502 system. This is also observed for protein drug that we will show in the following section.

- **Effect of ageing of BB system**

The ageing effects on the release kinetics were investigated in this section. The formulations loaded with metoclopramide salt and base were stored individually at 10 °C and 25 °C, and in vitro release tests were conducted 27 days later. The formulation ageing at 10 °C would be warmed to room temperature naturally before being injected into buffer, while half of the formulation aged at 25 °C was directly injected into buffer as gel, and another half was heated to 65 °C for 10 minutes to “melt” the gel structure before being injected into buffer.

Similar release kinetics was found between formulation aged at 10 °C and the fresh one. For the samples aged at 25 °C, the release rates from aged solutions that were melted before injection into buffer were similar to those seen with fresh solutions, whereas the aged, non-melted solutions showed a much faster release rate. This is true for both kinds of drugs (see Fig. 4-47 and Fig. 4-48).

Figure 4-47 Metoclopramide salt release over time from freshly prepared PLGA (Resomer RG502)/BB systems or systems aged at 10°C or 25°C for 27 days. Release from the samples aged at 25°C was also measured after melting. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Figure 4-48 Metoclopramide base release over time from freshly prepared PLGA (Resomer RG502)/BB systems or systems aged at 10°C or 25°C for 27 days. Release from the samples aged at 25°C was also measured after melting. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Above results showed the effect of gel formation on drug release. Thus, gel formation prior to injection actually leads to a faster release rate, presumably because the gelation process induces an increase in “pore” size where the “pores” are richer in solvent than the micro-phase separated polymer-rich regions. As expected, aging at low temperature (10 °C), where no gelation occurs, does not change the rate of drug release.

4.4.2 Low Molecular Weight Drugs: metoclopramide salt/base from other solvent systems

- Effect of solvent

Figure 4-49 Metoclopramide salt released from PLGA (Resomer RG502) solutions made up of different solvents, the formulation is PLGA/solvent/drug = 50/50/5 by weight. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Benzyl benzoate (BB) was first employed in the in situ formed depot system to alter the phase inversion dynamics and therefore the drug release kinetics. As emphasized in section 4.3.2 and 4.3.3, BB has very limited solubility in buffer and gelation upon ageing correlates well with in vitro depot formation, thus indicating that the depot formation mechanism is fundamentally different from the nonsolvent induced phase separation process. In this section, the release kinetics of metoclopramide salt and base were compared in three solvents solvent system. They are benzyl benzoate (BB), triacetin and N-methyl-2-pyrrolidone (NMP), among which NMP is fully water miscible and triacetin has a solubility of ~7% in water, while benzyl benzoate is a hydrophobic solvent, which has limited solubility in ppm as we measured with HPLC in section 4.3.2.

Fig. 4-49 shows the release kinetics of the salt form of metoclopramide into buffer. Both NMP and triacetin show the classic tri-phasic release pattern, which consists of an initial slow release, followed by an acceleration region, then a plateau and further acceleration (inflection). The release from the PLGA/BB system follows a smoother, 2-phase release profile. We explain the observations as follows:

- 1). For NMP and triacetin, a skin of almost pure PLGA forms upon contact with buffer. The initial slow release period (up to 200hrs) is diffusion of salt drug through this PLGA skin into buffer.

In the case of BB, due to the limited solubility of BB in buffer, there is nearly no change in the composition of the surface layer of the depot. In this case, the diffusion of drug is faster in this BB “plasticized” PLGA.

2). The release rate increases dramatically for the NMP and triacetin systems at about 100 and 200 hours individually, but not for the BB system. As degradation has not yet started for salt-containing systems at these time-points, we attribute this rise to diffusion of NMP/triacetin through the PLGA “skin”, creating liquid-filled, connected polymer-lean pathways for drug diffusion. This result parallels that of the liquid-liquid demixing (Fig. 4-32). Such acceleration is also observed by Brodbeck et al¹⁹ for lysozyme released from triacetin and ethyl benzoate systems.

3). Acceleration occurs at around 600—800 hours for all systems, most certainly due to the onset of polymer degradation. Release beyond this point is a contribution of diffusion and erosion, as pathways are created by polymer leaching out into the water phase.

When we change the nature of the drug, from the hydrophilic salt to the more hydrophobic base, the release kinetics is dramatically altered (see Fig. 4-50). Noting that base addition accelerates polymer degradation (see Fig. 4-26 and 4-27), we offer the following explanation for the observed features:

1). Up to 200 hours (before polymer degradation start), release of drug is faster from the BB system compared to the NMP/triacetin systems. This is again attributed to the different diffusion rates through a PLGA skin vs. a BB “plasticized” PLGA skin, as for the salt.

2). At about 200 hours, erosion starts to play a role for the NMP and triacetin systems, resulting in accelerated drug release.

Figure 4-50 Metoclopramide base released from PLGA (Resomer RG502) solutions made up of different solvents, the formulation is PLGA/solvent/drug = 50/50/5 by weight. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Figure 4-51 Release kinetics of metoclopramide salt and base from PLGA (Resomer RG502)/triacetin system, the formulation is PLGA/BB/drug = 50/50/5 by weight. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Figure 4-52 Release kinetics of metoclopramide salt and base from PLGA (Resomer RG502)/NMP system, the formulation is PLGA/BB/drug = 50/50/5 by weight. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Overall, the differences between salt and base release rates are higher for the hydrophobic (BB) (Fig. 4-40) and partially hydrophobic (triacetin) (Fig. 4-51) systems, and almost neglectable for the NMP system (Fig. 4-52). Again, the differences are pronounced from 0—800 hours, as seen from Fig.4-40, and are attributed to the relatively higher solubility of base in the matrix, and the acceleration of degradation by base.

- **Systems of non-gelled polymers**

Contrary to the BB system, all the polymers (those that do not form depot in BB system as well as those that do) proceed via phase inversion and thus solidify into depots in NMP system.

Fig. 4-53 shows metoclopramide salt release from three PLGA/NMP systems, i.e., A137-28 (PDLA, L/G 100/0, -COOH terminated), A137-29 (PDLGA, L/G 75/25, -COOH terminated) and RG502 (PDLGA, L/G 50/50, -COC₂H₅ terminated). Compared to the RG502 system, the initial slow release period in the other two polymer system is shorter and the release rate is also higher, indicating the diffusion of drug through the PLGA skin is easier in these two systems, which we believe to be due to the more hydrophilic carboxylic acid terminal groups of these two PLGA systems. Due to the slow hydrolysis of lactide compared to glycolide, the erosion phase did not appear during the whole experiment period in the PDLA system.

Figure 4-53 Metoclopramide salt released from PLGA solutions made up of PLGA with different L/G ratio, the formulation is PLGA/NMP/drug = 50/50/5 by weight. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Further we investigated another non-gelling polymer DL655FK, which has the same L/G ratio as RG502 but with higher molecular weight. Fig. 4-54 shows metoclopramide salt and base release from this NMP depot. Compared with RG502/NMP system, this higher polymer molecular weight system gave an increased release rate for both metoclopramide salt and base. This result contradicts the finding of Eliaz et al¹³, who have stated that higher molecular weight of polymer and higher content of polymer in polymer solution will tend to give higher solution viscosities, thus decreasing the diffusion rate.

Figure 4-54 Release kinetics of metoclopramide salt/base from PLGA (DL655FK)/NMP system, the formulation is PLGA/BB/drug = 50/50/5 by weight. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Examining the process of depot formation in these two systems during in vitro, we believe that the reason may lie in the morphology change during phase inversion. The high molecular weight PLGA solution showed a shrinking front when in contact with water that we believe to be due to the existence of entanglements of polymer chains in solution when the molecular weight is high enough, and the low molecular weight polymer PLGA solution showed swelling morphology instead of shrinking (Fig. 4-55).

RG502/NMP

DL655FK/NMP

Figure 4-55 Photographs of PLGA/NMP solutions in vitro at different time point: A: before in vitro; B: phase inversion after 1 minute; C: phase inversion after 4 hours; D: phase inversion after 110 hours.

As stated above, all polymers studied in this report do form depots when injected into buffer from NMP solutions, presumably via phase inversion.

However, only a limited number of polymers can be formulated into a depot system from BB solutions, further confirming that the depot formation pathways are quite different for NMP and BB solutions. It appears that gel formation is a pre-requisite for depot formation in the PLGA/BB system. Without gel formation, release of drug is generally much faster.

4.4.3 High Molecular Weight Drugs: bovine serum albumin (BSA)/lysozyme from BB system

Controlled release of therapeutic proteins from biodegradable poly (lactide-co-glycolide) depots has received much attention. The most critical problems to overcome for the protein delivery formulations are: (1) to minimize the instability of protein during the formulation, storage, and release periods, and (2) to achieve a constant release of the protein having sufficient biological activity. In this section, bovine serum albumin (BSA) and chicken egg lysozyme were used as model protein drugs. The release kinetics from different formulations was studied. Size exclusion chromatography (SEC) was used to analyze the released BSA, bicinchoninic acid (BCA) protein assay was used for comparison.

- **Stability of protein**

The protein instability events such as aggregation, absorption and hydrolysis affect the protein release kinetics, and vice versa. In this section, two determination methods were used, i.e., bicinchoninic acid (BCA) protein assay

to analyze the total protein released, and size-exclusion chromatography (SEC) to detect the protein aggregation and the amount of protein released. The reaction principle of BCA kit was shown in section 3.2.4.

Figure 4-56 Size-exclusion chromatograms (SEC) of native BSA solution and BSA released at different time points from RG502/BB/BSA (50/50/10) formulation, t1: 3 hours; t10: 406.8 hours; t20: 989.2 hours, RG502/BB analyzed as reference sample.

Native BSA solutions in pH7.0 buffer and the released samples at different time points were collected and subjected to SEC analysis to examine the extent of protein aggregation. PLGA/BB sample without drug was used as reference (Fig. 4-56). The native BSA solution has a small fraction of dimer, which elutes an early shoulder peak (peak 2) in addition to a major monomer BSA peak (peak 1); the ratio of dimer/monomer was measured to be 8/100 with R squared value 0.996. The same phenomenon was also found by G. Crotts and M.J. Hunter et al, and it is due to the preparation (Cohn) procedure^{211,212}.

Figure 4-57 BSA dimer/monomer ratio in released samples from RG502/BB/BSA (50/50/10), analyzed by SEC.

The relative content of dimer BSA was higher in the release samples during the early stage of in vitro experiment than that of native BSA solution, but it decreased following the in vitro time (see Fig. 4-57). Due to the complexity of our formulation, we could not distinguish the degraded oligomers fraction of BSA due to hydrolysis.

The dimeric aggregation of BSA has been well documented in the literature²¹³⁻²¹⁵. Acidity, commonly developed due to accumulation of acidic degradation products upon PLGA hydrolysis, is agreed to be an important factor to cause protein aggregation and peptide bond hydrolysis. However, according to Fig. 4-57, we do not see the increase of dimer content when the extent of polymer degradation increased.

Fig. 4-58 and 4-59 showed the protein release kinetics from different formulations, pH 7.0 buffer was used as receptor liquid. The release curves were plotted from BCA protein assay and SEC, which calibrated with fresh native BSA solutions. The two results overlapped very well for the early stage. After approximately 400 hours in vitro, the released BSA quantified by BCA assay kit began to deviate from that of SEC. The latter is lower and the deviation increased with time. These results further reflect the universal problem in the PLGA-protein system. As the in vitro experiment precedes, the degree of PLGA degradation increased, which created an acidic environment that induces aggregation of protein and hydrolysis of peptide bonds. As the SEC results above reflect a combination of monomer and dimer of BSA, we infer that oligomers also exist in the BCA value, but are not picked up by SEC. and relative to the deviation between these two methods.

As we changed the receptor liquid from buffer to HPLC grade water, the effect is even more exaggerated (Fig. 4-60). Since water does not have buffer capability when subject to pH change, we can imagine the degradation of PLGA when immersed in water is faster than in buffer. Due to the same reason, the aggregation and hydrolysis of protein is much higher than those in buffer system. The same phenomenon was also observed in lysozyme system (Fig. 4-61).

Figure 4-58 BSA released from PLGA (RG502)/BB system, pH 7.0 phosphate buffer used as acceptance liquid, measured by SEC and BCA assay, the formulation is PLGA/solvent/drug = 50/50/10 by weight. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Figure 4-59 BSA released from PLGA (RG502)/BB system, pH 7.0 phosphate buffer used as acceptance liquid, measured by SEC and BCA assay, the formulation is PLGA/solvent/drug = 50/50/20 by weight. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Figure 4-60 BSA released from PLGA (RG502)/BB system, HPLC grade water used as acceptance liquid, measured by SEC and BCA assay. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Figure 4-61 Lysozyme released from PLGA (RG502)/BB system, HPLC grade water used as acceptance liquid, measured by SEC and BCA assay. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

- Effect of protein loading

Figure 4-62 Effect of Bovine serum albumin loading on the release kinetics from PLGA (RG502)/BB system, measured by BCA assay. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

BSA is loaded into the PLGA/BB solution by dispersing solid BSA particles throughout the matrix. For a protein loaded polymer matrix, the rate of release depends on the molecular weight of the protein, the size of the dispersed particles, then the protein loading, and the molecular weight of the polymer. The protein is released slowly when the matrix is immersed in buffer. The diffusion of protein from the pure polymer phase is negligible and the release of the protein out of the matrix is assumed to be diffusion through a tortuous, water-filled network of pores. These pores can come from water uptake by matrix, from the protein leaving the matrix, and for degradable matrix, from degraded products diffusing out of the matrix. According to Bawa et al²¹⁶ in a

polymer slab system, the protein release rate has the relationship with the effective diffusion coefficient as:

$$\frac{M_t}{M_\infty} = 1 - \frac{8}{\pi} \sum \frac{1}{(2n+1)^2} \exp\left\{-\frac{D_{eff} (2n+1)^2 \pi^2 t}{L^2}\right\} \quad (4.17)$$

$$\text{and } D_{eff} = \frac{D_0}{F\tau} \quad (4.18)$$

where τ is the tortuosity and F is the shape factor.

Increasing the protein loading will increase the release rate by decreasing the tortuosity, and providing the protein molecules with less tortuous routes to the exterior of the matrix, thus increasing the diffusion coefficient and therefore the release rate²¹⁷. This is true only after ~400 hours for the PLGA/BB/BSA system with drug loading varying from 50/50/10 and 50/50/20 by weight ratio. The release rate (M_t/M_0) did not show obvious difference in the first 400hours (Figure 4-62), and is due mainly to diffusion through water-filled channels created by water uptake (not by leaching out of protein. Please see Figure 4-33 for water uptake).

So we believe the initial release is mostly through water channels set up by absorbed water, and the later release is through channels created by existing proteins.

- **Effect of terminal groups**

Terminal groups affected the degradation and dynamic viscosity of PLGA and its BB solution. Fig. 4-63 showed the release kinetics of BSA from two PLGA

systems, i.e., RG502H/BB system in which RG502H is terminated by carboxylic acid group, and RG502/BB system in which RG502 is terminated by ethyl ester.

Figure 4-63 Effect of terminal group of PLGA on the release kinetics of BSA from BB system, where RG502 is terminated by ethyl ester while RG502H is terminated by carboxylic acid, measured by SEC. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Similar to the observations with low molecular weight drug, the acceleration phase due to polymer degradation appeared much earlier in RG502H/BB system (about 200 hours), which indicates earlier degradation with the carboxylic acid terminated polymer. The release rate in the early stage (before polymer degradation phase) is slower from the RG502H system compared to the RG502 system. At the moment, we are unable to identify the exact functions of the terminal groups. We do suspect, however, that the limited

amount of drug released during the earlier stage might associate with the carboxyl acid terminal groups, and need further confirmation.

- **Effect of co-encapsulated additives**

Figure 4-64 Effect of additives on the release kinetics of BSA from PLGA (RG502)/BB system. The formulation is PLGA/BB/BSA/additive = 50/50/10/2. Measured by SEC. Data are shown as average of 3 repetitions, standard deviation not shown.

As seen from the above results, BSA release from RG502/BB system followed a smooth profile without initial burst, while the BSA showed incomplete release and instability during the later stages. In this section, a series of additives including low molecular weight sugar, salt and synthetic drug were

formulated into RG502/BB/BSA solutions together with BSA, in order to improve the release of BSA from this system.

All these additives were milled into fine powder ($<100\mu$) before encapsulation and exist as suspension in the depot. Fig. 4-64 shows the effects of different additives on the BSA release kinetics. Glucose and sodium chloride showed the best effect, which increase the release rate by approximate 80%. Lactose is also helpful for BSA release. Metoclopramide salt, the low molecular weight drug we used in above section showed an interesting effect. It improved BSA release in the early stage up to approximate 600 hours, and then decreased the release rate to a very low level.

When the molecular weights of all these formulations are measured at this time point (i.e. 600 hours), we found these additives including glucose, lactose and sodium chloride accelerated the PLGA degradation slightly. However, the PLGA in the formulation containing metosalt degraded much faster than the other samples. The metoclopramide salt released from the same samples was also determined by UV spectrometry, Fig. 4-65 showed the accelerated release of metosalt due to degradation coincident with the retarded release of BSA. As we discussed in above section, the stability of the protein is strictly related with the degradation product of PLGA, therefore the most possible reason for the retarded release of BSA in the later stage is due to accelerated PLGA degradation that hydrolyzed the protein, and the protein was released as oligomers, which could not show in the SEC results. Recovering the BSA in

the final depot, we found the amount of protein remaining is limited and far lower than the calculated amount, which confirmed the above conjecture.

Glucose has shown a substantial effect on improving the release rate of BSA by a remarkable percentage. Fig. 4-66 shows by increasing glucose level, the release rate of BSA is largely increased.

Figure 4-65 Kinetics of BSA and metoclopramide salt released from PLGA (RG502)/BB/BSA/metosalt (50/50/10/2) system. Measured by SEC. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Figure 4-66 Effect of glucose as an additive on the release kinetics of BSA from PLGA (RG502)/BB/BSA system. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Figure 4-67 BSA release kinetics measured by Size exclusion chromatography (SEC) and bicinchoninic acid (BCA) assay. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

Sugar as additive in protein-encapsulated microparticles/implants has been reported by different researchers. Griebenow et al²¹⁸ reported that addition of lactulose and lactose into both water phases during water-in-oil-in-water encapsulation resulted in reduction in the preparation induced lysozyme aggregation and inactivation. Schwendeman et al²¹⁹ found co-encapsulating glucose induced reduction in initial burst of octreotide acetate from PLGA microspheres by a complex effect. Sucrose was also found to increase the release rate and enhance the stability of BSA by increasing water uptake of the PLGA implant²²⁰.

In our system, the release of protein from the PLGA/BB system is mostly through the pores that were created by the protein molecules, degradation products diffusing out, and the water coming in. Glucose and the other low molecular weight additives most likely function as water channel creators. They diffuse out of the PLGA/BB depot faster than BSA and lead to creation of water channels. These water channels are favorable for protein diffusion out by increasing the hydration of suspended BSA solid particles. Further addition of hydrophilic low molecular weight additives can also increase the connectivity of pores, thus increase the release rate.

We did not observe the obvious effect of stabilization of glucose on BSA, as we observed an even larger deviation between results from BCA and SEC (compare Fig. 4-58 and 4-67). For results analyzed by BCA, we even see a second accelerated release due to polymer degradation, similar to that in metoclopramide salt system.

The above results indicate that the protein release rate and efficiency can be increased by using low molecular weight hydrophilic additives, which can neutralize the acidic environment and thus suppress the hydrolysis of protein, and increase the number of pores and the pore connectivity to facilitate the protein release.

The results also indicate that although the metoclopramide salt has less effect on the hydrolysis of PLGA than its counterpart base form, it has a larger effect on hydrolysis than those neutral additives such as sodium chloride, glucose, etc.

4.4.4 High Molecular Weight Drugs: bovine serum albumin from other systems

- **Release from NMP System**

Fig. 4-68 shows the in vitro release kinetics of BSA from NMP systems with PLGAs of different L/G ratio. The release curve followed the typical profile of rapidly NIPS system mentioned by McHugh et al, i.e., a large burst effect followed by a prolonged period of slow release of protein. Mechanistically, the burst effect is due to the rapid release of a major fraction of protein that resided in or near the pores formed during phase inversion, followed by slow release of protein trapped in the hardened polymer matrix. Protein release differs substantially from the release of metoclopramide from the same system (Fig. 4-49).

Figure 4-68 Bovine serum albumin (BSA) released from PLGA/NMP solutions made up of PLGAs of different L/G, the formulation is PLGA/NMP/drug = 50/50/10 by weight. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

• Release from Triacetin System

Fig. 4-69 shows the release kinetics of BSA from the triacetin formulations. It is seen to follow a more sustained and smooth release profile than the NMP system. No initial burst was found. The release curves of A137-28 (L/G 100/0), A137-29 (L/G 75/25) and RG502H (L/G 50/50) implied the effect of degradation on the release rate, the formulation of PLGA with L/G ratio of 50/50 showed faster release rate than the other two systems. The same effect also act on the difference between RG502H (terminated by carboxylic acids) and RG502 (terminated by ethyl ester) system, the BSA was released slowly

in the first 400 hours, and is followed by a burst release due to degradation from the RG502 system.

Figure 4-69 Bovine serum albumin (BSA) released from PLGA/triacetin solutions made up of PLGAs of different L/G, the formulation is PLGA/NMP/drug = 50/50/10 by weight. Data are shown as average of 3 repetitions, standard deviation shown as error bar.

The above results are consistent with that of McHugh et al in their lysozyme system, except that the lysozyme was released faster than BSA, which we also found in BB system (compare Fig. 4-58 and Fig. 4-61).

Among the three simple polymer/solvent/drug formulations of BB, triacetin and NMP, the highest drug release efficiency is found in the triacetin system, which we thought to be due to the lower water miscibility of triacetin. Both “pure PLGA skin” in NMP system and “plasticized” PLGA in BB system are

somewhat hydrophobic, while the triacetin modified the hydrophilicity of the depot, which is favorable for protein drug release.

Compared with the release rate of low molecular weight drug, protein drug displays incomplete release from all these systems, due to its chemical and physical instabilities. Clearly, protein stability in such degrading systems, especially in the presence of water, is a concern and warrants further study.

Chapter 5

CONCLUSION

PLGA/BB solutions used as injectable in-situ formed depots for drug delivery have been extensively characterized. We have evaluated both the degradation and the flow properties during storage and after injection into buffer. In addition, we have studied the mechanism of gel formation during ageing and correlated it to depot formation after buffer injection. This mechanism is contrasted with the NIPS mechanism of depot formation in other PLGA solutions, such as PLGA/NMP and PLGA/triacetin. Finally, drug and protein release were studied as a function of polymer concentration, drug type and concentration, and solution type.

5.1 Flow Studies

Decrease of dynamic viscosity and modulus was observed in both BB and NMP solutions over ageing time, which indicates molecular degradation, according to

$$\eta_0 = KM^\alpha$$

During ageing at 25°C and 37°C, some PLGA/BB solutions exhibit both gelation and degradation and develop a three-dimensional network structure. This is evidenced by the crossover behavior of the storage and loss modulus. Viscosity measurements show that structure formation precedes molecular degradation.

Gelation causes the increase of dynamic viscosity and modulus, the storage modulus G' increases even faster than loss modulus G'' until a 3-D network structure is formed. On the contrary, the degradation leads to decreases in both dynamic viscosity and modulus. This effect contributes to the slower rate of initial increase in viscosity (due to gelation) or even decrease of viscosity during the later stage.

5.2 Gel Formation

PLGA/BB systems that undergo gelation during ageing also gel upon buffer injection. Gelation upon ageing correlates well with “in vitro” depot formation, indicating that the depot formation mechanism is fundamentally different from phase inversion, as observed for the same polymer in a good water-miscible solvent, such as NMP.

The nature of the gels formed in these PLGA/solvent systems was studied using MDSC. Network formation appears to be a thermoreversible process, with a heat of transition associated with it, which is observed to increase as a function of time of ageing.

In conjunction with the microstructures of the polymers characterized by ^1H and ^{13}C NMR, some interesting facts about these “depot”-forming systems were clearly established:

1. Depot formation in these systems appears to be due to entirely to the formation of an ordered three-dimensional structure, and not due to phase inversion.

2. The three-dimensional structure formed is most likely due to formation of ordered aggregates or microcrystallites.
3. The aggregation is thermoreversible, and is associated with an enthalpy.
4. The ordered structure is found to form only for polymers that have glycolide block lengths above about 2.9 (number-average). For polymers with glycolide lengths below this value, no gel formation is observed.
5. The kinetics of gel formation is dependent on microstructural details, as well as on the nature of the solvent. Gels are formed in benzyl benzoate (BB), ethyl benzoate (EB), ethyl acetate (EA), tetrahydrofuran (THF) and dichloromethane (DCM) etc, but cannot form gel in acetonitrile (ACN), N-methyl-2-pyrrolidone (NMP) and triacetin.
6. The observed melting temperatures for the gels are much lower than the melting temperatures for the homopolymer of poly (glycolide), and are attributed to the much shorter glycolide segments, and to the presence of solvent in the microcrystallite.

5.3 Degradation

Results from gel permeation chromatography (GPC) support the rheological conclusion on the degradation of PLGA solution aged at different temperatures.

The encapsulated drugs also affect the hydrolysis of the polymer. Metoclopramide base accelerates the rate of hydrolysis of polymer, while metoclopramide

hydrochloride salt shows less effect than its counterparts. Drug-containing depots degrade faster than those containing other neutral additives such as sodium chloride, glucose, or sucrose. Protein drugs, due to their amphoteric properties, could buffer the carboxyl groups produced by hydrolysis and alter the microenvironment, and thus alter the degradation kinetics.

Further, terminal groups also contribute to the rate of hydrolysis, the degradation rate of ethyl ester terminated PLGA is found to be approximately 2.8 times lower than those uncapped PLGAs (terminated by carboxylic acid).

5.4 Controlled release

Drug release from PLGA solutions is controlled by a complex effect of degradation and diffusion. Metoclopramide (salt and base) release kinetics is affected by several variables.

The release of salt drug from BB system follows the classical 2-phase profile including early diffusion-control, followed by a distinct acceleration due to polymer degradation (erosion-control or erosion/diffusion control). The base drug has relative higher solubility in PLGA/BB depot, and the onset of polymer degradation also occurs earlier when base drug is used, thus the overall base drug release is faster than the salt drug.

For hydrophilic solvents, such as NMP, use of base or salt gives similar release rates (5% loading), while for less hydrophilic solvents, such as triacetin, the

degradation phase is accelerated by base drug, leading to faster release of base drug.

For protein drug, a distinct difference is observed in the release amount of BSA quantified by SEC and BCA assay, especially in the later stages (after about 400 hours). This indicates a common-observed problem in the PLGA-protein system, i.e., the PLGA degradation creates acidic environment that leads to aggregation of protein and the eventual hydrolysis of peptide bonds. The deviation was also found in lysozyme system. The deviation is exaggerated when HPLC grade water is used instead of buffer.

Similar to the metoclopramide salt, BSA released from the BB system followed a smooth, near zero-order profile, but the release efficiency is very low (less than 20% without additive). Low molecular weight neutral salts such as sodium chloride, or sugars such as glucose and sucrose can increase the release rate and efficiency several times, while additives that accelerated PLGA degradation have little effect or even a reverse effect. The function of additives is to create water channels. The fast leaching-out of the additives leads to creation of water channels, which are favorable for protein hydration and efflux. (We did not observe any obvious effect of stabilization of these additives on BSA, as the deviation between SEC and BCA assay is still observed.)

The release of BSA from the NMP system displays the typical profile of a NIPS system, i.e., a rapid release of a major fraction of protein that resided in or near the pores formed during phase inversion, followed by slow release of protein trapped in the hardened polymer matrix. However the triacetin system shows the prolonged

release of BSA without initial burst and the highest release efficiency among the three systems.

Compared with the release rate of low molecular weight drug, the protein drug displays incomplete release from all these systems, due to its chemical and physical instabilities.

Chapter 6

FUTURE SUGGESTIONS

The major suggestions for future research on PLGA/BB system used as injectable depot are summed up as follows:

1. Conduct in vivo studies to confirm the mechanism of depot formation, to understand the degradation and drug release kinetics.
2. To further understand the exact nature of structure formation (gelation) in this random copolymer system. This will include SALS studies.
3. To optimize formulations for protein drug release.

Experiments proposed for objective 1:

- a) Since the only in vivo result reported in the literature was on the RG502/BB system, which can form gel during ageing and depot upon buffer injection, and could not further confirm the mechanism we proposed for the depot formation of BB system. BB solutions from different PLGAs (or PLA) should be injected in vivo, especially those solutions that do not gel during ageing. Correlation of gelation during ageing with depot formation upon buffer injection should be further studied.

- b) The toxicity of polymers, solvents should be evaluated, both systematically and locally. The toxicity study will control the injection amounts relative to the size (weight) of the animals.
- c) In vivo drug release should be evaluated and compared to the in vitro data.

Experiments proposed for objective 2:

- a) Study gelation of PLGA in other solvents, such as ethyl acetate, THF, etc, since the solubility in BB solution is narrow. Gel point (critical concentration) should be found in these systems, and the kinetics of gelation may change.
- b) Synthesize tailored copolymers with different microstructure, e.g., synthesize copolymers from diblock, multiblock or random copolymer with different microstructure, in order to study the mechanism of gelation.
- c) Study with more experimental methods (such as SAXS or SANS) to investigate the gel domain structure.

Experiments proposed for objective 3:

- a). Study with more experimental methods to quantify/qualify the protein stability/structure. A SDS-PAGE for protein can be used to distinguish the protein aggregation and part of degradation (effective within a certain molecular weight), an even more complicated experiment can be done to harvest the proteins with different molecular weight and quantify.

Proteins such as lysozyme can be characterized by its enzyme activity.

All these experiments will be conducted on the released protein in buffer, recovered from the depot.

b). Optimize the formulation in order to increase the protein stability, release efficiency and the release profile.

c). Triacetin system, which showed a better release profile and efficiency for protein drug (BSA) should be studied in more detail for protein drugs.

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APPENDIX

Method for Calculation of Glycolide segment length:

$$L_A = P_1(A) / P_2(AB)$$

Since any sequence has a successor or predecessor which can only be either an A or a B,

$P_1(A) = P_2(\underline{A}A) + P_2(A\underline{B}) = P_2(\underline{A}A) + P_2(B\underline{A})$, where letter/underlined letter represent the successor/predecessor, and

$$P_2(AB) = P_2(BA)$$

$$\text{So } L_A = P_1(A)/P_2(AB) = \{P_2(AA) + P_2(AB)\}/P_2(AB) = P_2(AA)/P_2(AB) + 1$$

In this case,

$$L_G = P_2(GGGG)/P_2(GGLL) + 1 = A_{GGGG}/A_{GGLL} + 1$$

Since L_G is actually the length of a GG segment, rather than a G segment.

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